ASSESSMENT OF INGENIOUS FERMENTATION PRACTICE OF KOCHO IN DIFFERENT AREA OF ETHIOPIA

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Abstract
The enset plant is one of the fourth agricultural systems in Ethiopia. Its cultivation and fermentation tradition is unique and important food sources for Ethiopia. However, the fermentation practices of kocho differs from area to area, sometimes even from household to household. In Southern, Western and Southwestern part of Ethiopia at four different areas (Dilla, Ginchi, Woliso and Wolkite) traditional practices were investigated using open-ended questionnaires and interview. Lactic acid bacteria was also isolated and characterized both phenotypic and genotypic method. Selection of matured plants, scraping and pulverization of edible part of the plant and processing area preparation were quite similar in all study areas. Fermentation practices such as the fermentation type, length of fermentation, storage and application of traditional starter culture were the major difference among the study areas. A total of 137 bacterial isolates were collected and identified in both phenotypic and genotypic characteristics. The results indicated that Lactobacillus were the most dominant genera during kocho fermentation and Lactobacillus plantarum and Lactobacillus brevis being the prevalent species. Lactobacillus paracasei/casei, Lactobacillus fermentum, and Lactobacillus paracollinoides/collinoides were also the isolated species during kocho fermentation. Difference fermentation practices in enset processing area, length of fermentation period, enset variety, and environmental condition could be leads to have inconsistence end quality of kocho and different microbial succession. The culture-dependent results showed that lactic acid bacteria are the responsible microbe during kocho fermentation, and kocho prepared in different areas and using different processing methods varied in the types of LAB.

Keywords: Enset, kocho, fermentation type, fermentation length, sampling area, lactic acid bacteria

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1. INTRODUCTION

Enset is a multipurpose crop and provides food for more than 13 million people in Ethiopia (Guzzon and Muller, 2016). It's one of the fourth agricultural systems in Ethiopia (Brandt et al., 1997). Its cultivation and fermentation tradition is unique and important food sources for Ethiopia. The quarter of Ethiopian population those were inhabited in south and south western part were used as staples or co-staples food sources. Relative to other crops its highly productive, drought tolerate and obtained throughout the years and stored without the need of refrigerator (Birmeta et al., 2004), where it makes a major contribution to food security of the country. Regions where enset is used as staple food are usually less affected by the recurrent drought periods that occur in Ethiopia (Brandt et al., 1997).

Kocho is food product produced by decorticating and fermenting of enset parts. Similar to other fermented food it can inhibit growth of pathogenic bacteria, extending product shelf-life while ensuring consumer safety and it can be stored for years.

Despite these advantages, enset plant processing for preparation of food is time consuming, unhygienic, need long fermentation period, low in protein, and have strong odor. This are considered as a main reason for limited cultivation of enset plant in the rest of other parts of the countries (Bosha et al., 2016). Annual production of enset plant in Ethiopia is 6543 kg/ha and 4.5 million tons of kocho are available as standing stock (Sahle et al., 2018). Due to its productivity, biological, economical and food security issues need to increased cultivation of enset plant to non enset growing

regions of Ethiopia. Thus accurate understanding and introduction of these processes in both enset growing and non-growing regions can help to improve, standardize and increase the utilization of the process in order to contribute to food security of the country (Hunduma and Ashenafi, 2011). Different studies have been reviewed traditional enset processing practices in southern Ethiopia (Zerihun and Brihanu, 2015), and west shewa zone of the country (Hunduma and Ashenafi, 2011). However, the fermentation method and length of fermentation of kocho differs from area to area, sometimes even from household to household. Thus, this study investigates traditional enset fermentation practices in Southern, Western and Southwestern part of Ethiopia at four different areas (Dilla, Ginchi, Woliso and Wolkite). Meanwhile, lactic acid bacteria was isolated and characterized for starter culture development.

2. MATERIALS AND METHODS

2.1. The study areas

The four study areas are shown in Figure 1. Wolkite town was selected from south west part of Ethiopia at Abeshiga wered. The town has a latitude and longitude of 8°17′N37°47′E and an elevation between 1910 and 1935 meters above sea level. In southern part of Ethiopia study area was selected in Gedeo zone at district of Gedebe. The zone is located 365 kms south to the capital of the country Addis Ababa. The zones cover land area of 1,347 square kilometers and it lies at an altitude ranging from 1350 to 3000 m.a.s.1. Its latitude: 6° 7' 38 .17" Longitude: 38° 16’ 37.78". Woliso and Ginchi town were selected from south west shewa and west shewa zone of Ethiopia, respectively. Woliso is the administrative center of south west shewa zone, located 114 km from Addis Ababa; it has a latitude and longitude of 8°32′N 37°58′E with an elevation of 2063 meters above sea level.

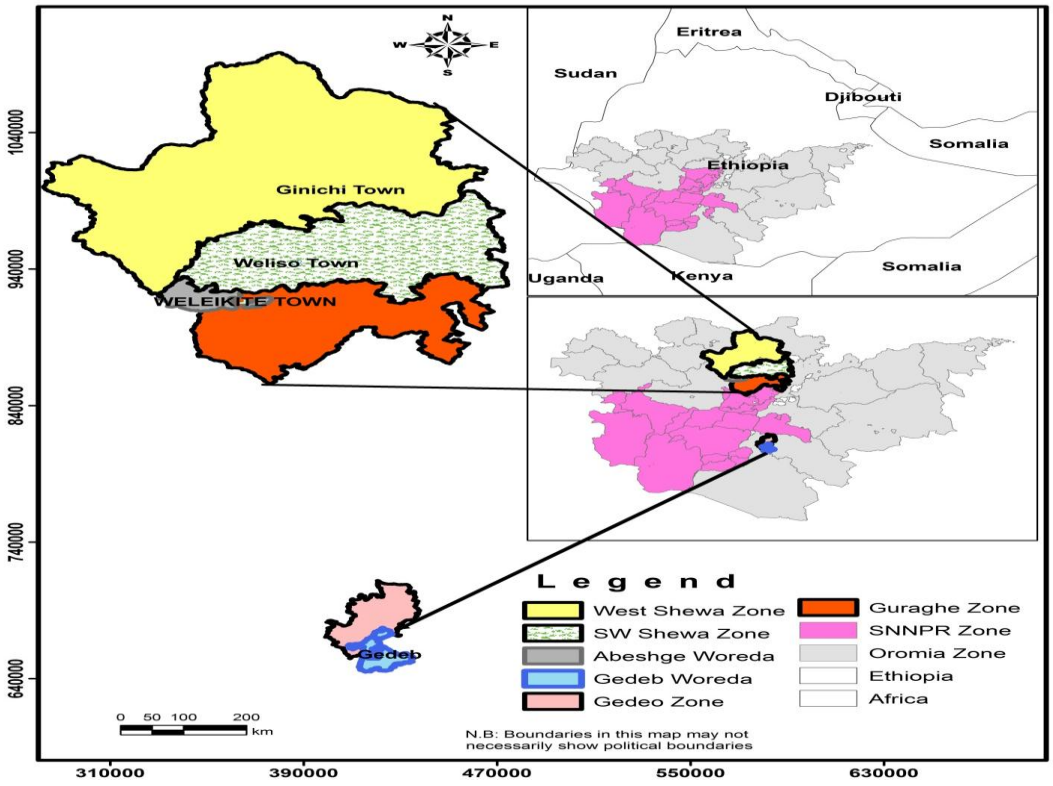

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Fig.1. Map of the study areas

Ginchi is a district in west shewa zone of Ethiopia. Ginchi is located 82 km from Addis Ababa and lies at elevation of 2,236 meters above sea level.

2.2. Data collection

Assessment of indigenous fermentation practices for the production of kocho was conducted mainly through: i) open-ended questionnaires, ii) individuals interviews iii) literatures reviewed from published . A total of 40 knowledgeable woman, farmers and agriculture expertise were participate for providing of information. Respondent were interviewed in households and market places. The questionnaires and interviews were focused on enset cultivation, indigenous fermentation practices, equipment used for preparation of enset plant, and enset varieties.

2.3. Enumeration and isolation Lactic acid bacteria

Serial dilution (up to 10^{-7}) of 10 g of kocho samples were used for enumerations of LAB using the de Man, Rogosa and Sharpe (MRS) (Hi media). A volume of 0.1 ml of diluted sample was spread onto the agar plates. The plats were incubated at 30 °C for 48 h. For characterization of LAB, colonies were selected randomly from plates at higher dilution and repeatedly strike out until purity. Cultures were stored in MRS broth containing 15% of glycerol at -80 °C.

2.4. Phenotypic characterization

LAB strains were characterized by determination of cell morphology using phase contrast microscopy, Gram staining, catalase test, gas (CO_2) production from glucose, and oxidase test used to determine the cytochrome oxidase enzyme. Oxidase test was carried using a drop of Gordon and McLead's reagent on Whatman filter paper. For the isomers test of LAB the commercial kit for D - and L- lactate dehydrogenase test (R-biopharm Enzyamtic Bioanalysis, Germany) was used according to the manufacturer's instruction. Arginine hydrolysis test was also carried out to investigate ammonia production from arginine. For all isolated strain, acid production was determined by measuring the pH of the culture after 24 and 48 h (Kostinek et al., 2005).

2.5. Genotypic characterization

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 DNA concentration was measured using a Qubit 2.0 fluorometer (Thermo Fisher Scientific) and stored at -20 °C until analysis. The 16S rRNA gene of all strains was amplified by PCR as described by Kostinek et al. (2005), bi-directionally sequenced and analysed as described by (Danylec et al., 2018).

3. RESULTS AND DISCUSSION

The range of altitude of the study areas are 1350-3000 m above sea levels. Enset plant is cultivated in areas extending from 1500 to 3300 meter altitude with annual average minimum and maximum temperatures are 8 and 22-27 °C, respectively (Karin, 2002; Bacha and Taboge, 2003). This indicated that those study areas were suitable for the growth of the plant. The previous studies also reported that,enset plant growth in all parts of Ethiopia (Brandt et al., 1997). The study areas used different languages, enset plant preparation, fermentation process, and preservation methods. Ensete is the genus name of a plant, although in the study area identified through different vernacular terms such as *Warqe*, *Aset* and *workicha* in Oromiya, Guraga and Gedeo zone, respectively as a local name. Similarly, the main enset growing areas are inhabited by more than 10 related ethnic groups, having different languages, agronomic practices, processing methods and different traditional utensils used for processing (Tsegaye and Struik, 2000).

3.1. Identification of mature enset plant

For preparation of enset based food products the first stages was a selection of matured plants. According to study participates; enset plant can be matured at a range of 3-7 years.

However, its maturity depends on soil fertility and environmental condition. In the previous studies, agricultural practices, type of cultivar and altitude also reported as maturity factories (Mohammed and Tariku, 2012).

Appearance of inflorescence and counting of plant ages mostly uses as maturity indicator. For recognized the age, plant on the same stags plotted on the same area, new growth planted near to the house and matured plants are found further away. Experienced women selected matured plant by maturity indicators or calculating the age of the plants and based on its location. An exposed corm, drying leaves and leaves sheaths also used as maturity indications in others areas (Zerihun and Brihanu, 2015).

Birmeta et al. (2004) said the best time for harvesting of plant is 9-14 years; the true stem emerges through the leaf sheaths and produces inflorescences, fruits and seeds. However, the enset plant is usually harvested before it reaches maturity, before or just at flowering. Particularly in Guraga zone harvested before flowering, because it said flowering process would deplete carbohydrate (Pijls et al., 1995), thus decreased food value of the plant. Previous study also indicated that harvesting of plant too young and too old is reduced starch content of the food (Mohammed and Tariku, 2012).

In the study areas ranges from 10-50 number of matured plants harvested yearly per households, in Ginchi relatively less number of plants were harvested. Enset plant yields 20- 40 kg of kocho, however, the study participates indicated that it depending on the size and maturity of the plant. An average of 16.2 kg and 23.4 kg of unsqueezed and squeezed kocho, respectively is produced per plant (Sahle et al., 2018).

3.2. Enset processing practices at different areas

In Wolkite enset plant uses as a staples food. The study participants listed some of enset cultivars used for food production and medicinal activities, such as: *Nechewa, ye'ka'siwe, goriya, lemat, amaratiy, ferziy, ka'nichewe, badedan, kibinar, gara, ye'shera, qiniqo,* and *astare.* The varieties of enset plant have been identified by color, size and structure of leaves, pseudostems, and other parts of a plant. The number of varieties may obtained on the same yard depend on farm size. Similarly, growth attributes, disease resistance, fiber quality and maturity times were also used as criteria for classification of plant (Yemataw et al., 2016).

However, the same variety may have different names or different varieties may have the same name in different ethnic groups (Zerihun and Brihanu, 2015).

December to January was an appropriate time for harvesting of enset plant in Wolkite area. According to the respondents drying season was more appropriate time for processing of the plant. The strength and moisture content of a plant differs in season. In rainy season the plant holds more water and reduced its thickness.

Beside the quality of the plant, processing area also becomes muddy and occasionally flooded. Thus, dried season was more appropriate time for the production of good quality kocho. Previous study also indicated that plant which processed in dried season produced high quality and yield of kocho than rainy season (Zerihun and Brihanu, 2015). However, it can be processed throughout the year as long as fresh enset leaves have been available for use at some critical steps during the processing (Hunduma and Ashenafi, 2011; Zerihun and Brihanu, 2015).

After selection of matured enset plant, the men are removed all the leaves, older leaf sheaths and small adventitious roots using locally made knife (Fig. 2. A-C).

After dug out of the plant, corm part of the plant and lower part of the mid ribs of the leaves were detached from the pseudostem and the remaining leave sheaths were separated down to the true steam (D-F). The corm was cleaned with locally made knife for removing of soils from its surfaces (G).

Next steps carried out strictly by Women.


A
B
C
D
E
F
G
H
I
J
K
L

Figure 1. Enset processing : A) select matured plant, B) removed fresh leaves, C) removed leave sheath, D) dig out the plant, E) detached corm, F) detached inner leave sheath, G) clean corm, H) preparation of processing areas, I-J) decortications of pseudostem, K) pulverization of corm, L-M) surface fermentation N) pit preparation O) pit fermentation

Unlike to other crops, the post-harvest and trading activities concerning enset are mainly owned by women (Chaka, 2016), it is considered taboo for men to be involved in this activity. Smooth working area prepared using enset leaves of which the convex side of mid ribs are removed with knife (H). For scraping of pseudostem, a plank of wood *(wattar) was inclining at angle of 45° on other standing enset plant. Then prepared sheaths in peeling the inner surface and split lengthwise to the workable size. The single leave sheaths was then putted on the plank of wood and secured by raising women one leg and pressing it with their hell* (I). *Using a bamboo splits (*sibisa*), the women from sitting position scrapes lower part of the sheaths to separates the fleshy part from the fiber. After turned upside down, reaming leaves sheaths secured with fiber extracted from the first scraping with twisted around her foot. Then, the remaining fleshy part scraped* (J). *Scraping from sitting position is very common in most areas* (Hunduma and Ashenafi, 2011). *The corm part also pulverized using* wood of which the zigzag sharp-edged end (zyeeba'nzyeebye) (K).This process turns the corm in to smaller grated pieces.

Finally two mixtures were prepared: The grated pieces of corm and fleshy scraped sheath obtained from inner part of the corm and leaf sheaths were mixed for preparation of light white colored kocho. The chopped inner part of mid rib (Fig. 2. H) was mixed with the outer part of corm and leaf sheath for preparation of dark colored kocho ("Zanzeeye"). Kocho which is light white colored prepared from the innermost leaf sheaths and corm is more acceptable than the black one which obtained from the outer leaf sheath and corm part. Mohammed and Tariku (2012) stated that the quality of kocho is influenced by the processed part of leaf sheath and corm , and the skill of the processor. The two mixtures was then covered with fresh leaves and allowed to ferment for 9 days at ambient temperature with turning, mashing and changing of the covered leaves (Fig.L-M). This was then remixed, buried in a pit lined with dried leave sheathes and plastics. The uses of plastics bag for lined the inner part of the pit was the unique and modified method in this area. Top part of the pit were laid with plastic, fresh leaves and finally loaded with heavy stones to create air tight condition (Fig.N-O). In Wolkite pit fermentation beside used as a storage of kocho for long period of time. If it preserved in good manner and changing covering layers frequently, it can bury in a pit for more than 3 years. However, if there a shortage of food it can be consumed after 10 days of fermentation. Due to less acidity and palatability nature most participates prefers kocho which fermented for short period of time. Pijls et al. (1995) also stated that, kocho can be kept for a period of up to 7 years, and is readily available throughout the year.

In Ginchi enset and other root crops are not staples food. They are the secondary importance crops. Enset named warqe in the Ginchi. Similarly, in west shewa and southwest shewa zones in Oromia region, enset plant named warqe (Chaka et al., 2016). Enset preparation and processing activities lay to women, and quite similar to Wolkite (Supplementary 1). After decortications and pulverization of edible parts, they mixed and wrapped with fresh leaves and left to ferment at ambient temperature for three weeks. This was then remixed, buried in a pit covered with dried and fresh leaves, and finally loaded with heavy stones to create air tight condition. During drying season they kept as such for more than two months, then after they take off and kept at home in plastic bags until it consumed (Supplementary 2).

In Wolkite and Ginchi surface fermentation were used for the first nine days and three weeks, respectively followed by pit fermentation for months or year. Similarly, previous study reported at the mid altitude sites, they have been used surface fermentation for two weeks followed by pit fermentation (Hunduma and Ashenafi, 2011). Surface fermentation locally thought initiate fermentation processes. Similarly, Gashe (1987) stated that fermented mass left at ambient for a days coulde be used to initiate fermetation processes.

In Dilla, enset plant mostly harvested at the dried season. Most activates such as identification of mature enset plant, surface preparation for processing of enset, pulverization, decortications, mashing and changing of fresh leaves are similar to the above mentioned areas. Equipment used for processing also quite similar to Wolkite and Ginchi. However, in this area they practiced traditional starter culture for initiation of fermentation process, and they only use surface fermentation for 15 days or 1 month depend on the need of consumption (Supplementary 3). Traditional surface fermentation has two phases: for immediate consumption, traditional starter culture (Gamama) was added immediately in mass of pulp and left at ambient temperature for 15 days by creating air tight condition. For long term consumption a mass of pulp left to fermented for 15 days, and then it was mixed with starter culture and ferment again for the next 15 days . When it ready for household or market consumption they wrapped with dried leaf sheets (Supplementary 4). The previous studies reported that digging of pit for fermentation of the mass in the pit are basically similar in all enset traditions (Gashe, 1987; Tsegaye and Struik, 2001; Hunduma and Ashenafi, 2011). However, in Dilla those processes are not a common practices.

For preparation of traditional starter culture in Dilla matured corm, previously prepared traditional starter culture (back slopping) or rotten banana and enset part is might be needed. It prepared 10-20 days before processing of plant and inoculated at a day of processing. After cleaning, slightly pulverizing and chopping of the matured corm, it was polished with previously fermented kocho (back slopping) or rotten banana and pseudostem. Then it was covered with fresh and dried leaves and left to ferment at ambient temperature for 10-20 days with exposed to sun for 5-12 h at 5 days intervals. The study participates stated that the amount and the maturity states of traditional starter cultures have effects on the sensory quality of kocho. The small amount and highly matured starters causes moldy and bad smell, respectively. Thus, appropriate amount and maturity period of traditional starter culture helps to enhance sensory quality of the product. Kocho quality also depend on enset cultivars, *Nifo, Astara*, and *kake*, are consumed by farmers mainly due to its fast fermentation process, quality of kocho, drought and diseases intolerance or productivity.

Enset processing practices at Woliso was started with preparation of pit (Supplementary 5). Pit preparation, also identified as a major processing step in Wolkite and Ginchi. However, in Woliso the pit was prepared on the same day when the process of scraping and decortications of parts of the plant were takes place. The previous study also reported that, in

high altitude pit and area preparation was done side by side. However, in mid altitude pit prepared about two week after processing of plant parts (Hunduma and Ashenafi, 2011).Similar to the above areas, the inner part of the pit was usually lined with dried leave sheaths or fresh leaves. Lining of pit with enset leaves was done to collect and prevent the juicy part from leaking into the ground (Gashe, 1987).

A traditional starter culture preparation and pit fermentation of the scraped pseudostem were carried out on the first day of enset processing. The corm part of the plant was pulverized with wood zig zag edge and acts as a mortal for preparation of traditional starter culture. Pulverized corm, rotten inflorescence, *Commelina latifolia*, *Lippia abyssinica*, *Ocimum Sanctum* and other herbs were the main ingredients for preparation of traditional starter culture. Bosha et al. (2016), also reported that different herbs such as *Commelina latifolia*, *Rumex abyssinicus*, and pre-fermented pseudostem base, garlic, onion, ginger, table salt and ripe banana were used for the preparation traditional starter culture. Those herbs thought improved flavor and help to maintain light white color of kocho. The prepared spices and pulverized corm were mixed and left to ferment for 2-3 week in the reaming part of corm. Afterward, a mass of partially fermented kocho which is buried in the pit was taken off and mixed with the traditional starter culture, than again left to ferment for 2-3 months in a pit. The mass of a pulp become ready for consumption within 2 weeks after addition of traditional starter cultures. In the previous time the mass of pulp was preserved in the pit for years, however nowadays due to environmental conditions it only preserved for months, and then removed from the pit and place in the plastic bag until consumed. In Woliso without inflorescence kocho might not be prepared, thought it enhanced fermentation process. Cultivars such as: *awegn, fersa, be'sheliga, sebara,* and *badedat* were mostly utilized for preparation of kocho. Kocho prepared from *badedat* cultivars relatively has good tastes, and from *be'sheliga* and *sebara* light white kocho have been produced.

3.3. Food preparation from fermented enset (kocho)

In ordered to prepared baked kocho, the dough to be baking is processed as follows. The fermented enset pulp cut with a big locally made knife to minimize the amount of fiber that escape into the food during scraping. However, nowadays the fermented pulp was washed with water. Then it was squeezed to leach out white liquid, drain off the water to get a pure white pulp without fiber and to improve its color. Finally the fermented enset can then be baked into pancake like bread using hot plates and consumed with kale, and animal based sauces. A pancake like bread is the most common, which is eaten with milk and cabbage. It is also very popular at restaurants that served together with the Ethiopian traditional food of kitfo (uncooked finely minced meat mixed with spicy butter)(Mohammed and Tariku, 2012). According to the responses of most participates, they chosen kocho with light white color, less acidic, free from fiber, have a good flavor and smell. The quality of kocho depends on the age of the harvested plant, the variety of plant, the harvesting season, and the part of leaf sheath and corm (Mohammed and Tariku, 2012).

3.4. Identification of Isolated Strains

A total of 205 bacterial strains were isolated from kocho samples on MRS agar and striking out reputedly for purification. Then after, 137 isolate were selected which became blue-purple after gram staining, because it considered as gram-positive bacteria. The isolates were selected from Wolkite (n=70), Dilla (50) and Woliso (n=17) area samples (Table 1-3). Isolates from Ginchi samples were not selected due to gram staining results. The selected 137 isolates were also characterized both phenotypic and genotypic methods.

Table 1. Phenotypic characterization of LAB isolated from Wolkite area samples

Strain number	CO_2 from glucose	Gram reaction	Catalase	Oxidase	Morphology	Isomer			pH		Arginine test
						D	L	DL	24h	48h	
I1A	+	+	-	-	Rods	-	-	+	4.87	4.83	+
I1B	-	+	-	-	Rods	-	-	+	3.87	3.85	-
I2A	-	+	-	-	Rods	-	-	+	3.95	3.83	-
I2B	+	+	-	-	Rods	-	-	+	5.00	4.52	+
I3	-	+	-	-	Rods	-	-	+	4.01	3.98	-
I4A1	-	+	-	-	Rods	-	-	+	4.01	3.74	-
I4B	+	+	-	-	Rods	-	-	+	5.08	4.12	+
I5	-	+	-	-	Rods	-	+	-	4.10	3.91	-
I6	-	+	-	-	Rods	-	-	+	4.27	3.93	-
I7A	+	+	-	-	Rods	-	-	+	5.08	4.62	+
I7B	-	+	-	-	Rods	-	+	-	3.90	3.78	-
I8	-	+	-	-	Rods	-	+	-	4.09	3.82	-
I9	-	+	-	-	Rods	-	+	-	4.02	3.91	-
I10	-	+	-	-	Rods	-	-	+	4.08	3.94	-
I11	-	+	-	-	Rods	-	-	+	3.97	3.90	-
I12	+	+	-	-	Rods	-	-	+	4.93	4.90	+
6-1	-	+	-	-	Rods	-	-	+	3.99	3.96	-
6-2	-	+	-	-	Rods	-	-	+	3.98	3.85	-
6-3	-	+	-	-	Rods	-	-	+	4.06	3.94	-
6-4A	-	+	-	-	Rods	-	-	+	4.00	3.86	-
6-4B	-	+	-	-	Rods	-	-	+	3.92	3.78	-
A1	-	+	-	-	Rods	-	+	-	3.99	3.90	-
A2	+	+	-	-	Rods	-	-	+	5.00	4.46	+
A3	+	+	-	-	Rods	-	-	+	4.74	4.29	+
A5	-	+	-	-	Rods	-	+	-	4.19	3.88	-
A6	+	+	-	-	Rods	-	-	+	5.01	4.48	+
A7A	+	+	-	-	Rods	-	-	+	5.04	4.53	+
A7B	+	+	-	-	Rods	-	-	+	5.04	4.55	+
A8	-	+	-	-	Rods	-	-	+	3.72	3.68	-
A9A	+	+	-	-	Rods	-	-	+	5.05	4.50	+
A9B	-	+	-	-	Rods	-	-	+	3.73	3.71	-
20-1	-	+	-	-	Rods	-	-	+	5.0	5.0	-
20-2A	-	+	-	-	Rods	-	-	+	3.77	3.71	-
20-2B	+	+	-	-	Rods	-	-	+	5.04	4.53	+
20-3	+	+	-	-	Rods	-	-	+	5.05	4.52	+
20-4A	-	+	-	-	Rods	-	-	+	3.86	3.76	-
20-4B	+	+	-	-	Rods	-	-	+	4.90	4.58	+
20-5	-	+	-	-	Rods	-	-	+	3.82	3.72	-
20-6	-	+	-	-	Rods	-	-	+	3.74	3.73	-
1-1	-	+	-	-	Rods	-	-	+	3.79	3.75	-
1-2A	-	+	-	-	Rods	-	-	+	3.83	3.77	-
1-2B	+	+	-	-	Rods	-	-	+	4.05	4.03	+
1-3	-	+	-	-	Rods	-	-	+	3.78	3.74	-
1-4	-	+	-	-	Rods	-	-	+	3.76	3.73	-
1-5A1	+	+	-	-	Rods	-	-	+	5.10	4.62	-
1-5A2	+	+	-	-	Rods	-	-	+	5.08	4.78	-
1-5B	+	+	-	-	Rods	-	-	+	4.90	4.54	-
1-6	-	+	-	-	Rods	-	-	+	3.78	3.76	-
1-7A	-	+	-	-	Rods	-	-	+	3.79	3.77	-
9-1A	+	+	-	-	Rods	-	-	+	5.07	4.66	-
9-1B	-	+	-	-	Rods	-	-	+	3.85	3.78	-
9-4A1	-	-	+	-	Rods	-	+	-	4.31	4.06	+

9-4A2	-	-	+	-	Rods	-	+	-	4.52	4.10	+
9-4B	-	+	-	-	Rods	-	+	-	4.90	4.63	-
9-5A	+	+	-	-	Rods	+	-	-	4.55	4.35	+
9-5B	-	-	+	-	Rods	-	+	-	5.01	4.53	+
9-6A	-	+	-	-	Rods	-	-	+	3.79	3.76	-
9-6B	+	+	-	-	Rods	-	-	+	5.01	4.53	+
10-1	+	+	-	-	Rods	-	-	+	5.09	4.58	+
10-2	+	+	-	-	Rods	-	-	+	5.07	4.57	±
10-41A	-	+	-	-	Rods	-	-	+	3.80	3.77	-
10-41B	-	+	-	-	Rods	-	-	+	3.78	3.74	-
10-42A	-	+	-	-	Rods	-	+	-	3.90	3.75	-
10-42B	-	+	-	-	Rods	-	+	-	3.90	3.72	-
15P3	-	+	-	-	Rods	-	-	+	3.96	3.81	-
9P4	-	+	-	-	Rods	-	-	+	3.86	3.81	-
21N	-	+	-	-	Rods	-	+	-	3.98	3.90	-
21P21A	-	+	-	-	Rods	-	-	+	3.86	3.72	-
21P21B	-	+	-	-	Rods	-	+	+	3.80	3.74	-
49N2	-	+	-	-	Rods	-	-	-	4.17	3.94	-

Table 2. Phenotypic characterization of LAB isolated from Dilla area samples

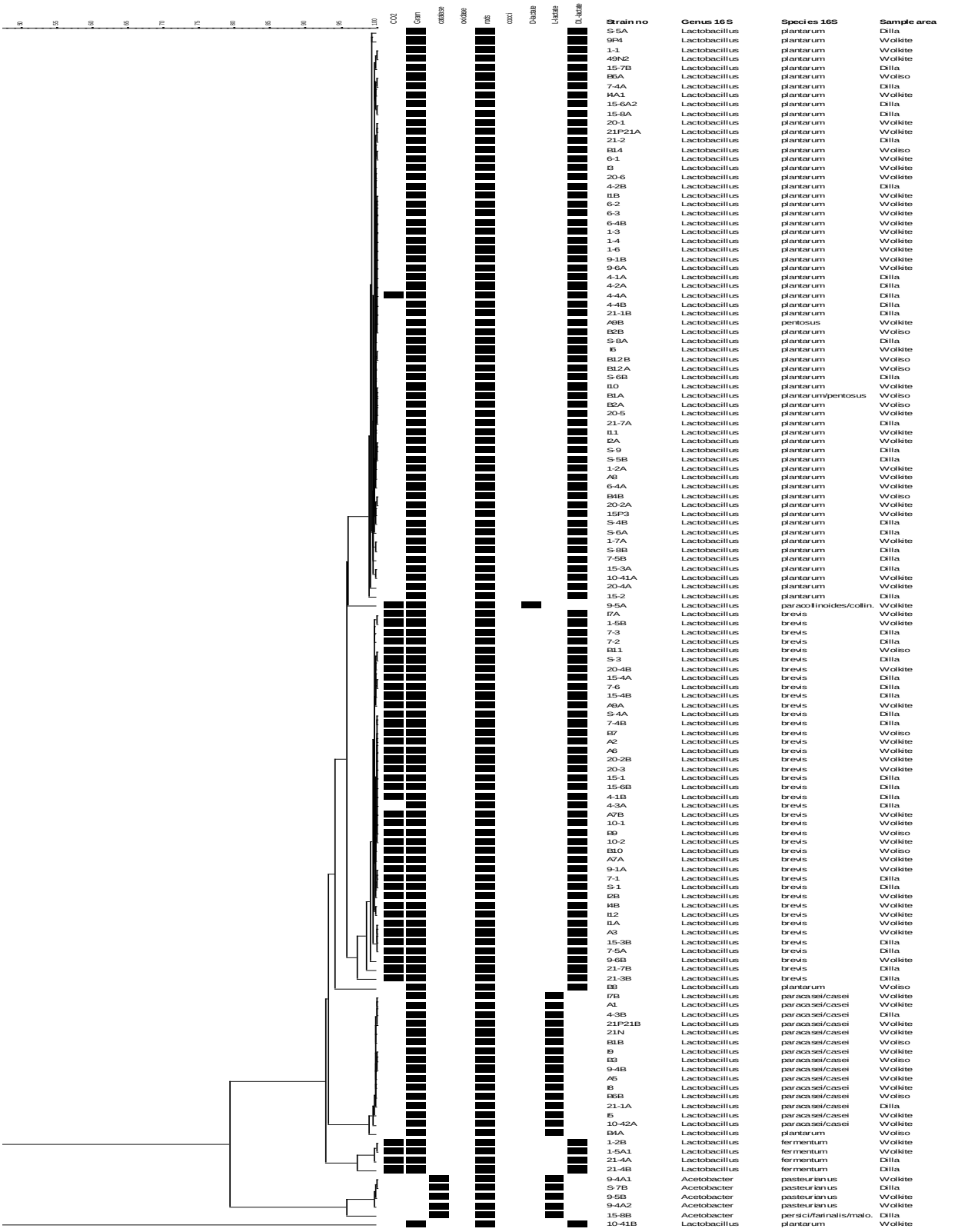
Strain number	CO_2 from glucose	Gram reaction	Catalase	Oxidase	Morphology	Isomer			pH		Arginine test
						D	L	DL	24h	48h	
15-1	+	+	-	-	Rods	-	-	+	4.85	4.81	±
15-2	-	+	-	-	Rods	-	-	+	3.97	3.81	-
15-3A	-	+	-	-	Rods	-	-	+	3.99	3.68	-
15-3B	+	+	-	-	Rods	-	-	+	4.82	4.24	+
15-4A	+	+	-	-	Rods	-	-	+	3.92	3.84	±
15-4B	+	+	-	-	Rods	-	-	+	3.90	3.85	±
15-6A2	-	+	-	-	Rods	-	-	+	3.78	3.72	-
15-6B	+	+	-	-	Rods	-	-	+	4.62	4.22	+
15-7A	-	+	-	-	Rods	-	-	+	4.16	4.14	-
15-7B	-	+	-	-	Rods	-	-	+	4.10	3.90	-
15-8A	-	+	-	-	Rods	-	-	+	4.15	4.14	-
15-8B	-	-	-	-	Rods	-	+	-	3.98	3.94	-
4-1A	-	+	-	-	Rods	-	-	+	3.96	3.86	-
4-1B	+	+	-	-	Rods	-	-	+	5.03	4.84	+
4-2A	-	+	-	-	Rods	-	-	+	3.92	3.91	-
4-2B	-	+	-	-	Rods	-	-	+	3.89	3.87	-
4-3A	-	+	-	-	Rods	-	-	+	4.78	4.16	±
4-3B	-	+	-	-	Rods	-	+	-	3.94	3.83	-
4-4A	+	+	-	-	Rods	-	-	+	4.10	3.92	-
4-4B	-	+	-	-	Rods	-	-	+	4.00	3.78	-
S-1	+	+	-	-	Rods	-	-	+	4.97	4.91	+
S-3	+	+	-	-	Rods	-	-	+	4.99	4.87	±
S-4A	+	+	-	-	Rods	-	-	+	4.96	4.79	+
S-4B	-	+	-	-	Rods	-	-	+	3.93	3.75	-
S-5A	-	+	-	-	Rods	-	-	+	3.86	3.70	-
S-5B	-	+	-	-	Rods	-	-	+	3.91	3.89	-
S-6A	-	+	-	-	Rods	-	-	+	3.99	3.68	-
S-6B	-	+	-	-	Rods	-	-	+	3.91	3.89	-
S-7A	-	+	-	-	Rods	-	-	+	3.97	3.80	-
S-7B	-	-	+	-	Rods	-	+	-	3.84	3.76	-
S-8A	-	+	-	-	Rods	-	-	+	3.81	3.77	-
S-8B	-	+	-	-	Rods	-	-	+	3.95	3.90	-
S-9	-	+	-	-	Rods	-	-	+	4.03	3.89	-

7-1	+	+	-	-	Rods	-	-	+	4.75	4.15	-
7-2	+	+	-	-	Rods	-	-	+	4.98	4.59	+
7-3	+	+	-	-	Rods	-	-	+	4.99	3.72	+
7-4A	-	+	-	-	Rods	-	-	+	3.86	3.84	-
7-4B	+	+	-	-	Rods	-	-	+	4.03	3.91	+
7-5A	+	+	-	-	Rods	-	-	+	5.12	4.81	+
7-5B	-	+	-	-	Rods	-	-	+	3.98	3.80	-
7-6	+	+	-	-	Rods	-	-	+	4.06	3.93	+
21-1A	-	+	-	-	Rods	-	+	-	3.88	3.69	-
21-1B	-	+	-	-	Rods	-	-	+	3.79	3.77	-
21-2	-	+	-	-	Rods	-	-	+	3.87	3.84	-
21-3A	-	+	-	-	Rods	-	-	+	3.91	3.87	-
21-3B	+	+	-	-	Rods	-	-	+	5.00	4.62	+
21-4A	+	+	-	-	Rods	-	-	+	4.87	4.66	-
21-4B	+	+	-	-	Rods	-	-	+	4.89	4.75	-
21-7A	-	+	-	-	Rods	-	-	+	3.97	3.72	-
21-7B	+	+	-	-	Rods	-	-	+	4.82	4.76	+

Table 3. Phenotypic characterization of LAB isolated from Woliso area samples

Strain number	CO_2 from glucose	Gram reaction	Catalase	Oxidase	Morphology	Isomer			pH		Arginine test
						D	L	DL	24h	48h	
B1A	-	+	-	-	Rods	-	-	+	5.02	4.90	+
B1B	-	+	-	-	Rods	-	+	-	5.04	4.94	+
B2A	-	+	-	-	Rods	-	-		4.04	3.88	-
B2B	-	+	-	-	Rods	-	-	+	4.00	3.71	-
B3	-	+	-	-	Rods	-	+	-	4.01	3.81	-
B4A	-	+	-	-	Rods	-	-	+	4.09	3.98	-
B4B	-	+	-	-	Rods	-	-	+	3.97	3.75	-
B6A	-	+	-	-	Rods	-	-	+	3.89	3.82	-
B6B	-	+	-	-	Rods	-	+	-	3.98	3.89	-
B7	+	+	-	-	Rods	-	-	+	4.34	4.07	-
B8	-	+	-	-	Rods	-	-	+	3.96	3.77	-
B9	+	+	-	-	Rods	-	-	+	4.36	4.11	+
B10	+	+	-	-	Rods	-	-	+	4.11	3.92	-
B11	+	+	-	-	Rods	-	-	+	4.40	4.03	+
B12A	-	+	-	-	Rods	-	-	+	3.83	3.77	-
B12B	-	+	-	-	Rods	-	-	+	3.85	3.71	-
B14	-	+	-	-	Rods	-	-	+	3.97	3.81	-

All the isolated strains were rod-shaped, oxidase and catalase-negative, 43 strains were able to hydrolyze arginine and change its color to bright orange and five strains which were catalase-positive and later identified as AAB (Table 1-3, Fig. 6). Thus, in total, a number of 132 LAB strains were isolated from all kocho samples. LAB are gram-positive, rods and cocci shapes, catalase and oxidase negative, and ferment hexose sugar for the production of lactic acid (Edward, 2010). The pH in MRS broth after growth for 24 h and 48 h ranged from 3.74 to 5.12 and 3.68 to 5.0, respectively. Among those 47 strains obligatory heterofermentative strains produced CO_2 from glucose fermentation and DL-lactate, except one strain which produced D-lactate and 85 facultative heterofermentative strains producing L- and DL-lactate and did not produced CO_2 from glucose were identified. Within the group of obligatory heterofermentative, the majority of strains was identified as *Lactobacillus brevis* (n=41), few strains as *L. fermentum* (n=5) and one strain as *L. paracollinoides/collinoides* (Fig. 6).



Strain no	Genus 16S	Species 16S	Sample area
S-5A	Lactobacillus	plantarum	Dilla
9P4	Lactobacillus	plantarum	Wolkite
1-1	Lactobacillus	plantarum	Wolkite
49N2	Lactobacillus	plantarum	Wolkite
15-7B	Lactobacillus	plantarum	Dilla
B5A	Lactobacillus	plantarum	Woliso
7-4A	Lactobacillus	plantarum	Dilla
I4A1	Lactobacillus	plantarum	Wolkite
15-6A2	Lactobacillus	plantarum	Dilla
15-8A	Lactobacillus	plantarum	Dilla
20-1	Lactobacillus	plantarum	Wolkite
21P21A	Lactobacillus	plantarum	Wolkite
21-2	Lactobacillus	plantarum	Dilla
B14	Lactobacillus	plantarum	Woliso
6-1	Lactobacillus	plantarum	Wolkite
I3	Lactobacillus	plantarum	Wolkite
20-6	Lactobacillus	plantarum	Wolkite
4-2B	Lactobacillus	plantarum	Dilla
B8	Lactobacillus	plantarum	Wolkite
6-2	Lactobacillus	plantarum	Wolkite
6-3	Lactobacillus	plantarum	Wolkite
6-4B	Lactobacillus	plantarum	Wolkite
1-3	Lactobacillus	plantarum	Wolkite
1-4	Lactobacillus	plantarum	Wolkite
1-6	Lactobacillus	plantarum	Wolkite
9-1B	Lactobacillus	plantarum	Wolkite
9-6A	Lactobacillus	plantarum	Wolkite
4-1A	Lactobacillus	plantarum	Dilla
4-2A	Lactobacillus	plantarum	Dilla
4-4A	Lactobacillus	plantarum	Dilla
4-4B	Lactobacillus	plantarum	Dilla
21-1B	Lactobacillus	plantarum	Dilla
A9B	Lactobacillus	pentosus	Wolkite
B2B	Lactobacillus	plantarum	Woliso
S-8A	Lactobacillus	plantarum	Dilla
I6	Lactobacillus	plantarum	Wolkite
B12B	Lactobacillus	plantarum	Woliso
B12A	Lactobacillus	plantarum	Woliso
S-6B	Lactobacillus	plantarum	Dilla
I10	Lactobacillus	plantarum	Wolkite
B1A	Lactobacillus	plantarum/pentosus	Woliso
B2A	Lactobacillus	plantarum	Woliso
20-5	Lactobacillus	plantarum	Wolkite
21-7A	Lactobacillus	plantarum	Dilla
I11	Lactobacillus	plantarum	Wolkite
I2A	Lactobacillus	plantarum	Wolkite
S-9	Lactobacillus	plantarum	Dilla
S-5B	Lactobacillus	plantarum	Dilla
1-2A	Lactobacillus	plantarum	Wolkite
A8	Lactobacillus	plantarum	Wolkite
6-4A	Lactobacillus	plantarum	Wolkite
B4B	Lactobacillus	plantarum	Woliso
20-2A	Lactobacillus	plantarum	Wolkite
15P3	Lactobacillus	plantarum	Wolkite
S-4B	Lactobacillus	plantarum	Dilla
S-6A	Lactobacillus	plantarum	Dilla
1-7A	Lactobacillus	plantarum	Wolkite
S-8B	Lactobacillus	plantarum	Dilla
7-5B	Lactobacillus	plantarum	Dilla
15-3A	Lactobacillus	plantarum	Dilla
10-41A	Lactobacillus	plantarum	Wolkite
20-4A	Lactobacillus	plantarum	Wolkite
15-2	Lactobacillus	plantarum	Dilla
9-5A	Lactobacillus	paracollinoides/collin.	Wolkite
I7A	Lactobacillus	brevis	Wolkite
1-5B	Lactobacillus	brevis	Wolkite
7-3	Lactobacillus	brevis	Dilla
7-2	Lactobacillus	brevis	Dilla
B11	Lactobacillus	brevis	Woliso
S-3	Lactobacillus	brevis	Dilla
20-4B	Lactobacillus	brevis	Wolkite
15-4A	Lactobacillus	brevis	Dilla
7-6	Lactobacillus	brevis	Dilla
15-4B	Lactobacillus	brevis	Dilla
A9A	Lactobacillus	brevis	Wolkite
S-4A	Lactobacillus	brevis	Dilla
7-4B	Lactobacillus	brevis	Dilla
B7	Lactobacillus	brevis	Woliso
A2	Lactobacillus	brevis	Wolkite
A6	Lactobacillus	brevis	Wolkite
20-2B	Lactobacillus	brevis	Wolkite
20-3	Lactobacillus	brevis	Wolkite
15-1	Lactobacillus	brevis	Dilla
15-6B	Lactobacillus	brevis	Dilla
4-1B	Lactobacillus	brevis	Dilla
4-3A	Lactobacillus	brevis	Dilla
A7B	Lactobacillus	brevis	Wolkite
10-1	Lactobacillus	brevis	Wolkite
B9	Lactobacillus	brevis	Woliso
10-2	Lactobacillus	brevis	Wolkite
B10	Lactobacillus	brevis	Woliso
A7A	Lactobacillus	brevis	Wolkite
9-1A	Lactobacillus	brevis	Wolkite
7-1	Lactobacillus	brevis	Dilla
S-1	Lactobacillus	brevis	Dilla
I2B	Lactobacillus	brevis	Wolkite
I4B	Lactobacillus	brevis	Wolkite
I12	Lactobacillus	brevis	Wolkite
I1A	Lactobacillus	brevis	Wolkite
A3	Lactobacillus	brevis	Wolkite
15-3B	Lactobacillus	brevis	Dilla
7-5A	Lactobacillus	brevis	Dilla
9-6B	Lactobacillus	brevis	Wolkite
21-7B	Lactobacillus	brevis	Dilla
21-3B	Lactobacillus	brevis	Dilla
B8	Lactobacillus	plantarum	Woliso
I7B	Lactobacillus	paracasei/casei	Wolkite
A1	Lactobacillus	paracasei/casei	Wolkite
4-3B	Lactobacillus	paracasei/casei	Dilla
21P21B	Lactobacillus	paracasei/casei	Wolkite
21N	Lactobacillus	paracasei/casei	Wolkite
B1B	Lactobacillus	paracasei/casei	Woliso
I9	Lactobacillus	paracasei/casei	Wolkite
B3	Lactobacillus	paracasei/casei	Woliso
9-4B	Lactobacillus	paracasei/casei	Wolkite
A5	Lactobacillus	paracasei/casei	Wolkite
I8	Lactobacillus	paracasei/casei	Wolkite
B6B	Lactobacillus	paracasei/casei	Woliso
21-1A	Lactobacillus	paracasei/casei	Dilla
I5	Lactobacillus	paracasei/casei	Wolkite
10-42A	Lactobacillus	paracasei/casei	Wolkite
B4A	Lactobacillus	plantarum	Woliso
1-2B	Lactobacillus	fermentum	Wolkite
1-5A1	Lactobacillus	fermentum	Wolkite
21-4A	Lactobacillus	fermentum	Dilla
21-4B	Lactobacillus	fermentum	Dilla
9-4A1	Acetobacter	pasteurianus	Wolkite
S-7B	Acetobacter	pasteurianus	Dilla
9-5B	Acetobacter	pasteurianus	Wolkite
9-4A2	Acetobacter	pasteurianus	Wolkite
15-8B	Acetobacter	persici/farinalis/malo.	Dilla
10-41B	Lactobacillus	plantarum	Wolkite

Fig.6. Dendrogram obtained through clustering of the strains numbers using origin, phenotypic methods and 16S rRNA gene sequencing

Of this 19 (46.3%) of *L. brevis* were isolated from Wolkite samples, 18 (43.9%) from Dilla and 4 (9.7%) from Woliso . The group of facultative heterofermentative strains consisted mostly of strains belonging to the *L. plantarum*-group (n=69). The *L. plantarum*-group consists of the species *L. plantarum, L. paraplantarum* and *L. pentosus* which cannot be separated by phenotypic methods and 16S rRNA gene sequencing reliably (Hammes and Hertel, 2003). Of this 33 (48.5%) were isolated from Wolkite samples, 26 (38.2%) from Dilla and 9 (13.2%) from Woliso samples. *L.plantarum* – group was the most abundantly isolated species in all area kocho samples. Among the facultative heterofermentative strains, 16 could be identified as *L. paracasei.* Of this 11(68.8%) were isolated from Wolkite, 2(12.5 %) from Dilla and 3 (18.8%) from Woliso area samples.

In addition to LAB, five gram-negative and catalase positives strains could be isolated on MRS agar which was targeted only for the isolation of LAB. These strains were identified as *Acetobacter pasteurianus* and *A. persici/farinalis/malorum.* Weldemichael et al (2019) also reported the presence of AAB in kocho. During cocoa bean fermentation AAB are known to be predominant next to LAB, and *A. pasteurianus* was detected as the predominant species (Illeghems et al., 2012).

L. plantarum, L.brevis and *L. paracasei* are found in all kocho samples. This indicates that LAB could be responsible bacteria for kocho fermentation. *L. fermentum* and *Acetobacteria* were isolated from Wolkita and Dilla area samples. This indicates that *L. fermentum* and *Acetobacteria* are not common in Woliso area samples (Table 3).

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 (Kostinek et al., 2005; Swain et al., 2014; Weldemichael et al., 2019). Particularly *L.plantarum, L. brevis* and *L. casei* were the common *Lactobacillus* species on some of fermented cereals and cassava products such as Fufu, Ogi, Iru and Ugba (Olasupo et al., 1997).

Similarly, in this study *L. plantarum, L. brevis, L. paracasei/casei* and *L. fermentum* were also isolated. However, *Leuconostoc* that were reported in starchy fermented food including kocho are not confirmed in this study. The reason could be differences in geographic regions, processing conditions, and in raw material. Another possibility could be the uniqueness of microbiota of *E. ventricosum* plants. In the literature, *L. mesenteroides* was reported to occur in *in vitro E. ventricosum* clones, but not in field growing clones (Birmeta, 2004). Ali (2011) also reported that the lower number of lactic acid cocci is probably due to their inability to compete with lactic acid bacilli in mixed cultures. *L. paracollinodes/collinoides* and *Acetobacteria spp.* were also identified in this study as responsible microbes during enset fermentation. *L. paracollinodes/collinoides* is obligatory heterofermentative lactic acid bacteria found in brewery environment (Suzuki et al., 2004) and fermented vegetable such as table olives (Capozzi et al., 2017). *Acetobacteria spp* also have been reported in fermentation of west Africa fermented food such as Ogi (fermented maize) and Burkutu (fermented sorghum), in manufacturing of acidic beers, and several traditional fermented food and slight acidic beverages like water and milk kefir, kombucha, lambic beer, cocoa and cider (Achi, 2005; De Roos and De Vuyst, 2018; Li et al., 2014). However, there is a limited knowledge about their occurrence and functional role in spontaneously fermented food (De Roos & De Vuyst, 2018). The main reasons are their cultivation, isolation, and identification is cumbersome and can occur in a viable but non-culturable state at anaerobic environment (Papalexandratou et al., 2013).

4. CONCLUSIONS

According to this study, some of traditional enset processing practices such as; selection of matured plants, scraping and pulverization of edible part of the plant and processing area

preparation were quite similar in all sites. However, fermentation practices such as the fermentation type, length of fermentation, preservation and application of traditional starter culture were the major difference among the study areas. Environmental conditions have effect on the length of fermentation time. Difference fermentation practices in enset processing area, length of fermentation period, enset variety, and environmental condition could be leads to have inconsistence end quality of kocho and different microbial succession. The strain isolation results indicated that AAB and LAB could be the response species during kocho fermentation. kocho prepared in different areas and using different processing methods varied in the types of LAB. The result of this study can be used as information to improve, standardize and scale-up the process for industrial production. The study of the LAB in kocho also contributes to the characterisation of kocho and helps to improve the knowledge of the fermentation process. Moreover, this information could be used in the future to select appropriate starter cultures for an improved, controlled fermentation of kocho.

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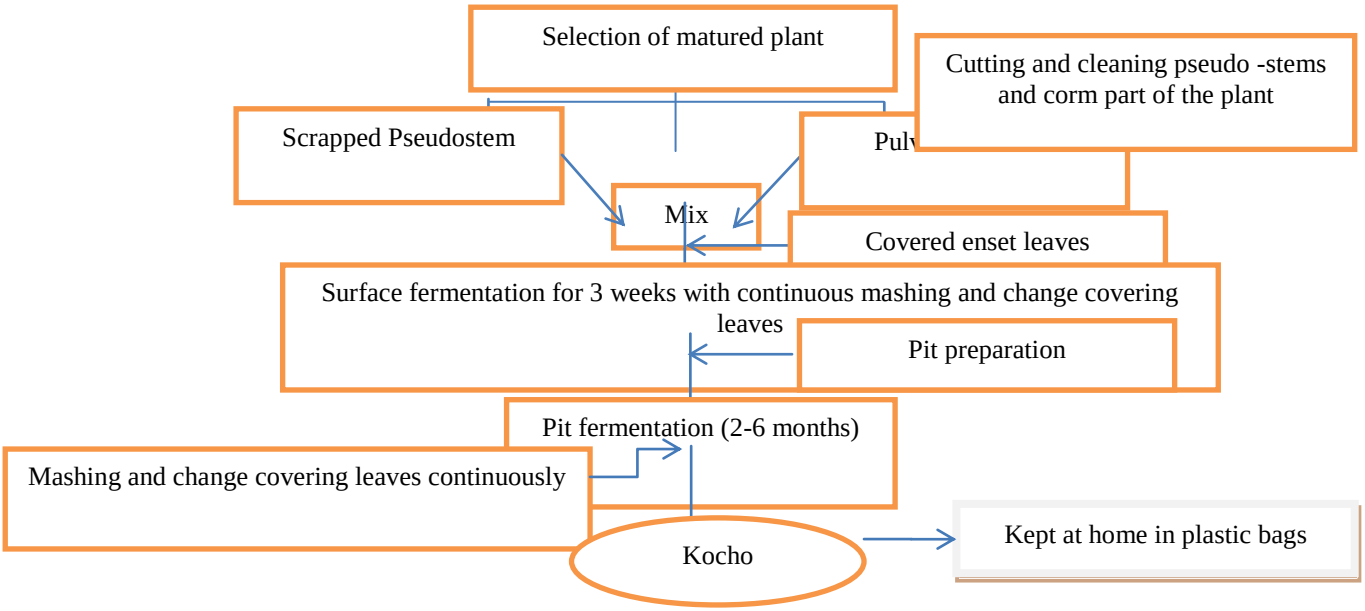
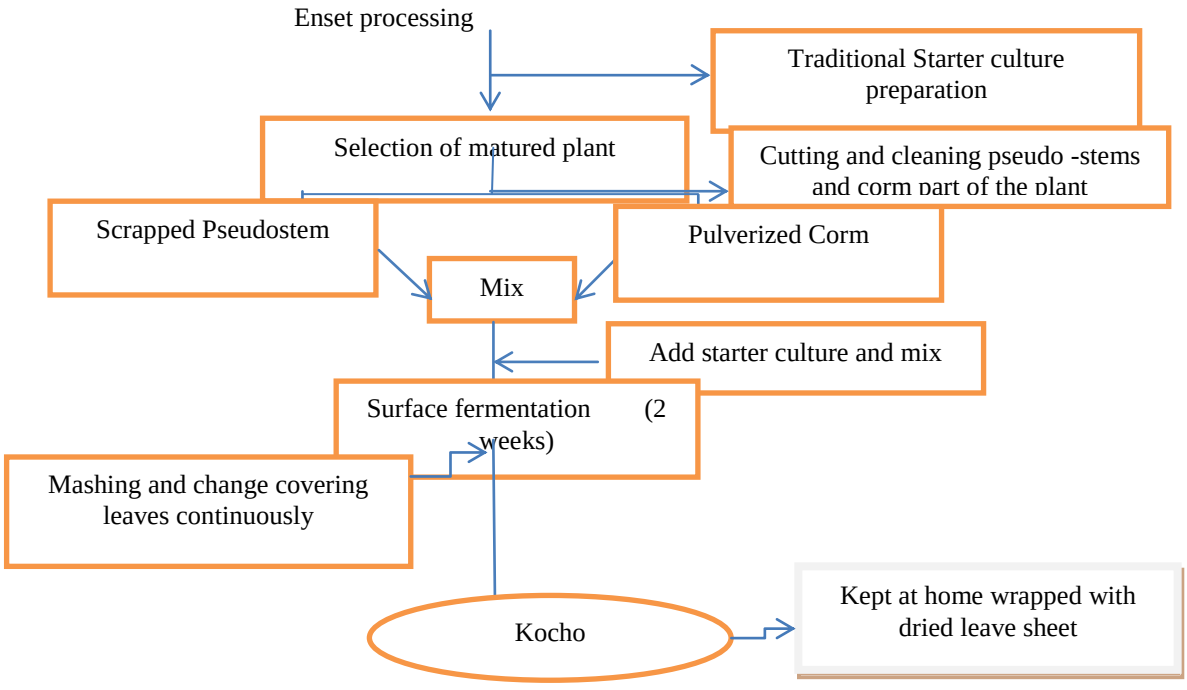
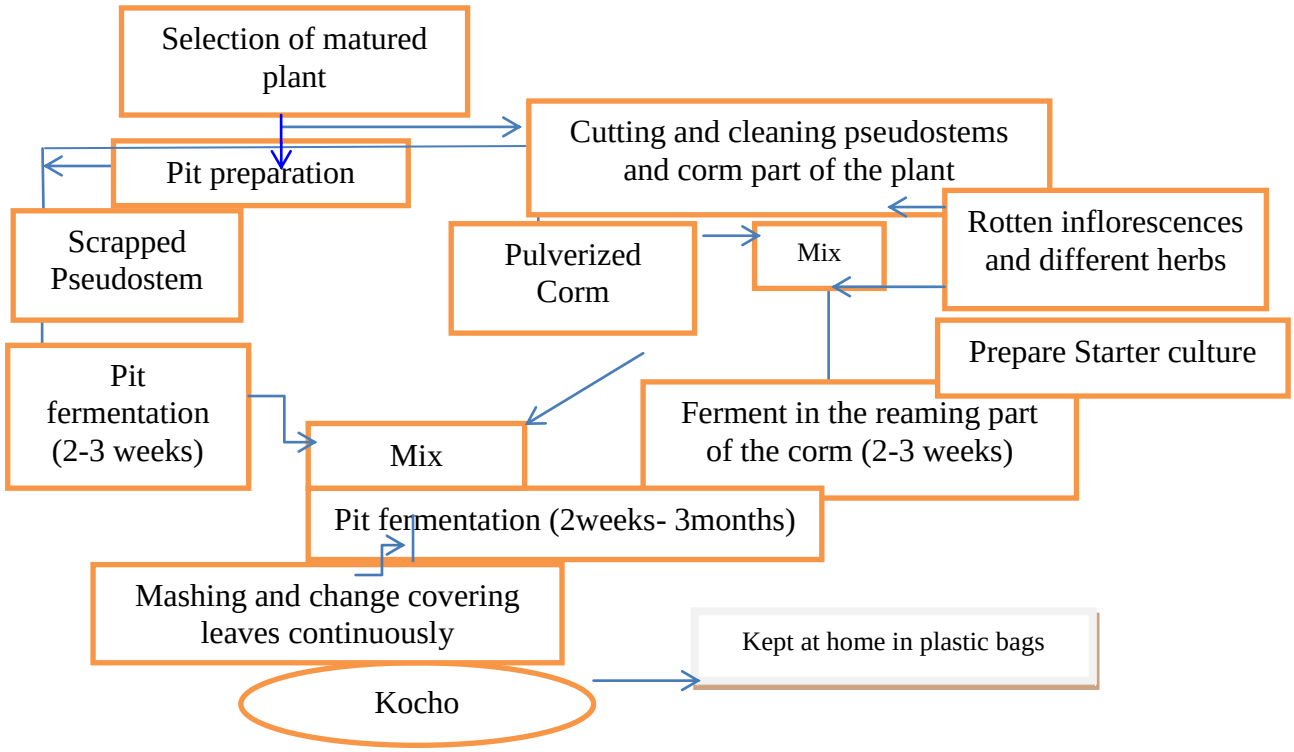


Supplementary 1: Diagrammatic representationof kocho production process in Ginchi



Supplementary 3. Diagrammatic representation of kocho production process in Gedeo zone



Supplementary 4. Diagrammatic representation of kocho production process in Woliso

Supplementary 2. Fermented kocho storage in plastic bags

Supplementary 5. Preservation of fermented kocho with dried leaf sheets