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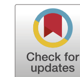

Fermentation of African nightshade leaves with lactic acid bacterial starter cultures

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ABSTRACT

The interest in the consumption of African indigenous leafy vegetables increased in African countries, e.g. Kenya, within the last years. One example of African indigenous leafy vegetables is African nightshade (*Solanum scabrum*) which is nutritious, rich in proteins and micronutrients and therefore could contribute to a healthy diet. African nightshade has several agricultural advantages. However, the most important disadvantage is the fast perishability which leads to enormous post-harvest losses. In this study, we investigated the fermentation of African nightshade as a post-harvest processing method to reduce post-harvest losses. The two lactic acid bacterial starter strains *Lactiplantibacillus plantarum* BFE 5092 and *Limosilactobacillus fermentum* BFE 6620 were used to inoculate fermentations of African nightshade leaves with initial counts of 10^6–10^7 cfu/ml. Uninoculated controls were conducted for each fermentation trial. Fermentations were performed both in Kenya and in Germany. The success of the inoculated starter cultures was proven by the measurement of pH values and determination of lactic acid concentration. Lactobacilli strains dominated the microbiota of the starter inoculated samples in contrast to the non-inoculated controls. This was supported by classical culture-dependent plating on different microbiological media as well as by the culture-independent molecular biological methods denaturing gradient gel electrophoresis and 16S rRNA gene high-throughput amplicon sequencing. We could demonstrate that the use of the selected starter cultures for fermentation of African nightshade leaves led to controlled and reliable fermentations with quick acidification. Thus, controlled fermentation with appropriate starter cultures is a promising method for post-harvest treatment of African nightshade leaves.

1. Introduction

Hunger is increasing in almost all African regions and the prevalence of undernourished people is at almost 20% (FAO et al., 2019). However, hunger may not only be characterised by a lack of calories but also by micronutrient deficiencies. This so-called hidden hunger affects about 2 billion people globally (Burchi et al., 2011; Grebmer et al., 2014; Tulchinsky, 2010) with the majority of the affected found in Sub-Saharan Africa (Muthayya et al., 2013). The World Health Organization advises consumption of 400 g of fruit and vegetables per day (WHO, 2004). However, the average consumption in Sub-Saharan African countries is far below these recommendations (Ruel et al., 2005).

The consumption of African Indigenous Leafy Vegetables (AILV) could contribute to enhance the uptake of micronutrients. AILV have several advantages, i.e. they present a source of cheap proteins, minerals and vitamins and therefore contribute to a healthy diet. AILV are more nutritious and contain more vitamins and minerals compared to their exotic counterparts e.g. cabbage (Wafula et al., 2016). Moreover, they have medicinal properties and have been cultivated in Western Kenya for a long time (Abukutsa-Onyango, 2007). They also show important agricultural advantages like resistance to pests and diseases and they can be cultivated during the dry season when other crops fail to grow (Aworh, 2018). However, the production of AILV has been neglected in Western Kenya due to several factors: major drawbacks are poor seed quality, drought, poor marketing strategies and postharvest losses due to perishability (Abukutsa-Onyango, 2007). Postharvest losses of AILV in Kenya can amount up to 50% (Gogo et al., 2016). Furthermore, negligence and lack of awareness contribute to the low production levels in

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Western Kenya (Mnzava, 1997). However, within the last years, the interest in and acceptance of the consumption of AILV increased in Kenya and other African countries (Aworh, 2018). The postharvest processing technologies that can be applied to AILV comprise, e.g. air-drying, sun-drying, blanching and fermentation (Muchoki et al., 2007). Fermentation, a thousands of year old method of food preservation, cannot only extend the shelf-life and improve the safety but could also improve palatability and sensory characteristics of food products (Holzapfel, 1997). Fermentation may also improve micronutrient bioavailability and enrichment of vitamins depending on the responsible bacterial strains (Holzapfel, 2002). In addition, food fermentation has been acknowledged by the Food and Agriculture Organization of the United Nations (FAO) as an important postharvest processing method especially for developing countries (Deshpande et al., 2000). In Africa, food fermentation is commonly conducted on a small scale, household-level which is characterised by the use of simple, non-sterile equipment, chance or natural inocula and unregulated conditions resulting in food of unpredictable quality and sensory fluctuations (Wafula et al., 2016).

Fermentation can be improved and regulated by the use of starter cultures. Especially lactic acid bacterial starter (LAB) cultures play a major role in the production of safe and healthy foods due to the production of lactic acid and several metabolites, e.g. acetic acid, hydrogen peroxide and bacteriocins (Holzapfel, 2002). There is limited information on the use of LAB starter cultures for the fermentation of AILV in Sub-Saharan Africa. However, some studies dealt with the development of starter cultures for other traditionally fermented African food products such as the fermented cassava products Gari (Huch et al., 2008) and Kivunde (Kimaryo et al., 2000). Unlike the fermentation of milk and of starchy roots like cassava, maize, sorghum and millet (Franz et al., 2014), the fermentation of vegetables is not common in Africa (Oguntoyinbo et al., 2016c). Muchoki et al. (2007) and Kasangi et al. (2010) reported that the fermentation of cowpea leaves in combination with solar-drying is a promising method for small scale producers to enhance the storability and nutritive quality.

In our study, we investigated the fermentation of AILV, i.e. African nightshade (*Solanum scabrum*) leaves with LAB starter cultures. African nightshade is one of the most commonly consumed AILV in East and West Africa (Wafula et al., 2016) and is an excellent source of minerals especially potassium and iron, proteins and carotenoids (Kamga et al., 2013). We selected well-characterised LAB strains from previously fermented African food products (Huch et al., 2008; Mathara et al., 2004) as starter cultures in order to obtain reliable fermentations of consistent quality and to reduce postharvest losses. The ability of the applied starter cultures to establish themselves as dominant lactic acid bacterial populations during the fermentation was investigated both on a phenotypic and a molecular level including 16S rRNA gene high-throughput amplicon sequencing.

2. Material and methods

2.1. Growth and preparation of plant material

Seeds of African nightshade (*Solanum scabrum*) were obtained within the collaborative research project HORTINLEA (Horticultural Innovation and Learning for Improved Nutrition and Livelihood in East Africa) from the AVRDC (World Vegetable Centre, Arusha, Tanzania). At the Max Rubner-Institut (MRI) in Germany, plants were cultivated in low fertilized peat-based substrate in a climatic chamber for four weeks at 20/25 °C day/night temperature and 40–70% day/night relative humidity with 12.5 h of artificial light. Then plants were transferred to a greenhouse for three to four weeks under conditions of 18–40 °C day/night temperature, 18–77% day/night relative humidity with additional light provided to compensate for daylight intensity variations. At the Jomo Kenyatta University of Agriculture and Technology (JKUAT) in Kenya, plants were cultivated in open fields for six to eight weeks (17–28 °C, 40–50% relative humidity). At both locations, leafy vegetables were harvested by hand-picking and delivered to the laboratory for processing. The leaves were washed with tap water and air-dried in a salad spinner.

2.2. Preparation of starter inoculum

The starter strains *Lactiplantibacillus* (*La.*) *plantarum* BFE 5092 and *Limosilactobacillus* (*Li.*) *fermentum* BFE 6620 are LAB strains which were previously isolated from fermented African products (Huch et al., 2008; Mathara et al., 2004). It should be noted that the *Lactobacillus* taxonomy was revised recently (Zheng et al., 2020) and that both starter strains formerly belonged to the genus *Lactobacillus*. These strains show good acidification abilities, are able to produce bacteriocins (Cho et al., 2010a, 2010b; Kostinek et al., 2007) and are well characterised including whole genome sequencing (Oguntoyinbo et al., 2016a; Wafula et al., 2017). They do not harbour acquired antibiotic resistance genes which was checked with ResFinder 3.2 (Zankari et al., 2012). In addition, they were previously successfully applied in fermentation of the AILV African kale (Oguntoyinbo et al., 2016b). The strains were grown in MRS (De Man, Rogosa and Sharpe medium, Merck, Darmstadt, Germany) at 30 °C overnight. For the preparation of the inoculum, 2 ml of fresh overnight cultures were centrifuged at 13,414 ×*g*, 10 min, and washed twice with quarter-strength Ringer's solution (Merck KGaA, Darmstadt, Germany).

2.3. Fermentation of African nightshade leaves

Fermentations were performed in 5 l clay pots (MRI) or in 5 l stainless steel pots (JKUAT) with a combined inoculum of both starter cultures *La. plantarum* BFE 5092 and *Li. fermentum* BFE 6620. For this, 1 kg of leaves and 3 l of brine were used. The brine consisted of a combination of salt and sugar solution, 3.0% each. Common table salt and retail sugar were purchased at local stores both in Germany and Kenya. Weights were used to keep all plant material under the liquid surface. Inoculation and sampling were performed under sterile conditions. The starter-batches were inoculated with the combined inoculum of each approximately 10^6–10^7 cfu/ml. An uninoculated batch (control) was performed within each fermentation trial. The fermentations were carried out in triplicate at the MRI and in quadruplicate at the JKUAT (Table 1) with plant material from different cultivations for each fermentation trial. Fermentation pots were incubated at 25 °C (MRI) or ambient temperature of ca. 25 °C (JKUAT). Statistical analyses of pH values and $\log_{10}$-transformed microbial counts were performed using the R software (version 4.0.0) (R Core Team, 2019) by performing ANOVA analysis, followed by two-sided Bonferroni-corrected Tukey test. Statistical results of relevant pairwise comparisons are given in Supplementary File 1.

2.4. Sampling and analysis of fermentation brine

Samples were taken at 0, 24, 48, 72 and 144 h and analysed for pH value, microbial counts on different media and lactate concentration. Moreover, the microbial diversity was investigated using culture-independent methods, i.e. DGGE (denaturing gradient gel

Table 1
Overview of all fermentation trials performed with African nightshade.

Location	With starter cultures	Without starter cultures (control)
JKUAT, Kenya	NS 1.1	NS 1.2
JKUAT, Kenya	NS 2.1	NS 2.2
JKUAT, Kenya	NS 3.1	NS 3.2
JKUAT, Kenya	NS 4.1	NS 4.2
MRI, Germany	NS 7.1	NS 7.2
MRI, Germany	NS 8.1	NS 8.2
MRI, Germany	NS 9.1	NS 9.2

electrophoresis) and 16S rRNA gene high-throughput amplicon sequencing. Microbial counts were enumerated by plating 10-fold serially diluted fermentation brine on VRBD (Violet Red Bile Dextrose) agar for enterobacteria, Std1 (Standard1) agar for total aerobic, mesophilic bacteria, MRS (de Man, Rogosa and Sharpe) agar for LAB and malt glucose agar with addition of antibiotics (ampicillin 100 μg/ml, chloramphenicol 25 μg/ml, erythromycin 50 μg/ml, streptomycin 50 μg/ml, tetracycline 50 μg/ml, vancomycin 25 μg/ml) (MRI) or potato-dextrose supplemented with 10% tartaric acid (14 ml/l) (JKUAT) for yeasts and moulds. All media were purchased at Merck. The concentration of lactate was determined using the lactate test kit (Boehringer Mannheim/ R-Biopharm AG, Darmstadt, Germany) according to the instructions of the manufacturer.

The total DNA of the fermentation liquid was isolated according to Pitcher et al. (1989). For DGGE, a part of the 16S rRNA gene was amplified using the eubacteria universal primer pairs 338f GC (5′ACTCCTACGGGAGGCAGCAG3′) and 518r (5′GACGGGCGGTGTGTACA3′) for DGGE. Amplification was performed using a Primus 96 advanced thermal cycler with the following reagents: PCR buffer, $MgCl_2$ (25 mM) and Taq Polymerase (1.5 U) (Genaxxon Bioscience, Ulm, Germany), BSA (New England Biolabs, Frankfurt, Germany), dNTP mix (1.25 mM, Peqlab, Erlangen, Germany), 2.5 μl of each primer (Thermo Fisher Scientific, Ulm, Germany) and DNA template (100 ng). Reaction mixtures were subjected to a touchdown PCR: 94 °C 5 min; 10 × 94 °C 30 s, 62–52 °C 1 min, 72 °C 30 s; 22 × 94 °C 30 s, 56 °C 1 min, 72 °C 30 s and 72 °C 7 min. DGGE was performed on 8% polyacrylamide gels with a gradient of 35% to 70% urea using the DCode Electrophoresis Kit for DGGE and the DCode system (Bio-Rad, Munich, Germany). Electrophoresis was performed at 70 V, 60 °C for 16 h. After staining with SYBR gold, PCR products were visualized under a UV transilluminator (FluorChem 5500, Alpha Innotech, San Leandro, USA). DGGE patterns were analysed using the BioNumerics software version 7.6 (Applied Maths, Sint-Martens-Latem, Belgium). Clustering was performed with the Pearson correlation coefficient and the unweighted pair-group method using arithmetic averages clustering algorithm (UPGMA) (Sneath and Sokal, 1973).

The 16S rRNA gene high-throughput amplicon sequencing was performed and analysed as recently described (Weldemichael et al., 2019), based on the modified Illumina Sample Preparation Guide (Illumina, 2013):

Briefly, the V3 and V4 region of the 16S rRNA genes were amplified using the following primers modified with Illumina adapters: 16S Metafw (5′-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG- 3′) and 16S Metarev (5′- GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C- 3′) (Klindworth et al., 2013). PCR was carried out in 25 μl volumes containing 12.5 μl ALLin™ HotStart Taq Mastermix 2× (highQu GmbH), 1 μl of each primer (10 pmol/μl), 0.5 μl sterile water (Sigma-Aldrich Chemie GmbH, Germany) and 10 μl DNA template (5 ng/μl). PCR conditions were 95 °C 1 min, 20 cycles of 95 °C 15 s, 55 °C 15 s, 72 °C 15 s. PCR products were cleaned with Mag-Bind RxnPure plus magnetic beads (Omega Bio-tek Inc., USA). Index PCR was performed with Nextera XT index kit (Illumina, Inc., USA). PCR was carried out in 50 μl volumes containing 10 μl 5× HF buffer, 8 μl dNTP mix (1.25 mM), 5 μl Nextera XT primer 1 (N70X), 5 μl Nextera XT primer 2 (S50X), 5 μl purified PCR product, 0.5 μl Phusion Hot Start Flex DNA Polymerase (2 U/μl; New England Biolabs) and 16.5 μl sterile water. PCR conditions were 98 °C 3 min, 8 × 98 °C 30 s, 55 °C 30 s, 72 °C 15 s, 72 °C 5 min. PCR products were again cleaned with Mag-Bind RxnPure plus magnetic beads. DNA concentration was determined using the Qubit dsDNA HS Assay Kit on a Qubit 2.0 fluorometer (ThermoScientific, Darmstadt, Germany). Paired-end sequencing (2 × 300 bp) was performed on a MiSeq benchtop sequencer (Illumina, Inc., USA) according to the manufacturer's instructions with the supplementation of PhiX for quality issues and as an internal control.

The 16S rRNA gene high-throughput amplicon sequencing raw reads were analysed as follows: Reads were trimmed using a custom Perl script. Afterwards, the remaining reads were processed using mothur 1.40.0 (Schloss et al., 2009) according to the MiSeq SOP (Kozich et al., 2013). In brief, bi-directional reads were merged into contigs, while sequences containing any ambiguous bases or homopolymer regions over 12 nt were excluded. The sequences were collapsed in unique copies and aligned against the SILVA v132 database (Quast et al., 2013; Yilmaz et al., 2014), which was also used for taxonomical classification. It should be noted that this database does not cover the latest changes of the *Lactobacillus* taxonomy (Zheng et al., 2020). Performing pre-clustering, up to four mismatches were allowed. Chimera sequences were identified using VSEARCH algorithm (Rognes et al., 2016). Remaining sequences were classified taxonomically (Wang et al., 2007). Other sequences including unknown as well as sequences belonging to archaea, eukaryota, chloroplasts, and mitochondria were excluded. Sequences were clustered in OTUs at a 0.03 similarity cut-off. Statistical analyses were performed using the R software (version 3.6.1) (R Core Team, 2019) with packages phyloseq (McMurdie and Holmes, 2013) and vegan (Oksanen et al., 2008) in RStudio (RStudio Inc., Boston, USA). Alpha diversity was determined as the inverse Simpson index (Simpson, 1949) while beta diversity was calculated as Jensen-Shannon divergence (JSD) and visualized via principal coordinate analysis (PCoA). In addition, the mean relative abundances of OTUs that have been assigned to the 17 most abundant genera were calculated. For visualization, ggplot2 (Wickham, 2016) and RColorBrewer (Neuwirth, 2011) were used and graphs were finalized in Inkscape 0.92. Furthermore, the relative abundances of the 100 most abundant OTUs were calculated. In addition, the consensus sequences of the two most abundant OTUs were subjected to BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.5. Data availability

All data on microbial counts, pH values and total lactic acid concentrations have been deposited at OpenAgrar [https://doi.org/10.25826/20191211-135551]. All 16S rRNA gene fastq files have been deposited in the SRA of NCBI associated with accession number PRJNA595463 [http://www.ncbi.nlm.nih.gov/bioproject/595463].

3. Results

3.1. Culture dependent approach

At the beginning of the fermentations performed at MRI, the mean count of LAB in the starter culture inoculated batches was ca. 1×10^7 cfu/ml (Fig. 1A). This mean count increased to ca. 4×10^8 cfu/ml within 24 h and remained at this level until 72 h. After 144 h the mean count decreased to 2×10^7 cfu/ml which is comparable to the beginning of the fermentation. At JKUAT, the LAB mean count in the starter inoculated batches showed a similar trend, however the LAB mean count increased significantly within the first 24 h (Supplementary File 1). The inoculum at the beginning was slightly lower with a mean count of 3×10^6 cfu/ml, which increased significantly to 8×10^8 cfu/ml within 24 h and decreased slightly to 1×10^8 cfu/ml until the end of the fermentation (144 h). In the control batches performed without the addition of starter cultures, the mean count of LAB at the beginning of the fermentation was 5×10^1 cfu/ml at MRI and 4×10^1 cfu/ml at JKUAT. At both MRI and JKUAT the mean counts of LAB in the control batches increased during the entire course of the fermentation; even increased significantly within the first 24 h (Supplementary File 1). However, the mean counts of LAB increased quicker and to slightly higher values at JKUAT with 1×10^8 cfu/ml within 24 h and 4×10^8 cfu/ml within 48 h. At MRI the mean counts of LAB were 4×10^6 cfu/ml after 24 h and ca. 2×10^8 cfu/ml after 48 h. At the end of the fermentation, the mean LAB counts in the control batches were 1×10^8 cfu/ml (JKUAT) and 3×10^7 cfu/ml (MRI).

The pH development reflects the ability of acidification by LAB

during the course of the fermentation. It can clearly be observed that the pH dropped more quickly in the starter inoculated batches from a starting value of pH 7.0 (MRI) and 6.0 (JKUAT) to pH 3.6 at both locations significantly within 24 h of fermentation (Fig. 1B; Supplementary File 1). This result was also in good agreement with the measured lactate concentrations. The lactate concentration calculated as total lactate (mixture of D- and L-lactate) was near the detection level at 0 h in all samples, i.e. below 0.03 g/l. Within 24 h, total lactate increased to 1.79 g/l (MRI) and 1.97 g/l (JKUAT) in the starter inoculated batches in contrast to 0.20 g/l (MRI) and 0.63 g/l (JKUAT) in the control batches (results not shown). The pH (pH 3.6 after 24 h) remained at this low level and even dropped slightly lower to pH 3.5 within 48 h and pH 3.4 until the end of the fermentation in the starter inoculated batches (Fig. 1B). In contrast in the control batches, the pH dropped significantly, but more slowly to pH 5.3 (MRI) and pH 5.0 (JKUAT) within 24 h and pH 4.8 (MRI) and pH 4.3 (JKUAT) within 48 h. Comparable to this, the lactate concentrations were 3.13 g/l (MRI) and 3.31 g/l (JKUAT) in the starter inoculated batches and 0.48 g/l (MRI) and 1.59 g/l (JKUAT) in the control batches after 48 h (results not shown). The lactate concentrations increased to 5.08 g/l (MRI) and 4.01 g/l (JKUAT) in the starter inoculated batches and to 4.39 g/l (MRI) and 4.45 g/l (JKUAT) in the control batches until the end of fermentation. The mean counts of aerobic, mesophilic bacteria which were determined on Std1 medium resembled the counts of LAB determined on MRS agar both at JKUAT and at MRI. This applies for all sampling points in the starter culture inoculated batches and for the sampling points 48 h, 72 h and 144 h in the non-inoculated control batches (Fig. 1C). The mean counts on Std1 in the control batches both at MRI and at JKUAT at the beginning of the fermentation were 2×10^4 cfu/ml and 3×10^4 cfu/ml, respectively. The mean counts of LAB at 0 h were much lower in comparison to the counts on Std1 suggesting that at the beginning of the fermentation other bacterial strains than LAB were present in the control batches. Within 24 h to 48 h of fermentation, it can be assumed that these strains were reduced due to the dominance of LAB. The mean counts detected on VRBD medium of starter inoculated batches at MRI showed that enterobacteria were present with 2×10^1 cfu/ml at 0 h (Fig. 1D). The mean count increased to 7×10^3 cfu/ml within 24 h, however not significantly (Supplementary File 1). Afterwards, the mean counts decreased continuously throughout the course of the fermentation to levels below the detection limit. The same trend was observed for the mean counts of enterobacteria in fermentations performed at JKUAT with starter cultures. However, the mean counts were slightly higher with 3×10^2 cfu/ml, 6×10^3 cfu/ml and 1×10^1 cfu/ml at the beginning of the fermentation, after 48 h and at the end of fermentation. In contrast, the mean counts on VRBD in the control batches were clearly higher throughout the course of the fermentation both at MRI and JKUAT. The mean counts increased significantly to 3×10^7 cfu/ml (MRI) and 2×10^8 cfu/ml (JKUAT) within 24 h and remained at a high level at least until 72 h with 8×10^6 cfu/ml (MRI) and 6×10^6 cfu/ml (JKUAT). No yeast and moulds could be detected in the fermentations performed at MRI. Only single colonies were observed in the fermentations performed at JKUAT and no differences between starter inoculated and control batches were detected (results not shown).

3.2. Culture-independent approach

To determine the impact of the starter cultures on the microbiota during the fermentation process, the culture-independent, molecular biological method DGGE was conducted with the fermentation brine. The cluster analysis of DGGE fingerprints revealed that the patterns of the starter inoculated batches differed from the patterns of the control batches both at MRI and at JKUAT (Fig. 2). In addition, the starter inoculated batches showed a homogenous band pattern in contrast to the control samples, in which the band patterns were very diverse. The fingerprints of all control samples from MRI clustered together at a correlation of $r = 44.8\%$ except samples NS 7.2 0 h and NS 8.2 0 h. The

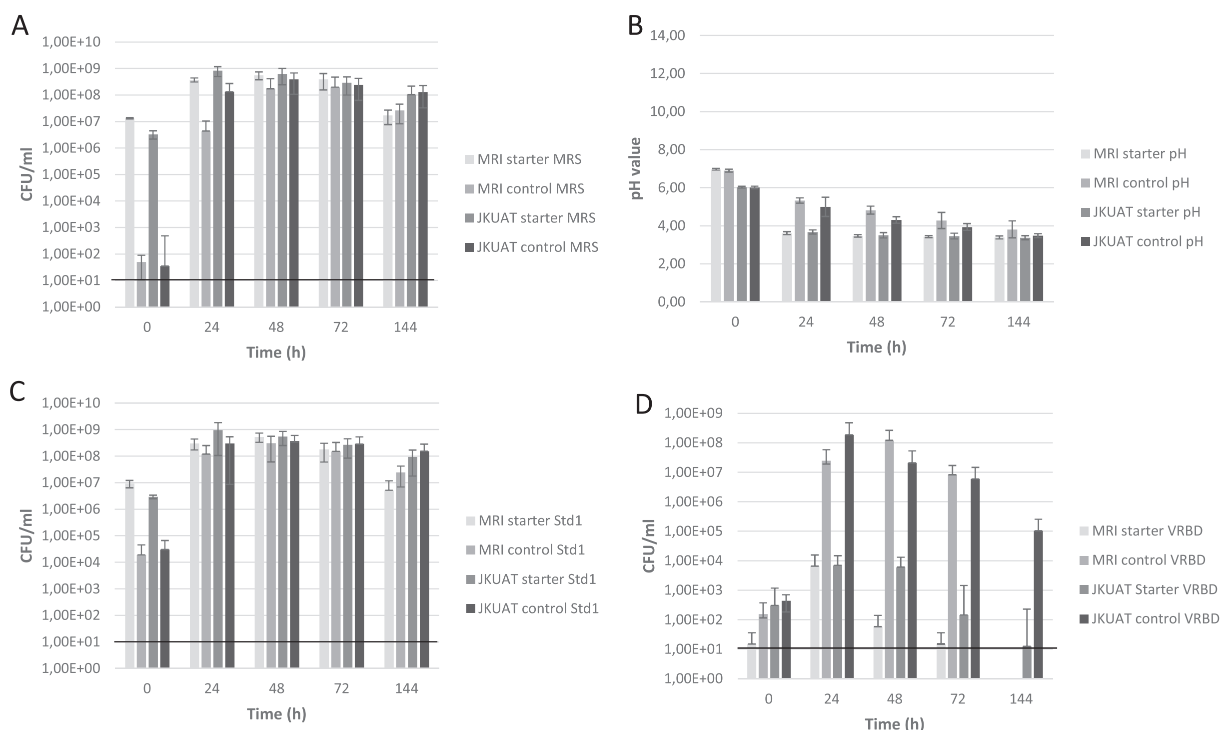

Fig. 1. Mean counts and standard deviations (CFU/ml) of lactic acid bacteria, aerobic, mesophilic bacteria, and enterobacteria of the fermentation brine were detected on MRS (A), Std1 (C), and VRBD agar (D), respectively. In addition, the mean pH values of the brine and standard deviations are given (B). Fermentations of African nightshade were performed with the addition of the starter cultures *La. plantarum* BFE 5092 and *Li. fermentum* BFE 6620 at MRI in Germany and at JKUAT in Kenya. In addition, control fermentations without the addition of starter cultures solely based on the action of the autochthonous microbiota were performed at both locations. The mean counts were calculated based on three (MRI) or four (JKUAT) independent biological replicates.

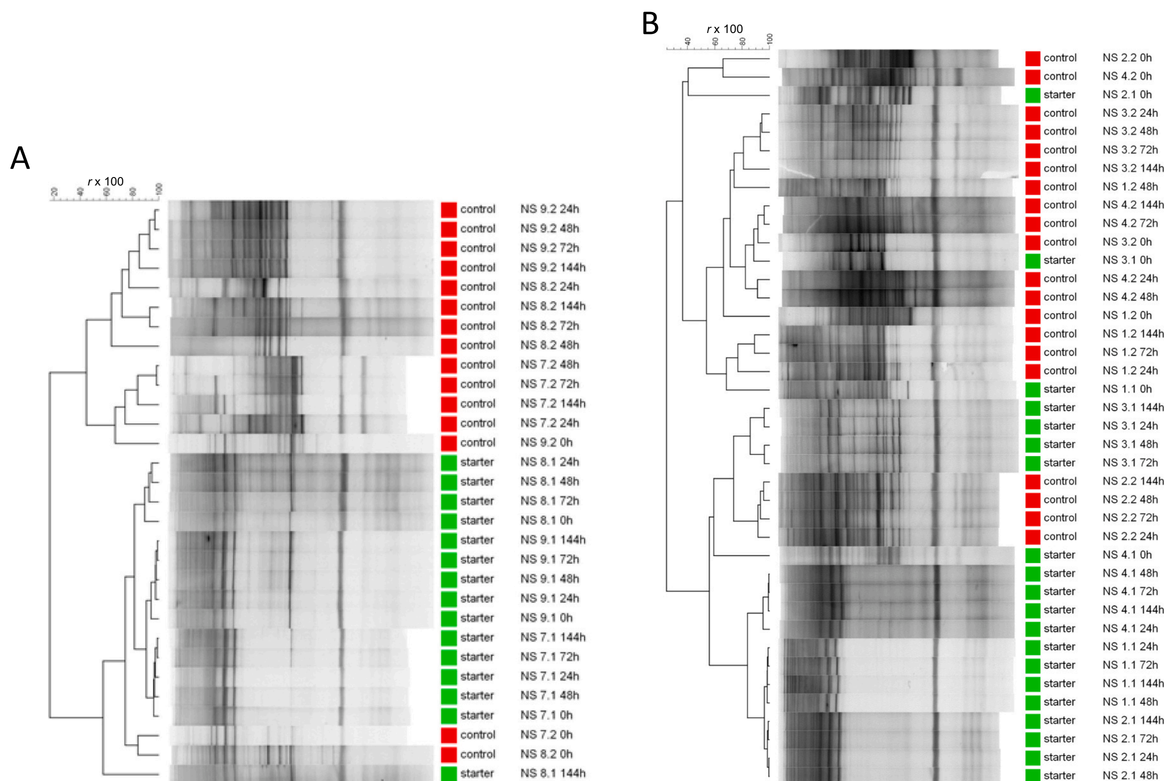

Fig. 2. Cluster analysis of DGGE profiles of the brine of nightshade fermentations. Three independent fermentations were performed at MRI (A) and four fermentations at JKUAT (B). Fermentations were conducted both with starter cultures (starter, labelled in green) and without starter cultures (control, labelled in red). Cluster analysis was performed using the Pearson correlation coefficient and UPGMA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

fingerprints of all samples of starter inoculated batches clustered at a higher correlation of $r = 81.5\%$ except sample NS 8.1 144 h (Fig. 2A). These correlation values showed that the control batches comprised a much higher diversity of bacteria than the starter inoculated batches which confirmed that the addition of starter cultures resulted in a reliable and controlled fermentation. The separate clustering of the fingerprints of samples NS 7.2 0 h and NS 8.2 0 h can be explained as these samples stemmed from the beginning of the fermentations. Comparable to fermentations at MRI, at JKUAT the control and starter inoculated samples can be distinguished by DGGE (Fig. 2B). However, samples NS 2.1 0 h and NS 3.1 0 h which stemmed from starter culture inoculated batches clustered in between control samples which can be explained as these samples were obtained at the beginning of the fermentation where a stable microbiota has not been established yet. Similar to fermentations at MRI, the starter inoculated batches showed similar patterns and samples of all sampling points except 0 h clustered narrowly together. For example, the samples of fermentations NS 1.1, NS 2.1 and NS 4.1 clustered at $r = 83.9\%$ and samples of NS 3.1 clustered at $r = 86.0\%$ with each other.

16S rRNA gene high-throughput amplicon sequencing was conducted as a second culture-independent molecular biological method to investigate the influence of the starter cultures on the microbiota involved in the fermentation process. Fig. 3A shows the development of the alpha diversity during the fermentation process, grouped into control and starter samples at JKUAT as well as MRI. The trend of the development of the alpha diversity is comparable between fermentations conducted at JKUAT and MRI. The alpha diversity of samples originating from the controls was more variable and increased throughout the fermentation period. It can be clearly stated, that the alpha diversity of control samples was much higher compared to starter samples. In addition, the alpha diversity of starter samples was less diverse and decreased over the fermentation process.

The majority of the variance (53.5%) of the beta diversity is explained by the first coordinate of the PCoA (Fig. 3B), plotted on the x-axis. The samples were further split on the y-axis which explains additional 12.1% of the variance. All samples from starter-inoculated fermentations of sampling points 24 h and afterwards, including some samples of starter-inoculated fermentations at even 0 h, were located in a narrow cluster. This indicates that these samples shared a high similarity in their beta diversity. In contrast, all other samples, i.e. samples of control batches at all time points and some starter inoculated batches at 0 h showed a high level of variation and were distributed over the plot.

Fig. 3C shows the relative abundances of the OTUs that have been assigned to the 17 most abundant genera. It can be confirmed that the composition of the microbiota of the control batches is much more diverse in contrast to the starter inoculated batches both at JKUAT and MRI. In addition, the control samples performed at JKUAT and at MRI differed in the composition of the microbiota. Unfortunately, no DNA could be isolated from control samples at 0 h of fermentations performed at MRI which can be explained by a very low presence of bacteria at the beginning of the fermentation. The microbiota of the control batches at the beginning of the fermentation performed at JKUAT consisted of OTUs belonging mainly to *Psychrobacter*, *Pseudomonas*, *Pantoea*, *Erwinia* and *Enterobacteriaceae* whereas the control batches performed at MRI consisted of OTUs belonging to *Staphylococcus*, *Peptostreptococcaceae*, *Enterobacteriaceae*, *Clostridium* and *Bacillus*. The control batches performed at JKUAT sampled at 24 h and afterwards showed that *Weissella* and *Lactococcus* were present. OTUs assigned to *Lactobacillus* increased whereas *Lactococcus*, *Enterococcus* and Gram-negative bacteria like *Pantoea*, *Kosakonia* and *Enterobacteriaceae* decreased throughout the course of the fermentation as expected. An increase of OTUs assigned to *Lactobacillus* was also observed for the control batches performed at MRI. The starter inoculated batches performed at MRI were strongly dominated by OTUs assigned to *Lactobacillus* at 0 h which may be

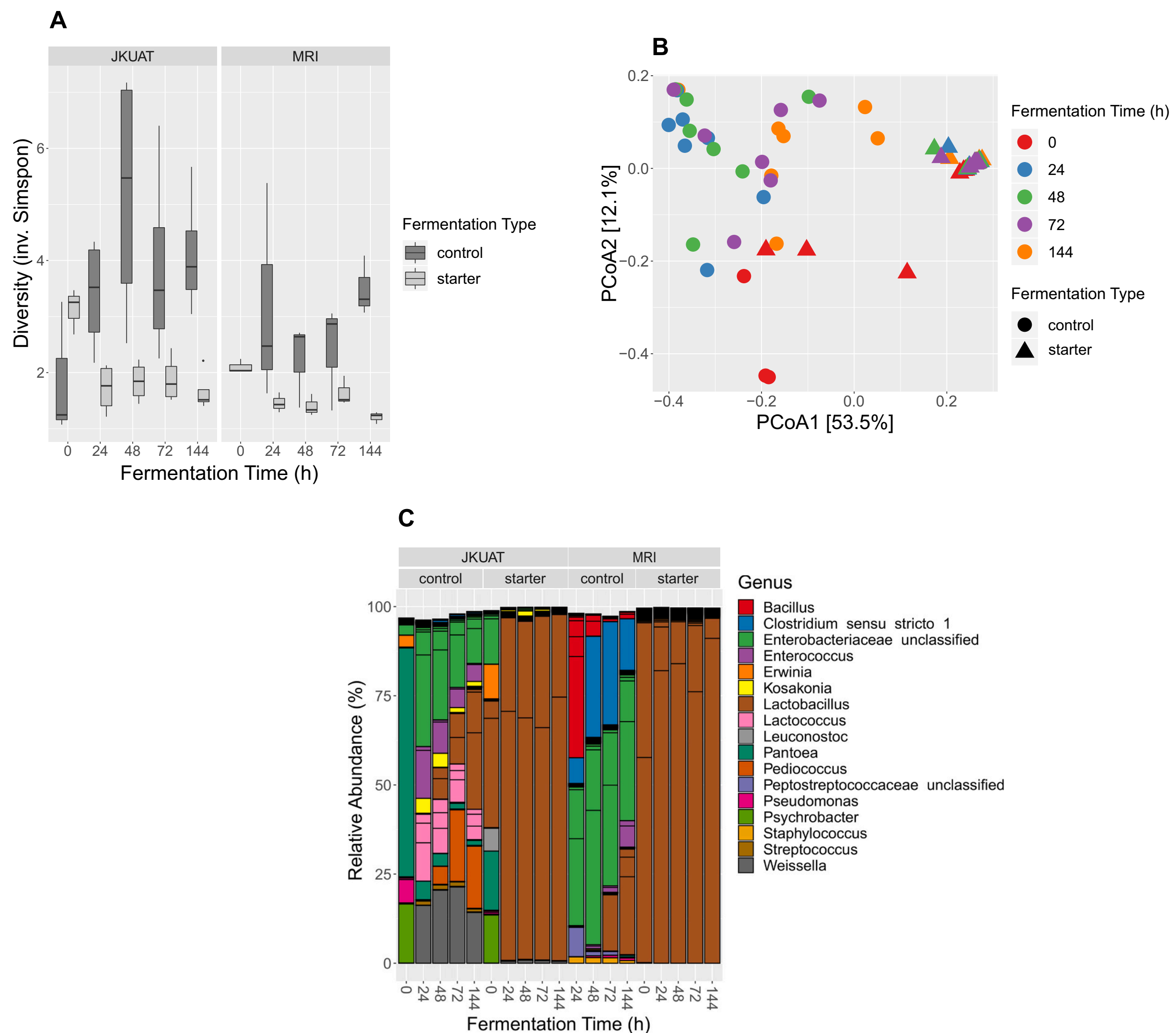

Fig. 3. Results of the 16S rRNA gene high-throughput amplicon sequencing obtained from fermentations of African nightshade performed with the addition of starter cultures and without starter cultures (control): Alpha diversity of the bacterial microbiota of the brine according to the inverse Simpson index (A). Beta diversity of the bacterial microbiota of the brine based on the Jensen-Shannon divergence (B). Mean relative abundances of OTUs that have been assigned to the 17 most abundant genera of the bacterial microbiota of the brine (C).

explained by the slightly higher inoculum of the starter cultures at MRI and the lower presence of autochthonous microbiota. Again, it should be underlined that the applied database did not cover the latest changes of *Lactobacillus* taxonomy. Therefore, OTUs assigned to *Lactobacillus* also comprise sequences of the inoculated starter strains. During the course of the fermentation, the starter inoculated batches at 24 h and afterwards consisted nearly only of OTUs which were assigned to *Lactobacillus*, both of fermentations performed at JKUAT and at MRI. After the end of the whole fermentation period at sampling point 144 h, the mean relative abundances of OTUs assigned to *Lactobacillus* represented 34.0% (±14.5%) and 29.6% (±16.2%) for the control fermentations conducted at JKUAT and MRI, respectively. When starters were added to the fermentations the mean relative abundances of OTUs assigned to *Lactobacillus* were 97.6% (±2.0%) and 97.7% (±0.9%) at JKUAT and MRI, respectively (Fig. 3C). The consensus sequences of the two most abundant OTUs which constituted the majority of *Lactobacillus*-OTUs were identified as *Li. fermentum* (100% query coverage, E value 0.0, 97.65% identity) and *La. plantarum* (100% query coverage, E value 0.0, 97.89% identity).

The results of alpha and beta diversity as well as the mean abundances of OTUs complement the other results of our study and underline that the inoculation of the starter cultures *La. plantarum* BFE 5092 and *Li. fermentum* BFE 6620 led to reliable and fast acidified fermentations with a less diverse microbiota dominated by lactobacilli strains.

4. Discussion

Fermented foods have a long tradition and are widespread all over the African continent (Holzapfel, 1997). Fermentation is an important processing method which often comprises the increase of the shelf life, enhanced micronutrient supply, improved palatability and digestibility (Oguntoyinbo et al., 2016c). Africa has a wide variety of fermented foods which include for example plant-derived products based on the fermentation of maize, sorghum, millet, and cassava (Franz et al., 2014). However, the fermentation of AILV is underutilized although AILV have the potential for fermentation. Thus, fermentation of AILV could contribute to improve the food supply and food security of the African people (Oguntoyinbo et al., 2016b, 2016c). In Africa, the majority of small-scale fermentation is still conducted spontaneously without the addition of well-characterised starter cultures (Holzapfel, 2002). These fermentations rely on the activity of autochthonous microorganisms which includes a certain risk for failure as the initiation of spontaneous

fermentations takes between 24 and 48 h (Holzapfel, 2002). During this initial phase diverse microorganisms are involved and compete for nutrients. The inoculation of appropriate starter cultures, e.g. LAB, leads to shortening of this initial phase of the fermentation which reduces the risk of failure (Holzapfel, 2002).

In this study, we investigated the fermentation of the AILV African nightshade both in Germany as well as in Kenya. The study design was chosen to determine the dominance of the selected starter cultures against the autochthonous microbiota which was expected to differ between both locations. Two previously selected, well-characterised LAB starter strains were applied (Cho et al., 2010a, 2010b; Kostinek et al., 2007; Oguntoyinbo et al., 2016a; Wafula et al., 2017). The inoculation of these two LAB starter cultures *La. plantarum* BFE 5092 and *Li. fermentum* BFE 6620 and a fermentation brine consisting of 3% sugar and 3% salt led to a successful fermentation of African nightshade leaves. The starter cultures were inoculated at 10^6 to 10^7 cfu/ml at the beginning of the fermentation and counts increased up to 10^8 cfu/ml within 24 h. In contrast, the samples of the non-inoculated control fermentations showed LAB counts of 10^1 cfu/ml at the beginning of the fermentations. These LAB counts increased both in the starter inoculated batches as well as in the control batches to counts of 10^8 cfu/ml, however much more slowly in case of the control fermentations. Similar results of LAB counts were described for the fermentation of various African fermented products (Kostinek et al., 2005; Weldemichael et al., 2019; Yousif et al., 2010). Oguntoyinbo et al. (2016b) fermented the AILV African kale with 2.5% NaCl with the same starter culture strains *La. plantarum* BFE 5092 and *Li. fermentum* BFE 6620 and with a comparable amount of 10^7 cfu/ml in the inoculum. The counts of LAB in the starter inoculated batches increased to counts of 10^8 cfu/ml within 24 h whereas the non-inoculated controls showed LAB counts of 10^1 cfu/ml at the beginning and ca. 10^8 cfu/ml at the end of the fermentation (Oguntoyinbo et al., 2016b). Kasangi et al. (2010) fermented cowpea leaves spontaneously with 3% glucose and detected counts of LAB of ca. 10^6 cfu/ml after 3 days of fermentation.

In our study, the growth of LAB in the starter inoculated batches as well as in the controls corresponded well with the increase in the lactate concentration and the development of the pH value. The pH value decreased faster in the starter inoculated batches. A pH value of 3.6 was obtained within 24 h whereas in the control batches the pH was between pH 5.3 and pH 5.0 after 24 h. Fast acidification is an important factor concerning food safety as putrefactive, Gram-negative bacteria including pathogens and some fungi are inhibited by lactic acid (Holzapfel, 2002). A pH value of 4.2 or less is regarded as an important factor for food safety (Holzapfel, 2002). The lactate concentrations determined in our study increased from near the detection level at the beginning to concentrations of 4–5 g/l at the end of the fermentations which corresponds well with the study of Oguntoyinbo et al. (2016b).

As the counts determined on Std1 agar were very similar to the counts obtained for LAB except for the control samples from the beginning of the fermentation, we assume that LAB strains comprised the majority of bacteria grown on Std1 medium which was also found by Oguntoyinbo et al. (2016b). In our study, enterobacteria were detected in all samples including fermentations with and without starter cultures at low counts at the beginning of the fermentations. These counts increased faster and to higher counts in the control samples in comparison to the starter-inoculated samples. Again, similar results were obtained within the study of Oguntoyinbo et al. (2016b).

The culture independent molecular biological approach of our study including DGGE and 16S rRNA gene high-throughput amplicon sequencing showed that the starter inoculated batches were characterised by a homogenous microbiota consisting mostly of strains belonging to the lactobacilli throughout the whole fermentation period. In contrast, the microbiota of the control batches differed in the composition not only during the chronological course of the fermentation but differed also in the replicates. In addition, control fermentations were also different whether the fermentations were performed in Kenya or in Germany. Oguntoyinbo et al. (2016b) found also that spontaneous fermentations of African kale leaves were rather variable. This was shown by the variable production of lactic acid and corresponding pH values as well as the composition of the microbiota which can be confirmed by our study. Samples of controls of 24 h and afterwards of fermentations performed at JKUAT were characterised by LAB, i.e. *Weissella*, *Lactococcus*, *Lactiplantibacillus*, *Limosilactobacillus* and *Enterococcus*. However, Gram-negative bacteria, i.e. *Pantoea*, *Kosakonia*, *Erwinia* and other enterobacteria were present and could be detected even at the end of the fermentation among the 17 most abundant genera. Samples of controls of fermentations performed at MRI during the first 48 h consisted of strains belonging to *Staphylococcus*, *Peptostreptococcaceae*, *Enterobacteriaceae*, *Clostridium* sensu stricto as well as *Bacillus*. During the course of the fermentation LAB strains, i.e. lactobacilli and *Enterococcus* increased. However, *Enterobacteriaceae*, *Clostridium* and *Bacillus* strains were still abundant at the end of the fermentation. The occurrence of enterobacteria, clostridia and *Bacillus* strains may present a health and safety risk as these bacterial groups include also pathogens and opportunistic pathogens e.g. *Pantoea agglomerans* (Delétoile et al., 2009), *Clostridium perfringens* (Chukwu et al., 2016) or *Bacillus cereus* (Schoeni and Wong, 2005). In the study of Oguntoyinbo et al. (2016b), the control samples consisted mostly of OTUs belonging to Proteobacteria.

In contrast to the diverse microbiota of the control batches, we could show that the application of the two LAB starter cultures led to a microbiota which was dominated by OTUs assigned to *Lactobacillus* with a mean relative abundance of ca. 98%. The predominant OTUs were assigned to *La. plantarum* and *Li. fermentum*. Similar to the results of our study, Oguntoyinbo et al. (2016b) showed that in the starter-inoculated batches of the fermentation of African kale leaves the bacterial community was dominated with 91% to 100% by OTUs belonging to the family *Lactobacillaceae*.

5. Conclusions

In this study, we could show by using a polyphasic approach comprising phenotypic and molecular biological analyses including the characterisation by 16S rRNA gene high-throughput amplicon sequencing that the addition of the lactic acid bacterial starter cultures *La. plantarum* BFE 5092 and *Li. fermentum* BFE 6620 led to a controlled and reliable fermentation of African nightshade leaves. Thus, fermentation of the African nightshade leaves by the selected starter cultures represents an adequate method to reduce postharvest losses. The inoculation of these starter cultures resulted in a faster, deeper and stable reduction of the pH during the fermentation period. The microbiota of the starter-inoculated batches was strongly dominated by OTUs assigned to *Lactobacillus* after 24 h and until the end of the fermentation whereas the non-inoculated control samples consisted of a diverse microbiota including undesired, putrefactive or potentially pathogenic bacterial genera.

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Declaration of competing interest

The authors declare that they have no conflicts of interest.

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References

Abukutsa-Onyango, M., 2007. The diversity of cultivated African leafy vegetables in three communities in Western Kenya. Afr. J. Food Agric. Nutr. Dev. 7, 1–15.
Aworh, O.C., 2018. From lesser-known to super vegetables: the growing profile of African traditional leafy vegetables in promoting food security and wellness. J. Sci. Food Agric. 98, 3609–3613.
Burchi, F., Fanzo, J., Frison, E., 2011. The role of food and nutrition system approaches in tackling hidden hunger. Int. J. Environ. Res. Public Health 8, 258.
Cho, G.-S., Hanak, A., Huch, Melanie, Holzapfel, W.H., Franz, C.M.A.P., 2010a. Investigation into the potential of bacteriocinogenic *Lactobacillus plantarum* BFE 5092 for biopreservation of raw Turkey meat. Probiotics Antimicro. 2, 241–249.
Cho, G.-S., Huch, M., Hanak, A., Holzapfel, W.H., Franz, C.M.A.P., 2010b. Genetic analysis of the plantaricin EFI locus of *Lactobacillus plantarum* PCS20 reveals an unusual plantaricin E gene sequence as a result of mutation. Int. J. Food Microbiol. 141, S117–S124. Supplement.
Chukwu, E.E., Nwaokorie, F.O., Coker, A.O., Avila-Campos, M.J., Solis, R.L., Llanco, L.A., Ogunsola, F.T., 2016. Detection of toxigenic *Clostridium perfringens* and *Clostridium botulinum* from food sold in Lagos, Nigeria. Anaerobe 42, 176–181.
Delétoile, A., Decré, D., Courant, S., Passet, V., Audo, J., Grimont, P., Arlet, G., Brisse, S., 2009. Phylogeny and identification of *Pantoea* species and typing of *Pantoea agglomerans* strains by multilocus gene sequencing. J. Clin. Microbiol. 47, 300–310.
Deshpande, S., Salunkhe, D., Oyewole, O., Azam-Ali, S., Battcock, M., Bressani, R., 2000. Fermented Grain Legumres, Seeds and Nuts: A Global Perspective. FAO Agricultural Service Bulletin, Rome.
FAO, IFAD, WW UNICEF, 2019. The State of Food Security and Nutrition in the World 2019. Safeguarding Against Economic Slowdowns and Downturns. Licence: CC BY-NC-SA 3.0 IGO. FAO, Rome.
Franz, C.M.A.P., Huch, M., Mathara, J.M., Abriouel, H., Benomar, N., Reid, G., Galvez, A., Holzapfel, W.H., 2014. African fermented foods and probiotics. Int. J. Food Microbiol. 190, 84–96.
Gogo, E., Opiyo, A., Ulrichs, C., Huyskens-Keil, S., 2016. Postharvest treatments of African leafy vegetables for food security in Kenya: a review. Afr. J. Hortic. Sci. 9, 31–40.
Grebmer, K.V., Saltzman, A., Birol, E., Wiesmann, D., Prasai, N., Yin, S., Yohannes, Y., Menon, P., Thompson, J., Sonntag, A., 2014. Global Hunger Index: The Challenge of Hidden Hunger. Welthungerhilfe, International Food Policy Research Institute, and Concern Worldwide, Bonn, Washington, D.C., and Dublin.
Holzapfel, W., 1997. Use of starter cultures in fermentation on a household scale. Food Control 8, 241–258.
Holzapfel, W.H., 2002. Appropriate starter culture technologies for small-scale fermentation in developing countries. Int. J. Food Microbiol. 75, 197–212.
Huch, M., Hanak, A., Specht, I., Dortu, C.M., Thonart, P., Mbugua, S., Holzapfel, W.H., Hertel, C., Franz, C.M.A.P., 2008. Use of *Lactobacillus* strains to start cassava fermentations for Gari production. Int. J. Food Microbiol. 128, 258–267.
Illumina, 2013. 16S Metagenomic Sequencing Library Preparation.
Kamga, R.T., Kouamé, C., Atangana, A.R., Chagomoka, T., Ndango, R., 2013. Nutritional evaluation of five African indigenous vegetables. J. Hortic. Res. 21, 99–106.
Kasangi, D.M., Shitandi, A.A., Shalo, P.L., Mbugua, S.K., 2010. Effect of spontaneous fermentation of cowpea leaves (*Vigna unguiculata*) on proximate composition, mineral content, chlorophyll content and beta-carotene content. Int. Food Res. J. 17, 721–732.
Kimaryo, V.M., Massawe, G.A., Olasupo, N.A., Holzapfel, W.H., 2000. The use of a starter culture in the fermentation of cassava for the production of "kivunde", a traditional Tanzanian food product. Int. J. Food Microbiol. 56, 179–190.
Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., Glockner, F.O., 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res. 41, e1.
Kostinek, M., Specht, I., Edward, V.A., Schillinger, U., Hertel, C., Holzapfel, W.H., Franz, C.M., 2005. Diversity and technological properties of predominant lactic acid bacteria from fermented cassava used for the preparation of Gari, a traditional African food. Syst. Appl. Microbiol. 28, 527–540.
Kostinek, M., Specht, I., Edward, V.A., Pinto, C., Egounlety, M., Sossa, C., Mbugua, S., Dortu, C., Thonart, P., Taljaard, L., Mengu, M., Franz, C.M., Holzapfel, W.H., 2007. Characterisation and biochemical properties of predominant lactic acid bacteria from fermenting cassava for selection as starter cultures. Int. J. Food Microbiol. 114, 342–351.
Kozich, J.J., Westcott, S.L., Baxter, N.T., Highlander, S.K., Schloss, P.D., 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Appl. Environ. Microbiol. 79, 5112–5120.
Mathara, J.M., Schillinger, U., Kutima, P.M., Mbugua, S.K., Holzapfel, W.H., 2004. Isolation, identification and characterisation of the dominant microorganisms of kule naoto: the Maasai traditional fermented milk in Kenya. Int. J. Food Microbiol. 94, 269–278.
McMurdie, P.J., Holmes, S., 2013. Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 8 (e61217).
Mnzava, 1997. Vegetable crop diversification and the place of traditional species in the tropics. In: L, G. (Ed.), Proceedings of the IPGRI International Workshop on Genetic Resources of Traditional Vegetables in Africa: Conservation and Use. IPGRI, Rome, pp. 1–15.
Muchoki, C., Imungi, J.K., Lamuka, P., 2007. Changes in Beta-carotene, Ascorbic Acid and Sensory Properties in Fermented, Solar-dried and Stored Cow-pea Leaf Vegetables.
Muthayya, S., Rah, J.H., Sugimoto, J.D., Roos, F.F., Kraemer, K., Black, R.E., 2013. The global hidden hunger indices and maps: an advocacy tool for action. PLoS One 8, e67860.
Neuwirth, E., 2011. RColorBrewer: ColorBrewer palettes. R package version 1.0-5. Retrieved 4 January, 2014.
Oguntoyinbo, F.A., Cho, G.-S., Brinks, E., Fiedler, G., Kabisch, J., Koberg, S., Bockelmann, W., Neve, H., Kang, Y.-G., Yun, D., Kim, A.-R., Narbad, A., Franz, C.M.A.P., 2016a. Draft genome sequence of *Lactobacillus plantarum* BFE 5092 isolated from Maasai fermented milk. Genome Announc. 4.
Oguntoyinbo, F.A., Cho, G.-S., Trierweiler, B., Kabisch, J., Rösch, N., Neve, H., Bockelmann, W., Frommherz, L., Nielsen, D.S., Krych, L., Franz, C.M.A.P., 2016b. Fermentation of African kale (Brassica carinata) using *L. plantarum* BFE 5092 and *L. fermentum* BFE 6620 starter strains. Int. J. Food Microbiol. 238, 103–112.
Oguntoyinbo, F.A., Fusco, V., Cho, G.S., Kabisch, J., Neve, H., Bockelmann, W., Huch, M., Frommherz, L., Trierweiler, B., Becker, B., Benomar, N., Galvez, A., Abriouel, H., Holzapfel, W.H., Franz, C.M., 2016c. Produce from Africa's gardens: potential for leafy vegetable and fruit fermentations. Front. Microbiol. 7, 981.
Oksanen, J., Kindt, R., Legendre, P., O'Hara, B., Simpson, G., Solymos, P., Stevens, M., Wagner, H., 2008. The Vegan Package.
Pitcher, D.G., Saunders, N.A., Owen, R.J., 1989. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett. Appl. Microbiol. 8, 151–156.
Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 41, D590–D596.
R Core Team, 2019. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing.
Rognes, T., Flouri, T., Nichols, B., Quince, C., Mahé, F., 2016. VSEARCH: a versatile open source tool for metagenomics. PeerJ 4, e2584.
Ruel, M.T., Minot, N., Smith, L., 2005. Patterns and Determinants of Fruit and Vegetable Consumption in Sub-Saharan Africa: A Multicountry Comparison. Eletronic publication World Health Organization (WHO).
Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B., Thallinger, G.G., Van Horn, D.J., Weber, C.F., 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl. Environ. Microbiol. 75, 7537–7541.
Schoeni, J.L., Wong, A.C.L., 2005. Bacillus cereus food poisoning and its toxins. J. Food Prot. 68, 636–648.
Simpson, E.H., 1949. Measurement of diversity. Nature 163, 688.
Sneath, P.H.A., Sokal, R.R., 1973. Numerical Taxonomy: The Principles and Practice of Numerical Classification. Freeman and Co Ltd.
Tulchinsky, T.H., 2010. Micronutrient Deficiency Conditions: Global Health Issues, 32, pp. 243–255.
Wafula, E.N., Franz, C.M.A.P., Rohn, S., Huch, M., Mathara, J.M., Trierweiler, B., 2016. Fermentation of African indigenous leafy vegetables to lower postharvest losses, maintain quality and increase product safety. Afr. J. Hortic. Sci. 9, 1–13.
Wafula, E.N., Brinks, E., Becker, B., Huch, M., Trierweiler, B., Mathara, J.M., Oguntoyinbo, F.A., Cho, G.-S., Franz, C.M.A.P., 2017. Draft genome sequence of *Lactobacillus fermentum* BFE 6620, a potential starter culture for African vegetable foods, isolated from fermented cassava. Genome Announc. 5, e00801–e00817.
Wang, Q., Garrity, G.M., Tiedje, J.M., Cole, J.R., 2007. Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl. Environ. Microbiol. 73, 5261–5267.
Weldemichael, H., Stoll, D., Weinert, C., Berhe, T., Admassu, S., Alemu, M., Huch, M., 2019. Characterization of the microbiota and volatile components of kocho, a traditional fermented food of Ethiopia. Heliyon 5, e01842.
Wickham, H., 2016. ggplot2: Elegant Graphics for Data Analysis. Springer.
World Health Organization, 2004. Global Strategy on Diet. Physical Activity and Health, Geneva.
Yilmaz, P., Parfrey, L.W., Yarza, P., Gerken, J., Pruesse, E., Quast, C., Schweer, T., Peplies, J., Ludwig, W., Glockner, F.O., 2014. The SILVA and "all-species living tree project (LTP)" taxonomic frameworks. Nucleic Acids Res. 42, D643–D648.
Yousif, N.M., Huch, M., Schuster, T., Cho, G.S., Dirar, H.A., Holzapfel, W.H., Franz, C.M., 2010. Diversity of lactic acid bacteria from Hussuwa, a traditional African fermented sorghum food. Food Microbiol. 27, 757–768.
Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmussen, S., Lund, O., Aarestrup, F.M., Larsen, M.V., 2012. Identification of acquired antimicrobial resistance genes. J. Antimicrob. Chemother. 67, 2640–2644.
Zheng, J., Wittouck, S., Salvetti, E., Franz, C.M.A.P., Harris, H.M.B., Mattarelli, P., O'Toole, P.W., Pot, B., Vandamme, P., Walter, J., Watanabe, K., Wuyts, S., Felis, G. E., Gänzle, M.G., Lebeer, S., 2020. A taxonomic note on the genus *Lactobacillus*: description of 23 novel genera, emended description of the genus *Lactobacillus* Beijerinck 1901, and union of *Lactobacillaceae* and *Leuconostocaceae*. Int. J. Syst. Evol. Microbiol. 70, 2782–2858.