Selection and characterisation of the predominant *Lactobacillus* species as a starter culture in the preparation of kocho, fermented food from enset

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Abstract Enset (*Ensete ventricosum* (Welw.) Cheesman) (false banana) plant is a multipurpose traditional crop widely cultivated in the south and southwestern Ethiopia. A study was conducted to determine the predominant microbes from kocho for subsequent use as a starter culture. Accordingly, a total of 40 lactic acid bacteria associated with kocho were characterized both at the phenotypic and genotypic level. *Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactobacillus paracasei/casei* and *Lactobacillus fermentum* were the isolated strains during kocho fermentation. Most *L. plantarum* showed rapid acidification and the higher growth rate than *L. brevis*. Based on these results *L. plantarum* (n = 10) and *L. brevis* (n = 3) were selected as possible starter strains and applied to enset pulp in laboratory scale. These starter strains showed fast pH reduction, increased microbial load than control sample and the possibility of a single strain in the fermentation of enset pulps for the production of kocho.

Keywords Enset · Kocho · Starter strain · *Lactobacillus plantarum* · *Lactobacillus brevis*

Introduction

Fermentation from a biochemical point of view is defined as the metabolic process of deriving energy from organic compounds without the involvement of an exogenous oxidizing agent (Bourdichon et al., 2012). Food fermentation is an ancient practice used for preservation of food, removal of toxic substance, and improving the nutritional value and organoleptic quality of the food (Edward et al., 2010). Fermented foods mostly the products of carbohydrate rich raw materials constitute a significant element of African diets. Gari from cassava (Edward et al., 2010), kocho from enset (Bosha et al., 2016), ogi and mahewu from maize (Franz et al., 2014) and ingera from teff (Senait et al., 1997) are examples of traditional fermented foods in Africa.

Enset (*Ensete ventricosum* (Welw.) Cheesman) (false banana) plant is a multipurpose traditional crop in Ethiopia (Brandt et al., 1997). Over 80% of *E. ventricosum* plants are cultivated and consumed as a food source in the central, south and southwestern parts of Ethiopia (Hunduma and Ashenafi, 2011). Its cultivation and fermentation tradition is unique and it is important food sources for 15 million Ethiopia people (Yemataw et al., 2014). Relative to other crops, it's highly productive, drought tolerate, obtained throughout the whole year and stored without the need of refrigerator (Birmeta, 2004), making it important crop for Ethiopia where 25–35% of population is undernourished (Bosha et al., 2016).

Kocho is the food produced by decorticating and fermenting enset parts. Like other fermented food items, it can

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inhibit the growth of pathogenic bacteria, extending product shelf-life while ensuring consumer safety. Currently, about 123,479,334 of enset plants are harvested all over the country with the yield of 31,625,631.77 quintals of kocho (CSA, 2017). Its abundance, economic and food security benefits have led to increased cultivation of enset to non enset growing regions of Ethiopia. Despite these advantages, enset plant processing for preparation of food is time-consuming, fermentation time and techniques differs from area to area, sensory attribute is very variable and it has a strong odor (Gashe, 1987; Hunduma and Ashenafi, 2011). These are considered as the possible reasons for the limited cultivation of enset in other parts of the country. Moreover, the culture and tradition of other areas does not force them to focus on enset as they have many other cereal and pulse crops to cultivate and consume. Whereas, the above mentioned areas are highly densely populated and lack adequate lands to cultivate other crops and thus obliged to depend on enset cultivation. Kocho is rich in carbohydrates and some minerals (Ca and Zn). It is however very poor in fat, protein, and vitamin A (Bosha et al., 2016; Minaleshewa and Chandravanshi, 2008). Thus, it fulfill most of the nutritional requirements for the people that depend on it for living as sources of food, in addition to its used as feed and fiber sources.

Previous studies on enset fermentation mainly focused on the microbiological counts on the selected agar media and phenotypic identification of randomly selected isolates. Those studies reported a group of lactic acid bacteria (LAB) are responsible for the desired changes of the final product (Gashe, 1987; Hunduma and Ashenafi, 2011). However, phenotypic method alone is not reliable for characterisation of some *Lactobacillus* in the species level (Marroki et al., 2011). In naturally fermented vegetables, *Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactobacillus lactis*, *Leuconostoc mesenteroides*, *Lactobacillus fermentum*, and *Weissella paramesenteroides* are the most commonly isolated species of lactobacilli (Edward et al., 2010; Montet et al., 2014; Steinkraus, 2002). Thus, identification of *Lactobacillus* species from fermented enset can help to pursuit industrial important culture and to optimize and improve fermentation process through the development of starter culture.

Hence, the objective of this study was to characterise *Lactobacillus* isolated from fermented enset using phenotypic and genotypic method and to investigate growth potential and colonial relationship of the dominant *Lactobacillus* species in ordered to select strains for a starter culture to be used in the production of fermented kocho. To the best of our knowledge, there is no detailed reports on characterisation of the *Lactobacillus* species associated with enset fermentation.

Materials and methods

Microbiological sampling

Kocho, fermented product of enset, was prepared in Wolkite, Ethiopia. Wolkite was selected because kocho is a staple diet around Wolkite. The process started with a selection of matured enset plant. It was then cleaned and separated into different parts. The inner part of the leaf sheaths (pseudostem) was scraped to separate the fiber from the remaining plant parts and mixed with pulverized corms. Thereafter, they used the traditional surface fermentation method for the first 9 days, followed by pit fermentation for several months until need for consumption.

Kocho samples (500 g each) at different fermentation periods (10 days–10 months) were collected in a sterile glass bottle with an ice-box and transported from Wolkite to Addis Ababa, Ethiopia for microbiological analysis. About 10 g of samples obtained from the same households at different fermentation period was homogenized with 90 ml sterile distilled water using orbital shaker at 250 rpm for 30 min. A volume of 0.1 ml of the diluted sample was spread on De Man, Rogosa and Sharpe agar plates (MRS) (10^{-2} – 10^{-7}) in duplicates to obtained LAB. The MRS plates were incubated anaerobically at 30 °C for 48 h. Colonies were selected randomly from plates of highest dilution and repeatedly streaked out until purity. For further investigation, MRS slants were transported from Addis Ababa to Max Rubner Institute (MRI, Germany).

Phenotypic characterisation

Bacterial strains isolated from 5 kocho samples were checked for catalase activity and gram reaction using 3% of H_2O_2 and KOH (Merck, Darmstadt, Germany), respectively. Cell morphology was observed using phase contrast microscopy at 1000× magnification (Leica, Wetzlar, Germany), and oxidase activity and production of CO_2 from glucose in MRS broth containing inverted Durham tubes was tested using standard methods. The configuration of D (–) and L (+) isomers of lactic acid produced from glucose was determined by the enzymatic method using the commercial kit (R-biopharma Enzymatic Bioanalysis, Germany).

Genotypic characterisation

The total genomic DNA of all strains was isolated using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The 16S rRNA gene of all strains was then amplified using PCR.

The amplicon was performed using ALLin HotStart Taq Mastermix, 2× (HighQu, Kraichtal, Germany) in 50 µl total reaction volume. The reaction volume containing 25 µl of master mix, 2.5 µl of each primer (16Sseqfw and 16Sseqrev primer), 10 µl of sigma H₂O, and 10 ng/µl of DNA sample. Amplifications were conducted in a pectSTAR 96 Universal Thermocycler (VWR International GmbH, Darmstadt, Germany) with the following program: denaturation for 1 min at 94 °C, annealing for 1 min at 56 °C and extension for 2 min at 72 °C in 32 cycles. To check effective amplification, PCR products were subjected to gel electrophoresis on a 1% (w/v) agarose gel using 1× TBE buffer. The gels were stained in ethidium bromide and visualized with Gel doc. After purification of PCR product bi-directionally sequenced and analysed as described by Danylec et al. (2018).

RAPD-PCR

The DNA of the predominant strains was amplified using PCR which was performed using ALLin HotStart Taq Mastermix, 2× (HighQu, Kraichtal, Germany) in 50 µl total reaction volume. The volume of each M13 (5'-GAG GGT GGC GGT TCT-3') and LB2 (5'-GGT GAC GC-3') primer was 2.5 µl and amplified in 40 and 29 cycles, respectively. Then after, the PCR products were separated by electrophoresis on 200 ml of 1.8% agarose gel with 1× TAE buffer and gel for 3.5 h at 100 V. The gels were stained with ethidium bromide for 1 h, decoloring for 30 min, and visualized with a UV transilluminator. Gel images were captured using Molecular Imager Gel Doc XR⁺ system (Bio-RAD, USA). The digitized images were normalized and subsequently analyzed using the Bionumerics software package (Kostinek et al., 2005).

Bioscreen C technique

The optical density of all strains was measured by using Bioscreen C (LabSystems Helsinki, Finland). An overnight culture diluted to obtain about 7 log CFU/ml⁻¹ was distributed in triplicate into honeycomb microplates with a total volume of 300 µl (30 sample + 270 MRS growth media) per well. The control tubes contained MRS growth medium without inoculum. The optical density (OD) of a cell suspension was measured at 580 nm at every 30 min intervals over a 24 h period. The temperature was maintained at 30 °C after the sample plate was preheated for 10 min. Data obtained from the Bioscreen were imported into Microsoft Excel for data capturing and to generate growth curve. Results were reported as detection times (t_d).

Acid production

An overnight culture (1%) of LAB test strains were inoculated into MRS broth and grown aerobically at 30 °C. The cultures were centrifuged and the pH of the supernatant was measured at 0, 6, 24 and 48 h for determination of acid production. For acid production test MRS broths were prepared from a single batch in which pH was adjusted and then allotted into tubes before sterilization (Kostinek et al., 2005).

Preparation of starter strains

Thirteen LAB strains (10 *Lactobacillus plantarum* and 3 *Lactobacillus brevis*) were selected as a starter strains for fermentation of enset pulp in the production of kocho. MRS broth was inoculated with 10% of overnight culture and incubated at 30 °C for 48 h. The cultures were centrifuged (MSE memmer, London) at 45,000×g for 10 min and the pellets were collected to measured biomass production after drying at 105 °C for 24 h. After weighing the pellets, 2% glycerol and 10% skim milk was added as cryoprotection (Hubalek, 2003). The cell material with cryoprotectants added was then dried using freeze-dryer (Mini Lyodel, India), packaged into sterile aluminium bags and stored at −20 °C. The viability of the cell in the pellets before and after freeze-dried was done in duplicate on MRS agars.

Peeled enset pulps used for this study were obtained from Wolkite area, Ethiopia, of which 200 g of roughly sterilized pulp was inoculated with each freeze-dried strains at an initial concentration of approximately 7 log CFU/g after rehydration for 10 min in 500 ml of buckets. All buckets were sealed tightly and incubated at room temperature for 6 days for the production of kocho in laboratory scale. For microbial analysis and acid production, samples were taken at 0, 2, 4 and 6 fermentation days.

Results and discussion

Microbiological analysis of kocho

The isolated 43 bacterial strains from kocho samples on MRS agar were characterised using phenotypic and genotypic methods. Phenotypic characteristics indicated that all strains were rods in shape and negative to oxidase test (Table 1). Among those, three strains were gram-negative and catalase-positive. This indicated that they were not LAB. LAB are gram-positive, rods and cocci, catalase and oxidase negative, and ferment hexose sugar for the production of lactic acid (Makarova et al., 2006; Schleifer et al., 1995). Thus, a total number of 40 LAB strains were

Table 1 Phenotypic and genotypic properties of strains isolated from kocho samples at different fermentation durations

Fermentation period	Strain number	Morphology	Gram reaction	Catalase	Oxidase	CO ₂ from glucose	D-lactate	L-lactate	DI-lactate	Genotypic identification
10 days	A1	Rods	+	—	—	—	—	+	—	<i>L. paracasei/casei</i>
	A2	Rods	+	—	—	+	—	—	+	<i>L. brevis</i>
	A3	Rods	+	—	—	+	—	—	+	<i>L. brevis</i>
	A5	Rods	+	—	—	—	—	+	—	<i>L. paracasei/casei</i>
	A6	Rods	+	—	—	+	—	—	+	<i>L. brevis</i>
	A7A	Rods	+	—	—	+	—	—	+	<i>L. brevis</i>
	A7B	Rods	+	—	—	+	—	—	+	<i>L. brevis</i>
	A8	Rods	+	—	—	—	—	—	+	<i>L. plantarum</i>
	A9A	Rods	+	—	—	+	—	—	+	<i>L. brevis</i>
	A9B	Rods	+	—	—	—	—	—	+	<i>L. pentosus</i>
20 days	20-1	Rods	+	—	—	—	—	—	+	<i>L. plantarum</i>
	20-2A	Rods	+	—	—	—	—	—	+	<i>L. plantarum</i>
	20-2B	Rods	+	—	—	+	—	—	+	<i>L. brevis</i>
	20-3	Rods	+	—	—	+	—	—	+	<i>L. brevis</i>
	20-4A	Rods	+	—	—	—	—	—	+	<i>L. plantarum</i>
	20-4B	Rods	+	—	—	+	—	—	+	<i>L. brevis</i>
	20-5	Rods	+	—	—	—	—	—	+	<i>L. plantarum</i>
	20-6	Rods	+	—	—	—	—	—	+	<i>L. plantarum</i>
30 days	1-1	Rods	+	—	—	—	—	—	+	<i>L. plantarum</i>
	1-2A	Rods	+	—	—	—	—	—	+	<i>L. plantarum</i>
	1-2B	Rods	+	—	—	+	—	—	+	<i>L. fermentum</i>
	1-3	Rods	+	—	—	—	—	—	+	<i>L. plantarum</i>
	1-4	Rods	+	—	—	—	—	—	+	<i>L. plantarum</i>
	1-5A1	Rods	+	—	—	+	—	—	+	<i>L. fermentum</i>
	1-5A2	Rods	+	—	—	+	—	—	+	<i>L. fermentum</i>
	1-5B	Rods	+	—	—	+	—	—	+	<i>L. brevis</i>
	1-6	Rods	+	—	—	—	—	—	+	<i>L. plantarum</i>
	1-7A	Rods	+	—	—	—	—	—	+	<i>L. plantarum</i>
9 months	9-1A	Rods	+	—	—	+	—	—	+	<i>L. brevis</i>
	9-1B	Rods	+	—	—	—	—	—	+	<i>L. plantarum</i>
	9-4A1	Rods	—	+	—	—	—	+	—	<i>A. pasteurianus</i>
	9-4A2	Rods	—	+	—	—	—	+	—	<i>A. pasteurianus</i>
	9-4B	Rods	+	—	—	—	—	+	—	<i>L. paracasei/casei</i>
	9-5A	Rods	+	—	—	+	+	—	—	<i>L. paracollinoides/collinoides</i>
	9-5B	Rods	—	+	—	—	—	+	—	<i>A. pasteurianus</i>
	9-6A	Rods	+	—	—	—	—	—	+	<i>L. plantarum</i>
	9-6B	Rods	+	—	—	+	—	—	+	<i>L. brevis</i>
10 months	10-1	Rods	+	—	—	+	—	—	+	<i>L. brevis</i>
	10-2	Rods	+	—	—	+	—	—	+	<i>L. brevis</i>
	10-41A	Rods	+	—	—	—	—	—	+	<i>L. plantarum</i>
	10-41B	Rods	+	—	—	—	—	—	+	<i>L. plantarum</i>
	10-42A	Rods	+	—	—	—	—	+	—	<i>L. paracasei/casei</i>
	10-42B	Rods	+	—	—	—	—	+	—	<i>L. paracasei/casei</i>

isolated from kocho samples. From the 40 strains, 18 strains produced CO₂ from glucose fermentation and DL-lactate except one isolate (9-5A) which produced D-lactate, indicating that they were obligatory heterofermentative. Twenty-two strains produced L- and DL-lactate, but not CO₂ from glucose fermentation. This indicated that they were facultative heterofermentative.

The genotypic investigation also showed that 40 strains were grouped in genus *Lactobacillus* and three strains were *Acetobacteria*. Among those *Lactobacillus* strains, 17 facultative heterofermentative strains which produced DL-lactate were grouped into *Lactobacillus plantarum*, and 5 strains which produced L-lactate were grouped into *L. paracasei/casei*. The obligatory heterofermentative strains that produced DL-lactate were grouped into *L. brevis* (n = 14) and *L. fermentum* (n = 3), and the strain that has produced D-lactate was grouped as *L. paracollinodes/collinoides*.

Different studies reported that *Leuconostoc* and *Lactobacillus* species such as *L. plantarum*, *L. brevis* and *L. casei*, *L. fermentum*, *L. rhamnosus*, *L. sakei*, *L. fallax*, and *L. buchneri* have been isolated from fermenting vegetables or plant products (Gashe, 1987; Kostinek et al., 2005; Yousif et al., 2014). Similarly, in this study *L. plantarum*, *L. brevis*, *L. paracasei/casei* and *L. fermentum* were isolated from kocho. However, *Leuconostoc* that were reported in starchy fermented food including kocho is not confirmed in this study. This is possibly a result of differences in processing condition, geographic regions, raw materials, and enset varieties. Furthermore, *L. paracollinodes/collinoides* and *Acetobacteria* spp. were also identified in this study as responsible microbes during enset fermentation. *L. paracollinodes/collinoides* are found in brewery environment (Suzuki et al., 2004) and fermented vegetables such as table olives (Capozzi et al., 2017). Acetic acid bacteria (AAB) have been reported in fermentation of West African fermented food such as Ogi (fermented maize) and Burkutu (fermented sorghum), in manufacturing of acidic beers, and slight acidic beverages like water and milk kefir, kombucha, lambic beer, cocoa and cider (Achi, 2005; De Roos and De Vuyst, 2018). However, there is limited knowledge about their occurrence and functional role in spontaneously fermented food (De Roos and De Vuyst, 2018). The main reasons are that their cultivation, isolation, and identification are cumbersome and that they can occur in a viable but non-culturable state at anaerobic environment (Papalexandratou et al., 2013). Moreover, most microbial analysis studies designed on isolation of LAB using MRS growth media. In this study, AAB were isolated on MRS agar which was only directed for the isolation of LAB. If an appropriate growth media for AAB is used, it can be the dominated microbes during enset fermentation. Thus, for a clear understanding

of the microbial community during food fermentation, different growth media need to be used.

Biochemical changes

Lactobacillus plantarum (n = 17) and *L. brevis* (n = 14) were selected as possible starter strains on the basis of their prevalence in the kocho fermentation. The initial cell concentration, acid production, maximum OD and detection time of those strains were summarized in Table 2. The pH reduction of *L. plantarum* strains except for one strain (20-1) ranges from 4.76 to 5.3 and 3.72 to 3.86 at 6 and 24 h fermentation times, respectively. At 48 h, the pH ranges from 3.68 to 3.78 (Table 2). Maximum pH reduction observed after 6 h fermentation period and become constant until 48 h fermentation time. The pH reduction of *L. brevis* at 6 and 24 h fermentation period ranges from 5.33 to 5.49 and 4.74 to 5.09, respectively. At 48 h, the pH ranges from 4.29 to 4.66. These results indicate that *L. plantarum* species relatively have better acid production with short fermentation period than *L. brevis*. These could be because of the fact that facultative heterofermentative bacteria convert glucose primarily into lactic acid while obligatory heterofermentative bacteria convert glucose into lactic acid, CO₂, and ethanol (Konig and Frohlich, 2009). Kostinek et al. (2005) also stated that *L. plantarum* strains were characterized by faster and stronger pH reduction than obligatory heterofermentative, and should be selected as a possible starter strain. According to Holzapfel (2002), a fast lowering of the pH is one of the criteria for starter strain introduced for small-scale fermentation.

The optical density (OD) of *L. plantarum* and *L. brevis* strain measured with Bioscreen C are shown in Fig. 1. The OD measurements using a spectrophotometer and/or an automated system used to examine the growth of bacteria cell (Lewis et al., 2014). Bioscreen C is an automated system used for measuring growth dynamics of bacterial over time in a 100 well plate. The OD measurement using a Bioscreen C system showed that most *L. plantarum* were characterised by high OD production at 24 h fermentation periods. However, *L. plantarum* 20-4A, 1-2A, 20-1 and 10-41B produced low OD, required long detection time (> 18 h) and covered small 24 h fermentation area than others (Fig. 1). Detection time (t_d) for a turbidimetric instrument defined as the time required for the Bioscreen recording a 0.05 increase in OD from the initial value (Mckellar and Knight, 2000), would help to calculate specific growth rate and estimate lag phase duration of a population.

Lactobacillus brevis species presented relatively lower OD value and a long detection time (14–16.45 h). Initial OD values and cell counts of all *L. plantarum* and *L. brevis* strains were found at the same ranges. However, the

Table 2 Acid production, detection time and maximum OD (580 nm) of the predominant *Lactobacillus* strains

Fermentation period	Strain code	Genotypic identification	pH				Max. OD	Initial count (logCFU/ml) ^a	DT (h)
			0 h	6 h	24 h	48 h			
Control	C	–	5.53	5.53	5.53	5.53	0.23	ND	24:00
10 days	A2	<i>L. brevis</i>	5.53	5.38	5.00	4.46	0.90	9.49	16:00
	A3	<i>L. brevis</i>	5.53	5.33	4.74	4.29	0.81	9.46	14:07
	A6	<i>L. brevis</i>	5.53	5.39	5.01	4.48	0.95	9.45	15:07
	A7A	<i>L. brevis</i>	5.53	5.38	5.04	4.53	0.96	9.34	15:15
	A7B	<i>L. brevis</i>	5.52	5.39	5.04	4.55	0.93	9.23	15:45
	A8	<i>L. plantarum</i>	5.46	4.76	3.72	3.68	1.97	9.79	12:37
	A9A	<i>L. brevis</i>	5.53	5.40	5.05	4.50	0.98	9.25	15:07
	A9B	<i>L. plantarum</i>	5.52	4.93	3.73	3.71	1.94	9.75	13:00
20 days	20-1	<i>L. plantarum</i>	5.52	5.20	5.00	5.00	0.22	ND	24:00
	20-2A	<i>L. plantarum</i>	5.53	4.85	3.77	3.71	1.85	9.67	12:52
	20-2B	<i>L. brevis</i>	5.52	5.41	5.04	4.53	1.02	9.27	14:45
	20-3	<i>L. brevis</i>	5.54	5.41	5.05	4.52	0.97	8.95	14:00
	20-4A	<i>L. plantarum</i>	5.51	5.32	3.86	3.76	0.46	9.55	21:22
	20-4B	<i>L. brevis</i>	5.55	5.33	4.90	4.58	0.85	9.57	14:00
	20-5	<i>L. plantarum</i>	5.49	4.94	3.82	3.77	2.12	8.90	09:45
	20-6	<i>L. plantarum</i>	5.46	4.89	3.74	3.73	2.00	9.95	12:37
30 days	1-1	<i>L. plantarum</i>	5.49	4.89	3.79	3.75	1.89	9.55	14:07
	1-2A	<i>L. plantarum</i>	5.50	5.27	3.83	3.77	0.77	9.20	18:30
	1-3	<i>L. plantarum</i>	5.50	4.83	3.78	3.74	1.81	9.61	13:45
	1-4	<i>L. plantarum</i>	5.49	4.92	3.76	3.73	1.68	9.63	13:37
	1-5B	<i>L. brevis</i>	5.57	5.41	4.90	4.54	0.85	8.89	16:45
	1-6	<i>L. plantarum</i>	5.50	4.93	3.78	3.76	1.73	9.46	14:07
	1-7A	<i>L. plantarum</i>	5.53	4.78	3.79	3.77	1.78	9.95	12:52
9 months	9-1A	<i>L. brevis</i>	5.54	5.49	5.07	4.66	0.89	9.43	14:00
	9-1B	<i>L. plantarum</i>	5.52	4.81	3.85	3.78	1.77	9.27	13:45
	9-6A	<i>L. plantarum</i>	5.49	4.76	3.79	3.76	1.92	9.72	13:15
	9-6B	<i>L. brevis</i>	5.55	5.42	5.01	4.53	0.87	9.69	14:52
10 months	10-1	<i>L. brevis</i>	5.55	5.42	5.09	4.58	1.01	9.79	14:00
	10-2	<i>L. brevis</i>	5.55	5.43	5.07	4.57	0.94	9.84	14:22
	10-4 _{1A}	<i>L. plantarum</i>	5.49	5.20	3.80	3.77	1.28	9.69	16:30
	10-4 _{1B}	<i>L. plantarum</i>	5.47	5.07	3.78	3.74	0.53	9.63	20:15

ND: below the limited value

^aIndicates the analysis is done in duplicate

automated Bioscreen C results show most *L. plantarum* recorded high OD and required short detection time (t_d) than *L. brevis*.

RAPD-PCR

RAPD-PCR analysis was done to determine clonal relationships of isolated strains from five kocho samples. RAPD-PCR is one of the molecular techniques considered as important for the specific characterisation of LAB isolated from different sources (Ruiz et al., 2014). It is also used to determine possible clonal relationships among the

strains in order not to choose multiple isolates during the selection of starter culture (Kostinek et al., 2007).

RAPD-PCR analysis showed that among 14 *L. brevis*, 10 were (labeled in green) cluster very narrowly together and have visually a very uniform pattern in both primers (Fig. 2, Supplementary Fig. 1). This indicated that one *L. brevis* strain occurring at the beginning of fermentation (10 days) and lasting until the end (10 months) of fermentation. These bacteria may be environmentally abundant in enset processing area. Three *L. plantarum* strains (labeled in red) clustered together. They presented at the beginning (20 days) and after 10 months; two *L. plantarum*

Fig. 1 The growth rate of *Lactobacillus* strains

strains also presented at 10 days and after 1 month (labeled in blue). The strains originated from the same sample such as 20-2A and 20-5 (labeled light blue), and 1-3, 1-6 (labeled yellow) were showed presumptively clonal relation. Thus, RAPD-PCR results clustered 31 *Lactobacillus* strains into 17 strains groups.

Properties of starter cultures

Among 17 *Lactobacillus* groups of strains, *L. plantarum* ($n = 10$) and *L. brevis* ($n = 3$) were selected as possible starter strains in onset fermentation on the basis of OD and pH results. The selection of starter strain is dependent on different variables such as growth potential, cell concentration and preservation techniques used (Edward et al., 2010). The starter strains were preserved using a freeze-drying technique with the use of skimmed milk (10%) and glycerol (2%) as cryoprotectants. Selection of culture format is the most vital stage to preserve the cultures for long period (Parthuisot et al., 2003), and freeze-drying technique is one of the commonly used culture formats. Skimmed milk at a median of 10% was more frequently used cryoprotection for freeze-dried LAB, sometimes in a combination with other cryoprotective against (Hubalek, 2003).

The cell concentration, biomass production and the survival rate of starter strains after freeze-drying are shown

in Table 3. *L. brevis* strain 10-1 produced higher biomass (1.12 g/l) than *L. brevis* strains of 20-4B and 9-1A. However, *L. brevis* 20-4B had higher survival rate after freeze-drying (52.1%) than *L. brevis* of 10-1 and 9-1A (21%). The biomass production of *L. plantarum* strains ranges from 0.68 to 2.64 g/l. The survival rate of *L. plantarum* strains after freeze-drying ranges from 11.3 to 83.3%. *L. plantarum* strains 1-7 and 10-41A showed the lowest biomass production among others *L. plantarum* strains. However, their survival rate after freeze-drying ranges from 60.7 to 83.3%. This finding is supported by other studies which reported that the survival of LAB after freeze-drying seemed to be strain dependent (Edward et al., 2010; Fguiri et al., 2017). The main reasons for this variation could be the nature of the strains, the age of the culture, cell concentration, the type of cryoprotectants used, and cell damage at the cell wall and cell membrane.

Decorticated and pulverized onset pulps obtained from Wolkite area were fermented through inoculation of each freeze-dried strains, to test the ability of each strain for kocho production. The change in pH and viable cell count are shown in Table 4. The control sample changes its pH from the initial 6.23–5.64 at the 6 days of fermentation. The pH change of samples inoculated with *L. brevis* and *L. plantarum* after 6 days of fermentation ranges from 5.01 to 5.13 and 3.98 to 4.63, respectively. The viable cell count of a control sample at the 6 days of fermentation was 4.13 log

Fig. 2 Clustering as a composite dataset with primers M13 and Lb2 with Pearson coefficient and UPGMA

CFU/g. The viable cell count of a sample inoculated with starter strain ranges from 4.86 to 7.0 CFU/g at the 6 days of fermentation. *L. plantarum* 9-6A showed fast pH reduction and viable cell count at the 6 days of fermentation. Inoculation of strains relatively improves acid production and concentration of viable cell than a control sample. Similarly, in cassava fermentation starter culture reduced fermentation time by a fast decrease in pH when compared to the controls (Edward et al., 2010). Most African fermented food used a spontaneous fermentation process. This process takes a relatively long time and there is also the growth of contaminating microorganism from different sources (Holzapfel, 2002). The use of selected starter strains can decrease the long fermentation time and

reduce the risk of contamination in spontaneously fermented foods.

In conclusion, the result of this study indicated that *Lactobacillus* spp and *Acetobacter* are involved in kocho fermentation. Particularly, *L. plantarum* and *L. brevis* strains were abundant in all samples from the initial to the later stage of fermentation. Thus, those dominated LAB strains could be a potential starter culture for enslet fermentation. Particularly *L. plantarum* strain is more attractive than *L. brevis* to be a starter culture for enslet fermentation due to its fast pH reduction and microbial growth potential in the growth media and exemplary fermentation. Additionally, this study gives a great awareness about the possibility of single starter strain for fermentation of enslet pulp for the production of kocho.

Table 3 Viable cell count, biomass production and survival rate of selected starter strains (values are the means of duplicate analysis)

Strains	Cell count before freeze dried (CFU/ml)	Biomass (g/l)	Cell count in after freeze-dried (CFU/g)	Survival rate (%)
<i>L. brevis</i>				
10-1	3.2×10^{10}	1.12	6.8×10^9	21.3
20-4B	1.4×10^{10}	0.46	7.3×10^9	52.1
9-1A	4.1×10^{10}	0.42	8.8×10^9	21.5
<i>L. plantaurum</i>				
A8	4.8×10^{10}	1.80	1.59×10^{10}	33.1
A9B	9.2×10^{10}	1.88	1.64×10^{10}	17.8
20-5	7.6×10^{10}	2.64	2.98×10^{10}	39.2
20-6	1.0×10^{11}	2.22	2.47×10^{10}	24.7
9-1B	1.9×10^{10}	1.80	2.14×10^9	11.3
9-6A	2.3×10^{10}	2.44	3.90×10^9	17.0
1-1	3.2×10^{10}	1.44	1.10×10^{10}	34.4
1-3	9.8×10^{10}	2.16	1.98×10^{10}	20.2
1-7	1.2×10^{10}	1.60	1.00×10^{10}	83.3
10-41A	1.4×10^{11}	0.68	8.50×10^{10}	60.7

Table 4 pH reduction and viable cell count of starter strain in fermented kocho (values are the means of duplicate analysis at different samples)

Strain	pH				Count(log CFU/g)			
	0	2 days	4 days	6 days	0	2 days	4 days	6 days
Control	6.23	6.04	5.92	5.64	ND	2.04	3.90	4.13
<i>L. brevis</i>								
10-1	5.97	5.81	5.4	5.01	2.36	3.32	4.47	4.23
20-4B	6.02	5.94	5.4	5.08	2.17	2.25	4.63	5.18
9-1A	6.21	6.05	5.7	5.13	2.14	4.04	3.42	4.86
<i>L. plantaurum</i>								
A8	6.50	5.63	5.0	4.34	3.07	5.01	5.34	6.23
A9B	6.20	5.80	4.8	4.32	2.64	4.63	4.53	5.91
20-5	6.15	5.90	5.3	4.63	2.87	4.73	5.64	5.55
20-6	6.13	5.55	4.9	4.25	3.14	5.27	5.62	6.83
9-1B	5.85	5.12	4.3	3.98	3.21	5.14	6.31	6.00
9-6A	6.22	5.72	4.84	4.28	2.92	4.98	5.87	7.0
1-1	6.5	5.42	4.6	4.30	3.20	5.45	5.15	5.27
1-3	6.10	5.49	5.12	4.31	2.74	4.47	5.51	6.23
1-7	5.92	5.23	4.47	4.11	3.45	5.20	6.18	6.20
10-41A	6.23	6.0	5.5	4.91	2.06	3.69	4.13	4.92

ND: below the limited value

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Appendix

The percentage survival of the strain after the freeze-drying process was calculated as:

$$\text{Survival (\%)} = N/N_0 \times 100$$

where N = viable cell count after freeze-drying; N_0 = viable cell count before freeze drying.

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