

The Effects of Mandillo (*Crassocephalum Macropappum*) on the Physico-chemical Profile and Microbial Dynamics during Enset (*Ensete Ventricosum* Welw.) Fermentation

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Abstract

Fermentation of Enset has been known for a long time to produce a starchy and nutritionally enriched food product referred as *Kocho*. However, due to uncommon sensory attributes for non-Enset consuming societies, a short shelf life and a long fermentation period, *Kocho* has been ignored worldwide and limited to only some regions of Ethiopia. To improve its sensory attributes and enhance its fermentation rate, Shekacho society in Ethiopia uses the stem of *Mandillo* as a starter culture ingredient. Therefore, this study was initiated to determine the effects of *Mandillo* (*Crassocephalum macropappum*) on the microbial dynamics and physicochemical properties of Enset (*Ensete Ventricosum Welw.*) fermentation products. Microbial enumeration and isolation were carried out following standard methods on suitable culture media. Various fermentation parameters were determined analytically. Molecular identification of LAB and yeasts was based on 16S and 18S rRNA genes sequencing, respectively. In this study, significant ($p < 0.05$) differences were observed between control (Koki) and experimental *Kocho* (Kom) samples. The lactic acid bacterial (LAB) count increased by 23.3 folds on day 37 of fermentation of Enset with *Mandillo*. Similarly, the yeast count increased by 2.6 folds on day 29 of the fermentation period. On the final (45 day), lactic acid and acetic acid contents increased by 103.90% and 40.04%, respectively whose cumulative effect resulted in a lowering of pH by 0.65. The titratable acidity increased by 64.34%. The 16S rRNA gene sequence analysis assigned LAB to *Lactobacillus* spp. Accordingly, 82.14% strains identified as *Lactobacillus*, 9.82% isolates identified as *Leuconostoc*, and 8.04% isolates reported unidentified LAB strains in *Kocho*. Similarly, the major yeast strains were molecularly characterized as *Candida boidinii* (26%), *Wickerhamomyces* sp. UFLA (16%), *Candida* sp. MM 4018 (8%), but some yeast strains (28%) remained also an unidentified. The current findings revealed that *Mandillo* exhibited significant effect on the microbial dynamics of Enset fermentation with overall improvement of the final product.

1. Introduction

Fermentation is one of the oldest forms of food preservation technology in the world. Indigenous fermented foods such as bread, cheese, and wine have been prepared and consumed for thousands of years. The first fermented foods consumed were probably fermented fruits because of their abundance. There is reliable information that fermented drinks were being produced over 7000 years ago in Babylon (Iraq), 5000 years ago in Egypt, 4000 years ago in Mexico, and 3500 years ago in Sudan [1, 2]. Bread-making probably originated in Egypt over 3500 years ago [3]. Fermentation of milk started in many places, with evidence of fermented products in use in Babylon over 5000 years ago. There is also evidence of fermented meat products being produced for King Nebuchadnezzar of Babylon [4].

China is thought to be the birthplace of fermented vegetables and the use of *Aspergillus* and *Rhizopus* molds to make food [5]. In Africa, fermented cassava products like *Gari* and *Fufu* are the major components of the diets of more than 800 million people, and in some areas, these products constitute over 50% of the consumed food products [6, 7].

Food fermentation is a biochemical process that involves lactic acid fermentation to yield organic acids, alcohols, aldehydes, ketones, bacteriocins, and other metabolites in which lactic acid bacteria and yeasts play a major role [8]. According to Saeed et al. [8], there are many factors that affect the growth and metabolic activities of lactic acid bacteria and yeasts populations and their functions. Some of these factors are biochemical and physical environments such as temperature, pH, acidity, salt concentrations, and the nutritional profile of fermented foods, oxygen content, co-existing microorganisms, and food preservatives. Nutritional factors include sugars, peptides, free amino acids, minerals, and vitamins that may affect microbial dynamics [9].

Traditional fermented foods are important to preserve nutrients and provide diversified flavors, aromas, and textures that enrich the human diet [10]. Generally, fermentation improves taste, nutritional value, digestibility, and significantly lowers the content of anti-nutrients [11–13]. The other benefits of locally fermented foods are enhancement of organoleptic properties, provision of nutritional quality, detoxification, production of antibiotics, and ensuring food security [14]. Fermented foods have many other health benefits. For instance, the antimicrobial property of lactic acid bacteria in fermented herbs increases by about 80–170% compared to the crude herbal extract [15, 16]. The author further noted that herbals have selective inhibition potential and can selectively suppress common human pathogens like *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhi*, *Psuedomonas aeruginosa*, *Bacillus cereus*, *Bacillus subtilis*, *Klebsiella* species, and many others [17].

Among locally fermented Ethiopian foods, *Kocho* is a starchy and mineral-rich product obtained through a long fermentation period of Enset plant (*Ensete ventricosum* Welw.) [18, 19]. Due to its high product potential (300 quintals per hectare per year) on a dry weight basis [20], drought tolerance, and multipurpose values, Enset has been regarded as a priority food security crop in Ethiopia [21, 22]. For instance, regions where Enset is used as a staple food are usually less affected by the recurrent drought [23, 24]. Enset is also known by the name "tree against hunger" due to its extraordinary resourcefulness [25]. However, Enset cultivation and *Kocho* consumption are ignored worldwide activities that are only practiced in limited regions of Ethiopia. Out of the total populations of Ethiopia, *Kocho* is a staple food for about 15–20% in mixed subsistence farming systems [26–30].

The principal reasons for the decreased popularity of Enset are its uncommon and unacceptable sensory attributes for non-Enset-consuming people that encompass its short shelf life, its long fermentation period, and a lack of awareness [31]. Evidently, these defects can be improved through good fermentation practices and the use of natural product preservatives [32, 33].

Multiple lines of evidence have witnessed the common practice of using plant parts as medicine, drugs, and food sources [34–36]. These plants may also be added to food as spices and bio-preservative substances to improve flavor, odor, taste, and color to increase the safety, quality, and shelf life

of a product [37–39]. The components of such plants may possess characteristic flavors and antioxidant, anticancer, antitumor, and antimicrobial activities [33, 40, 41].

In Ethiopia, the Shekacho society is unique in applying *Mandillo* (*Crassocephalum macropappum*) plant as a starter culture ingredient to enhance Enset fermentation and to improve *Kocho* sensory attributes [42, 43, 44, 40]. Various investigations have been conducted concerning Enset in the areas of its cultivation, productivity [20], microbial dynamics of its fermentation [47], microbial spoilage and accompanying changes [48], food safety issues [49], biochemical changes during fermentation and the effect of altitude on microbial succession [50], chemical composition, and degradability in different morphological fractions [51]. Moreover, mineral content [52] and its absorption inhibitors [53], improving the indigenous processing of *Kocho* using different vernaculars of Enset [54], differences between pits and jars for the fermentation of *Kocho* produced [21], and the effect of *Mandillo* on nutrition and sensory attributes of *Kocho* [40] were partly investigated. However, to the best of our knowledge, there is no prior data on the effect of *Mandillo* addition during Enset fermentation processes on microbial dynamics, fermentation rate and quality of *Kocho*. Thus, the major purposes of this research were, therefore, to evaluate the significant differences between experimental and control groups of fermented *Kocho* in microbial dynamics and chemical changes as a result of the addition of *Mandillo* stems.

2. Materials and Methods

2.1. Kocho samples collection

Kocho samples (n = 11) with different fermentation periods were collected for analyses of microbial and other parameters. A total of 5 samples from Enset fermentation pits from 0–9 days, and 6 samples from jars within 11–45 days were collected. Each day analysis was conducted following standard methods [53, 54].

The fermentation process for microbial analysis was conducted in two batches. Each batch was designated as initiation period (Ko), control (Koki), and experimental (Kom) group. All the three groups were done in triplicate. Initiation period group (Ko) was fermented without starter cultures. The starter culture of the control group (Koki) was without *Mandillo* stem. *Mandillo* stems were added to the starter culture of the experimental group (Kom) [55].

2.2. Enset fermentation processes

Fermentation took place in three batches including a preliminary observation. Batch I was planned for qualitative analysis of indigenous knowledge of Shekacho society in Enset fermentation practice and to evaluate the effect of *Mandillo* qualitatively [56, 57]. Batch II and III were to evaluate both quantitative and qualitative outcomes in chemical profiles and, microbial dynamics as a result of added *Mandillo* stems. All the partially fermented (Ko), control (Koki), and experimental *Kocho* (Kom) samples were analyzed [56]. In all batches, fermentation was initiated at Masha town from days 0–9 in pits (Ko) until the starter culture was ready on the 9th day. The starter cultures were added to the partially fermented *Kocho* samples on the 11th day. Fermentation from days 11–45 of the Koki and Kom sample groups took place in fermentation jars sealed with polyethylene bags at the Food Microbiology Laboratory of the Center for Food Science and Nutrition, Addis Ababa University, Ethiopia. The fermentation processes took place at room temperature in the research Laboratory (14–18°C). *Kocho* samples for microbial and changes in pH, total titratable acidity, lactic acid, and acetic acid analyses were collected from the fermentation pits and jars on days 0, 3, 5, 7, 9, 11, 15, 21, 29, 37, and 45. The samples were kept at 2–4°C for further analysis [58].

2.3. pH Measurement

The pH of the fermenting *Kocho* was determined using a SensION MM 150 pH meter (MACH Switzerland) with a reference glass electrode. The pH meter was calibrated prior to each reading with standard buffers of pH 4.00, 7.00, and 10.00 (Oyediji et al., 2013).

2.4. Titratable Acidity (TTA) Analysis

The TTA was determined by using the supernatant of 10 g sample dissolved in 90 mL of distilled water and titrated against 0.1 N NaOH to the phenolphthalein end point. The relative lactic acid equivalent is the amount of NaOH consumed in mL. Each milliliter of 1N NaOH is equivalent to 9.008 mg of lactic acid [59, 60].

$$\% \text{ Acidity} = \frac{(\text{mL of NaOH} \times 0.009) \times 100}{\text{Sample weight in g}}$$

2.5. Lactic Acid and Acetic acid HPLC Analysis

Organic acids produced during *Kocho* fermentation from days 0, 3, 5, 7, 9, 11, 15, 21, 29, 37, and 45 were extracted as described by Sanchez-Machado et al. [61]. A 10 g sample was dissolved in 90 mL of deionized water. The content was homogenized and heated in a water bath at 65°C for 5 min to prevent the lactic acid bacteria from continuing the fermentation process during the analyses. The samples were vortexed for 1 min and neutralized to pH 7 by 0.1 N NaOH. The tubes were placed in ultrasonic bath for 5 min to facilitate the extraction of the organic acids. The homogenates were centrifuged at 4000 rpm for 10 min using a low-speed centrifuge. The acid-containing supernatants were filtered through cellulose acetate membrane with a pore size of 0.45 µm (Millipore HAWP 02500), and 1.5 mL of each filtrate was filled into vials in triplicate. A 20 µL

was injected into the HPLC system (Shimadzu, Kyoto, Japan) column (Aminex HPX-87H, 300 x 7.8 mm, Bio-Rad Laboratories, Richmond, USA, UV detector) at 14°C, mobile phase 0.005N H₂SO₄, at 210 nm. Standard curves were prepared from pure and known concentrations of a mixture of acetic and lactic acids (0.10 mg/L, 0.25 mg/L, 0.50 mg/L, 0.75 mg/L, and 1.00 mg/L). A 1.00 mg/L single standards' solutions were run to know the retention time of the respective acids.

2.6. Enumeration of total aerobic mesophiles, endospore formers, coliforms and staphylococci

A 10 g sample from each group of *Kocho* (Ko, Koki and Kom) was aseptically removed from the pits and jars and homogenized with 90 mL of sterile distilled water using an orbital shaker (SSL1) at 250 rpm for 30 min. A tenfold serial dilution was made. From the appropriate dilution factor, 0.1 ml of each aliquot was aseptically taken and spread plated on respective pre-dried agar media (de Man, Rogosa, and Sharpe (MRS) agar, potato dextrose agar (PDA), plate count agar, violet red bile agar and mannitol salt agar) for LAB, yeasts, total aerobic mesophiles/spore formers, coliforms, and staphylococci, respectively [62]. Aerobic spore formers were counted on plate count (PC) agar after appropriate dilution was heat-treated at 80°C for 10 min in a water bath, and spread-plated. A 50 mg/L of Chloramphenicol (Sigma) was added to PDA to inhibit bacterial growth. Inoculated plates were incubated at 30°C for 24–48 h. LAB-inoculated plates were incubated under anaerobic conditions in GasPak jars (GasPak System, BBL) at 30°C for 24–48 h. PDA-inoculated plates were aerobically incubated at 28°C for 24–72 h [63].

2.7. Isolation and Identification of LAB and Yeasts

Ten to twenty presumptive colonies of LAB and yeasts were randomly picked from MRS agar and PDA (Fig. 1A and B), respectively for isolation of the fermentative microbes. The isolated microbes were purified on their respective culture media by repeatedly streaking on appropriate solid media. For presumptive identification of lactic acid bacteria and yeasts, overnight cultures of 340 LAB and 340 yeast isolates in MRS and potato dextrose broth media were used, respectively [9, 64]. All the LAB isolates were subjected to different morphological, physiological, and biochemical tests to be identified to the genus level. Only gram-positive (gram staining and 3% KOH) bacteria with catalase-negative reactions (3% H₂O₂), none motile (on MRS agar medium) [65–67], and that produced acid from glucose (in MRS broth) were purified by successive streaking on MRS agar media and maintained [68, 67]. These preliminary tests enabled to classify the isolates (n = 288) to genus level following established protocols in the Bergey's manual.

The pure isolates were sub-cultured every month on respective agar slants and maintained at 2–4°C until use [64]. A total of 288 isolates of LAB with different shapes (rods and cocci) were tested for tolerances to various temperatures (5°C, 10°C, 15°C, 37°C, and 45°C), NaCl % (2, 4, 6.5, and 8 w/v) and pH values (3.3, 3.9, 4.4, 6.5, and 9.6 [69, 70]. The LAB isolates further classified as hetero and homofermentative in MRS broth using inverted Durham's tubes. A total of 155 yeast isolates were separated from molds [71]. The presumptive identification of LAB and yeasts was confirmed by 16S rRNA and 18S rRNA genes sequencing analyses of representative microbes of 120 LAB and 50 yeast isolates, respectively [72, 73].

2.8. Molecular Identification of LAB and Yeasts

2.8.1. Genomic DNA Extraction

A. Lactic acid bacteria

From 288 LAB isolates, only 120 of them were selected systematically and randomly [65] by focusing their fermentative features. Generally, all the isolates with a low population size that looked unique were considered for the genomic DNA extraction. In addition, the most dominant isolates were randomly picked proportional to their population to represent the group without disregarding their potential for fermentation. Overnight-grown LAB were used for genomic DNA extraction. Accordingly, a 100 µL lysine buffer was added to 1.5 mL Eppendorf tube. A 100 mg/mL of 2.0 µL of lysozyme and 20 mg/mL of 2.0 µL of proteinase K were added. Loopful LAB colonies on a plate were taken for each LAB isolate and separately added to the buffer solution in Eppendorf tube and vortexed. The suspension was heated in a water bath at 65°C for 30 min and then boiled at 100°C for 10 min. The tube was centrifuged at 13,000 rpm for 10 min. The supernatants of each isolate were recovered, checked for quality and maintained at -20°C for further use. A 3.0 µL of the template DNA was used for the PCR run [74].

B. Yeast isolates

Out of 155 yeast isolates, 50 of them were selected systematically and randomly [74] by focusing on their fermentative abilities. All rare and unique isolates with excellent fermentative features were considered. Furthermore, dominant and fermentative groups were randomly picked proportional to their population to represent the group for DNA extraction [76]. Overnight-grown pure yeast colonies were used for molecular methods. A 100 µL lysine buffer was added to a 1.5 mL Eppendorf tube. A 2.5 unit/mL of 1.0 µL lyticase and 20 mg/mL of 2.0 µL proteinase K were added. A loopful of yeast colonies on a PDA plate were removed and added to the buffer solution. The suspension was vortexed and heated in a water bath at 65°C for 30 min. Thereafter, it was boiled at 100°C for 10 min. The suspension of each isolate was separately centrifuged at 13000 rpm for 10 min. The quality of the DNA was checked and stored at -20°C for subsequent activities. A 3.0 µL of the supernatant was used for the PCR run [76].

2.8.2. Polymerase Chain Reaction (PCR) Processes of DNA and Amplicon Sequencing of LAB and Yeast

A 3.0 µL of genomic DNA of LAB and yeast isolates were used for PCR processing. PCR was run using SolGent's PCR series (Korea) [77]. PCR ingredients included 3.0 µL of DNA sample, 1.0 µL of 10 mole of each forward primer 27F (5'AGA GTT TGA TCC TGG CTC AG3') and reverse primer 1492R (5'GGT TAC CTT GTT ACG ACT T3') for LAB and forward primer ITS1 (5'TCC GTA GGT GAA CCT GCG G3') and reverse primer ITS4 (5'TCC TCC GCT TAT TGA TAT GC3') for yeast [78], and a 12.5 µL of SolgTM 2X EF-Taq PCR Smart Mix 1 (Cat. No. SEF01-M50h). RNase free sterile distilled water filled to 25 µL of total PCR volume. PCR was performed under the following conditions: preheating was done at 95°C for 15 min for 1 cycle, denaturation at 95°C for 20 s, annealing at 55°C for 40 s and extension at 72°C for 15 min run for 35 cycles. The 16S/18S rRNA gene was amplified with the universal bacterial and fungal primer pairs [79]. The purified PCR products (amplicon) were sequenced according to the protocol of the company, SolGent Co. Ltd (Daejeon, Korea) [80].

2.8.3. Phylogenetic analysis of LAB and Yeast isolates

The 16S (LAB) and 18S (yeasts) rRNA, gene sequences were first clustered by BLAST comparison [81, 82] and sorting identical strains from each phylogenetic tree of highly fermentative 11 LAB and 8 yeast isolates. A fourth method of clustering, Bayesian rRNA Classifier Version 2.10 software at confidence threshold of 80% [83, 84] was used. Related sequences were obtained and aligned by Pairwise sequence alignments in MEGA11 program using ClustalW algorithm and phylogenetic tree was constructed by the neighbour-joining method program available online [85, 86]. A total of 500 bootstrapped values were sampled to determine a measure of support for each node on the consensus tree [87–89].

2.9. Data analysis

Statistical analysis in all cases was performed in triplicate, and the values were averaged over the three measurements. The standard deviation was also calculated using IBM/SPSS/Statistics/20, by means of one way ANOVA followed by Duncan tests and MEGA 11 bioinformatics software was used for molecular analysis. The significance of differences was defined at the $p < 0.05$ level.

3. Results and Discussion

3.1. Evaluation of some fermentation parameters

As shown in Table 1, the effect of *Mandillo* on Enset fermentation processes was clearly observed between the experimental (Kom) and control (Koki) *Kocho* groups. In experimental *Kocho* samples (Kom), the pH linearly decreased to less than 4.6, while TA increased as previous verified by several investigators [90, 91, 56, 92]. On the final day of *Kocho* fermentation, lactic acid and acetic acid contents increased by 103.90% and 40.04%, respectively, this resulted in a lowering of pH by 0.65 with total TA increment by 64.34% [55].

These results highlight the potential role of *Mandillo* in influencing the fermentation process of Enset and its impact on the production of lactic acid and acetic acid. The acidity parameters (total TA, lactic acid, and acetic acid) are strongly related and responsible for the safety, tartness, and shelf-life extension of *Kocho*, as food is said to be microbially safe when the pH is less than 4.4, where most food-borne spoilers and pathogenic microorganisms cannot survive or are totally inhibited [93, 90].

Table 1
pH, titratable acidity (TA), lactic acid (LA) and acetic acid (AA) in the experimental *Kocho* samples with *Mandillo* (Kom) and control *Kocho* samples without *Mandillo* (Koki) during *Enset* plant fermentation

Sample type	Fermentation day	pH	TA	LA (mg/100g)	AA (mg/100g)
<i>Control group (Koki)</i>	11	4.57±0.05	3.95±0.55	255.53±0.03	28.57±0.03
	15	4.59±0.05	6.15±0.15	195.85±0.07	53.04±0.07
	21	4.61±0.01	6.65±0.55	312.82±0.07	98.48±0.09
	29	4.67±0.01	6.80±0.60	245.52±0.06	100.64±0.02
	37	4.75±0.04	7.15±0.35	205.43±0.01	121.30±0.08
	45	4.49±0.02	7.95±0.45	215.55±0.08	175.16±0.05
<i>Experimental group (Kom)</i>	11	4.95±0.19	1.85±0.05	308.47±0.09	94.74±0.01
	15	4.72±0.07	3.20±0.30	264.43±0.05	80.55±0.02
	21	4.43±0.01	7.85±0.65	366.06±0.03	126.20±0.01
	29	4.25±0.04	10.70±0.50	378.92±0.09	143.53±0.08
	37	4.18±0.08	11.77±0.95	418.32±0.05	169.87±0.02
	45	4.10±0.02	9.90±0.40	399.26±0.05	207.82±0.01

3.2. Microbial Enumeration

The other point observed in the present finding was that the food-borne spoiler and pathogenic microbial populations, i.e., total aerobic plate count, spore formers, coliforms, and staphylococci significantly ($p < 0.05$) decreased. Moreover, *Staphylococcus aureus* was not identified throughout the fermentation period in both cases [56, 68]. The case of staphylococci could not be justified as only the effect of added *Mandillo* since the fermentation environment may not be convenient to the growth of pathogens/spoilage microbes [64, 18, 56, 94].

In the experimental group, during the final fermentation day, no growth of coliforms detected and spore formers were also rarely encountered [68]. However, all the results observed in Table 2 promote the safety, long shelf life, and overall improved quality of the experimental *Kocho* (Kom), with a significant ($p < 0.05$) difference compared to the control *Kocho* (Koki).

The addition of *Mandillo* during *Enset* fermentation resulted in a significant reduction of total aerobic bacteria, with some lowered by 50 times in total aerobic bacteria and no growth in coliforms [95]. These similar phenomena were also reported by Gashe [56] and Idris et al [68]. However, LAB and yeast populations increased as fermentation days extended [56] due to addition of *Mandillo*. This in turn enhances fermentation rate and may produce alcohol, acids, and other fermentation byproducts like bacteriocin, which are responsible for the increment of shelf-life, food safety, and improved organoleptic qualities of the fermented *Kocho* [96].

Table 2

Total aerobic plate counts, spore formers, coliforms and staphylococci in the experimental *Kocho* samples with *Mandillo* (Kom) and control *Kocho* samples without *Mandillo* (Koki) during *Enset* plant fermentation (Unit : CFU/ml)

Sample type	Fermentation day	Aerobic plate count	Spore formers	Coliforms	Staphylococci
Control <i>Kocho</i> Samples (Koki)	11	$2.80 \pm 0.1 \times 10^4$	$5.05 \pm 0.1 \times 10^2$	$1.80 \pm 0.0 \times 10^4$	ND
	15	$3.00 \pm 10.0 \times 10^6$	$4.90 \pm 0.1 \times 10^2$	$1.20 \pm 0.0 \times 10^4$	ND
	21	$2.75 \pm 5.0 \times 10^6$	$3.90 \pm 0.1 \times 10^2$	$4.50 \pm 0.0 \times 10^3$	ND
	29	$3.65 \pm 5.0 \times 10^6$	$7.35 \pm 0.4 \times 10^2$	$1.20 \pm 0.0 \times 10^3$	ND
	37	$5.7 \pm 1.0 \times 10^5$	$2.60 \pm 0.1 \times 10^2$	$1.01 \pm 0.0 \times 10^3$	ND
	45	$1.26 \pm 0.1 \times 10^5$	$2.30 \pm 0.0 \times 10^1$	$1.6 \pm 0.0 \times 10^1$	ND
Experimental <i>Kocho</i> Samples (Kom)	11	$3.30 \pm 0.1 \times 10^4$	$2.90 \pm 0.0 \times 10^1$	$2.50 \pm 0.0 \times 10^4$	ND
	15	$2.05 \pm 0.5 \times 10^5$	$2.9 \pm 0.01 \times 10^1$	$7.20 \pm 0.0 \times 10^3$	ND
	21	$3.55 \pm 0.5 \times 10^5$	$5.9 \pm 0.01 \times 10^1$	$2.60 \pm 0.0 \times 10^3$	ND
	29	$6.60 \pm 1.0 \times 10^5$	$7.7 \pm 0.03 \times 10^1$	$5.9 \pm 0.0 \times 10^2$	ND
	37	$2.38 \pm 0.1 \times 10^4$	$3.1 \pm 0.01 \times 10^1$	$2.6 \pm 0.0 \times 10^2$	ND
	45	$2.5 \pm 0.01 \times 10^3$	$4.00 \pm 0.1 \times 10^0$	ND	ND
Legend: ND = Not detected					

There were also significant ($p < 0.05$) differences between control and experimental *Kocho* samples on day 21 of fermentation of Enset with *Mandillo*, LAB count increased by 23.3 folds, and similarly, yeast count increased by 2.6 folds on day 29 of the fermentation (Table 3). On the final fermentation day, as the pH value lowered the population of yeast decreased by a factor of one tenth due to less pH tolerance (Tables 3). When compared with other findings, the population of yeast (this study) was not high. This might be linked to an anaerobic environment where the fermentation took place in jars that discourages the growth of yeast [56, 21, 97, 48].

In the meantime, the minimum number of yeast and maximum number of LAB populations, specifically from the homo-fermentative species, have large contributions to the maximum production of lactic acid. This is because the yeast group of microorganisms are responsible for favoring the production of alcohol, which may be changed to acetic acid with the help of acetobacter in an aerobic environment [98, 18]. The high ratio of homo-fermentative to hetero-fermentative lactic acid bacteria indicates the reason why lactic acid production is higher in experimental *Kocho* samples than that of the controls [99]. This difference is rational when considering that the fermentation environment is an anaerobic condition (in jars) that favors facultative anaerobic LAB such as *Lactobacillus plantarum*. Considering the comparatively more anaerobic environment than the previous Enset fermentation processes by other researchers, it might be concluded that the conversion of alcohol to acetic acid by acetobacter could be very low [56, 21, 48].

The concentration of lactic acid in the experimental *Kocho* samples was also significantly greater than that in the control *Kocho* samples that coincided with the abundance of LAB species in the experimental *Kocho* samples. In addition, the decrease in non-LAB communities during fermentation courses creates less competition for nutrients among LAB and other microorganisms to favour LAB multiplication [100].

Table 3

Lactic acid bacteria (LAB) and yeast population in the experimental *Kocho* samples with *Mandillo* (Kom) and control *Kocho* samples without *Mandillo* (Koki) during *Enset* plant fermentation

Day of fermentation	LAB population		Ratio of LAB (Kom: Koki)	Yeast population		Ratio of Yeast (Kom: Koki)
	Kom (CFU/ml)	Koki (CFU/ml)		Kom (CFU/ml)	Koki (CFU/ml)	
11	3.46± 0.06×10 ⁷	2.41± 0.01×10 ⁶	14.7:1	1.97± 0.14×10 ⁵	1.15± 0.05×10 ³	1.7:1
15	5.44± 0.09×10 ⁷	8.59± 0.05×10 ⁶	6.6:1	4.88± 0.35×10 ⁵	2.88±0.5×10 ⁵	1.7:1
21	2.83± 0.13×10 ⁸	1.16± 0.00×10 ⁷	23.3:1	9.77±0.3×10 ⁵	7.85± 0.25×10 ⁵	1.2:1
29	8.48± 0.47×10 ⁸	1.16± 0.77×10 ⁸	7.4:1	6.85± 0.35×10 ⁵	2.78± 0.16×10 ⁵	2.6:1
37	9.30± 0.30×10 ⁸	7.55± 0.13×10 ⁸	1.2:1	2.26± 0.01×10 ³	1.59± 0.23×10 ⁵	0.01:1
45	7.40± 0.29×10 ⁸	6.01± 0.12×10 ⁸	1.2:1	1.35± 0.15×10 ³	1.38± 0.75×10 ⁵	0.01:1

Even though this part needs further research work to optimize fermentation parameters and the potential application of *Mandillo* in other food fermentation processes, it may be recommended to use *Mandillo* as a starter culture ingredient in both lactic acid and acetic acid production in the food and beverage industries [101, 102].

3.3. Isolation and Characterization of LAB and Yeast

A total of 340 LAB and 340 yeast colonies were isolated from MRS agar and PDA media, respectively. The colonies of LAB grown were mostly white, grayish, creamy, flat, and spherical, with entire margins (Fig. 1A). LAB isolates were also subjected to different morphological, physiological, and biochemical tests [21, 103], and 288 isolates were presumptively identified as LAB (Table 4). All the 288 LAB isolates were gram-positive, catalase-negative, non-spore formers, non-motile, and acid producers from glucose.

The results suggest that the entire LAB belonged to two genera. Among the 288 LAB, 76 isolates reported as cocci and 212 of them as rods. In addition, 208 isolates produced gas from glucose, which can be discussed from the facultative nature of the dominant isolate, *Lactobacillus plantarum* (67.57%) that showed both hetero- and homo-fermentative characteristics in glucose fermentation processes that is in agreement with the report of Siezen and Hylckama [103]. A total of 80 isolates did not produce gas from glucose and were considered strictly homo-fermentative LAB similar to the investigation of Liu and Dong [105]. Most of these anaerobic LAB isolates were found in the experimental *Kocho* samples only (Table 3) [106]. This result also has a positive correlation to the high concentration of lactic acid production in the experimental *Kocho* samples (Table 1) similar to the report made by Siezen and Hylckama [103].

The yeast colonies were white and creamy in color with budding, smooth surfaces, and bipolar (Fig. 1B). Among the isolates, only 45.59% were confirmed as yeasts.

Table 4

Physico-chemical characterization of LAB isolates of initiation period (Ko), control *Kocho* samples without *Mandillo* (Koki) and experimental *Kocho* samples with *Mandillo* (Kom) during *Enset* fermentation

Samples with <i>M. luteus</i> (R01) during Enset fermentation													
No of isolates	Morphology		Gas production from sugar		Growth temperature				Catalase test	Gram's reaction	KOH string test	Spore forming test	Motility test
288 (100%) LAB	212 (73.61%)	76 (26.39%)	208 (72.22%)	80 (28.78%)	70 (24.51%)		218 (75.69%)		-	+			
					15°C	45°C	15°C	45°C					
:	Rod	Cocci	+		+	-	+	±					

3.4. Molecular Identification of LAB and Yeast Based on 16S and 18S rRNA genes Sequences

The 16S rRNA gene sequence and phylogenetic tree analysis categorized all of the 112 LAB strains into clusters of 4 different species and 1 group, of which 92 (82.14%) strains identified as *Lactobacillus*, 11 (9.82%) isolates identified as *Leuconostoc*, and 9 (8.04%) isolates as unidentified LAB strains (Fig. 2). This finding, except the unidentified LAB strains is also consistent with other previous investigations that showed the dominance of *Leuconostoc mesenteroides* followed by *Lactobacillus* species in the natural fermentation processes of Enset [48, 56].

Compared with GenBank database, the nucleotide sequences of 16S rRNA and 18S rRNA gene identified the potential LAB and yeasts into species level, according to levels of homology of nucleotide ranging from 99–100% similarity indices for both LAB and yeasts. The bacterial and yeast isolates from this study were designated (Fig. 2 and Fig. 3), respectively. This finding suggests that in both the LAB and yeasts, some of sequences obtained revealed low similarities to sequences in Genebank and suggest novel species to present.

In the present study, from the dominant species isolated as LAB (n = 112 strains), identified from minimum population 4 (3.57) to maximum 78 (69.64%). The microorganisms further classified as 11 (9.82%) *Leuconostoc mesenteroides* strains found only in initiation period, specifically, *Lactobacillus* species as 78 (69.64%) isolates identified with different *Lactobacillus plantarum* strains, 10 (8.93%) isolates identified with *Lactobacillus casei* and *paracasei* strains, and 4 (3.57%) isolates identified with *Lactobacillus brevis* strain, however, the remaining 9 (8.04%) isolates were not identified.

Among these species of LAB, *Leuconostoc mesenteroides* was reported to initiate fermentation and lactic acid production during the early stage of Enset fermentation [56]. During the initiation period, all of the isolates of genus *Leuconostoc* were hetero-fermentative LAB, which produced gas from glucose and created an anaerobic condition that is suitable for the development and succession of other *Lactobacillus* species [107].

As the result of 16S rRNA sequence analysis showed, *Lactobacillus* species started the succession of *Leuconostoc* genus before the addition of starter culture (during initiation) on day 9 of the fermentation period. During the pre-screening work of LAB isolates, the strains diversity in the initiation period (Ko) was less than the control *Kocho* (Koki) and the experimental *Kocho* samples (Kom) (Table 5). Accordingly, for LAB cluster analysis, 14, 45, and 53 strains, respectively, from each group were systematically and randomly picked (Table 5) [107].

Table 5

Percentage (%) of lactic acid bacteria found in the initiation period (Ko), control *Kocho* samples without *Mandillo* (Koki) and experimental *Kocho* samples with *Mandillo* (Kom) during *Enset* fermentation

Type of bacteria ^{a)}	Total strain (112)		Ko (14)		Koki (45)		Kom (53)	
	No. of strain	% of strain	No. of strain	% of strain	No. of strain	% of strain	No. of strain	% of strain
<i>Lactobacillus brevis</i>	4 ^a	3.57	1 ^b	-	-	-	4	3.57
<i>Lac. paracasei</i>	10	8.93	-	-	-	-	10	8.93
<i>Lac. plantarum</i>	78	69.64	3	2.68	42	37.50	33	29.46
<i>Leu. mesenteroides</i>	11	9.82	11	9.82	-	-	-	-
Unidentified LAB strains	9	8.04	-	-	3	2.68	6	5.36
Total	112	100%	14	12.5%	45	40.18%	53	47.32%
^{a)} , Type of bacteria indicate bacteria exhibiting the highest identity with a microorganism isolated in this study ^{b)} , no strains found								

The representative strains of LAB identified as 2 different species in Ko, minimum 2 species in Koki, and minimum 4 species in Kom samples (Table 5). In the Table 5, among the 4 species clusters identified and the group unidentified, minimum of 3 types of species were found only in the experimental samples of *Kocho* (Kom) (Table 5). These LAB species exhibited varied (3.57 to 82.14) identity percentages with *Lactobacillus brevis* strain (3.57%), and *Lactobacillus paracasei* strains (8.93%), are special to the experimental *Kocho* samples. However, *Lactobacillus plantarum* strains (69.64%), and unidentified LAB strains (8.04%) of LAB were common to the control and experimental *Kocho* samples.

In addition to the high population and diversified species of LAB in the experimental group, it is important to further elaborate the special properties of the 2 species of LAB found only in the experimental samples (Table 5) with respect to *Mandillo*. For instance, a *Lactobacillus paracasei* strain was the highest in population on the final fermentation day when the pH of the experimental *Kocho* sample was 4.10 with acidity increment [108]. In the previous findings, it was explained that the bacteria grow well at pH 3.7–4.5 [109], and survived at low pH of 1.5–2.5 (Edward and Farnworth, 2008), which is consistent with the present research outcome. The second dominant LAB species found only in experimental samples were different *Lactobacillus plantarum* strains, which can grow at pH 4–4.5 and are homo-fermentative LAB that may be responsible for the higher concentration of lactic acid produced in experimental samples than the control samples (Table 1) [110, 111, 105, 108].

The 18S rRNA gene sequences analysis of 50 isolates of yeast, phylogenetic tree analysis and BLAST comparison showed that the isolates were more or less evenly distributed in both Koki and Kom samples (Table 6). The majority of the yeast strains were among the *Pichia kluyveri* (16%), *unclassified Pichia* (14%), *Wickerhamomyces xylosica* (16%), *Candida boidinii* strain PMM10-1634L (30%), *Williopsis* sp. (10%), *Kazachstania exigua* (2%) and *unclassified* fungi (12%). However, *Pichia kluyveri* was more dominant in Kom than in Koki samples, whereas *Williopsis*_sp. and *Kazachstania exigua* group were observed only in Koki and the unclassified fungi group encountered in Ko and Koki. The yeast isolates are expected to be new strains where further identification work is required.

In natural fermentation, there are different trends in microbial dynamics and succession based on fermentation place, time, and biochemical and biophysical environments all over the world ([112]. For example, bread products are made by a long fermentation of dough using naturally occurring lactobacilli and yeasts [113]. For example, the yeast used for leavening bread will differ depending on the physical and chemical environments. Actually, many different species of yeast including *Saccharomyces cerevisiae*, *Kazanchastania exigua*, *Candida milleri*, *Candida humilis*, and *Kazachstania exigua*, were used for leavening bread worldwide [114–116].

Table 6
Percentage (%) of yeast distributed in the initiation period (Ko), control *Kocho* samples without *Mandillo* (Koki) and experimental *Kocho* samples with *Mandillo* (Kom) during *Enset* fermentation

Name of the groups	No. isolates	% of isolates	Number of isolates /sample groups/fermentation day															
			Ko (9 isolates)		Koki (20 isolates)						Kom (21 isolates)							
			0–9	%	11	15	21	29	37	45	%	11	15	21	29	37	45	%
<i>Pichia Kluyveri</i> 18s	8	16	-	-	-	-	-	1	-	-	2	-	-	3	1	-	3	14
<i>Unclassified Pichia</i>	7	14	-	-	-	-		2	1	1	8	-	-	1	-	-	2	6
<i>Wickerhamomyces xylosica</i>	8	16	1 ^a	2	1	-	2	-	-	-	6	2	2	-	-	-	-	8
<i>Candida boidinii</i> strainPMM10-1634L	15	30	-	-	-	-	-		4	4	16	-	-	-	2	5	-	14
<i>Williopsis</i> _Sp.	5	10	5	10	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Kazachstania Exigua</i>	1	2	1	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Unclassified</i> Fungi	6	12	2	4	1	2	1	-	-	-	8	-	-	-	-	-	-	-
a), Type of yeast indicated exhibiting the highest identity with a microorganism isolated in this study. b), no strains found																		

3.5. Conclusion

The addition of *Mandillo* stem created a favorable environment for useful microorganisms such as LAB and yeast species by suppressing (or inhibiting) the growth of total aerobics, coliforms, staphylococci, and spore formers. *Mandillo* also enhanced the growth rate and populations of LAB and yeast species and diversified the species during Enset fermentation. Intensive research has to be conducted to use *Mandillo* stem in fermentation process of Enset for production of quality *Kocho* products.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Data Availability and materials

All data and materials are within the manuscript.

Competing interests

The authors declare that there is no competing interest.

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Authors' Contributions

NM, TM, SKP, YK and GD Conceived and designed the experiments, AGR and DM designed the experiments, collect samples, performed the laboratory works, analyzed the data and wrote the paper.

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Figures

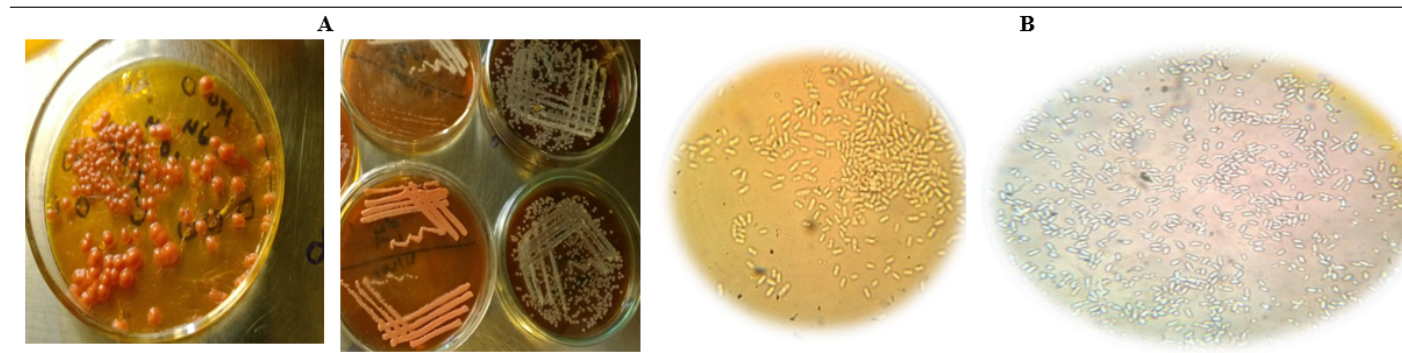


Figure 1

Structure of physically identified LAB Culture (A) and Yeast cells (B) under light Microscope.

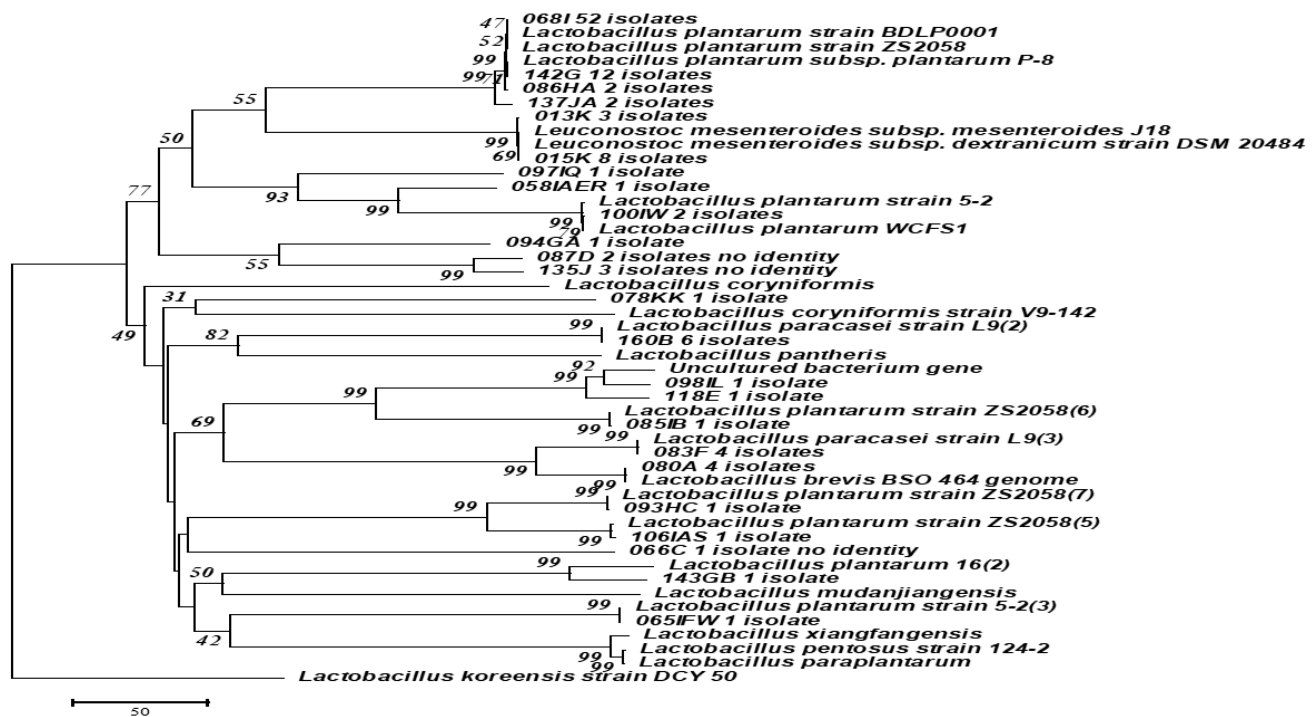


Figure 2

Phylogenetic tree of LAB isolated from fermented Kocho. The evolutionary history was inferred using the Neighbour Joining method. The confidence probability estimated using the bootstrap test (1000 replicates). There were a total of 1331 positions in the final dataset. The phylogenetic tree was reconstructed using MEGA 6. The sequence of *Lac. koreensis* was used as an out-group representative.

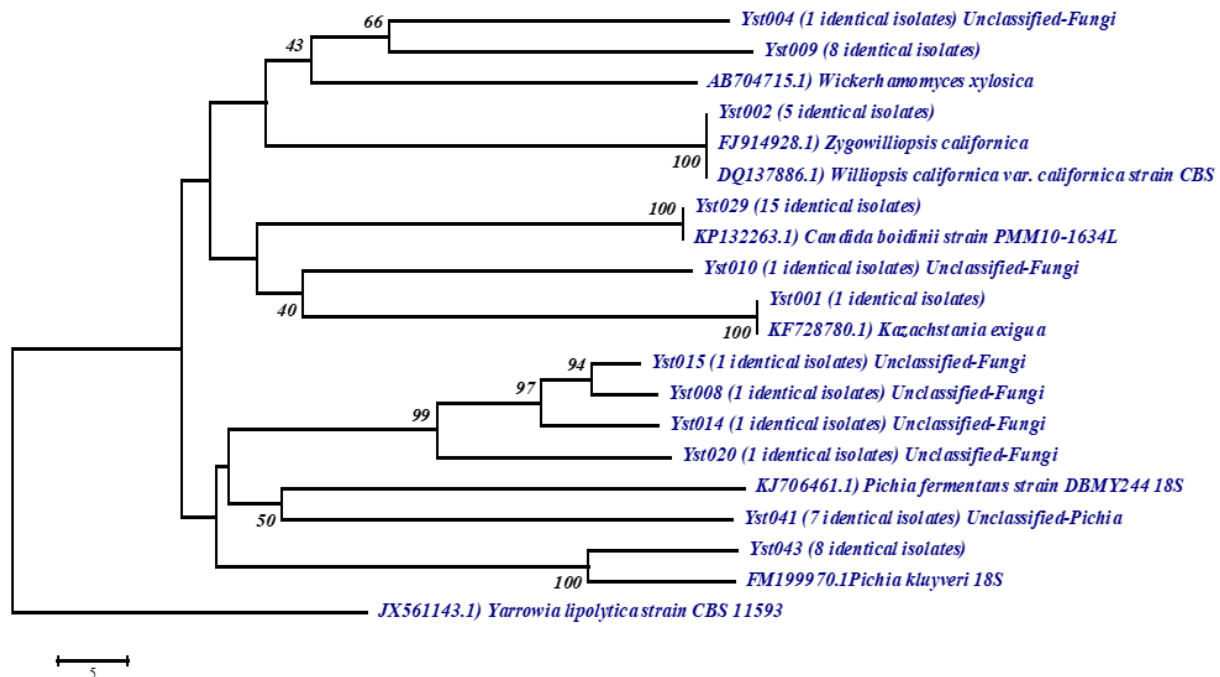


Figure 3

Phylogenetic tree of yeast isolated from fermented Kocho. The evolutionary history was inferred using the Neighbour Joining method. The confidence probability estimated using the bootstrap test (1000 replicates). There were a total of 367 positions in the final dataset. The phylogenetic tree was reconstructed using MEGA 6. The sequence of *Yarrowia lipolytica* was used as an out-group representative

