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Research article

Physicochemical and microbial community dynamics of *Kocho* fermented from different enset varieties in South West Ethiopia

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ABSTRACT

Enset (Ensete ventricosum (Welw.) Cheesman) is an indigenous multipurpose plant in Ethiopia. More than 20 % of people in Ethiopia rely on *enset* for their subsistence livelihood. Its fermentation produces a starchy food named *Kocho*, which is yet poorly studied. In this study, physicochemical and microbial community dynamics of *Kocho* fermented from different enset varieties (Maziya, Genna, and Arkiya) were collected at Dawro Zone (Southern Ethiopia). Samples were collected at various fermentation times (days 1–60) for physicochemical and microbial (culture-dependent and culture-independent) characterization. Results showed that increasing fermentation time has a significantly strong positive ($R^2 = 0.768$, $p = 0.004$) correlation between titrable acidity, and a significantly strong negative association with pH ($R^2 = -0.715$, $p = 0.009$), moisture ($R^2 = -0.982$, $p < 0.05$), ash ($R^2 = -0.932$, $p < 0.05$), fat ($R^2 = -0.861$, $p < 0.05$), fiber ($R^2 = -0.981$, $p < 0.05$) and carbohydrate ($R^2 = -0.994$, $p < 0.001$) contents. An increasing or decreasing trend of physicochemical parameters observed during enset fermentation is significantly associated with microbial community dynamics. Shifts of microbial community observed during culture-dependent analysis were also confirmed by metagenomic results. During fermentation, Firmicutes (39–68 %) > Proteobacteria (7–53 %) > Cyanobacteria (7–24 %) were dominant phyla in the three enset varieties. Gamma (traditional starter culture) is dominated by *Lactobacillus plantrum* and *Lactobacillus manihotivorans* most probably the two species that play a significant role in initiating enset fermentation.

1. Introduction

Enset is a perennial herbaceous drought-tolerant and multipurpose plant in Ethiopia that ensures food security in the rural livelihood of the country, particularly in the southern regions of Ethiopia [1,2]. *Kocho*, *Bulla,* and *Amicho* are important food types prepared from enset *(Ensete ventricosum* (Welw.) Cheesman) corm and pseudo-stem [1,3]. *Kocho* is the most prominent food product of

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enset. The final *Kocho* product quality is mainly affected by the activity of fermenting microbes [4]. Enset fermentation is also affected by geographical location, where an extended fermentation time (a year) is required in the cooler regions than in warmer regions, which is ready to use within 15–30 days [5].

Previous studies reported in detail about changes in proximate composition, hydrolysis, proteolysis, and lipolysis that occur as significant biochemical and microbial changes during the fermentation process of *Kocho* [4–7]. During enset fermentation, lactic acid bacteria (LAB) are more abundant through the entire process than other microbial flora [8,9]. It has been reported that the pH drop through fermentation time is due to lactic acid produced by LAB [5,6]. A decrease in pH subsequently inhibited many microbes in the *Kocho* production process. On top of enjoying their lactic acid products to create a favorable environmental condition, the LAB tolerated and existed until the last phase of fermentation could be also due to anaerobic conditions in the pit favoring their growth. In addition to LAB, enset fermentation also contains spore formers (aerobic and anaerobic), Enterobacteriaceae, and yeast [5].

In most part of Ethiopia, *Kocho* is fermented naturally without defined starter cultures to initiate the fermentation process. The undefined traditional starter cultures are used in some areas of the country containing undesirable microbial groups [7]. For instance, Gamma is traditional starter culture in the study area. It is a traditional back slopping from previously fermented enset that can be used as the raw material for the next fermentation step. Gamma as a starter culture is mainly used in facilitating *Kocho* fermentation like yeasts used in bread. Although Gamma as a starter culture is frequently used in most parts of the Dawro Zone, the microbial community is not yet studied. Gamma starter also used in Sidama region, however, the ingredients added for sensory quality makes slightly different from Dawro. In Dawro, "qutsaruwa" (prepared from enset leaf sheaths and buried with fresh enset leaves for about 15 days) is added in Gamma to raise the sensory quality. According to Karsa et al. [7], mature enset corms were used as major raw materials to prepare a traditional starter culture (Gamancho) in the Sidama region.

The traditional *Kocho* production process in the study area is a long, labor-intensive, and time-consuming process, which is almost done by females (Fig. S1) require up to three months to obtain a quality *Kocho* product from enset [10]. In the study area, microflora involved in a traditionally added starter culture (Gamma) and at different fermentation times is poorly understood. Physicochemical parameter changes, which could be influencing microbial dynamics during fermentation also poorly understood in the study area. This study was designed to evaluate the physicochemical and microbial community dynamics of *Kocho* fermented from different enset varieties collected from Dawro Zone, Southern Ethiopia. The specific objectives were to: (i) select the best three varieties of enset used for *Kocho* production in the society; (ii) assess change in physicochemical parameters at various fermentation periods; and (iii) evaluate the microbial community shifts at various fermentation period using culture-dependent and culture-independent techniques. We hope this study will provide insights into the microbial community dynamics and physicochemical parameters change of *Kocho* made from different enset varieties.

2. Materials and methods

2.1. Study area

The study was carried out in Dawro Zone, which is the potential enset cultivating area in the South Western (in 2021 newly formed) Region of Ethiopia. Dawro is located about 500 km Southwest of Addis Ababa, the capital city of Ethiopia (Fig. S2). Based on the Central Statistical Agency of Ethiopia [11], the Dawro Zone has a total population of 489,577 (249,263 and 240,314 men and women, respectively).

Dawro Zone, with an area of 4436 square km, has ten districts and one town administration that lies in three (Kolla, Woyna Dega, and Dega) agroecological regions. The study was conducted in Essera district kebeles (Kambo, Oki and Delba), where the annual average temperature and rainfall are between 17.6 and 27.5 $^{\circ}C$ and 1401–1800 mm ranges, respectively. Farmers produce enset (as staple food), wheat, barley, peas, beans, and coffee. They also engaged in animal husbandry such as livestock ranching and beekeeping.

2.2. Experimental design and fermentation techniques

2.2.1. Informants and experimental design

The general flow chart which explains enset variety selection, fermentation, isolation, screening and characterization of the best isolates, is described in (Fig. S3). In addition to consulting different kinds of literature, a survey based on the questionnaire was carried out on 30 informants/respondents in three (Kambo, Oke, and Delba) Kebeles of Essera district, Dawro Zone, to get information about general enset utilization and preparation technique. Before data collection was carried out through questionnaires, a brief group discussion (in the local language, Dawrogna) was held with the informants to explain the objectives, benefits, risks and general procedures of the survey. The questionnaires were prepared in Amharic (most commonly used language) and also translated to Dawrogna when necessary. This was carried out to acknowledge the respondents' cooperation in preserving traditional knowledge of the study area and build their confidence in providing reliable information about enset type identification, cultivating ways, food preparation techniques, fermentation mechanisms they followed and the like. Once the objective was briefly explained, the respondents were asked to sign a prior informed consent form. Ethical approval was also obtained from the Dawro zone administration and kebele association heads of Kambo, Oke, and Delba. The questionnaires were administered with the help of translators who were conversant in Dawrogna. The questionnaires were designed to obtain the respondents' overall information, which has been shown in the supplementary section (Text Supplementary 1).

After listing down all the known enset varieties from respondents, 10 key informants were also selected from the three kebeles and enquired to rank the varieties based on the information provided. The ten most enset varieties were ranked according to a given

criterion (Table 1). Finally, based on the sum of all respondent's results, the three most frequently used enset varieties (Genna, Arkiya and Maziya) were selected based on product quality and quantity for further fermentation studies.

2.2.2. Enset processing steps

After selecting the three most frequently used enset varieties, they were traditionally processed and stored in three different pits. Harvesting matured enset variety is the pioneering step in enset preparation (Fig. S1). After harvesting, the older leaf sheath removal, internal leaf sheath (~20 cm lengths) peeling from the pseudo-stem down to the true stem and separating the true stem from the underground corm was done. In such a way, the prepared enset is chopped into pieces for further processing. Women stand to decorticate, and the pseudo-stem is attached with fibers on a wooden pole (locally called *Koqa*). After decortications and pulverization, which occur at the same time, granted corm (*Godiya*) and scarped leaf sheath (*Qashincha*) mass were mixed well in the newly prepared pit. The mixed mash is named "*Shucuwa*" in the study area. The traditional starter (Gamma) prepared earlier was added to the mixed mash (*Shucuwa*). Gamma is prepared from the processed mixture of the fine-cut pieces of the inner pseudo-stem of enset and different ingredients, such as herbs, tree leaves, aromatic plants, and rotten enset leaf sheaths (i.e., quxaruwa) and the preparation is left for 15–30 day to be fermented before use [12]. Based on family status, the fermented mash (*Kocho*) is ready to use (Fig. S1) after 1–3 months.

2.3. Sample collection and analysis

For physicochemical and microbial dynamics characterization, samples were collected (Fig. S3) on different (1, 15, 30, and 60) days from the three enset varieties (Genna, Arkiya, and Maziya) using a sterilized beaker and sterilized spoons. The traditional starter (Gamma) sample was also collected for microbial study using a sterilized beaker. Soil samples were also collected parallel to enset sample used for *Kocho* fermentation for various (pH, organic matter, conductivity, and moisture) physicochemical parameter analyses following standard methods [13]. For example, to determine the moisture content, 5 g homogenized sample were taken and put in an oven at 105 °C for 24 h. The soil pH was also determined using a digital pH meter (PH 1100H, VWR International, Darmstadt, Germany). The samples were transported to Arba Minch University Advanced Microbiology Laboratory in an ice box and kept in the refrigerator (~4 °*C*) until analysis.

2.3.1. Enset physicochemical parameter analysis

Using fresh samples, moisture content was determined according to the Association of Official Analytical Chemists (AOAC, 2000) and expressed on a wet weight basis. However, other physicochemical parameters (pH, titrable acid, fat, fiber, ash, protein, and carbohydrate) of the samples were determined following the standard proximate analysis methods [14] and values expressed in dry weight basis. After milling into a fine powder, samples were sieved (0.425 mm sieve size), packed (with polyethylene bags), and stored until required for further analysis.

The pH, and titrable acid of the *Kocho* sample were measured according to the standard methods [14] mentioned above. The total (carbohydrate and protein) and crude (fat and fiber) content of the samples were also analyzed using standard methods [14]. To measure the ash content crucible glass without the sample was dried in an oven, cooled, and weighed. Then, 2 g of the sample was placed on crucible glass, dried in an oven (at 105 °*C*) for an hour and carbonized with the blue flame of a Bunsen burner until the contents changed to black. Then, the crucible was transferred to a Muffle furnace and further ignited at a temperature of 550 °*C*. The weight of the crucible with ash was measured using analytic balance and total ash values were expressed as percentages.

2.3.2. Microbial analysis

2.3.2.1. Culture-dependent analysis. A 10 g fermenting *Kocho* of Arkiya, Genna, and Maziya samples was taken aseptically from three

Table 1
Preference ranking of ten most frequently used enset.

Enset varieties (V)	Key informants (n = ~10)												
	HY	KQ	BQ	AQ	FT	EM	DR	D-R	FQ	SQ	OA	Total (%)	Rank
Genna[a]	8.3	10	8.2	8.3	7.2	8.9	2.6	3.0	6.3	8.4	8.5	**79.7**	1
Maziya[a]	7.0	8.6	2.2	5.4	5.0	3.3	10.0	9.8	10.0	5.1	9.7	**76.1**	2
Arkiya[a]	6.1	8.4	8.7	9.8	8.8	2.4	2.6	3.4	1.8	8.5	8.6	**69.1**	3
Yeka	2.5	8.9	9.2	8.9	9.2	3.7	3.4	2.3	3.6	8.8	7.9	68.4	4
Boza	9.7	2	1.5	2.1	5.7	9.9	1.8	9.1	8.6	5.5	8.2	64.1	5
Shododinya	7.1	5.5	4.1	2.1	1.5	2.0	8.6	7.8	8.4	5.6	6.8	59.5	6
Bargiya	5.5	3.5	6.6	5.7	3.7	6.0	6.8	4.4	6.3	3.2	6.4	58.1	7
Agena	1.6	6.5	6.6	6.8	6.2	5.3	6.1	2.6	5.3	6.3	3.8	57.1	8
Gia	3.6	4.8	4.1	2.4	1.8	7.7	8.5	8.0	2.9	2.6	5.6	52.0	9
Xella	3.5	3.9	3.7	4.5	6.2	4.5	4.8	5.6	2.7	3.1	1.9	44.4	10

[a] = Selected Varity, HY=High yield, QQ = *Kocho* quality, BQ= *Bulla* quality, AQ = *Amicho* quality, FT= Fermentation time, EM = Early maturity, DR = Disease resistance, D-R = Drought resistance, FQ= Fiber quality, SQ= Sensory quality, OA= Overall acceptance. Single value (given out of ten) in each cell of the Table indicates the average frequency number of ten respondents.

pits. Samples were homogenized with sterile water (90 mL) using a shaker (BSOR 104, Germany) adjusted at 250 revolutions per minute (rpm) for an hour [6,15,16]. Different selective media for specific microbe growth was initially prepared, and ten-fold serial dilution was done. Culturing of total viable bacterial counts on plate count agar (30 °*C* for 3 days), Enterobacteriaceae on violet red bile glucose medium (37 °*C* for 24 h), LAB on de Man Rogosa Sharpe medium (30 °*C* for 48 h), yeasts, and moulds on potato dextrose agar with 25 mg/L chloramphenicol (25 °*C* for 3–5 days) were used to isolate the microbes [17]. *Clostridium* endospores were also counted by transferring 100 μL aliquot of a sample taken from different (10^{-4} to 10^{-6}) dilutions to a heat shock treatment (15 min at 75 °*C*), followed by plating onto reinforced Clostridium agar (RCA) and anaerobic incubation at 37 °*C* for 24 h using anaerobic jars, gas generating kits and indicator strips as described elsewhere in a previous study [18]. All plates with 30–300 colonies were counted, and all microbial counts were expressed as shown in Eq [1].

$$\text{Microbial counts} = \frac{\text{CFU}}{\text{S}(mL)} \quad (1)$$

Where CFU is the colony forming unit, S is the sample added in mL.

Morphologically distinct colonies were picked and transferred to new media plates to obtain pure colonies for further characterization. A total of 68 isolates were obtained, and all the pure isolates were labeled and stored in the refrigerator for further analysis.

2.3.2.2. Culture-independent analysis. For metagenomic analysis, samples were taken on the initial day (1st day) and the 30th day from enset mash. Samples of Gamma were also collected for microbial consortia analysis. After the bacteria DNA was extracted with Nucleo Spin Soil Kit (Macherey-Nagel, Germany), its concentration was measured using a Qubit dsDNA BR Assay kit (Invitrogen, USA).

Details on the primers (forward and reverse) used, the amount of reaction volume, the polymerase chain reaction cycling conditions, and library construction were followed methods used by Guadie et al. [19]. After sequencing done by Illumina HiSeq platform (BGI, Shenzhen, China), raw data was pre-processed by removing adapters, and trimming low-quality reads. Sequences were also further subjected to chimera check by Uchime (v4240) to detect chimeric sequences generated by PCR amplification. Finally, at 97 % similarity index, operational taxonomic units (OTUs) using USEARCH (v7.0.1090) were constructed and compared with the Ribosomal Database Project, which is known as the most applied tool in taxonomic assignments that allow classification at the genus level.

2.4. Statistical analysis

Descriptive statistics was used to analyze interview and guided field walk data. The data was analyzed using SPSS software. The sample collection techniques were done under triplicate conditions and analysis results were expressed as mean and standard error. Tukey's hook test was used to determine multiple mean comparison tests and significance values were considered at $p \leq 0.05$.

3. Results and discussion

3.1. Indigenous knowledge and practice on enset preparation

According to information from the study area, enset, maize, teff, yam, and bean are the most commonly cultivated crops **(Table S1).** All of the respondents have enset farms in their corresponding gardens and other farmlands. Most (80 %) respondents also know more than ten enset varieties. In the study area, enset is utilized mainly for food (70 %), livestock feed/medicine (16.7 %), and socio-cultural and economic (13.3 %) purposes. According to respondents, they use previously fermented enset (called Gamma) for fermentation of enset (Table S1). They prepare it from the inner part of the pseudo-stem, cut it into pieces, the plant leaf (katiya), and enset midrib (*Qutsaruwa*) with red or yellow colored edge covered tightly for a long time. The mixture was added into the holes formed at the bottom part of enset after pulverization and covered tightly (Fig. S1). The mass was left for about a month to ferment and produce a flavor that was ready to use as a traditional starter, which is consistent with another study reported in Ethiopia [12].

Although >90 enset varieties are estimated to present in Dawro Zone, Buquniya, Shamara, Atuma Boza, Maca Boza, Agena, Aguntha, Hoiya, Goshindya, Tuzuma, Amya, Katanya, Maxaxiya, Suytya, Bargiya, Qartiya, Gia, Cica, Sharka, Koziya, Xelia, Yeqa, Locingya, Musa-arkiya, Maziya, Shododinya and Genna are the most familiar enset varieties. Table 1 describes the preference ranking of the ten most frequently used enset varieties based on perceived importance in the study area. The rating results (Table 1) were the average numbers given by ten selected key informants. Accordingly, Boza (9.7) and Genna (8.3) varieties have higher yields than others, while the Maziya (9.7) variety has the highest overall acceptance followed by Arikya (8.6) and Genna (8.5). Overall, the enset varieties Genna (79.7 %), Maziya (76.1 %), and Arkiya (69.1 %) were found to be the most frequently used and have greater overall acceptances in the study area (Table 1). Based on this preferential ranking result, the three enset varieties were selected for further fermentation evaluation purposes. These enset varieties were grown at a soil pH of 6.06 ± 0.05, organic matter content of 2.84 %, a conductivity of 3063.3 ± 15.27 μS/cm, and a moisture content of 75.6 %.

3.2. Physicochemical composition of Kocho

Different physicochemical properties considered in the current study have great potentials to enhance the quality of *Kocho* final product [20]. Moisture, pH, fat, ash, crude fiber, and total carbohydrate decreased, while TA and crude protein increased with increasing fermentation time (Table 2). There is a significant difference ($p < 0.05$) between various physicochemical parameters (moisture content, pH, TA, ash, fat, fiber, protein, and carbohydrate contents) and fermentation times within the same variety and among varieties (on the same day).

3.2.1. Titrable acidity and pH

The values of TA (0.21–0.46 %) and pH (7.20–4.15) from different day (day 1 to day 60) fermented enset variety samples have been found to an increasing and decreasing trends, respectively which could be due to organic acid (acetic acid, malic acid and lactic acid) production from fermenting material during fermentation [6,21].

3.2.2. Ash content

The ash content of fermented *Kocho* was found to be between 5.15 and 5.95 % at day 1 and 2.30–2.61 % at day 60. After one month of fermentation, the ash results were between 3.36 % and 3.78 %, which is similar to other studies [15,22] reported the same range of ash value (3.40–3.80 %). However, the report of Weldemichael et al. [23] indicated a very small amount of average ash content, which ranged from 0.98 to 1.06 %. This difference could be due to enset varieties used, fermentation time, traditional processing activities, and geographical variation of the study area. According to Yirmaga [22], the ash result is significantly affected by the fermentation time.

3.2.3. Carbohydrate, protein, fat, and fiber contents

As the fermentation period progresses (days 1–60), the carbohydrate content of the *Kocho* sample has significantly decreased (Table 2, Fig. 1a). For instance, Arkiya was reduced from 85.21 ± 0.36 % (day 1) to 68.12 ± 0.28 % (day 30) and 49.52 ± 0.37 % (day 60). Genna also reduced from 86.99 ± 0.38 % (day 1) to 66.17 ± 0.30 % (day 30) and 47.90 ± 0.18 % (day 60). Similar to the current result, Yimarga [22] also reported the lowest value (33.80 %) carbohydrate content at day 30, which was 44.08 % after 10 days of fermentation result. The reduction of carbohydrate content is most probably due to microbial enzymatic degradation and depolymerization of carbohydrates to their smaller forms as fermentation time extends.

As shown in Table 2 and Fig. 1b, the protein contents were found to vary between different varieties of enset, which shows increasing trends through fermentation time (days 1–60) in the order of Arkiya (0.83–1.09 %) > Genna (0.36–0.72 %) > Maziya (0.20–0.70). Although fermentation enhances protein content, the results still suggest that the lower content of protein in Genna and Maziya verities *Kocho* required complement with protein-rich foods such as meat, peas, and beans. The protein results identified in the current study are much smaller than the study carried out by others [9,10,22], but it is comparable to Bosha et al. [15].

The fat results identified in this study decreases as fermentation time increases from day 1 to day 60 (Fig. 1c); particularly the decline was relatively higher in Arkiya (6.22–2.43 %) than in Genna (4.87–2.31 %) and Maziya (5.04–1.91 %) varieties. In general, the fat-decreasing trends through fermentation time most probably due to the increasing activities of the lipolytic enzymes during fermentation that catalyze the triacylglycerol content of fat into fatty acid and glycerol [24]. This indicates that considering inherent parameters like incubation time, pH, and fermentation temperature are essential for enhanced *Kocho* fermentation.

Table 2
Physicochemical composition of *kocho*.

Enset variety	Time (day)	Physicochemical parameters (%, except for pH)							
		pH	TA	Moisture	Ash	Fat	Fiber	Protein	Carbohydrate
				WB	DB				
Arkiya	1	5.20 ± 0.15^{a}	0.26 ± 0.03^{a}	73.69 ± 0.25^{a}	5.18 ± 0.03^{a}	6.18 ± 0.04^{a}	3.82 ± 0.03^{a}	0.85 ± 0.02^{a}	85.21 ± 0.36^{a}
	15	4.81 ± 0.15^{b}	0.28 ± 0.03^{b}	60.73 ± 0.31^{b}	4.83 ± 0.15^{b}	6.17 ± 0.04^{b}	2.83 ± 0.02^{b}	1.05 ± 0.00^{b}	75.62 ± 0.30^{b}
	30	4.23 ± 0.15^{c}	0.29 ± 0.01^{c}	56.90 ± 0.21^{c}	3.72 ± 0.04^{c}	4.92 ± 0.05^{c}	2.14 ± 0.03^{c}	1.05 ± 0.00^{c}	68.12 ± 0.28^{c}
	60	4.14 ± 0.26^{d,a}	0.31 ± 0.04^{d}	41.47 ± 0.17^{d}	2.47 ± 0.04^{d}	2.86 ± 0.04^{d}	1.26 ± 0.02^{d}	1.09 ± 0.00^{d}	49.52 ± 0.37^{d}
Genna	1	7.19 ± 0.01^{a}	0.21 ± 0.00^{a}	74.92 ± 0.67^{a}	5.90 ± 0.04^{a}	4.84 ± 0.03^{a}	3.68 ± 0.03^{a}	0.38 ± 0.02^{a}	86.99 ± 0.38^{a}
	15	6.12 ± 0.01^{b}	0.28 ± 0.00^{b}	60.52 ± 0.43^{b}	5.20 ± 0.03^{b}	4.08 ± 0.03^{b}	2.76 ± 0.08^{b}	0.52 ± 0.01^{b}	72.21 ± 0.68^{b}
	30	4.49 ± 0.03^{c}	0.29 ± 0.00^{c}	56.52 ± 0.45^{c}	3.74 ± 0.06^{c}	3.94 ± 0.03^{c}	2.19 ± 0.03^{c}	0.65 ± 0.03^{c}	66.17 ± 0.30^{c}
	60	4.22 ± 0.04^{d,b}	0.31 ± 0.00^{d}	41.71 ± 0.59^{d}	2.57 ± 0.04^{d}	2.32 ± 0.01^{d}	1.25 ± 0.03^{d}	0.69 ± 0.03^{d}	47.90 ± 0.18^{d}
Maziya	1	5.14 ± 0.04^{a}	0.26 ± 0.00^{a}	72.75 ± 0.42^{a}	5.37 ± 0.04^{a}	5.02 ± 0.02^{a}	4.33 ± 0.03^{a}	0.21 ± 0.01^{a}	87.35 ± 0.39^{a}
	15	4.66 ± 0.04^{b}	0.28 ± 0.00^{b}	59.87 ± 0.27^{b}	4.96 ± 0.41^{b}	4.94 ± 0.04^{b}	3.25 ± 0.04^{b}	0.58 ± 0.02^{b}	73.59 ± 0.22^{b}
	30	4.48 ± 0.04^{c}	0.34 ± 0.00^{c}	57.44 ± 0.35^{c}	3.38 ± 0.02^{c}	3.34 ± 0.04^{c}	2.48 ± 0.02^{c}	0.63 ± 0.02^{c}	66.21 ± 0.56^{c}
	60	4.28 ± 0.03^{d,c}	0.43 ± 0.03^{d}	41.60 ± 0.49^{d}	2.31 ± 0.01^{d}	1.93 ± 0.02^{d}	1.25 ± 0.02^{d}	0.68 ± 0.02^{d}	47.12 ± 0.20^{d}

Values are Mean ± SD. ^{a,b,c,d} Different superscripts within the same column from the same accession indicate significant differences ($p < 0.05$). Same superscripts within the same column from different enset variety that have been sampled at the same day indicate insignificant differences ($p < 0.05$). TA = titrable acidity, WB = wet basis, DB = dry basis.

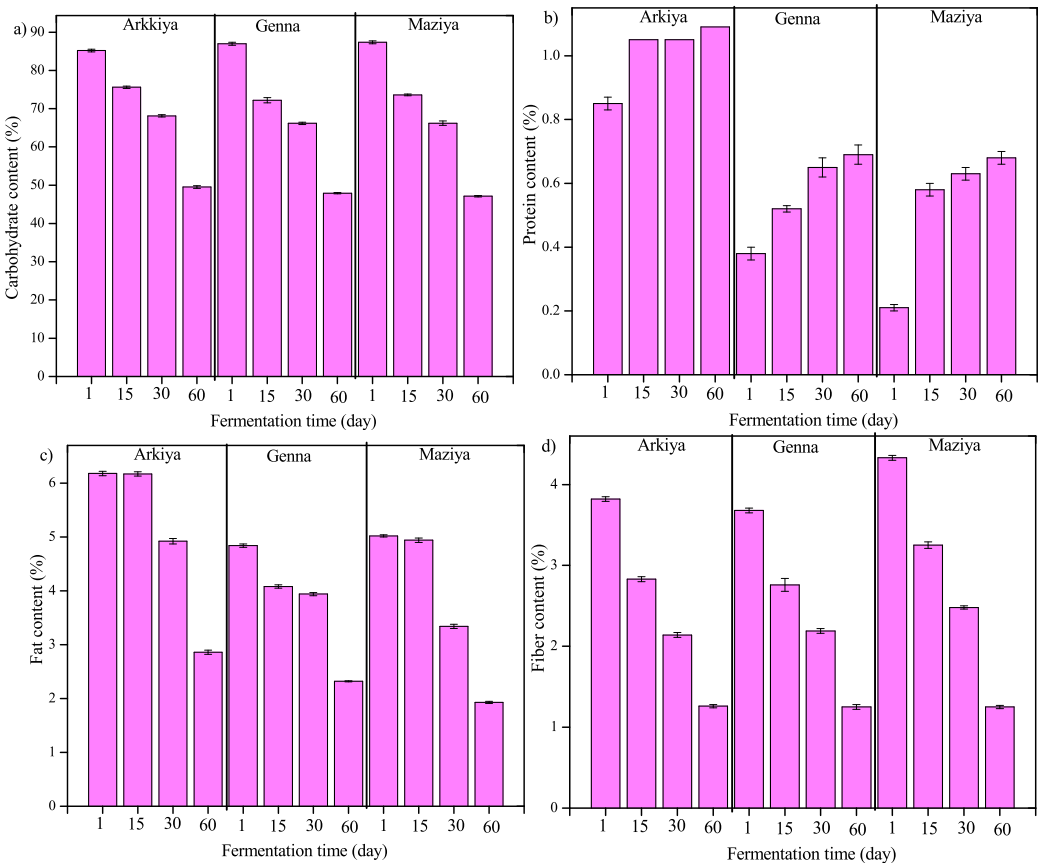

Fig. 1. Different nutrient content from different enset varieties at different fermentation times (a) carbohydrate, (b) protein, (c) fat, and (d) fiber.

As fermentation time elongated from day 1 to day 60, the fiber results of *Kocho* also progressively declined from 3.85 to 1.24 % for Arkiya, 3.71 to 1.22 % for Genna, and 4.36 to 1.23 % for Maziya (Table 2, Fig. 1d), which could be associated with fermenting microbes exist in the system. The microbes could be synthesized cellulase enzyme that solubilizes the cellulose, the structural material in the enset plants [24,25].

3.2.4. Moisture content

As shown in Table 2, the moisture contents had shown a decline in values for the three enset varieties when fermentation time was in progress [day 1 (72.33–75.61 %) > day 30 (56.95–60.14 %) > day 60 (41.30–42.30 %)]. Similarly, Andeta et al. [6] and Gashe [5] also observed a moisture content drop in *Kocho* samples from 78.32 to 83.01 % (three varieties) and 84 % at day 1–68.40–73.09 % and 60 % at day 60, respectively. According to Bekele [26], the moisture content of bulla sample was significantly varied with the four enset varieties (Yanbule, Gewada, Zereta, and Messina) investigated. The moisture results difference observed between varieties and fermentation periods could be associated with genetic varieties of the enset used for fermentation purposes, enset management at the farm, leakage, microbes using water for metabolic and growth activity, or/and high temperature created under anaerobic fermentation that leads evaporation [6,7,22]. Reducing moisture content is not favorable for the growth of some pathogenic organisms like Clostridia, is an advantage to be safe for consuming fermented *Kocho*.

3.3. Culture-dependent and culture-independent microbial analysis during enset fermentation

3.3.1. Effect of fermentation time and enset variety on culture-dependent microbial analysis

To figure out which microorganism was involved initially and showed increasing or decreasing changes during *Kocho* production, culture-dependent/independent analysis techniques were employed. As shown in Tables 2, 3, and S2, the load of microbes was changed with fermentation time, which is in line with other previous studies [6,27].

3.3.1.1. Lactic acid bacteria dynamics. There is a significant ($p = 0.004$) strong positive ($R^2 = 0.768$) correlation between titrable acidity and fermentation time. However, fermentation has a significantly strong negative correlation with pH ($R^2 = -0.715$, $p = 0.009$), moisture content ($R^2 = -0.982$, $p < 0.05$), ash ($R^2 = -0.932$, $p < 0.05$), fat ($R^2 = -0.861$, $p < 0.05$), fiber ($R^2 = -0.981$, $p <$

0.05) and carbohydrate ($R^2 = -0.994$, $p < 0.001$). The counts of LAB were significantly ($R^2 = -0.645$, $p = 0.023$) affected by fermentation time, while there was no statistical difference ($R^2 = 0.319$, $p = 0.312$) in their counts observed between enset varieties. The LAB count reached the highest level at day 30 for Arkiya (8.15 log cfu/g), Genna (8.06 log cfu/g), and Maziya (7.99 log cfu/g) varieties (Table 4). Such a steady and prominent growth of LAB throughout the fermentation time, reflects their ability to survive at very low pH ($R^2 = 0.382$, $p = 0.213$), and anaerobic condition created inside tightly packed fit [7]. As it was also reported in another study [28], the diversity of LAB, particularly *Lactobacillus* started to increase, while the pH of the Gray Sufu fermentation rapidly declines. Although LAB is insignificantly affected by pH drop, their steadily increasing relation with carbohydrate ($R^2 = 0.610$, $p = 0.035$) and ash ($R^2 = 0.665$, $p = 0.018$) content decrease through fermentation time is found to be significant and strongly positive.

As mentioned in the review of another study [29], the ingredients and herbs like "*Kutsaruwa*" and "*Katiya*" (their leaf) which were added to *Kocho* for flavor development may possess the characteristic flavors and antimicrobial activities that enhance the LAB count. Such plants play a prominent role in facilitating fermentation and creating an appropriate environment for the proliferation of LAB by stunting the growth of some food-borne pathogens and spoiler microorganisms. The simultaneous increase in LAB and yeasts' numbers throughout the fermentation period may be also attributed to their symbiotic association. In another study [30], it was reported that LAB produce and create an acidic environment conducive to yeast proliferation, while the yeasts provide vitamins and other growth factors such as amino acids for the LAB. The relative lower counts of LAB after two months than a month of fermentation were observed which could be due to nutrient (i.e., carbohydrate) depletion (Tables 2–4).

3.3.1.2. Yeast and mould dynamics. In this study, yeast and mould counts were increased significantly ($p < 0.05$) from days 1–15. Their counts were increased from 2.53, 2.60, and 2.85 log cfu/g on day 1–8.04, 8.09, and 7.99 log cfu/g on day 15 for Arkiya, Genna, and Maziya varieties, respectively (Table 4). This result is in line with Hunduma and Ashenafi [31] which have been reported <3 log cfu/g counts of yeast and mould at the initial period of fermentation and reached a maximum of 5 log cfu/g at 82 days. Attainment of maximum yeast and mould population after day 15 is presumably due to the regular mixing and aeration of *Kocho* mass in the study area. Some studies indicated the reduction and low number in yeast and mould count during *Kocho* production could result from the tightly packed and sealed pit [7]. These pits create anaerobic conditions by preventing enough oxygen availability and increasing the level of acidity inside the pit as fermentation time increases [5]. The other yeast and mould increasing reason most likely due to an increases in protein content, which has shown positive strong ($R^2 = 0.773$) significant ($p = 0.003$) associated with their counts during an extended time of enset fermentation (Tables 3 and 4).

3.3.1.3. Enterobacteriaceae dynamics. The counts of Enterobacteriaceae declined (7.04–7.8 to <1.00 log cfu/g) significantly ($p < 0.05$) as fermentation time elongated which is consistent with the results of the culture-independent analysis (Fig. 2). The decrease in Enterobacteriaceae count (Table 4) during fermentation of the varieties could be due to the domination of acid-producing microbes in the system [15,22]. A strong negative significant correlation was observed between Enterobacteriaceae and fermentation time ($R^2 = -0.984$, $p = 0.000$) and titrable acidity ($R^2 = -0.740$, $p = 0.006$), while the relation was significantly strong positive ($R^2 = 0.723–0.981$, $p \leq 0.008$) with pH, moisture, ash, fat, fiber and carbohydrate contents (Table 3).

The production of acidity by LAB during fermentation reduces the pH, creates unfavorable conditions for Enterobacteriaceae survival, and finally leads to their disappearance [22,32]. Natural fermentation helped in the elimination of Gram-negative, pathogenic rods and spoilers like Enterobacteriaceae, especially coliform, from the food, which is unable to proliferate at low pH value (pH < 4) [7,30]. A pH of 3.5–4.0 has been reported to inhibit Enterobacteriaceae and other Gram-negative bacteria [33].

3.3.1.4. Aerobic plate count dynamics. The aerobic plate counts increased from 7.04, 7.07, and 7.01 log cfu/g at the initial to 8.32, 8.29, and 8.22 log cfu/g at day 60 for Arkiya, Genna, and Maziya varieties, respectively. There is a significant strong positive correlation with fermentation time ($R^2 = 0.845$, $p = 0.001$) and titrable acidity ($R^2 = 0.681$, $p = 0.015$), and a negative ($R^2 = \geq$ -0.776, $p \leq 0.003$) association with pH, moisture, ash, fiber and carbohydrate content with aerobic plate counts (Table 3).

Table 3
The relation between various physicochemical parameters and different microbial counts.

		Fer-Tim	pH	TA	Moisture	Ash	Fat	Fiber	Protein	Carboh	En-Var
Fer-Tim	R^2	1.000	**−0.715**	**0.768**	**−0.982**	**−0.932**	**−0.861**	**−0.981**	0.521	**−0.994**	0.000
	P-value	NC	**0.009**	**0.004**	**0.000**	**0.000**	**0.000**	**0.000**	0.082	**0.000**	1.000
Enterobac	R^2	**−0.984**	**0.723**	**−0.740**	**0.960**	**0.914**	**0.867**	**0.981**	−0.460	**0.975**	−0.042
	P-value	**0.000**	**0.008**	**0.006**	**0.000**	**0.000**	**0.000**	**0.000**	0.133	**0.000**	0.897
YM	R^2	0.308	−0.421	0.379	−0.432	−0.252	−0.150	−0.464	**0.773**	−0.381	−0.053
	P-value	0.330	0.173	0.224	0.161	0.429	0.642	0.129	**0.003**	0.221	0.870
Clostrid	R^2	−0.159	−0.238	0.031	0.109	0.094	0.226	−0.030	0.453	0.122	−0.166
	P-value	0.622	0.457	0.924	0.736	0.771	0.479	0.926	0.139	0.706	0.607
LAB	R^2	**−0.645**	0.387	−0.369	0.575	**0.665**	0.468	0.524	−0.291	**0.610**	0.319
	P-value	**0.023**	0.213	0.237	0.050	**0.018**	0.125	0.080	0.359	**0.035**	0.312
APC	R^2	**0.845**	**−0.812**	**0.681**	**−0.776**	**−0.863**	**−0.744**	**−0.894**	0.447	**−0.815**	0.000
	P-value	**0.001**	**0.001**	**0.015**	**0.003**	**0.000**	**0.006**	**0.000**	0.145	**0.001**	1.000

Fer-Tim = Fermentation time, Enterobacteriaceae, YM=Yeast and mould, LAB=Lactic Acid Bacteria, APC = Aerobic plate count, TA = Titrable acidity, Carboh = Carbohydrate, EN-Var = Enset variety.

Table 4
Change in microbial count during *Kocho* production.

Enset variety	Microbes	Fermentation period (day) and count (log cfu/g)			
		1	15	30	60
Arkiya	Enterobacteriaceae	7.08	4.62	2.51	<1.00
	Yeast and mould	2.53	8.04	7.79	5.73
	Clostridium	5.87	6.89	8.29	5.23
	Lactic acid bacteria	7.29	7.88	8.15	6.03
	Aerobic plate count	7.04	7.21	7.93	8.32
Genna	Enterobacteriaceae	6.29	4.52	2.44	<1.00
	Yeast and mould	2.60	8.09	7.86	5.68
	Clostridium	5.35	6.73	8.06	5.17
	Lactic acid bacteria	7.61	7.95	8.10	6.81
	Aerobic plate count	7.07	7.14	7.98	8.29
Maziya	Enterobacteriaceae	6.20	4.51	2.59	<1.00
	Yeast and mould	2.85	7.99	7.88	5.81
	Clostridium	5.48	6.2	7.99	5.07
	Lactic acid bacteria	7.68	7.92	8.04	6.94
	Aerobic plate count	7.01	7.16	7.83	8.22

This result is in line with another study [31] that showed aerobic plate counts attained maximum count after day 20 of enset fermentation, and their proliferation increased with fermentation time. This study is also consistent with another finding [34] that evaluated the effects of adding starter cultures on sausage product quality which have increased aerobic bacteria counts as fermentation time extended and reached a maximum count (8 log cfu/g) at the end of the fermentation period. This signifies that the organisms were able to grow by utilizing the substrates.

3.3.1.5. Clostridium dynamics. The counts of *Clostridium* spores showed a significant difference ($p < 0.05$) through fermentation time (Table 4), which confirms the results obtained from culture-independent analysis (Fig. 2). On day 1, their counts were 5.87, 5.35, and 5.48 log cfu/g and reached maximum count on day 30 (8.29, 8.06, and 7.99 log cfu/g) for Arkiya, Genna, and Maziya varieties, respectively. This could be their tolerance to acidic environments. Solventogenic *Clostridium* organisms accumulate organic acids, butyrate, and acetate associated with lower pH during exponential growth. These are thought to trigger the sporulation process in the presence of excess nutrients [35].

Moreover, *Clostridium* species are known for producing the butyrous smell of *Kocho* because they convert lactic acid to butanoic acid at low pH [5]. In this study, the lowest count was attained on day 60 for the three varieties, which agrees with Andeta et al. [6] result observed in the fermented enset sample. The result is also consistent with the study of Gashe [5], which indicated that *Clostridium* and *Bacillus species* increased their count till the first two weeks of enset fermentation, but decreased thereafter.

3.3.2. Culture-independent microbial analysis

3.3.2.1. Microbial diversity analysis. The culture-independent results are shown in Table 5. A total of 388 OTUs were identified from all *Kocho* samples analyzed. In the current study, Gamma ($S_{ob} = 55232$) starter culture contained the richest microbial abundance than the three enset varieties ($S_{ob} = 36793–55456$). The richness of species observed (S_{ob}) also increased as fermentation time extended for the respective varieties (Table 5). Indeed, microbial community richness in a given sample has been reported differs due to fermentation time, plant varieties, and physicochemical dynamics [6,36], which is consistent with the current study. Specially, pH, moisture, and temperature were reported as the main environmental factors that can shape microbial abundance existence in a given ecology [37]. In this study, Chao1 and Shannon indices were also shown an increment with fermentation time (days 1–30). Except for Arkiya, microbial community diversity (Shannon index) decreased from day 1 to day 30 (Table 5), which could be related to the pH drop observed (7.12–4.46 for Genna, and 5.18 to 4.44 for Maziya at day 30) during an extended fermentation time (Table 2). This result is in agreement with another study [38] that observed the dynamics and diversity of microbial community succession during Suan Yu fermentation, indicating the predominance of many microbial strains drops as the pH value drop.

3.3.2.2. Microbial community structure analysis. At the phylum (Fig. 2a) level, the Gamma starter is almost dominated by three bacterial groups [Cyanobacteria (45 %) > Proteobacteria (33 %) > Firmicutes (21 %)]. Seven well-known bacterial phyla were also detected in the three enset varieties at the starting (first-day) and mid (30th day) fermentation time. Samples collected from the beginning of *Kocho* production were dominated by three phyla [Firmictus (39–68 %) > Proteobacteria (7–53 %) > Cyanobacteria (7–24 %)] (Fig. 2a). Although Firimictus is the dominant phylum in the three varieties at the initial fermentation day, it has decreased as fermentation time extended from the initial time to day 30 (68–19 % in Arkiya, 38 to 34 % Genna and 64 to 39 % in Maziya varieties). In contrast, Cyanobacteria (7–24 to 21–53 %), Bacteriodetes (0.3–2.3 to 6–29 %) and Actinobacter (0.3–6.0 to 3–11 %) communities were increased with increasing fermentation time (Fig. 2a). Except for the Genna variety (a decline from 52 to 6 %), Proteobacteria also showed an increment for Arkiya (7–18 %) and Maziya (9–12 %) varieties in the day 30 sample.

Consistent with our study, others [37,39] were also identified four bacterial phyla (Proteobacteria, Cyanobacteria Actinobacteria,

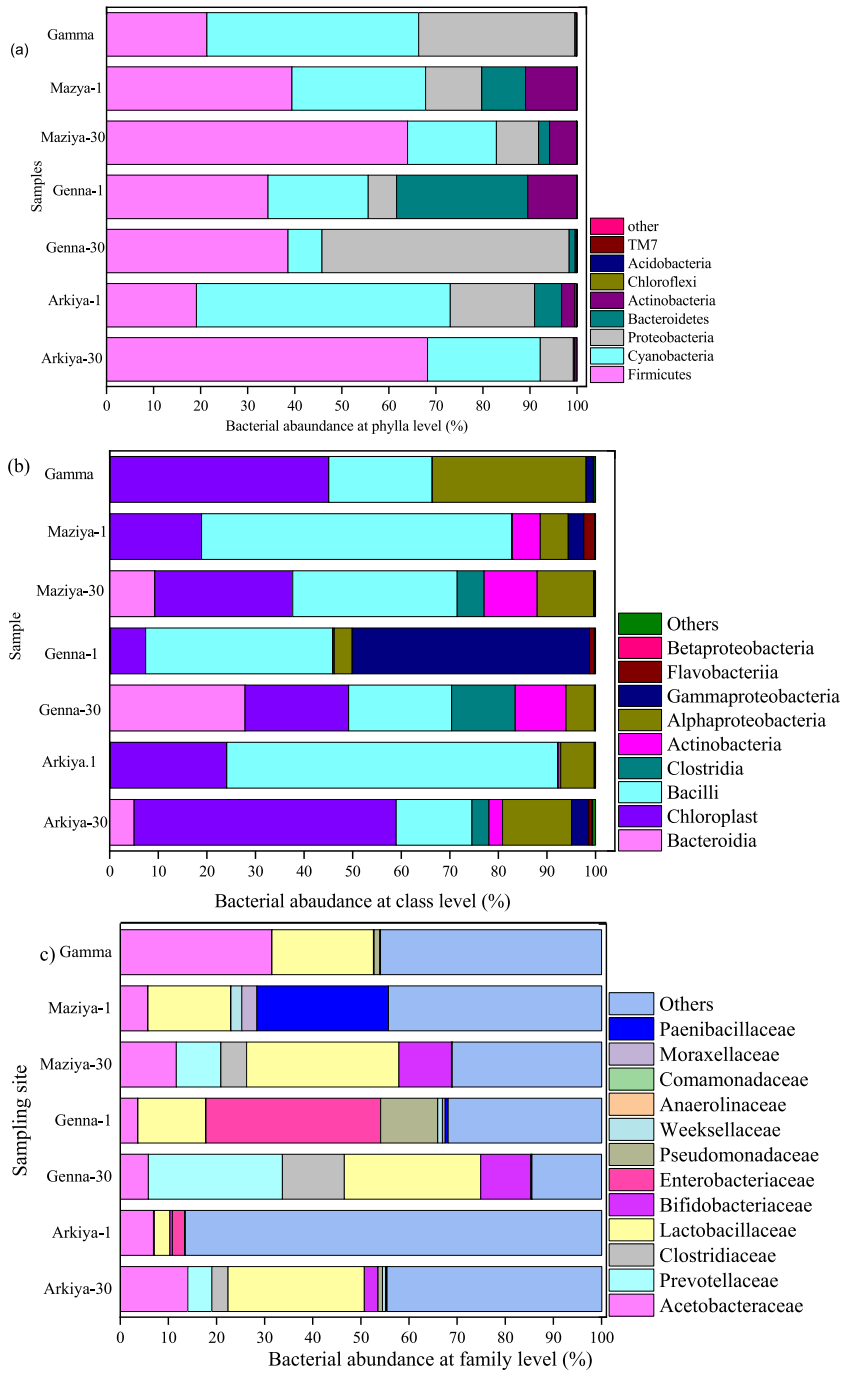

Fig. 2. Microbial community changes at different time with three enset varieties (a) phylum level, (b) class level, and (c) order level.

and Bacteriodetes) highly dominating plant tissue and fermenting mass. In our recent investigation [36] about enset bacterial wilt, we found four bacterial phyla (Firimictus > Cyanobacteria > Proteobacteria > Bacteriodetes) dominating the healthy enset, which is consistent with the initial fermentation time phyla detected (Fig. 2a).

At the class level (Fig. 2b), the relative abundance of the bacterial community also showed a visible variation between the initial fermentation day and the extended fermentation time of *Kocho* samples. In the Gamma sample, Chloroplast (45 %), Alphaproteobacteria (31 %), and Bacilli (21 %) were the dominant class of bacteria. Similarly, Chloroplast, Bacilli, Alphaproteobacteria, Actinobacteria, Gammaproteobacteria, and Betaproteobacteria were found to be the most relative abundance of bacterial communities identified in the three enset varieties. However, these bacteria classes were different from samples collected initially and after a month of fermentation time. For instance, the Chloroplast community increased and reached a maximum on day 30 in Arkiya (24–54 %),

Table 5
Bacterial alpha diversity for Gamma and various enset varieties through fermentation time.

Sample (enset varieties)	Diversity index			
	S_{Ob}	Chao1	Shannon	Coverage
Gamma[a]	55232	55344	1.658	0.9987
Maziya-1	47879	47920	2.325	0.9990
Maziya-30	50736	50786	2.314	0.9990
Genna-1	16164	16297	2.327	0.9975
Genna-30	53241	53305	2.251	0.9992
Arkiya-1	36793	36955	1.766	0.9997
Arkiya-30	55456	55464	1.816	0.9972

[a] = Traditional starter culture, S_{ob} = Species observed.

Genna (7–21 %), and Maziya (19–28 %) varieties. In the same manner, Alphaproteobacteria (4–7 to 6–14 %), Clostridia (0.06–0.10 to 4–13 %) and Actinobacteria (0.3–6.0 to 11.0 %) communities were increased as fermentation time increased in the three enset varieties. However, the community of Gammaproteobacteria significantly decreases from 48.0 to 0.1 % in Genna enset variety. Although the decline of Gammaproteobacteria is not larger, it has decreased from 3.20 to 0.06 % in the Maziya variety at the end of one-month fermentation period. This increasing or decreasing trend of the different classes may be related to the proximate composition of enset mainly due to pH, titrable acidity, and temperature, which showed significant changes during fermentation time. It also indicates that enset variety is one factor for bacterial community dynamics. This study is consistent with our recent study [36], which indicated that bacterial community shifts (zero to the third stage of enset disease) were related to soil and enset plant physicochemical composition. In another study [40], microbial community shifts were also observed during chili pepper fermentation, which is associated with physicochemical parameter changes like volatile metabolites.

At the genera level, the Gamma sample was dominated by *Acetobacter* (29 %) and *Lactobacillus* (21 %) bacterial communities, but it is free from *Clostridium*. *Clostridium* was more abundant in samples collected on day 30 than the initial fermentation day for all the three varieties. It was increased from 0.09 to 3.50 %, 0.1–5.50 %, and 0.06–13.00 % in Arkiya, Maziya, and Genna varieties, respectively. This is due to their higher spore counts when fermentation extends because spore formers reach fairly high numbers during the first 15 days of fermentation and show active growth in the fermenting enset [5]. *Clostridium* resists an acidic environment as their spore germinate to new cells or sporulate, and spore counts increase during enset fermentation [8]. As the genus *Clostridium* contains pathogenic species and 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 [5].

At the family level (Fig. 2c), Enterobacteriaceae showed a decreasing trend through fermentation time (very strong negative significant correlation; $R^2 = -0.984$, $p < 0.05$) in Arkiya (0.08–2.54 %), Genna (36.30–0.12 %), Maziya (36.30–0.03 %), which is supporting the culture-dependent analysis observed in Table 4. Enterobacteriaceae are associated with methylglyoxal detoxification and mixed acid fermentation pathways, which involve the production of lactate, acetate, succinate, and ethanol [16,21]. This bacterial family declining trend is most probably associated with the decreasing trends observed in pH, moisture, ash, fiber, and carbohydrate contents (Table 3). Opposite to Enterobacteriaceae, other groups of bacteria analyzed for culture-dependent techniques (Table 4) also showed an increasing trend at the family level (Fig. 2c). For instance, Clostridiaceae and Lactobacillaceae increased from 0.01 to 0.02 to 3.38–12.88 %, and 3.23–17.18 to 28.32–31.60 % in the three varieties, respectively.

The Gamma starter sample was dominated by *Lactobacillus plantrum* species followed by *Lactobacillus manihotivorans*, suggesting that these bacterial species are most probably responsible for *Kocho* production and could be the potential candidate to develop starter culture. Andeta et al. [6] also reported *Lactobacillus plantrum* and *Lactobacillus mesenteroides* as the dominant LAB in the initial phases of enset fermentation and suggested to be the potential candidate for developing starter culture. *Rahnella aquatilis* also one of the species identified in the Enterobacteriaceae family, which could play a significant role in enset fermentation. Indeed, *Rahnella aquatilis* is reported as a bacteria that efficiently metabolizes (ferments) carbohydrate-containing foods [41].

Our investigation on *Kocho* fermented from different enset varieties has limitations. The study lacks information on modern enset processing and storage materials that help to produce *Kocho* at a commercial scale. Gamma is prepared from the processed mixture of the fine-cut pieces of the inner pseudo-stem of enset and different ingredients, such as herbs, tree leaves, aromatic plants, and rotten enset leaf sheaths (i.e., quxaruwa), and the preparation is left a month to be fermented before use. However, the antimicrobial, preservative, and other roles of quxaruwa need further investigation. Developing a commercial and safe starter culture that increases the overall acceptance of *Kocho* and replaces the traditional (Gamma) starter culture was also lacking in this study. Despite these limitations, the current study provides information about the physicochemical and microbial community dynamics of *Kocho* fermented from different enset varieties in South West Ethiopia where data has not been previously published. Moreover, studying the microbial community from the Gamma sample through culture-independent techniques is also unique to this study to provide information about traditional starter culture.

4. Conclusions

This study revealed that an extended time of fermentation resulted in microbial and physicochemical parameter dynamics. Except

for protein and titrable acidity, other physicochemical parameters (pH, moisture ash, fat, fiber, and carbohydrates) were reduced through enset fermentation time in the three varieties. Except for Enterobacteriaceae, most other microbial (LAB, aerobic plate count, *Clostridium,* and yeast) counts were progressively increased with fermentation time increased, unless nutrition is a limiting factor. Although the culture-independent analysis did not considered day 60 sample like culture-dependent analysis, microbial diversity and community analysis results were found to be consistent with the culture-independent analysis results. Gamma (traditional starter culture), which is found to be free from *Clostridium*, is dominated by *Lactobacillus plantrum* and *Lactobacillus manihotivorans* most probably the two species that play a significant role in initiating the *Kocho* production process. These two bacterial species also increased through fermentation time in the three enset varieties indicates that *Lactobacillus plantrum* and *Lactobacillus manihotivorans* could be used as potential candidates to develop starter cultures for *Kocho* production.

Data availability statement

Data included in article/supplementary material/referenced in article.

CRediT authorship contribution statement

Melesse Tadesse: Writing – original draft, Methodology, Investigation, Formal analysis. **Fidelis Odedishemi Ajibade:** Writing – review & editing, Visualization, Validation. **Mengist Minale:** Writing – review & editing, Visualization, Validation, Data curation. **Addisu Mekonnen:** Writing – review & editing, Visualization, Validation. **Awoke Guadie:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Methodology, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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