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Phenotypic and genotypic characterization of lactic acid bacteria isolated from spontaneously fermented vegetable amaranth

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Lactic acid bacteria (LAB) are Gram-positive, non-spore-forming, catalase-negative cocci or rod-shaped bacteria that produce lactic acid as a major fermentation product. They are also involved in the production of fermented foods. They have applications in industry and human health, such as food preservation and probiotics. The aim of this research was to isolate, characterize, and classify indigenous lactic acid bacteria from fermented vegetable amaranth, a leafy vegetable native to Africa. The isolates' 16S rRNA gene was amplified using bacterial universal primers 27F and 1492R. From fermented vegetable amaranth, a total of 15 LAB were isolated were grouped into the genera *Lactobacillus*, *Lactococcus*, and *Weissella* based on 16S rRNA gene analyses. *Lactobacillus plantarum* dominated vegetable amaranth fermentation, accounting for 60% of all isolates.

Key words: Lactic acid bacteria, vegetable amaranth, fermentation, 16S rRNA.

INTRODUCTION

Fermentation is one of the oldest food processing techniques. It is a technology that millions of people in the developing world depend on to preserve their food at prices that are affordable to the average consumer (Kalui et al., 2010). Fermented food items are a subset of foods that are distinguished by various carbohydrate breakdowns in the presence of probiotic microorganisms (Mulaw et

al., 2019). Africa has a wide variety of fermented foods which include plant-based products from maize, sorghum, millet and cassava (Franz et al., 2014). It has been suggested that vegetables are a good source of lactic acid bacteria growth (Wu et al., 2020). Vegetables also have high contents of vitamins, minerals, dietary fiber, and antioxidant compounds (Wu et al., 2020).

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Fermented foods are now known not only as a source of nutrients, but also as functional foods that offer health benefits against food-borne illnesses in addition to their nutritional value (Mulaw et al., 2019). Fermented vegetables contain a variety of microorganisms, including LAB, which produce lactic acid and, in rare cases, bacteriocins, which are essential for food preservation (Viridiana et al., 2018). In recent years, the probiotic potential of LAB isolated from fermented vegetable has been investigated (Viridiana et al., 2018). The microorganisms present during vegetable fermentation are very diverse and may considerably affect the quality and safety of the final product (Bautista-Gallego et al., 2020). The microbiota that is initially present in lactic acid fermentation processes comes primarily from the material, though other factors such as brines, ingredients used, and the industry's environment have an effect (Bautista-Gallego et al., 2020).

Lactic acid bacteria are a group of Gram-positive, aerotolerant, acid-tolerant, generally non-sporulating rod or cocci organisms that play an important role in food fermentation by inhibiting pathogenic microorganisms (Anjum et al., 2014). Lactic acid bacteria are present in a wide range of environments, including plant materials, various animal products, human and animal gastrointestinal and urogenital tracts, soil, and water (Ruiz Rodríguez et al., 2019). Lactic acid bacteria (LAB) use the Embden Meyerhof Parnas (EMP) pathway to ferment sugars and generate lactic acid as a final product (Anjum et al., 2014). Homofermentative lactic acid bacteria produce only lactic acid as a major product of fermentation, whereas heterofermentative lactic acid bacteria produce CO₂, hydrogen peroxide, acetic acid, and alcohol in addition to lactic acid (Anjum et al., 2014). Organic acids and other low molecular weight substances produced by these microorganisms have been shown to improve food nutritional, sensory, technical, as well as protection and shelf-life properties (Oguntoyinbo et al., 2016a). Due to their versatile metabolism, LAB has been widely used as starter cultures and probiotics for these purposes (Naeem et al., 2012; Ruiz Rodríguez et al., 2019). Heterofermentative microorganisms usually perform the first steps of vegetable fermentation, producing lactic and acetic acids, which contribute significantly to the final product's flavor and aroma (Breidt et al., 2013). Then, because of their ability to produce lactic acid, which causes a greater decrease in pH but prevents the growth of other microbial classes, they are replaced by more acid-tolerant homofermentative microorganisms (Montet et al., 2014).

*Amaranth*s are a diverse group of food crops with some grown as edible grains and others as edible leaves (Achigan-Dako et al., 2014). Amaranth belongs to the family Amaranthaceae (Zehring et al., 2015). Vegetable amaranth is one of the most widely consumed vegetables in Asia and Africa, and it plays a significant role in the supply of essential proteins and minerals (Achigan-Dako

et al., 2014). The consumption of amaranth as a vegetable is mainly exclusive in Africa and Asia, whereas the grain is popular all over the world (Zehring et al., 2015). Amaranth leaves contain secondary plant metabolites such as saponins, flavonoids, betalains, tannins, etc. (Zehring et al., 2015).

Smallholder farmers in rural and peri-urban areas in Africa depend heavily on indigenous leafy vegetables for food protection. Post-harvest losses, on the other hand, may be as high as 50% due to poor production conditions (Abukutsa-Onyango, 2007). Although fermentation of vegetables, particularly cabbage, is common in Europe, fermentation of leafy indigenous vegetables is not yet common in Africa (Oguntoyinbo et al., 2016a). As a result, there appears to be a case for stepping up efforts in Africa to research and introduce this form of biological preservation system for leafy vegetables (Oguntoyinbo et al., 2016a). As a result, spontaneous fermentation was produced and monitored in this study to identify the LAB that enable fermentation and can be used as a viable approach for the preservation and enhancement of the protection of African leafy vegetables.

MATERIALS AND METHODS

Sampling and preparation of sampling materials

Amaranthus dubius was obtained from Agrifood Organics, Ruiru, Kenya. The plant was cultivated for six to eight weeks (22-30°C). Hand-picking was used to harvest the vegetables, which were then shipped to the laboratory for processing. The leaves were destemmed, washed with tap water, and air-dried with paper towels on a clean and sterilized work bench.

Fermentation of vegetable amaranth

Fermentation was performed in 5-L stainless steel buckets. 1 kg of leaves and 3 L of salt and sugar solution were used. The solution consisted of a combination of salt and sugar, 3.0% each. Common table salt and retail sugar were purchased at local stores in Kenya. These components were sterilized by autoclaving for 15 min at 121°C. Weights were used to hold all plant material below the surface of the liquid. Inoculation and sampling were done in a sterile setting. The fermentations were done in duplicates at a constant temperature of 25°C (Stoll et al., 2021).

Sampling and analysis of fermentation brine

Samples were taken and analyzed for pH and microbial counts at 0, 24, 48, 72, and 144 h on MRS and M17 agar plates. The microbial diversity was investigated using amplicon sequencing of the 16S rRNA gene. 1 ml samples from different stages of vegetable amaranth fermentation brine were added to 9 ml quarter-strength Ringer's solution and vortexed to isolate LAB. To obtain the typical LAB associated with fermenting vegetable amaranth, the samples were further diluted in a 10-fold dilution sequence and 10 µl aliquots were spread-plated onto MRS agar (De Man, Rogosa, and Sharpe, M641) and M17 (M929) agar. Under aerobic conditions, plates were incubated at 30°C for 24-48 h (Stoll et al., 2021). For further characterization, colonies were selected at random from the highest

dilution agar plates. The strains were then grown aerobically in MRS broth at 30°C and streaked to ensure purity. All media were purchased at Himedia (Mumbai, India). The isolates stock cultures were stored in MRS broth with 20% glycerol at -80°C (Abdou et al., 2018). The isolates were divided into groups based on phenotypic and biochemical characteristics, and their identity was confirmed through 16S rRNA gene sequencing.

Phenotypic characterization

Presumptive lactic acid bacteria were phenotypically identified using phase-contrast microscopy at 100x magnification (Shimadzu CX41, Japan), as well as standardized tests including catalase activity, gas output from glucose in MRS broth, growth at different NaCl concentrations (4 and 6.5%), and growth at different temperatures (15 and 45°C).

Determination of cell morphology and Gram status

Overnight cultures were introduced on microscopic slides and visualised under a light microscope at 100x magnification. Gram status was determined using 3% KOH as described by Mulaw et al. (2019).

Catalase test

Overnight cultures were introduced on a microscopic glass slide and two drops of 3% hydrogen peroxide added and thoroughly blend as described by Mulaw et al. (2019). The development of gas bubbles during a positive catalase test indicates that the test bacterium is producing catalase enzyme. The absence of gas bubbles indicates a negative catalase test.

Gas production from glucose fermentation

The aim of this test was to determine the homofermentative and heterofermentative properties of LAB isolates. Inverted Durham tubes with 1% glucose were used to measure CO₂ output from glucose in modified MRS broth. Separately, 50 µl LAB culture was inoculated with 9 ml MRS broth in separate tubes containing 1% glucose and inverted Durham tubes. The test tubes were then incubated for 5 days at 30°C. The presence of gas bubbles in Durham tubes over the course of 5 days indicated that the isolates produced CO₂ from glucose fermentation (Mulaw et al., 2019).

Growth at different temperatures

Growth at 15 and 45°C are the most frequently used for the classification of bacilli. To determine the growth at given temperatures, the MRS broth was used. 50 µl of overnight cultures were inoculated into 9-ml test media, incubated at 15 and 45°C and observed for five days for color and growth. Negative control was set using a 9-ml test tube containing the broth without inoculating with the LAB cultures (Mulaw et al., 2019).

Growth at different NaCl concentrations

The resistance of LAB isolates to various NaCl concentrations was tested. For this purpose, 4 and 6.5 % NaCl concentrations were used for testing. Test tubes containing 5 ml of modified MRS broth containing bromocresol purple indicator were prepared according to

the concentrations needed and were inoculated separately with 50 µl of each overnight culture. The Test tubes were then incubated at 30°C for 5 days (Mulaw et al., 2019).

Genotypic characterization

Genomic DNA was extracted from overnight cell cultures grown in MRS broth using Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, USA) according to the manufacturer's protocols. The final DNA concentration and purification were determined using a NanoDrop spectrophotometer (PCR^{max} Lambda, Staffordshire, United Kingdom) and DNA quality was checked by 1% agarose gel electrophoresis. PCR amplification of 16S rRNA gene for presumptive LAB strains was done using bacterial universal primers 27 F:5'-AGA GTT TGA TCC TGG CTC AG-3' and 1492 R:5' GGT TAC CTT GTT ACG ACT T-3'. PCR was performed in a 50-µl reaction containing 25 µl One Taq® 2X Master Mix with standard buffer (New England Biolabs), 1 µl forward primer, 1 µl reverse primer, and 22 µl RNase free water. Then 49 µl of the mixture was added into a sterile PCR tube, and 1 µl of gDNA was added and used as a template. The condition of amplified gene fragment: pre-denaturation of the target DNA at 96°C for 4 min followed by 30 cycles at 94°C for 1 min, primer annealing at 51.5°C for 1 min, and 30 s and primer extension at 68°C for 8 min. PCR was completed with 10 min elongation at 68°C followed by cooling to 4°C. The reactions were carried out in a thermal cycler (ProFlex PCR systems). The size of the 16S rRNA gene PCR products was confirmed by electrophoresis on a 1% (w/v) agarose gel stained with GelRed and visualized using a Uvitec Cambridge gel documentation system (Uvitec, UK). PCR products were purified using the QIAquick PCR purification Kit (Qiagen, Germany) according to the manufacturer's instructions. The purified amplicons were Sanger sequenced at Human Genomics Macrogen Europe (Macrogen Europe B.V, Amsterdam, Netherlands).

Phylogenetic analysis

The 16S rRNA gene sequences of the bacterial isolates were viewed for quality checks and edited using ChromasPro 2.1.8 software package. They were then compared with available standard sequences of bacteria lineages in the public nucleotide sequence databases in the National Center for Biotechnology Information (NCBI) using nucleotide blast (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to find closely related bacterial 16S rRNA gene sequences. The 16S rRNA gene sequences of the isolates and those of the unknown closely related bacteria strains were aligned using MEGA (Molecular Evolutionary Genetics analysis) 7.0 software package (<https://www.megasoftware.net>) and phylogenetic trees were constructed using Maximum Likelihood method based on the Kimura 2-parameter model (Kimura M 1990) with MEGA (Molecular Evolutionary Genetics analysis) 7.0 software package (Kumar et al., 2016) (<https://www.megasoftware.net>). The tree topologies were evaluated using the bootstrap resampling method (Felsenstein, 1985) based on 1000 replicates. *Escherichia coli* was used as an outgroup.

Statistical microbial analysis

The microbial enumeration results were expressed as the mean and standard deviation of duplicate experiments of the counts and sampling sites using two-way ANOVA (GraphPad Prism version 8.4.2 software, GraphPad LLC, San Diego, California, USA), at a significance level of p<0.05.

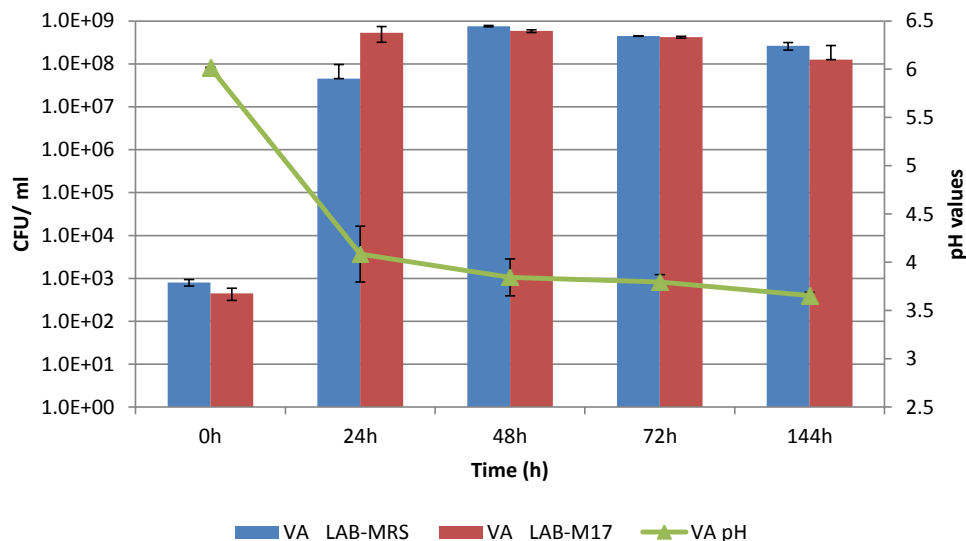


Figure 1. Mean counts and standard deviation (CFU/ml) of lactic acid bacteria of the vegetable amaranth fermentation brine on MRS (orange) and M17 (blue) agar plates. Also, the mean pH values and standard deviations are given (grey). On the basis of duplicate independent fermentations, mean counts were determined. VA stands for vegetable amaranth.

RESULTS

Selection and enumeration of presumptive lactic acid bacteria

The microbial counts varied between the sampling points (Figure 1). The highest microbial counts were recorded between 24-48h. The results also showed a decrease in microbial counts between 72-144h. The ability of LAB to acidify during fermentation is reflected in the pH production. Within 24h of fermentation, the pH had reduced significantly from a pH 6.0 to pH 4.0. Within 48 h, the pH had reduced to pH 3.8, and by the end of the fermentation, it had dropped to pH 3.6. The counts of LAB on MRS and M17 agar were log 2 CFU/ml at the beginning of the fermentation and increased to log 7 and 8 CFU/ml after 24 h of fermentation, respectively. From 24 h to the end of the fermentation, the LAB counts ranged from log 7 to log 8.9 CFU/ml, suggesting that LAB constituted the majority of the isolates on these media (Figure 1). Classic macroscopic techniques of color, type, shape, and elevation of pure colonies were used to morphologically characterize presumptive LAB. Most colonies were able to grow within 2-4 days of incubation at 30°C. Gram's reaction, form, and catalase activity of bacterial species were also investigated. All of the isolates were Gram-positive and catalase-negative. The presumptive LAB strains are shown in Table 1.

Phenotypic characterization

In total, 15 Gram-positive, catalase-negative presumptive

lactic acid bacteria were isolated from fermentation brine samples using MRS and M17 agar. Eight coccus-shaped isolates and seven rod-shaped isolates were among the presumptive lactic acid bacteria.

Morphological and physiological characterization

All isolates were identified according to their morphological and physiological properties (Table 1) and were able to grow at 4% NaCl salt concentration. Only 10 were found growing at 6.5% NaCl concentration. Examining the potential of the isolates to grow at 15 and 45°C showed that all isolates were able to grow at 15°C and only 12 isolates grew at 45°C. From the 15 isolates, 8 isolates produced gas from glucose. Thus, 8 isolates were found to be heterofermentative and 7 isolates were homofermentative (Kostinek et al., 2008).

Identification and phylogenetic analysis of isolates

Isolates were blasted on the Blastn search and showed similarities with percentage identities of 98 to 100% (Figure 2). Based on the Blastn search performed against the GenBank and phylogenetic analysis, nine isolates NS489A (MF992229.1), NS442B (MF992227.1), N8481(MN420754.1), N8243 (MT613638.1), M444B (KX057551.1), M723B (MF992227.1), JK248A (MN640561.1), JK 248B (MF992227.1) and JK444A (KX649074.1) were affiliated with *Lactobacillus plantarum*, Four isolates, JK482 (MT613468.1), JK481 (MT613505.1), JK721 (MT613505.1) and JK487

Table 1. Colony morphologies and molecular identities of lactic acid bacteria strains.

Sample ID	Cell shape	Gram stain	Catalase	Closest relative	% Identity	Accession number	GC content
JK 482	Rods	+	-	<i>Weissella cibaria</i>	100	MT613468.1	46
JK 481	Rods	+	-	<i>Weissella cibaria</i>	99.63	MT613505.1	47
JK 444A	Rods	+	-	<i>Lactobacillus plantarum</i>	100	KX649074.1	52
JK 248B	Rods	+	-	<i>Lactobacillus plantarum</i>	100	MF992227.1	52
JK 248A	Rods	+	-	<i>Lactobacillus plantarum</i>	98.94	MN640561.1	51
JK 244	Cocci	+	-	<i>Lactococcus garvieae</i>	98.22	MT611574.1	51
M 723B	Rods	+	-	<i>Lactobacillus platarum</i>	99.06	MF992227.1	49
M 444B	Rods	+	-	<i>Lactobacillus plantarum</i>	99.27	KX057551.1	50
JK 721	Cocci	+	-	<i>Weissella cibaria</i>	99.82	MT613505.1	37
JK 487	Rods	+	-	<i>Weissella cibaria</i>	100	MT613505.1	50
N8 243	Rods	+	-	<i>Lactobacillus plantarum</i>	99.72	MT613638.1	48
N8 729	Cocci	+	-	<i>Lactococcus garvieae</i>	100	MT604790.1	51
N8 481	Rods	+	-	<i>Lactobacillus plantarum</i>	96.75	MN420754.1	48
NS 442B	Rods	+	-	<i>Lactobacillus plantarum</i>	98.67	MF992227.1	50
NS 489A	Rods	+	-	<i>Lactobacillus plantarum</i>	99.76	MF992229.1	48

(MT613505.1) were affiliated with *Weissella cibaria*, and two isolates, JK244 (MT611574.1) and N8729 (MT604790.1) were affiliated with *Lactococcus garvieae*.

DISCUSSION

Fermented foods have a long history in Africa and are found all over the continent. Fermentation is a popular processing method for extending shelf life, increasing micronutrient availability, improving palatability, and improving digestibility (Wafula et al., 2016).

Amaranths, or *Amaranthus* spp., are a non-grass plant belonging to the *Amaranthaceae* family. Plants in this genus are important not only as vegetable and grain crops, but also as a source of vegetable protein for dry-land agriculture (Niveyro et al., 2012). Amaranth vegetable is one of the most popular leafy vegetable in Africa (Achigan-Dako et al., 2014). In many temperate and tropical regions, amaranth is widely grown as a green, leafy vegetable and grain crop. Amaranths have C₄ photosynthesis and develop quickly in hot, dry environments. They also tolerate a wide range of unfavorable abiotic conditions, such as high salinity, acidity, or alkalinity, making them ideal for subsistence farming. Amaranth has the potential to have a positive effect on malnutrition if it is implemented (Maughan et al., 2011). Amaranths contain high contents of essential micronutrients such as calcium, iron, vitamin c, folic acid and b-carotene hence they have excellent nutritional value (Achigan-Dako et al., 2014).

Lactic acid bacteria are a group of microorganisms that produce lactic acid as a result of carbohydrate fermentation. They are normally considered microorganisms with no pathogenic activities. They are widely

used in the production of fermented dairy and non-dairy food products such as yoghurt (*Streptococcus* spp and *Lactobacillus* spp), cheese (*Lactobacillus* spp and *Lactococcus* spp), and sauerkraut (*Leuconostoc* spp) (Biosci et al., 2014). The vegetable amaranth was chosen for this study because it is an indigenous vegetable that is high in micronutrients, as well as being readily available and inexpensive. The minerals and vitamins content in AILVs are higher than those present in most exotic vegetables (Gido et al., 2017). In this study, we investigated the natural fermentation of the AILV vegetable amaranth in Kenya.

The study design was chosen to determine the dominance of lactic acid bacteria involved in the natural fermentation of this vegetable. Vegetable amaranth leaves fermented successfully in a fermentation brine containing 3% sugar and 3% salt. The aim of this study was to discover the diversity of the LAB population found in fermented vegetable amaranth. In present study, LAB counts were log 2 at the beginning of the fermentation and increased to log 7 and 8 respectively by 24h of fermentation. This was because, within the first 24h of the fermentation, the bacteria were at the exponential or log phase of growth whereby the cells are dividing and doubling in number. The LAB counts ranged from log 7 to log 8.9 from 24h of fermentation till the end of the fermentation. In line with this growth, there was a reduction in pH from pH 6.0 to pH 4.0 within the first 24h, and the pH further decreased to pH 3.6 by 144h of the fermentation. Lactic acid bacteria are acid-tolerant and produce acid and bacteriocin which reduces the pH and also inhibits the growth of pathogenic organisms (Masud and Anwaar, 2002; Oguntoyinbo et al., 2016a; Oluwajoba et al., 2013). A pH value of 4.2 or less is regarded as an important factor for food safety (Holzapfel, 2002).

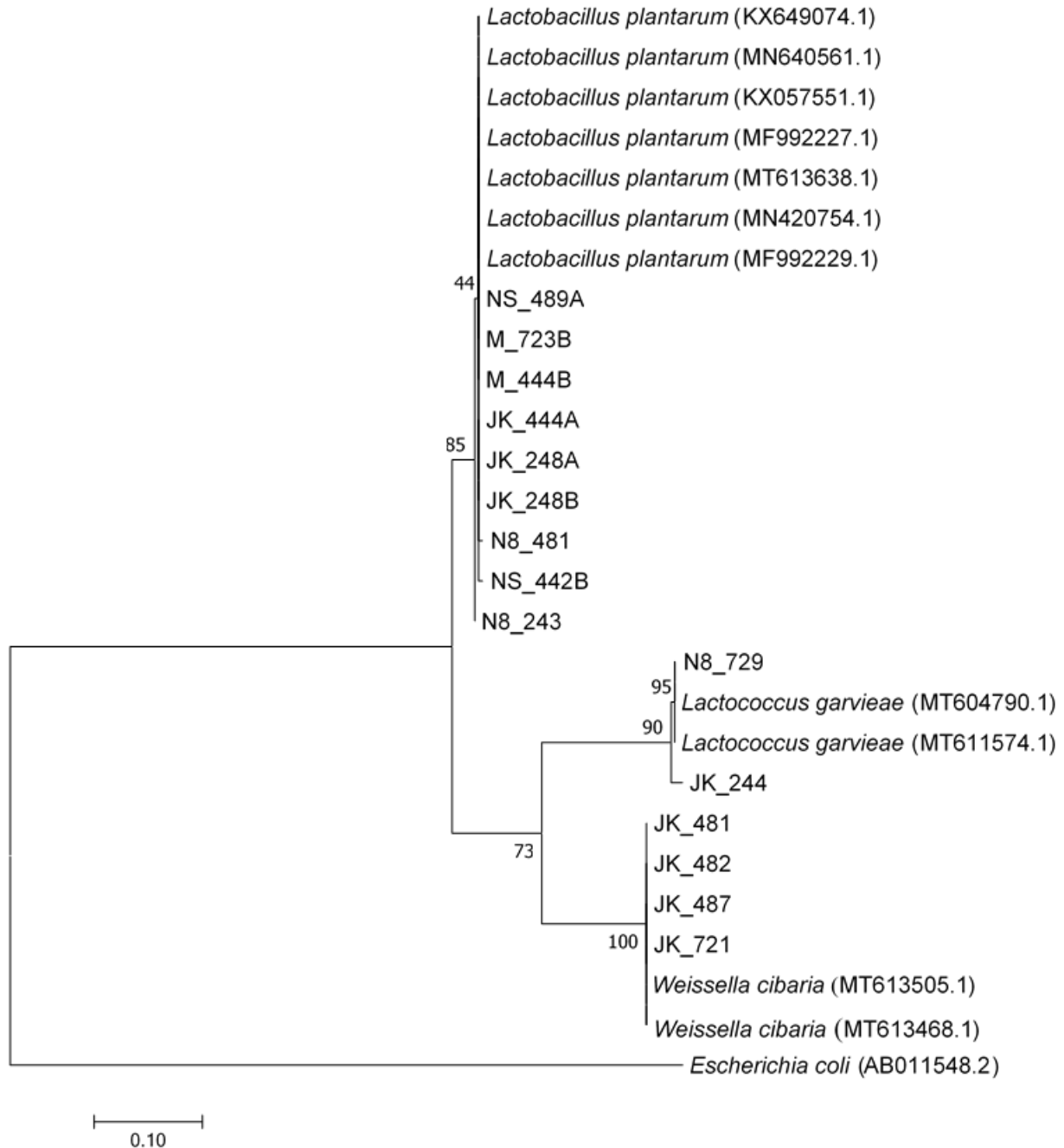


Figure 2. Phylogenetic tree based on 16S rRNA gene sequences showing the relationship among the lactic acid bacterial isolates and between representatives of other related taxa. The scale bar indicates 0.10 substitutions per nucleotide position. The number beside the node is the statistical bootstrap value. Besides the bacterial name is the GenBank accession numbers.

Reduction of pH due to lactic acid bacteria fermentation between underneath 4.0 is critical to hinder the development of various pathogenic organisms (Oguntoyinbo et al., 2016a).

The 16S rRNA gene sequencing showed that the fermentation batches were characterized by a diverse microbiota consisting of strains belonging to the genus

Lactobacillus, *Weissella* and *Lactococcus* (Oguntoyinbo et al., 2016a) and also found that spontaneous fermentation of African kale leaves was rather variable. The sequence data based on a constructed phylogenetic tree identified the isolates as *L. plantarum*, *L. garvieae*, and *W. cibaria*. Nine isolates were affiliated with *L. plantarum*. Four isolates were affiliated with *W. cibaria*

which have been described by Kang et al. (2016) as a Gram-positive, non-pore-forming, non-motile, hetero lactic acid-fermenting, and catalase-negative bacillus that cannot produce dextran from sucrose. Two isolates were affiliated with the genus *Lactococcus* with all two isolates associated with *L. garvieae*. Some genera of LAB isolated from this work like *Lactobacillus* and *Weissella* were also isolated in the previous study of Park et al. (2010) involving the use of 16S rRNA gene sequencing analysis to identify LAB diversity from fermented kimchi (a vegetable dish in Korea). According to the work carried out by other authors, Corsetti et al. (2001), Emerenini (2013), Sánchez et al. (2000), *L. plantarum* in plant materials was dominating the LAB flora. Similar to most other studies on non-dairy fermentation, *L. plantarum* strains were the LAB most dominant in our study (60% isolates). In addition, the study of LAB throughout the fermentation period clearly showed that *L. plantarum*-group strains occurred throughout the fermentation period (Huch et al., 2008). *L. plantarum* is the most common species found in fermented vegetables, owing to its ability to withstand the high saline and acidity content of fermented vegetables such as cucumber, sauerkraut, and olives (Behera et al., 2018). The results of the present study showed strains of *Lactobacillus* species from traditional fermented vegetable amaranth is important and more recurrent in vegetable amaranth fermentation and these findings can help us to have a better fermentation of this vegetable.

Conclusion

This work provides a microbiological and molecular study of fermented vegetable amaranth. The results of this study showed which strain of lactic acid bacteria is present in the fermentation of vegetable amaranth. Lactic acid bacteria were isolated from the fermentation of vegetable amaranth. The molecular results identified the lactic acid bacteria as *L. plantarum*, *W. cibaria*, and *L. garvieae* with *L. plantarum* being the most abundant species present. The purpose of this study was to use 16S rRNA sequencing to profile and taxonomically identify bacteria isolated from fermented vegetable amaranth. *L. plantarum* was the most dominant preceding lactic acid bacteria followed by *W. cibaria* and finally *L. garvieae*. According to the findings of this study, fermented vegetable amaranth contains a variety of lactic acid bacteria organisms. These bacterial strains could be used in a variety of industrial and commercial settings. As a result, further research is required to evaluate the isolates' functional properties as potential probiotics and starter cultures. Specifically, the molecular and metagenomic procedures would allow for a thorough examination of the function of LAB during fermentation, as well as microbial variations during processing, geographical and varietal effects.

Ethical approval

This study did not use human or ethical subjects, and as such, ethical approval was not required.

CONFLICT OF INTERESTS

The authors have not declared any conflicts of interests.

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