Effects of Postharvest-Processing Technologies on the Safety and Quality of African Indigenous Leafy Vegetables

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“No man has a chance to enjoy permanent success until he begins to look in a mirror for the real cause of all his mistakes”. Napoleon Hill.

List of publications

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List of abbreviations

ADP	Adenosine diphosphate
AILVs	African indigenous leafy vegetables
ANOVA	Analysis of variance
APS	Ammonium persulfate
ATP	Adenosine triphosphate
AVRDC	The World Vegetable Centre
$_aw$	Water activity
BFE	Federal Research Centre for Nutrition
BLAST	Basic local alignment search tool
Bp	Base pair
BPLS	Brilliant-green phenol-red lactose Sucrose
BPW	Peptone buffered water
CFU	Colony forming units
DGGE	Denaturing gradient gel electrophoresis
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphate
EDTA	Ethylenediaminetetra-acetic acid
FAO	Food and Agricultural Organization
FLD	Fluorescence detection
fw	forward
G + C	Guanine + Cytosine
g	Acceleration due to gravity
H_2O_2	hydrogen peroxide
HPLC	High performance liquid chromatography
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KOH	potassium hydroxide
LAB	Lactic acid bacteria
Log_{10}	Logarithm
MAP	Modified atmosphere packaging

MG-A	Malt-glucose-antibiotics
MKTTn	Muller-Kauffmann tetrathionate-novobiocin
MRI	Max Rubner-Institut
MRS	de Man Rogosa and Sharpe
NADH	Nicotinamide adenine dinucleotide
NCBI	National Centre of Biotechnology Information
OD	Optical density
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
RAPD	Randomly amplified polymorphic DNA
Rev	reverse
RID	Refractive index detection
Rpm	Revolutions per minute
rRNA	ribosomal ribonucleic acid
RVS	Rappaport Vassiliadis soya
SDS	Sodium dodecyl sulphate
SSA	Sub-Saharan Africa
Std.I	Standard nutrient medium I
TAE	Tris ethylenediamine tetra-acetic acid
TEMED	N'-tetramethylethylenediamine
UPGMA	Unweighted pair group method with arithmetic mean
USD	US Dollar
UV	Ultraviolet
V	Volts
VRBD/G	Violet red bile dextrose/glucose
WHO	World Health Organization
XLD	Xylose lysine deoxycholate
DM	Dry mass

Zusammenfassung

Afrika ist reich an vielfältigem Blattgemüse, die große Mengen Vitamine, Proteine und Mineralien enthalten, was Unterernährung unter der armen Bevölkerung lindern könnte. Das warme und feuchte Wetter macht diese Produkte anfällig für schnellen Verderb aufgrund der vorherrschenden schlechten Produktionsbedingungen sowie für den Verderb bei Transport, Lagerung und Vermarktung. Lebensmittel-Verarbeitung und -konservierung ist ein großes Problem vieler Entwicklungsländer. Es sollten geeignete Methoden der Lebensmittel konservierung zur Erhaltung von Qualität, Sicherheit und Hygiene entwickelt werden, die preisgünstig und lokal einsetzbar sein müssen. Die Fermentation von Lebensmitteln führt zur Ansäuerung durch mikrobielle Produktion von organischen Säuren wie Acetat und Laktat, sowie zur Freisetzung von anderen antimikrobiellen Verbindungen, wie Bakteriozinen. Dies führt zu einem dramatischen Anstieg der Haltbarkeit von Lebensmitteln und damit zu verbesserter Sicherheit, Qualität und Verfügbarkeit der Lebensmittel für die menschliche Ernährung. Das Ziel der vorliegenden Arbeit war, am Beispiel der Fermentation von afrikanischen Nightshade Blattgemüse, die Auswirkungen von Fermentation und Solar-Trocknungsverfahren auf die Sicherheit und die Qualität zu charakterisieren und um Nachernteverluste zu minimieren.

Zur Optimierung der Fermentationsbedingungen für Blattgemüse wurden verschiedene Milchsäurebakterienstämme, isoliert aus afrikanischen fermentierten Lebensmitteln, unter Einsatz verschiedener Salz- oder Salz-Zucker-Konzentrationen bei der Säuerung der Produkte getestet. Alle getesteten Starterstämme zeigten in Kombination mit einer 3% Salz-Zucker-Lösung eine schnelle und stabile pH-Reduktion in der Lebensmittelfermentation.Unter diesen Bedingungen wurden *Lactobacillus plantarum* BFE 5092 und *Lactobacillus fermentum* BFE 6620 als Starterkulturen für die Gärung von Nightshade Blattgemüse eingesetzt (Versuche beim MRI Karlsruhe sowie und in JKUAT Kenia in 5-Liter Fermentationsbehältern). Um detailliertere Informationen über den Einfluss der Starterkulturen auf die autochthone Mikrobiota zu erhalten, wurden die aerobe mesophile Gesamtkeimzahlen sowie die Milchsäurebakterienzahlen zu verschiedenen Fermentationszeitpunkten bestimmt. Der Nachweis von Enterobakterien, Hefen und Schimmelpilzen ermöglichte die Beurteilung des Levels der Kontamination des fermentierten Lebensmittels mit Verderbs- und /oder pathogenen Mikroorganismen. Die Bestimmung von pH, Milchsäure, Saccharose und D- Glucose über Enzym-Assays erlaubte, die Fermentationsdynamik zu charakterisieren.

Die Verwendung von *Lb plantarum* BFE 5092 und *Lb fermentum* BFE 6620 als Starterkultur hatte einen signifikanten Einfluss auf die bakterielle Zusammensetzung der Nachtschatten Fermentationen: der pH-Wert wurde durch die Freisetzung von Milchsäure schnell abgesenkt, daher wurde das Wachstum von Verderbs- und pathogenen Mikroorganismen gehemmt, welches zu Produkten mit verbesserter Sicherheit und Qualität führte. Fermentationen mit und ohne Starterkultur Zusatz wurden mittels denaturierender Gradienten-Gelelektrophorese (DGGE) und Metagenomics Analysen mit Hochdurchsatz 16S rRNA-Amplicon Sequenzierung sowohl bei MRI und JKUAT charakterisiert. Es wurde deutlich, dass die Verwendung der Starterkultur *Lb plantarum* BFE 5092 und *Lb fermentum* BFE 6620 Auswirkungen auf die Biodiversität und die Dynamik der natürlich vorhandenen Microbiota bei der Fermentation von afrikanischem Nightshade Blattgemüse hatte.

Der Wachstumserfolg der Starterkulturstämme in den Fermentationen wurde in beimpften und ungeimpften Kontrollversuchen durch Randomly-Amplified-Polymorphic DNA Analyse (RAPD) und 16S-rRNA-Sequenzierung untersucht. Die Analysen zeigten, dass die eingesetzten Starterkulturen die Fermentationen mit schneller Säuerung einleiteten (und damit sicher machten), die aber später von indigenen Milchsäurebakterien überwachsen wurden.

Die Auswirkungen der Fermentation und Solartrocknung auf Sicherheit, Ernährungsqualität und sensorische Eigenschaften des Gemüses wurden durch Nährstoffanalyse, sensorische Eigenschaften und Challenge Tests mit humanpathogenen *Salmonellen* und *Listerien* untersucht. Gärungen, die mit Starterkulturen und Krankheitserregern angeimpft worden waren, zeigten einen schnellen und tiefe Absenkung des pH innerhalb von 24 h bis unter pH 4,0 aufgrund der hohen Keimzahlen der Milchsäurebakterien und der damit verbundenen starken Milchsäureproduktion. *Listeria monocytogenes* wurde innerhalb von 48 h (pH <3,6) vollständig gehemmt, *Salmonella* Enteritidis erst nach 144 h (pH <3,5).

Obwohl die Gärung des Gemüses zur Reduktion von wasserlöslichen Vitaminen führte, enthielt das fermentierte Produkt nach wie vor noch eine erhebliche Menge an Vitamin B_1, B_2 und C, in ausreichend hoher Konzentration, um die tägliche empfohlene Zufuhr dieser Mikronährstoffe zu ergänzen. Andererseits hatte Solartrocknung einen großen Einfluss auf den Vitamin C Gehalt und konnte auch nicht die Sicherheit und Qualität des Produktes gewährleisten. Fermentation der afrikanischen Nachtschattenblätter schien die sensorischen Eigenschaften von Farbe, Geschmack, Geruch und Aussehen zu verbessern. Generell gab es eine gute Akzeptanz des fermentierten

Nightshade Gemüses. Ein entsprechendes Verbraucherbewusstsein vorausgesetzt könnte Fermentation eine Option für ein Lebensmittel mit verbesserter Sicherheit, Qualität und Haltbarkeit sein. Fermentation von Gemüse in Afrika sollte zukünftig ein Forschungsschwerpunkt sein, da der Kontinent mit einer sehr reichen Auswahl an einheimischem Gemüse ausgestattet ist, die reich an Spurenelementen sind und deren Aufnahme zu einer besseren Gesundheit der Menschen beitragen könnte.

Abstract

Africa is endowed with rich varieties of indigenous leafy vegetables with high levels of vitamins, proteins and minerals, which can help to alleviate malnutrition among poor populations. The warm and moist weather makes these products prone to faster spoilage due to poor production conditions, decay of products during transport, storage and marketing. Food processing and preservation is one of the primary problems facing many developing countries. Therefore, proper methods of food preservation, which ensure quality, safety and hygiene, but are cheap and can be conducted locally, are necessary. Fermentation technology causes the acidification of food by microbial production of organic acids such as lactate and acetate, as well as the production of other antimicrobial compounds such as bacteriocins. This leads to a dramatic increase in the shelf life of food, as well as to improved safety, quality and availability of nutrition. The aim of this study was to determine the effects of fermentation and solar drying methods on the safety and quality of African indigenous leafy vegetables in order to minimize postharvest losses, specifically for the fermentation of African nightshade leaves.

To establish and optimize fermentation conditions, various lactic acid bacteria strains previously isolated from African fermented food products and different salt or salt-sugar concentrations were tested for rapid acidification of the product. The results showed that all the tested starter strains in combination with a 3 % salt-sugar solution resulted in a fast and stable pH reduction. Hence, 3% salt-sugar solution was established as the optimal fermentation condition with *Lactobacillus plantarum* BFE 5092 and *Lactobacillus fermentum* BFE 6620 used as a starter cultures for nightshade fermentation both at MRI, Karlsruhe, Germany and in JKUAT, Kenya, in 5 litre fermentation vessels. In order to gain more detailed information about the influence of the inoculated starter cultures on the background autochthonous microbiota in the fermentations, total aerobic mesophilic and lactic acid bacteria counts were determined to give an overview of the growth kinetics, while enterobacteria and yeast and moulds counts were used to give an overview of possible microbial contamination with potential spoilage and/or pathogenic microorganisms. The determination of pH, lactate and sucrose/D-glucose by enzyme assays were used to study fermentation dynamics. The use of *Lb. plantarum* BFE 5092 and *Lb. fermentum* BFE 6620 starter strains had a significant influence on the bacterial composition in nightshade fermentation, this was noted by a rapid and stable reduction of the pH and accumulation of lactic acid. Hence, the growth of spoilage pathogenic microorganisms was inhibited, resulting in products with improved safety

and quality. Denaturing gradient gel electrophoresis (DGGE) and metagenomics analyses using high throughput 16S rRNA amplicon sequencing were investigated on both starter cultures inoculated and uninoculated batches performed both at MRI and JKUAT. It was clear that the use of starter culture *Lb. plantarum* BFE 5092 and *Lb. fermentum* BFE 6620 had an impact on the biodiversity and dynamics of the background microbiota during fermentation of vegetable African nightshade.

The success of the inoculated selected starter strains during fermentation was investigated on both starters inoculated and uninoculated trials by randomly amplified polymorphic DNA (RAPD) analysis and 16S rRNA sequencing. The analysis showed that the starter culture *Lb. plantarum* BFE 5092 and *Lb. fermentum* BFE 6620 may have initiated the fermentation, but were later overgrown by indigenous LAB initially present on the plant materials. The effects of fermentation and solar drying on safety, nutritional quality and sensory attributes were evaluated by basic nutrient analysis, assessing sensory attributes of fermented leaves and challenge tests with the human pathogenic *Salmonella* and *Listeria*. The results showed that the fermentations where pathogens were co-inoculated with starter cultures led to a fast and deep reduction of the pH within 24 h to < 4.0, due to increased lactic acid bacteria counts and lactic acid production by these bacteria. Concomitant to this, *Listeria monocytogenes* was completely inhibited within 48 h (pH < 3.6), while *Salmonella* Enteritidis inhibition occurred after 144 h (pH < 3.5). Even though fermentation led to reduction of water-soluble vitamins, the fermented product still contained a significant amount of vitamin B_1, B_2 and C, enough to supplement the daily-recommended intake. Solar drying, on the other hand, had a huge impact on vitamin C. Moreover, solar drying did not guarantee the safety and quality of the product. Fermentation of African nightshade seems to improve the sensory attributes of colour, taste, smell and appearance. There was a general acceptance of the fermented nightshade leaves. Hence, with proper awareness, fermentation might be an option for the local consumers for ensuring product with improved safety, quality and shelf life. Thus, fermentation of vegetables in Africa should be given priority, since the continent is endowed with a rich variety of indigenous vegetables, which are rich in micronutrients and whose uptake could contribute to a better health of the people

1.0 Introduction

1.1 General introduction

The main challenge faced by agricultural researchers, policy makers and economic developers is how to safely feed 9.1 billion people by the year 2050 (Parfitt et al., 2010). Even though attention has focussed towards increasing food production by around 50 % to 70 %, the equally important complementary factor of reducing food postharvest loss has been overlooked (Hodges et al., 2011). According to the FAO-World Bank (2010) and (Prusky, 2011), about one third of the food produced around the world goes to waste, representing about 1.3 billion tons of food, which stands in stark contrast to the 870 million people in developing nations who do not have enough food (Gustavsson et al., 2011; Niewiara 2016). According to FAO-IFAD-WFP (2015), 72 out of the 129 developing countries have reached the Millennium Development Goal 1 (MDG) target 1c. However, hunger still affects about one in nine people (795 million people) around the world. A report by the World Bank recently revealed that each year, 150 kg of food produced is lost per person in Sub-Saharan Africa (SSA) (FAO, 2011). It is estimated that grains of four billion USD value are affected per year in SSA. This report showed the extent of how food losses surpassed the value of food aid received by SSA in the last 10 years (World Bank, 2011). Therefore, reducing food losses will offer an important gateway of not only improving nutrition but also alleviating poverty in the SSA region.

There are roughly 780 million undernourished people living in developing nations, sub-Saharan Africa is the region with the highest prevalence of hunger (FAO-IFAD-WFP, 2015). Hidden hunger affects about 2 billion people globally (WFP, 2017) with the majority of the affected found in SSA, as well as India and Afghanistan (Muthayya et al., 2013). However, as the population continues to grow, especially in these areas, the water, farming land, forest and energy resources are being depleted fast and global warming is adversely affecting the agricultural productivity (Pimentel et al., 2013). Africa is endowed with rich varieties of indigenous leafy vegetables (ILV), which contains high levels of vitamins, minerals and protein, and which constitute a valuable source of nutrition in rural areas, where they can help alleviate malnutrition among the poor populations (Oguntoyinbo et al., 2016). However, Africa also has warm and moist weather, which promotes massive postharvest losses, reaching between 30-50 % (Shiundu & Oniang'o, 2007). These losses are attributed to poor production conditions (Abukutsa-Onyango,

2007), as well as to decay of products during transport, storage and marketing (Muchoki et al., 2007). The annual losses due to food spoilage and waste in developing countries amounts to USD 310 billion, with 65 % of the lost food occurring in production, processing and other postharvest stages (Pedrick, 2012). Moreover, there is also a lack of quality control and food safety regulations, which calls for urgent regulatory policies and mechanisms (HCDA, 2008).

The commonly used, local preservation methods for African indigenous vegetables include blanching, air-drying, solar-drying (Nguni & Mwila, 2007) and fermentation (Muchoki et al., 2007). Although drying is one solution to the problem of perishability, it does not satisfy the needs of supplying for a large population of consumers, particularly urban dwellers (Smith & Eyzaguirre, 2007). Drying has been an African way of processing various foods to make them available during dry periods, when the supply is short. This method can increase the shelf life but may also alter the nutrient quality (Smith & Eyzaguirre, 2007). Therefore, food processing and preservation is one of the central problems facing developing countries. In Africa, particularly in Kenya, this is the major problem with many indigenous vegetables, resulting in wastage during the in-season and limited supply during the off-season (Abukutsa-Onyango et al., 2005). This is accompanied by high prices (Habwe & Walingo, 2008), because most locally available vegetables are seasonal and thus not available all year round. Therefore, proper methods of food preservation, which will ensure quality, safety and hygiene, but which are cheap and can be conducted locally, are necessary.

1.2 Food preservation

Preservation of food is based on the principle of preventing or delaying growth of microorganisms (Caplice & Fitzgerald, 1999). The shelf-life of food is influenced by interrelated factors such as storage temperature, endogenous enzymes, atmospheric oxygen, moisture, light and presence of microorganisms, acting either singly or combined and which can result in food decay or spoilage (Adams & Moss, 2002). Therefore, food spoilage is defined as any alteration that renders the food unfit for human consumption (Britannica, 2015). Modern food preservation methods have been able to bridge the gap between different harvest times and seasons, making a constant food production and supply possible (Warriner et al., 2009). The international exchange of goods has also increased (Warriner et al., 2009). The global fruit and vegetable production has increased by 94 % from 1980 to 2004 (Olaimat & Holley, 2012). The commonly used preservation techniques applied today for preventing or delaying food spoilage are through reduction of water activity,

reduction of temperature, reduction of pH and application of high temperatures. These methods can either be physical, chemical or biological in nature (Adams & Moss, 2002; Gould, 1996).

1.2.1 Physical methods of food preservation

These methods employs thermal processes such as cold, heat or irradiation and aim to slow down or completely inhibit microbial activities (Adams & Moss, 2002). Examples of physical methods in food preservation include cooling and freezing, heat treatment, low water activity, drying and ionization radiation (Adams & Moss, 2002; Gould, 1996). Storage at low temperatures inhibits microbial activities which are generally temperature dependent (Jay et al. 2005), while heat treatment causes the inactivation or killing of microorganisms through pasteurization and sterilization. Pasteurization involves heating processes at 60–80 ^{o}C for up to a few minutes to eliminate harmful pathogens associated with a product. Sterilization, on the other hand, involves applying temperatures between 115 ^{o}C and 135 ^{o}C, resulting in complete inactivation of all microorganisms and their spores (Jay et al., 2005; Adams & Moss, 2008). Lowering the water activity involves desiccation or drying. Water activity (a_w) is the amount of "free state" water in a product and can be defined by the ratio of the water vapour pressure of food substrate to the vapour pressure of pure water at the same temperature (Jay et al., 2005). This method removes or binds moisture making it unavailable for microbial metabolic activities, thus controlling the growth of spoilage microorganisms (Sofos, 1993). The use of ionizing radiation involves exposure of food to different kinds of UV rays to increase their durability and to kill pathogenic microorganisms. Ionizing radiation can affect microorganisms directly, by interacting with key molecules within the microbial cell, or indirectly through the inhibitory effects of free radicals produced by the radiolysis of water (Adams & Moss, 2002; Jay et al., 2005).

1.2.2 Chemical preservation methods

These methods apply antimicrobial substances to control the growth of microorganisms in foods. The commonly used chemical methods of food preservation include the use of preservatives such as smoking and salting. Preservatives are substances capable of inhibiting, retarding or arresting the growth of microorganisms in food and may either be biocidal that kill the target microorganisms, or biostatic, in which case they prevent their growth. These agents are usually organic acids such as lactic acid, acetic acid and citric acid, or inorganic acids, such as nitrite and

sulphite and they are generally used together with sodium chloride (Adams & Moss, 2002; Leroy & De Vuyst, 2004). Smoking of foods is a natural method of preservation method. Smoke contains ingredients such as formic acid, acetic acid, formaldehyde, phenol, and cresol with biostatic and biocidal effects (Adams & Moss, 2002). Most methods such smoking are not reliable in preservation against spoilage microorganisms, unless used in combination with other preservation methods such as pickling, salting or heating (Adams & Moss, 2002).

1.2.3 The concept of hurdle technology

Microbial food safety and stability is based on the combined application of preservation technologies called hurdles, which involve reduction in water activity, lowering temperature, reduction of pH, addition of competitive microorganisms and addition of preservatives and the technique for applying different hurdles is known as hurdle technology (Leistner, 1994; Singh & Shalini, 2016). The concept of hurdle technology was developed to address the consumer demand for less heavily preserved foods and more natural and fresh food products (Gould, 1996). The safety and stability of food depends on a variety of chemical, physical and microbiological reactions within the food (Gorris, 1999). Food spoilage due to microorganisms can only occur when the food matrix and the environmental conditions support their growth and survival. In cases where the water activity or pH is below critical limits within the food, microorganisms will not survive (Gorris, 1999; Leistner, 1994). Therefore, intelligent use of hurdle technology in food preservation guarantees safety and stability and additionally it maintains the organoleptic, nutritional quality and economic viability of the food products (Chirife & Favetto, 1992; Leistner, 1992; Singh & Shalini, 2016).

1.2.4 Biological preservation methods

The effect of the biological preservation method is based mostly on the antimicrobial metabolites produced by microorganisms such as organic acids (e.g. lactic or acetic acids) and bacteriocins (Bourdichon et al., 2012; Jay et al., 2005; O'Sullivan et al., 2002). Preservation of food is based on the use of microorganisms as starter or protective cultures by converting fermentable sugars into organic substances in the process called fermentation (Bourdichon et al., 2012). Fermentation causes the acidification of foods by the microbial production of the organic acids. Moreover, specific bacteria are capable of producing other antimicrobial compounds such as

bacteriocins, which may inhibit competing non-starter lactic acid bacterial strains or other autochthonous bacteria in the food matrix, including pathogens. This may lead to a dramatic increase in the shelf-life, as well as safety of foods (Cogan et al., 2007; Kalui et al., 2009).

1.3 African indigenous leafy vegetables

Indigenous vegetables are cultivated in many parts of the world. Study surveys indicating that over 7,000 species of wild plants are being used and amongst these, the indigenous vegetables are included (Schönfeldt & Pretorius, 2011). According to Abukutsa (2003), Adebooye & Opabode (2004) and Schönfeldt & Pretorius (2011), the underutilised and neglected African plant species could help addressing the problem of food security and income generation among many rural poor populations. There are two main categories of vegetables in urban and peri-urban SSA, i.e. exotic and indigenous (or traditional). The exotic vegetables are those that originated from outside of the continent, while the indigenous or traditional African vegetables occur naturally in the respective environment. AILVs can be defined as plants that have been part of the food systems in SSA for generations, or are those that have their natural habitat on SSA and whose leaves, young shoots and flowers are consumed (Abukutsa-Onyango, 2010).

The most common exotic vegetables found in SSA include cabbages (*Brassica oleracea*), green beans (*Phaseolus vulgaris*), onions (*Allium* spp.), carrots (*Daucus carota*), tomatoes (*Lycopersicon esculentum*), lettuce (*Lactuca sativa*), and chards (*Beta vulgaris*) (Shackleton, 2003). In terms of important indigenous vegetables, there is variation in the utilization of plant families by different countries and regions, with the common genera being *Amaranthus*, *Agathosma*, *Bidens*, *Cleome*, *Chenopodium*, *Corchorus*, *Crotalaria*, *Cucurbita*, *Ipomoea*, *Solanum*, *Vernonia* and *Vigna* (Coetzee & Reinten, 1999; Shackleton, 2003). However, in West and Central Africa, the most popular indigenous leafy vegetables are pumpkin (*Cucurbita* spp.), wild spinach (*Amaranthus* spp.), sweet potato leaves (*Ipomoea batatas*) and okra (*Abelmoschus esculentus*). While in East and Southern Africa, the African nightshades (*Solanum* spp.), wild spinach (*Amaranthus* spp.), jude mellow (*Corchorus olitorius*), spider plant (*Cleome* spp.), cowpea (*Vigna unguiculata*), African kale (*Brassica carinata*), cassava leaves (*Manihot esculenta),* slender leaves *(Crotalaria ochroleuca)* and pumpkin leaves (*Cucurbita* spp.) are mostly consumed (Coetzee et al., 1999; Shackleton, 2003).

1.3.1 Diversity of African indigenous leafy vegetables

There are about 13,000 plant species which are utilized as food and the Plant Resources of Tropical Africa (PROTA), reported an estimated 6,376 useful indigenous African plants, of which 397 are vegetables (Mwangi & Kimathi, 2006; Smith & Eyzaguirre, 2007). In Kenya, for example, out of the 800 plants used as food crops, 210 are used as vegetables (Mwangi & Kimathi, 2006). Currently, AILVs commonly consumed in East and West Africa include cowpea leaves (*Vigna unguiculata*), baobab leaves (*Adansonia digitata*), amaranth (*Amaranthus viridis*), spider plant (*Cleome gynandra*), jude mallow (*Corchorus olitorius*), moringa leaves (*Moringa oleifera*), African nightshade (*Solanum scabrum*), cassava leaves (*Manihot esculenta*), pumpkin leaves (*Cucurbita* spp.), slender leaves (*Crotalaria ochroleuca*), African kale (*Brassica carinata*) and sweet potato leaves (*Ipomoea batatas*) (Coetzee et al., 1999; Shackleton, 2003). According to Lyimo et al. (2003), at least thirty types of indigenous vegetables are commonly consumed in rural areas of Tanzania.

1.3.2 Nutritional benefits of indigenous vegetables

There is little published data on the scale of production of AILV, one report quotes that the total production of leafy vegetables in Cameroon in 1998 was estimated to be 93,600 tons, of which 21,549 tons were the 'bitter leaf' *Vernonia amygdalina* (Smith & Eyzaguirre, 2007). According to Mwangi and Kimathi (2006), the consumption for AILVs just in the city area of Nairobi, Kenya, increased from 31 tons in 2003 to 600 tons in 2006. Indigenous vegetables are inexpensive, easily accessible and contain high amounts of health-promoting compounds such as anti-oxidants, minerals and vitamins, that are important in fighting off infectious diseases and maintenance of good health (Abukutsa-Onyango, 2003; Schönfeldt & Pretorius, 2011). Table 1.1 shows nutrient composition of some of the mostly consumed African indigenous vegetables vs. exotic leafy vegetables, and it depicts the nutritive value of these vegetables to the local population.

Table 1.1: Nutrient composition of fresh weight edible AILVs vs. exotic leafy vegetables in mg/100 g (Maundu et al., 1999, Abukutsa-Onyango 2003).

Indigenous vegetables	**Protein %)**	**Ca**	**Fe**	**Vit A**	**Vit C**
Amaranthus spp. (amaranths)	4.0	480	10	10.7	135
Cleome gynandra (spider plant)	5.1	262	19	8.7	144
Solanum villosum (nightshade)	4.6	442	12	8.8	131
Vigna unguiculata (cowpea)	4.7	152	39	5.7	8.7
Cucurbita moschata (pumpkin)	3.1	40	2.1	3.9	170
Corchorus olitorius (jute mallow)	4.5	360	7.7	6.4	187
Exotic vegetables					
Brassica oleracea var. acephala (kale)	-	187	32	7.3	93
B. oleracea var. capitata (cabbage)	1.4	44	--	1.2	33
Lactuca sativa (lettuce)	1.2	62	2.2	0.04	18
Spinacia oleracea (spinach)	2.3	93	32	5.1	28

The intake of AILVs can help reduce malnutrition among the rural poor due to their high levels of vitamins, proteins, minerals and certain amino acids (Imungi, 2002; Imungi & Potter, 1983). These vegetables can be prepared together with other starchy foods that represent affordable nutrition to the poor segment of the population (Mnzava, 1997; Abukutsa-Onyango, 2003). Indigenous vegetables contain equivalent or higher minerals, protein and vitamin than their exotic counter parts (Odhav et al., 2007). Actually, on average 100 g of fresh indigenous vegetable contains enough amounts of vitamins, calcium and iron that would supplement 100 % daily requirement and 40 % protein (Abukutsa-Onyango 2003). These vegetables also contain substantial amounts of proteins, vitamin A, B, C and E, β-carotene, iron, calcium, magnesium, zinc and phosphorus (Lebotse, 2010; Muchoki et al., 2007; Smith & Eyzaguirre, 2007; Tumwet et al., 2014). A good example is vegetable amaranth. A study by Uusiku et al. (2010) found that it is much more nutritious when compared to cabbage, which is exotic to Kenya. There is also a positive correlation between the consumption of these vegetables and the treatment of hypertension, gastrointestinal diseases and gout (Kimiywe et al., 2007). Therefore, consumption of AILVs will play a crucial role in averting chronic malnutrition among many SSA countries, as well as fulfilling the WHO global initiative on fruits and vegetable consumption (Smith & Eyzaguirre, 2007).

1.4. *Solanum* species

1.4.1 General description

Solanum (Black nightshade or garden huckleberry) belongs to the family *Solanaceae* with about 102 genera and nearly 2,500 species. The term nightshade collectively refers to a diverse group of plants with considerable economic importance as medicine and food (Edmonds & Chweya, 1997). The most important plants are eggplant (*Solanum melongena*), potato (*Solanum tuberosum*), and tomato (*Solanum lycopersicum*). *Solanum nigrum* is native to North Africa, Europe and West Asia, and is renowned for its poisonous berries and leaves (Shackleton et al., 2009).

The species that are commonly used as leafy green vegetables in most parts of SSA are described below. *Solanum americanum* Mill has relatively thin branches and is easily distinguishable from other species by their small, usually glossy leaves and small, shiny green fruits that turn purple-black when ripe (Shackleton et al., 2009) The species is mainly found in warm humid areas, particularly in coastal environments (Shackleton et al., 2009). *Solanum eldoretii* is found in the Kenyan highlands and in Northern Tanzania. It has small fruits and the mature ones are green, unlike other *solanum* group, for which the mature fruits turn purple-black (Shackleton et al., 2009). *Solanum retroflexum* is usually found across the Sahel, horn of Africa and East Africa, especially Tanzania, but it is commonly used as vegetable in Southern Africa (Rensburg & Averbeke, 2007; Shackleton et al., 2009). *Solanum villosum* Mill has orange berries and can be distinguished from the others by its orange to yellow berries, which are edible. It is commonly found in dry environments, although this species is known in East Africa and is both wild and cultivated (Rensburg & Averbeke, 2007; Shackleton et al., 2009).

1.4.2 *Solanum scabrum* Mill (African nightshade)

This is largest species with broad leaves and is cultivated widely in many tropical African regions, East, South and South East Asia, South pacific, North America and the Caribbean and is non-poisonous (Shackleton et al., 2009). It is believed to have been introduced to East and South Africa from humid West Africa. It is one of the most popular and promising vegetables among nightshade species in East, Central and West Africa. It grows to 1.5 m height and can be distinguished by rapid growth and broad leaves with large purple black berries or fruits of ca. 0.6 cm across (Abukutsa-onyango, 2015). The leaves are ovate to ovate- lanceolate, mostly 8-15 cm

long, entire or angularly lobed, undulate and glossy. The petiole measures up to 3 cm long and they have white or yellowish flowers (Fig.1.1). This species also shows varied diversity in terms of growth habits, leaf colour and levels of bitterness (Abukutsa-onyango, 2015; Shackleton et al., 2009).

Leaves

Flowers

Immature berries

Mature berries

Figure 1.1: African nightshade plant with flowers and berries photographed at JKUAT fields, Nairobi, Kenya.

Ecologically, African nightshade grows well in a wide range of soils both in low and highland regions and it requires well-aerated soil with high water retention ability, high organic matter content and with a pH range of 5.5 to 6.8 (Abukutsa-onyango, 2015). The crop usually

requires moderate rainfall with a temperature range of 18 ^{o}C to 30 ^{o}C and with high light intensity (Abukutsa-onyango, 2015). The plant is susceptible to frost, but can tolerate partial shading. African nightshade can be propagated directly from seeds or by use of stem cuttings. Seeds can be used directly, or raised in the nursery beds and then transplanted (Abukutsa-onyango, 2015). This species requires a lot of nutrients, especially nitrogen, for growth and organic manure from farmyard is usually recommended (Abukutsa-onyango, 2015). The edible parts are usually the young shoots and leaves and are blanched, boiled, fried or cooked with other vegetables. Previous studies by Maundu et al. (1999) and Kamga et al. (2013), showed that African nightshade is an excellent source of beta-carotene, vitamin A, C, E, iron, iodine, zinc, potassium and protein. This is important in addressing malnutrition among the poor, as well as the issue of chronic disorders.

1.5 Postharvest processing technologies

Production of high-quality fresh produce depends on sound production practices, proper handling during harvest, appropriate postharvest handling, and storage (Schippers, 2002). The challenge is usually the ability to maintain a certain level of freshness. Thus, a grower who can meet these challenges is able to expand marketing opportunities and has a better chance to compete in the marketplace (Earles & Bachmann, 2000). Due to high perishability, leafy vegetables have a very short shelf life of about 3 days (Acedo, 2010). They deteriorate very quickly in quality and flavour after harvesting and the extent of postharvest losses can be serious if the crop is handled poorly, with the result that this creates marketing chain problems (Schippers, 2002).

Postharvest processing is mainly the transformation of a perishable food into a more reliable one with a long shelf life (FAO, 2016). Processing helps to maintain a constant supply of food during times of scarcity (Habwe et al., 2008). In most African countries, vegetables are rarely processed, due to the lack of preservation equipment for e.g. canning, freezing and dehydration (Mepba et al., 2007). Once foods are harvested, they are sometimes sun-dried, which results in poor product quality that is usually characterized by varying moisture levels, as well as high microbial loads which affect it’s storability and safety (Mepba et al., 2007). Processing and preservation thus is one of the central problems facing developing countries, which characteristically have high food wastage during the in-season and only a limited supply during the off-season. This is accompanied by high prices for fresh produce, which is based on the fact that availability is seasonally dependent (Habwe et al., 2008). There is a need for alternative processing treatments of locally consumed,

cooked preparations as to prevent postharvest losses, while at the same time promoting nutrient retention and microbial safety (Mepba et al., 2007). Such technologies include on-farm evaporative coolers and modified atmosphere packaging (MAP), and these should be explored for implementation (Yumbya et al., 2014). The local preservation methods commonly used in Africa so far are mostly based on blanching, air-drying, solar-drying (Nguni & Mwila, 2007) and fermentation (Muchoki et al., 2007).

1.6 Microbial classification and community studies

1.6.1 Conventional taxonomy

The traditional way for classification of LAB is based on the phenotypic characteristics such as determination of cell morphology, ability for gas production from glucose fermentation, as well as establishing growth and survival at different temperatures. The ability to tolerate different concentration of NaCl, as well as growth at varying pH ranges, the production of lactic acid isomers and analysis of cell wall proteins patterns and the methyl esters of their fatty acids (Sharpe, 1979). The combined application of the above features with other studies has proved useful in classification and identification LAB (Hasting & Holzapfel, 1987). Phenotypic methods are not completely accurate, they lack reproducibility and depend entirely on the microbial growth. They are also labour intensive, time consuming and have poor discriminatory power (Farber, 1996).

1.6.2 Molecular based taxonomy

There are different genotyping techniques used for either species identification or differentiating of strains of LAB to the clonal level. The major advantages of these DNA-based typing methods are their ability to distinguish different bacteria to the species or even strain level and they have a wider range of application (Farber, 1996). Even today, closely related strains with similar phenotypical characteristics can be accurately and reliably distinguished by DNA-based techniques, such as randomly amplified polymorphic DNA (RAPD), DGGE, PFGE, TGGE, real-time PCR, RFLP and amplification rDNA restriction analysis among others (Mohania et al., 2008).

1.6.2.1 Microbial community analyses by denaturing temperature/gradient gel electrophoresis (DGGE/TGGE)

The general principle of DGGE and TGGE is the ability to separate DNA of the same length fragments based on differences in their nucleotide base sequence and the respective different DNA melting points. DGGE is based on the principal of separation of a mixture of DNA fragments using polyacrylamide gels that consist of linear denaturing gradient formed by urea and formamide (Muyzer & Smalla, 1998). The high mol % GC DNA fragments melt at higher denaturant region of the gradient, while TGGE separation takes advantage of the linear temperature gradient. The 16S rRNA gene sequences from bacterial species in a mixed culture are first amplified using conserved bacterial primers that bracket a hypervariable region of the 16S rRNA gene, producing amplicons of the same length, but with differing sequences that are specific to a given species (Muyzer & Smalla, 1998). These methods are used in the phylogenetic profiling of complex microbial communities within different environments, without necessarily depending on the cultivability of the microorganisms. They create band patterns in a polyacrylamide gel that represents the microbial community profiles, with each band representing the 16S rRNA of a hypothetical single bacterial strain. The comparison of the various samples allows the identification of the bands characteristics (Muyzer & Smalla, 1998; Cetecioglu et al., 2012).

1.6.2.2 Pulse Field Gel Electrophoresis (PFGE)

This method employs an alternating electric current that is periodically switched in three directions; one that runs through the central axis of the gel and the two that run at 60-degree angle either side. There is equal pulse time for each direction, thus resulting to a forward movement of the large chromosomal DNA fragments obtained from restriction digests with rare-cutting enzymes, that generates fingerprint profiles that can be explored for bacterial identification (Holzapfel et al., 2001; O'Sullivan & Kullen, 1998). This method is usually more time-consuming than other DNA fingerprinting techniques. Nonetheless, PFGE generated profiles represents a whole genome with superior discriminatory power. This technique can differentiate microorganisms to the subspecies/strain level (Kimura et al., 1997; O'Sullivan & Kullen, 1998).

1.6.2.3 RAPD PCR fingerprinting

Randomly Amplified Polymorphic DNA (RAPD) PCR is a modification of conventional PCR in which a single, short and random oligonucleotide primer anneals and primes at several

locations throughout the genome. Unlike conventional PCR analysis, RAPD-PCR does not require prior knowledge of the target organism DNA sequence. In RAPD analysis, the targeted amplified sequence is unknown and a primer with an arbitrary sequence (a 10-base pair sequence randomly generated by computer) is used. After these primers have been synthesized, they are used in PCR reactions with low-stringency annealing conditions, which results in the amplification of randomly sized DNA fragments (Spano et al., 2002). This method can be used to characterize and identify LAB strains. However, the reproducibility of RAPD PCR patterns is occasionally poor hence; performing this method needs a lot of care (Spano et al., 2002).

1.7 Fermentation

Fermentation is among the oldest technologies known to man for extending the shelf-life, palatability and improving sensory characteristics of food for more than 6,000 years (Holzapfel, 1997; Smid & Hugenholtz, 2010). Food fermentation involves the conversion of carbohydrates to alcohol and carbon dioxide or organic acids (mostly lactic and acetic acids) using bacteria and/or yeasts, under aerobic or anaerobic conditions (Stiles & Holzapfel, 1997). Fermentation of food can be done in three ways, namely spontaneous (natural) fermentation, backslopping and controlled fermentation. In natural or spontaneous fermentation, the conditions are set to favour the desired or the most adaptive microorganisms to grow which produce by-products of growth that help to outgrow others and dominate in the fermentation (Holzapfel, 1997). In case the fermentation experiment is unstable or the indented microorganism might not grow, then controlled fermentation is used. In this approach, specific LAB are isolated, characterised and then maintained for further use as starter cultures (Zulu et al., 1997). The starter cultures can be used as single or a combination of different strains under optimal growth conditions, which results in products of predictable quality and desirable organoleptic characteristics (Zulu et al., 1997; Oguntoyinbo et al., 2016).

Backslopping involves the use of the residues from the previous fermentation batch of good quality for inoculation, thus resulting in accelerated initial fermentation time and controlled desired changes (Holzapfel, 1997). Backslopping ensures the predominant strains present in the previous successful fermentation are used to inoculate fresh raw material and take control over the fermentation. This strategy is used in the production of various fermented foods, such as sauerkraut, cucumbers and sourdough (Leroy & De Vuyst, 2004; Stiles & Holzapfel, 1997), or for the

production of products for which the microbial ecology and the role of succession in the microbial populations are not known (Leroy & De Vuyst, 2004; Mogensen et al., 2002).

Lactic acid bacteria play a big role in the rapid production of lactic acid, which in turn causes a rapid acidification of the raw material. The fermentation of the raw material offers numerous benefits, several of which are vital for survival and safe nutrition of populations among local communities (Steinkraus, 1995; Stiles & Holzapfel, 1997). Here, fermented foods are treasured as major dietary constituents, primarily because of their stability and elevated shelf life under ambient conditions, and for their safety and traditional acceptability (Lahtinen et al., 2011). The benefits of food fermentation include: 1) enhancement of food flavour, texture and aroma development and reduction of food spoilage, 2) aiding in food preservation due to the accumulation of lactic acids, acetic acid, CO_2, bacteriocins and low molecular metabolites from carbohydrate metabolism, 3) biological enrichment of the food substrates with essential amino acids, proteins, fatty acids and vitamins, 4) improved toxicological safety of products by degradation of antinutritive factors (e.g., linamarin and mycotoxins) and 5) cooking time and fuel requirements are reduced through fermentation (Kostinek et al., 2005; Leroy and De Vuyst, 2004; Steinkraus, 1995; Stiles & Holzapfel, 1997).

1.7.1 Lactic acid bacteria

Lactic acid bacteria belong to phylum Firmicutes with low guanine-cytosine content (32-53 mol %) in the DNA. They are grouped in the *Bacillus* class (class three) of the Firmicutes, together with the Clostridia (class one) and the Mollicutes (class two). Based on the 16S rRNA gene comparative nucleotide sequence analysis, the Firmicutes can be distinguished from Actinobacteria as the latter have a high guanine-cytosine content (≥ 55 mol %) in the DNA (Holzapfel & Wood, 2014). LAB are described as Gram-positive rods or cocci, they are acid tolerant, devoid of cytochromes and porphyrins and therefore are catalase- and oxidase-negative, anaerobic or aero-tolerant, non-spore-forming and mostly non-motile (Stiles & Holzapfel, 1997; Jay et al., 2005; Holzapfel & Wood, 2014). They are usually fastidious microorganisms that require rich and complex nutrients for growth. They produce lactic acid as the main by-product of sugar fermentation. They are ubiquitous commonly occurring in food-related environments such as wine, meat, milk, fruits, vegetables and cereals and are frequently used as starter cultures during food fermentation. Additionally, they are found in animals and humans, especially in the gastrointestinal

tract, mouth and mucous membranes as normal flora (Axelsson, 2004; Majheniì & Matijašiè, 2001; Vaughan et al., 2002). Through lactic acid production and the rapid drop in the pH, the LAB are able to prevail in a nutrient-rich environment against other microorganisms (Leroy & De Vuyst, 2004). Among LAB, the genus *Lactobacillus* is the most diverse group that includes a high number of generally recognized as safe (GRAS) species whose strains contribute immensely in food microbiology and human nutrition (Salvetti et al., 2012). Taxonomically, the genus *Lactobacillus* comprises >150 well-described species and it belongs to the family *Lactobacillaceae* together with other important genera like *Lactococcus*, *Pediococcus, Streptococcus*, *Enterococcus, Leuconostoc*, *Tetragenococcus*, *Aerococcus*, *Oenococcus*, *Fructibacillus*, *Carnobacterium* and *Weissella* (Holzapfel & Wood, 2014; O'Sullivan et al., 2002; Salvetti et al., 2012).

1.7.2 Classification of lactic acid bacteria

The classification of LAB into different genera is largely based on cell morphology, mode of glucose fermentation, growth at different temperatures, configuration of the lactic acid produced, ability to grow at high salt concentrations, and acid or alkaline tolerance (Axelsson, 2004). An important characteristic used in the differentiation of the LAB genera is the mode of glucose fermentation under standard conditions, i.e. non-limiting concentrations of glucose and growth factors (amino acids, vitamins, and nucleic acid precursors) and limited oxygen availability. Under these conditions, LAB can be divided into two groups: the homofermentative and the heterofermentative groups (Axelsson, 2004; Adams & Moss, 2008).

1.7.3 Homofermentative lactic fermentation

In the homofermentative metabolism, LAB ferment hexoses to lactic acid via glycolysis (Embden-Meyerhof-Parnas) pathway (Fig.1.2). In the presence of excess glucose and limited oxygen, LAB convert 1 molecule of glucose to 2 molecules of pyruvate, nicotinamide adenine dinucleotide is simultaneously oxidized with pyruvate reduction to yield 2 molecules of lactic acid and 2 molecules of ATP per mole of glucose in the presence of the enzyme lactate dehydrogenase.

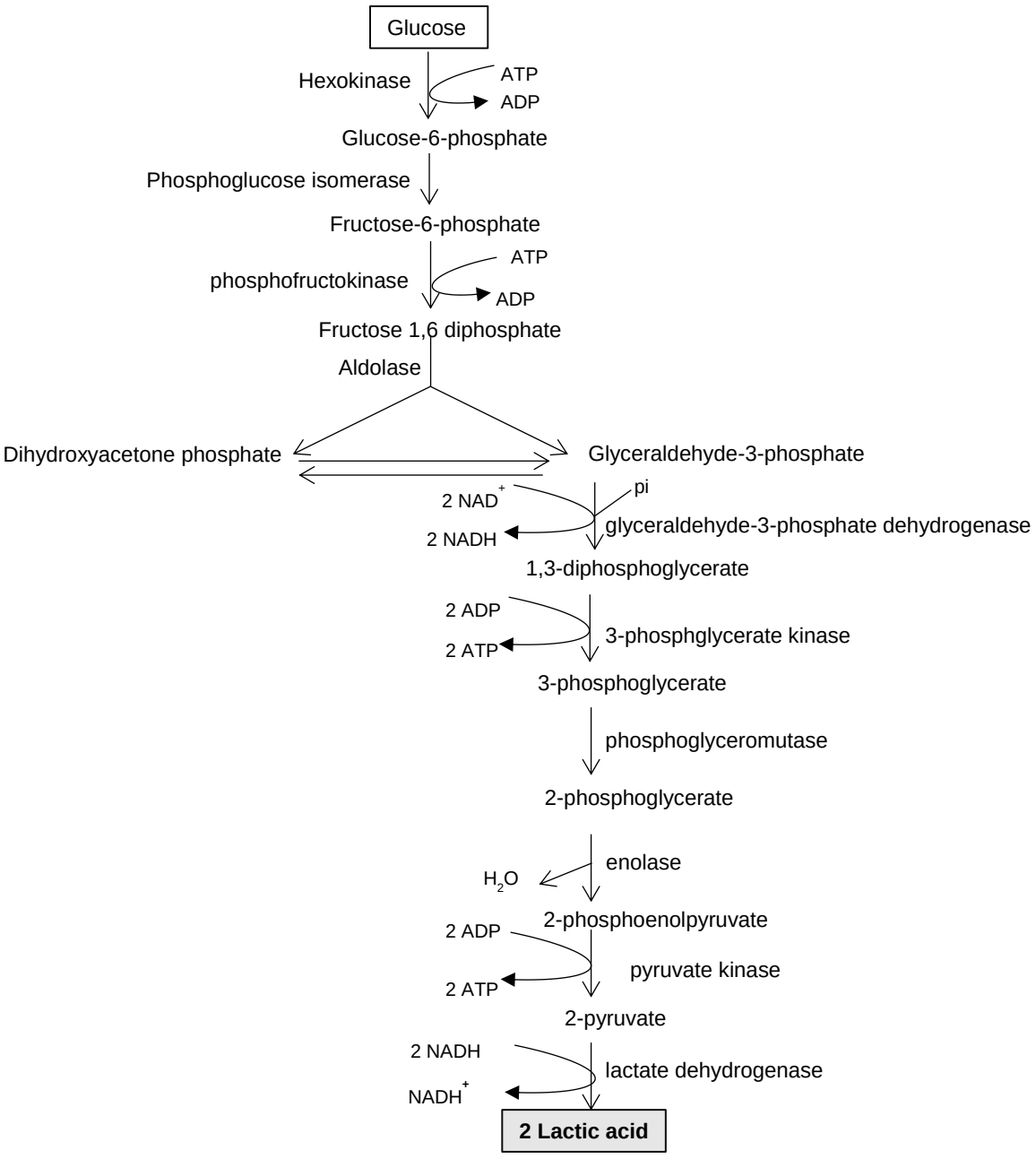

Figure 1.2: Homofermentative lactic fermentation through Embden-Meyerhorf-Parnas (EMP) glycolytic pathway.

1.7.4 Heterofermentative lactic fermentation

Heterofermentative LAB lack the necessary glycolytic enzymes aldolase and triosephospate isomerase. Therefore, fermentation takes place via the pentose phosphate pathway. Hexose (glucose) is transformed into pentose by an oxidation and a decarboxylation sequence by heterofermentative lactic acid bacteria. The pentose is cleaved into glyceraldehyde phosphate and acetyl phosphate in the presence of the phosphoketolase enzyme. The triose phosphate is converted

into lactate by a similar reaction mechanism as in the case of glycolysis, to yield two molecules of ATP. The fate of acetyl phosphate depends on the presence or absence of electron acceptors, their absence results in the reduction of acetyl phosphate to ethanol, at the same time, two molecules of NAD^+ are generated from NADH. However, in the presence of oxygen, NAD^+ is generated through NADH oxidases and peroxidases, leading to conversion of acetyl phosphate to acetic acid, yielding two molecules of ATP. Figure 1.3 below illustrates heterofermentative lactic acid fermentation via the pentose phosphate pathway (Hutkins, 2007; Adams & Moss, 2008; Holzapfel & Wood, 2014). Examples of heterofermentative LAB are species belonging to the genera *Leuconostoc*, *Oenococus*, *Weissella* and some species of the genus *Lactobacillus.*

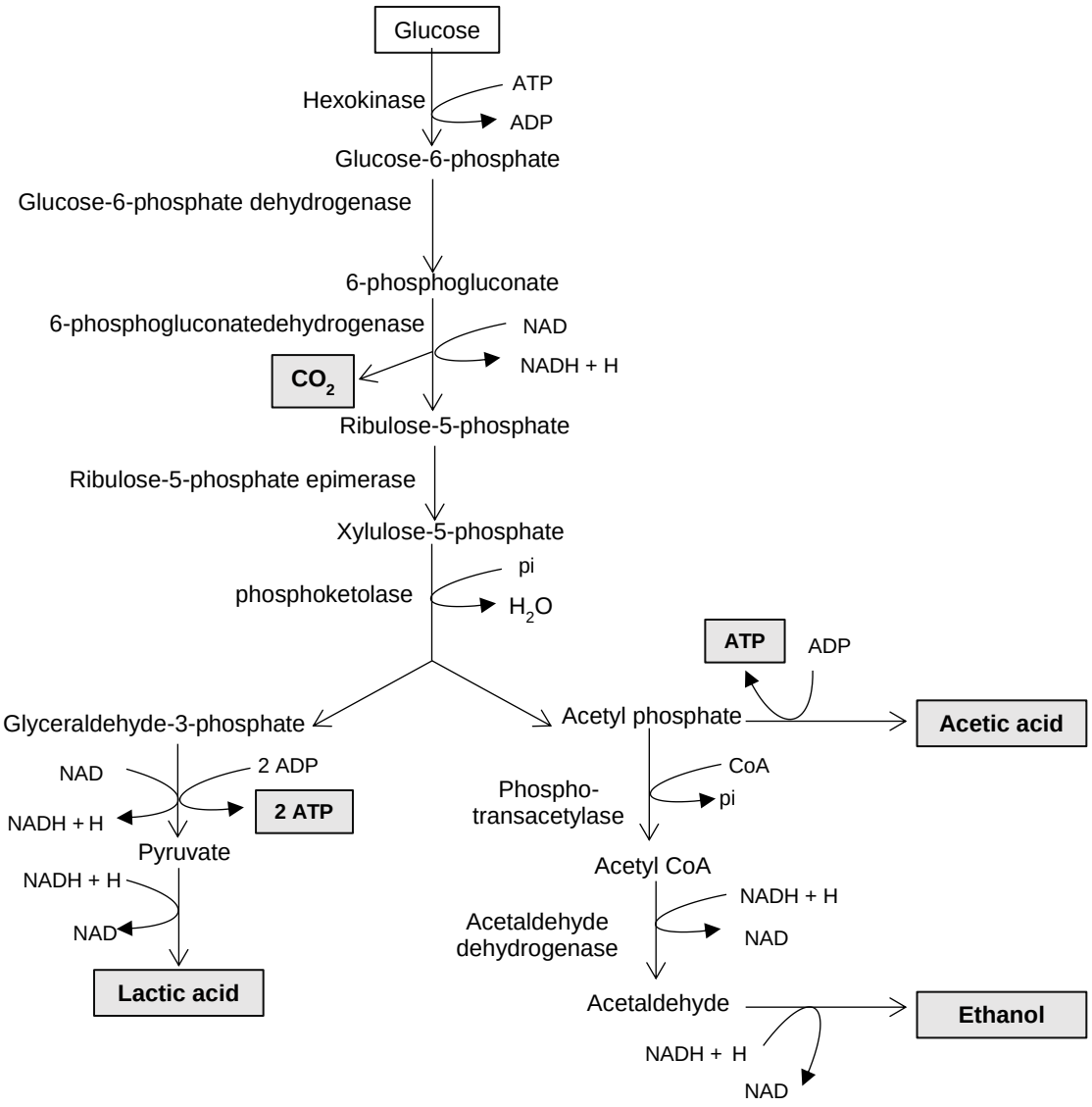

Figure 1.3: Heterofermentative lactic acid fermentation through the pentose phosphate pathway.

1.7.5 Lactic acid bacteria starter cultures in food fermentation

As LAB are natural inhabitants of most food products, their influence on fermentations spans for many years. Production of fermented foods in ancient times was based on spontaneous fermentation as a result of development of natural microorganisms present in the raw material, the varieties of the starting material and the microbial capacity determined the quality of the end product (Leroy & De Vuyst, 2004). Spontaneous/natural fermentation was optimized by inoculation of the fresh material with a small amount of material from a previously performed successful fermentation in a process called backslopping (Holzapfel, 1997). This process resulted in

proliferation of the mostly dominant and well-adapted strains to the substrate (Holzapfel, 1997). Backslopping represented an empirical way of using selected starter cultures for shortening fermentation time and thus reducing fermentation failures (Holzapfel, 1997; Leroy & De Vuyst, 2004). This method works for any type of fermented food, and is currently used for production of beer, vinegar, sauerkraut/ pickles and sourdough bread at household level, in addition to the preferred use of this method in the less developed countries. Natural fermentation in conjunction with backslopping is very successful in production of unique products with good qualities. However, modern production of industrialized fermented food and beverages demands a more controlled process, with consistency in product quality and predictability in production timelines, as well as strict quality controls for ensuring food safety (Hutkins, 2007).

Therefore, there has been a development towards the deliberate use of LAB starter cultures in order to reduce processing time and to produce standardized and microbiologically safe product with specific properties (Caplice & Fitzgerald, 1999; O'Sullivan et al., 2002). Starter cultures comprise a microbial preparation with a large number of (different) microorganisms which can be added to a raw material, to accelerate a fermentation process and to dominate the existing populations. Fermentation thus improves the product functionality and shelf life, and enhances the sensory qualities, as well as increases the product's economic value (Holzapfel, 1997; Hutkins, 2007). Starter cultures can be classified based on their composition; the single strain starter is used as a single, pure strain culture, while mixed strains are naturally occurring starters of an undefined combination of different bacterial species. The composition is based on dynamic equilibrium between different starter bacteria and subject to change during use. The last group is the multiple strain starter, which consist of specific mixture of a few of different species of bacteria, or different strains of one species (Schaechter, 2009). There are currently twelve LAB genera, although only four are commonly used as starter cultures in food and beverage fermentation. These include species of the genera *Lactococcus*, *Leuconostoc*, *Streptococcus* and *Lactobacillus,* with the dairy LAB representing the largest group. The main functions of LAB starter cultures in food fermentation are to cause acidification of milk or cheese via fermentation of lactose (milk sugar), to improve or modify the texture of foods as well as to improve or modify the product's flavour. The main principle behind LAB fermentation is the acidification of food, which plays an inhibitory role towards pathogenic and other food spoilage microorganisms (Holzapfel, 1997; Steinkraus, 1994; Hutkins, 2006).

1.7.6 Food fermentation in Europe

There are many varieties of fermented foods in Europe, with the majority belonging to the group of fermented animal proteins and vegetables. For example, meat is fermented to produce a variety of products, while fermentation of milk gives many different types of cheeses, depending on the method of production. There are also many fermented breads, e.g., from wheat and rye, apples are fermented into cider, while grapes are fermented into a different range of red and white wines. The type of food substrate and LAB involved in European fermented foods and beverage are summarised in table 1.2 below (Adams and Ross, 2008; Batt and Tortorello, 2014; Leroy and De Vuyst, 2004).

Table 1.2: Types of European fermented foods and beverages and the microorganisms involved in these fermentations

Fermented product	Fermented substrate	Microorganisms involved
Dairy products		
Hard cheeses without eyes	Milk	*Lactococcus lactis* subsp. *lactis, Lc. lactis* subsp. *cremoris*
Cheeses with small eyes	Milk	*Lc. lactis* subsp. *lactis*, *Lc. lactis* subsp. *lactis* var. *diacetylactis*, *Lc. lactis* subsp. *cremoris*, *Leuconostoc mesenteroides* subsp. *cremoris*
Swiss- and Italian-type cheeses	Milk	*Lactobacillus delbrueckii* subsp. *lactis, Lb. helveticus, Lb. casei, Lb. delbrueckii* subsp. *bulgaricus, Streptococcus thermophilus*
Butter and buttermilk	Milk	*Lc. lactis* subsp. lactis, *Lc. lactis* subsp. lactis var. *diacetylactis*, *Lc. lactis* subsp. *cremoris*, *Leuc. mesenteroides* subsp. *cremoris*
Yoghurt	Milk	*Lb. delbrueckii* subsp. *bulgaricus*, *Strep. thermophilus*
Probiotic milk	Milk	*Lb. casei, Lb. acidophilus, Lb. rhamnosus, Lb. johnsonii, Bifidobacterium lactis, Bifidobacterium bifidum, Bifidobacterium breve*
Kefir	Milk	*Lb. kefir, Lb. kefiranofacies, Lb. brevis*
Koumiss	Milk	*Lb. delbrueckii* subsp. *bulgaricus*
Fermented vegetable products		
Sauerkraut	Cabbage	*Leuconostoc mesenteroides, Lb. brevis, Lb. plantarum, Pediococcus acidilactici*
Pickles	Vegetables and fruits	*Leuc. mesenteroides, Ped. cerevisiae, Lb. brevis, Lb. plantarum*
Fermented olives	Olives	*Leuc. mesenteroides, Lb. pentosus, Lb. plantarum*.
Fermented vegetables	Vegetables	*Ped. acidilactici*, *Ped. pentosaceus*, *Lb. plantarum*, *Lb. fermentum*
Capers	Capers fruits (*Capparis spinosa* L)	*Lb. plantarum, Lb. pentosus, Lb. fermentum, Lb. brevis, Lb. paraplantarum, Enterococcus faecium, Ped. pentosaceus*

Fermented meat products		
Fermented sausage	Various meat	*Lb. sakei*, *Lb. curvatus*
Peperoni	Pork, beef	Lactic acid bacteria, micrococci, yeast
Salami	Pork	Lactic acid bacteria, micrococci
Bacon	Slices of cured pork, beef	LAB, yeast, micrococci
Chorizo	Pork	Lactic acid bacteria
Ham	Cured pork	LAB, yeast, micrococci
Fermented grain products		
Sourdough	Rye, wheat	*Lb. sanfranciscensis*, *Lb. farciminis*, *Lb. fermentum*, *Lb. brevis*, *Lb. plantarum*, *Lb. amylovorus*, *Lb. reuteri*, *Lb. pontis*, *Lb. panis*, *Lb. alimentarius*, *Weissella cibaria*, yeast
Fermented fish products		
Fermented fish products	Fish	*Lb. alimentarius*, *Carnobacterium piscicola*

1.7.7 Fermentation in Africa

1.7.7.1 Food and health situation in Africa

According to the FAO, about 795 million people are undernourished globally. However, hunger and malnutrition are prevalent among majority of the poorest sub Saharan countries (FAO et al., 2015). Hunger is based on the lack of calories as result of insufficient or lack of consumption of enough food that supplements the body with the required energy and nutrition. Micronutrient deficiencies are the so-called 'hidden-hunger' and affect approximately 2 billion people worldwide (Burchi et al., 2011). Good nutritional status is vital for the proper functioning of the body immune system, however, malnutrition tends to weaken the natural immunity, hence increasing disease progression and even death. Children are mostly affected by chronic malnutrition, since they have increased nutritional requirements, concurrent with a less developed immune system. Due to this fact, simple infectious diseases, as a result of poor hygienic conditions, usually result in death (Black & Bryce, 2003). Malnutrition is suspected to contribute more than one third of worldwide deaths of all children (Bain et al., 2013; Kalipeni, 2000; Nannan et al., 2007). It is estimated that in 2013, about 6.3 million children under 5 years died, of which 2.9 million were from Africa (WHO, 2013).

1.7.7.2 African fermented foods

The African continent has a wide variety of traditional fermented foods; especially those based on plant substrate materials. In Africa, fermentation has an age old history with the richest

variety of fermentations being the lactic acid fermented foods. These foods usually have a large impact on the nutritional health and social economic status of the people (Franz et al., 2014). There are three different traditional food habits in the world that are based on staple cereal dietary cultures: i.e. the eastern food culture diets of cooked-riced, the Western and Australian food culture of wheat/barley-based breads/loaves and the African and south American culture of maize/sorghum/millet porridges (Fusco et al., 2017; Tamang & Kailasapathy, 2010). Fermentation of African foods is usually performed using minimal technology often on a small scale and household basis, characterized by the use of simple, non-sterile equipment, chance or natural inoculas, unregulated conditions, sensory fluctuations, poor durability and unattractive packing of the processed products, resulting in food of unpredictable quality (Oguntoyinbo et al., 2016; Wafula et al., 2016).

The variety of foods and diverse cultures encountered in Africa make it difficult to select a specific type of food for the whole population. Each area has its own regional favourite foods that depends on customs, tradition and religion (Adebayo et al., 2010). The fermentation processes for these foods constitute a vital knowledge for the indigenous food preservation, acquired from observations and experience, and passed on from generation to generation (Aworh, 2008; Chelule et al., 2010). Cereals and root tubers are the most important source of food in Africa, constituting over 80 % of the average diet (Onwueme & Sinha, 1991). With increasing industrialization and urbanization, efforts are presently geared towards the development of large-scale, factory production facilities for these foods, where the quality of the finished product will be assured (Agarry et al., 2010). The most popular African fermented raw foods are crops, cereals, oil seeds, roots and milk (Oyewole, 1997). Fish, meat and vegetables are also fermented in Africa, but not as frequently as in Europe and Asia (Lee, 1997; Oyewole, 1997). Milk fermentation in Africa is mostly practiced in the Savannah, Sahara, Northern, and in rift valleys of East Africa regions, usually with addition of wood ash, blood or leafy vegetables in fermentation vessels that are usually treated with wood smoke (Fusco et al., 2017). Fermentation of milk products (e.g., yoghurt, cheese) are not similar to those typically found in Europe or North America (Fusco et al., 2017; Olasupo et al., 2010).

Fermented food in Africa can be classified on the basis of the substrate used for their preparation or the nature of the finished products (Oguntoyinbo et al., 2016; Olasupo et al., 2010) into the following: 1) fermented, non-alcoholic, cereal based products, 2) starchy root crops, 3)

fermented animal protein, 4) fermented vegetable proteins 5) alcoholic beverages and 6) fermented fruits and leafy vegetables Table 1.3 (Fusco et al., 2017). In the fermented non-alcoholic cereals, the main substrates used in fermentation are usually maize, millet and sorghum, which are fermented through natural/spontaneous fermentation (without addition of starter culture) or without backslopping (Fusco et al., 2017). They usually account for up to 80 % of the total energy consumption in many African countries. Fermented foods serve as a staple diet for adults and as weaning foods for infants (Fusco et al., 2017). Fermentation is usually carried out by lactobacilli, particularly *Lb. plantarum, Lb. fermentum, Lb. delbrueckii, Pediococcus* and *Weissella* spp., and occasionally yeast and moulds are involved (Tab.1.3). LAB fermented cereal-based African foods maybe classified based on two factors: i.e. the raw cereal ingredients used for preparation, examples include: maize-based foods such *mahewu* (South Africa), *ogi* (Nigeria / West Africa), *kenkey* (Ghana), *uji* (east Africa), *potopoto* (Congo), gowé and *mawe* (Benin); b) millet based foods such as *kunu-zaki* (Northern Nigeria), *mbege* (Tanzania), dégué and *ben-saalga* (Burkina Faso); c) sorghum based foods such as *ogi-baba* (West Africa), *ting* (Botswana), *injera* (Ethiopia) *bogobe* (Botswana), *humulur* (Sudan), *kisra* (Sudan) and *hussuwa* (Sudan);and d) wheat-based foods such as *bouza, kishk* or *kishj* Egypt (Tab.1.3), or the fermented product texture (Nout, 2009; Oyewole, 1997; Olasupo et al., 2010).

In the African fermented starchy roots, cassava (*Manihot esculenta*) is the main substrate for making a wide range of fermented products like *gari*, *fufu* and *lafun*, consumed commonly in West Africa and *agbelima* in Ghana (Caplice & Fitzgerald, 1999; Kostinek et al., 2007), *kivunde* and *chikwangue* in East Africa (Kimaryo et al., 2000; Padonou et al., 2009) (Tab.1.3). The common LAB associated with starchy roots fermentation usually are *Leuconostoc, Lb. plantarum* and *Lb. fermentum* strains (Kostinek et al., 2008). Cassava fermentation facilitates a detoxification process by breaking down cyanogenic glucosides (linamarin and lotaustralin) which occurs by endogenous linamarase activity, resulting in safe final products (Kostinek et al., 2008) Fermentation of animal proteins in Africa is mostly associated with milk, although fish fermentation is also practiced at a lesser scale (Fusco et al., 2017). The common LAB associated with milk fermentation includes; *Lactococcus lactis, Streptococcus thermophilus, Lb. plantarum, Lb. paracasei, Lb. acidophilus*, and *Lb. delbrueckii* and yeasts such as *S. cerevisiae* and *Candida* spp. (Franz et al., 2014; Gonfa et al., 2001; Mathara et al., 2004). In Africa, milk fermentation is done on a rural and tradition-based, small–scale level, to convert milk into varying products with extended shelf life. Different milk

products are produced from different parts of Africa, for example: *Amasi* is a traditional fermented milk consumed in South Africa and Zimbabwe and it's prepared by fermenting raw milk for several days in calabashes made of gourd, or in stone jars (Chelule et al., 2010). In Ethiopia, raw milk is traditionally fermented into products such as *ergo* (fermented sour milk), *ititu* (fermented milk curd), *kibe* (local butter), *neterkibe* (*kibe* or traditional *ghee*), *ayib* (cottage cheese), and *arera* (sour defatted milk) (Gonfa et al., 2001; Fusco et al., 2017). In Kenya, the Maasai consume *kule naoto*, a fermented product from Zebu cow milk (Mathara et al., 2004). Other African fermented animal protein products are shown in table 1.3 below.

Fermented legumes and oilseeds are usually fermented Africa vegetable proteins (Fusco et al., 2017). *Bacillus* spp. are commonly involved in fermentation via alkaline hydrolysis of the proteins to amino acids and ammonia (Olasupo et al., 2010). Alkaline-fermented food condiments in Africa are prepared from seeds from several wild trees (Achi, 1992; Ogunshe et al., 2007; Ouoba et al., 2004), as well as from various cultivated plant seeds. Raw materials used for vegetable protein fermentations in Africa include soy beans, roselle (*Hibiscus sabdariffa*) seeds, Bambara groundnut, melon (*Citrullus vulgaris*) cotton seeds (*Gossypium hirsutum* L.), castor oil bean (*Ricinus communis*), African locust bean (*Parkia biglobosa*), African mesquite (*Prosopis Africana*), cotton seed (*Gossypium hirsutum*) and African oil bean (*Pentaclethra macrophyla*), Saman tree (*Albizia saman*) and Baobab tree (*Adansonia digitate*) (Fusco et al., 2017)) (Tab.1.3). Africa is rich in different fruits and leafy vegetables containing high amounts of nutrients and micronutrients (Oguntoyinbo et al., 2016). It is conceivable, therefore, that efforts for the fermentation of e.g. cowpea, sorghum, spider plant, nightshade or kale leaves are intensified, in order to preserve the nutrients and prevent postharvest losses of such highly perishable products. Some examples of known African leaf fermentations are shown in table 1.3. The reported microorganisms associated with these fermentations were a mixture of a variety of yeasts and bacteria such as lactobacilli, micrococci, staphylococci and propionibacteria. This point to the fact that in these fermentations a predominating lactic acid microbiota cannot establish itself and that the microorganisms, which predominate in the fermentation, may possibly depend on which microorganisms were initially present on the raw materials.

Table 1.3: Types of African fermented food with the microorganisms involved in fermentation (alcoholic beverages are not included)

Fermented product	Region of production	Raw substrate	Microorganisms involved in fermentation
African fermented cereal-based non-alcoholic foods			
Ogi	Nigeria, Benin	Maize, sorghum or millet	*Ped. pentosaceus*, *Lb. fermentum Lb. plantarum*, yeasts
Koko and Kenkey	Ghana	Maize, sorghum or millet	*Weissella confusa*, *Lb. fermentum*, *Lb. salivarius*, *Pediococcus* spp., yeasts
Mahewu (magou)	South Africa	Maize, sorghum or millet	*Lb. delbrueckii*, *Lb. bulgaris*, *Leuconostoc* spp.
Uji	East Africa	Maize, sorghum or millet	*Lb. plantarum*, *Lb. paracasei*, *P. pentosaceus*, *Leuconostoc* spp.
Kisra	Sudan	Sorghum	Lactic acid bacteria
Injera	Ethiopia	Sorghum	*Candida guillermondii*
Ting	Botswana	Sorghum	*Lb. fermentum*, *Lb. plantarum*, *Lb. rhamnosus*
Obusera	Uganda	Millet	Lactic acid bacteria
Mawe	Benin	Maize, sorghum or millet	*Lact. lactis*, *Ped. pentosaceus*, *Lb. plantarum*
Hussuwa	Sudan	Sorghum	*Lb. fermentum*, *Ped. acidilactici*
Bogobe	Botswana	Sorghum	Unknown
Kunu-Zaki	Nigeria	Millet, sorghum	*Lb. fermentum*, *P. pentosaceus*, *W. confusa*, *Enterococcus faecalis*
Potopoto	Congo	Maize	*Lb. gasseri*, *Lb. plantarum*, *Lb. acidophilus*, *Lb. delbrueckii*, *Lb. reuteri*, *Lb. casei*, *Bacillus* spp., *Enterococcus* spp.
Dégué	Burkina Faso	Millet	*Lb. gasseri*, *Lb. fermentum*, *Lb. brevis*, *Lb. casei*, *Enterococcus* spp.
Ben Saalga	Burkina Faso	Millet	*Lb. plantarum* and other lactic acid bacteria
Fermented starchy root products			
Gari	West Africa	Cassava	*Lb. plantarum*, *Leuc. fallax*, *Lb. fermentum*, *W. paramesenteroides*, *Lb. brevis*, *Strep. lactis*
Fufu	Nigeria	Cassava	*Ped. pentosaceus*, *Lb. fermentum*, *Lb. plantarum*
Kivunde	Tanzania	Cassava	*Lb. plantarum*, other LAB, yeast
Lafun	Nigeria	Cassava	*Lb. fermentum*, *Lb. plantarum*, *W. confusa*, yeast
Chikawngue	Zaire	Cassava	LAB, yeast
Cingwada	East and Central	Cassava	Unknown

	Africa		
Kocho	Ethiopia	Ensette or Abyssinian banana (*Ensette* ventricosum)	LAB, yeast
Agbelima	Ghana	Cassava	*Lb. plantarum, Lb. brevis, Lb. fermentum, Leuc. mesenteroides, Bacillus* sp., yeast
Fermented animal proteins			
Nono (milk curd)	N. West Africa	Milk	Lactic acid bacteria
Maziwa lala	East Africa	Milk	*Lactococcus lactis, Strep. thermophilus*
Leban (sour milk)	Morocco	Milk	Lactic streptococci (lactococci), *Leuc. lactis*, *Leuc. mesenteroides* subsp. *Cremoris*
Wara	West Africa	Milk	*Lac lactis, Lactobacillus* spp.
Ergo	Ethiopia	Milk	*Lactobacillus* spp., *Lactococcus* spp.
Kule naoto	Kenya	Milk	*Lb. plantarum, Lb. fermentum, Lb. paracasei, Lb. acidophilus, lactococci,* leuconostocs and enterococci
Sethemi	South Africa	Milk	Lactobacilli, lactococci, yeast
Guedj	Senegal	Fish	*Lac. lactis*
Bonome (stink fish)	Ghana	Fish	Unknown
Fermented vegetable proteins			
Dawadawa or iru	West Africa	African locust bean (*Parkia biglobosa*), Soybean	*Bacillus subtilis, B. licheniformis*
Ogiri	Nigeria	Melon (*Citrullus vulgaris*)	*Bacillus* spp., (predominant), *Proteus, Pediococcus*
Ogiri-nwan	Nigeria	Fluted pumpkin bean (*Telfaria occidentalis*)	*Bacillus* spp.
Ogiri-igbo	Nigeria	Castor oil seed (*Ricinus communis*)	*Bacillus subtilis, B. megaterium, B. firmus*
Ogiri-saro.(sigda)	Sierra Leone, Sudan	Sesame seed	*Bacillus* spp.
Ogiri-okpec/okpehe	Nigeria	Mesquite *(Prosopsis africana*)	*Bacillus* spp.
Ugba.(Apara)	Nigeria	African oil bean (*Pentaclethra macrophylla*)	*Bacillus subtilis, Micrococcus* spp.

Owoh	Nigeria	Cotton seeds (*Gossypium lursutum*)	*Bacillus* spp.
Bukalga	Niger, Mali, Sudan, Burkina Faso	Kartade, red sorrel (*Hibiscus sabradiffa*)	*Bacillus subtilis*
Fermented fruits and leafy vegetables			
Kawal	Sudan	Fresh leaves of *Cassia obtusifolia*	*Bacillus subtilis* and *Propionibacterium* spp. (dominant), *Lb. plantarum, Candida krusei* and *Saccharomyces* spp.
Ntoba mbodi	Congo	sun-drying cassava leaves and papaya leaves	*Micrococcus varians, Bacillus macerans, Bacillus subtilis, Staphylococcus sciuri* and *Staphylococcus xylosus*
Agadagidi	Nigeria	ripe plantain (*Musa paradisiaca*) pulp	*Leuconostoc and Streptococcus* (dominant) *Bacillus, Micrococcus* and yeast
mudetemwa	Zimbabwe	fruits of the sand apple (*Parinari curatellifolia*	
palm wine	Africa	palm sap of *Rafia guinensis* and *Borassus akeassi*i	*Micrococcus, Leuconostoc, Streptococcus, Lactobacillus, Acetobacter, Serratia, Aerobacter (Klebsiella), Bacillus, Zymomonas and Brevibacterium* and *yeasts*
Masau fermented fruit pulp	Zimbabwe	*Ziziphus mauritiana* fruit (locally called masau)	*Lactobacillus agilis, Lb. plantarum* (dominant), *Lactobacillus bifermentans, Lactobacillus minor, Lactobacillus divergens, Lactobacillus confusus, Lactobacillus hilgardii, Lactobacillus fructosus, Lb. fermentum, Streptococcus* spp. and yeasts

1.7.7.3 Fermentation of African leafy vegetables

1.7.7.3 Fermentation of African leafy vegetables

There is limited information on the use of LAB starter cultures in the fermentation of AILVs in SSA. There are, however, various studies on the development of starter cultures for other fermented foods consumed in other countries, such as *uji* (Mbugua et al., 1984), *gari* (Kostinek et al., 2008), *kivunde* (Kimaryo et al., 2000) and *ogi* (Teniola & Odunfa, 2001). The fresh leafy vegetables are usually processed immediately and consumed. Sun drying is the common preservation method in Africa for leafy vegetables. However, this method results in the loss of vital nutrients. The study by Muchoki et al. (2007) fermented cowpea leaves, using the plants naturally occurring microbiota as source of starter microorganisms for 21 days. The fermentation showed a positive effect in the maintenance of various nutrients. There are recent studies involving prevention and control of postharvest losses of AILVs. Such studies included the fermentation of African kale (*Brassica carinata*), cowpea (*Vigna unguiculata)* and African nightshade (*Solanum scabrum*) leaves with previously isolated LAB from other fermented African food products as starter cultures. The results showed that controlled fermentation of kale, cowpea and nightshade offers a promising avenue to prevent spoilage and to improve the shelf life and safety (Wafula et al., 2015; Oguntoyinbo et al., 2016).

A generalised scheme for vegetable fermentation entails the harvesting of the vegetables followed by sorting and destemming, proper washing to remove soil and insects, and cutting of the vegetables into small pieces, or using it whole (Fig.1.4). Fermentation can be performed in three ways, i.e. either with 2.5-10 % salt, in non-salt solution, or with a mixture of 3-5 % salt-sugar brined solution. The vegetables are fermented in vessels, with addition of the brine solution pre-warmed at 60 ^{o}C. Fermentation of vegetables occurs spontaneously by the natural LAB surface microbiota, such as autochthonous *Lactobacillus, Leuconostoc* and *Pediococcus* spp. (Steinkraus, 2002) at 25-30 ^{o}C for up to 30 days (Fig.1.4). Lactic acid fermentation represents the easiest and the most suitable way for increasing the daily consumption of fresh vegetables (Swain et al., 2014; Wafula et al., 2016).

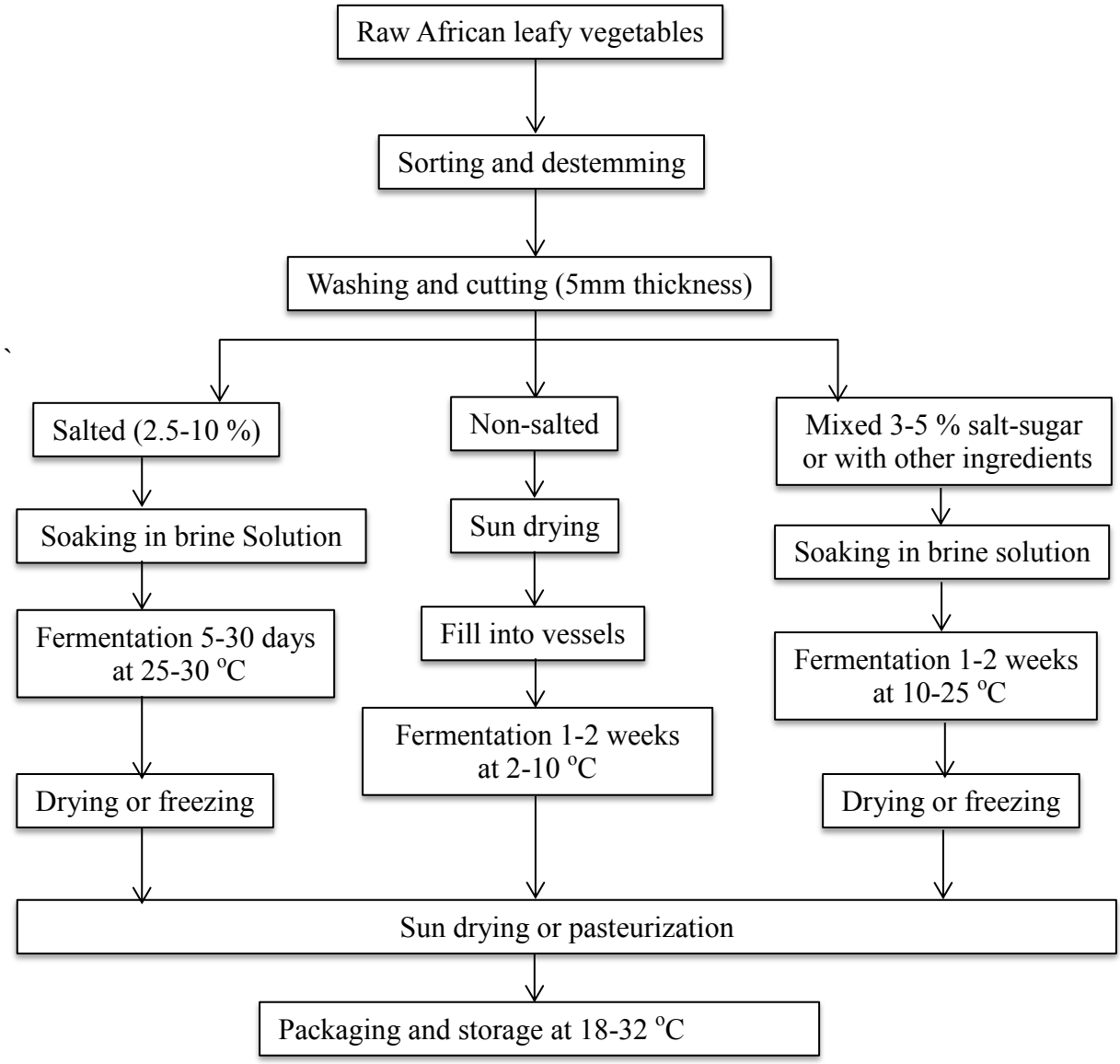

Figure 1.4: Generalised scheme for vegetable fermentation processes (Swain et al., 2014).

1.7.7.4 Importance of vegetable fermentation in Africa

Food fermentation plays an important role in most developing countries from nutrition, health, social and economic perspectives. The climatic conditions in many parts of Africa are usually not optimal for long-term storage and stability of fresh foods (Caplice & Fitzgerald, 1999). Therefore, fermentation is the simplest way of improving the nutritional, shelf life, sensory and functional qualities (Blandino et al., 2003).

Although most vegetables, especially the grains and legumes, are rich in dietary nutrients, their nutritive value is limited by the presence of several anti-nutritional and toxic substances, including oligosaccharides (especially raffinose, stachyose and verbascose) that are the main cause of

flatulence problems. Therefore, legume fermentation results to nutritional benefits as result of LAB breakdown of flatulence causing, indigestible oligosaccharides into absorbable organic acids that are of health benefit to the human body (Granito et al., 2005).

Certain probiotic LAB have been shown to prevent human diarrheal diseases, since they can temporarily modify the composition of intestinal microbiota and strengthen the host immune system (Franz et al., 2014; Mathara et al., 2004), thus preventing the growth of pathogenic enterobacteria. LAB also produce antifungal inhibitory compounds, which are mainly organic acids (Sauer et al., 2013). Research has shown that LAB fermentation is an effective way of removing Gram-negative bacteria from food products, since they are more sensitive to fermentation processing, i.e. to organic acids and low pH (Oguntoyinbo et al., 2016; Mensah, 1997; Motarjemi, 2002; Wafula et al., 2015). LAB thus play a defining role in the preservation and microbial safety of fermented foods, promoting the microbial stability of the final products of fermentation. In most African countries, where resources for cooking and food preservation are scarce, fermentation is highly recommended technique, especially in rural areas. This process is known to alter the composition of food and to soften its texture in such a way, that cooking will need minimal time and energy (Holzapfel, 1997).

Muchoki et al. (2007) fermented cowpea leaves in 16 kg batches for 21 days and then heat treated and solar dried the fermented leaves. The fermentation, heat-treatment and drying were shown to retain substantial levels of β-carotene and ascorbic acid. Kasangi et al. (2010) also fermented cowpea leaves and studied the fermentation kinetics. By adding 3 % glucose to the leaves, a fermentation with the highest concentration of lactic acid of 0.6 % and with a low pH of 4.7 could be obtained. The study concluded that fermentation, in conjunction with solar drying, has potential for small-scale farmers to enhance the product keeping quality, as well as nutrient quality (Kasangi et al., 2010). Cowpea, African nightshade and kale leaves were fermented in 1 litre beakers for 5 days to determine the role of the starter cultures *Lb. plantarum* BFE 5092 and *Lb. fermentum* BFE 6620 to inhibit the growth of pathogens *Listeria monocytogenes* and *Salmonella* Enteritidis. Fermentation was conducted on 100 g of the leaves and 3 % salt + 3 % sugar brine solution and inoculated with 1 x 10^7 cfu/ml starter cultures and 1 x 10^3 cfu/ml pathogens. The results showed that fermentation of African indigenous vegetables with selected starter cultures

inhibits the growth of pathogens within the first 3 days of fermentation (Wafula et al., 2015; Oguntoyinbo et al., 2016).

1.8 Drying techniques in food preservation

Food losses in the developing world reach up to 50 % of the fruits and vegetables produced and 25 % of the harvested food grain (Burden & Wills, 1989). Food drying is a very simple ancient preservation method. It is the most accessible and widespread processing technology that can reduce wastage of a harvest surplus, allow storage for food shortages, and in some cases facilitate export to high-value markets. Drying makes produce lighter, smaller, and less likely to spoil. There are two main methods of drying i.e. sun and solar drying (Szulmayer, 1971).

1.8.1 Sun drying

Sun drying of food is the most widespread method of food preservation in many African countries due to the abundance of solar radiance for the most part of the year. Drying of fruits and vegetables by means of the sun has been practiced from ancient times (Szulmayer, 1971). This method involves direct exposure of the product under the sunlight. The advantages of this method are that it needs low operating costs and capital without any expertise (Szulmayer, 1971). However, sun drying is prone to product contamination, theft or damage from birds, rats or insects (Szulmayer, 1971). There is intermittent or slow drying without the protection from rain and rain allows the growth of harmful microorganism due to high moisture content, leading to products with unreliable quality. Sun drying is also labour intensive and requires large spaces (Fuller, 1991; Szulmayer, 1971). Direct exposure of some fruits and vegetables under sunlight has profound negative effects on nutrient composition, especially vitamins, and even colour (Ukegbu & Okereke, 2013). There is also lack of standardised operational procedures, since sun drying depends on uncontrolled factors (Fuller, 1991; Szulmayer, 1971).

1.8.2 Solar drying

Solar drying is an alternative to traditional sun drying and it involves the use of dryers with solar radiation as a heat source. Solar drying works on the principle that the uptake of moisture by air is limited by the absolute (maximum) humidity and it is temperature dependent. Therefore, when air passes over a moist food it will take up moisture until fully saturated, i.e. until absolute

humidity has been reached. Nevertheless, the capacity of the air for taking up this moisture is temperature dependent. Which means that the higher the temperature, the higher the absolute humidity, and thus the larger the moisture uptake. By warming the air, the amount of moisture in it remains the same, but the relative humidity falls; and the air takes up more moisture from its surrounding (Fuller, 1991; Szulmayer, 1971).

The use of solar drying has more advantages compared to sun drying, especially when correctly designed solar dryers are used. The drying rate in solar dryers is usually higher when compared to sun drying, because the air is heated up to 10-30 ^{o}C above ambient, which also causes the air to move faster through the dryer and reduces its humidity (in addition to preventing the settling of insects) (Bindu et al., 2016). The faster drying decreases the risk of microbial spoilage, at the same time improving product quality. Hence, the solar drying systems allows higher throughput with reduced drying areas. However, too rapid drying can lead to incomplete drying, resulting in product hardening, which in turn encourages microbial growth and spoilage (Bindu et al., 2016; Sontakke & Salve, 2015). Construction of a solar dryer is usually cheap, because locally available materials are used. Thus, solar drying can be useful in areas where fuel or electricity is expensive, or where there is less space for sun drying (Rosselló et al., 1990; Szulmayer, 1971). Product evaluation of solar dried foods indicate that in most cases, the physical properties such as flavour and vitamin A and C retention were better than in the case of sun dried foods (Ukegbu & Okereke, 2013).

1.9 General objectives

To determine the effects of fermentation and solar drying methods on the safety and quality of AILVs, in order to minimize postharvest losses.

1.9.1 Specific Objectives

1) To select and identify appropriate *Lactobacillus* starter culture strains for optimal fermentation of nightshade based on their growth performance, acid production and product pH lowering capabilities.
2) To determine the success of these selected *Lactobacillus* starter cultures to establish themselves as dominant LAB in nightshade fermentations.
3) To determine community-level effects of the selected *Lactobacillus* starter cultures on the background autochthonous microbiota in nightshade fermentations by denaturing gradient gel electrophoresis (DGGE) and by metagenomic analyses using high-throughput 16S rRNA gene amplicon sequencing.
4) To determine effects of nightshade fermentation on safety and nutritional quality of the product by basic nutrient analyses and challenge tests with the human pathogenic bacteria *Salmonella* and *Listeria.*
5) To determine the effects of solar drying on the nutritional quality and microbial safety of African nightshade.
6) To determine the sensory characteristics of fermented AILVs products.

2.0 Materials and methods

2.1 Materials

2.1.1 Preparation of samples

African nightshade seeds used in this project were obtained from the AVRDC- The World Vegetable Centre (Arusha, Tanzania) within the framework of the HORTINLEA (Horticultural Innovation and Learning for Improved Nutritional and Livelihood in East Africa) project. African nightshade (*Solanum scabrum*) plants were cultivated in 5 L pots in low fertilized, peat-based substrate for sowing, potting and pricking out vegetables and ornamental plants. The culture substrate had the following nutrient composition; pH value ($Cacl_2$) 5.8, salinity g/l (water) 1.2, peat H2-H4 and H4-H8 (%) 100, nitrogen (N) mg/l 140, phosphate (P_2O_5) mg/l 160, potassium (K_2O) mg/l 180, magnesium (Mg) mg/l 150 and sulphur (S) mg/l 120 (Gramoflor Vechta, Germany).

Nightshade was grown at the Max Rubner Institute's climatic chamber (Fig.2.5a) for four weeks at 20/25 ^{o}C day/night temperature, 40-85 % day/night relative humidity with 12.5 h of artificial light and three to four weeks under greenhouse conditions (Fig.2.5b) at 18-40 ^{o}C day/night temperature, 18-77 % day/night relative humidity with additional light provided to compensate for daylight intensity variations.

Nightshade plants were also cultivated at the Jomo Kenyatta University of Agriculture and Technology (JKUAT) in the open fields (Fig.2.5c and d) for 6-8 weeks. JKUAT is situated in Juja, Kiambu County, Central, Kenya. The area is semi-humid to semi-arid with annual rainfall of 600-1100 mm, temperature, 17-28 ^{o}C and 40-50 % relative humidity. The soil type is nitisols, the vegetable nightshade were cultivated during the dry seasons of December to April. The used soil was supplemented with composite farmyard manure with application of drip-line irrigation 12 hours a day. The leafy vegetables were then harvested by hand picking (without gloves) and they were immediately delivered to the laboratory immediately for processing.

Figure 2.5: African nightshade photographed at MRI Karlsruhe, Germany climatic chamber (a) greenhouse (b) and JKUAT open field (c-d) Nairobi, Kenya.

2.1.2 Preparation of leaves for fermentation

The nightshade leaves were handpicked from the greenhouse (without gloves). The freshly collected vegetables leaves were thoroughly washed in a plastic trough under cold running tap water (Fig.2.6a) to remove impurities and insects (using gloves). They were then dried using a salad spinner to reduce drying time (Fig.2.6b and d) after which a layer of paper towel was spread on the disinfected stainless surface, the washed leaves were spread in a single layer on the paper towel, followed by creating a new layer of paper towel over the leaves, gently tapping. A new layer of the paper towel was put on another layer of leaves. The process was repeated until all the leaves were

covered with a layer of paper towel and gently tapped. Afterward the paper towels were removed layer by layer from the dried leaves and collected in a clean basket (Fig.2.6c).

Figure 2.6: Washing (a) and drying of nightshade leaves with salad spinner (b & d) and paper towels (c).

2.1.3 Preparation of fermentation vessels

Fermentation was done as submerged fermentation in a salt or salt/sugar brine (see below). To optimise fermentation conditions, four different experimental designs were used. The first approach was carried out in 50 ml Schott bottles, the second was done in 100 ml Schott bottles, the third fermentation was done in 1000 ml glass beakers and the last fermentation was carried out in 5 litre big fermentation (crock) pots (Fig.2.7), with either special metal plates for glass beakers (Fig.2.8c) or stones (crock pots) used for weighing down the leafy vegetables (Fig.2.8a-b). This avoided floating of the leaves to the surface (air interface), which prevented the growth of moulds during fermentation. Fermentation at JKUAT was carried out in 5 litre aluminium culture buckets (Fig.2.9a) with a sieve mesh (Fig.2.9b) with water in 1000 ml beaker wrapped in sterile

polyethylene bags to weigh down the leaves (Fig.2.9c). Prior to fermentation experiments, all the vessels were sterilised by autoclaving at 121 ^{o}C for 15 min.

Figure 2.7: Fermentation pots (5 litre pots/crocks) used at the MRI in Karlsruhe, Germany.

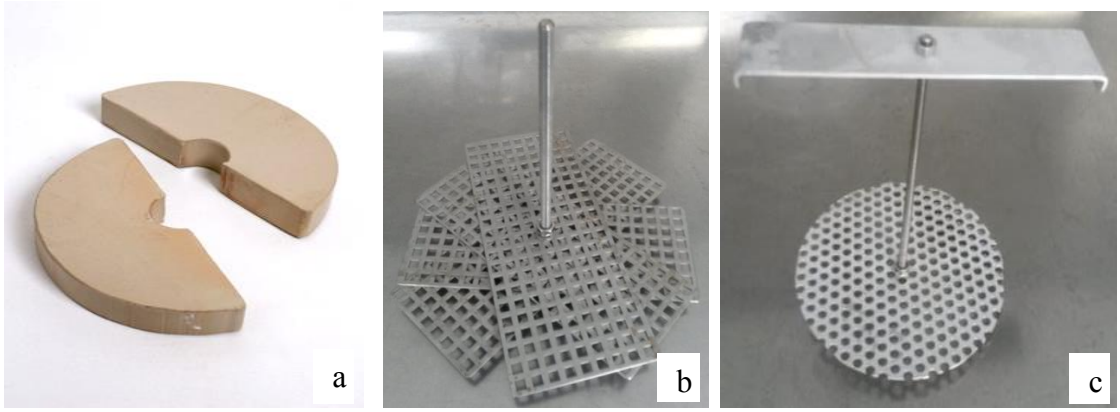

Figure 2.8: Metal mesh (b-c) and stones (a) to weigh down leaves during fermentation.

Figure 2.9: Fermentation buckets (a) with a sieve mesh (b) with water in 1000 ml beaker wrapped in sterile polyethylene bags to weigh down the leaves (c), JKUAT in Nairobi, Kenya.

2.1.4 Chemicals

2.1.4.1 Chemicals and reagents for nutrient analysis

The chemicals and reagents used for nutrient analysis, including safety data information according to the Gobally Harmonised System of Classification and Labelling of Chemicals (GHS) are described in annex 2A and 2B.

Table 2.4: Working solutions for vitamin B_1 and B_2 analysis

	Reagent/solution	**Concentration**	**Preparation**
2.4.1	Hydrochloric acid (HCl)	0.2 Mol/l	200 ml 1 M HCl (Annex 2A) were diluted in 800 ml of bidistilled water (2.4.8) and filled up to 1000 ml.
2.4.2	Hydrochloric acid (HCl)	0.1 Mol/l	100 ml 1 M HCl (Annex 2A) were diluted in 900 ml of bidistilled water (2.4.8) and filled up to 1000 ml.
2.4.3	Sodium acetate	2.5 Mol/l	205 g sodium acetate (Annex 2A) were dissolved in 500 ml bidistilled water and filled up to 1000 ml.
2.4.4	Sodium hydroxide	15 %	150 g sodium hydroxide (Annex 2A) were dissolved in small amount of bidistilled water (2.4.8) and filled up to 1000 ml.
2.4.5	Acetic acid	10 %	10 ml acetic acid (Annex 2A) were diluted in 90 ml bidistilled water (2.4.8) to make 100 ml.
2.4.6	Ammonium phosphate buffer (pH 3.5)	10 mMol/l with ion pair reagent	1.15 g ammonium dihydrogen phosphate (Annex 2A) were dissolved in approximately 700 ml bidistilled water (2.4.8), the pH was adjusted to 3.5 with 10 % acetic acid (2.4.5) (approximately 5.4 ml were required), 1 g tetraethyl ammonium chloride (Annex 2A) and 1 g sodium heptane sulfonate (Annex 2A) were added and dissolved. Bidistilled water (2.4.8) was used to fill up to 1000 ml.
2.4.7	Taka-Diastase enzyme suspension	25 mg/ml	The total amount of the enzyme suspension was prepared according to the number of samples [50 mg of the enzyme Taka-Diastase (2.4.7)] was dissolved in 2 ml bidistilled water (2.4.8). The enzyme was completely dissolved with magnetic stirrer at room temperature. This enzyme was prepared each day of the experiment. Taka-Diastase contains phosphatase active at 45-50 ^{o}C at pH 4.0-4.5 necessary to digest protein and starch.
2.4.8	Bidistilled water	HPLC quality	The LaboStar^{TM} system (Annex 3C) was fed with distilled water, sterilization was done

			through reverse osmosis in a system fitted with (0.2 µm) sterile filters.

Table 2.5: HPLC mobile phase for vitamins B_1 and B_2

	Reagent/solution	**Concentration**	**Preparation**
2.5.1	Ammonium phosphate buffer : methanol	80 %:20 % (v:v)	800 ml aqueous ammonium phosphate buffer solution (2.4.6) was mixed with 200 ml methanol (Annex 2A) in a brown glass bottle, it was mixed and degassed with ultra-sound.
2.5.2	Post- column reagent, K_3 (Fe (CN_6)	0.4 %	200 mg of potassium ferricyanide-III (Annex 2A) were weight and transferred quantitatively into a 500 ml volumetric flask and was dissolved in 50.0 ml of 15 % sodium hydroxide solution (2.4.4). Bidistilled water (2.4.8) was added up to 500 ml. This reagent was prepared each day of the experiment.

Table 2.6: Standard solutions for vitamins B_1 and B_2

	Reagent/solution	**Concentration**	**Preparation**
2.6.1	Thiamine stock solution	1000 µg/ml	100 mg of thiamine chloride- hydrochloride (Annex 2A) were dissolved in 0.1 Mol/l hydrochloric acid (2.4.2), it was then transferred into 100 ml volumetric flask and filled up to the mark. NB: The stock solution was stable at 4 ^oC for 2 months.
2.6.2	Thiamine standard solution1	100 µg/ml	10.0 ml of thiamine stock solution (2.6.1) was pipetted into 100 ml volumetric flask and filled up to the mark with 0.1 Mol/l hydrochloric acid (2.4.2).
2.6.3	Riboflavin stock solution	400 µg/ml	100 mg of riboflavin (Annex 2A) were dissolved into 30 ml 0.1 Mol/l hydrochloric acid (2.4.2), which was then transferred into a brown 250 ml volumetric flask. It was filled up to the mark with bidistilled water (2.4.8). The stock solution was stable at 4 ^oC for 2 months.
2.6.4	Riboflavin standard solution1	100 µg/ml	25 ml riboflavin stock solution (2.6.3) were transferred in a 100 ml brown volumetric flask then diluted with 30 ml 0.1 Mol/l hydrochloric acid (2.4.2)

			and filled up to the mark with bidistilled water (2.4.8).
2.6.5	Standard mix solution	$(B_1)=(B_2)=1\mu g/ml$	1000 µl of both vitamin B_1 standard solution1 (2.6.2) and vitamin B_2 standard solution1 (2.6.4) were pipetted into a 100 ml brown volumetric flask, which was then filled with 0.1 Mol/l hydrochloric acid (2.4.2) up to the mark.
2.6.6	Mix heated standard	$(B_1)=(B_2)=1$ g/ml	1000 µl vitamin B_1 standard solution1 (2.6.2) and vitamin B_2 standard solution1 (2.6.4) were pipetted into a 100 ml brown volumetric flask, they were mixed with 50 ml of 0.1 Mol/l hydrochloric acid (2.4.2) and autoclaved at 120 ^oC for 30 min. After autoclaving, the solution was cooled then filled to the mark with 0.1 Mol/l hydrochloric acid (2.4.2).

Table 2.7: Working solutions and reagents for determination of vitamin C

	Reagent/solution	**Concentration**	**Preparation**
2.7.1	m-phosphoric acid/ acetic acid solution	7.5 %/20 %	75 g m-phosphoric acid (Annex 2A) were completely dissolved in 600 ml bidistilled water (2.4.8) and transferred into a 1000 ml conical flask. 200 ml acetic acid (Annex 2A) were added and filled to the mark with bidistilled water (2.4.8). It was stable at room temperature for approximately 1 month.
2.7.2	Diluted m-phosphoric acid/ acetic acid solution	3 %/8 %	400 ml m-phosphoric acid/acetic acid solutions (7.5 %/20 %) (2.7.1) were diluted in 1000 ml bidistilled water (2.4.8).
2.7.3	Sodium acetate solution	2.5 Mol/l	205 g of sodium acetate (Annex 2A) were dissolved in 500 ml bidistilled water (2.4.8) and filled up to 1000 ml mark.
2.7.4	L (+)-ascorbic acid stock solution	1 mg/ml	100 mg of L-ascorbic acid (Annex 2A) were transferred into a 100 ml flask with a m-phosphoric acid /acetic acid solution (2.7.1) and was filled to the mark. The

			solution was freshly prepared before use.
2.7.5	L (+)–ascorbic acid standard solution	20 to 60 µg/ml	2 ml, 4 ml and 6 ml of L-ascorbic acid stock solution (2.7.4) were pipetted in a 100 ml volumetric flasks and diluted with m-phosphoric acid/ acetic acid solution (2.7.1) to the mark. This resulted in standard solutions with concentration 20 µg/ml, 40 µg/ml and 60 µg/ml respectively. The solution was freshly prepared before use.
2.7.6	L (+)- ascorbic acid standard solution for externa calibration l	10 to 60 µg/ml	2 ml of L-ascorbic acid standard solution (2.7.5) were mixed with 2 ml acetonitrile (Annex 2A) and ultra-filtered using a 0.2 µm membrane filter (Annex 4).
2.7.7	Ammonium dihydrogen phosphate	5.75 g/l	5.75 g of Ammonium dihydrogen phosphate (Annex 2A) were dissolved in bidistilled water (2.4.8) in a 1000 ml graduated flask and filled up to the mark with bidistilled water (2.4.8).

Table 2.8: Mobile phase for vitamin C HPLC

	Reagent/solution	**Concentration**	**Preparation**
2.8.1	Acetonitrile : 50 mM Ammonium dihydrogen phosphate	70 %:30 % (v:v)	700 ml of acetonitrile (Annex 2A) were mixed with 300 ml of 50 mM ammonium dihydrogen phosphate (2.7.7) in a brown glass bottle, mixed again and degassed through ultrasonication.
2.8.2	Post-column reagent	0.3 g 1,2-Phenylenediamine /100 ml	1.5 g 1,2-phenylenediamine (Annex 2A) were mixed with 10 ml 2.5 M sodium acetate (2.7.3) followed by addition of 25 ml dilute m-metaphosphoric acid/acetic acid (3 %/8 %) (2.7.2). Bidistilled water was added to a 500 ml mark. The solution was transferred into a brown glass bottle.

Table 2.9: Working solutions for vitamin E analysis

	Reagent/solution	**Concentration**	**Preparation**
2.9.1	Methanol:acetone:water	54:40:6(v:v:v)	54 ml methanol (Annex 2A) were mixed with 40 ml acetone (Annex 2A) and 6 ml bidistilled water (2.4.8).
2.9.2	Eluent A (methanol:water)	91:9 (v:v)	91 ml of methanol (Annex 2A) were mixed with 9 ml bidistilled water (2.4.8).
2.9.3	Eluent B (tert-methyl-butylether:methanol:water	80:18:2 (v:v:v)	80 ml of tert-methyl-butylether (Annex 2A) were mixed with 18 ml methanol (Annex 2A) and 2 ml bidistilled water (2.4.8).

Table 2.10: Working solutions for protein analysis

	Reagent/solution	**Concentration**	**Preparation**
2.10.1	Sodium hydroxide solution	40 %	400 g sodium hydroxide (Annex 2A) were dissolved in 1000 ml bidistilled (2.4.8).
2.10.2	Boric acid solution	2 %	20 g boric acid stock solution (Annex 2A) were diluted in 980 ml bidistilled (2.4.8).
2.10.3	Sulphuric acid	1 Mol/l	54.4 ml sulphuric acid stock solution (Annex 2A) were added to 250 ml bidistilled water (2.4.8). The final volume was adjusted to 1000 ml with bidistilled water (2.4.8).
2.10.4	Sulphuric acid solution	0.05 Mol/l	50 ml 1 Mol/l sulphuric acid (2.10.3) were diluted in 950 ml of bidistilled water (2.4.2) and filled up to 1000 ml
2.10.5	Taschiro indicator		0.3 g methyl red (Annex 2A) and 0.15 g methylene blue (Annex 2A) were dissolved in 115 ml 96 % ethanol (Annex 2A).

Table 2.11: Working solutions for determination of soluble sugars

	Reagent/solution	Concentration	Preparation
2.11.1	Acetonitrile	70 %	550.2 g (700 x 0.786 (specific gravity acetonitrile)) acetonitrile (Annex 2A) were mixed with 300 ml bidistilled water (2.4.8) and degassed.
2.11.2	Standard solutions	2 mg/ml	200 mg of fructose (Annex 2A), glucose (Annex 2A), and sucrose (Annex 2A) were added into 100 ml volumetric flask and filled to the mark with bidistilled water (2.4.8).
2.11.3	Calibration solution 1	1 mg/ml	Mix 1 ml of stock solution (2.11.2) with 1 ml bidistilled water (2.4.8).
2.11.4	Calibration solution 2	0.5 mg/ml	Mix 1 ml of the stock solution(2.11.2) with 3 ml bidistilled water (2.11.8).
2.11.5	Calibration solution 3	0.25 mg/ml	Mix 1 ml of the stock solution (2.11.2) with 7 ml bidistilled water (2.4.8).

2.1.4.2 Chemicals and reagents for molecular biological work

The chemicals and reagents used for molecular biological work, including safety data information according to the Gobally Harmonised System of Classification and Labelling of Chemicals (GHS) are described in annex 2A and 2B.

Table 2.12: Working solutions for genomic DNA isolation by Pitcher et al. (1989)

	Solutions and reagents	Composition/ preparation
2.12.1	0.5 M EDTA (pH 8)	186.1 g disodium EDTA (Annex 2A) were dissolved in 800 ml distilled water and pH adjusted to pH 8.0 with 5 Mol/l NaOH (Annex 2A). It was then filled up to 1000 ml mark with distilled water and then autoclaved.
2.12.2	1 M Tris-HCl (pH 8)	121.1 g Tris base (Annex 2A) were dissolved in 800 ml distilled water, 1 Mol/l HCl (2.12.11) was used to adjust the pH to pH 8.0 and the solution was then filled up to 1000 ml mark volumetric flask with distilled water and then autoclaved.
2.12.3	1 x TE buffer (pH 8)	1.0 ml 1 Mol/l Tris-HCl solution (2.12.2) (pH 8) and 0.2 ml of 0.5 Mol/l EDTA (2.12.1) (pH 8.0) were mixed with 100 ml distilled water and then autoclaved.
2.12.4	1x TE buffer + 0.5 % NaCl	0.5 g of NaCl (Annex 2A) was dissolved in 100 ml of 1 x TE buffer (2.12.3) (pH 8.0) and autoclaved.
2.12.5	7.5 M ammonium acetate	57.81 g of ammonium acetate (Annex 2A) were dissolved in 100 ml of distilled water. The solution was filtered with a sterile filter (0.2 µm pore size)

		and stored at 4 ^{o}C.
2.12.6	Chloroform-2-pentanol	A chloroform (Annex 2A) and 2-pentanol (Annex 2A) mixture was prepared in the ratio 24:1 (v/v). It was stored with protection against light.
2.12.7	GES reagent	60 g of guanidium thiocyanate (Annex 2A) were dissolved in 20 ml sterile distilled water (2.12.10) and mixed by continuous stirring (pH 8.0) followed by addition of 20 ml of 0.5 M EDTA (2.12.1) at 65 ^{o}C. The solution was cooled to room temperature, and then 5 ml of 10 % Sarkosyl solution (Annex 2A) were added and filled up to 100 ml mark with sterile distilled water (2.12.10). The solution was filtered through 0.45 µm membrane filter.
2.12.8	TERMLS solution	0.15 g lysozyme (Annex 2A), 2.0 g of sucrose (Annex 2A), 250 µl of 1 Mol/l Tris-HCl (2.12.2) (pH 8.0), 200 µl of 0.5 Mol/l EDTA (2.12.1) at pH 8.0, 100 µl mutanolysin (Annex 2A) (1.000 U), 25 µl of RNAse (Annex 2A) in 9.42 ml sterile distilled water (2.12.10). The solution was sterilized by filtration and stored at -20 ^{o}C.
2.12.9	Isopropanol	Used without dilution (Annex 2A).
2.12.10	Sterile distilled water (for PCR)	It was prepared by autoclaving distilled water at 121 °C for 15 min
2.12.11	1 Mol/l HCl	8.21 ml of stock solution (Annex 2A) were slowly added to 25 ml distilled water. The volume was adjusted to 100 ml with distilled water.
2.12.12	10 % Sarkosyl solution	10 g of Sarkosyl solution were dissolved in 90 ml d distilled water.

2.1.4.3 Chemicals and reagents for PCR analysis

Table 2.13: Solutions and reagents for PCR-DGGE with manufacturer and specifications

	Solution and reagents	**Manufacturer**
2.13.1	10 x PCR buffer without $MgCl_2$	Genaxxon Bioscience GmbH, Ulm, Germany
2.13.2	Taq polymerase (5 U/µl)	Genaxxon Bioscience GmbH, Ulm, Germany
2.13.3	Bovine Serum Albumin (10 mg/ml)	Biolabs New England
2.13.4	dNTP mix (dATP, dCTP, dATP, dTTP), 100 mM	Peqlab Biotechnology, Erlangen, Germany
2.13.5	$MgCl_2$ (25 mM)	Genaxxon Bioscience GmbH, Ulm, Germany
2.13.6	Primer 338f-GC (10 pmol/µl) (Tab.2.21.1)	Thermo Fisher Scientific GmbH, Ulm, Germany
2.13.7	Primer 518r (10 pmol/µl) (Tab.2.21.1)	Thermo Fisher Scientific GmbH, Ulm, Germany
2.13.8	Sigma water	Sigma-Aldrich, Steinheim, Germany

Table 2.14: Solutions and reagents for RAPD- PCR with manufacturer and specifications

	Solution and reagents	**Manufacturer**

2.14.1	Allin ™ Hot Start Taq Mastermix, 2X	HighQu GmbH, Kraichtal, Germany
2.14.2	Primer M13, 10 pmol/µl (Tab.2.21.2)	Thermo Fisher Scientific, Waltham, MA, USA
2.14.3	PCR water	HighQu GmbH, Kraichtal

Table 2.15: Solutions and reagents for atpD PCR genes with manufacturer and specifications

	Solution and reagents	**Manufacturer**
2.15.1	Allin ™ Hot Start Taq Mastermix, 2X	HighQu GmbH, Kraichtal, Germany
2.15.2	Primer atpD A1 fw (10 pmol/µl) (Tab.2.21.4)	Thermo Fisher Scientific, Waltham, MA, USA
2.15.3	Primer atpD A2 rev (10 pmol/µl) (Tab.2.21.4)	Thermo Fisher Scientific, Waltham, MA, USA
2.15.4	PCR water	HighQu GmbH, Kraichtal

Table 2.16: Solutions and reagents for 16S rRNA PCR genes with manufacturer and specifications

	Solution and reagents	**Manufacturer**
2.16.1	10X PCR buffer with $MgCl_2$ (15 mM)	Genaxxon bioscience GmbH, Ulm
2.16.2	dNTP mix (dATP, dCTP, dGTP, dTTP), 100 mM	VWR International GmbH, Erlangen (Peqlab)
2.16.3	Primer 16S seq fw (10 pmol/µl) (Tab.2.21.3)	Thermo Fisher Scientific, Waltham, MA, USA
2.16.4	Primer 16S seq rev (10 pmol/µl) (Tab.2.21.3)	Thermo Fisher Scientific, Waltham, MA, USA
2.16.5	DF-Taq DNA polymerase, 5 U/µl	Genaxxon bioscience GmbH, Ulm
2.16.6	Sigma water	Sigma-Aldrich, Steinheim, Germany

Table 2.17: Solutions and reagents for 16S rRNA metagenomics for MiSeq-based high throughput sequencing

	Solutions and reagents/kits	**Manufacturer**
2.17.1	Phusion HotStart Flex DNA polymerase	New England Biolabs, Frankfurt, Germany
2.17.2	Mag-Bind® RxnPure Plus	Omega bio-tek Inc., Norcross, USA
2.17.3	Primer 16S fw meta (10 pmol/µl) (Tab.2.21.5)	Thermo Fisher Scientific, Waltham, MA, USA
2.17.4	Primer 16S rev meta (10 pmol/µl) (Tab.2.21.5)	Thermo Fisher Scientific, Waltham, MA, USA
2.17.5	Allin ™ Hot Start Taq Mastermix, 2X	HighQu GmbH, Kraichtal, Germany
2.17.6	PhiX CONTROL V3	Illumina Inc, Munich, Germany
2.17.7	MiSeq Reagent Kit v3 (600 cycle)	Illumina Inc, Munich, Germany
2.17.8	Nextera XT Index Kit 24 Indices – 96 Samples	Illumina, Inc, Munich, Germany

2.1.4.4 Solutions and reagents for gel electrophoresis

Solutions and reagents for general gel electrophoresis, PFGE, DGGE used, including safety data information according to the Gobally Harmonised System of Classification and Labelling of Chemicals (GHS) are described in annex 2A and 2B.

Table 2.18: Working solutions for the agarose gel electrophoresis

	Solutions and reagents	**Preparation**
2.18.1	1.5 % agarose gel	0.9 g agarose (Annex 2A) was dissolved in 60 ml of 1X TAE buffer (2.18.7).
2.18.2	1.8 % agarose	3.6 g agarose (Annex 2A) were dissolved in 200 ml of 1X TAE buffer (Tab.2.18.7).
2.18.3	50 x TAE buffer	242 g Tris-HCl (Annex 2A) was weighed in 1litre Schott bottles; 7.43 g $EDTANa_2$ (Annex 2A) were added, followed by 500 ml distilled water, then 57.1 ml of acetic acid (Annex 2A) was added and the mixture was thoroughly mixed with a magnetic stirrer. The volume was adjusted to 1 litre by addition of distilled water. The solution was stored at room temperature.
2.18.4	DNA size marker (0.1 – 10.0 kb)	33.3 µl 6X purple dye were mixed with 146.7 µl 10 mM Tris HCl (1x), and 20 µl DNA ladder 1000 µg/ml (1:10 dilution).
2.18.5	DNA size marker (100 bp/500 bp)	30 µl 100 bp marker, 30 µl 500 bp marker and 60 µl blue running dye (bromophenol blue) were mixed.
2.18.6	Loading buffer	250 mg were of bromophenol blue were dissolved in 50 ml of 1 x TE buffer (pH 8.0) followed by addition of 50 ml glycerol. The ratio of loading buffer to the sample was 1:10 during electrophoresis.
2.18.7	TAE buffer, 1X	20 ml of 50X TAE buffer (2.18.3) were dissolved in 980 ml distilled water.

Table 2.19: Working solutions for PFGE

	Solutions and reagents	**Composition and preparation**
2.19.1	WB_I buffer (pH 7.2)	WB_1 buffer was prepared by dissolving 0.12 g Tris (Annex 2A). (10 mM), 0.12 g NaCl (Annex 2A) (20 mM), 1.86 g $EDTANa_2$ (Annex 2A) (50 mM) in 90 ml of distilled water and the pH was adjusted to 7.2 with 5 Mol/l NaOH and volume adjusted to 100 ml

		then autoclaved.
2.19.2	LB_1 buffer (pH 7.6)	LB_1 buffer was prepared by dissolving 0.04g Tris (Annex 2A) (6 mM), 2.92g NaCl (Annex 2A) (1 M), 1.86 g $EDTANa_2$ (Annex 2A) (100 mM), 0.5 g SDS (Annex 2A) (1 %), 0.1 g deoxycholate (Annex 2A) (0.2 %) in 40 ml distilled water and the pH was adjusted to 7.6 with 5 Mol/l NaOH and volume was adjusted to 50 ml and then autoclaved.
2.19.3	LB_2 buffer (pH 8.0)	LB_2 buffer was made by dissolving 1.86 g $EDTANa_2$ (Annex 2A) (100 mM), 0.5 SDS (Annex 2A) (1 %), 0.1 g deoxycholate (Annex 2A) (0.2 %) in 40 ml distilled water, the pH was adjusted to 8.0 with 5 Mol/l NaOH and the volume was adjusted to 50 ml and was then autoclaved.
2.19.4	WB_2 buffer (pH 8.0)	WB_2 buffer was prepared by dissolving 0.27 g Tris (Annex 2A) (20 mM), 1.86 g $EDTANa_2$ (Annex 2A) (50 mM) in 80 ml distilled water. The pH was adjusted to 8.0 with 5 Mol/l NaOH and filled to 100 ml mark with distilled water and then autoclaved.
2.19.5	TE buffer (pH 8.0)	This buffer was prepared by dissolving 0.68 g Tris (Annex 2A) (10 mM), 0.19 g $EDTANa_2$ (Annex 2A) (1 mM) in 450 ml distilled water and the pH set to 8.0 and final volume was adjusted to 500 ml then autoclaved.
2.19.6	SDS (10 %)	2.0 g SDS (Annex 2A) were dissolved in 20 ml of distilled water, then autoclaved.
2.19.7	Lysis buffer (pH 8.2)	Lysis buffer was prepared by dissolving 0.61 g Tris (Annex 2A), 1.90 g EDTA (Annex 2A) and 1.05 g N-lauryl sarcosine (Annex 2A) in 100 ml of distilled water, followed by autoclaving.
2.19.8	10X TBE buffer (pH 8.0)	This buffer was made by dissolving 121.1 g Tris (Annex 2A), 0.76 g EDTA (Annex 2A) and 61.83 g boric acid (Annex 2A) in 500 ml of distilled water. The pH was adjusted to 8.0 and filled to the mark of a 500 ml volumetric flask with distilled water which was followed by autoclaving.

2.1.4.5 Antibiotics

List of working antibiotics for malt-glucose agar and their preparation including safety data information according to the Gobally Harmonised System of Classification and Labelling of Chemicals (GHS) are described in annex 2A and annex 8.

2.1.5 Culture media

List of culture media, used in this study, their suppliers, composition and preparation as used at the MRI, Karlsruhe Germany (Annex 9A), JKUAT, Nairobi Kenya (Annex 9C) and for challenge test in nightshade fermentations with selected pathogens (Annex 9B).

2.1.6 Devices and equipment

2.1.6.1 Analytical equipment

The list of equipment used for analytical experiment are described in annex 3A-G while the HPLC separation systems are described in annex 3H.

2.1.6.2 Devices and equipment used for fermentation and molecular biological work

The list of devices and equipment used for fermentation and molecular biological work are described in annex 5.

2.1.7 Solutions for fermentation

Table 2.20: Solutions used for the fermentation of African leafy vegetables

	Solutions	**Composition/preparation**
2.20.1	2.5 % salt solution	25 g table salt (REWE Group) was dissolved in 1 litre tap water. The solution was autoclaved for 15 min at 121 °C.
2.20.2	2.5 % salt and 2.5 % sugar solution	25 g table salt and 25 g of sugar (REWE Group) were dissolved in 1 litre tap water. The solution was autoclaved for 15 min at 121 °C.
2.20.3	3.0 % salt and 3.0 % sugar solution	30 g table salt and 30 g sugar (REWE Group/ Nzoia sugar company ltd/ Kensalt ltd) were dissolved in 1 litre tap water. The solution was autoclaved for 15 min at 121 °C.

2.1.8 Enzyme kits and molecular biological kits

The enzyme kits used in this study are listed in annex 7.

2.1.9 Primers

Table 2.21: List of used primers, sequence size, melting temperature (Tm) and target group

	Name	**Sequence (5' → 3')**	**Products size (bp)**	**Tm (^{o} C)**	**Target group**
2.21.1	338f-GC	5'-GC-Clamp[a]-ACTCCTACGGGAGGCAGCAG-3'	220	56	16 S rRNA gene of eubacteria (universal)
	518r	5'-ATTACCGCGGCTGCTGG-3'			
2.21.2	M13	5'-GAGGGTGGCGGTTCT-3'	Variable	40	Random polymorphic sequences
2.21.3	16s seq fw	5'-ATAGTTTGATCMTGGCTCAG-3'	1500	56	Universal (16S rRNA gene)
	16s seq rev	5'GGNTACCTTGTTACGACTTC-3'			
2.21.4	atpD A1 fw	5'RT [dI] AT[dI] GG [dI] GC [dI] GT [dI] RT [dI] GAY GT-3'	884	50	atpD subunit gene of enterobacteria
	atpD A2 rev	5'TCR TC [dI] GC [dI] GG [dI] ACR TA[dI] AY [dI] GCY TG-3'			
2.21.5	Metafw	5′-TCG TCG GCA GCG TCA GATGTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG- 3'	1500	50	16S rRNA V3 and V4 region
	Metarev	5'-GCT TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGACTA CHV GGG TAT CTA ATC C- 3'			

Key: [a]GC-Clamp: CGC CCG CCG CGC GCG GCG GC GGG GCG GGG GCA CGG GGG G; Letters: H = A/C/T, M= A/C, N= A/C/G/T, R= A/G, V = A/C/G, Y= C/T; dI= Deoxyinosine universal base pairs IC/IA/IT/IG.

2.1.10 Bacterial strains used in fermentation

Table 2.22: List of strains used in fermentation, culture medium and temperatures used for propagation

	Strain name	**Origin**	**Culture media**	**Growth temperature**
2.22.1	*Lactobacillus plantarum* BFE 6710	Fermenting cassava, Benin	MRS	30 ^{o}C (aerobically)
2.22.2	*Lactobacillus plantarum* BFE 5092	Fermented milk, Kenya	MRS	30 ^{o}C (aerobically)
2.22.3	*Pediococcus acidilactici* BFE 2300	Fermenting cassava, Benin	MRS	30 ^{o}C (aerobically)
2.22.4	*Leuconostoc mesenteroides* subspecies *mesenteroides* BFE 7668	Fermented milk, Kenya	MRS	30 ^{o}C (aerobically)
2.22.5	*Lactobacillus fermentum* BFE 6074	Fermenting cassava, Benin	MRS	30 ^{o}C (aerobically)
2.22.6	*Lactobacillus fermentum* BFE 6620	Fermenting cassava, Benin	MRS	30 ^{o}C (aerobically)
2.22.7	*Lactobacillus fermentum* BFE 6639	Fermenting cassava, Benin	MRS	30 ^{o}C (aerobically)
2.22.8	*Lactobacillus fermentum* BFE 6700	Fermenting cassava, Benin	MRS	30 ^{o}C (aerobically)
2.22.9	*Lactobacillus fermentum* BFE 6662	Fermenting cassava, Benin	MRS	30 ^{o}C (aerobically)
2.22.10	*Lactococcus lactis* BFE 902	Fermented milk, Kenya	MRS	30 ^{o}C (aerobically)
2.22.11	*Weissella paramesenteroides* LC11	Fermenting cassava, Benin	MRS	30 ^{o}C (aerobically)
2.22.12	*Weissella paramesenteroides* LC18	Fermenting cassava, Benin	MRS	30 ^{o}C (aerobically)
2.22.13	*Listeria monocytogenes* SLCC 8210, sv1/2a	Agrotechnical Research Institute	PALCAM	37 ^{o} C
2.22.14	*Salmonella enterica* Serovar. Enteritidis S 489	MRI-Kulmbach	XLD	37 ^{o}C

2.2 Methods

2.2.1 Selection of appropriate starter culture strains for optimal fermentation

This section describes the selection of appropriate lactic acid starter culture strains for fermentation of nightshade leaves and the optimisation of fermentation conditions that were necessary for the subsequent fermentation trials. Proper attention was particularly given to the development of acid production by measuring the pH from different experimental trials. Each experimental trial was inoculated with a different starter strain. Preparation of the African nightshade leaves was performed according to the description in section 2.1.2. The clean dry leaves were cut into small pieces before the beginning of the experimental trials. Ten grams each were weighed into nineteen 50 ml Schott bottles (Fig.2.10). Thirty ml of 3.0 % salt -sugar solution which was pre-warmed at 60 ^{o}C was mixed with the leaves, while in a another other trial, only a 3.0 % salt solution was used, in the ratio of 1+3 (1 part of the leaves and 3 parts of the autoclaved 3.0 % salt-sugar solution or only the 3.0 % salt solution) The inoculated leaves were left to cool at room temperature followed by inoculation with different overnight starters cultures at 10^{6}-10^{7} cfu/ml. The control approaches were not inoculated with starter cultures

Table 2.23: Starter cultures used for nightshade fermentation trials with salt/salt-sugar solution

	Starter cultures	**Salt/Sugar- sugar solution**
2.23.1	*Lb. plantarum* BFE 6710	3.0 % salt
2.23.2	*Lb. plantarum* BFE 5092	3.0 % salt
2.23.3	*Ped. acidilactici* BFE 2300	3.0 % salt
2.23.4	*Leuc. mes. mes.* BFE 7668	3.0 % salt
2.23.5	*Lc. lactis* BFE 902	3.0 % salt
2.23.6	Control (uninoculated)	3.0 % salt
2.23.7	*Lb. plantarum* BFE 6710	3.0 % salt-sugar solution
2.23.8	*Lb. plantarum* BFE 5092	3.0 % salt-sugar solution
2.23.9	*Ped. acidilactici* BFE 2300	3.0 % salt-sugar solution
2.23.10	*Leuc. mes. mes.* BFE 7668	3.0 % salt-sugar solution
2.23.11	*Lc. lactis* BFE 902	3.0 % salt-sugar solution
2.23.12	*Lb. fermentum* BFE 6074	3.0 % salt-sugar solution
2.23.13	*Lb. fermentum* BFE 6639	3.0 % salt-sugar solution
2.23.14	*Lb. fermentum* BFE 6620	3.0 % salt-sugar solution

2.23.15	*Lb. fermentum* BFE 6700	3.0 % salt-sugar solution
2.23.16	*Lb. fermentum* BFE 6662	3.0 % salt-sugar solution
2.23.17	*W. paramesenteroides* LC 11	3.0 % salt-sugar solution
2.23.18	*W. paramesenteroides* LC 18	3.0 % salt-sugar solution
2.23.19	Control (uninoculated)	3.0 % salt-sugar solution

The experimental trials were incubated at 25 ^{o}C; the samples were collected at 0 h, 24 h, 48 h, 72 h and 144 h for pH determination under a sterile air clean bench.

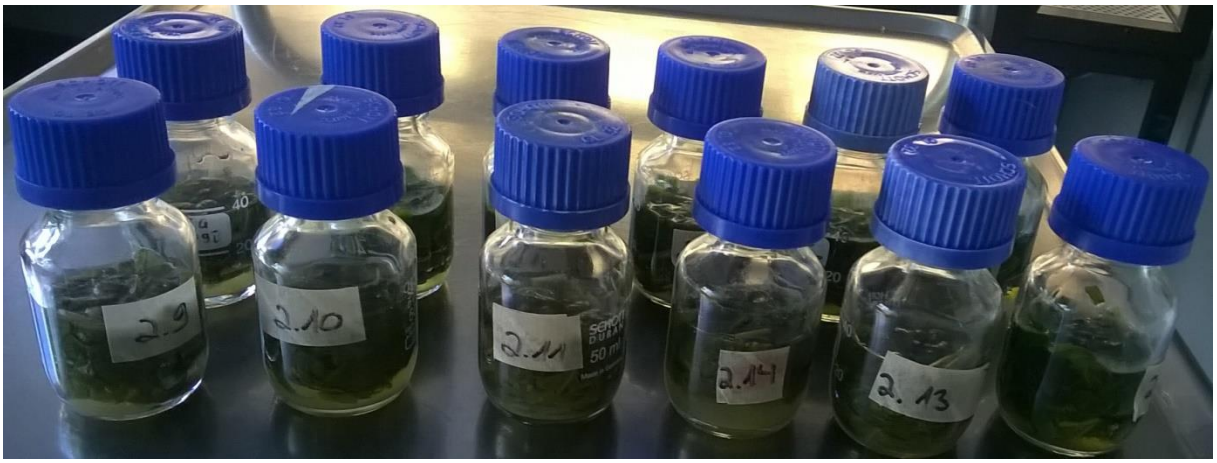

Figure 2.10: Nightshade fermentation trials in 50 ml Schott bottles photographed at MRI Karlsruhe, Germany.

2.2.2 Fermentation of vegetable nightshade in 100 ml Schott bottles with different starter culture strain combinations

In this section, 100 ml Schott bottles were used as a pilot trial for the combination of the best performed single starter strains in 3.0 % salt-sugar solution, 2.5 % salt- sugar and 2.5 % salt solutions in the fermentation of African nightshade leaves. Preparation of the vegetable leaves was performed according to the description in section 2.1.2. Twenty grams of clean dry leaves were weight into 100 ml Schott bottles (Fig.2.11) Sixty ml of sterile brine solutions pre-warmed at 60 ^{o}C was added (ratio of 1+3, i.e. 1 part of the leaves and 3 parts of the autoclaved brine solutions) as shown in the table below. The fermentation set-ups were left to cool at room temperature, followed by inoculation with different overnight starters' cultures at 10^{6}-10^{7} cfu/ml. The control fermentations were not inoculated with the starter culture strains.

Table 2.24: Starter strain combinations used for nightshade fermentation trials with salt/salt-sugar solution

	Starter cultures	**Salt/ sugar- sugar solution**
2.24.1	*Lb. plantarum* BFE 5092 + *Lb. fermentum* BFE 6620	3.0 % salt-sugar
2.24.2	*Lb. plantarum* BFE 5092+ *W. paramesenteroides* LC11	3.0 % salt-sugar
2.24.3	Control (uninoculated)	3.0 % salt-sugar
2.24.4	*Lb. plantarum* BFE 5092 + *Lb. fermentum* BFE 6620	2.5 % salt-sugar
2.24.5	*Lb. plantarum* BFE 5092+ *W. paramesenteroides* LC11	2.5 % salt-sugar
2.24.6	Control (uninoculated)	2.5 % salt-sugar
2.24.7	*Lb. plantarum* BFE 5092 + *Lb. fermentum* BFE 6620	2.5 % salt
2.24.8	*Lb. plantarum* BFE 5092+ *W. paramesenteroides* LC11	2.5 % salt
2.24.9	Control (uninoculated)	2.5 % salt

The fermentations were incubated at 25 ^{o}C; the samples were collected at 0 h, 24 h, 48 h, 72 h and 144 h for pH determination and enumeration of enterobacteria, total aerobic count, total lactic acid bacteria (LAB) count and total yeast and moulds counts under the sterile clean bench.

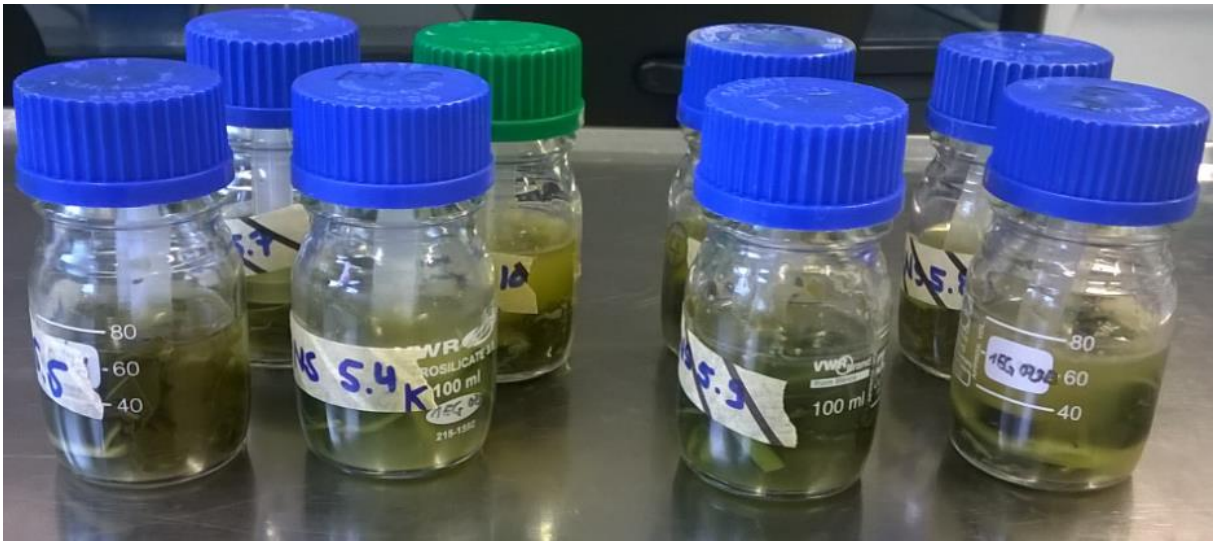

Figure 2.11: Nightshade fermentation trials with starter culture combination in 100 ml Schott bottles photographed at MRI Karlsruhe, Germany.

2.2.3 Fermentation of vegetable nightshade in 5 L pots/crocks at MRI and 5 L culture buckets at JKUAT

The scaled-up fermentations in 5 litre clay crock pots with the selected starter culture strains was performed as test for practical usability in household setup with *Lb. plantarum* BFE 5092 and *Lb.*

fermentum BFE 6620 as starter cultures were finally selected. This experiment was performed both at MRI (Fig.2.13) and at JKUAT (Fig.2.12). The procedure and the fermentation conditions were the same. The preparation of the leaves for the fermentation was carried out as described in section 2.1.2. The clean and dry leaves were used whole (without cutting) and 1 kg of the leaves was weighed into 2 sterile fermentation crock pots (Fig.2.7) and pressed down with autoclaved metal mesh (JKUAT) (Fig.2.9) or stones (MRI) (Fig.2.8). The pots were transferred into a sterile bench. Three litres of sterile salt and sugar solution (3.0 % each) pre heated at 60 ^{o}C in a water bath was added making the mixing ratio of 1+3. The batches were allowed to cool to room temperature, then the fermentation set-ups were inoculated with overnight cell cultures of *Lb. plantarum* BFE 5092 and *Lb. fermentum* BFE 6620 with approximately 10^{6}-10^{7} cfu/ml, while the control batch was not inoculated.

Figure 2.12: Fermentation of nightshade leaves in 5 L culture buckets at JKUAT, Nairobi, Kenya.

Figure 2.13: Fermentation of nightshade leaves in 5 L pots at MRI, Karlsruhe, Germany.

The pots were incubated at 25 ^{o}C and carefully swirled upon incubation to mix the fermentation brine and materials. Then, 700 µl of brine samples were collected daily (0 h, 24 h, 48 h, 72 h and 144 h) for pH determination, while 10 ml of the fermentation brine was centrifuged and the pellet was frozen for DNA isolation. The supernatant was frozen and was kept for determination of lactate and sucrose content, as well as total acidity by titration. Microbial enumeration was done on VRBD agar, Std.I agar, MRS agar and MG+ antibiotics agar under a sterile air clean bench. After the fermentation period of 144 h, the fermented leaves were immediately lyophilized for basic nutrient analysis and determinations of dry mass, ash, vitamin B_1/$B_{2,}$ C, E, protein, and soluble sugars. Fermentations were done three times (MRI) or four times (JKUAT) on different occasions from different harvests.

2.2.4 Microbial enumeration during fermentation in 5 L vessels

The progress of the fermentation was determined by microbial enumeration on different types of media. The enumeration of enterobacteria was performed on VRBD agar, malt-glucose agar with antibiotics incorporation was used to determine the yeast and moulds count, the total aerobic and mesophilic count was carried out on Std-1 agar while the development of the lactic acid bacteria count was determined by MRS agar. The experiment was performed in duplicate. Five ml of the fermentation solution were transferred into a sterile 9 ml test tube and vortexed properly to mix. One millilitre of the mixture was transferred to 9 ml test tube containing quarter-strength Ringer's

solution to make serial 10-fold dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9}. These preparations were thoroughly mixed by vortexing. 10 µl or 100 µl of the aliquots from different dilutions were transferred to Petri dishes containing VRBD, Std.I, MRS and MG+A agar (media preparation (Annex 9A-9C) and spread plated. The counting was performed in duplicate with the detection limit of 1 x 10^2 cfu/ml. This was followed by incubating the agar plates at respective temperatures (Std.I and VRBD at 37 ^{o}C, MRS at 30 ^{o}C and MG+A at 25 ^{o}C). The incubated plates were counted daily for up to 6 days to determine the bacterial colony forming (CFU), using the following formula:

$$\text{Colony forming unit (CFU/ml)} = \frac{\text{Number of colonies}}{\text{Volume of inoculum}} \text{ X Dilution factor}$$

2.2.5 Determination of the ability of selected lactic acid bacteria to inhibit pathogenic bacteria in vegetable nightshade fermentations (challenge studies)

2.2.5.1 Reactivation of the strains

The lactic acid bacteria used in this experiment were *Lactobacillus plantarum* BFE 5092 and *Lactobacillus fermentum* BFE 6620. The cryopreserved starter culture strains (100 µl) was inoculated in 9 ml MRS broth and incubated at 30 ^{o}C, while *Listeria monocytogenes* SLCC 8210 (serovar 1/2a) and *Salmonella* Enteritidis S 489 were used as pathogens and were inoculated in 9 ml standard I broth and incubated at 30 ^{o}C. The overnight cultures from broth were streaked out on their respective agar plates as follows: *Salmonella* Enteritidis (standard I and XLD), *Listeria monocytogenes* (Palcam and standard I) and *Lb. plantarum* BFE 5092 and *Lb. fermentum* BFE 6620 were streaked onto MRS agar. The plates were incubated at 30 ^{o}C. Colonies were visually evaluated to ensure they were pure. The pure plates were stored at 4 ^{o}C. One colony from each plate was inoculated in the respective broth medium and incubated overnight. The initial cell number of overnight cultures was determined by drop plate method, dilutions -5/-6/-7 were plated in duplicates each with 10 µl.

2.2.5.2 Preparation of the materials

The media used in this experiment were prepared according description in annex 9B while section 2.17 (Tab.2.20) describes the used solutions

2.2.5.3 Preparation of enrichment media

The enrichment was done in test tubes of 10 ml each with buffered peptone water (BPW), Rappaport Vassiliadis soy (RVS) broth, Mueller Kauffmann tetrathionate novobiocin (MKTTn), iodine potassium iodide solution and novobiocin supplement were prepared for *Salmonella* Enteritidis enrichment. While 1.5 L of Palcam broth + supplement was prepared for *Listeria monocytogenes* enrichment. For *Salmonella* selective enrichment, 100 ml MKTTn base was boiled in Duran bottles (MKTTn base can be stored at 3 ± 2 ^{o}C for up to 4 weeks) and cooled while stirring to 45 ^{o}C, 10 ml were distributed in 10 test tubes. Then one vial of lyophilised novobiocin was dissolved in 5 ml of sterile double distilled water (stored at 2-8 ^{o}C). Novobiocin solution (100 µl) was added to 10 ml MKTTn base, 200 µl of iodine-potassium iodide solution was added to 10 ml MKTTn broth followed by stirring to evenly mix the broth and stored at 2-8 ^{o}C up to 6 days.

2.2.5.4 Enumeration media

Standard I agar plates were prepared for enumeration of total aerobic count, MRS agar for lactic acid count, VRBD agar for total enterobacteria count, PDA with 10 % tartaric acid for yeast and moulds, Xylose lysine deoxycholate (XLD) agar for *Salmonella* while palcam agar for enumeration of *Listeria* (Annex 9B).

2.2.5.5 Preparation for fermentation

The nightshade leaves were handpicked from the greenhouse (without the use of gloves). Then 500 g of freshly collected vegetables leaves were thoroughly washed in stainless steel baskets under cold running tap water to remove impurities and insects (using gloves) and were dried as described in section 2.1.2 (Fig.2.6). Then 100 g of the washed leaves were weighed in sterile 1 litre glass beakers and immediately put under clean bench (laminar flow). The sterile 1 litre glass beaker with aluminium foil and metal mesh were put under clean bench. The aluminium covers and metal mesh inserts were removed from sterile glass beakers. The leaves were pushed down with metal mesh insert. The brine solution (3 % salt+3 % sugar) was pre- warmed at 60 ^{o}C in a water bath followed by addition of 300 ml to the batches and covered with aluminium foil. The batches were allowed to cool to room temperature then inoculated with 1 x 10^{7} cfu/ml starter cultures and 1 x 10^{3} cfu/ml pathogens. The experiment was performed in 4 batches: Batch 1 was a control with only the leaves together with 3 % salt +3 % sugar solution, batch 2 consisted of leaves, selected starter cultures *Lactobacillus plantarum* BFE 5092 and *Lactobacillus fermentum* BFE 6620 at a count of 1 x 10^{7}

cfu/ ml (each) with 3 % salt + 3 % sugar solution, batch 3 constituted of leaves, the selected starter cultures *Lactobacillus plantarum* BFE 5092 and *Lactobacillus fermentum* BFE 662) at 1 x 10^7 (each) and the pathogenic strains *Listeria monocytogenes* SLCC 8210 sv1/2a and *Salmonella* Enteritidis S 489, with a bacterial cell number of 1 x 10^3 cfu/ ml (each) in 3 % salt + 3 % sugar solution, while batch 4 consisted of 100 g leaves, the pathogenic strains of *Listeria monocytogenes* SLCC 8210 and *Salmonella* Enteritidis S 489 with bacterial cell number of 1 x 10^3 cfu/ ml each and with 3 % salt + 3 % sugar solution, without the starter strains.

The batches were incubated at 25 ^oC and samples for microbiological enumeration were taken immediately at 0 h and from 24 h to 168 h of fermentation. Sampling was done after manually swirling the beakers to mix the fermentation solution with plant material. For sampling, 10 ml of the solution was collected in sterile test tubes. The samples were serially diluted in a 10-fold dilution series depending upon the batch and the microorganism to be enumerated i.e. total aerobic count on Std.I agar the dilutions were made from 10^o to 10^{-7}, enterobacteria (VRBD agar) 10^o to 10^{-4}, LAB (MRS agar) 10^0 to 10^{-7}, yeast and mould (PDA) 10^o to 10^{-3}, *Listeria monocytogenes* (Palcam agar) 10^o to 10^{-3} and for *Salmonella* Enteritidis (XLD agar) 10^o to 10^{-3}. The serial diluted samples were thoroughly vortexed and inoculated in duplicates as follows; enumeration of total aerobic count, enterobacteria, LAB and yeast and moulds, 100 µl undiluted and 500 µl diluted were spread plated, while for *Salmonella* and *Listeria* both 100 µl undiluted and 500 µl diluted were spread plated. The agar plates were incubated as follows; VRBD for 24 h anaerobically in an anaerobic jar at 37 ^oC, Std.I for 24 h at 37 ^oC, PDA for 72 h at 25 ^oC, MRS for 48 h at 30 ^oC aerobically, Palcam for 24 h/48 h at 30 ^oC and XLD for 24 h/ 48 h at 30 ^oC aerobically. The pH was determined daily by sampling 600 µl from each batch in a 2 ml Eppendorf tunes. The samples from the batches with pathogens were heated for 10 min in a heating block at 100 ^oC to kill the bacteria, followed by cooling the samples to room temperature before measuring the pH.

An enrichment was done for batches inoculated with pathogens when the cell numbers were expected to be low or undetectable by plate counting. For the batch inoculated with *Salmonella*, pre-enrichment was done by incubating 25 ml of the nightshade fermentation brine with 225 ml buffered peptone water at 37 ^oC for 24 h. After 24 h, a selective enrichment was performed in MKTTn broth by mixing 10 ml Mueller Kauffmann Tetrathionate Novobiocin broth with 1 ml pre-enrichment and incubating at 37 ^oC for 24 h, and in Rappaport Vassiliadis soy broth (10 ml RVS broth plus 100 µl pre-enrichment) and incubating this enrichment at 41.5 ^oC for 24 h in a shaking

incubator (180 rpm). Following the incubation periods, 10 µl of the sample were streaked out on XLD and BPLS agar. For *Listeria*, 25 ml nightshade fermentation brine was incubated with 225 ml of Palcam enrichment broth at 30 ^{o}C for 24 h in a shaking incubator (180 rpm) and 10 µl of the enrichment was later streaked out on a Palcam agar plate. The fermentations were carried out in triplicate from separate nightshade harvests, and from different occasions.

The bacterial colony forming (CFU) was calculated using the following formula:

$$\bar{C} = \frac{\sum C}{n_1 \times 1 + n_2 \times 0.1} \times \frac{1}{d}$$

$\bar{C}$ = Weighted arithmetic mean of the number of colonies

ΣC = Sum of all colonies Petri dishes, which were included for the calculation (lowest and higher countable dilution)

n_1 = Number of Petri dishes of the lowest countable dilution

n_2 = Number of Petri dishes of the higher dilution

d = Factor of the lowest countable dilution (n_1 related dilution)

2.2.6 Isolation of presumptive LAB from 5 L fermentation crock pots

During nightshade fermentation, presumptive LAB colonies were isolated from MRS agar plates at 0 h, 48 h and 144 h with starter strains inoculated batch and the control (natural fermentation without starters) from MRI, while from JKUAT both the lactic acid and enterobacteria colonies were isolated from the control fermentation. The purity of the isolates was checked by repetitive streaking on fresh MRS agar for lactic acid bacteria and VRBG agar for enterobacteria at least 3 times.

2.2.7 Phenotypic characterization of predominant lactic acid bacteria

2.2.7.1 Long term preservation of the isolates

All bacteria cultures were cryopreserved in the medium containing 15 % glycerol (Annex 2A). The bacteria were stored at –75 ^{o}C for long-term storage.

2.2.7.2 Determination of cell morphology

Cell morphology was determined microscopically using phase contrast at x 1000 magnification. Cells were determined to be either of rod or coccoid morphology.

2.2.7.3 Gram reaction

The isolates Gram reaction was determined using 3 % potassium hydroxide (3 % KOH). Where one drop of 3 % KOH was introduced onto a microscopic slide; a loopful of overnight cell cultures was added and thoroughly mixed. The positive KOH reaction was seen when a viscous substance as a result of cell lysis pulled along the wire loop when raised up, hence indicating Gram-negative results. A negative KOH reaction was recorded if cells did not lyse and nothing was pulled along the wire loop, indicating Gram positive results.

2.2.7.4 Catalase test

This test was performed using 3 % hydrogen peroxide (H_2O_2). One drop of 3 % H_2O_2 was put on the slide; a loopful of the overnight cultures was introduced and thoroughly mixed. The production of gas bubbles/ effervescence indicated positive result, while lack of gas bubbles was evidence of negative result.

2.2.7.5 Gas production from glucose fermentation

MRS broth containing inverted Durham tubes was used to determine CO_2 production abilities of the lactic acid bacterial isolates. About 100 µl of overnight cultures were inoculated into 9 ml MRS broth containing inverted Durham tubes and incubated for 5 days at 30 °C. Production of CO_2 was observed as an accumulation of gas in the inverted tubes and indicates an obligately heterofermentative metabolism.

2.2.7.6 Growth at different temperatures

This experiment was conducted in MRS broth where 100 µl of overnight cultures were inoculated into 9 ml freshly prepared MRS broth and this was followed by incubation at 10 ^{o}C and 45 ^{o}C.

2.2.7.7 Growth in 6.5 % NaCl concentration

Salt tolerance may be used to distinguish between enterococci, lactococci/vagococci and streptococci. NaCl tolerance was tested in MRS broth containing 6.5 % NaCl. 100 µl of overnight cultures were inoculated in this broth, followed by incubation at 30 ^{o}C for two days.

2.2.7.8 Determination of D/L lactic acid production

D-lactic acid / L-lactic acid concentrations produced were by enzymatic analysis determined according to the procedure described in section 2.15.1.

2.2.8 Isolation and characterisation of enterobacteria from nightshade fermentation

Enterobacteria were isolated from the control fermentation at JKUAT. Isolates were checked for purity by repetitive streaking on fresh VRBG agar plates for at least three times. The pure isolates were cryo-preserved in 15 % glycerol at -75 ^{o}C. Determinations of cell morphology, Gram reaction, and testing for the production of catalase were done as described above (2.2.7.2, 2.2.7.3., 2.2.7.4).

2.2.9 Molecular characterization

2.2.9.1 Genomic DNA extraction by Pitcher method

Genomic DNA was extracted by a method described by Pitcher et al (1989) from bacteria cells isolated from different fermentation sampling points of 0 h, 24 h, 48 h, 72 h and 144 h for bacterial community composition studies during fermentation. Ten ml of the fermentation brine sample was transferred into a falcon tube and centrifuged at 7500 × g for 10 min at 4 ^{o}C. The supernatant was discarded and the pellet was dissolved in TE + 0.5 % NaCl (Tab.2.12.4). The dissolved pellet was transferred to 1.5 ml Eppendorf tube and centrifuged at 15,000 x g for 10 min at 4 ^{o}C and the supernatant was discarded. The cells were re-suspended in 100 µl TERMLS (lysozyme, sucrose, Tris-HCl EDTA, mutanolysin, RNAse) (Tab.2.12.8) then mixed gently by carefully pipetting up and down several times. The lysis of the bacterial cell walls was done by incubation for 1 hour at 37 °C, followed by addition of 500 µl of GES (guanidium thiocyanate, EDTA, sarkosyl) (Tab.2.12.7) and gently mixed then incubated on ice for 5 min. This resulted in cell lysis and denaturation of the proteins. Then 250 µl of ice-cold 7.5 M ammonium acetate (Tab.2.12.5) was added and mixed by gentle inversion of the tubes and incubated on ice for 10 min. This was followed by addition of 500

µl of chloroform-2-pentanol (24: 1) Tab.2.12.6). The mixture was then mixed by inversion of the Eppendorf tubes followed by centrifuging at 15,000 x g for 10 min at 4 °C, thereby producing two phases. The upper aqueous phase containing the DNA, the interphase containing the proteins and the lower phase containing chloroform. The upper phase with the DNA was carefully transferred to a new 1.5 ml Eppendorf tube. The DNA was precipitated by adding 460 µl ice-cold isopropanol (Tab.2.12.9) followed by gentle mixing of the samples before incubating for 20 min at -20 ^{o}C. It was then centrifuged at 15,000 x g for 10 min at 4 °C and the supernatant discarded. 460 µl of 70 % ethanol was added and centrifuged at 15,000 x g for 5 min at 4 °C. The supernatant was carefully discarded to avoid disturbing the pellet. The pellets were then air-dried at room temperature for 10-15 min under the sterile bench. The dried pellet was dissolved in 120 µl 10 mM Tris-HCl (pH 8).

2.2.9.2 Genomic DNA extraction by Bacterial DNA Kit

DNA was also isolated from bacterial cells isolated from nightshade fermentations at 0 h, 48 h and 144 h for genotypic analysis (RAPD-PCR) and 16S rRNA gene sequence analysis using E.Z.N.A. ® Bacterial DNA Kit (Annex 7) according to the manufacturer's instructions.

2.2.9.3 Determination of DNA concentration

2.2.9.3.1 Determination of DNA concentration by spectrophotometric method

For the measurement of the DNA concentration using a photometer, the DNA was diluted 1:10 with MQ water (7 µl of 1:10 diluted DNA was added to 63 µl MQ water) in a cuvette and UV at a wavelength of 260 nm. An OD_{260} of 1.0 was considered to approximately equate to concentration of 50 µg/ml dsDNA. Based on the measured value, the DNA concentration of DNA used as matrix for PCR reactions were diluted to a concentration of 10 ng/µl in sterile water

2.2.9.3.2 Determination of DNA concentration by Qubit fluorometer

The Qubit working solution was prepared by diluting the Qubit reagent 1: 200 in Qubit buffer. The assay tubes were prepared so that the tubes for standards assay contained 190 µl while those for the samples contained between 180-199 µl. 10 µl was added in each standard assay tube, while the sample varied from 1-20 µl. The samples were then vortexed for 2-3 seconds, followed by incubation for 2 min at room temperature. The tubes were inserted into the Qubit 2.0 Fluorometer and readings were taken. DNA concentration was calculated as described above.

2.10 DNA amplification

2.10.1 PCR amplification of the 16S rRNA genes for eubacteria for denaturing gradient gel electrophoresis (DGGE)

The gene encoding the 16S rRNA was amplified by PCR using Eubacteria universal primer pair (Tab.2.21.1) Amplification was performed using a Primus 96 advanced thermal cycler (Annex 5) in a 50 µl mixture containing 5.0 µl PCR buffer without $MgCl_2$ (Tab.2.13.1), 4 µl $MgCl_2$ (25 mM) (Tab.2.13.5), 5 µl BSA (1.10) (Tab.2.13.3), 5.0 µl of dNTP´s mix (1.25 mM) (Tab.2.13.4), 2.5 µl of primer 338f GC (Tab.2.13.6), 2.5 µl of primer 518r (Tab.2.13.7) reverse primer, 0.3 µl of Taq polymerase (Tab.2.13.2), 10 µl of DNA template (10 ng/µl) and 14.7 µl sigma PCR water (Tab.2.13.8). Reaction mixtures were subjected to a touchdown PCR as described in annex 1D.

2.10.2 RAPD-PCR amplification using M13 primer

Randomly Amplified Polymorphic DNA (RAPD) is a modification of PCR in which a single, short and random oligonucleotide primer is able to anneal and prime at several locations throughout the genome. Unlike conventional PCR analysis, RAPD does not require prior knowledge of the target organism DNA sequence and the use of a random primer leads to the generation of unspecific, randomly amplified PCR products.

Total genomic DNA was used as a template for amplification of the 16S rRNA gene. The gene encoding the 16S rRNA was amplified using primer M13 (Tab.2.21.2). Amplification was performed in a Primus 96-advanced Peqlab, thermal cycler (Annex 5). Amplification was carried out in a 50 µl mixture containing 25 µl ALLin™ Hot Start Taq Mastermix, 2X (Tab.2.14.1), 5 µl M13 primer (Tab.2.14.2), 10 µl sigma PCR water (Tab.2.14.3) and 10 µl DNA template (10 ng/µl). PCR amplification was done according to annex 1A.

2.10.3 Pulsed field gel electrophoresis (PFGE) analysis

The method was used as a strain-typing tool in order to assess the success of the two inoculated starter cultures, i.e. *Lactobacillus plantarum* BFE 5092 and *Lactobacillus fermentum* BFE 6620, in establishing themselves as dominant microbial populations in the African nightshade fermentation. The DNA from the *Lb. fermentum* bacteria was isolated according to the method described by

Gosiewski et al. (2012). Overnight cell cultures (500 µl) from MRS broth were centrifuged at 7500 rpm for 5 min in 1.5 ml Eppendorf tubes and later the pellet was washed in 1 ml WB_1 buffer (Tab.2.19.1) at pH 7.2 and centrifuged at 7500 rpm for5 min.

The measurement of the bacteria cell OD was performed by spectrophotometry and the bacterial cell concentration was adjusted to between OD 2.5 to 3.0 with WB_1 buffer to obtain a cell density of ca.10^7-10^8 cfu/ml. The diluted cell suspension was held at 50 ^oC on a shaking incubator at 80 rpm. Then 2 % InCert agarose (Annex 2A) was prepared, followed by adding 150 µl of the pre-cooled to 50 ^oC InCert agarose solution to the suspension and vortexing briefly. Then 100 µl InCert agarose-bacterial solution was poured in three to five small blocks and left to polymerise for 10 min at 4 ^oC. The 500 µl of LB_1 buffer (Tab.2.19.2) at pH 7.6 with 12.5 µl lysozyme (100 mg/ml) (Annex 2A) plus 20 µl Mutanolysin (1000 U/ml) (Annex 2A) were prepared in 2 ml Eppendorf tube. Using a flamed spatula, the small blocks were pressed into the Eppendorf tubes and incubated at 37 ^oC for 16 hours at 80 rpm.

The blocks were washed with 1 ml WB_1 buffer (Tab.2.19.1) followed by addition of 500 µl LB_2 buffer (Tab.2.19.3) at pH 8.0 containing 25 µl of proteinase K (20 mg/ml) (Annex 2A) to the blocks and incubated for 16 hours at 50 ^oC at 80 rpm. The blocks were washed 3 times with 1 ml WB_2 buffer for 30 min (Tab.2.19.4) at pH 8.0. This was followed by washing the blocks 2 times for 30 min in 1ml TE buffer (Tab.2.19.5) at pH 8.0. The blocks were stored in 1 ml TE buffer at 4 ^oC for up to 6 months. DNA restriction enzymatic digestion was done by adding 267.5 µl sigma water plus 30 µl CutSmart buffer (Annex 2A) in 2 ml Eppendorf tubes, followed by adding one small block in each Eppendorf tube with flamed spatula. The blocks were incubated at room temperature for approximately one hour and later 2.5 µl of *Asc*I enzymes (Annex 2A) was added and incubated at 37 ^oC for 16 hours at 80 rpm.

Pulsed field gel electrophoresis (PFGE) was performed by first preparing 0.5x TBE buffer. Then 1 % low melting Biozym gold agarose (Annex 2A) was prepared in 150 ml 0.5x TBE buffer. The agarose was heated for 2.5 min in the microwave to dissolve then pre-cooled at 50 ^oC in the water bath. The PFGE chamber was prepared by adding 2 litres of 0.5x TBE buffer and pre-cooled at 14 ^oC. The gel blocks were placed on the front side of the gel comb wells with flamed spatula ensuring that they slightly protruded below the edge of the comb. The gel chamber was prepared and the edges were sealed with melting agarose. The comb was inserted with the gel blocks; approximately 150 ml of the pre-cooled agarose was poured on the gel tray, sparing at least 2 ml of the melting

agarose. It was let to polymerise, after which the comb was carefully removed and the empty wells were filled with meting agarose at 50 ^{o}C. The gel was allowed to polymerise and the gel casting frame was removed together with polymerized gel from the chamber. One strain was selected and used as marker throughout the experiment, this strain was used on each gel in order to normalise the gels upon computerised image analysis. The gel was put in the PFGE chamber (CHEF-DR III system Bio-Rad). It was allowed to stand for about 15 min, followed by running at 14 ^{o}C with a pulse time of 1.0–25 secs, voltage 5.5 V/cm, at an angle of 120^{o} for 24 hours. After running, the gel was stained for 1 hour in ethidium bromide and de-stained for 30 min in distilled water and visualised under UV-transilluminator.

The DNA from the *Lb. plantarum* strains was isolated according to methods of Graves & Swaminathan, (2001) with modifications. Cells from 2 ml of an overnight culture in MRS broth were washed once in TE buffer [10 mM Tris (Annex 2A), 10 mM EDTA (Annex 2A), pH 8.0]. The pellet was resuspended in 500 µl TE buffer and diluted to obtain a suspension of OD_{610} = 1.85 using a SmartSpecPlus Spectrophotometer (Annex 5). A volume of 180 µl of the cell suspension was mixed with 60 µl lysozyme solution (100 mg/ml, Annex 2A), 9 µl mutanolysin solution (1000 U/ml, Annex 2A) and 51µl TE buffer. The mixture was incubated on a shaking platform (Peqlab) at 37 ^{o}C, 60 rpm for 45 min. After lysis, 300 µl of the cell suspension was mixed with an equal volume of PSP solution [1.2 % InCert agarose (Annex 2A), 10 % sodium dodecyl sulphate, proteinase K (20 mg/ml, Annex 2A)]. The mixture was transferred to plug molds (Bio-Rad) which were stored at 4 ^{o}C for 15 min to complete polymerisation. The agarose plugs were transferred into Falcon tubes (15 ml sterile plastic tubes, Annex 6) containing 4 ml of buffer [50 mM Tris pH 8.0, 50 mM EDTA pH 8.0, 1 % sodium lauryl sarcosine (Sigma)] and 30 µl Proteinase K solution (20 mg/ml). The Falcon tubes were incubated overnight at 54 ^{o}C and 60 rpm in a shaker/incubator (Infors, Bottmingen, Switzerland). The next day, the solution was removed and the gel plugs were washed twice with preheated sterile distilled water (54 ^{o}C) for 10 min, followed by two washes with preheated TE buffer. After washing, the plugs were stored at 4 ^{o}C in 5 ml TE buffer until restriction enzyme digest. Restriction enzyme digest was done by transferring the plugs into a sterile 1.5 ml Eppendorf tube and digested with *Not*I-HF (Annex 2A) according to the manufacturer's instructions. DNA was restricted using an enzyme concentration of 40 U per plug for 24 h at 37 ^{o}C and 180 rpm. Preparation of agarose gel was done as described above. The switch times were 0.1-

10 s and the run time was 28 h. After electrophoresis, the gels were stained and visualised as described above.

2.10.4 PCR amplification of 16S rRNA genes

Total genomic DNA was used as a template for amplification of the 16S rRNA genes of the isolates from nightshade fermentation. PCR amplification was performed using primer pair combination primers 16s seq fw and 16S seq rev (Tab.2.21.3) Amplification was performed in Primus 96 advanced Peqlab, thermal cycler (Annex 5) in relation to positions 8–27 and 1511–1491 of the corresponding 16S rRNA gene of *Escherichia coli* respectively (Brosius et al., 1978). DNA was amplified in 50 µl mixture containing 0.30 µl gene script Taq (Tab.2.16.5), 2.5 µl 16s seq fw primer (Tab.2.16.3), 2.5 µl 16s seq rev primer (Tab.2.16.4), 10 µl template DNA (10 ng/µl), and 6.0 µl of dNTP's mix (1.25 mM) (Tab.2.16.2), 5.0 µl 10 X PCR buffer with $Mgcl_2$ (Tab.2.16.1) and 23.7 µl of PCR water (Tab.2.16.6). PCR amplification was done according to annex 1C.

2.10.5 PCR amplification of enterobacteria using atpD primers

Total genomic DNA was used as a template for amplification of the atpD gene. DNA was amplified using atpD A1 fw forward and atpD A2 rev reverse primer (Tab.2.21.4). Amplification was performed in a Primus 96 advanced Peqlab, thermal cycler (Annex 5). Amplification was carried out in a 50 µl mixture containing 25 µl ALLin™ Hot Start Taq Mastermix, 2X (Tab.2.15.1), 2.5 µl atpD A1 fw primer (Tab.2.15.2), 2.5 µl atpD A2 rev primer (Tab.2.15.3), 10 µl sigma PCR water (Tab.2.15.4) and 10 µl DNA template (10 ng/µl). The mixture was amplified according to annex 1B.

2.10.6 DNA purification for 16S rRNA sequencing for strains identification

The amplified PCR products were purified with the Mag-Bind® RXNPure plus Magnetic Beads (Annex 7) according to the manufacturer's instructions.

2.10.7 Sequencing of atpD and 16S rRNA genes of LAB and enterobacteria isolates

Sequencing of the purified products was carried out by a commercial service provider LGC Genomics group (Berlin, Germany) based on Sanger sequencing for precise characterization of Enterobacteriaceae isolates with atpD primer and 16S seq for Lactobacillaceae isolates.

2.11 Agarose gel electrophoresis

2.11.1 Agarose gel electrophoresis of amplified eubacterial 16S rRNA genes for denaturing gradient gel electrophoresis

The agarose gel electrophoresis was used to verify the standard PCR products of their size and purity. The amplicons from the PCR size standard were loaded on a 1.5 % agarose gel. The gel was prepared as described in tab.2.18.1. The PCR products were prepared by mixing 5 µl of the amplified products with 2 µl loading buffer. Six µl of the mixture of two markers 100 bp and 500 bp (Tab.2.18.5) was used as the DNA size standard. This was followed by electrophoresis at 100 V for 60 min in 1 x TAE buffer (Tab.2.18.7). The DNA bands could be detected under UV transilluminator (Annex 5).

2.11.2 Agarose gel electrophoresis for amplified RAPD PCR products profiling

Agarose gel (w/v) (1.8 %) was prepared as described in tab.2.18.2. Fifteen µl of each RAPD-PCR product was subjected to electrophoresis at 100 V for 3.5 hours. After running, the gel was stained in 500 ml distilled water containing 2 µl ethidium bromide (Annex 2A) for 1 hour, followed by destaining in distilled water for 30 min. The RAPD gel was viewed under UV transilluminator.

2.11.3 Evaluation of band patterns and analysis of sequences

Phenotypic and genotypic data were analyzed as described in section 2.20.

2.12 Determining effects of starter cultures on the background microbiota during nightshade fermentation by denaturing gradient gel electrophoresis (DGGE)

The DNA was isolated from fermentation samples at 0 h, 24 h, 48 h, 72 h and 144 h using the modified method of Pitcher et al. (1989) (section 2.2.9.1). PCR amplification was performed with eubacteria 16S rRNA gene specific primer pair and was subjected to touchdown PCR amplification (Annex 1D). The amplified PCR products were separated on a polyacrylamide vertical gel The DGGE gradient of 35 % and 70 % of urea and formamide respectively (Tab.2.25) were used in this experiment.

2.12.1 Treatment of the glass plates

The two glass plates of the gel holding unit were thoroughly washed with detergent and rinsed with distilled water. Under the fume hood, 500 µl Repel saline solution was evenly spread onto the small glass plate and wiped with Kleenex tissue and left to stand for 5 minutes, after which 2 ml of 100 % ethanol was evenly spread onto the glass and let to air dry. The larger glass was treated with only 100 % ethanol and no Repel saline solution was used. The plate assembly was done in a Biorad casting stand according to the manufacturer's (Annex 5) instruction manual.

2.12.2 Preparation of 8 % polyacrylamide gel

The preparation of the polyacrylamide gel (PAA) was prepared as shown in the table below.

Table 2.25: Composition of polyacrylamide gels for DGGE.

Components	35 %	70 %
Urea	3.67 g	7.35 g
Formamide (ml)	3.5 ml	7.0 ml
TAE 50 X (ml)	0.5 ml	0.5 ml
Acrylamide/ bis-acrylamide (37:5:1) 40 % (ml)	5.0 ml	5.0 ml
TEMED	55 (µl)	55 (µl)
Ammonium persulfate (10 %)	95 (µl)	95 (µl)
Distilled water	Add to 25 ml	

As shown from the above table, two 50 ml falcon tubes were labelled according to the gradient percentage. Urea was weighed as shown in table 2.25 and dissolved in 5 ml distilled water, followed by addition of the other reagents as shown in table 2.25. The final volume was adjusted to 25 ml by addition of distilled water. The components were mixed by shaking the tube and the tube was immediately put on the ice. The preparation of 10 % ammonium persulfate (APS) was done by dissolving 0.1 g/ ml of distilled water and it was immediately cooled on ice.

2.12.3 Casting the PAA gel

Addition of 95 µl ammonium persulfate (Annex 2A) (radical initiator) and TEMED (Annex 2A) (catalyst) was done first in the low percentage (35 %) gradient gel since the gel polymerises slowly.

The gel was cast as described by the Biorad user manual's instructions using a gradient mixer. Care was taken to avoid gas bubbles entering the gel. The comb was quickly inserted taking care not to form gas bubbles under the gel wells. After polymerisation, the comb was carefully removed and the wells rinsed with the distilled water using a syringe.

2.12.4 Running of the gel

The DCode apparatus was filled with 7 litres of freshly prepared 1x TAE buffer (140 ml 50x TAE buffer in 6860 ml of distilled water) (Annex 2B) The gel holder was removed from the buffer tank; it was fixed in the gel holder and set back in the buffer tank. The controller system was placed on the buffer tank; the buffer was preheated at 60 ^{o}C for 1 hour. The PCR products were prepared by mixing 45 µl of the DCode 2x gel loading dye with 45 µl of successfully amplified PCR products. Forty (40 µl) of the mixture was applied using gel loading tips. The controller was placed on the system again; the DCode was then connected to the power unit. The gel was allowed to run at 70 V and 60 ^{o}C for 16 hours.

2.12.5 Staining the gel

After running the gel, the controller was switched off and removed from the buffer tank. The buffer tank was emptied; the gel holder was removed from the buffer tank. The gel was removed from the tank and stretched on the gel tray. The gel was carefully removed from the glass plate and transferred to a tray containing staining solution (300 ml distilled water or 1x TAE buffer with 5 µl of SYBR gold (Annex 2A). The gel was stained for at least 30 minutes then further de-stained in fresh 300 ml distilled water for at least 30 minutes. The DGGE gel was viewed under UV transilluminator.

2.13 Determination of the bacterial community composition by high through-put sequencing

Following the method described by Pitcher et al.(1989) in section 2.2.9.1, total genomic DNA was extracted from the bacterial communities that inhabited the nightshade fermentation solutions at 0 h, 24 h, 48 h, 72 h and 144 h. Gene specific primers were used to target the 16S rRNA V3 and V4 region with Illumina adapter overhang for the amplification. The PCR was carried out in a 25 µl mixture containing 12.5 µl ALLin™ HotStart Taq Mastermix 2X (Tab.2.17.5), 1 µl 16S Metafw primer (Tab.2.17.3), 1 µl 16S Metarev primer (Tab.2.17.4) 0.5 µl sigma PCR water (Tab.2.16.6)

and 10 µl DNA template (5 ng/µl). The reaction mixtures were subjected to amplification conditions as described in annex 1E. Successful amplification and the size of the PCR products were confirmed on 1.5 % agarose gel (Tab.2.18.1). The PCR products were cleaned with Mag-Bind RxnPure plus magnetic beads (Annex 7) according to the Illumina Sample Preparation Guide. The purified PCR products were used as template for index PCR to attach dual indices with the Nextera XT index kit (Annex 7). This PCR was carried out in 50 µl mixtures containing 10 µl 5X 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 (unadjusted), 0.5 µl Phusion Hot Start Flex DNA Polymerase (2 U/ µl) (Tab.2.17.1) and 16.5 µl sterile water (Tab.2.16.6). The reaction mixtures were amplified as described in Annex 1F. The index PCR products were cleaned with Mag-Bind RxnPure plus magnetic beads (Annex 7) according to Illumina Sample Preparation Guide. The final DNA concentration was determined using the Qubit dsDNA HS Assay Kit on a Qubit 2.0 fluorometer (Annex 7). The final DNA concentration was adjusted to 4 nM with 10 mM Tris buffer at pH 8.5. The normalized samples were finally pooled by aliquoting and mixing 5 µl from each sample. The resulting sample library was transferred and stored at -20 °C.

2.13.1 Library denaturation and MiSeq sample loading

Denaturation of DNA was done by combining 5 µl of 4 nM pooled library with 5 µl freshly prepared 0.2 N NaOH. The sample solution was mixed briefly and then centrifuged at 280 x g for 1 minute at 20 ^{o}C, followed by incubation for 5 minutes at room temperature. Afterwards, the library was diluted by adding 990 µl of pre-chilled HT1 buffer. This resulted in a concentration of 20 pM in 1 mM NaOH. 300 µl of the library were further diluted with 300 µl pre-chilled HT1 buffer, resulting to a final library concentration of 10 pM with 0.5 mM NaOH. The denatured and diluted library was placed on ice before proceeding.

2.13.2 Setting and denaturing PhiX control library

2.5 µl of 10 nM PhiX library (Annex 7) first were diluted by adding 3 µl of 10 mM Tris at pH 8.5 resulting in a 4 nM PhiX library. The PhiX control library was further denatured and diluted equivalent to the procedure described above resulting in the same concentration as the sample library.

2.13.3 Combining amplicon library and PhiX control

60 µl of denatured and diluted PhiX control library and 540 µl of denatured and diluted amplicon library were combined in a microcentrifuge tube. The combined, final sequencing library was incubated on at 96 ^{o}C for 2 minutes. After incubation, the tube was inverted 1-2 times to mix and immediately placed and stored at least for 5 minutes on ice.

2.13.4 MiSeq wash

This was done by selecting MiSeq home screen and selecting PERFOM WASH. The maintenance wash run was performed by preparing approximately 1 litre tween 20 for 3 wash cycles. The wash bottles were emptied and approximately 250 ml of 0.5 % tween was poured in the wash bottle. Each well of the wash cartridge was filled with about 3 ml of 0.5 % Tween 20 from a spray bottle. After each wash cycle, the wash cartridge and bottle were prepared and filled.

2.13.5 Preparation of sample sheet

This was done by opening Illumina experimental manager, the sample sheet was created by selecting MiSeq, targeted sequencing was selected under category with metagenomics 16S rRNA as the selected application. The reagent cartridge code was entered, then NexteraXT sample prep Kit was added with 2 index reads. The name of the experiment, user and date were entered. The type of read was paired-end with two cycle reads of 301 cycles (2 x 301). The metagenomics 16S rRNA workflow was set to use adapter trimming as specific settings. The index 1 (N701-N/12) and index 2 primers were selected and automatically inserted and the saved on the USB stick.

2.13.6 Preparation of reagent cartridge

The reagent cartridge was thawed overnight in refrigerator at 2-8 ^{o}C. The reagent cartridge was inverted ten times to mix the thawed reagents, and properly inspected to ensure that all positions are thawed. The foil seal covering the reservoir labelled ‘’load samples’’ was cleaned with a low-lint lab tissue.

2.13.7 Loading sample libraries

The foil seal was pierced with a clean 1 ml pipette, 600 µl of the prepared libraries (section 2.13.3) were pipetted into the MiSeq reagent cartridge. The MiSeq reagent cartridge was stored on ice until ready to use.

2.13.8 Set up sequence run

Start new MiSeq: on MiSeq home screen, the MiSeq Control Software (MCS) was closed by shutting down the windows and switching on again (to remove/stop "Temp'' files). The prepared sample sheet was uploaded into MiSeq by selecting manage files and choosing Sample sheet. The Sample sheet was browsed, opened and saved. To start the running, SEQUENCE run was selected on the Home Screen; the box "Use Base Space for storage and analysis" was checked. The email and password were entered in MyIllumina account.

2.13.9 Cleaning and loading flow cell

The flow cell was rinsed with bidistilled water until both the glass and plastic cartridge were thoroughly rinsed of excess salts. The flow cell was dried with low-lint lab tissue and cleaned again with low-lint cloth moistened with 70 % ethanol and dried with the lint cloth. The flow cell compartment door was raise and the release button pressed to the right of the flow cell latch thus opening the flow cell latch. The flow cell was inserted and the flow cell compartment door closed. The reagent compartment door was opened, the sipper handle was raised was raised until it locked into place. The wash bottle and the PR2 bottle were removed and emptied and new PR2 buffer was inserted. The sipper handle was slowly lowered to ensure that the sippers lower into the PR2 and waste bottles. The reagent chill door was opened and the washing cartridge removed. The MiSeq reagent cartridge was briefly dried, and slide into the reagent chiller until the cartridge stops. The reagent compartment door was closed.

Starting the run: The sample sheet was selected, the review windows opened automatically, then the Pre-Run Check window opened , the system performed a check of all components if no errors are found, the Start Run was selected.

2.14 Determination of draft genome sequence of *Lb. plantarum* BFE 5092 and *Lb. fermentum* BFE 6620

The genomic DNA of *Lb. fermentum* BFE 6620 DNA was isolated using the peqGOLD bacterial DNA kit (Annex 7). The sequencing library were prepared with an Illumina Nextera XT library prep kit (Annex 7) according to the manufacture's instruction. The genome sequencing was done with an Illumina MiSeq sequencer with 2 x 251 paired-ends as above. The draft genome of the *Lb. plantarum* BFE 5092 genome was previously sequenced and assembled (Oguntoyinbo et al., 2016).

2.15 Determination of enzymes from fermentation solution

2.15.1 D-lactic acid/L-lactic acid

D-lactic acid /L-lactic acid was determined by UV test using the Enzyme Kit (Annex 7). The test was performed according to the manufacturer's instructions, without any modifications. D/L lactic acid determination in foodstuffs is based on the principle of oxidation of D-lactate dehydrogenase (D-LDH), D-lactic acid (D-lactate) to pyruvate by nicotinamide-adenine dinucleotide (NAD). The oxidation of L-lactic acid requires the presence of the enzyme L-lactate dehydrogenase (L-LDH).

(1) D-lactate + NAD^+ $\longleftrightarrow$ pyruvate + NADH + H^+

(2) L-lactate + NAD^+ $\longleftrightarrow$ pyruvate + NADH + H^+

The equilibrium of these reactions lies on the side of lactate. By trapping pyruvate in the subsequent reaction catalysed by the enzyme glutamate-pyruvate transaminase (GPT) in the presence of L-glutamate, the equilibrium can be displaced in favour of pyruvate and NADH.

Pyruvate + L-glutamate $\xleftrightarrow[\text{GPT}]{}$ L-alanine + 2-oxoglutarate

The amount of NADH formed in the above reactions is equivalent to the amount of D-lactic acid and L-lactic acid respectively. The increase in NADH is determined photometrically by means of its light absorbance measured at 340 nm.

2.15.2 Sucrose / D-glucose

Sucrose/D-glucose was determined by the UV test with the enzyme kit (Annex 7). The test was performed according to the manufacturer's instructions also without any modifications. Sucrose/D-glucose concentration is determined before and after enzymatic hydrolysis. Determination of D-

glucose before inversion is done at pH 7.6 in the presence of enzyme hexokinase (HK) which catalyses the phosphorylation of D-glucose by adenosine- 5'-triphosphate (ATP) with simultaneous formation of adenosine-5'-diphospate (ADP).

$$\text{D-Glucose + ATP} \longrightarrow \text{Glucose-6-P + ADP}$$

In the presence of glucose-6-phosphate dehydrogenase (G6P-DH), the D-glucose-6-phosphate (G6P) formed is specifically oxidised by nicotinamide-adenine dinucleotide phosphate (NADP) to D-gluconate-6-phosphate with the formation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH).

$$\text{Glucose-6-P + NADP}^{+} \xrightarrow{\text{G-6-P-DH}} \text{D-gluconate-6-phosphate + NADPH + H}^{+}$$

The amount of NADPH formed is equivalent to the amount of D-glucose and is determined based on its absorbance at 340 nm. The enzymatic inversion of sucrose at pH 4.6 is hydrolysed by the enzyme-β-fructosidase (invertase) to D-glucose and D-fructose.

$$\text{Sucrose + } H_2O \xrightarrow{\beta\text{-Fructosidase}} \text{glucose + D-fructose}$$

The content of sucrose is calculated from the difference of the D-glucose concentrations before and after enzymatic inversion.

2.15.3 Total titratable acids

Titration is a chemical process used in ascertaining the amount of constituent substance in a sample, e.g. acids, by using a standard counter-active reagent, e.g. an alkali (NaOH). The total titratable acidity (TTA) was measured by titrating a mixture of 10 ml of the juice from fermented vegetables and 100 ml of distilled water to pH 8.1 using 0.1 M sodium hydroxide solution with an automatic Schott titroline easy machine (Annex 5) according to the manufacturer's instructions The results were calculated as acid in gram/litre using the following formula;

$$\text{g/l acid} = \frac{\text{Titre x acid factorx 100 x 10}}{\text{10 ml (sample)}}$$

NB: Multiplication factor for lactic acid = 0.009 (OECD, 2009).

2.16 Solar drying of vegetable nightshade

The vegetable nightshade was cultivated and harvest as described in section 2.1.1. They were properly sorted to remove unwanted materials, then thoroughly washed with tap water to remove dust and insects. Fresh weight were taken and recorded, the leaves were then spread into the solar drier chamber to dry at 37-40 ^{o}C. During drying the weights were taken after every 24 hours until a constant weight was achieved, generally drying took 2-3 days. The dried leaves were packaged into zip lock air tight polyethylene bags and stored at 10 ^{o}C and 25 ^{o}C for further analysis.

2.16.1 Storage experiments at 10 ^{o}C and 25 ^{o}C

After solar drying, the leaves were divided into 2 portions of 300 g. They were packaged in zip lock airtight polyethylene bags. The batches were stored at either 10 ^{o}C and 25 ^{o}C respectively, for a period of 4 weeks. Microbial analyses were done (see below), after which the nutrient contents were determined to assess the effect of solar drying and storage temperature on nutrient and chemical composition.

2.16.2 Microbial analysis of fresh and solar dried nightshade leaves after storage

Microbiological analysis was performed both on fresh leaves immediately after harvesting and on solar dried stored samples once a week for one month. The experiment was conducted by weighing 10 g of each sample followed by addition of 90 ml of sterile Ringer solution. The samples were homogenised in a stomacher mixer for 3 minutes. The dilution was done by mixing and transferring 1 ml of the homogenate to 9 ml of sterile Ringer solution. The dilution series were prepared from 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9}. The dilutions were vortexed thoroughly to mix, and then 10 µl or 100 µl of the aliquots were used spread out onto 4 different media plates. Enterobacteria were determined by VRBG agar, total aerobic mesophilic counts were evaluated on Std.I agar, while lactic acid bacteria counts were determined by MRS agar. The yeast and moulds were enumerated on PDA agar. The inoculation was done in duplicates for reproducibility of the results. The plates were incubated at their respective temperatures (Std.I and VRBG at 37 ^{o}C, MRS at 30 ^{o}C and PDA at 25 ^{o}C). The plates were counted after 24 hours to determine the colony forming units (CFU) as described in section 2.2.4.

2.17 Freeze drying of vegetable nightshade leaves

Freeze drying or lyophilisation is the process of extraction of water from frozen material. The drying process takes place by avoiding the liquid state through sublimation, or direct conversion from ice to vapour. A water-soluble product which will have the same characteristics as the original product can be obtained after addition of water to the freeze-dried product. Since the drying process takes place in a frozen state at very low temperature, denaturation of proteins is prevented. Other chemical components will be qualitatively and quantitatively unchanged. Freeze drying of the vegetable samples was performed in Christ ALPHA 1-4 LD plus freeze drier (Annex 3). The leaves were harvested and prepared as described in section 2.1.2. They were cut into small pieces and 150 g was put into 5 x 7-inch sealable plastic bags and kept at -20 ^{o}C for 24 hours or longer. The frozen samples were removed from the freezer; small holes were made on the samples bags to facilitate liquid evaporation during freeze drying. The samples were loaded into the freezing unit shelves and the drying chamber was closed. The drain valve was closed, the machine was switched on, the pump was warmed, the coil was frozen and main drying initiated. Drying took 3-4 days, depending on the volume of the sample to be dried. After drying the samples were removed from the chamber, put in airtight sealable plastic bags, and stored at room temperature or at -20 ^{o}C. The samples were then sent to MRI, Karlsruhe, for chemical and nutrient analyses

2.18 Nutrient and chemical analyses

2.18.1 Determination of dry mass

A metal dish with 30-35 g sea sand with a lid were dried at 103 ^{o}C for 2 hours in the oven and cooled in a desiccator. 1.5 g of the samples were weighed onto the dishes, and mixed with the sand. The dishes were placed in the oven with the loosened covers at 103±2 ^{o}C. They were heated for 16 hours (overnight) until constant weight was achieved. The dishes were covered while still in the oven and transferred to the desiccator to be weighed as soon as they reached room temperature (AOAC, 2005.950.46).

Calculation

$$\text{Weight loss g/100 g sample}: \quad W = \frac{m_a - m_b}{m} \times 100 \qquad (1)$$

$$\text{Dry matter g/100 g sample}: \quad 100 - W \qquad (2)$$

Where

m: Weight of sample (g)

m_a: Weight of sample with dish and sand before drying

m_b: Weight of sample with dish and sand after drying

2.18.2 Determination of total ash

A platinum dish was heated in the furnace and cooled in a desiccator to room temperature. 1.5–2.0 g of freeze dried samples were weighed onto the platinum dish. The dish and the sample were heated for 2 hours at 103 ^{o}C (until the water was expelled). To remove the volatile organic matter, the platinum dish was heated slowly over the flame until swelling had stopped. The platinum dish was transferred into the furnace and heated at 550±5 ^{o}C for 15 hours (overnight). The platinum dish was removed from the furnace and transferred into a desiccator to cool for 30 min. The weight was taken as soon as the dish reached room temperature (AOAC, 2005.923.03).

Calculation

g Ash/100 g wet sample: $$A = \frac{m_b - m_a}{m} \times 100 \qquad (1)$$

g Ash/100 g dry matter: $$\frac{A}{DM} \times 100 \qquad (2)$$

where:

m: Weight of sample (g)
m_a: Weight of platinum dish (g)
m_b: Weight of platinum dish with ash (g)
DM: Dry matter g/100 g sample

2.18.3 Determination of Vitamin B_1 and B_2 by HPLC

Determination of Vitamin B_1 and B_2 from green leafy vegetables was performed with HPLC after acid hydrolysis and enzymatic preparation. Freeze dried vegetable nightshade powder (1.5 g) was weighed into a 150 ml conical flask followed by addition of 10 ml 1 Mol/l hydrochloric acid (Annex 2A) and 50 ml bidistilled water (Tab.2.4.8). The samples were loaded into a basket; the hydrolysation was done by heating the samples in an autoclave at 120 ^{o}C for 30 min. Subsequently, the samples were cooled to approximately 20 ^{o}C in a cooling water bath and transferred into a 100 ml volumetric flask and filled with bidistilled water (Tab.2.4.8) up to the mark. The sample extracts were filtered through a prepleated filter paper into a polyethylene container with a screw cap. 5.0

ml of the extract were transferred into a 20 ml volumetric flask; 7.5 ml of 0.1 Mol/l hydrochloric acid (Tab.2.4.2) were added to bring the total volume to 12.5 ml. The pH was adjusted to 4.0 with 2.5 Mol/l sodium acetate solution (Tab.2.4.3). 2.0 ml Taka-Diastase-suspension (Tab.2.4.7) were added, followed by incubating the samples in a water bath for 18 hour at 45 °C.

After enzymatic treatment, the flasks were removed from the water bath and samples were cooled to 20 ^{o}C. The pH was adjusted to 3.0 using 1 Mol/l hydrochloric acid (Annex 2A). The magnetic stirrer bars were removed and the flasks were filled with bidistilled water (Tab.2.4.8) up to 20 ml mark. The solution was thoroughly mixed and 5.0 ml of this mixture were put into a 5 ml syringe fitted with a 0.2 µm membrane filter (Annex 4). The first drops of the filtrate were allowed to run out; approximately 1.5 ml of the samples were filtered into the HPLC vials. Filtration was done in duplicates and an extra vial was frozen for subsequent HPLC analysis.

Determination of vitamin B_1 and B_2 was performed with an Agilent 1100 series HPLC system with modification by Bognar (1992) to detect both vitamin B_1 and B_2 simultaneously as described in annex.3H.1.

Four calibration standard solutions (0.25 µg/ml, 0.5 µg/ml, 1.0 µg/ml and 2.0 µg/ml) were used to cover the expected range of riboflavin (vitamin B_2) and thiamine (vitamin B_1) contents of the measured sample. The measurements were taken at the beginning and end of the HPLC to establish calibration curves. The initial volume of the sample prepared for measurement was 20 ml. Therefore, the sample conversion in µg/100 g was calculated using the following formula:

$$W = \beta \text{ x4 x 100 ml x } \left(\frac{100}{20}\right) \text{x E}$$

$$W = \frac{(\beta \text{ x } 2000)}{E}$$

Where:

W: Content of analyte in the starting sample material (g/ 100g)

β: Content of analyte in the sample measured solution (g/ 20 ml)

E: Initial weight of the starting sample material (g)

2.18.4 Determination of ascorbic acid (vitamin C)

Determination of vitamin C was done according to Bognar & Daood (2000). The method can detect L-ascorbic acid and L-dehydroascorbic acid. It works by principle of an in-line oxidation of L-ascorbic acid (AA) to L-dehydroascorbic acid (DHAA) and separation is performed on a short column of activated charcoal as shown in figure 2.14. The derivatization of DHAA is done with 1,2-phenylenediamine solution in heated capillary reactor into fluorescent quinoxaline compounds and are detected fluorometrically. Freeze dried nightshade powder (0.5–2.0 g) was weighed in a 100 ml volumetric flask and 40 ml meta-phosphoric/acetic acid (7.5 %/20 %) solution (Tab.2.7.1) (extraction solvent) were added and filled to the mark with bidistilled water (Tab.2.4.8). A magnetic stir bar was added and the solution was mixed for 5 min at a maximum speed. A portion of the sample extract was filtered through a prepleated filter paper into a polyethylene 50 ml conical flask. (Process can be accelerated by centrifugation before filtration). 2.0 ml of the sample extracts were transferred into a 20 ml volumetric flask, followed by the addition of 2.0 ml acetonitrile (Annex 2A), and was then thoroughly mixed. Approximately 4.0 ml of the mixture was transferred into a 5 ml syringe fitted with a 0.2 µm membrane filter (Annex 4). The first drops of the filtrate were allowed to run out, and then approximately 2 ml of the sample was filtered into the HPLC vials for subsequent analysis.

Chromatographic analysis of vitamin C was performed with Shimadzu HPLC system (Tokyo, Japan) according to the description in annex 3H.2.

The calibration curve based on external standards was prepared using three calibration solutions (20 µg/ml, 40 µg/ml and 60 µg/ml) to analyse the expected range of L-ascorbic acid content of the measured sample. The measurements were taken at the beginning and end of the HPLC to establish the calibration line and to calculate the regression factors.

The concentration of vitamin C in mg/100 g in the nightshade samples was calculated according to the formula below.

$$W_{AA} = \frac{y_{AA}}{m} \times \frac{V}{10}$$

Where:

W_{AA} L-ascorbic acid content in mg/100 g sample

V Total volume of the sample extracts solution in 100 ml

M Weight of sample in gram

$\mathbf{y_{AA}}$ Total concentration of L-ascorbic acid in µg/ml in the final sample extract determined with external calibration.

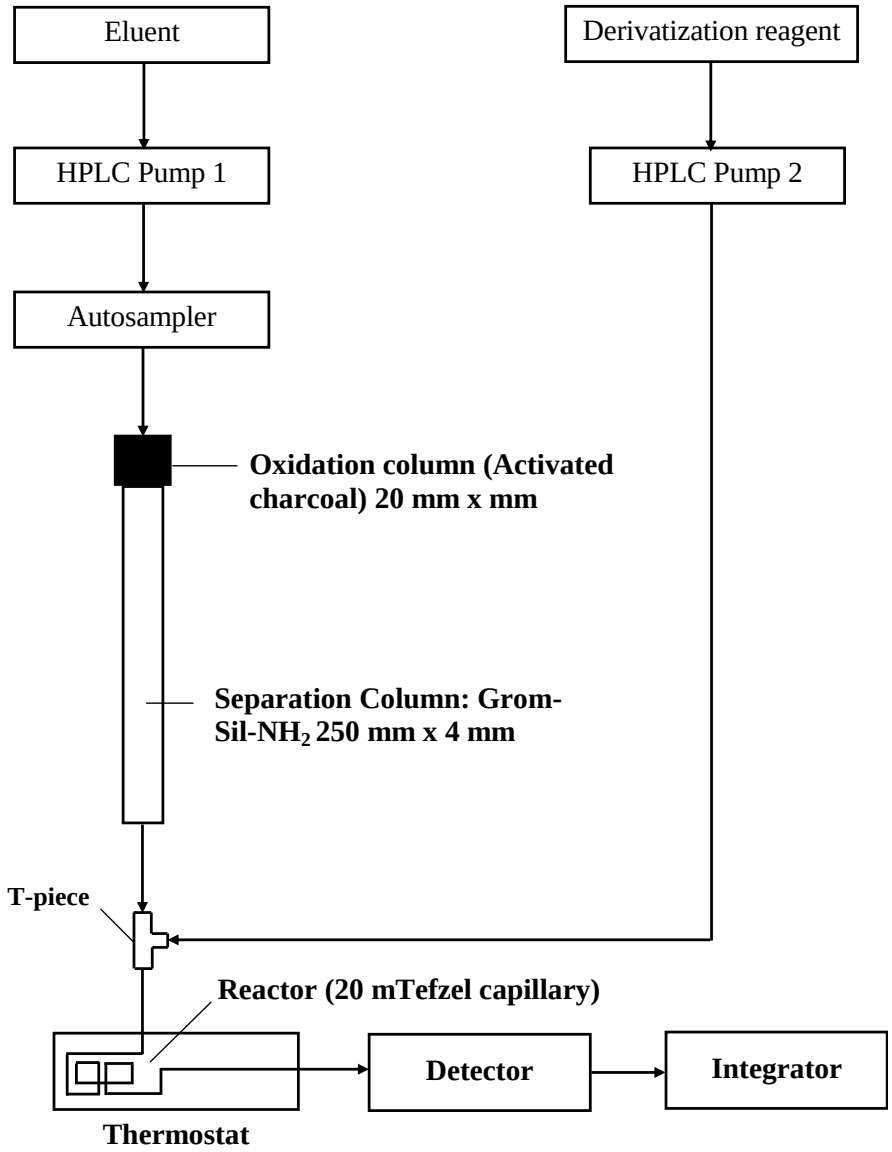

Figure 2.14: HPLC-system scheme for the determination of total vitamin C.

2.18.5 Extraction and determination of tocopherol equivalents (vitamin E)

Vitamin E is found in foods in four main forms, α-, β-, γ and δ-tocopherols. Tocopherols and tocotrienols are collectively known as tocochromanols and these are a naturally occurring group of antioxidants. A direct extraction method as described by Knecht et al. (2015) was used to determine

tocopherols and tocotrienols equivalents. Freeze dried vegetable nightshade powder (0.1 g) was weight into 50 ml centrifuge tubes (PP, Corning Inc. New York, USA) followed by the addition of 10 ml acetone (containing 0.025 % butylhydroxytoluene (BHT) (Annex 2A), the sample was subjected to ultra-sonication for 2 minutes and further extracted for 30 seconds by using a vortex mixer (Corning Inc.) at maximum speed. The sample was centrifuged at 3600 x g for 2 minutes at 8 ^{o}C, the acetone extract was collected in 50 ml volumetric flask. The extraction process was repeated two times without initial ultra-sonication. The extracts were combined together and the volume was adjusted up to 50 ml with acetone (containing 0.025 % BHT). This was followed by transferring a 10 ml aliquot into 12 ml glass vial which was then dried under a stream of nitrogen. The dried extract was re-dissolved in 500 µl mixture containing methanol/acetone/water in the ratio of 54:40:6 (*v: v*) (Annex 2B) respectively. The solution was filtered through PTFE 0.2 µm membrane filter (Annex 4) into the HPLC vial for analysis. HPLC-FLD analysis of tocopherols was performed according to information in annex 3H.3.

The calibration curve based on external standards was prepared with seven calibration solutions (0.1 µg/ml, 0.5 µg/ml, 2 µg/ml, 5 µg/ml, 10 µg/ml, 20 µg/ml and 40 µg/ml) to cover the expected range of tocopherols of the measured sample. The measurements were taken at the beginning and end of the HPLC to establish the calibration curves.

Calculation of results

The β, γ and δ-tocopherols are calculated as α-tocopherol equivalent as shown in the formula below:

$$\text{Tocopherol } (\mu g/100mg) = \left(\frac{K\ x\ 50\ x\ 0.5\ x\ 100}{W}\right)$$

Where:

K: Sum total α-Tocopherol equivalent (1x α) + (0.4 x β) + (0.1 x γ) + (0.01 x δ) measured in the extract

W: Weight of the sample (mg)

50: Volume used to dissolve the sample (ml)

0.5: Volume used to measure the sample (ml)

2.18.6 Determination of raw protein by Kjeldahl-method

The Kjeldahl method (AOAC, 2005.955.04) was used for the determination of crude protein content. The method involved three stages: digestion, distillation and titration. Digestion process involves the conversion of nitrogen into ammonium sulphate by boiling with concentrated H_2SO_4. Four grams freeze-dried vegetable powder were weighed into 300 ml Kjeldahl digestion flask followed by addition of 15 ml of 98 % N_2-free sulphuric acid (Annex 2A) and 1 Kjeldahl-tablet (Annex 2A). The sample was digested by heating the flask in an inclined position at approximately 400 ^{o}C for ≥ 30 min under fume hood. It was cooled and diluted with 50 ml bidistilled water (Tab.2.4.8) and distilled in Erlenmeyer flask under the condenser distillation unit by adding 25 ml of 2 % boric acid (Tab.2.10.2) and 4 drops of taschiro indicator (Tab.2.10.5). The digestion flask was connected to the distillation unit; 70 ml of 40 % sodium hydroxide (Tab.2.10.1) were added to set free ammonia. The flask was removed and the tip of the condenser was washed with water. Titration was done with 0.05 Mol/l sulphuric acid (Tab.2.10.4) and the readings were taken when the colour changed from green to violet.

Calculation of total nitrogen

Nitrogen g/100 g sample:	$N = \frac{V \times 1.4008}{m \times 10}$	(1)
Protein g/100 g sample:	$P = N \times 6.25$	(2)
Protein g/100 g dry matter.	$\frac{P}{DM} \times 100$	(3)

Where:

V volume of 0.05 M sulphuric acid for titration in ml

m weight of sample in g

DM dry matter content of sample in g/100 g

2.18.7 Determination of soluble sugars by HPLC

The extraction and determination of soluble sugars (fructose, glucose and sucrose) from freeze dried grounded African indigenous vegetables were determined according to the method described by Van Den et al. (1986). Two grams of the freeze-dried powder were weighed into a 50 ml volumetric flask followed by the addition of approximately 40 ml bidistilled water (Tab.2.4.8). The

samples were mixed and heated at 60 ^{o}C for 30 min in a water bath. They were cooled to about 20 ^{o}C; bidistilled water was then added to the mark. The mixture was centrifuged at 3000 x g for 4 min. The supernatant was filtered through prepleated filter paper (Annex 4); about 10 ml of the filtrate were stored at –18 ^{o}C until further HPLC analysis. Prior to injection, 3 ml of acetonitrile (Annex 2A) were mixed with 1 ml of the sample solution in a 10 ml volumetric flask and filtered through a 0.2 µm membrane filter (Annex 4) directly into the auto sampler vial. The calibration solutions were prepared prior to injection by mixing 300 µl of each calibration solution with 900 µl acetonitrile into an auto sampler vials without filtration.

HPLC analysis was carried out into Agilent 1100 series HPLC system as described in annex 3H.4.Three calibration standard solutions; (0.25 mg/ml, 0.5 mg/ml and 1.0 mg/ml) were used to cover the expected range of fructose, glucose and sucrose concentrations of the measured sample.

Calculation of sugars (fructose/glucose/sucrose):

$$\text{Conc}\left(\frac{\text{g}}{100\text{g}}\right) = \frac{\text{D x 50 x 100}}{\text{W x 1000}}$$

Where:

D: concentration measured in final sample extract (mg/ml)

W: weight of the sample (g)

50: volume used to dissolve the sample (ml)

2.19 Sensory evaluation of fermented nightshade leaves

Sensory analysis of the fermented nightshade leaves was performed to evaluate the general knowledge and acceptability of the fermentation technology. In total 20 volunteer panellists, comprised of male and female students from the Department of Food Science and Technology (JKUAT), who were familiar and are regular consumers of vegetable nightshade, volunteered. The fermented and unfermented leaves were prepared by boiling for 5 minutes, followed by frying with onions and tomatoes and later was given to the panellists to determine the sensory attributes of colour, appearance, taste, feel in the mouth, smell and the general acceptability based on nine (9) point hedonic scale, where 1 represented “very bad” and 9 represented “excellent”. The panellists were also asked to indicate if they had the knowledge of fermented vegetables and if they could recommend them to others (sample questionnaire guide annex 10).

2.20 Data analysis

Data entry management and preliminary summaries such as averages was done in Microsoft excel spreadsheet. Colony counts from repeated fermentation experiments were subjected to descriptive statistics for the comparison of the means between nightshade fermentation with starter cultures and control. Phenotypic characteristics of the isolates were made to define the nature of each quantitative test either as positive or negative with the binary data (0, 1) were entered into the BioNumerics 7.0 program and the cluster analysis was performed using the Dice coefficient. The agarose gel bands of RAPD, PFGE and DGGE were selected and compared with each other to create cluster analysis using Pearson correlation, Dice similarity coefficient (*s*) and UPGMA clustering methods (Sneath & Sokal, 1973).

The 16S rRNA and atpD gene sequences of the bacteria isolates were analysed with SeqBuilder MegAlign and Chromas pro programs. The chromatograms peaks were viewed and edited by comparing the forward to the reverse sequences. The edited sequences were compared to the sequences in the public database with Basic Local Alignment Search Tool (BLAST) on the NCBI website to find closely related bacterial 16S rRNA/ atpD gene sequence and the results were recorded with their corresponding percentage similarities to identify the bacteria. The sequences of the corresponding similarities were further downloaded and compared with sequences of type strains from GeneBank in BioNumerics 7.0 program for pairwise alignment of the atpD/16S rRNA cluster analysis of fast algorithm.

The raw metagenomics data analysis was conducted online on BaseSpace Sequence Hub using the 16S Metagenomics Application (Illumina, Inc., USA). This application is based on the Ribosomal Database Project (RDP) Classifier published by Wang et al.(2007), where the reads are assigned to a modified version of the GreenGenes May 2013 reference taxonomy database.

The draft genome sequence of *Lb. fermentum* BFE 6620 was template-based assembled *de novo* using SPAdes version 3.10.1 (Bankevich et al., 2012). The genome sequence was annotated using the Rapid Annotations Subsystems Technology (RAST) (Aziz et al., 2008) and the NCBI server.

The results for the chemical analyses were given as the means and standard deviation of three independent measurements. The effect of fermentation and solar drying on nightshade leaves was tested using a one-way analysis of variance (Sigma plot version 13.0, Systat software Inc. Munich, Germany) at significance level of $p<0.05$ was performed for post hoc comparison using Tukey's

test. Sensory results were analysed with SPSS version 21 using Pearson Chi-square and means at p = 0.05.

3.0 Results

3.1 Selection of appropriate starter culture strains for optimal nightshade fermentation

To establish the best fermentation conditions, nightshade leaves were fermented with different LAB starter cultures, sugar, and salt concentrations. These LAB were previously isolated from fermented African food products. The first batch was fermented in only 3 % salt, while the other batch was in 3 % sugar and 3 % salt solution in 50 ml Schott bottles for 144 h. The uninoculated batch served as a control. Figure 3.15 illustrates the influence of varying salt or salt-sugar concentrations and the starter cultures used on the pH profile of the fermented nightshade leaves.

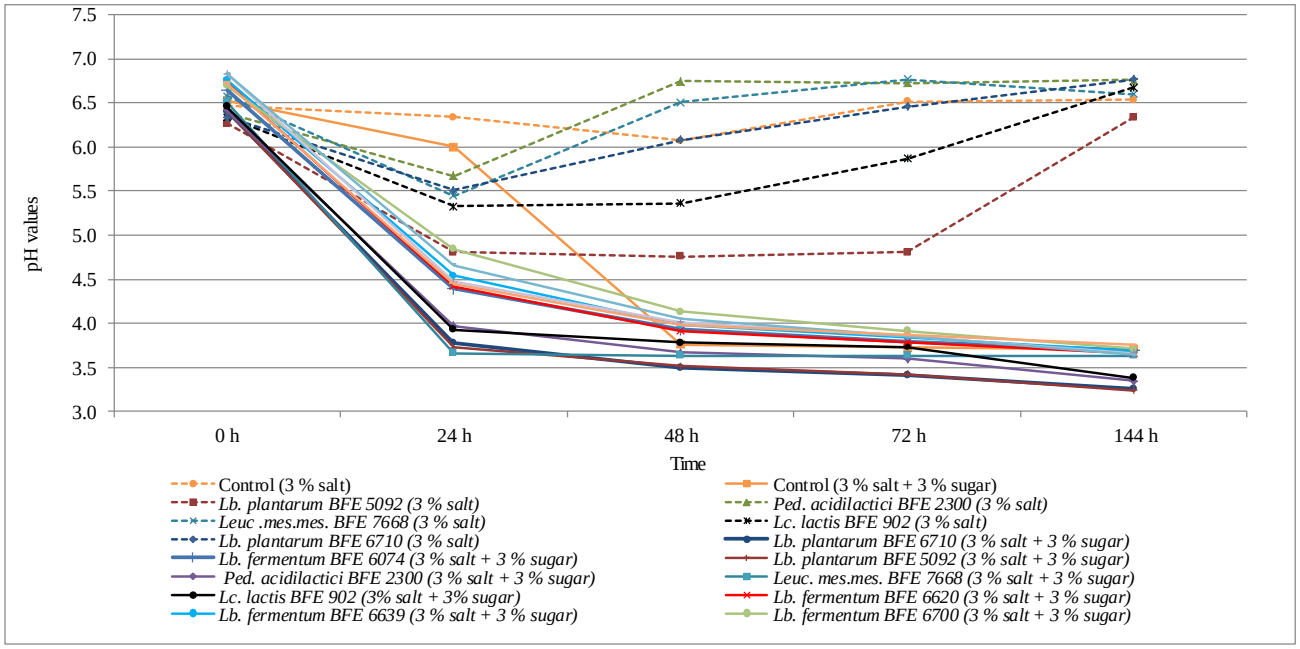

Figure 3.15: Influence of different starter culture strains and sugar/ salt solution on pH development in nightshade fermentation in 50 ml Schott bottles.

The results showed that at the beginning of the fermentation, the initial pH values in all batches ranged from 6.2 to 6.8. The inoculated batches with different starter strains and a combination of 3 % salt and 3 % sugar solution, showed a rapid decrease in the pH below 5.0 within 24 h. After 48 h, the control batch with sugar-salt solution had reached a pH below 4.0 (Fig.3.15). Therefore, the use of starter cultures in combination with sugar-salt solution, showed a rapid and deep drop in the pH throughout the fermentation time. After 144 h of fermentation, all batches fermented with sugar and salt, including the control batch, showed pH values below 3.8. The use of starter cultures resulted in

a stable and more controlled fermentation. The inoculated batches with 3 % salt solution, to which no sugar was added, showed varying results after 48 h. The lowest pH values reached only 4.76 to 6.75. Moreover, after 144 h of fermentation in 3 % salt solution, there was no clear pH trend, since the final pH values of fermentations were often higher than the initial pH, i.e. the pH increased rather than decreased (Fig.3.15). Therefore, the study showed that fermentation of nightshade leaves with starter cultures in conjunction with 3 % sugar-salt solution results in a faster and more reliable and stable reduction of the pH.

3.2 Fermentation of nightshade leaves with a combination of different starter culture strains and sugar/salt solution

To determine the best combinations of fermentation brine sugar/salt combinations and starter culture strains, a small-scale fermentation was performed with the homofermentative strain *Lactobacillus plantarum* BFE 5092 together with a heterofermentative strain, either *Lactobacillus fermentum* BFE 6620 or *Weissella paramesenteroides* LC11, which were selected based on their success for reducing the pH of nightshade fermentations in the experiments described above. The fermentations were performed in 100 ml Schott bottles in either each 3.0 % salt-sugar combination, each 2.5 % salt- sugar combination or only 2.5 % salt solution. The pH was determined after 0-144 h.

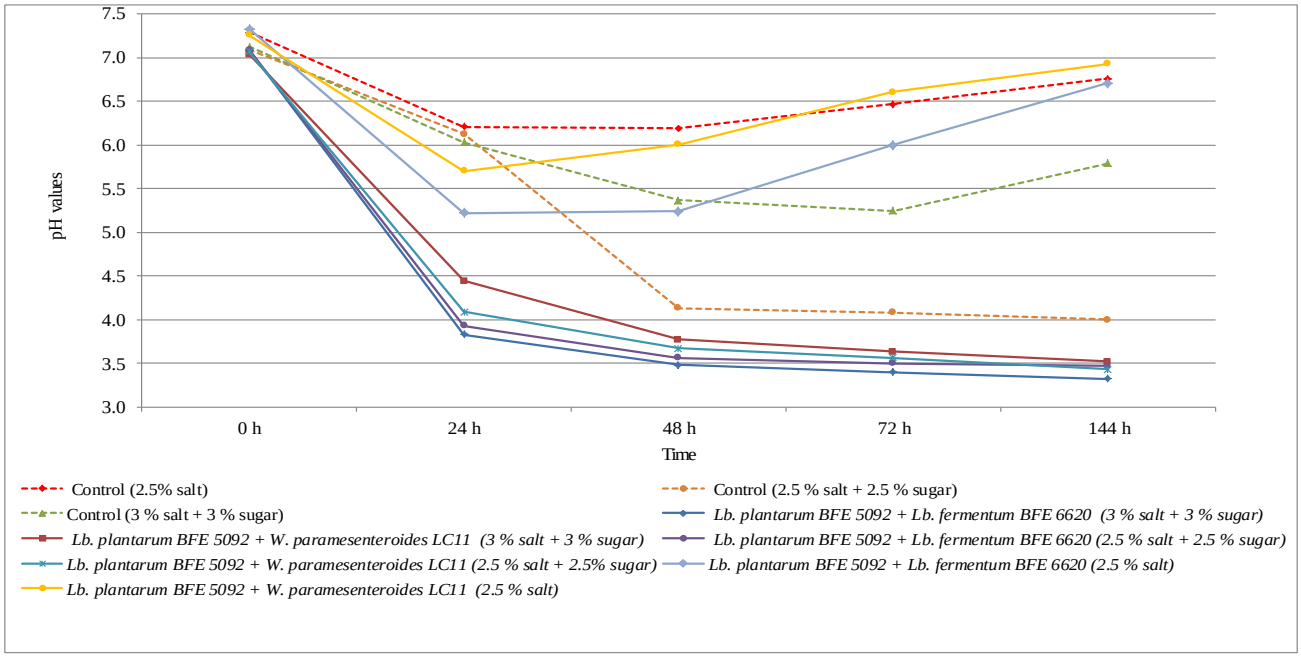

Figure 3.16: Influence of combined starter culture strains and sugar/ salt solution on pH development in nightshade fermentation in 100 ml Schott bottles.

Figure 3.16 shows that the combinations of *Lb. plantarum* BFE 5092 and *Lb. fermentum* BFE 6620 in both 2.5 % sugar-salt solution and 3.0 % sugar-salt solution showed the best result, as they dropped the pH below 4.0 within 24 h, and after 144 h of fermentation both batches had a pH below 3.5. However, a combination of *Lb. plantarum* BFE 5092 and *Lb. fermentum* BFE 6620 with 3.0 % sugar-salt solution, showed the deepest drop in the pH when compared to the other batches, and it showed the lowest pH value of 3.3 after 144 h fermentation, while the other batches had similar, but slightly higher lowest pH values, ranging from 3.4 to 3.5. LAB counts were higher with 3.0 % salt-sugar solution than with 2.5 % salt-sugar solution, there was also a complete inhibition of enterobacteria and yeast and moulds when using the 3 % salt-sugar solution. Thus, these conditions were chosen for subsequent fermentations of nightshade leaves. Therefore, the study showed that fermentation of nightshade leaves with a combination of *Lb. plantarum* BFE 5092 and *Lb. fermentum* BFE 6620 as starter cultures, in the presence of 3 % sugar-salt solution, resulted in a fast and deep reduction of the pH.

3.3 Nightshade fermentations in 5 litre pots at the MRI in Karlsruhe, Germany and at the JKUAT, Kenya, with the selected starter cultures *Lb. plantarum* BFE 5092 and *Lb. fermentum* BFE 6620

As described in section 3.2, the *Lb. plantarum* BFE 5092 and *Lb. fermentum* BFE 6620 starter culture strains were selected and used in subsequent fermentations as a two strain starter culture combination. Additionally, the 3.0 % sugar and 3.0 % salt solution was selected as fermentation brine for all further fermentations. The nightshade leaves were fermented in 5 litres fermentation crocks/buckets as described in section 2.2.3. The enumeration of bacteria and moulds were done on MRS agar, VRBD/G agar, Std.I agar and MG+A/PDA agar media, respectively, as described in section 2.2.4. The pH was determined during the entire fermentation period of 144 h. The influence of starter cultures on fermentation was investigated by formation of lactate and sucrose/D-glucose utilisation

3.4 Microbial enumeration from nightshade fermentation in 5 L pots at MRI, Karlsrune, Germany and JKUAT, Kenya

At the beginning of the fermentation, the mean LAB counts at MRI in the starter culture inoculated batches were ca. 1 x 10^7 cfu/ml. The mean count increased to about 10^8 cfu/ml after 24 h and

remained at this high level until 72 h after which it gradually decreased to 10^7 cfu/ml after 144 h of fermentation, the count was only slightly higher than the initial mean count at 0 h (Fig.3.17). The JKUAT starter culture inoculated batch had a similar trend compared to the MRI fermentation experiments. However, after 144 h of fermentation the count was higher as compared to those fermented at MRI (Fig.3.17).

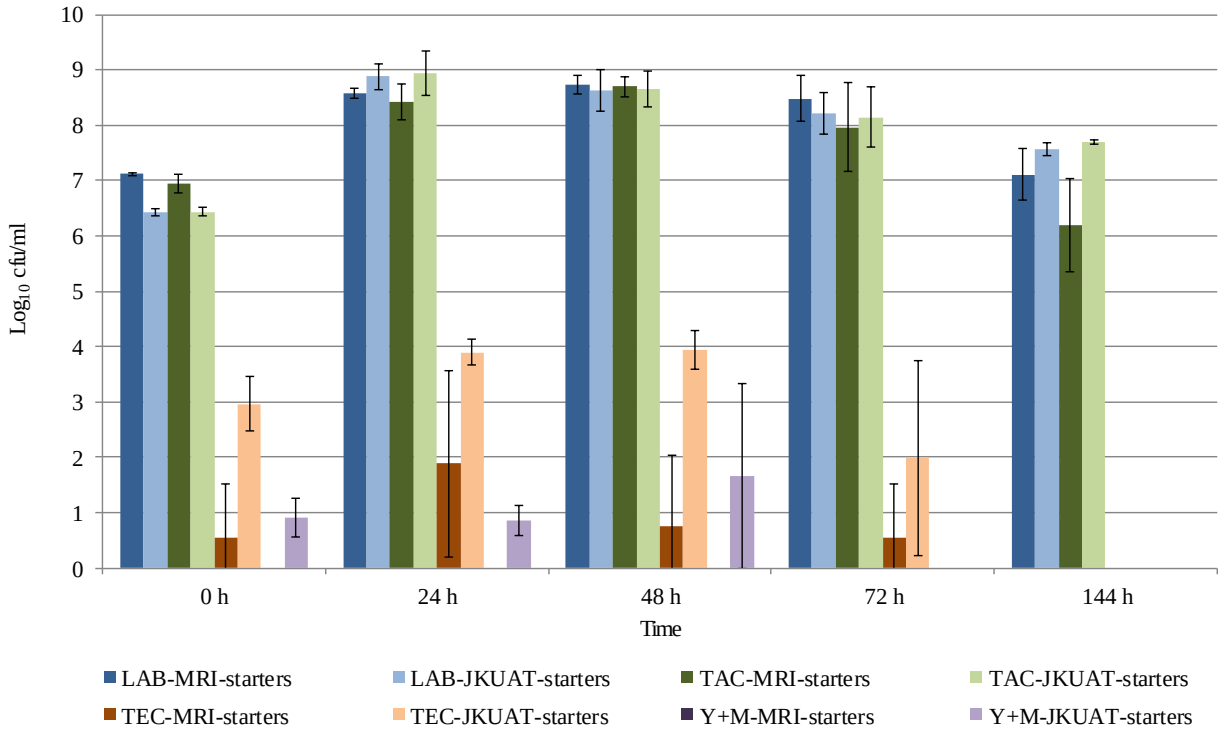

Figure 3.17: The mean lactic acid bacteria (LAB) counts, total aerobic mesophilic plate counts (TAC), total enterobacterial count (TEC) and yeast and moulds (Y+M) counts from (3 MRI, Karlsruhe, Germany and 4 JKUAT, Kenya) independent nightshade fermentation experiments inoculated with *Lb. plantarum* BFE 5092 and *Lb. fermentum* BFE 6620 for 144 h in 5 L crocks/buckets. The standard deviation is indicated.

In the control batches, the mean LAB count was about 10^1 cfu/ml (MRI) and 10^2 cfu/ml (JKUAT) after 0 h (Fig.3.18). However, after 24 h, the mean count increased to ca. 10^6 for MRI and JKUAT fermentations (Fig.3.18). Maximum growth of almost 10^9 cfu/ml was observed after 48 h for JKUAT and 10^7 cfu/ml for MRI fermentations (Fig.3.18). At 48 h the mean lactic acid bacterial count for JKUAT fermentations was comparable to those determined for the starter culture inoculated batches at the same sampling time (Fig.3.17). After 144 h, the mean LAB counts in the control were similar to those determined for the starter-inoculated batches at approximately 10^7

cfu/ml for MRI fermentation but were slightly higher for JKUAT fermentations at almost ca.10^8 cfu/ml (Fig.3.18).

The results in figure 3.17 shows that the mean total aerobic mesophilic plate count on standard I agar (Std.I) inoculated with starter culture for fermentation experiments done at MRI, Germany and JKUAT, Kenya, was similar to the mean LAB count**,** suggesting that the inoculated LAB starter cultures most likely constituted the highest number of growing bacteria on Std.I agar plates. However, in the control (uninoculated with starter cultures), the mean total aerobic count increased from 10^3 cfu/ml at 0 h to almost ca. 10^8 cfu/ml after 24 h for both the MRI and JKUAT fermentations. It remained at this high level for up to 72 h and then slightly decreased, reaching approximately 10^7 cfu/ml after 144 h for MRI fermentations. However, for the JKUAT fermentations, the count remained at this high level reaching ca. 10^8 cfu/ml after 144 h of fermentation (Fig.3.18) Therefore, the growth of total aerobic mesophilic counts from the control did not reflect that of the LAB starter cultures, and was approx. 1 log higher, hence the bacteria that grew on Std.I agar plates presumably were not only LAB, but also other bacteria.

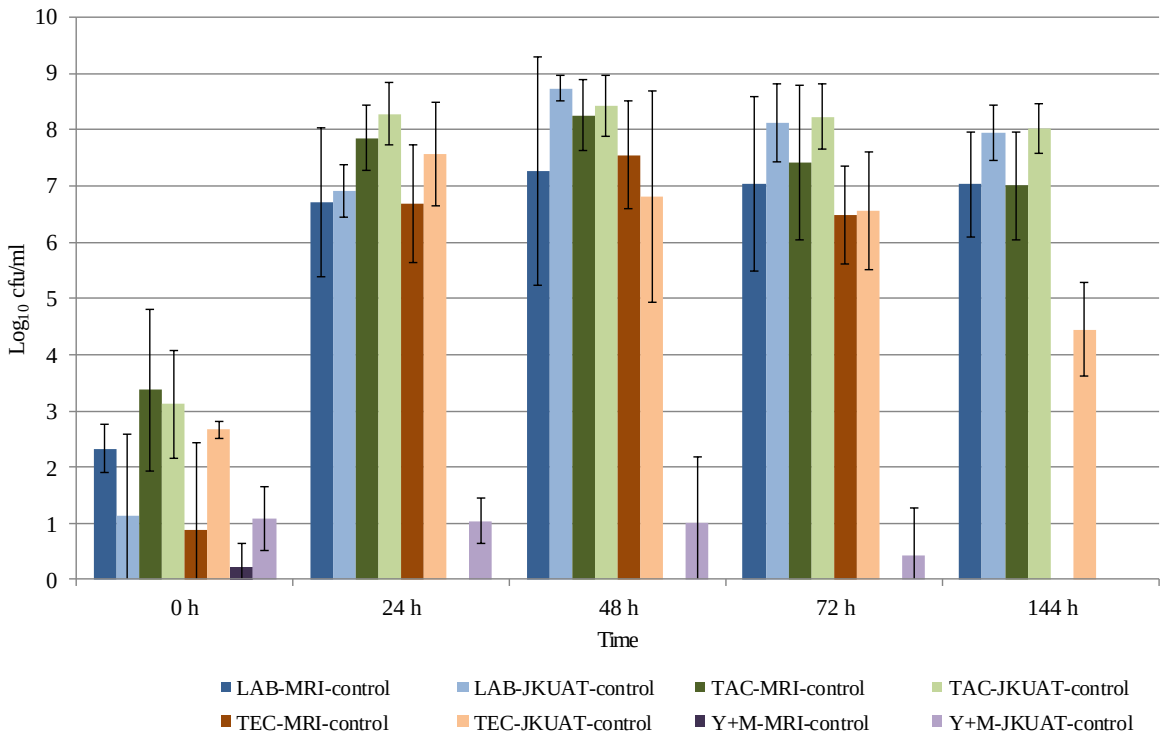

Figure 3.18: The mean lactic acid bacteria (LAB) counts, total aerobic mesophilic plate counts (TAC), total enterobacterial (TEC) counts and yeast and moulds (Y+M) counts from (3 MRI, Karlsruhe, Germany and 4 JKUAT, Kenya) independent nightshade fermentation experiments which were not inoculated with starter cultures (control) for 144 h in 5 L crocks/buckets. The standard deviation is indicated.

The mean enterobacterial count from the starter culture inoculated fermentation batches from MRI, was < 1 log cfu/ml at 0 h, which slightly increased to log 1.9 cfu/ml after 24 h and then gradually decreased below log 1.0 cfu/ml, until eventually no enterobacteria could be detected after 144 h of fermentation (Fig.3.17). However, for the JKUAT, Kenya starter culture inoculated batches, the mean enterobacterial count after 0 h was log 3.0 cfu/ml, which increased to log 3.9 cfu/ml after 24 h and remained at this level up to 72 h then decreased to ca. log 2.0 cfu/ml, until eventually enterobacteria could no longer be detected after 144 h (Fig.3.17).

In the control (uninoculated with starter culture) fermentations done at MRI the mean enterobacteria count increased from log 0.9 cfu/ml after 0 h to log 6.7 cfu/ml after 24 h. However, in this control fermentation the mean enterobacterial count increased further to log 7.5 cfu/ml after 48 h, before falling to log 6.5 cfu/ml after 72 h fermentation. Enterobacteria could not be detected after 144 h (Fig.3.18). For the JKUAT control fermentation batches, there was a higher enterobacterial count as compared to the one above. The mean enterobacterial count increased from log 2.7 cfu/ml after 0 h to log 7.1 cfu/ml after 24 h. However, the count decreased noticeably to log 6.2 cfu/ml after 48 h and remained at this level until 72 h, and finally reached a count of log 4.4 cfu/ml after 144 h (Fig.3.18).

The result also showed no yeast and moulds were detected from the starter culture inoculated batches for fermentation experiments done at MRI, Germany (Fig.3.17). However, in the control batches, the yeasts and moulds were detected at low numbers of below log 1.0 <cfu/ml (Fig.3.18). In the JKUAT fermentation batches, the results showed that the yeast and moulds were detected both in inoculated starters and uninoculated batches after 48h for inoculated batches and 72 h for the control (Fig.3.18).

3.5 Determination of pH, lactate and sucrose/D-glucose concentration from nightshade fermentation in 5 litres pots

The pH development during nightshade fermentation was used to determine the progress of the fermentation which was either inoculated with starter bacteria, or which was left to ferment spontaneously.

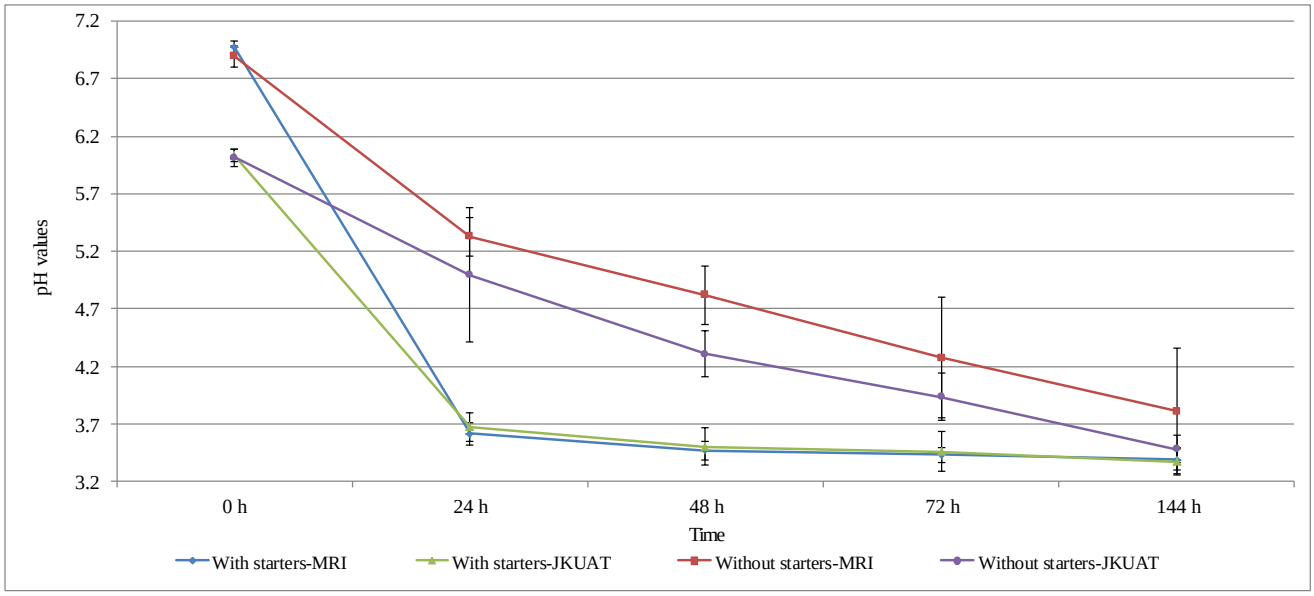

Figure 3.19: The development of pH from (3 MRI, Karlsruhe, Germany and 4 JKUAT, Kenya) independent nightshade fermentation experiments in 5 L pots with starter cultures combination *Lb. plantarum* BFE 5092 and *Lb. fermentum* BFE 6620 and without starter bacteria (control).

The figure above shows that at the beginning of the fermentation both at MRI (Karlsruhe, Germany) and JKUAT (Nairobi, Kenya), mean pH values of ca. 7.0 and 6.0 could be measured, respectively. However, after 24 h, the starter inoculated batch showed a sharp decrease in the mean pH to 3.6, which remained stable until 144 h, where the final pH was ca. 3.34 (Fig.3.19**)**. In contrast, the control fermentation showed only a slow reduction of the pH when compared to the starter-inoculated batch. The JKUAT fermentation showed a faster pH reduction as compared to one performed at MRI, with the mean pH was below pH 5.0 only after 24 h while the MRI fermentation, the was below 5.0 only after 48 h and from there, there was only a slow and gradual further decrease, to reach pH 3.8 and 3.4 after 144 h of fermentation for MRI and JKUAT respectively (Fig.3.19). Therefore, the results showed that the use of starter cultures led to a faster, deeper and stable pH reduction during the nightshade fermentations.

The progress of fermentation was determined by measuring lactic acid accumulation as described in section 2.15.1 during fermentation of both the starter culture inoculated *Lb. plantarum* BFE 5092 + *Lb. fermentum* BFE 6620 fermentation and the control batches during 144 h fermentation at the MRI, Karlsruhe, Germany and JKUAT, Kenya. While the total titratable acid was measured by titration as described in sections 2.15.3. Utilization of sucrose/D-glucose was determined as described in section 2.15.2.

The study showed that at 0 h, in both the starter culture inoculated and control (uninoculated) batches from MRI and JKUAT almost no lactate could be detected. The starter inoculated batch displayed a faster increase in the lactate concentration during fermentation when compared to the control. After 24 h, the starter inoculated batches had 1.8 g/l lactate (MRI) and 1.9 g/l lactate (JKUAT). The concentration of lactate increased steadily to 3.2 g/l lactate after 48 h both for MRI and JKUAT fermentations and finally reached 5.2 g/l and 4.0 g/l lactate concentrations for MRI and JKUAT fermentations, respectively (Fig.3.20a). The enzyme assay kit for the determination of lactate agreed with the total titratable acid data (Fig.3.20b). Spontaneous fermentation had only a slow increase in lactate concentration, recording only 0.3 g/l (MRI) and 1.4 g/l (JKUAT) after 48 h. However, after 72 h the lactate concentration increased noticeably to ca 2.0 g/l in both MRI and JKUAT fermentations and further increased to 4.4 g/l and 4.6 g/l after 144 h of the fermentation for MRI and JKUAT batches (Fig.3.20a).

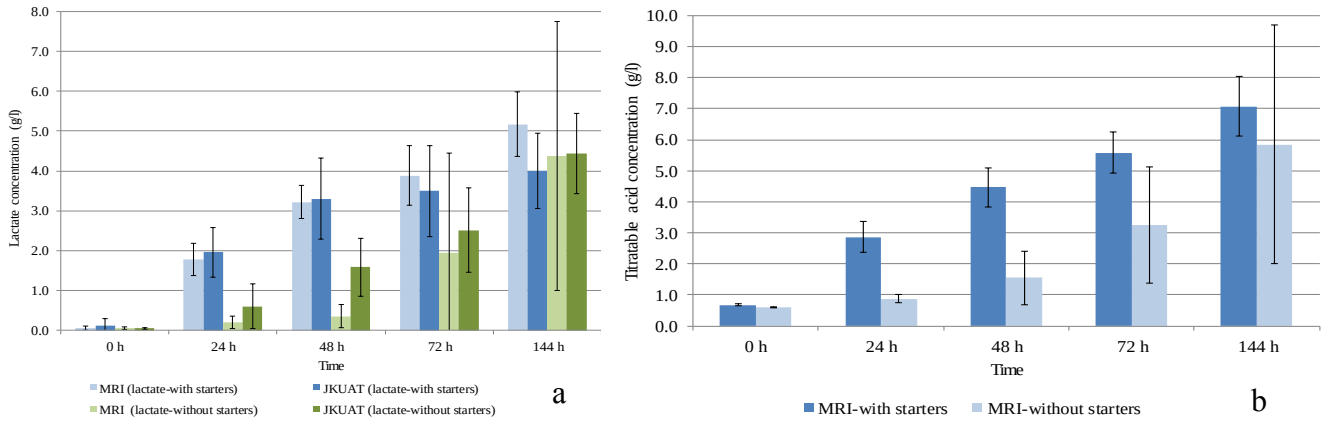

Figure 3.20: Development of mean lactate concentration derived from 3 (MRI, Karlsruhe, Germany) and 4 (JKUAT, Kenya) independent experiments of nightshade fermentations done either with starters *Lb. plantarum* BFE 5092 and *Lb. fermentum* BFE 6620 or without starter culture determined with either UV-method lactate determination kit (a) or determined by titration (b). Total titratable acid was not determined in JKUAT fermentations.

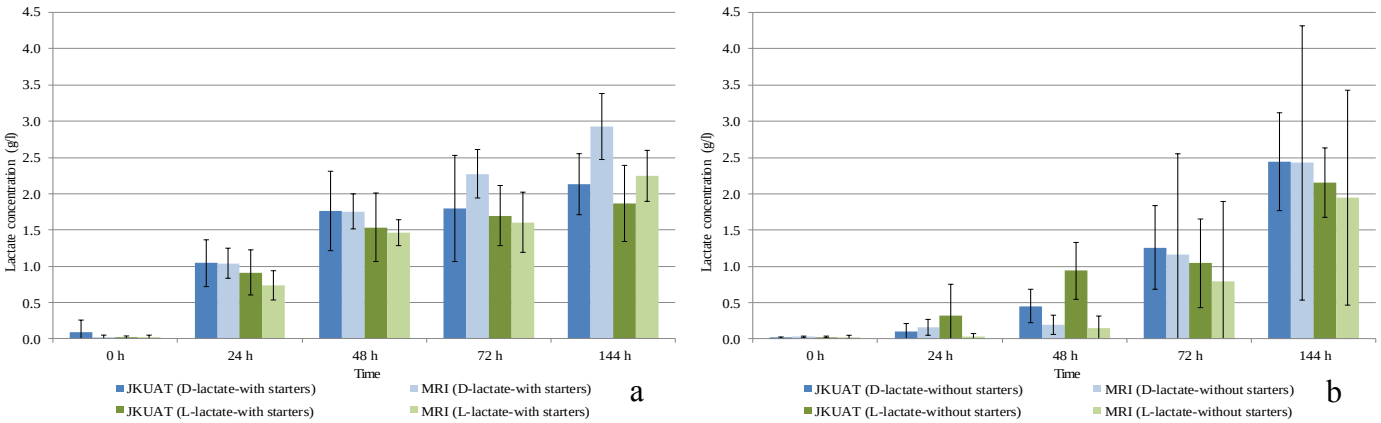

Figure 3.21: Mean concentration of lactic acid (D and L isomers, separately) determined from (3 MRI, Karlsruhe, Germany and 4 JKUAT, Kenya) independent nightshade fermentation experiments done either with starter cultures *Lb. plantarum* BFE 5092 and *Lb. fermentum* BFE 6620 (a) or without starter cultures (b) for 144 h .in 5 L pots. The standard deviation is indicated.

Determination of D- and L- stereoisomers of lactic acid in the batches inoculated with starter cultures had similar concentrations of D- and L- lactic acid for both MRI and JKUAT fermentations, respectively (Fig.3.21a). D- lactic acid was slightly higher ranging between 1.0 g/l to 2.9 g/l, while L-lactate ranged between 0.7 g/l to 2.2 g/l after 24 h to 144 h. MRI fermentations had higher D- lactate after 72 h to 144 h than JKUAT, while L- lactate was higher in the JKUAT fermentations at 24-72 h than those at MRI (Fig.3.21a). The control fermentation showed a similar trend as the fermentation inoculated with starter cultures with amounts of D- and L- lactate increasing after 24 h to 144 h of fermentation. D- lactate ranged between 0.1 g/l to 2.4 g/l, while L-lactate ranged between 0.04 g/l to 2.2 g/l (Fig.3.21b). In the JKUAT fermentations, L- lactic acid was higher than D- lactic acid at 24 h and 48 h (Fig.3.21b).

The mean sucrose /D-glucose concentration of nightshade fermentations inoculated with starter cultures done both at the MRI and JKUAT were almost in the same range of about 28.0 g/l at 0 h. The starter culture inoculated batches showed a noticeably faster sucrose /D-glucose metabolism when compared to their respective uninoculated batches, where after 24 h the concentration of sucrose/D-glucose were 10.1 g/l and 17.9 g/l for JKUAT and MRI fermentation respectively. It further decreased to 2.6 g/l and 10.4 after 72 h for JKUAT and MRI fermentation respectively. After 144 h of fermentation, the sucrose/D-glucose concentration was 1.0 g/l and 7.1 g/l for JKUAT and MRI experiments done with starter culture respectively (Fig.3.22). Nightshade fermentation at

JKUAT showed a faster sucrose/D-glucose decrease than the fermentations done at the MRI.

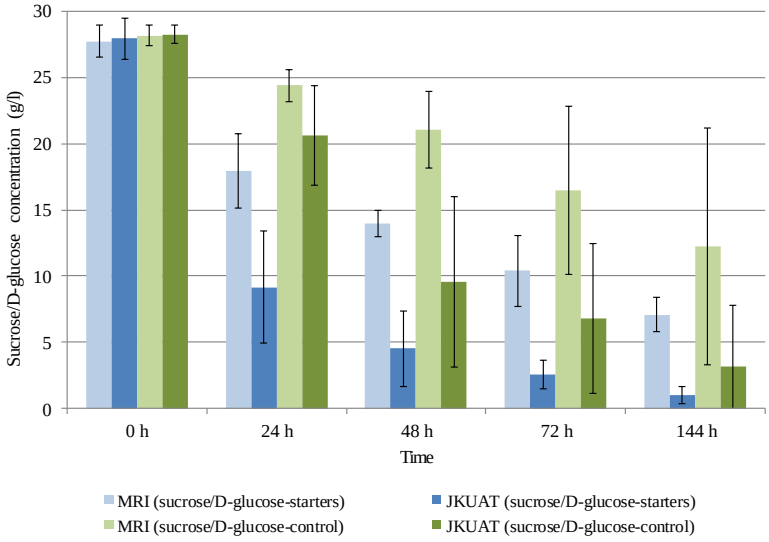

Figure 3.22: Sucrose/D-glucose concentration from (3 MRI, Karlsruhe, Germany and 4 JKUAT, Kenya) independent nightshade fermentation experiments done with either starter cultures *Lb. plantarum* BFE 5092 and *Lb. fermentum* BFE 6620 or without starter cultures at MRI, Germany and JKUAT, Kenya for 144 h in 5 L pots. The standard deviation is indicated.

In the control or uninoculated batches, sucrose/D-glucose metabolism was slow and after 24 h the concentration was 19.8 g/l and 24.4 g/l for JKUAT and MRI fermentations, which dropped to 21. 6 g/l, 21.1 g/l, 16.5 g/l and 12.2 g/l after 48 h, 72 h, and 144 h for MRI and 10.9 g/l, 8.6 g/l and 4.2 g/l after 48 h, 72 h and 144 h for JKUAT fermentations, respectively (Fig.3.22).

3.6 Challenge tests for determination of the ability of selected lactic acid bacteria to inhibit pathogenic bacteria in nightshade fermentations

Challenge tests were done to determine the ability of the selected *Lb. plantarum* BFE 5092 and *Lb. fermentum* BFE 6620 starter cultures to inhibit the growth of the pathogenic bacteria *Salmonella* Enteritidis S489 and *Listeria monocytogenes* SLCC 8210 serovar 1/2a. The fermentation of nightshade leaves was performed in 1000 ml beakers with 3 % sugar and 3 % salt solution (section 2.2.5). The experiment was performed in four batches, the first batch served as a control (no starters or pathogens were inoculated), the second batch was inoculated with the starter cultures *Lb. plantarum* BFE 5092 and *Lb. fermentum* BFE 6620 at 10^7 cfu/ml, while in the third batch starter cultures were inoculated at 10^7 cfu/ml together with the 2 pathogenic bacteria *Salmonella* Enteritidis S489 and *Listeria monocytogenes* SLCC 8210 each at 10^3 cfu/ml and the final batch was inoculated with only pathogens each at 10^3 cfu/ml. The batches were fermented for 144 h at 25 ^oC.

The pH and microbial enumeration was performed during the entire fermentation as described in section 2.2.5.3.

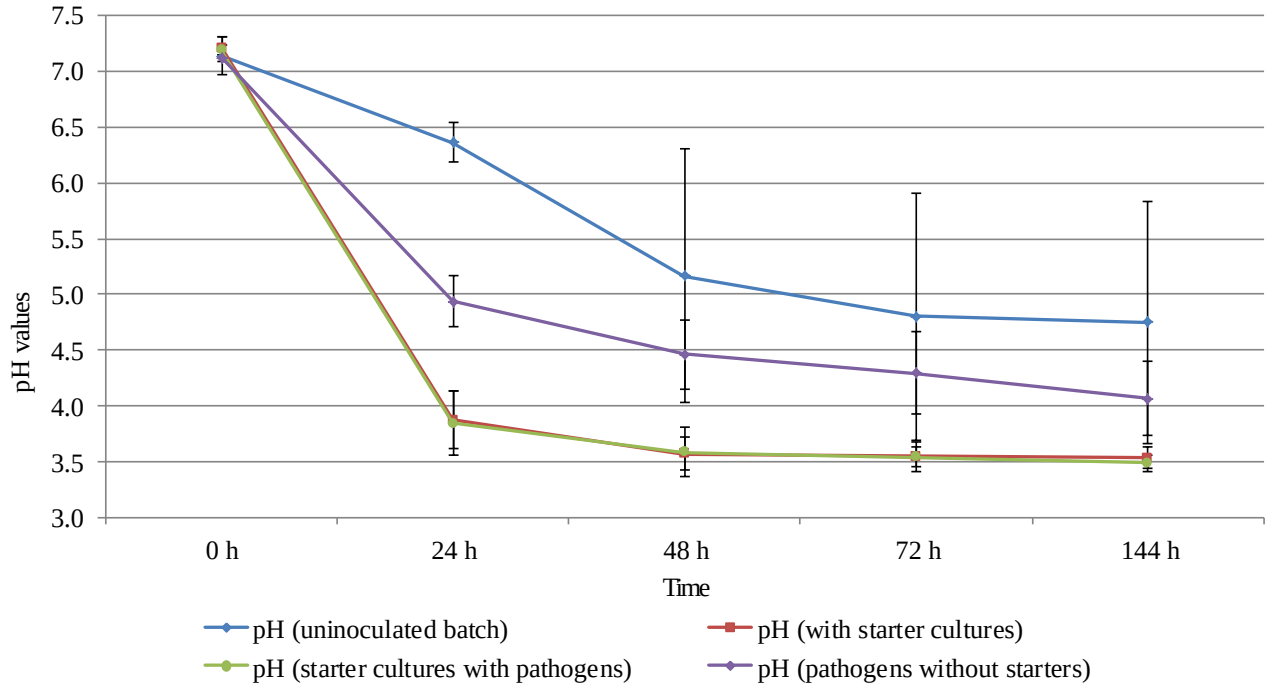

Figure 3.23: Mean pH values from three nightshade fermentation uninoculated (blue line), inoculated only with starter cultures (*Lb. plantarum* BFE 5092 and *Lb. fermentum* BFE 6620 (red line) inoculated with starters cultures in combination with pathogens (green line) and only pathogens without starters (purple line) during 144 h of fermentation in 1000 ml beakers at MRI, Karlsruhe, Germany. The standard deviation is indicated.

The fermentation of the all the batches had an initial pH of ca. 7.1 to 7.2. However, after 24 h of fermentation, the batches inoculated either with only the starter cultures, or inoculated with the starter cultures together with the pathogens, showed a sharp decrease of pH to below 3.9 (Fig.3.23). After 24 h, these pH values decreased further slowly and remained stable after ca. 72 h until 144 h, reaching a final pH of 3.48 for the batches inoculated with starter culture and pathogens, or a final pH of 3.53 (Fig.3.23) for the batches inoculated with only the starter cultures**.** However, in both the control (natural fermentation without starters inoculated) and the batches inoculated only with the pathogens, there was only a relatively slow reduction in the pH. Nevertheless, in the batch inoculated with only the pathogens, the pH still decreased to pH 4.9 and 4.5 after 24 h and 48 h, respectively. For the control batches, which were not inoculated with either starter bacteria or pathogens, the pH decreased only to pH 6.4 after 24 h. As the fermentation progressed, the

pathogens inoculated batch reached a final pH of 4.1 after 144 h, while the control batch without inoculated bacteria reached a final pH of 4.8 (Fig.3.23).

3.6.1 Microbial enumeration during nightshade fermentation in challenge tests with foodborne pathogens

Microbial enumeration was carried out to study the progress of fermentation of nightshade and the growth of the different starter bacteria and pathogens during the fermentation using different selective culturing media and incubation conditions as described in section 2.2.5.3. The fermentation trials were conducted in triplicates on separate occasions from different nightshade harvests. The mean yeast/moulds and bacterial counts from control (natural fermentation) and starter cultures inoculated batches are shown in Fig.3.24.

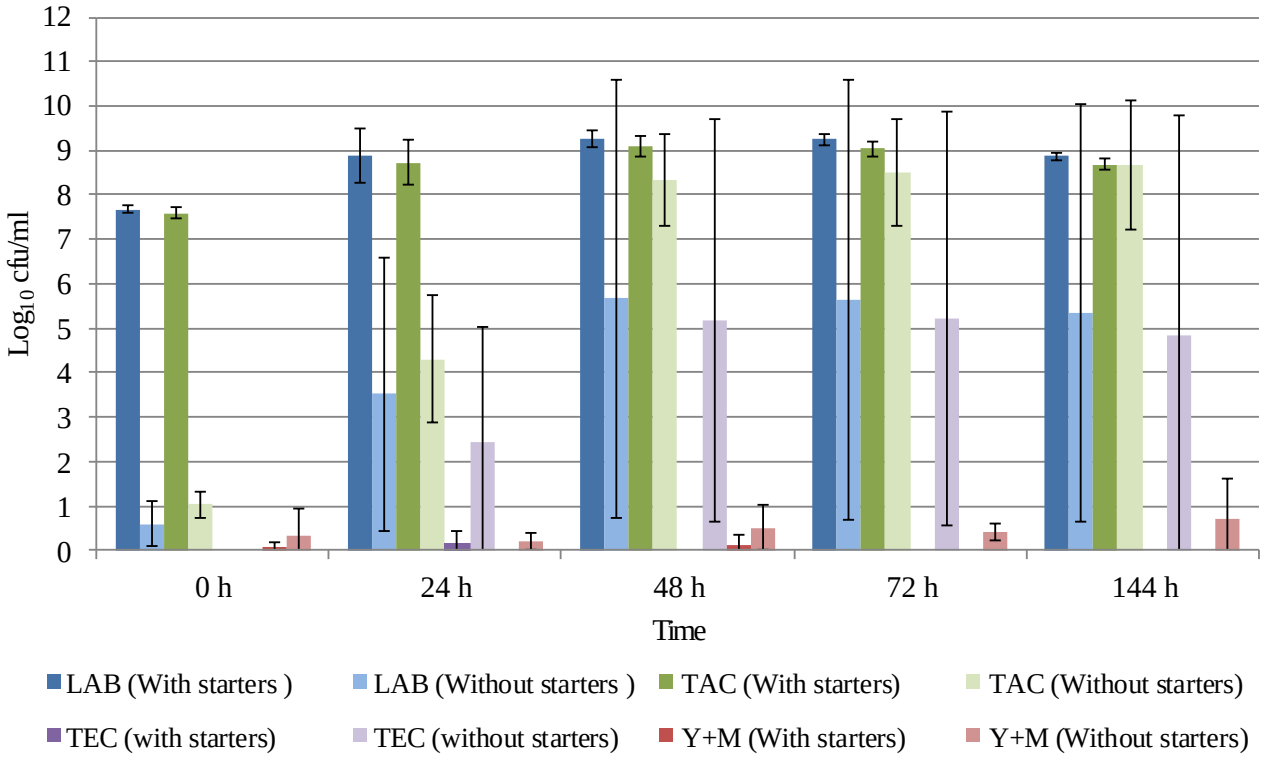

Figure 3.24: The mean lactic acid bacteria (LAB) counts, total aerobic mesophilic plate counts (TAC), total enterobacterial counts (TEC) and yeast and moulds (Y+M) counts from triplicate nightshade fermentations uninoculated with starter cultures (control) and inoculated with *Lb. plantarum* BFE 5092 and *Lb. fermentum* BFE 6620 at MRI, Karlsruhe, Germany for 144 h in 1000 ml beakers. The standard deviation is indicated.

The results showed that at the beginning of the fermentation, the mean LAB count in the starter culture inoculated batches was approximately 10^7 cfu/ml. The count increased rapidly to about 10^9 cfu/ml after 24 h, and remained at this approximate level for up to 144 h (Fig.3.24). In the control batches without inoculated starter bacteria, the mean LAB count was about 10^1 cfu/ml after 0 h, and increased to approximately 10^3 cfu/ml after 24 h. It further increased to over 10^5 cfu/ml and remained in this range until 144 h. The mean total aerobic mesophilic colony counts on Std.I agar from the batches that were inoculated with starter cultures showed almost similar mean counts compared to the LAB counts (Fig.3.24), suggesting that the inoculated LAB starter cultures most likely constituted the highest number of growing bacteria on Std.I agar plates. However, in the uninoculated controls, the mean total aerobic count was about 10^1 cfu/ml after 0 h and increased to about 10^4 cfu/ml after 24 h and reached almost similar high levels of approximately 10^8 cfu/ml after 48 to 144 h fermentation as in the case with the starter inoculated batch (Fig.3.24). The mean counts of enterobacteria from the fermentation batch inoculated with starter culture were below 10^1 cfu/ml after 24 h and no bacteria were detected afterwards. In the uninoculated control batches, the mean enterobacterial count was less than 10^1 cfu/ml at 0 h and the count increased to approximately 10^2 cfu/ml after 24 h, after which it further increased to about 10^5 cfu/m after 48-72 h, before decreasing again to approximately 10^4 cfu/ml after 144 h (Fig.3.24). The mean yeast and moulds count were below 10^1 cfu/ml for all of the fermentation batches.

The mean bacterial counts from the batches with pathogens co-inoculated with starter cultures and control batches inoculated only with pathogens are shown in Figure 3.25. This experiment was performed to determine the role of selected LAB starter cultures to inhibit the growth of pathogenic bacteria such as *L. monocytogenes* and *S.* Enteritidis, thus ensuring the safety of the vegetables. The nightshade fermentation trials were co-inoculated with approximately 1 x 10^7 cfu/ml *Lb. plantarum* BFE 5092 and *Lb. fermentum* BFE 6620 and 1 x 10^3 cfu/ml *Salmonella* Enteritidis and *L. monocytogenes*. The results in figure 3.25 show that at the beginning of the fermentation, the mean LAB count in the fermentation batches containing the starter culture which were co-inoculated with pathogens was approximately 1 x 10^7 cfu/ml. This mean rapidly increased to over 10^9 cfu/ml after 24 h and remained at this high level of ca. 10^9 cfu/ml up to 144 h of fermentation.

In the batch inoculated with pathogens only, the mean LAB count was much lower at about 10^2 cfu/ml after 0 h, and it increased to approximately 10^5 cfu/ml after 24 h and then remained in this range until 144 h of fermentation. The mean total aerobic mesophilic bacterial count on Std.I agar

from the fermentation batches inoculated with both starter cultures and the pathogens showed very similar mean counts when compared to the mean LAB counts (Fig.3.25), suggesting that the inoculated LAB starter cultures most likely were the highest number of bacteria growing on Std.I agar plates. However, in fermentation batches inoculated only with the pathogens, the mean total aerobic, mesophilic bacterial count was ca. 10^3 cfu/ml after 0 h and it increased to about 10^8 cfu/ml after 24 h, and remained at this high level cfu/ml for 48 to 144 h fermentation, and these counts were almost at similar levels as in the case with the fermentations inoculated with starter cultures together with pathogens (Fig.3.25).

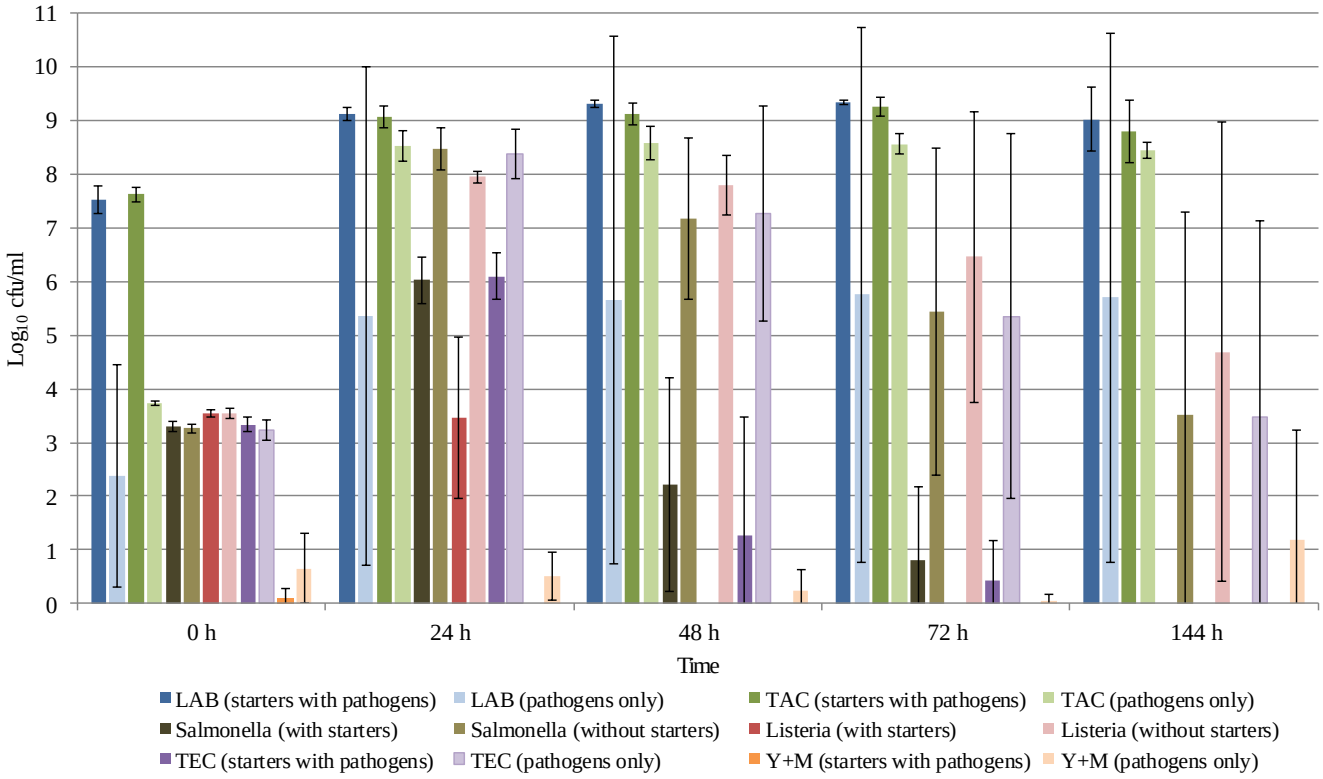

Figure 3.25: Lactic acid bacteria (LAB) counts, total aerobic mesophilic colony counts (TAC), *Salmonella* counts, *Listeria* counts and total enterobacteria counts (TEC) from triplicate nightshade fermentation with pathogens and starter cultures *Lb. plantarum* BFE 5092 and *Lb. fermentum* BFE 6620 at MRI, Karlsruhe, Germany for 144 h in 1000 ml beakers. The standard deviation is indicated.

The results also showed that in the batch with starter cultures co-inoculated with pathogens, the mean number of *Salmonella* Enteritidis increased from approximately 10^3 cfu/ml after 0 h to about 10^6 cfu/ml after 24 h, after which it decreased by 4 logs to about 10^2 cfu/ml after 48 h. It further decreased to log 0.8 cfu/ml after 72 h. After 144 h, no viable cells could be detected either after

directly plating from nightshade fermentation brine or after enrichment (Fig.3.25). However, in the fermentation batch inoculated with pathogens only, the mean *Salmonella* Enteritidis count increased from 10^3 cfu/ml after 0 h to ca. 10^8 cfu/ml after 24 h, it then decreased to ca. 10^7 cfu/ml after 48 h it further reduced to ca. 10^5 cfu/ml after 72 h and reached about 10^3 after 144 h of fermentation (Fig.3.25).

The mean number of *Listeria monocytogenes* from fermentations co-inoculated with starter cultures and pathogens was approximately 10^3 cfu/ml after 0 h and remained at this level for up to 24 h of fermentation. After 48 h, no viable cells were detected from samples plated directly from nightshade fermentation brine, or after enrichment (Fig.3.25). However, the mean *L. monocytogenes* count from fermentations only inoculated with pathogens increased from 10^3 cfu/ml after 0 h to ca. 10^8 cfu/ml after 24 h, remained at this high level up to 72 h, it then decreased to about 10^6 cfu/ml after 72 h, and reached ca. 10^4 cfu/ml after 144 h of fermentation (Fig.3.25). The mean enterobacterial count from fermentation with starter cultures co-inoculated with pathogens almost resembled the mean *Salmonella* Enteritidis count, suggesting that the majority of the bacteria on VRBD agar probably were *Salmonella* Enteritidis. However, the mean enterobacterial count from the batch inoculated with pathogens only increased from 10^3 cfu/ml after 0 h to 10^8 cfu/ml after 24 h, it further decreased by 1 log to 10^7 cfu/ml after 48 h then 10^5 cfu/ml after 72 h and reached 10^3 cfu/ml after 144 h fermentation (Fig.3.25).

3.7 Isolation of genomic DNA from fermentation brine and PCR amplification of 16S rRNA genes for DGGE

Genomic DNA was extracted by the method described by Pitcher et al. (1989) (section 2.2.9.1) from fermentation brine solutions sampled at 0 h, 24 h, 48 h, 72 h and 144 h. The genomic DNA was used for the amplification of the 16S rRNA genes using the universal eubacterial primer pair (338f GC and 514r). The PCR mixture was subjected to a touchdown PCR program and the amplified PCR products were visualized on 1.5 % agarose gel through electrophoretic analysis (section 2.11.1). The differences in eubacteria population among fermentation trials were detected by DGGE analysis. Figure 3.26 below shows an example of the amplified PCR products from nightshade fermentation solution. The starter cultures used in the fermentation were *Lb. plantarum* BFE 5092 and *Lb. fermentum* BFE 6620. The use of 16S rRNA gene primers resulted in a specific band from all samples with the approximate size of 220 bp on a 1.5% agarose gel.

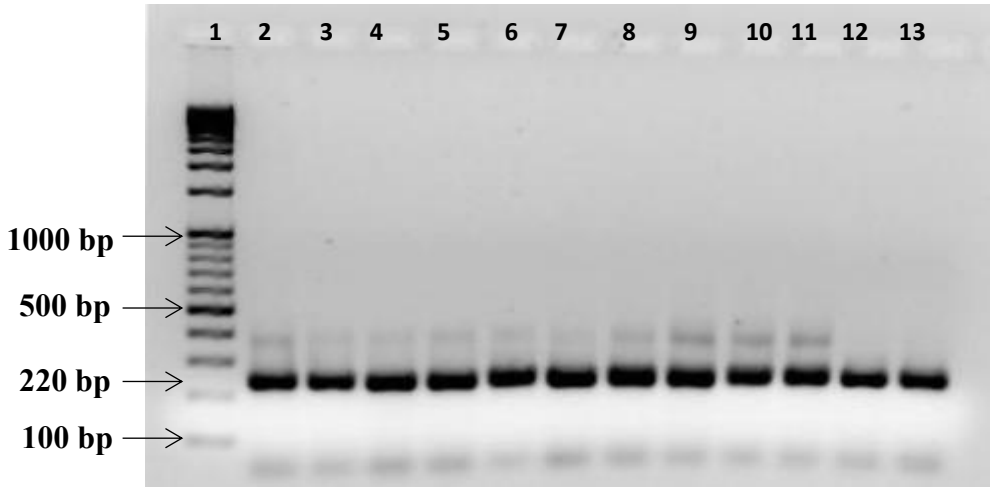

Figure 3.26: The PCR amplified 16S rRNA products from representative samples of nightshade fermentation with starter cultures using eubacteria primer pair 338f GC and 518r for DGGE analysis. Lane 1 (mixture of two DNA markers, 100 bp and 500 bp); 2 (0 h sample); 3 (24 h sample); 4 (48 h sample); 5 (72 h sample); 6 (144 h sample) for fermentation done with starter cultures, while lanes 7 (0 h); 8 (24 h); 9 (48 h); 10 (72 h); 11 (144 h) are control fermentations and lanes 12 (*Lb. fermentum* BFE 6620); lane 13 (*Lb. plantarum* BFE 5092) were from pure starter culture DNA.

3.7 1 Determination of bacterial community composition during nightshade fermentation by DGGE analysis (MRI/JKUAT)

The influence of the inoculated starter cultures on the background microbiota composition during nightshade fermentation was determined by DGGE analysis from fermentation brine solutions sampled at different time points. The amplified 16S rRNA gene fragment PCR products of the fermentation samples were separated on DGGE gels as shown in figures 3.27 and 3.28 below. The band patterns generated from individual samples represented the profiles of microbial communities, while the individual bands represented different 180 bp 16S rRNA gene fragments together with a 40 bp GC-clamp of each of the bacterial strains. The DGGE image profiles were analysed to create a phylogenetic fingerprint profile of complex microbial community during the fermentation. DGGE analysis with universal primer pair 338 GC/518r revealed the differences among various fermentation samples both in number and position as well as in the intensity of the bands.

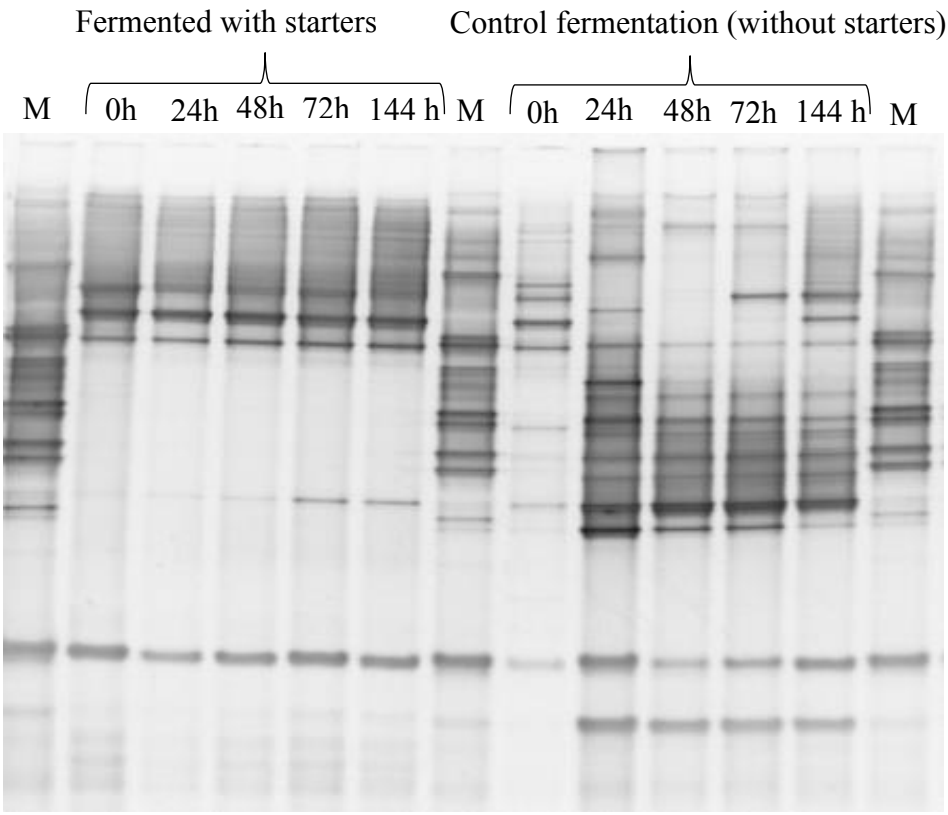

Figure 3.27: DGGE profiles of PCR amplified 16S rRNA gene fragments of batch seven fermented nightshade samples fermented at MRI,Karlsruhe, Germany for 144 h either without starter bacteria, or inoculated with starter bacteria, showing the eubacterial amplicons. The marker was selected from a JKUAT spontaneous/control fermentation obtained at 24 h fermentation.

The DGGE results from figure 3.27 above showed that MRI fermented nightshade with starter cultures, had the same banding patterns at all times from 0 h up to 144 h, which suggested that the use of starter cultures resulted in a stable microbial population. However, in the control fermentation (without starter cultures), less bands were amplified at 0 h and both the number of bands and their intensity increased after 24 h until 144 h fermentation, suggesting that microbial biodiversity increased after 24 h. However, as judging from a visual inspection of the band patterns, there were noticeable changes in the microbial composition at 144 h, which seemed to be different from those at 24 h, 48 h and 72 h of fermentation (Fig.3.27). The DGGE results of the fermentation performed at JKUAT showed that in the fermentation without starter cultures, there were numerous band patterns at 0 h. However, more bands were obtained from 24 h up to 144 h, indicating increased microbial biodiversity in the control fermentation. However, the results show that there were little changes in the microbial composition from 24 h until the end of fermentation (Fig.3.28).

On the other hand, for the starter-inoculated fermentation, 0 h recorded the highest number and diversity of the bands. Fewer band patterns were obtained after 24 h and these remained the same up to 144 h, which clearly showed the influence of the starter culture usage (Fig.3.28).

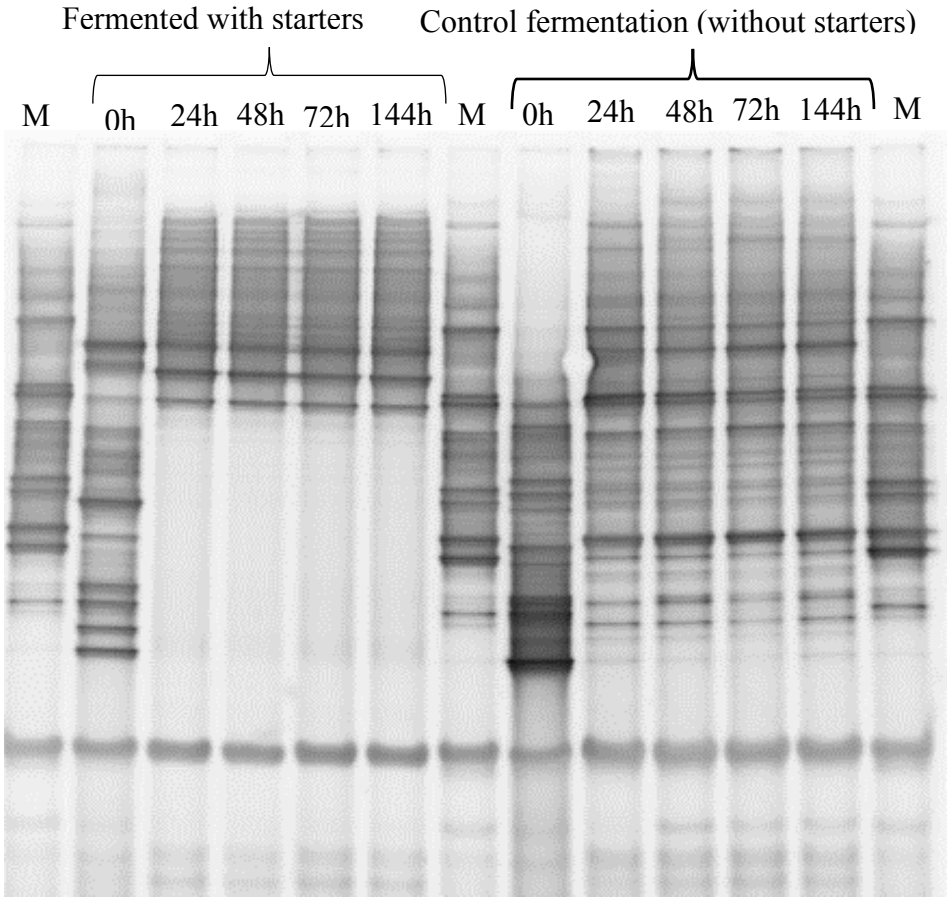

Figure 3.28: DGGE profiles of PCR amplified 16S rRNA gene fragments of batch two fermented nightshade samples fermented at JKUAT, Kenya for 144 h either without starter bacteria, or inoculated with starter bacteria, showing the eubacterial amplicons. The marker was selected from a JKUAT spontaneous/control fermentation obtained at 24 h fermentation.

The DGGE images were further analysed using the BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium), the band patterns of the samples were normalized based on the marker system and were subjected to cluster analysis (section 2.20) as to determine their similarities with each other and thus establish possible relationships. This cluster analysis of DGGE band patterns is shown in figures 3.29 and 3.30 below.

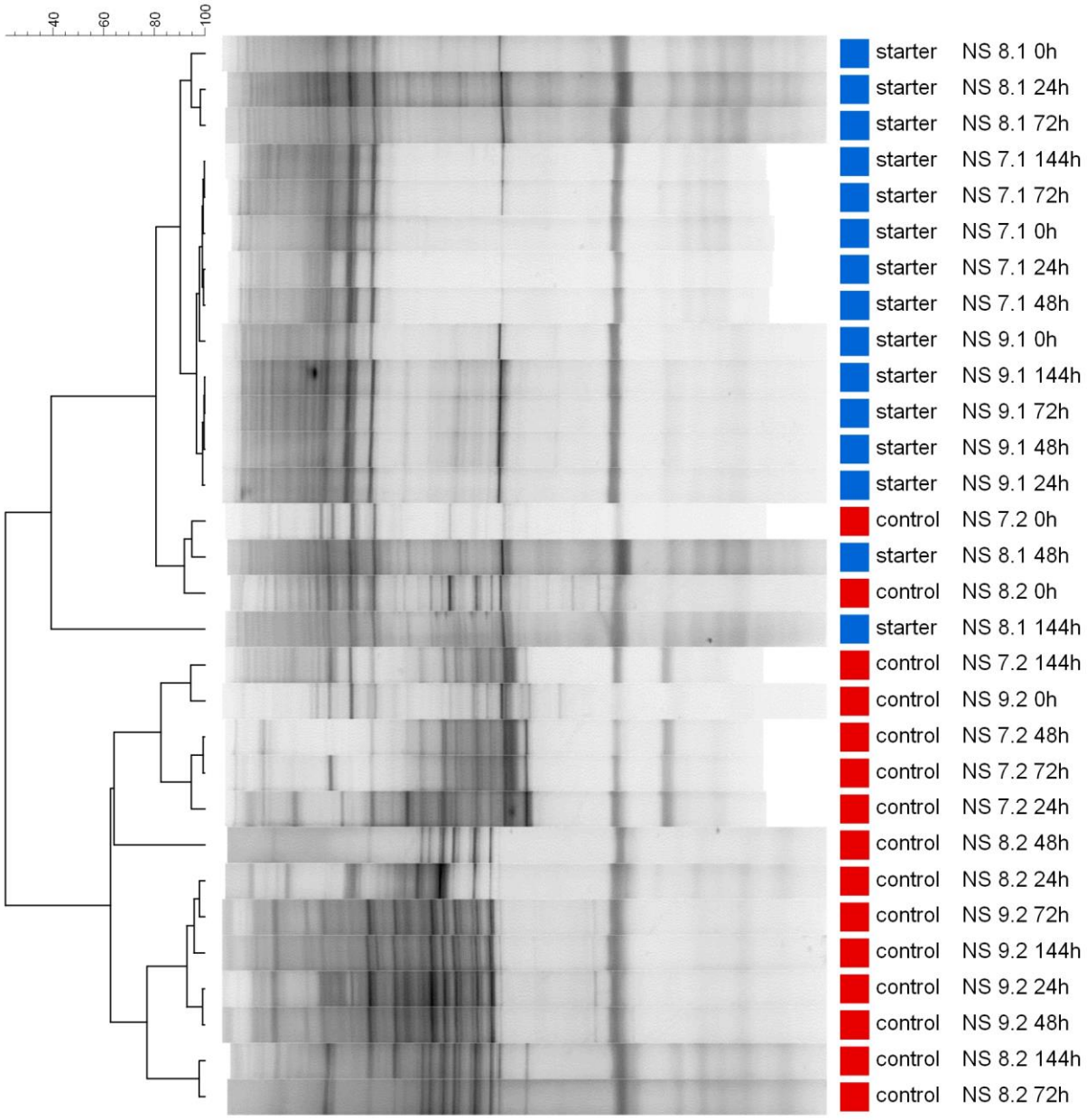

Figure 3.29: Cluster analysis of DGGE profiles of 3 independent nightshade fermentation without starter bacteria (control, red) or inoculated with starter bacteria (starter, blue) with the eubacteria universal primer at MRI, Karlsruhe, Germany.

The DGGE microbial community fingerprint profiles obtained from nightshade fermentation at the MRI clustered into two major groups i.e. those fermented with starter cultures and those stemming from uninoculated/ controls fermented without starters (Fig.3.29). The starter culture inoculated batches showed great similarity to each other at r = 90 %. An inspection of the bands showed same band pattern at sampling point 0 h to 144 h (Fig.3.29). The uninoculated batches were rather diverse

sharing a similarity to each other at *r* = 62 %, physical inspection of the bands showed unique diversity between each fermentations (Fig.3.29).

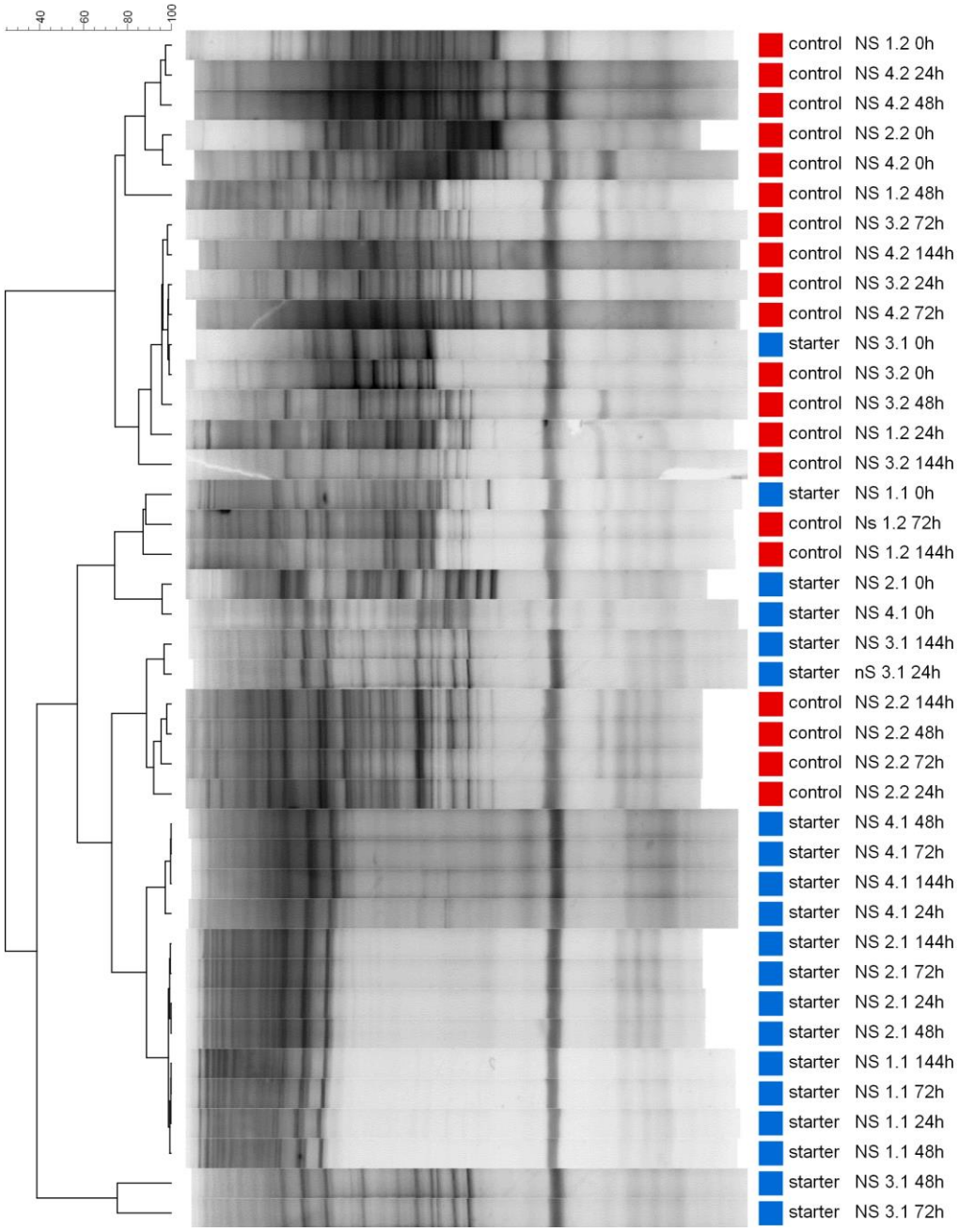

Figure 3.30: Cluster analysis of DGGE profiles of 4 independent nightshade fermentation without starter bacteria or inoculated with starter bacteria with the eubacteria universal primer at JKUAT, Kenya. The blue labelled clusters are starter culture inoculated while the red were uninoculated with starter cultures.

The JKUAT fermentations experiments showed the inoculated batches had higher similarity at *r* = 87 %, however, fermentation one and two with starter cultures showed the same band pattern at

sampling points 24 h up to 144 h, while fermentations three and four had showed diverse band patterns different to those in fermentations 1 and 2 from 24 h up to 144 h (Fig.3.30). However, the uninoculated fermentation two at sampling points 24 up to 144 h were clustered together with the starter-inoculated batch three at sampling points 24 h and 144 h at *r* = 87 % suggesting that at these particular sampling points, the LAB from the control fermentation were probably similar in composition as those from starter inoculated batch and thus dominated the fermentation (Fig.3.30), possibly indicating that the starter culture did not establish itself as the dominant LAB in the fermentation. The control fermentation one at 72 h and 144 h also clustered together with the starter inoculated fermentation one, two and four at sampling points 0 h. The control fermentation showed a unique and diverse band pattern from 0 h to 144 h and the shared a similarity with each other at *r* = 74 % indicating high microbial biodiversity (Fig.3.30). Interestingly, the starter inoculated fermentation three at sampling points 0 h, also clustered together with uninoculated group. This suggested that the inoculated starter cultures had not grown at these particular sampling points or that a high diversity of bacteria was already present, and thus the thus the isolated DNA was likely from the resident, autochthonousbacterial strains.

3.8 Determination of bacterial community composition by metagenomics

MiSeq high throughput amplicon sequencing of 16S rRNA genes was used to determine the relative composition of the bacterial community in the nightshade fermentation. The results show the diversity and composition of microbial communities at the genus level during the nightshade fermentation with or without starter cultures at 0 h, 24 h, 48 h, 72 h and 144 h done both at the MRI and at JKUAT.

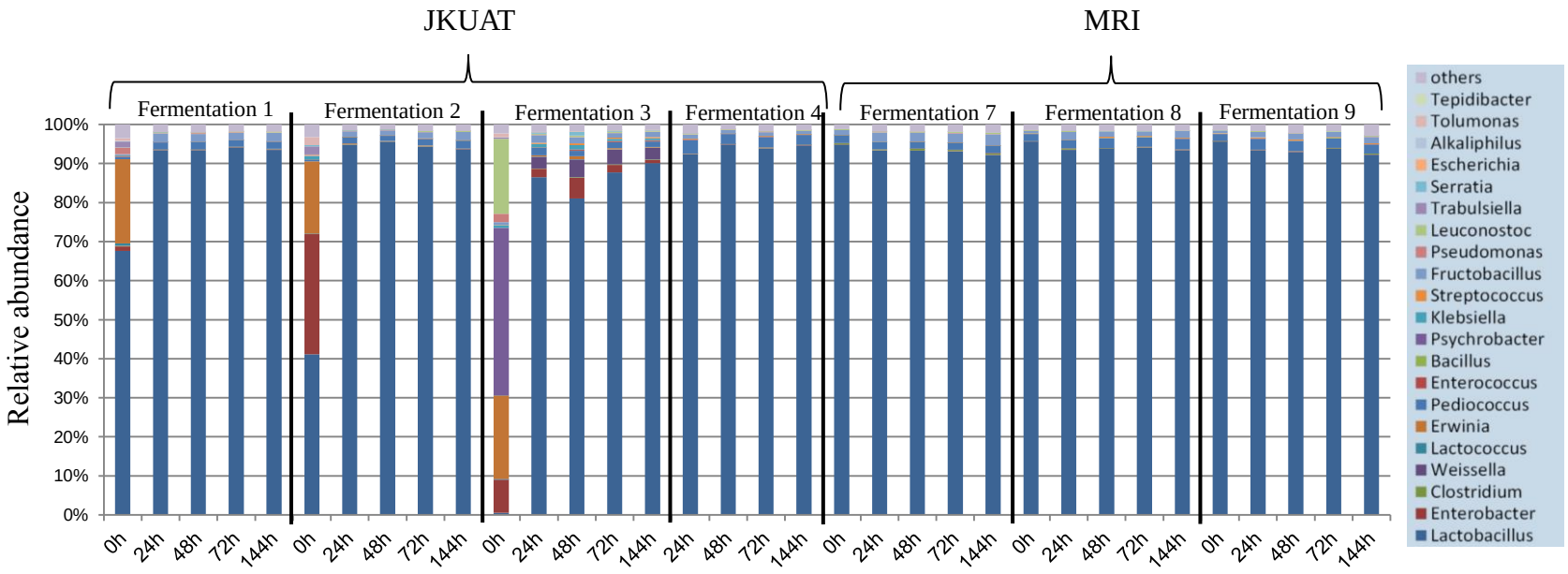

Figure 3.31: Relative abundance of the bacterial community at genus level during nightshade fermentation at JKUAT and MRI with starter culture.

The experiments performed at JKUAT with starter cultures showed that fermentation 1 at 0 h was dominated by the genera *Lactobacillus* (67.7 %), *Erwini*a (21.5 %), *Pseudomonas* (1.5 %), while other lesser genera together constituted 3.5 % of the total bacterial abundance (Fig.3.31). Fermentation 2 with starter cultures at 0 h showed that 41.2 % of the total bacterial community belonged to the genus *Lactobacillus*, *Enterobacter* (30.8 %), *Erwinia* (18.5 %), *Tolumonas* (2.1 %), *Trabulsiella* (2.1 %) and other lesser genera constituted 3.2 % of the total bacterial abundance (Fig.3.31). In the third fermentation the dominant genera at 0 h included *Psychrobacter* with 42.9 %, *Erwinia* (21.3 %), *Leuconostoc* (19.2 %), *Enterobacter* (8.4 %), *Pseudomonas* (2.1 %), *Lactobacillus* (0.6 %), and others (3.2 %) (Fig.3.31).

As the fermentation progressed from 24 h to 144 h, fermentations one, two, three and four were dominated by genus *Lactobacillus*, constituting between 81.1 % to 95.4 % of the total bacterial community, while the genus *Weissella* occurred in fermentation 3 and constitued between 3.0 to 4.5 % of the population. The genus *Pediococcus* occurred in all four fermentation accounting for 1.2 to 3.4 % of the total bacterial abundance (Fig.3.31). In contrast, the starter culture inoculated experiments conducted at MRI showed that all the three fermentation (7, 8 and 9) were dominated by the genus *Lactobacillus* already at 0 h and throughout the fermentation up to 144 h, where this genus constituted between 92.2 % to 95.6 % of the total bacterial abundance. The genus *Fructobacillus* constituted between 1.2 to 2.8 %, the genus *Pediococcus* constituted between 1.7 to 2.8 %, while the lesser genera together contributed between 1.2 % to 2.9 % of the total microbial composition (Fig.3.31).

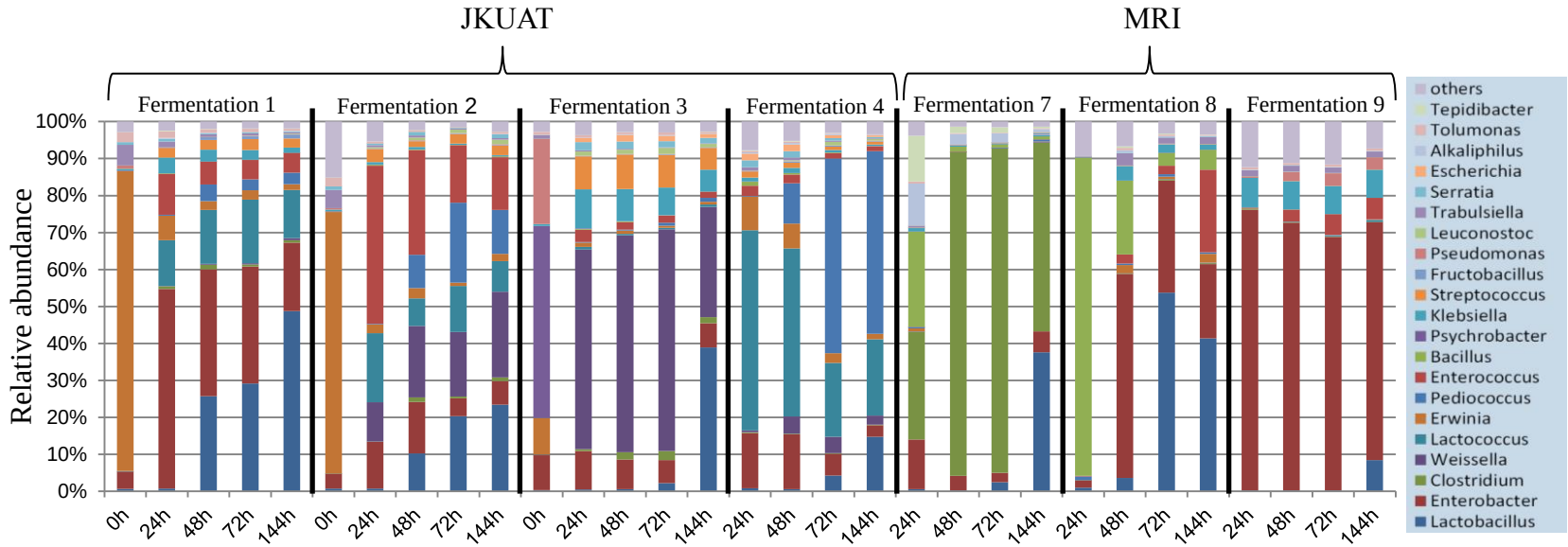

Figure 3.32: Relative abundance of the bacterial community at genus level during nightshade fermentation at JKUAT and MRI without starter culture

In contrast to the fermentation inoculated with starter bacteria, the control fermentation (without starters) performed at JKUAT showed quite diverse microbial profiles, in which the genus *Erwinia* dominated the fermentation 1 at 0 h, constituting 81.1 % of the total bacterial abundance. At 24 h to 144 h, the genera *Enterobacter* constituted between 18.5 to 54 %, *Lactobacillus* increased from 25.7 % (48 h) to 29.1 % (72 h) and finally 48.7 % (144 h). The genera *Lactococcus*, *Enterococcus* and *Pediococcus* collectively constituted 23.4 % (24 h), 25.3 % (48 h), 25.2 % (72 h) and 21.5 % (144 h) of the total bacteria present (Fig.3.32). In fermentation 2, the genus *Erwinia* dominated at time 0 h at 70.8 %, while *Weissella*, *Lactococcus*, *Enterococcus*, *Lactobacillus*, *Pediococcus* and *Enterobacter* were among the most abundant genera of bacteria present at 24 to 144 h. The predominant genera in the JKUAT fermentation three at 0 hwere *Psychrobacter* (52 %), *Pseudomonas* (23 %), *Erwinia* (10 %) and *Enterobacter* (9.4 %). However, the genus *Weissella* was the most predominant at 24 h to 72 h, constituting between 54 to 60 % and at 144 h, *Lactobacillus* (38.9 %) and *Weissella* (29.8 %) predominated. The genus *Streptococcus* constituted 8.9 % (24 h), 9.3 % (48 h), 8.2 % (72 h) and 5.9 % (144 h) of the total bacterial community (Fig.3.32). In the JKUAT fermentation four, the genus *Lactococcus* was the most predominant at 54.1 % after 24 h and 45.4 % after 48 h fermentation, while *Pediococcus* was the most abundant genus at 52.7 % after 72 h and 49.4 % after 144 h. The genus *Lactobacillus* accounted only for 14.7 % at 144 h and *Enterobacter* for 15 % after 24-48 h of fermentation of the total of the bacteria present (Fig.3.32).

The uninoculated MRI nightshade fermentation experiments were relatively different from those conducted at JKUAT, showing diverse results in which in fermentation 7 the 24 h-144 h samples were dominated by genus *Clostridium* ranging between 29.2 % to 87.7 % of the total bacterial abundance. The result showed that the abundance of *Lactobacillus* was below 3 % after 72 h, but then increased to 37.6 % after 144 h. The genera *Bacillus*, *Enterobacter*, *Tepidibacter* and *Alkaliphilus* constituted 25.8 %, 13.5 %, 12.4 % and 11.3 % respectively of the total bacterial abundance after 24 h, then decreased to below 6 % after 144 h (Fig.3.32).

In spontaneous fermentation 8 the abundance of members of the genus *Lactobacillus* was less than 0.1 % after 24 hfermentation, after which they increased to 3.6 % after 48 h and to 53.7 % after 72 h fermentation. Finally the abundance of lactobacilli decreased again to 41.3 % after 144 h. At the same time, fter 24 h the genus *Bacillus* dominated, reaching the bacterial abundance of 86.1 % but decreased to 20.0 % (48 h), 2.2 % (72 h) and 5.4 % (144 h). The genus *Enterobacter* reached 1.9 %,

53.3 %, 30.4 % and 20.1 % at 24 h, 48 h, 72 h and 144 h, respectively. The genus *Klebsiella* constituted 3.9 % (48 h), 2.2 % (72 h) and 1.4 % (144 h) of thebacterial sequences abundance. The genus *Enterococcus* constituted 2.5 % (48 h), 2.3 % (72 h) and 22.4 % (144 h), while the other lesser genera together constituted between 3.3 % to 9.3 % of the total bacterial community (Fig.3.32).

The genus *Enterobacter* dominated the spontaneous fermentation 9 with over 64.3 % total bacterial abundance at 24 h-144 h. The result showed that the genera *Lactobacillus* reached 8.4 % (144 h), *Enterococcus* 3.2 % (48 h), 5.6 % (72 h) and 5.8 % (144 h). The genera *Pseudomonas* and *Klebsiella* constituted between 0.3-3.4 % (48-144 h) and 7.6-8.0 % (24-144 h) respectively. Additionally other lesser genera together constituted 7.3-12.3 % from 24 h -144 h (Fig.3.32).

Therefore, in all the control fermentations done without starter bacteria, lactic acid bacteria did not predominate the bacterial populations.

3.9 Determination of draft genome sequences of *Lb. fermentum* BFE 6620

The *Lb. plantarum* genome was sequenced previously (Oguntoyinbo et al., 2016). *Lb. plantarum* BFE 5092 had a total of 2,909,131 paired–end sequence reads of 500 bp with 117-fold coverage and the 66 scaffolds were yielded by template guided assembly with A5 pipeline (Oguntoyinbo et al., 2016). The *Lb. plantarum* BFE 5092 genome was 3,285,094 bp in size with a 44.39 mol % G+C contents. The genome contained 3,111 protein-encoding sequences, 17 rRNAs and 70 tRNAs (Oguntoyinbo et al., 2016). The *Lb. fermentum* BFE 6620 genome was assembled *de novo* with SPAdes using 2 x 251 paired-ends. In total 2,429,489 paired-end sequence reads were obtained, with approximately 242 fold-coverage. The draft genome of *Lb. fermentum* BFE 6620 consisted of 149 contigs and the N_{50} was 35,982. The genome size was 1,982,893 bp and with a 52.1 mol% G+C contents. It contained 2,003 protein-coding sequences, 14 rRNAs, 54 tRNAs and 3 ncRNA. No acquired antibiotic resistance gene was found using Res finder server version 2.1 (Zankari et al 2012).

3.10 Determination of the success of inoculated starter strains during nightshade fermentation

The relative success of the inoculated starter culture to establish itself as a dominant microorganism in the fermentation was verified by genotyping at strain level. For this, colonies from MRS agar

plates of the highest dilution were randomly isolated from fermentation samples taken at 0 h, 48 h and 144 h fermentation. The isolates were streaked to ensure they were pure, then DNA was extracted from overnight cultures and the bacteria were genotyped by RAPD-PCR.

3.10.1 Phenotypic and characterization of the presumptive LAB isolates from MRI fermentation

Presumptive LAB colonies were isolated from MRS agar plates of the highest dilution after 0 h, 48 h and 144 h fermentation. The plates were incubated at 30 ^{o}C for 24-72 h. The random colonies were also selected from uninoculated fermentations performed at JKUAT from both MRS and VRBG agar plates in order to characterize the autochthonous microbiota occurring in the fermentations. In total, 164 strains were isolated from three nightshade fermentations performed at MRI, of which 30 strains were isolated from fermentation 7 from the batch inoculated with starter culture, while 25 were isolated from the uninoculated control. Thirty three strains were isolated from fermentation eight form the batch inoculated with starter cultures, while 25 strains were isolated from the uninoculated control batch. Finally, 30 strains were isolated from the starter culture inoculated batch from fermentation nine while 21 strains were isolated from the uninoculated control fermentation (Tab.3.26).

Phenotypic characterisation was based on cell morphology, Gram-reaction, catalase production, growth at different temperatures, gas production from glucose fermentation in MRS broth, salt tolerance in MRS broth with 6.5 % NaCl and the ability of the strains to form lactic acid isomers during glucose metabolism. The strains were unequivocally identified by sequencing of the 16S rRNA gene. In total, 164 strains were isolated from MRI nightshade fermentation on MRS agar plates. Sixteen strains did not grow on MRS agar upon subsequent cultivation. Six strains of the remaining 164 were catalase positive, of which four were Gram-negative, and thus were not considered presumptive LAB. Therefore, 158 isolates were further characterised**.**

3.10.2 Identification of the isolates by 16S rRNA/atpD genes for sequencing

Genomic DNA was extracted from both LAB and enterobacteria isolates according to the method described in section 2.2.9.2. Amplification of the 16S rRNA gene with 16S seq fw/rev primers yielded an amplification product of approximately 1500 bp (Fig.3.33). While amplification of atpD gene with A1/A2 primers yielded PCR product of approximately 884 bp (Fig.3.34).

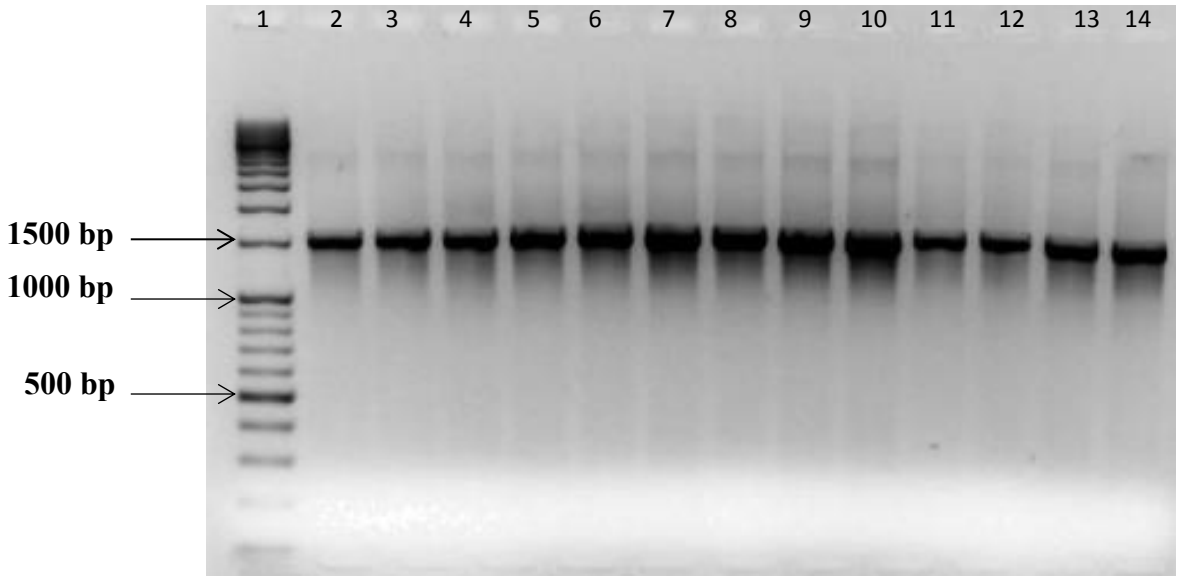

Figure 3.33: The amplified 16S rRNA gene PCR products from representative isolates among the LAB isolates from nightshade fermentation using primer pair 16S seq fw/rev primers. Lane 1 marker (mixture of 2 DNA size markers, 100 bp + 500 bp); Lane 2: B48C1; Lane 3: B48C3; Lane 4, B48C9, Lane 5: C48S6; Lane 6: C44S4, Lane 7: D24C2, Lane 8: D48C2, Lane 9: D48C6, Lane 10:D48C9, Lane 11: F44C2, Lane 12: F44C3, Lane 13: F44C5, Lane and 14: F44C7.

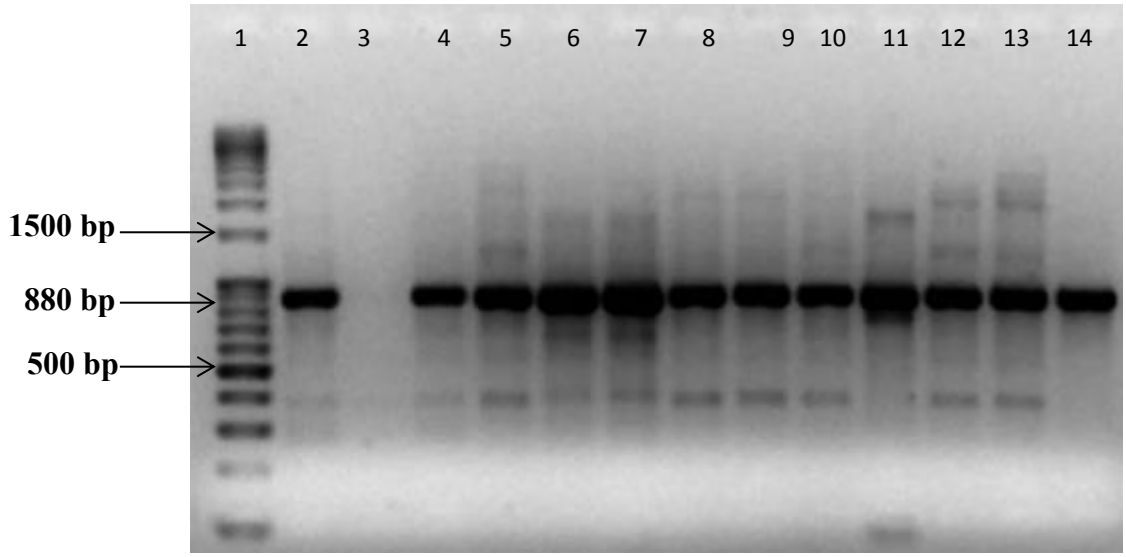

Figure 3.34: The amplified atpD gene PCR products from representative enterobacteria isolates from nightshade fermentation using primer pair atpD A1 fw and A2 rev primers. Lane 1 marker (mixture of two DNA size markers, 100 bp + 500 bp); Lane 2: JKN Eb0011; Lane 3: JKN Eb4811; Lane 4, JKN Eb4812, Lane 5: JKN Eb4813; Lane 6: JKN Eb4814, Lane 7: JKN Eb4815, Lane 8: JKN Eb7212, Lane 9: JKN Eb4411, Lane 10:JKC Eb7233, Lane 11: JKC Eb4421, Lane 12: JKN Eb4831, Lane 13: JKN Eb7241, Lane 14: JKC Eb4444.

The results showed that forty seven (47) strains (28.7 %) from nightshade fermentation were rod-shaped and produced gas from glucose metabolism. They also produced DL-lactate and thus were

characterised as heterofermentative rods, which belonged to either the genus *Lactobacillus* or *Weissella.* The 16S rRNA sequence were compared to 16S rRNA gene sequences in the GenBank database and these strains could all be identified as *Lb. fermentum* with sequence similarities between 99-100 % (Tab.3.27). Ninety-one strains (55.5 %) showed rod-shaped morphology, produced no gas from glucose metabolism but produced DL-lactate. Hence, these were considered to be homofermentative rods belonging to the *Lb. plantarum* group, which was confirmed by 16S rRNA sequence results, with similarities of corresponding sequences in the GenBank database reaching 98-100 %. Ten strains (6.1 %) from the control fermentation 9 showed coccus-shaped morphology, did not produce gas from glucose and produced L-lactate, thus they were considered homofermentative cocci belonging either to the genera *Enterococcus, Lactococcus* or *Streptococcus*. The 16S rRNA gene sequencse identified these isolates as *Enterococcus faecium,* with the sequence similarity of 99 %, while five strains (3.0 %) from control fermentation 8 showed coccus shaped morphology, neither produced gas from glucose nor grew in 6.5 % NaCl, but produced L-lactate, thus they were considered homofermentative cocci belonging either to the genera *Enterococcus, Lactococcus* or *Streptococcus*. The 16S rRNA sequence from NCBI database identified this cluster as *Enterococcus faecalis,* with the sequence similarity ranging between 98-100 %. Five strains (3.0 %) showed rod-shaped morphology, did not produce gas from glucose but produced L-lactate and thus were characterised as to homofermentative lactobacilli. The 16S rRNA sequence from NCBI identified this cluster as *Lactobacillus sakei,* with the sequence similarity reaching between 99-100 % (Tab.3.27).

The results also showed that two strains (1.2 %) from control fermentation 8 showed coccus-shaped cell morphology, but were catalase positive, produce L-lactate thus were considered as belonging to genus *Staphylococcus.* However, 16S rRNA gene sequence from NCBI database showed that the strain B48C2 was *Staphylococcus epidermidis* with 99 % sequence similarity while strain B48C6 was *Staphylococcus condimenti* with 99 % sequence similarity. Four strains (2.4 %) from control fermentation batch 8 were Gram-negative rods and were classified in family Enterobacteriaceae. Based on the atpD gene sequence, strain B48C1 could be identified as *Enterobacter ludwigii* with sequence similarity reaching 99 %, while strains B48C3, B48C4 and B48C9 could be identified as *Enterobacter cloacae* with sequence similarities ranging between 99-100 %, when compared to reference sequences in the NCBI Genbank Database.

Table 3.26: A summary of strains isolated at MRI with their respective fermentation trials.

	Fermentation 7		Fermentation 8		Fermentation 9		
	Starter (A)	**Control (B)**	**Starter (C)**	**Control (D)**	**Starter (E)**	**Control (F)**	**Total**
Total no of strains	**30**	**25**	**33**	**25**	**30**	**21**	
Lb. plantarum	15	19	17	19	15	5	90
Lb. curvatus						1	1
Lb. fermentum	15	1	16		15		47
Lb. sakei						5	5
E. faecalis				5			5
E. faecium						10	10
Enterobacter		3		1			4
Staphylococcus		2					2

Table 3.27: Summary of 16S rRNA and atpD gene sequence identification of the MRI strains.

MRI isolates n=164	**Occurence (%)**	**Identification (16S rRNA gene)**	**Identification (atpD gene)**	**BLAST (%)**
90	54.9	*Lactobacillus plantarum*		98-100
1	0.6	*Lacctobacillus curvatus=F44C10*		99
47	28.7	*Lactobacillus fermentum*		99-100
5	3.0	*Lacctobacillus sakei*		99-100
5	3.0	*Enterococcus faecalis*		98-100
10	6.1	*Enterococcus faecium*		99
1	1.2	*Staphylococcus epidermidis=B48C2*		99
1		*Staphylococcus condimenti=B48C6*		99
1	2.4	*Enterobacter ludwigii=B48C1*	*Enterobacter ludwigii*	100
1		*Enterobacter ludwigii=B48C3*	*Enterobacter cloacae=B48C3*	99-100
1			*Enterobacter cloacae=B48C4*	99
1			*Enterobacter cloacae=B48C9*	99

3.10.3 Phenotypic and genotypic characterization of JKUAT isolates

In total, 45 strains were isolated from control nightshade fermentation at JKUAT from both MRS agar (32 isolates) and VRBG agar (13 isolates). Phenotypic characterization of the 13 isolates from VRBG agar showed that the isolates were Gram-negative rods and catalase-positive, hence they were presumably Enterobacteriaceae. The atpD sequence analysis identified these strains as

Providencia rettgeri, *Enterobacter cloacae*, *Escherichia coli*, *Klebsiella oxytoca*, *Enterobacter mori*, *Enterobacter asburiae* and *Klebsiella* spp. (Fig.3.35) with sequence similarities ranging between 98-100 %, when compared to reference sequences in the NCBI Genbank Database (Tab.3.28).

The result showed that among the strains isolated from MRS agar, one strain was Gram-positive, coccus-shaped and catalase positive and produced L-lactate and was therefore considered to belong to the genus *Staphylococcus* (Fig.3.35). Indeed the 16S rRNA gene sequence showed that the strain JKN Lb0011 could be identified as *Staphylococcus hominis* with 99 % sequence similarity to a reference strain. Eleven strains were Gram-positive, rod-shaped morphology, catalase negative, produced no gas from glucose metabolism but produced DL-lactate thus were considered homofermentative rods belonging to the *Lb. plantarum* group that was confirmed by 16S rRNA gene sequence results, whose similarities reached 99 %. The results further showed that three strains were Gram-positive, rod-shaped morphology, catalase-negative and produced gas from glucose metabolism. They also produced DL-lactate and thus were characterised as heterofermentative rods, which belonged to either the genus *Lactobacillus* or *Weissella*. Based on the 16S rRNA gene sequencing results and comparison of the sequences to corresponding 16S rRNA genes in the GenBank databank showed that these strains could be identified as *Lb. fermentum*, with the sequence similarity reaching 99 %. Six strains were Gram-positive, coccus-shaped morphology, catalase negative, they did not produce gas from glucose but produced DL-lactate, thus were considered to be homofermentative cocci (Fig.3.35). The 16S rRNA gene sequence identified these isolates as *Pediococcus pentosaceus,* with the sequences having 99 % similarity (Fig.3.35) to reference sequences from the NCBI Genbank Database. One strain was Gram-positive, catalase negative, showed coccus-shaped morphology, produced gas from glucose metabolism and produced D-lactate, thus it was considered either heterofermentative cocci belonging to the genera *Leuconostoc* or *Weissella.* The 16SrRNA gene sequence analysis confirmed that this strain was a *Leuconostoc lactis* having 99 % sequence similarity (Tab.3.28) to sequences in the Genbank database. Seven strains were Gram-positive, cocci, catalase-negative, did not produce gas from glucose metabolism and produced L-lactate, thus these were characterised as belonging either to the genera *Enterococcus*, *Lactococcus* or *Streptococcus* (Fig.3.35). The 16S rRNA gene sequence from the NCBI database identified these strains as belonging *Enterococcus faecium* with the sequence similarity of 99 % to reference sequences, while two strains were identified as

Enterococcus mundtii, with the sequence similarity to reference sequences ranging at approx. 99 %. Additionally, one strain was identified as *Enterococcus durans* with sequence similarity of 99 % and finally, the remaining strain was closely related to *Enterococcus gallinarum* with 99 % sequence similarity (Tab.3.28) to reference sequences in the Genbank database.

The results further showed that two strains were Gram-positive, rod-shaped, catalase negative and produced gas from glucose metabolism and grew in 6.6 % NaCl and at 10 ^{o}C. They also produced D and L-lactate and thus were characterised as heterofermentative rods (Fig.3.35). The 16S rRNA sequence when compared to sequences in the NCBI database identified these two strains as *Weissella confusa* with 99 % sequence similarity to reference sequences. One strain produced D-lactate and was identified by 16S rRNA gene sequence as a *Weissella oryzae* strain with 99 % sequence similarity to reference serquences in the NCBI database (Tab.3.28).

Table 3.28: Summary of 16S rRNA and atpD gene sequence identification of the JKUATstrains.

JKUAT isolates n= 45	Identification (16S rRNA gene)	Identification (atpD gene)	BLAST (%)
6	*Pediococcus pentosaceus*		99
11	*Lactobacillus plantarum*		99
3	*Lactobacillus fermentum*		99
3	*Enterococcus faecium*		99
2	*Enterococcus mundtii*		99
1	*Enterococcus durans*		99
1	*Enterococcus gallinarum*		99
1	*Staphylococcus hominis*		99
2	*Weissella confusa*		99-100
1	*Weissella oryzae*		99
1	*Leuconostoc lactis*		99
1		*Providencia rettgeri*	99
1		*Enterobacter cloacae*	100
6		*Escherichia coli*	99-100
2		*Klebsiella oxytoca*	99-100
1		*Enterobacter mori*	100
1	*Klebsiella sp.*		99
1		*Enterobacter asburiae*	98

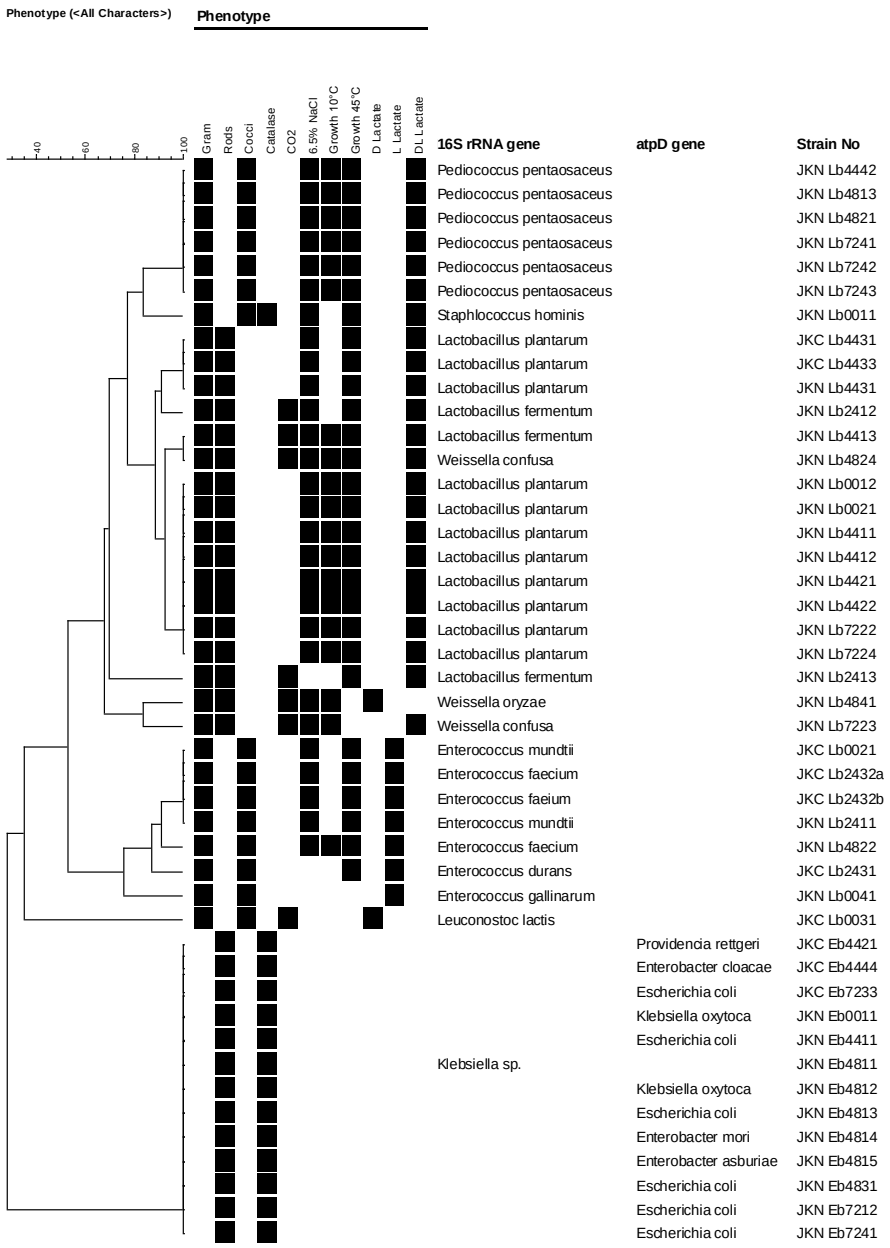

Figure 3.35: Phenotypic and 16S rRNA/atpD fingerprints for the JKUAT strains isolated from natural fermentation of nightshade leaves.

3.11 Randomly amplified DNA polymorphism (RAPD) PCR analysis of the isolates

RAPD-PCR was used to follow the success of the inoculated starter culture strains to establish themselves as dominant LAB during the MRI nightshade fermentations. The M13 primer was used for RAPD-PCR that generated profiles which were analysed by BioNumerics 7.0 program for cluster analysis see (section 2.20). This primer was successfully used since the bands generated were clearly visible as show in figure 3.36 below.

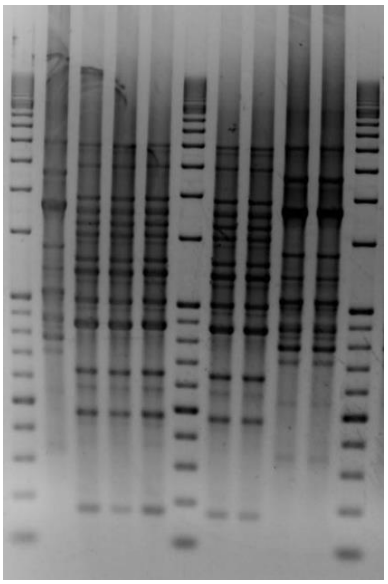

Figure 3.36: Agarose gel with RAPD-PCR products generated using the primers M13 from representative LAB isolates. Lanes 1, 6 and 11 are marker bands.

3.11.1 Investigation of the clonal relatedness among *Lb. fermentum* group strains isolated from four fermentation batches

A total of 47 heterofermentative rods that produced gas from glucose metabolism and DL-lactate were isolated from the MRI fermentation experiments after 144 h fermentation and amongst these, 46 strains were isolated from the batches inoculated with starter cultures including the *Lb. fermentum* BFE 6620 strain. The strains isolated from the fermentations possessed properties similar to the heterofermentative starter strain *Lb. fermentum* BFE 6620. Fifteen strains (31.9 %) were isolated from fermentation batch 7 (inoculated with the starter strain), 16 strains (34.0 %) from fermenentation batch 8 (inoculated with starter strains) while 15 strains (31.9 %) were isolated from fermentation batch 9 (inoculated with starter strain). Only one strain was isolated from fermentation batch 7 (uninoculated with starter strains) (Tab.3.26). To investigate whether these heterofermentative rod-shaped isolates were related to the reference strain *Lb. fermentum* BFE 6620, RAPD-PCR profiles of these isolates as well as the starter strain were analysed (Fig.3.37). The result showed that the 47 isolates clustered in five groups, of which 3 isolates clustered with the fingerprint of *Lb. fermentum* BFE 6620 inoculated starter strain at r = 65.5 % in group III, 16 isolates clustered in group II at r = 64.5 %, 21 isolates clustered in group I at r = 56.5 %, 4 isolates clustered in group IV at r = 79.5 % and 3 isolates clustered in group 5 at r = 24.5 % (Fig.3.37). A visual inspection of the band patterns showed that the fingerprints of the isolated strains and the

reference starter strain were visibly quite different (Fig.3.37). Contrary to different sub-clusters in the RAPD-PCR analysis, there were high similarities in the banding patterns within groups, which showed that some of the isolates were undoubtedly closely related.

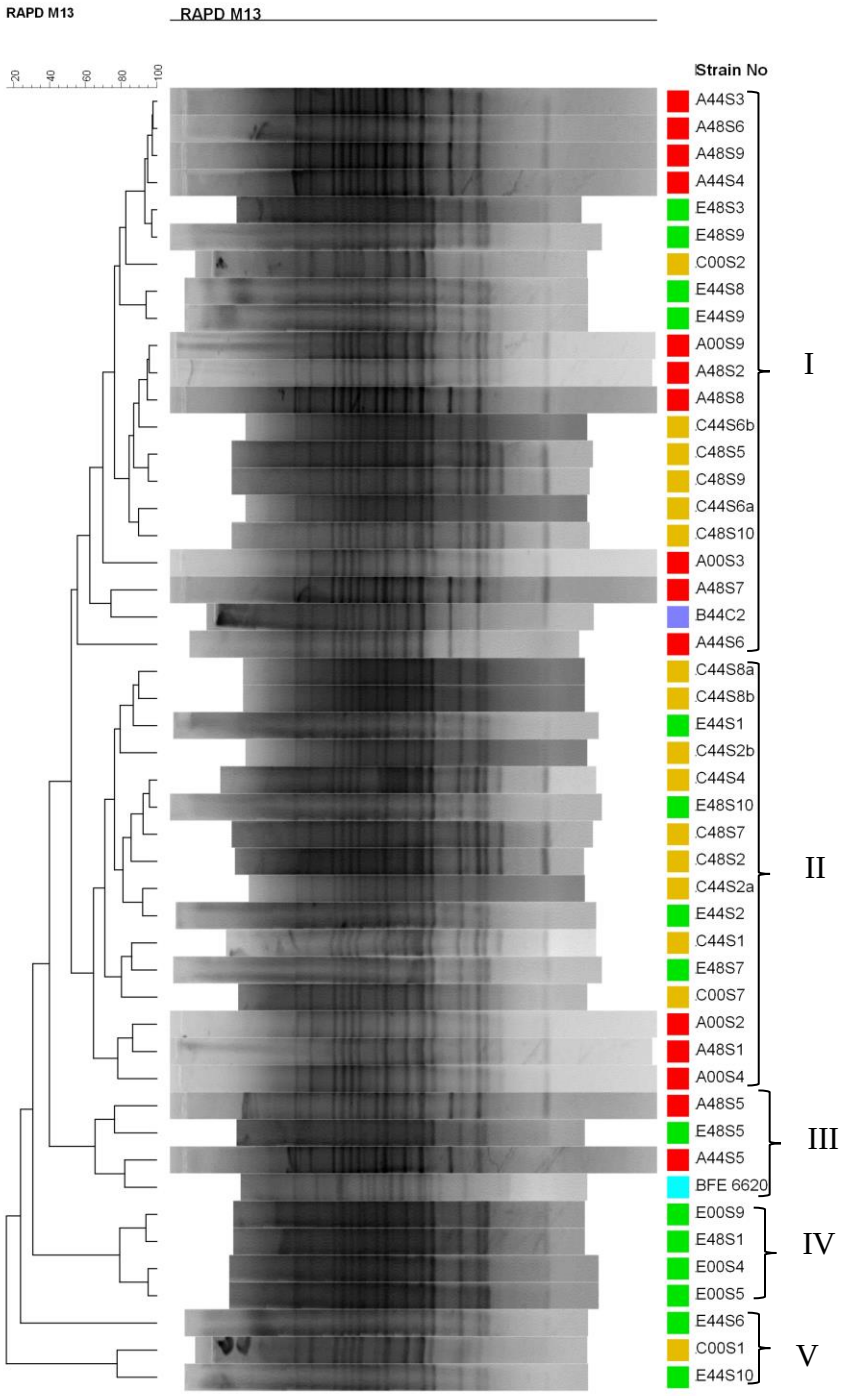

Figure 3.37: Dendrogram obtained by UPGMA of correlation value r of RAPD-PCR fingerprint patterns with primer M13 of 47 *Lb. fermentum* strains isolates from three MRI nightshade fermentation from batch 7 (starter = red), 7 (control = violet), 8 (starter= orange), 9 (starter = green) and inoculated starter (*Lb. fermentum* BFE 6620=light blue) after 144 h fermentation.

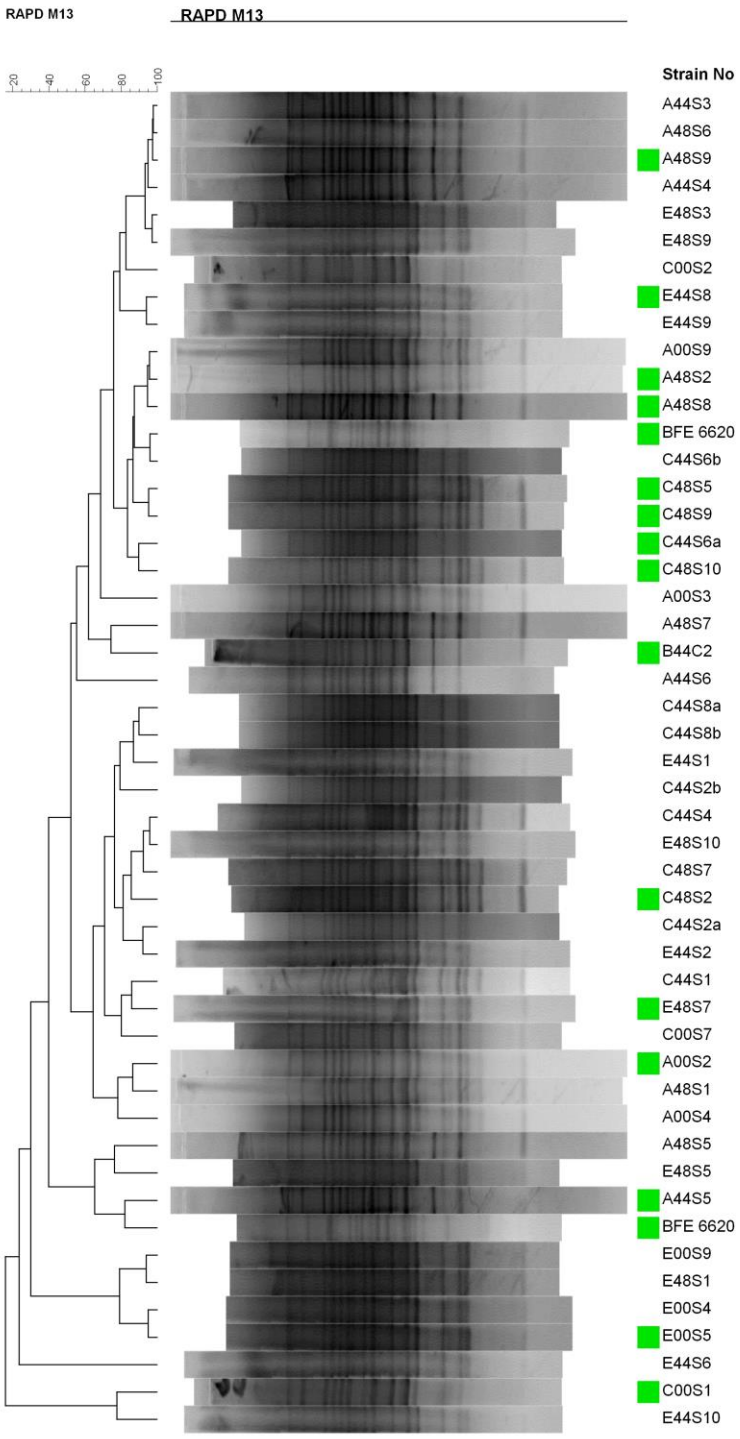

Figure 3.38: RAPD of 49 *Lb. fermentum* strains, by Pearson correlation coefficient, labelled according to selected for PFGE

To further determine the clonal relatedness between the isolates from nightshade fermentations inoculated with starters and the starter culture *Lb. fermentum* BFE 6620 used in the fermentation,

15 strains (BFE 6620, A48S2, A48S9, B44C2, C00S1, C44S6a, E44S8, E48S7, C48S10, C48S2, C48S5, A00S2, A48S8, C48S9 and A44S5), representative of several of the RAPD-PCR clusters were selected for PFGE analysis (Fig.3.38).

2.11.2 Pulsed field gel electrophoresis (PFGE) analysis of selected *Lb. fermentum* isolates

The PFGE was done in duplicate for three strains (*Lb. fermentum* BFE 6620, B44C2 and C00S1) to determine the reproducibility of the method. The duplicate fingerprint patterns of these three strains all clustered at S_D = 100 % (Fig.3.39). Nine strains (A48S2, A48S9, C44S6a, E00S5, E44S8, C48S7, C48S10, C48S2 and C48S5) isolated from the batches inoculated with *Lb. fermentum* BFE 6620 starter strain, clustered together with this reference starter strain in group I at S_D = 100 %, suggesting that these isolates were also *Lb. fermentum* strains. Strains (A00S2, A48S8, C48S9 and A44S5) clustered in-group II at S_D = 96 %, however, all the strains in group I have almost the same band pattern profile suggesting that they could be clonally related even though it seems that some bands did not cut well giving unclear band separation

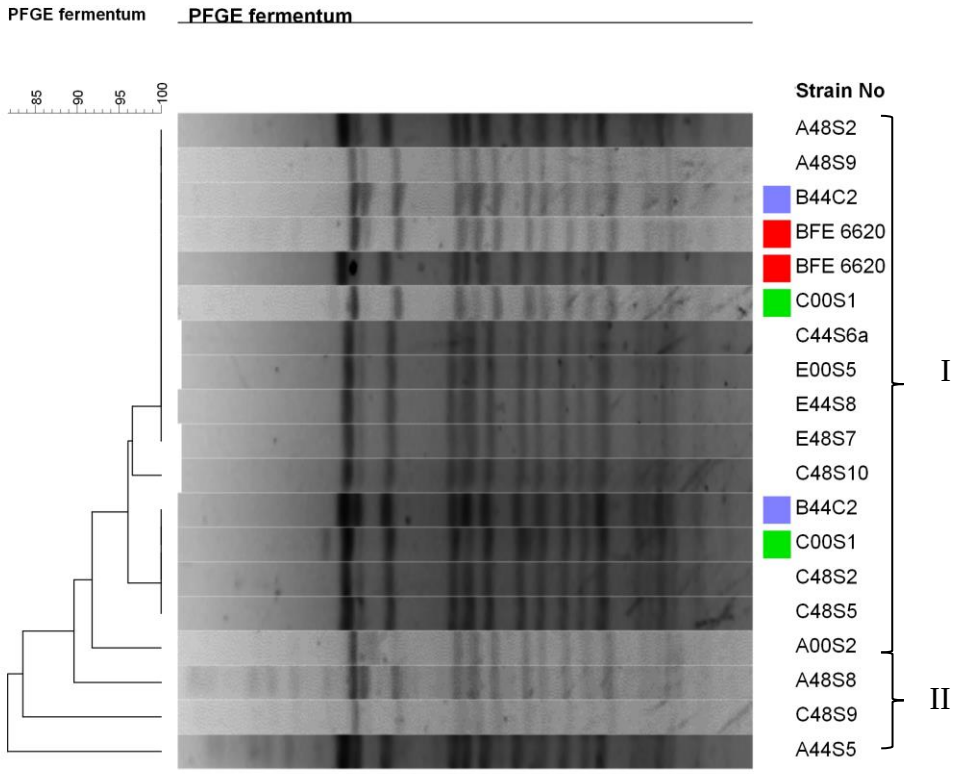

Figure 3.39: Dendrogram obtained by Dice coefficient of similarity value S_D of PFGE fingerprints for 15 selected *Lb. fermentum* isolates with starter strain *Lb. fermentum* BFE 6620 (the coloured labelled strains are duplicates).

2.11.3 Investigation of clonal relatedness of *Lb. plantarum* strains isolated from the three fermentation batches

A total of 90 presumptive *Lb. plantarum* group strains were isolated from all three MRI fermentation trials, of which 47 (52.2 %) strains were isolated from the starter inoculated batches and 43 (47.8 %) from non-inoculated batches. A total of fifteen strains (16.7 %) were isolated from batch A/ fermentation 7 (inoculated with the *Lb. plantarum* BFE 5092 starter strain) and nineteen strains (21.1 %) from batch B / fermentation 7 (non-inoculated with starter strain), seventeen strains (18.9 %) were isolated from batch C/ fermentation 8 (inoculated with the *Lb. plantarum* BFE 5092 starter strain) and nineteen strains (21.1 %) from batch D/fermentation 8 (non-inoculated with starter strain). From batch E/fermentation 9 which was inoculated with the starter strain *Lb. plantarum* BFE 5092, fifteen strains (16.7 %) were isolated, while five strains (5.6 %) were isolated from the non-inoculated batch F/fermentation 9 (Tab.3.26) as already mentioned in section 2.2.6. These results are in agreement with DGGE and metagenomics data, which showed the dominance of all starter inoculated batches with genus *Lactobacillus* in which case our starter strains belonged.

To investigate whether these presumptive *Lb. plantarum* strains were related to the *Lb. plantarum* BFE 5092 starter strain, RAPD-PCR profiles of these isolates, as well as the starter strain, were analysed (Fig.3.40). The RAPD-PCR was done in triplicate for the starter strain *Lb. plantarum* BFE 5092. The result showed that 90 isolates clustered in seven groups, of which 8 isolates clustered with the fingerprint of *Lb. plantarum* BFE 5092 inoculated starter strain at S_D = 52.1 % in group VI, 19 isolates clustered in group I at S_D = 83.2 %, 26 isolates clustered in group II at S_D = 84.2 %, 8 isolates clustered in group III at S_D = 71.2 %, 23 isolates clustered in group IV at S_D = 71.2 % while 4 isolates clustered in group V at S_D = 61.2 % and 2 isolates clustered in group VII at S_D = 44 % (Fig.3.40). The visual inspection of the band patterns of strains from various groups showed high similarities within individual groups, which indicated that some isolates were closely related. However, the band patterns of the strains in groups I, II, III, IV, V and VII were different when visually compared also to the band pattern of the inoculated reference strain (Fig.3.40).

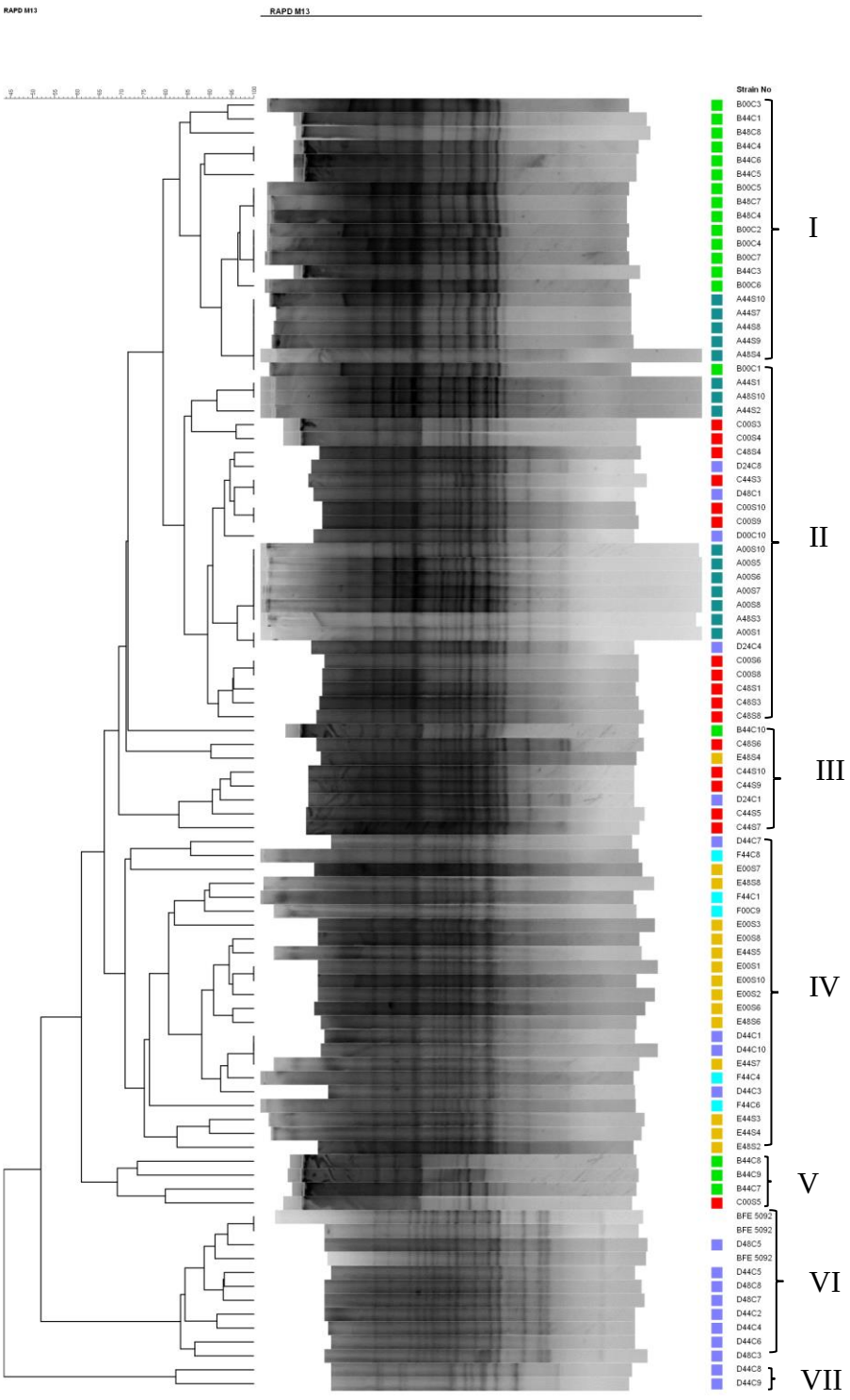

Figure 3.40: Dendrogram obtained by Dice coefficient of similarity value S_D value of RAPD-PCR fingerprint patterns with primer M13 of 90 *Lb. plantarum* strains from fermentation of nightshade from batch7 (starter = green), 7 (control = lemon green), 8 (starter = red), 8 (control = purple), 9 (starter = orange) 9 (control = sky blue) after 144 h fermentation.

3.11.4 Pulsed field gel electrophoresis (PFGE) analysis of selected *Lb. plantarum* isolates

To further determine the clonal relatedness between the isolates from nightshade fermentations inoculated with starters and the starter culture *Lb. plantarum* BFE 5092 used in the fermentation, 10 strains (BFE 5092, A44S1, A44S7, C00S5, C00S9, C48S3, C44S3, C48S4, E48S8, and E00S1), representative of several of the RAPD-PCR clusters were selected for PFGE analysis (Fig.3.41). Eight strains (A44S1, A44S7, C00S5, C00S9, C48S3, C44S3, C48S4 and E48S8) isolated from the batches inoculated with *Lb. plantarum* BFE 5092 starter strain, clustered together at S_D = 100 % while the inoculated starter strain clustered at S_D = 99.4 %, however, all the strains showed almost the same band pattern profile suggesting that they could be clonally related.

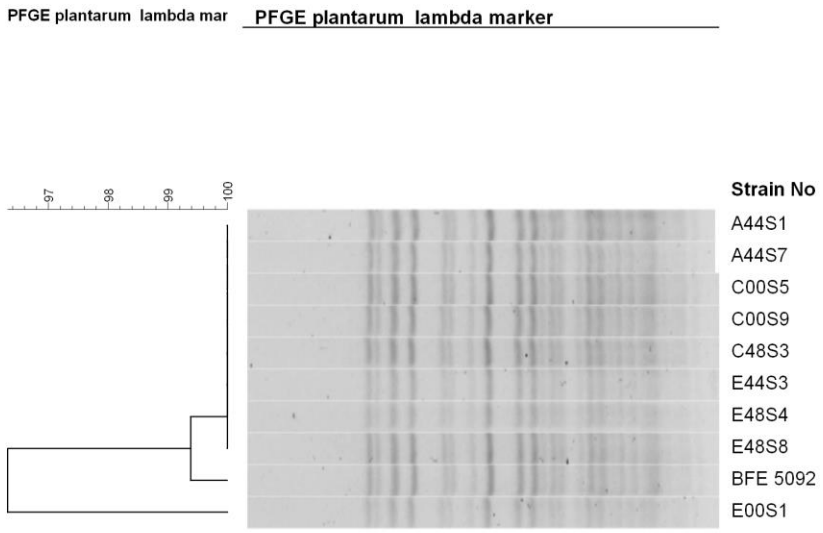

Figure 3.41: Dendrogram obtained by Dice coefficient of similarity value S_D of PFGE fingerprints for 9 selected *Lb. plantarum* isolates with starter strain *Lb. plantarum* BFE 5092.

3.12 Determination of nutrient contents from fresh, fermented and solar dried nightshade leaves

The changes in the composition of basic nutrients, vitamins (B_1, B_2 C, E), ash, protein and soluble sugars (fructose, glucose and sucrose) was performed on fresh, fermented and solar dried nightshade leaves both grown at MRI greenhouse and JKUAT open fields. The fermentation experiments were conducted in triplicates at MRI and JKUAT in 5 L fermentation vessels with starter cultures *Lb. plantarum* BFE 5092 and *Lb. fermentum* BFE 6620 or as uninoculated control fermentations without added starter cultures. The determination of nutrient composition on fresh,

fermented and solar dried nightshade leaves was done according to the description in section 2.18. The analysis of the result was done by one-way analysis of variance to determine the significance level among different groups.

3.12.1 Analyses of ash and protein contents

The result in figure 3.42 showed that fermentation of nightshade leaves with or without starter cultures led to an increase in ash content. The mean ash of freeze-dried fresh leaves was 14.6 g/100g dry mass (DM) for the leaves grown at MRI greenhouse, after fermentation the ash content increased to 22.4 g/100 g DM for starter fermented and 21.7 g/100 g for the spontaneous fermentation. The same trend was observed in the JKUAT grown and fermented leaves, where the mean ash of the fresh leaves was 14.6 g/100 g DM, after fermentation with starter culture the ash content increased to 25.0 g/100 g while the uninoculated fermented sample ash increased to 25.1 g/100 g (Fig.3.42). The result showed that solar drying did not affect ash content. One-way analysis of variance (ANOVA) showed a statistical significant difference between fermented and freeze-dried fresh samples ($p < 0.05$).

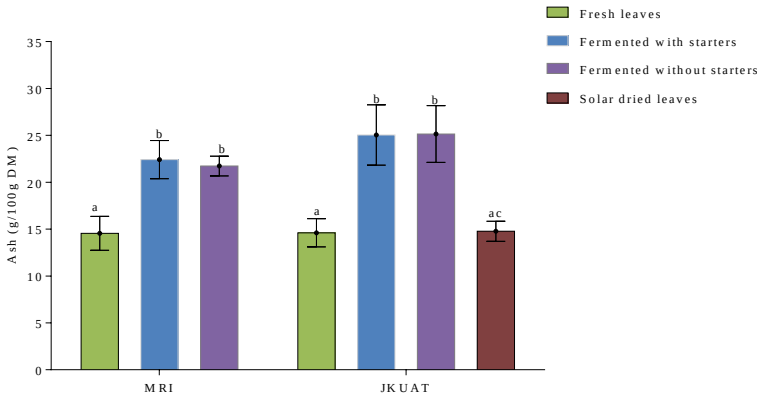

Figure 3.42: Effect of fermentation and solar drying on ash content of African nightshade leaves. Means followed by the same letter within a variable are not significantly different ($p \leq 0.05$) according to Tukey's test. The results of triplicate determination are shown with standard deviation

The result in figure 3.43 shows that for the leaves that were grown and fermented at MRI, Karlsruhe the fermentation decreased the protein content by about 15-33 %, while a 36-38.3 % reduction was observed for JKUAT, Kenya grown and fermented nightshade leaves. However, this reduction was not significantly different $p>0.05$. Solar drying did not affect protein content, the

statistical results showed no significant difference between solar dried and fermented leaves with starter cultures and uninoculated fermentation (Fig.3.43).

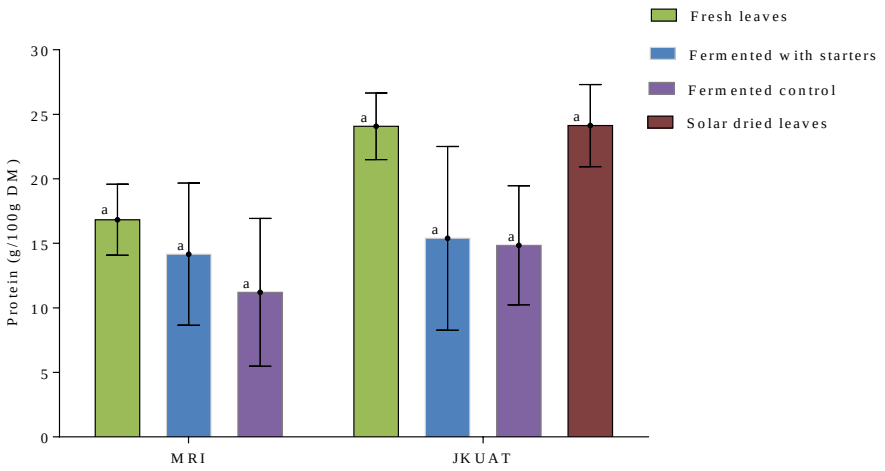

Figure 3.43: Effect of fermentation and solar drying on protein content of African nightshade leaves. Means followed by the same letter within a variable are not significantly different ($p \leq 0.05$) according to Tukey's test. The results of triplicate determination are shown with standard deviation

3.12.2 Analyses of vitamin C and E contents

The results showed that fermentation of African nightshade leaves with or without lactic acid bacteria starter cultures causes a decrease in vitamin C content. The freeze-dried fresh leaves grown in the MRI greenhouse had a mean value of 757.2 mg/100 g vitamin C content of DM, after fermentation for 144 h with starter cultures, the value decreased to 256.8 mg/100 g, representing ca. 66 % loss, while the control fermentation decreased to 142.5 mg/100 g, representing 81 % loss in vitamin C (Fig.3.44). For the experiment carried out at JKUAT, the freeze-dried fresh leaves had a mean value of 622.9 mg/100 g vitamin C content of DM, those fermented with starter cultures, vitamin C content decreased to 158.9 mg/100 g representing about 74.5 % loss, while the uninoculated fermentation had a mean value of 160.4 mg/100 g, representing 74.2 % decrease in vitamin C content. The results showed that solar drying led to a massive loss of vitamin C, whose mean value was 19.2 mg/100 g representing a total reduction of over 97 % (Fig.3.44).

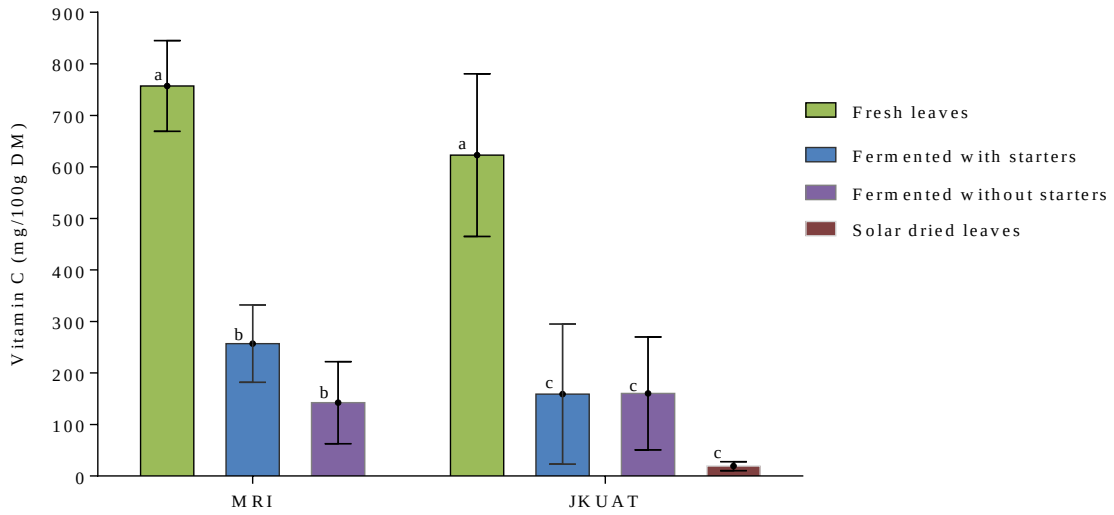

Figure 3.44: Effect of fermentation and solar drying on vitamin C content of African nightshade leaves. Means followed by the same letter within a variable are not significantly different ($p < 0.05$) according to Tukey's test.

Statistical analysis showed the differences in the mean values among treatment groups where, $p < 0.001$. Therefore, to identify the group or groups that differed from others, a multiple comparison was conducted by Tukey's method with overall significance level = 0.05. The comparison showed a significant difference between freeze-dried fresh and fermented leaves, as well as between freeze-dried fresh and solar dried leaves, with $p < 0.05$. However, the results showed there was no significant difference in vitamin C content between fresh vegetables grown at JKUAT, Kenya and fresh grown at MRI, Germany $p > 0.05$ (Fig.3.44).

The results for the vitamin E analyses showed that the fresh African nightshade leaves grown in the MRI greenhouse had a mean concentration of 45.7 mg/100 g vitamin E content of DM. After fermentation with starter cultures for 144 h, the value decreased to 37.0 mg/100 g, representing an approximate 19 % reduction, while the control fermentation decreased to 41.3 mg/100 g, representing a 10 % loss of vitamin E (Fig.3.45). For the experiment carried out at JKUAT, fresh leaves had a mean value of 35.8 mg/100 g vitamin E content of DM, those fermented with starter cultures the value increased to 44.5 mg/100 g vitamin E representing about 24 % gain, while uninoculated fermentation had a mean value of 48 mg/100 g representing a 34 % increase in vitamin E content. The results show solar drying led to an increase in vitamin E with the mean value 40.3 mg/100 g representing a total gain of approximately 13 % (Fig.3.45). Statistical analysis,

however, showed that there was no statistically significant difference between fermented, freeze-dried fresh and solar dried nightshade leaves in vitamin E content (p> 0.05)

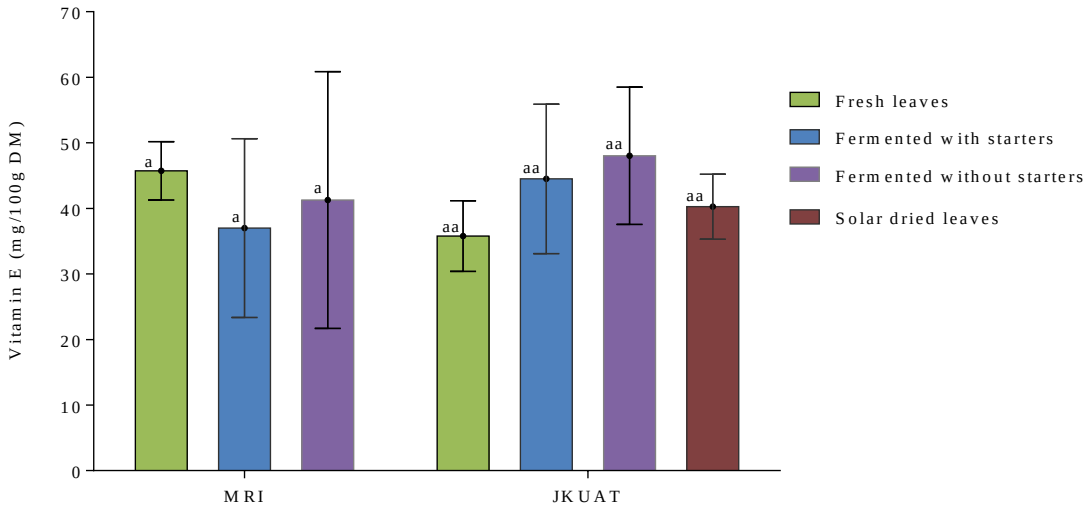

Figure 3.45: Effect of fermentation and solar drying on vitamin E content of African nightshade leaves. Means followed by the same letter within a variable are not significantly different ($p < 0.05$) according to Tukey's test.

3.12.3 Analyses of vitamin B_1 and B_2 contents

The extraction of vitamin B_1 and B_2 from food matrices involved separation from protein by acid hydrolysis and enzymatic dephosphorylation. These vitamins were quantified by HPLC with a fluorescence detection method.

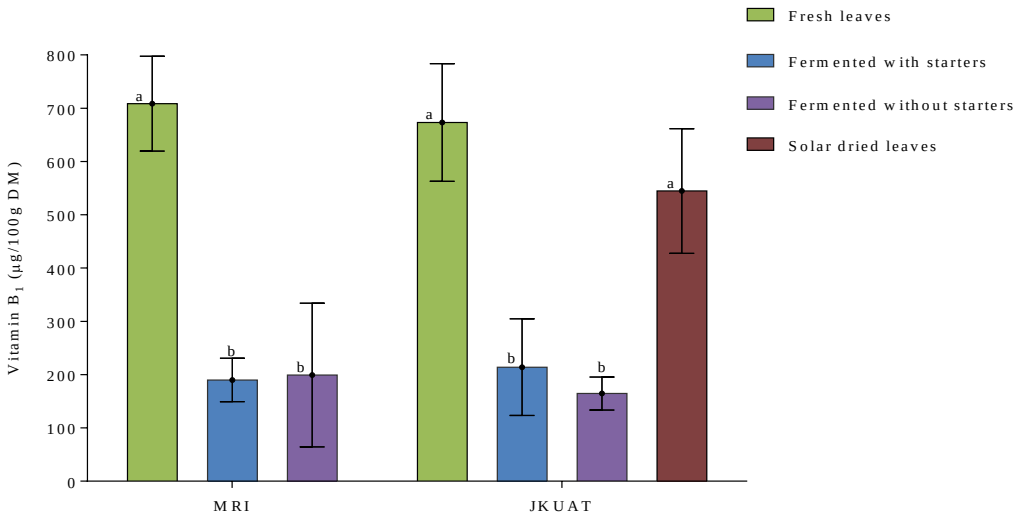

Figure 3.46: Effect of fermentation and solar drying on vitamin B_1 content of African nightshade leaves. Means followed by the same letter within a group are not significantly different ($p < 0.05$) according to Tukey's test.

Figure 3.46 above shows freeze-dried fresh nightshade leaves grown at MRI greenhouse had vitamin B_1 mean value of 708.5 µg/100 g DM. However, after fermentation with starter cultures, it decreased to mean value of 190.0 µg/100 g DM representing about 73 % reduction, while the control fermentation reduced to the mean value of 199.3 µg/100 g, representing a 71 % loss of vitamin B_1. The experiments carried out at JKUAT showed similar trend, where the fresh solar dried nightshade leaves had vitamin B_1 mean value of 673.3 µg/100 g DM, after fermentation with starter cultures vitamin B_1 decreased to 214.0 µg/100 g DM representing 68 % loss, while the control fermented leaves without starter vitamin B_1 decreased to 164.6.1 µg/100 g DM representing 76 % reduction in vitamin B_1 content. The results also revealed that solar drying of African nightshade causes a reduction of vitamin B_1 to mean value of 544.7 µg/100 g dry mass representing approximately 19 % loss (Fig.3.46). These differences were supported by statistical analysis, which showed the mean differences of vitamin B_1 among freeze-dried fresh and fermented leaves with or without starter cultures were greater than would be expected by chance, hence there was a statistically significant difference ($p = <0.001$). To identify the groups that differed, a post hoc multiple comparison procedure with Tukey method with overall significance level = 0.05 was performed. The comparison showed significant difference between freeze-dried fresh and fermented leaves with or without use of starter cultures, as well as freeze-dried fresh and solar dried leaves ($p<0.05$). However, the results revealed there was no significance difference in vitamin B_1 content between freeze-dried fresh nightshade leaves grown at MRI greenhouse and those grown at JKUAT open field. There was also no statistical difference between vitamin B_1 in freeze-dried fresh and solar dried nightshade leaves (Fig.3.46).

Freeze-dried fresh nightshade leaves grown in MRI greenhouse had a vitamin B_2 mean value of 1538.5 µg/100 g DM. However, fermentation with starter cultures caused the reduction of vitamin B_2 to mean value of 1065.5 µg/100 g DM, representing about 30 % loss, while uninoculated fermentation reduced vitamin B_2 to the mean value of 979.5 µg/100 g DM, representing a 36 % loss (Fig.3.47). The experiments carried out at JKUAT showed that the freeze-dried fresh nightshade leaves had vitamin B_2 mean value of 2211.9 µg/100 g DM, however, after fermentation with starter cultures the vitamin B_2 level decreased to 1165.3 µg/100 g DM, representing about a 47.3 % loss, while for the control fermented leaves vitamin B_2 concentration decreased to 1180.1 µg/100 g DM, representing an approximate 46.6 % reduction. The results also revealed that solar drying of

African nightshade causes a reduction of vitamin B_2 to mean value of 1895.3 µg/100 g DM, representing an ca. 13 % loss (Fig.3.47). These differences were supported by statistical analysis, which showed that the mean differences of vitamin B_2 among fresh leaves and fermented with or without starter cultures nightshade leaves were greater than would be expected by chance, hence there was a statistically significance difference. The post hoc multiple comparison procedure based on Tukey method was used with overall significance level = 0.05 and showed a significant difference between freeze-dried fresh and fermented leaves with or without use of starter cultures for the experiment performed at JKUAT ($p<0.05$). However, the results revealed there was no significance difference in vitamin B_2 content between freeze-dried fresh nightshade leaves grown at MRI greenhouse and those grown at JKUAT open field (Fig.3.47). There was also no statistical difference between freeze-dried fresh and solar dried nightshade leaves. Furthermore, there was no statistical significance between the means of freeze-dried fresh and fermented leaves performed at MRI. Thus, the results showed that even though fermentation causes a reduction in vitamin B_2 content in samples conducted at MRI, the reduction was not statistically significant $p>0.05$.

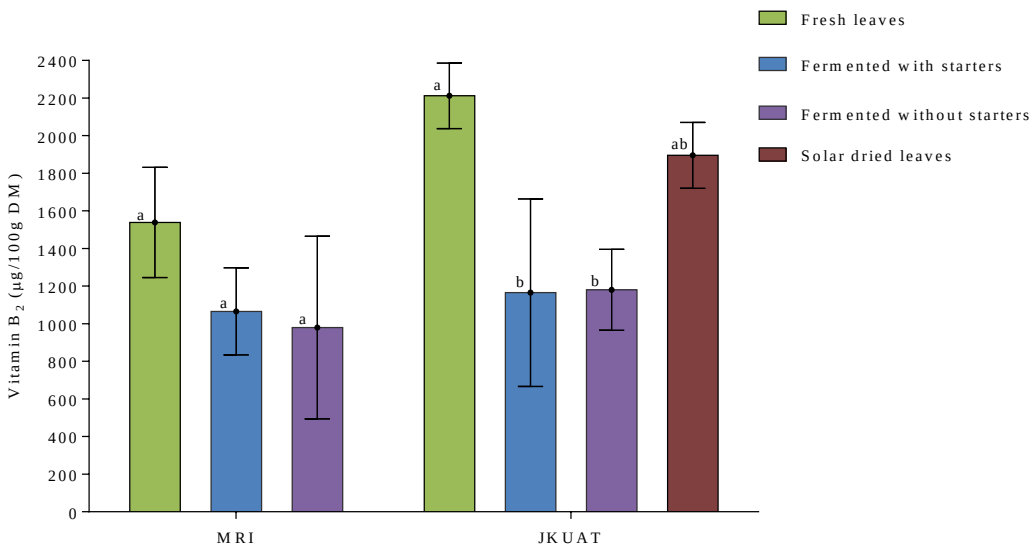

Figure 3.47: Effect of fermentation and solar drying on vitamin B_2 content of African nightshade leaves. Means followed by the same letter within a group are not significantly different ($p < 0.05$) according to Tukey's test.

3.12.4 Analyses of soluble sugars

The extraction and determination of sugars (fructose, glucose and sucrose) from freeze dried and ground up African indigenous vegetables were determined according to the method described by

Van Den et al. (1986). Figure 3.48 shows the mean concentration of fructose in fresh nightshade leaves grown at MRI greenhouse was 1.16 g/100 g DM. However, fermentation with starter cultures caused the reduction of fructose to the mean value of 0.59 g/100 g DM representing about 50 % loss, while uninoculated fermentation increased fructose to the mean value of 3.01 g/100 g DM, representing over 100 % increase (Fig.3.48). The experiments carried out at JKUAT showed that the freeze-dried fresh nightshade leaves had mean fructose concentrations of 1.65 g/100 g DM, however, after fermentation with starter, the cultures decreased the fructose concentration to 0.07 g/100 g DM, representing about 96 % loss (or utilisation), while for the control fermented leaves fructose increased to 1.95 g/100 g DM representing ca. 18 % increase. The results also revealed that solar drying of African nightshade causes an increase of fructose concentration to a mean value of 2.0 g/100 g DM, representing approximately 20 % gain (Fig.3.48). Statistical analysis showed no significant differences in the mean values between fermented, freeze-dried fresh and solar dried nightshade leaves in fructose concentration p> 0.05 (Fig.3.48).

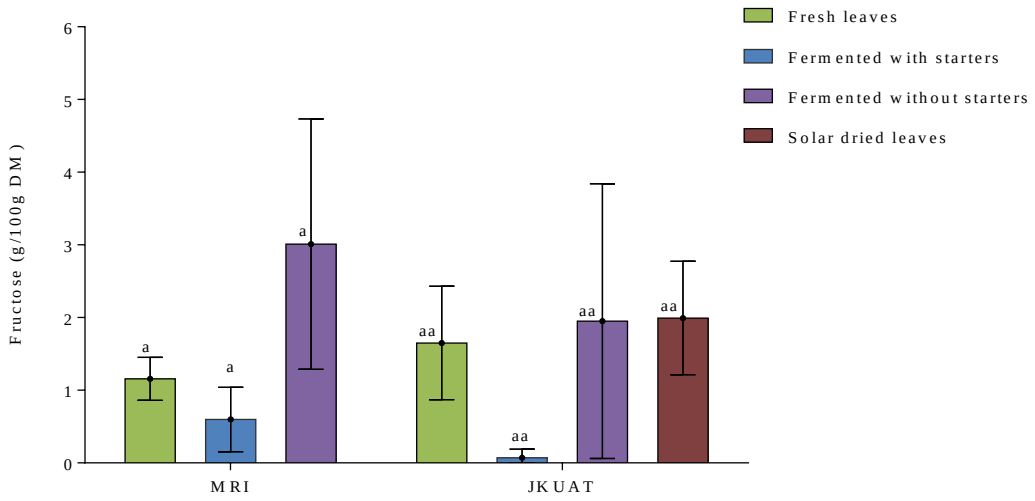

Figure 3.48: Effect of fermentation and solar drying on fructose content of African nightshade leaves. Means followed by the same letter within a variable are not significantly different ($p < 0.05$) according to Tukey's test.

The result for glucose concentration of fresh nightshade leaves grown at MRI greenhouse had a mean value of 1.4 g/100 g DM. However, after fermentation with starter cultures, the concentration of glucose decreased to 1.0 g/100 g DM, representing an about 30 % loss, while fermentation without starter cultures caused increased glucose concentration to 2.5 g/100 g DM, representing a ca. 79 % gain (Fig.3.49). The experiments carried out at JKUAT showed that the freeze-dried fresh nightshade leaves had glucose mean value of 3.5 g/100 g DM, however, after fermentation with or

without starter cultures, glucose was completely depleted to a mean concentration of 0.0 g/100 g DM. The results further revealed that solar drying of nightshade leaves caused increased glucose concentration to a mean value of 6.7 g/100 g DM representing approximately 92 % gain (Fig.3.49). Statistical analysis showed the differences in the mean values of glucose concentration among fresh, fermented leaves with or without starter cultures were not significant for the samples grown at MRI greenhouse $p>0.05$ (Fig.3.49). However, the samples grown at JKUAT field showed a significant difference between fresh and fermented leaves with or without starter cultures $p<0.05$, nevertheless no significant difference was observed between fresh and solar dried leaves $p>0.05$, but there was a significant difference between fermented with or without starter cultures and solar dried nightshade leaves $p<0.05$ (Fig.3.49).

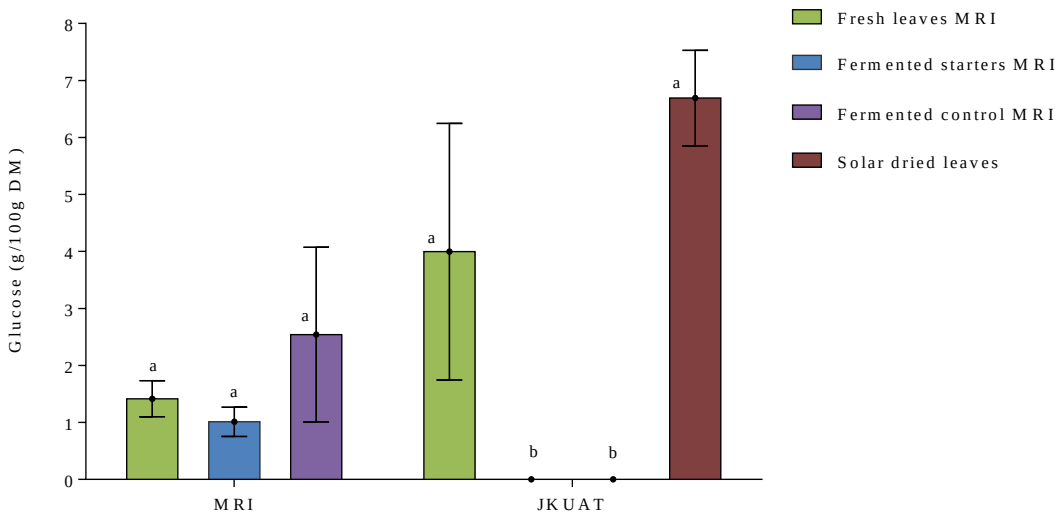

Figure 3.49: Effect of fermentation and solar drying on glucose content of African nightshade leaves. Means followed by the same letter within a variable are not significantly different ($p < 0.05$) according to Tukey's test.

The result for sucrose concentration of fresh nightshade leaves grown at MRI greenhouse had a mean value of 0.21 g/100 g DM. However, after fermentation with starter cultures, the concentration of sucrose increased to 0.33 g/100 g DM representing about 57 % gain, while fermentation without starter cultures caused an increase in sucrose concentration to 0.34 g/100 g DM representing approximately 62 % gain (Fig.3.50). The experiments carried out at JKUAT showed that the freeze-dried fresh nightshade leaves had sucrose mean value of 0.14 g/100 g DM, however, after fermentation with starter cultures sucrose increased to a mean of 0.15 g/100 g DM representing 4 % gain, while fermentation without starter cultures showed an increase to 0.25 g/100

g DM representing about 76 % gain. The results further showed that solar drying of nightshade leaves caused increased sucrose concentration to a mean value of 2.47 g/100 g DM representing > 100 % gain (Fig.3.50).

Statistical analysis showed the differences in the mean values of sucrose concentration among fresh, fermented leaves with or without starter cultures were not significant for the samples grown at MRI greenhouse $p>0.05$ (Fig.3.50). However, the samples grown at JKUAT field showed a significant difference in sucrose concentration among solar dried fermented with or without starter cultures and fresh leaves $p<0.05$. Nevertheless no significant difference was observed between fresh and fermented leaves with or without starter cultures $p>0.05$, there was also significant difference between solar dried and fermented leaves without starters $p>0.05$ (Fig.3.50).

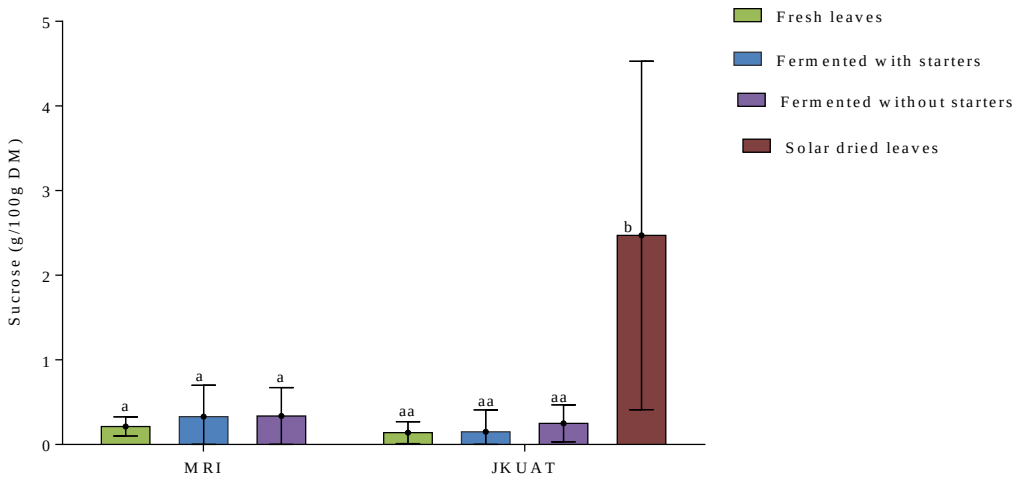

Figure 3.50: Effect of fermentation and solar drying on sucrose content of African nightshade leaves. Means followed by the same letter within a variable are not significantly different ($p < 0.05$) according to Tukey's test.

3.13 Sensory evaluation of nightshade fermented leaves with starter cultures

The African nightshade leaves were fermented for 144 h at 25 ^{o}C with starter culture *Lb. plantarum* BFE 5092 and *Lb. fermentum* BFE 6620, after which the fermented and fresh normally prepared (unfermented) leaves were subjected to 20 untrained panellists for sensory evaluation of colour, smell, appearance, taste, feel in the mouth and general acceptability as described in section 2.19. The mean values of sensory evaluation scores are summarised in the table below.

Table 3.29: Summary of sensory evaluation scores of freshly prepared and fermented African nightshade leaves

	Postharvest process		Gender		Recommendation (fermented vs unfermented	
Attribute	**Fermented**	**Unfermented**	**Male**	**Female**	**Yes**	**No**
Colour	6.8 ± 1.7^{a}	7.9 ± 1.0^{a}	6.1 ± 1.2^{a}	6.5 ± 2.0^{a}	7.1 ± 1.1^{a}	5.3 ± 1.9^{a}
Appearance	6.7 ± 1.5^{c}	8.5 ± 0.6^{d}	6.8 ± 1.2^{a}	6.5 ± 1.7^{a}	6.6 ± 1.7^{a}	6.8 ± 0.4^{a}
Smell	6.7 ± 1.2^{e}	8.0 ± 0.0^{f}	6.8 ± 0.6^{a}	6.6 ± 1.6^{a}	6.8 ± 0.8^{a}	6.3 ± 2.2^{a}
Taste	6.6 ± 1.1^{g}	8.0 ± 0.0^{h}	6.4 ± 1.0^{a}	6.6 ± 1.2^{a}	6.6 ± 1.1^{a}	6.5 ± 0.9^{a}
Mouth feel	6.3 ± 1.3^{i}	8.5 ± 0.5^{j}	6.8 ± 1.5^{a}	5.8 ± 1.0^{a}	6.1 ± 1.2^{a}	6.8 ± 1.9^{a}
Acceptability	5.3 ± 1.8^{k}	8.0 ± 0.7^{l}	5.8 ± 1.9^{a}	4.8 ± 1.6^{a}	5.8 ± 1.5^{d}	3.0 ± 0.8^{e}

- Values are means ±SD
- Means not sharing a superscript in a group (postharvest process/gender/recommendation) within a row are significantly different ($P<0.05$)
- A 9-point hedonic rating scale (9= Excellent; 1= Very bad)
- Gender; male (45 %), female (55 %)
- Recommendation; Yes (80 %), No (20 %)
- Knowledge of fermented vegetables; Yes (75 %), No (25 %)

The results showed that 55 % of the panellists were female while 45 % were male, 80 % of the panellists said would recommend eating fermented vegetables and 20 % declined to recommend and 75 % said they had knowledge of vegetable fermentation, while 25 % did not. The mean score value for colour of the normally prepared (unfermented) and fermented samples ranged from 6.8 (satisfying) to 7.9 (good) respectively ($p>0.05$). The mean score values for appearance, smell, taste and feel in the mouth of fermented and unfermented leaves ranged from 6.3 (satisfying) to 8.5 (very good)($p<0.05$).

The general acceptability of the fermentation technology was evaluated by the panellists comparing fermented and unfermented leaves with the mean score values ranging from 5.3 (moderate) to 8.0 (very good), respectively ($p<0.05$). The male and female panellists all said the appearance and smell of the fermented vegetables was good (mean score 6.5-6.8). The female panellists cited colour and taste as good (mean score 6.5-6.6), with the feel in the mouth as satisfying (mean score 5.8). On the other hand, the male panellists cited feel in the mouth as good (mean score 6.8) with taste and colour as satisfying (mean score 6.1-6.4). Those who said that they would recommend eating fermented vegetables cited the colour, smell, taste and appearance as good (mean score 6.6-

7.1), but said the feel in the mouth was satisfying. However, those that declined to recommend eating of fermented vegetables, cited colour as moderate (mean score 5.3 and further said fermented vegetables are insufficient (mean score 3.0) as compared to fresh or unfermented (Tab.3.29).

3.14 Storage experiments for fresh and solar dried nightshade leaves

The storage experiments were performed in duplicate to determine the effects of solar drying on the nutritional quality and microbial safety of African nightshade after storage. Microbial analysis were carried out for enumeration of total enterobacteria, total aerobic mesophilic bacterial count, lactic acid bacterial count and for yeast and moulds on both freshly harvested leaves and after solar drying, followed by storage at 10 ^{o}C and 25 ^{o}C for 4 weeks. Nutrient analyses were determined from fresh, after solar drying and after storage different temperatures.

The total enterobacterial counts were determined on VRBG agar plates with the detection limit for of 1 x 10^2 cfu/ml. The results in figure 3.51 showed that freshly harvested vegetables had a mean total enterobacterial count of about 1 x 10^5 cfu/ml. However, after solar drying and storage at 10 ^{o}C for one week, this mean count declined by almost 1 log, but later increased again to 10^5 cfu/ml and remained at this level also at the fourth week of storage (Fig.3.51). The same trend was observed for the leaves stored at 25 ^{o}C, which showed that after a week of storage the mean enterobacterial count was nearly at the same level as freshly harvested enterobacterial count at 10^5 cfu/ml. The counts remained unchanged until end of storage where they dropped by almost 1 log unit to 10^4 cfu/ml (Fig.3.51).

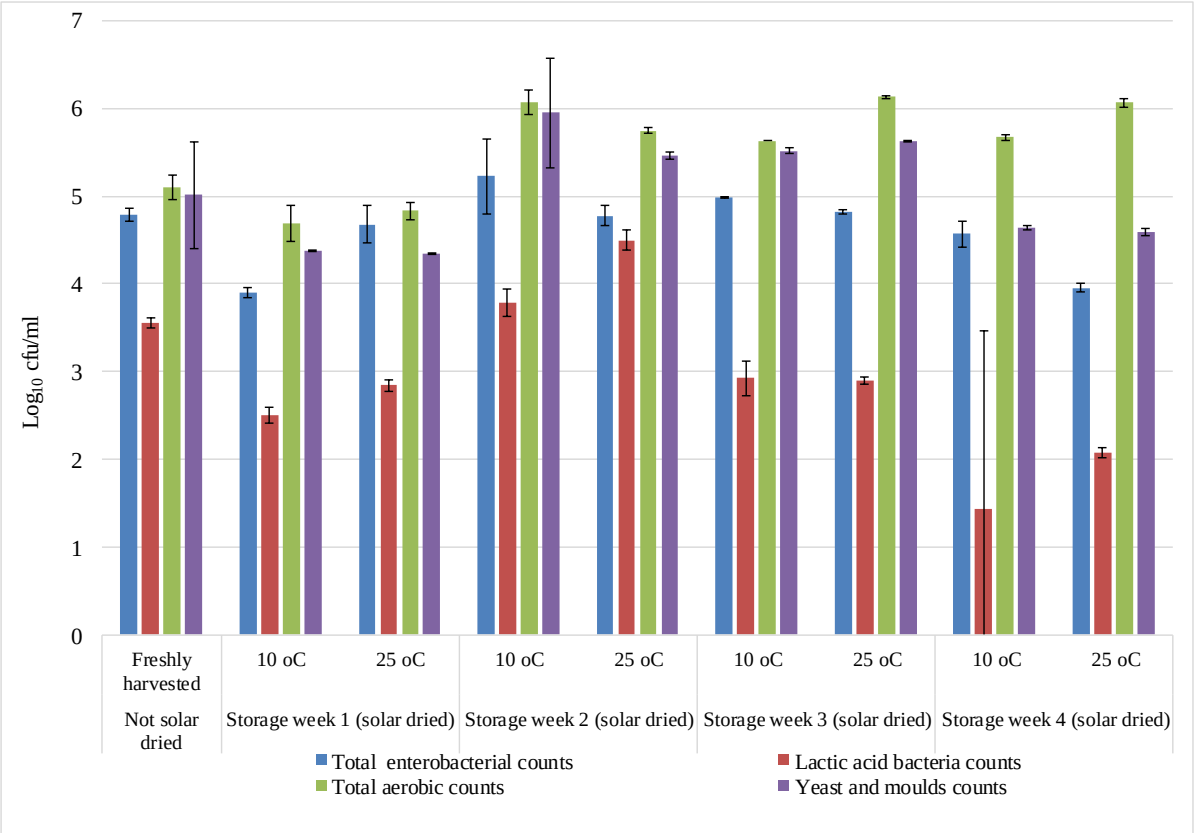

Figure 3.51: Mean lactic acid bacteria counts, total aerobic mesophilic colony counts, total enterobacteria counts and yeast and moulds counts from duplicate experiments of freshly harvested and solar dried nightshade leaves stored for 4 weeks at 10 ^{o}C and 25 ^{o}C at JKUAT, Kenya.

The mean LAB count from fresh leaves was ca.10^3 cfu/ml, which dropped to about 10^2 after one week storage both at 10 ^{o}C and 25 ^{o}C. The mean LAB count increased after week two at both 10 ^{o}C and 25 ^{o}C to ca. 10^3 cfu/ml and 10^4 cfu/ml respectively. This count decreased from week three to week four up to about 10^1 cfu/ml at 10 ^{o}C and 10^2 cfu/ml at 25 ^{o}C (Fig.3.51). The mean total aerobic, mesophilic counts in fresh leaves were 10^5 cfu/ml after one week of storage; the microbial counts at 10 ^{o}C and 25 ^{o}C were almost similar with fresh leaves. However, from week two to week four, the counts increased by about 1 log cfu both at 10 ^{o}C and 25 ^{o}C reaching highest count of 10^6 cfu/ml (Fig.3.51). The mean yeast and moulds count from fresh leaves was 10^5 cfu/ml, this count however, dropped to about 10^4 cfu/ml both at 10 ^{o}C and 25 ^{o}C after one week storage. The yeast and moulds reached highest count of 10^6 cfu/ml after two weeks at 10 ^{o}C. Weeks three and four saw the reduction of counts to about 10^5 at 10 ^{o}C and 25 ^{o}C, however, at the end of storage time, the yeast and moulds count were about 10^4 cfu/ml (Fig.3.51).

3.15 Effects of solar drying and storage temperature on nutrient composition

The changes in the composition of basic nutrients such vitamins (B_1, B_2, C, E, ash, protein and soluble sugars (fructose, glucose and sucrose) was performed on fresh, solar dried and solar dried and stored at 10 ^oC and 25 ^oC nightshade leaves grown at JKUAT open fields. The result in figure 3.52 showed that solar drying of nightshade leaves led to slight increase in ash content.

3.15.1 Analyses of ash and protein contents

The mean value of ash in fresh leaves was 13.3 g/100 g dry mass (DM) after solar drying the ash content increased to 16.4 g/100g DM, representing ca. 23 % gain. The storage of solar dried leaves at 10 ^oC and 25 ^oC led to increased ash contents of 17.0 g/100 g DM and 16.2 g/100 g DM respectively representing an increase of between 21-27.8 % in both cases. Statistical analysis using the Tukey's test showed no significant difference ($p > 0.05$) among fresh, solar dried and solar dried samples stored at either 10 ^oC or 25 ^oC . Solar drying and storage at 25 ^oC decreased the protein from 12-13.9 % while storage at 10 ^{oC} slightly increased it by about 5 % However, these reductions/gains were not significantly different ($p > 0.05$) (Fig.3.52).

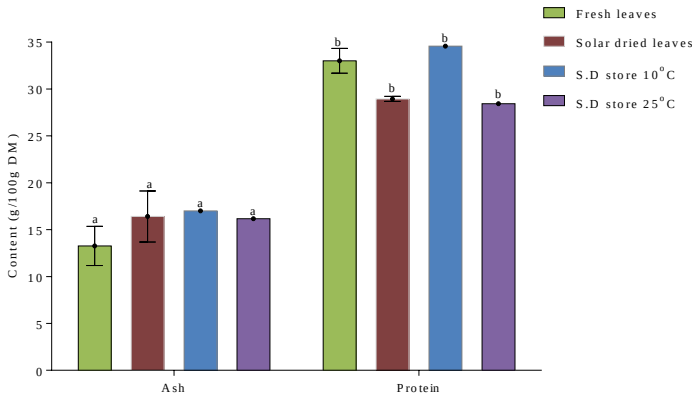

Figure 3.52: Effect of solar drying and storage temperature on mean ash and protein contents of African nightshade leaves. Means followed by the same letter within a variable are not significantly different ($p \leq 0.05$) according to Tukey's test.

3.15.2 Analyses of vitamin C and E contents

Solar drying and storage at 10 ^{o}C and 25 ^{o}C cause a reduction of water soluble vitamin C contents. The freeze-dried fresh leaves had a mean value of 206.2 mg/100 g vitamin C content of DM, after solar drying the value decreased to 54.5 mg/100 g representing ca. 73.5 % loss, storage at 10 ^{o}C let to a complete loss of vitamin C, while storage at 25 ^{o}C resulted in an 89.7 % loss (Fig.3.53). The results showed there was a significance difference in vitamin C content between fresh, solar dried and those stored at either 10 ^{o}C or 25 ^{o}C $p > 0.05$. The result showed no significance difference between solar dried leaves and stored at 10 ^{o}C or 25 ^{o}C $p< 0.05$.

The results for fat-soluble vitamin E showed that fresh leaves had a mean value of 22.7 mg/100 g vitamin E content of DM. After solar drying, the value increased to 25.1 mg/100 g representing about 10.6 % gain (Fig.3.54). The storage at 10 ^{o}C let to an increase to 26.5 mg/100 g representing a 16.7 % increase in vitamin E concentration, while storage at 25 ^{o}C resulted in a 2 % reduction. However, statistical analysis showed that there was no statistically significant difference of vitamin E content among freeze-dried fresh, solar dried leaves and solar dried leaves stored at either 10 ^{o}C or 25 ^{o}C ($p > 0.05$)

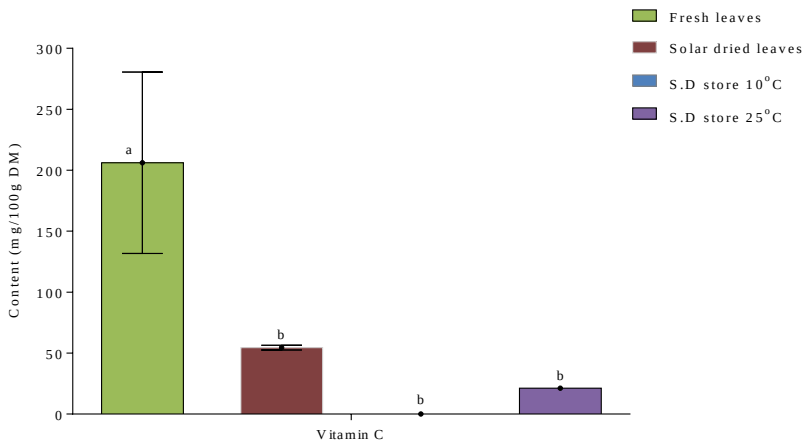

Figure 3.53: Effect of solar drying (S.D) and storage temperature on mean vitamin C contents of African nightshade leaves. Means followed by the same letter within a variable are not significantly different ($p \leq 0.05$) according to Tukey's test.

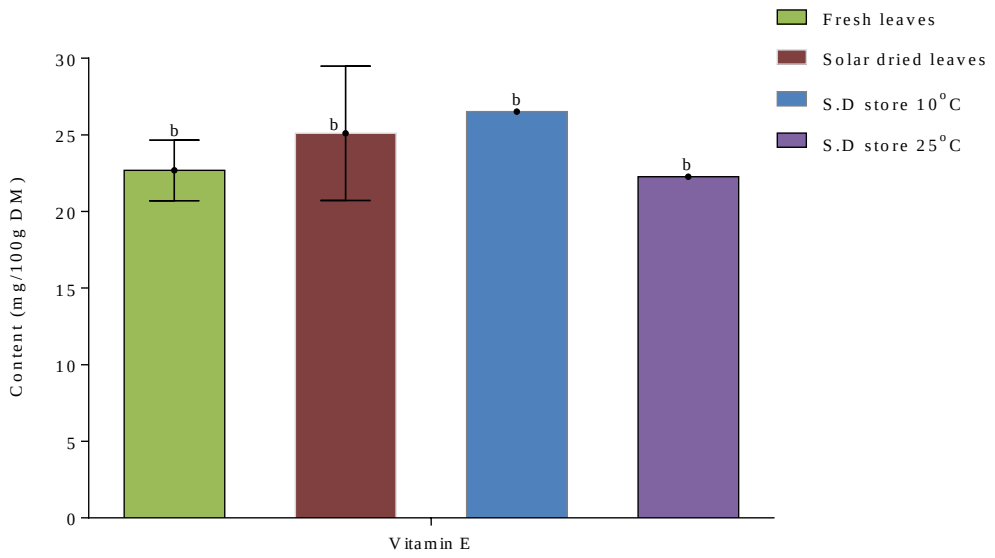

Figure 3.54: Effect of solar drying (S.D) and storage temperature on mean vitamin E contents of African nightshade leaves. Means followed by the same letter within a variable are not significantly different ($p \leq 0.05$) according to Tukey's test.

3.15.3 Analyses of vitamin B_1 and B_2 contents

The fresh nightshade leaves had vitamin B_1 mean value of 683.7 µg/100 g DM. However, after solar drying, the concentration decreased to a mean value of 474.3 µg/100 g DM representing about 30.6 % reduction, while storage at 10 ^{o}C and 25 ^{o}C showed mean values of 573.7 µg/100 g and 469.8 µg/100 g DM correspondingly, which represented reductions of 16 % and 31 %, respectively. Statistical analysis showed that there was a significance ($p < 0.05$) difference of vitamin B_1 content among freeze-dried fresh leaves and solar dried leaves. There was also a statistically significant ($p < 0.05$) difference between fresh and solar dried leaves stored at 25 ^{o}C , but not between fresh and solar dried leaves stored at 10 °C. Moreover, there was also a statistical difference ($p< 0.05$) between the solar dried leaves stored at the different temperatures of 10 ^{o}C and 25 °C. (Fig.3.55)

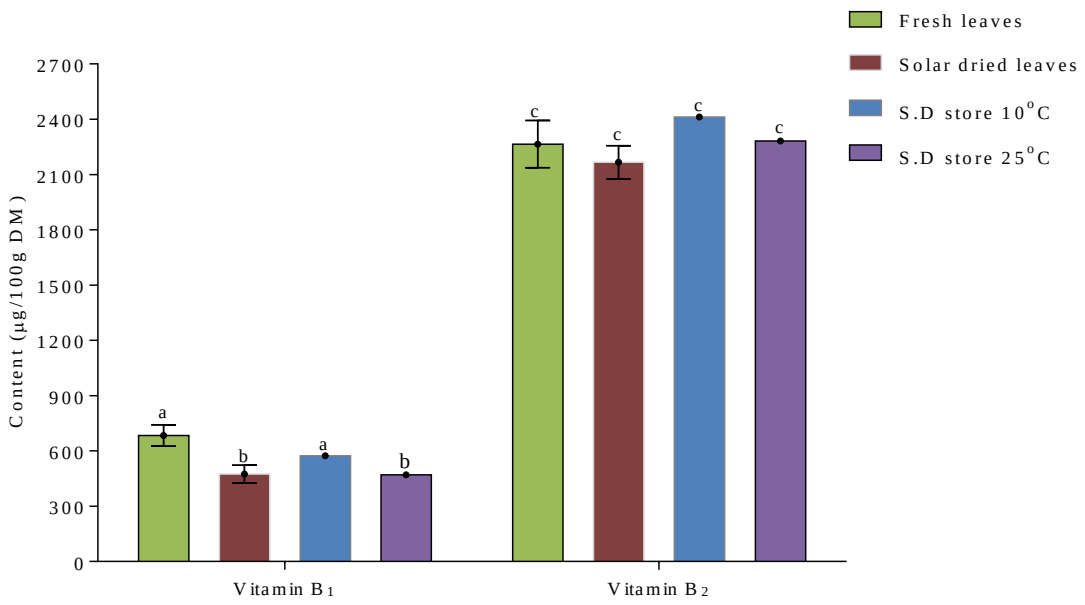

Figure 3.55: Effect of solar drying (S.D) and storage temperature on mean vitamin B_1 and B_2 contents of African nightshade leaves. Means followed by the same letter within a variable are not significantly different ($p \leq 0.05$) according to Tukey's test.

For vitamin B_2, freeze-dried fresh nightshade leaves had mean value of 2264.9 µg/100 g DM. After solar drying, it was determined to be somewhat less at a mean value of 2165.9 µg/100 g DM, representing a 4.4 % reduction, while storage at 10 ^{o}C and 25 ^{o}C mean values of 2412 µg/100g and 2282 µg/100 g DM, respectively, could be determined, which represented a small increase of 6.5 % and 1 % respectively (Fig.3.55). However, the results revealed there was no significance difference in vitamin B_2 content among freeze-dried fresh nightshade leaves, solar dried and those stored at either 10 ^{o}C or 25 ^{o}C ($p > 0.05$).

3.15.4 Analyses of soluble sugars

The mean concentration of fructose in fresh nightshade leaves was 2.0 g/100 g DM. However, solar drying caused the reduction of fructose to a mean value of 1.1 g/100g DM representing about 45 % loss, while storage at 10 ^{o}C and 25 ^{o}C decreased fructose to the mean value of 0.88 g/100g and 1.1 g/100 g DM, representing 56 % and 45 % loss, respectively. Statistical analysis showed no significant differences in fructose content among fresh nightshade leaves, solar dried and those stored at either 10 ^{o}C or 25 ^{o}C $p > 0.050$ (Fig.3.56).

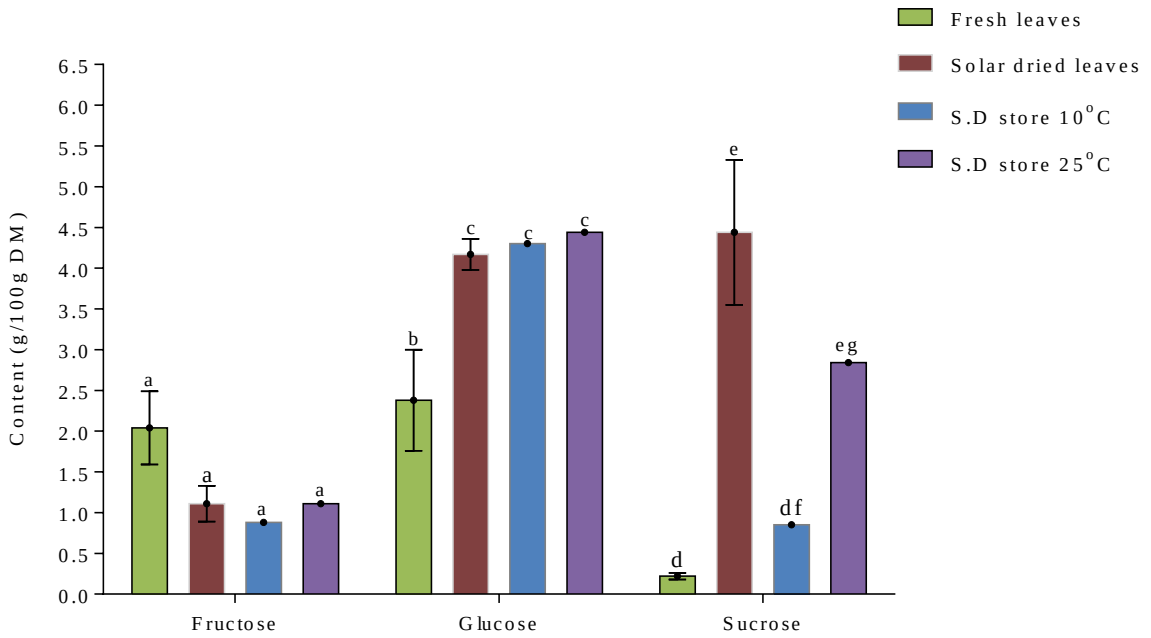

Figure 3.56: Effect of solar drying (S.D) and storage temperature on soluble sugars contents of African nightshade leaves. Means followed by the same letter within a variable are not significantly different ($p \leq 0.05$) according to Tukey's test.

The mean concentration of glucose in fresh nightshade leaves was 2.4 g/100 g DM. However, solar drying increased glucose to the mean value of 4.2 g/100 g DM representing about 75 % gain, while storage at 10 ^{o}C and 25 ^{o}C increase glucose to the mean value of 4.3 g/100 g and 4.4 g /100g DM, respectively, each representing a 79 % and an 83 % gain. Statistical analyses showed significant difference in glucose content between fresh nightshade leaves and solar dried and between fresh and solar dried and stored at either 10 ^{o}C or 25 ^{o}C ($p< 0.05$). However, no significant difference in glucose content was observed among, solar dried leaves and solar dried and stored at either 10 ^{o}C or 25 ^{o}C ($p> 0.05$) (Fig.3.56).

The result further showed that sucrose content in fresh nightshade leaves was 0.22 g/100g DM. However, solar drying glucose increased to the mean value of 4.4 g/100 g DM representing about over 100 % gain, while storage at 10 ^{o}C and 25 ^{o}C increased sucrose to the mean value of 0.85 g/100g and 2.8 g/100 g DM respectively representing a >50 % increase.

4.0 Discussion

The aim of this study was to determine the effects of fermentation and solar drying methods on the safety and quality of AILVs in order to minimize postharvest losses, specifically for the fermentation of African nightshade leaves. African nightshade is one of the widely consumed indigenous leafy vegetables in Africa due to its richness in vitamins, beta carotene, protein and minerals (Lebotse & Lyatuu, 2010; Smith & Eyzaguirre, 2007). However, in many African countries, indigenous leafy vegetables are consumed fresh or solar/sun dried, little is known about the fermentation of African leafy vegetables (Mepba et al., 2007; Oguntoyinbo et al., 2016). Muchoki et al. (2007) and Kasangi et al. (2010) conducted studies on the effects of spontaneous fermentation on the nutritional contents of cowpea leaves and reported positive effects of fermentation on nutrient retention, degradation of anti-nutritional factors as well as extension of shelf life of leafy vegetables. The most recent published data on fermentation of AILVs was on kale (*Brassica carinata*) and Oguntoyinbo et al. (2016) reported that fermentation of kale leaves with selected LAB starters provides a promising avenue to prevent spoilage and improve shelf life and safety.

The ability of different potential starter LAB to grow on nightshade leaves was first tested in a pilot-scale fermentation for the selection of suitable starter cultures. The fermentation conditions were first established and optimized in controlled trials and for this, various salt and sugar concentrations, fermentation vessels, and LAB strains were tested. The starter strains were checked for their ability to rapidly and deeply lower the pH as a result of their lactic acid production and the absence of the formation of slime and smell were considered important selection criteria (Gopal et al., 2005). There was a particular concern as to whether nightshade leaves contain enough fermentable sugars to support the microbial growth and lead to a satisfactory fermentation. Cabbage used in Sauerkraut production contains about 1.2 to 2.5 % of fermentable sugar (Harris, 1998) which is sufficient to carry out fermentation without additional sugar, while other green leafy vegetables with lower sugar content may require addition of sugar of about 1-3 % for fermentation (Kasangi et al., 2010; Oguntoyinbo et al., 2016; Viander et al., 2003). Inoculation with starter cultures contributes to rapid acidification under pH 4.2, which inhibits growth of a wide range of pathogenic bacteria, thus ensuring food safety (Holzapfel, 1997; Leroy & De Vuyst, 2004; Motarjemi, 2002; Oguntoyinbo et al., 2016). The lactic acid bacteria starter strains used in this

study stemmed from fermented cassava and were selected based on their previous established abilities to rapidly produce acids and bacteriocins (Cho et al., 2010; Kostinek et al., 2007).

In order to gain more detailed information about the inoculated starter cultures on the background autochthonous microbiota in the fermentations, DGGE and metagenomics analyses using high throughput 16S rRNA amplicon sequencing were investigated on both starter cultures inoculated and uninoculated batches performed both at MRI and JKUAT. The total aerobic mesophilic and LAB counts were determined to give an overview of the growth kinetics, while enterobacteria and yeast and moulds counts were used to give an overview of possible microbial contamination with potential spoilage and/or pathogenic microorganisms. The determination of pH and lactate and sucrose/D-glucose by enzyme assays were used to study fermentation dynamics. The success of the inoculated selected starter strains during fermentation was investigated on both starters inoculated and uninoculated trials by RAPD-PCR and 16S rRNA sequencing. The effects of fermentation on safety, nutritional quality and sensory attributes were evaluated by basic nutrient analysis, assessing sensory attributes of fermented leaves and challenge tests with the human pathogenic *Salmonella* and *Listeria*. While the effects of solar drying and storage temperature on the nutritional quality and microbial safety was studied by analyzing basic nutrients and total microbial enumeration. In this study nightshade leaves were fermented both at MRI (Karlsruhe, Germany) and JKUAT (Kenya) with the same experimental design and conditions.

Selection of suitable starter culture strains and establishment of fermentation conditions for nightshade leaves

The results showed that the addition of starter cultures led to a faster and deeper reduction of pH values as opposed to spontaneous fermentations (without the use of starter culture) (Fig.3.15). The use of starter cultures in a controlled experiment led to rapid reduction of the pH due to production of organic acids i.e. lactic acid and acetic acids, depending on the nature of the starter strain (hetero- or homofermentative). Furthermore, the influence of salt or salt-sugar concentration on the pH values was determined. For example, in the fermentation trials with only salt solution and without use of starter cultures, no stable pH reduction could be achieved (Fig.3.15). The study showed a combination of salt and sugar (3 % each) as brine led to the quickest and deepest pH-reduction kinetics. The starters reduced the pH< 5.0 within 24 h and further dropped to pH < 4.0 after 144 h. In control batches without starter cultures and with sugar-salt solution (3 % each), the

pH decreased slowly and after 24 still was > 6.0, but it dropped to pH <4.0 after 48 h and remained at this level until 144 h of fermentation (Fig.3.15). Therefore, the relationship between the usage of starter cultures and application of salt-sugar solution (3 % each) were clear. In Sauerkraut fermentation, it is known that the addition of 2-3 % salt can result in the rapid reduction of pH ranging between to 3.0-4.0 (Pundir & Jain, 2010). Moreover, Kasangi et al. (2010) demonstrated that by fermenting cowpea (*Vigna unguiculata*) leaves with the addition of 3 % glucose, it was possible to achieve pH lower than 4.7.

The nightshade leaves used in this experiment had approximately 0.9 g/100 g (0.9 %) for MRI grown and 1.8 g/100 g (1.8 %) for JKUAT grown (section 3.12.4) of the fermentable sugars (fructose, glucose and sucrose), which was clearly far too low to support growth of the LAB starter strains for a rapid acidification of the raw material. Therefore, 3 % (salt-sugar solution) was established as one of the conditions for nightshade fermentation in order to achieve a reliable fermentation. The starter cultures tested in this trial included *Lb. plantarum* BFE 5092, *Lb. plantarum* BFE 6710, *Leuc. mesenteroides* subsp. *mesenteroides* BFE 7668, *Lc. lactis* BFE 902, *Ped. acidilactici* BFE 2300, *Lb. fermentum* BFE 6639, *Lb. fermentum* BFE 6662, *Lb. fermentum* BFE 6620, *Lb. fermentum* BFE 6700, *Lb. fermentum* BFE 6674, *Weissella paramesenteroides* LC 11 and *W. paramesenteroides* LC 18. They were tested on their abilities to cause rapid acidification of the raw material and they all stemmed from previously fermented African food products (Kostinek et al., 2005). The experiments were carried out at room temperature at approximately 25 ^{o}C, since according to Muchoki et al. (2007), the best fermentation temperatures for African leafy vegetables are between 22 ^{o}C-25 ^{o}C.

The pH values of all tested starter cultures showed that the use of 3 % (sugar-salt solution) gave similar pH patterns (Fig.3.15). However, the *Lb. plantarum* BFE 5092, *Lb. fermentum* BFE 6620 and *W. paramesenteroides* LC 11 strains were selected based on how fast and deep they reduced the pH within 48 h. For example, *Lb. plantarum* BFE 5092 scored highest with deepest pH reduction of < 4.0 after 24 h, while, *Lb. fermentum* BFE 6620 and *W. paramesenteroides* LC 11 reduced the pH below 4.0 only after 48 h (Fig.3.15). Therefore, *Lb. plantarum* BFE 5092 and *Lb. fermentum* BFE 6620 were selected for the trials to test the influence of different sugar-salt concentrations (2.5 % and 3.0 % each) on the fermentation dynamics and the development of the starter and the autochthonous microbiota development. *Leuc. mesenteroides* subsp. *mesenteroides* BFE 7668 was not used in further experiments due to its classification as risk group two organism

(as human pathogen) in Germany. Based on the pilot small-scale starter culture combination trials, the use of the *Lb. plantarum* BFE 5092 and *Lb. fermentum* BFE 6620 starter strains and 3 % (salt-sugar solution) combination showed the quickest and deepest pH reduction below 4.0 within 24 h (Fig.3.16). LAB counts were higher with 3 % (salt-sugar solution) than with 2.5 % (salt-sugar solution), there was also a complete inhibition of enterobacteria and yeast and moulds with 3 % (salt-sugar solution) as brine within 24 h of fermentation (results not shown). Thus these conditions were selected for subsequent fermentations of nightshade leaves. Oguntoyinbo et al. (2016) showed that fermentation of kale (*Brassica carinata*) inoculated with starter strains *Lb. plantarum* BFE 5092 and *Lb. fermentum* BFE 6620, rapidly reduced the pH from 6.0 to 3.6 within 24 h without the addition of external sugar, as kale, different to the African nightshade in this study, contained sufficient levels of fermentable sugars to support the fermentation.

The ability of the starter strains to produce antimicrobial compounds may help to dominate the fermentation, indeed, *Lb. plantarum* BFE 5092 is known to possess genes for production of three plantaracins i.e. EF, JK and N (Cho et al., 2010) as could be confirmed by whole genome sequencing (Oguntoyinbo et al., 2016). In contrast, *Lb. fermentum* BFE 6620 does not possess bacteriocin genes as such genes could not be identified from whole genome sequencing data (Wafula et al., 2017). Also from the genome sequencing data, the bacteria are able to reduce indigestible alpha-galactoside i.e. raffinose and stachyose (Oguntoyinbo et al., 2016; Wafula et al., 2017) that are responsible for abdominal discomfort and flatulence (Granito et al., 2005). *Lb. fermentum* BFE 6620 can synthesise vitamin B_6 due to the presence of pyridoxamine 5'-phospate oxidase (Wafula et al., 2017). These are useful characteristics of good starter strains. However, genes for vitamin B_1 and B_2 production were not present on the chromosome of both starter strains.

Nightshade fermentation in 5 litre culture buckets at MRI/JKUAT with selected *Lb. plantarum* BFE 5092 and *Lb. fermentum* BFE 6620 starter strains

After successful small-scale fermentation trial testing of the starter strains and fermentation conditions, *Lb. plantarum* BFE 5092 and *Lb. fermentum* BFE 6620 were used in all subsequent fermentations both at MRI, Karlsruhe and in JKUAT, Kenya in 5 litre fermentation vessels in the presence of 3 % (salt-sugar solution). The LAB are known to grow on MRS agar as well as on Std.I agar media (De Man et al., 1960; Pathak et al., 2012). The comparison of LAB counts with the total aerobic mesophilic counts were used to determine the presumed contribution of inoculated LAB

starters in the fermentation. The results showed that the LAB cell counts corresponded to the total aerobic mesophilic counts, indicating that the aerobic total plate count constituted mostly of lactic acid bacteria. The use of *Lb. plantarum* BFE 5092 and *Lb. fermentum* BFE 6620 as starter cultures for nightshade fermentation led to a fast increase of the LAB count, reaching a maximum of > 1 x 10^8 cfu/ml after 24 h to 48 h. After this, the LAB counts remained constant up to 72 h, and then decreased to 1 x 10^7 cfu/ml which was slightly above the initial inoculation concentration (Fig.3.17). In association with this growth, there was a quick and deep pH decrease to below 3.7 within 24 h, and it remained at this low level until 144 h of the fermentation (Fig.3.19).

In the control/uninoculated fermentations, the increase in LAB and total aerobic mesophilic counts were much slower. Growth of LAB was observed after 24 h but reached maximum counts > 10^7 cfu/ml (MRI) and 10^8 cfu/ml (JKUAT) only after 48 h. After 144 h of fermentation, the LAB and total aerobic mesophilic counts were slightly higher than in the starter culture inoculated batch, due to the presence of autochthonous LAB that facilitated a 'natural' fermentation (Fig.3.18). As result of this delayed growth, the pH reduced much more slowly than the starter culture inoculated batch, staying above 4.0 after 24 h, but finally reached < 4.0 after 144 h of fermentation (Fig.3.19). The standard deviations of the pH values of the control fermentations (without starters) were rather high from their equivalent mean values, which indicated much more variability in the control fermentations (Fig.3.19). It is known that plant materials harbours different types of LAB microbiota, such as *Leuconostoc mesenteroides* and *Lactobacillus* spp. (Maria, 2005; McFeeters et al., 2013). The analysis of different white cabbage samples showed that 0.15 % to 1.5 % of the total bacterial population were LAB, although they were present in only small population, they have indispensable metabolic activities that enable them to dominate spontaneous vegetable fermentations (Maria, 2005; McFeeters et al., 2013). Other studies have already shown the involvement of LAB in spontaneous fermentation of different plant material. For example Kostinek et al. (2007) identified different LAB involved in spontaneous fermentation of cassava. The genera *Lactobacillus* and *Leuconostoc* play an important role in the spontaneous fermentation of kimchi, a Korean fermented vegetable (Kim & Chun, 2005; Lee et al., 2006). Sauerkraut is produced by means of spontaneous fermentation of cabbage also by LAB (Adams & Moss, 2008; Halász et al., 1999).

In the starter culture inoculated fermentation, enterobacteria were detected for up to 72 h. The MRI fermentation showed only low enterobacteria counts, reaching a maximum of about 1 x

10^2 cfu/ml only after 24 h (Fig.3.17), while in the JKUAT fermentation, maximum enterobacteria counts were higher reaching 1 x 10^4 cfu/ml after 24 h to 48 h (Fig.3.17). Interestingly, at this fermentation time points the LAB counts were highest with the pH below 4.0. The enterobacteria counts were reduced after 48 h and completely inhibited after 72 h, when the pH was reduced to < 3.5 (Fig.3.19). The reduction of the pH < 4.0 due to LAB fermentation is an important inhibitory factor against many pathogenic bacteria (Stringer, 2004). Kim (1996) reported that microbiological safety of food could be guaranteed if the growth of food pathogens is inhibited early in the growth phase of fermentation.

Contrary to the starter culture inoculated batches; spontaneous fermentation was characterized by high number of enterobacteria counts throughout the entire fermentation, and it can be assumed that the slow reduction in the pH could be responsible. Those fermented at MRI showed a complete inhibition of enterobacteria only after 72 h (Fig.3.18); at this point the pH was below 4.0. This study was in agreement with Oguntoyinbo et al. (2016) who reported that *Lb. plantarum* BFE 5092 and *Lb. fermentum* BFE 6620 starter strains are able to utilize simple sugars in African kale and quickly reduce the pH < 4.0 within 24 h. The JKUAT spontaneous fermentation showed that even though the pH was < 4.0, the enterobacteria could still be detected to about 10^4 cfu/ml after 144 h of the fermentation. This is exactly why *Lb. plantarum* BFE 5092 and *Lb. fermentum* BFE 6620 starter cultures were chosen for fermentation, because they produce sufficient lactic acid to bring about a rapid acidification which can inhibit spoilage and potentially pathogenic bacteria. Besides, *Lb plantarum* BFE 5092 has been reported to cause not only rapid acidification, but also the production of bacteriocins which may be responsible for additional antimicrobial effects (Cho et al., 2010; Kostinek et al., 2007). The above results clearly showed that fermentation of nightshade leaves without added starter cultures led to an unpredictable and slow decrease in pH as a result of an unpredictable growth and acid production of natural lactic acid bacteria. Furthermore, the growth of enterobacteria indicated that fermentation without starter bacteria also compromises the safety of these products, as other, potentially pathogenic bacteria, are not inhibited by sufficient growth and acid production of the lactic acid bacteria.

There were no yeast and moulds detected in MRI fermentation done with starter cultures, and these occurred only in the control fermentation at 0 h (Fig.3.18). Ahlberg et al. (2015) and Dalié et al. (2010) reported that LAB inhibits the growth of moulds and are able to binds aflatoxins to the cell surface, thus mitigating toxic effects of aflatoxins in food and feed. The JKUAT

fermentation showed that the yeast and moulds were present in starter-inoculated batches until 48 h with pH < 3.5, while in the control they were present up to 72 h with pH < 4.0. However, their counts were insignificant (Fig.3.18). It is known that anoxic conditions contribute to reduction of yeast and moulds. This was expected in this study, since the fermentation was a submerged fermentation conducted in closed, airtight vessels. Massawe & Lifa. (2010) reported-that fermentation of coffee with select LAB starter cultures, inhibited the growth of yeast and moulds after 72 h with the pH around 4.2. Nevertheless, due to every day opening of the vessels for sampling, contamination from the air could have occurred.

Carbohydrates are basic substrate for LAB metabolism; hence, the progress of inoculated starter cultures was studied by determining the sucrose/D-glucose metabolism. Plants based sugars in addition to household sugar-salt brine were used as source of sucrose. The results showed metabolism of sucrose in all fermented batches with or without starter cultures. However, in the starter-inoculated batches, metabolism of sucrose started at 24 h both for MRI and JKUAT fermentations (Fig.3.22). This was due the increase in LAB counts that resulted to rapid consumption of sucrose/D-glucose (Cho et al., 2006). The LAB counts after 24 h were above 1 x 10^8 cfu/ml and remained at this highest level until 72 h (Fig.3.17). The MRI based fermentation had about 7.0 g/l sucrose/D-glucose concentration after 144 h of fermentation, while in the JKUAT based fermentation, the metabolism of sucrose was much quicker than that of the MRI with the approximately 1.0 g/l sucrose/D-glucose recovered after 144 h (Fig.3.22). Metagenomics data by high throughput sequencing showed that in the JKUAT natural fermentation, diverse LAB belonging to genera *Lactobacillus*, *Pediococcus*, *Lactococcus*, *Enterococcus* and *Weissella* were among the most predominant bacteria present. These bacteria are known to predominate most traditional African fermented foods since they are well adapted, and are also able to utilize various sugars including sucrose, fructose and glucose to produce lactic acid by-product. The MRI natural fermentation on the other hand, showed that the genera *Clostridium*, *Bacillus* and *Enterobacter* were among the frequently isolated bacteria (Fig.3.32).

In the uninoculated fermentation, sucrose was metabolized slowly only after 48 h, which was as result of rapid growth of autochthonous LAB, whose counts were slightly above 10^7 cfu/ml (MRI) and 10^8 cfu/ml (JKUAT) after 144 h, and 12.2 g/l and 3.2 g/l of sucrose/D-glucose concentrations were recovered from MRI and JKUAT based fermentation respectively (Fig.3.22). The results hence showed sucrose was quickly metabolized into glucose and fructose, which were

readily available for LAB through glycolysis (Kandler, 1983). Therefore, it was clear that > 75 % of sucrose was utilized during MRI based fermentation while > 95 % was consumed by the inoculated starter cultures at JKUAT based fermentations. Sucrose/D-glucose was metabolized quickly in JKUAT than in MRI fermentations this could be speculated that LAB counts were higher in the JKUAT starter inoculated batch at 24 h and after 144 h that the MRI (Fig.3.17). The same trend was witnessed in spontaneous fermentations with JKUAT fermentation showing higher microbial count than the MRI fermentation (Fig.3.18). This was however, confirmed by DGGE and metagenomics data which showed more microbial diversity in JKUAT than in MRI fermentations (Fig.3.31 & 3.32). As discussed above, the presence of different naturally occurring LAB from JKUAT grown leaves as well as the fact that, JKUAT grown leaves had higher fructose and glucose concentration than those grown at MRI (Fig.3.48 & 3.49) may have been readily available for the fermenting bacteria facilitating their faster growth.

The results are in agreement with Oguntoyinbo et al. (2016), who reported *Lb. plantarum* BFE 5092 possesses 433 genes involved in sugar metabolism, especially those for phosphoenolpyruvate or phosphotransferase system for utilization of various sugars such as sucrose, fructose, glucose and maltose among others. The result by Wafula et al. (2017) showed that the *Lb. fermentum* BFE 6620 contained 88 different sugar coding genes, including PEP/PTS systems for utilization of sucrose, fructose, glucose, trehalose, a malolactic enzyme and a pyridoxamine 5'-phosphate oxidase (vitamin B_6 biosynthesis). Additionally *Lb. fermentum* strains have been shown to possess protein encoding genes necessary for carbohydrates metabolism, especially mannose-6- phosphate isomerase (Jimenez et al., 2010).

Metabolism of carbohydrates by LAB depends on the type of fermentation and which fermentation pathway is involved, i.e. whether the bacteria are homo or heterofermentative. In the homofermentative metabolism, LAB ferments sugars to lactic acid as the main end product either as D- or as L-lactate. While in heterofermentative, LAB ferments sugars to lactic acid, ethanol/acetate and CO_2. The inoculated starters were used to follow progress in the fermentation by determination of lactate accumulation. Lactate was detected after 24 h in both the MRI and JKUAT based fermentations inoculated with starter cultures. In both cases, D- and L-lactate were formed. This was due to faster growth of LAB (>1 x 10^8 cfu/ml) in association with the above mentioned quick and deep reduction of the pH < 4.0 after 24 h. As expected, the levels of lactic acid produced in the starter-inoculated batches increased from ca.1.8 g/l (MRI) and 2.0 g/l to about 5.2 g/l and 4.0 g/l for

MRI and JKUAT based fermentations respectively (Fig.3.20a). In both cases, D- and L-lactate enantiomers were produced (Fig.3.21a) which was in agreement with the fact that *Lb. plantarum* and *Lb. fermentum* produce a mixture of D- and L-stereoisomers of lactic acid (Caplice & Fitzgerald, 1999; Oguntoyinbo et al., 2016). In the spontaneous fermentation, appreciable amounts of lactate were detected after 48 h in both MRI and JKUAT based fermentations, which increased slowly but steadily until 144 h where 4.4 g/l of lactic acid was produced in both cases (Fig 3.20a). Just like in the inoculated batches, both D- and L-lactate enantiomers were produced in almost equal volumes for MRI based spontaneous fermentation, while in the JKUAT based fermentation, more L-lactate was produced at 24 h-48 h and more D-lactate at 72 h-144 h (Fig.3.21b). This results were supported by metagenomics data which showed that the JKUAT spontaneous fermentation genera *Lactococcus* and *Enterococcus* were among the most abundant LAB bacteria present at 24-48 h. These bacteria belong to homofermentative group that only produced L-lactate from sugar metabolism. Hence, presence of L-lactate could be explained by the fact that at this fermentation points more homofermentative LAB were most predominant (Sutic & Banina, 1990), as the fermentation progressed, metagenomics data showed that members of the genera *Lactobacillus*, *Weissella* and *Pediococcus* predominated at 24-144 h. The genus *Lactobacillus* is known to produce D, L or DL-lactate, *Pediococcus* produce L or DL-lactate while *Weissella* is known to produce D or DL-lactate, hence formation of more D-lactate suggested involvement of heterofermentative LAB strains in the fermentation (Benthin & Villadsen, 1995; Chang et al., 1999), especially heterofermentative rods or cocci such as, *Leuconostoc, Weissella* or other *Lactobacilli* (Axelsson, 2004; Endo et al., 2014).

Determination of bacterial community composition by DGGE and high throughput sequencing

DGGE and high throughput sequencing were used for biodiversity studies for the influence of inoculated starter cultures on the microbial composition during nightshade fermentation. The results showed the use of starter cultures gave greatest band similarities with stable continuous pattern after 24 h (Fig.3.27 & 3.28). This was in line with rapid growth of LAB counts and subsequent reduction of pH < 4.0 after 24 h. In the spontaneous fermentation, fewer bands were amplified at 0 h (when compared to the later time points in the fermentation) and the intensities of the bands increased after 24 h and remained stable up to 144 h of the fermentation (Fig.3.27 & 3.28). The comparison of different fermentations revealed greatest similarities of the bands in

starter cultures inoculated at 0-144 h for the MRI fermentation. Different fermentation batches showed similar identical bands that were present in each fermentation batch, as well as specific ones that could be found in specific batches (Fig 3.29). The starter culture inoculated batches showed great similarity to each other at *r* = 90 % The results also showed control fermentation batches seven and eight at 0 h, clustered together with the starter inoculated batches, this meant that the amplified DNA belonged to the naturally resident LAB (Fig 3.29). This could indicate that the starter culture did not establish itself as the dominant LAB in the fermentation.

The JKUAT starter inoculated fermentation showed similar results as MRIs, where comparison analysis revealed each batch was unique with similar identical bands that were present in each fermentation batch, as well as specific ones that could be found in specific batches (Fig.3.30). Hence it was assumed that apart from inoculated starters, other microorganisms could have participated in the fermentation. The similarity analysis revealed the inoculated batches shared approximately at *r* = 87 % with each other. However, control fermentation two at 24-144 h, clustered together with the starter inoculated group sharing similarity at *r* = 87 % with starter inoculated fermentation three at sampling 24 h and 144 h. This suggested that at these particular sampling points, the autochthonous LAB were properly similar in composition with those in the inoculated batch three (Fig.3.30), in which case the lactic starters may not have succeeded in becoming the dominant population. Also the uninoculated batches showed varying banding patterns from 24-144 h, all batches shared similarity at *r* = 87 % (JKUAT) and at *r* = 62 % (MRI) in the fermentations (Fig.3.29 & 3.30). This may indicate that when uninoculated the microorganisms which eventually dominate the fermentation will vary and this may depend on which microorganisms were initially present on the leaves.

The metagenomics analyses revealed that the starter inoculated fermentations were dominated by the genus *Lactobacillus* from 0-144 h, constituting between 92.2-95.6 % of the total bacterial abundance for MRI, and from 24-144 h constituting 81.1-95.4 % cumulative bacterial abundance for JKUAT fermentations (Fig.3.31). This was expected, as the inoculated starters belong to the genus *Lactobacillus* of which members have the ability to grow at high acidity (Jung et al., 2011). These results are in support of earlier data where the growth of LAB in the inoculated batches after 24 h to 144 h led to pH reduction and thus accumulation of lactic acid, which resulted in the inhibition of Gram-negative enterobacteria and thus dominating the fermentation. The lactate

analysis showed that presumably both starter strains were actively involved in the fermentation. This study was in agreement with Oguntoyinbo et al. (2016) who reported similar results for the fermentation of African kale with starters *Lb. plantarum* BFE 5092 and *Lb. fermentum* BFE 6620.

The results further showed the JKUAT starter inoculated fermentation one at 0 h was dominated by genus *Lactobacillus* (67.7 %) and *Erwinia* (21.5 %) total bacterial abundance, indicating a high initial enterobacterial load, as the enterobacteria occurred together at such high incidence with the starter strains. Fermentation two inoculated with starter strains, at 0 h constituted of the genera *Enterobacter* (30.8 %), *Erwinia* (18.5 %) with genus *Lactobacillus* accounting for 41.2 % of total bacterial abundance (Fig.3.31). In this case, the enterobacterial sequences occurred at a higher incidence as those for the inoculated starter strains. In the fermentation three at 0 h, the genera *Psychrobacter*, *Erwinia*, *Leuconostoc* and *Enterobacter* were the sequences occurring at the highest incidence, constituting 42.9 %, 21.3 %, 19.2 and 8.4 % of the bacterial abundance respectively (Fig.3.31) The genus *Lactobacillus* here only made up less than 1 % of the sequences, indicating a possibly heavily contaminated 0 h sample. Nevertheless, as mentioned above, the inoculated fermentations were again dominated by *Lactobacillus* after 24 h of fermentation (Fig.3.31). DGGE profiles from JKUAT spontaneous fermentation two (24-144 h) clustered with starter culture inoculated group (Fig.3.30). The metagenomics results showed that LAB belonging to genera *Lactobacillus*, *Weissella*, *Streptococcus*, *Lactococcus*, *Pediococcus* and *Enterococcus* together constituted 75.7 % (24 h), 75.9 % (48 h), 90 % (72 h) and 84.5 % (144 h) of the combined total bacterial abundance in this fermentation, which may explain why the DGGE fingerprinting showed some agreement with the fermentations with LAB inoculated starter profiles. Therefore, both the DGGE and metagenomics results suggested that the inoculated starters dominated the entire fermentation, which appeared to correspond positively to the fact that the lactate analysis showed both D-and L-lactate to be present and to be produced in almost equal proportion. Hence, addition of the starter cultures appeared to lead to a systematic reduction of other bacterial diversity within the fermentation, which contributed to predictability, reliability of the fermentation and microbial stability of the product.

The results showed spontaneous nightshade fermentation at JKUAT was governed by distinct population dynamics of six LAB genera, *Lactobacillus*, *Weissell*a, *Lactococcus*, *Enterococcus*, *Streptococcus* and *Pediococcus*. Among these genera *Weissella*, *Lactococcus*,

Enterococcus and *Streptococcus* were abundant at the early stages of the fermentation (24-72 h). Previous studies reported that these groups of LAB initiate the fermentation when the pH is low and later a more acid tolerant group i.e. *Lactobacillus* take over the fermentation. These results are in agreement with our earlier findings where the pH went below 4.0 after 48 h (Fig.3.19) due to LAB growth (Fig.3.18). Jung et al. (2011) reported that in Kimchi fermentation, *Leuconostoc* and other less acid tolerant bacteria dominate at the early stages of fermentation but were later replaced by species of the genera *Lactobacillus* and *Weissella* which are better adapted to grow at high acidity and low pH conditions (Cho et al., 2006; Lee et al., 2005).

Determine success of the inoculated selected starter strains in nightshade fermentation by RAPD-PCR and 16S rRNA sequencing.

Overall, the DGGE results and metagenomics results so far suggested that the use of starter cultures led to a controlled fermentation in which the starter culture strains probably established themselves in the fermentation and dominated the fermentation. However, DGGE can only determine relatedness of profiles and changes in profiles. i.e. in bacterial diversity, while metagenomics can only resolve bacterial diversity at the genus, but definitely not at the strain level. For this, RAPD-PCR and PFGE were used, to determine whether the starter cultures used successfully initiated and established themselves in the fermentations.

The results showed that the use of starter cultures *Lb. plantarum* BFE5092 and *Lb. fermentum* BFE 6620 for the fermentation of African nightshade had a noticeable effect on initiating the fermentation by creating favourable fermentation conditions. Accordingly, the LAB numbers were generally higher throughout the entire fermentation when compared to the spontaneous fermentations and the pH reduction was faster and deeper at the end of the fermentation than when compared to the uninoculated batches. The initial phenotypic profiling allowed a general characterization of the isolated LAB strains into two groups i.e. *Lb. plantarum* group strains homofermentative rods producing DL-lactate and with no gas from glucose metabolism (starter culture *Lb. plantarum* BFE 5092 group) and *Lb. fermentum* group strains or heterofermentative rods producing CO_2 and DL-lactate (starter culture *Lb. fermentum* BFE 6620 group) which, together with DGGE and RAPD-PCR results, indicated that the starters indeed established themselves well in the fermentation. Afterwards, the strains were checked for their identity by 16S rRNA gene sequencing (Tab.3.27) and were then investigated for clonal relatedness to the inoculated starter

strains by strain typing. In total, 164 strains were isolated from three fermentation batches and were grouped based on their phenotypic characteristics (section 3.10.1).

Based on phenotypic results The *Lb. plantarum* strains constituted the majority of the isolates (90), of which 52.2 % strains stemmed from the batch inoculated with *Lb. plantarum* BFE 5092 starter strain. The second most frequently isolated LAB group belonged to heterofermentative rods (47 isolates) and of these strains, 97.9 % originated from the fermentation batch inoculated with the *Lb. fermentum* BFE 6620 starter strain. However, performance based on each fermentation batch, indicated that both *Lb. plantarum* and *Lb. fermentum* isolates were equally involved in the fermentation (Tab.3.26). Though the *Lb. plantarum* isolates dominated the first phase of the fermentation (0-48 h), the *Lb. fermentum* isolates dominated the middle and last phase of the fermentation (48-144 h). The third largest group of strains were homofermentative cocci (15) all of which originated from the control fermentation the 16S rRNA gene sequence identified them as belonging to *Enterococcus faecium* and *E. faecalis* species (Tab.3.27). Previous studies reports that these strains are frequently isolated from fermenting foods (milk, cheese, sausages and vegetables) (Franz et al., 1999; Moreno et al., 2006; Yousif et al., 2005). However, recent studies suggest, *Enterococcus faecium* and *E. faecalis* isolates are characterised by the presence of antibiotic resistance determinants and/or virulence factors hence these strains are important nosocomial pathogens (Franz et al., 2011). The presence of these bacteria in the control fermentations might thus constitute a potential health risk.

The fourth largest group of strains were obligately homofermentative lactobacilli producing L-lactate, with five isolates which all stemmed from the control-uninoculated batch; 16S rRNA gene sequencing identified these as *Lactobacillus sakei*. The strains showed differing physiological properties from other members of genus *Lactobacillus* and they usually produce D- and L-lactic acid. *Lb. sakei* has been reported to change the ratios of both D (-) and L (+) isomers of lactic acid in the presence or absence of sodium acetate and particularly L (+) lactic acid is produced in the presence of acetate, while equal amounts of the two isomers are produced in the absence of acetate (Iino et al., 2001). Previous studies showed strains of *Lb. sakei* isolated from kimchi had similar physiological features (Lee et al., 2011). These isolates have also been previously isolated from fermenting sauerkraut (Vogel et al., 1993), vacuum packaged and smoked fish (Leroi et al., 1998; Lyhs et al., 1999) although it is best adapted to meat fermentation and its products (Hugas et al.,

1993; Vogel et al., 1993). Little is known about the involvement of *Lb. sakei* in fermentation of African foods, especially vegetables.

Two Gram-positive, catalase-positive coccus-shaped strains originated from the uninoculated batch and the 16S rRNA gene sequence identified them as *Staphylococcus epidermidis* and *S. condimenti*. Members of the genus *Staphylococcus* are common inhabitants of skin and mucosal membrane of human and animals as protective microbiota or as opportunistic nosocomial pathogens associated with infections of the immunocompromised patients (O'gara & Humphreys, 2001; Torok & Day, 2005). *S. condimenti* was first isolated from soy sauce mash and *S. hominis* is associated with human blood-stream infection (Misawa et al., 2015), while *S. epidermidis* is the primary cause of nosocomial bacteraemia and medical equipment-related infections (Chaves et al., 2005; Minto et al., 1999; Torok & Day, 2005). However, previous studies have reported involvement of *Staphylococcus* spp., especially *S. epidermidis* and *S. hominis* in Korean fermented fish products (Mi-na & Lee, 1996), as well as *S. xylosus* in the natural fermentation of sausages (Fiorentini et al., 2009). Little is known about fermentation of plant materials by *Staphylococcus*. The presence of these bacteria from the control and from an unknown source could, however, indicate a potential health risk. Four Gram-negative rods (identified as *Enterobacter ludwigii* and *E. cloacae*) were isolated from the uninoculated batch. They are widely found in nature as saprophytes or as pathogens (Mezzatesta et al., 2012). *E. cloacae* is frequently isolated from human clinical specimens. It is the most important opportunistic and multi drug resistant bacterial pathogen for humans and the leading cause of nosocomial infections among *Enterobacter* spp. (Davin-Regli & Pages, 2015). Recent studies have revealed the potential role of *E. ludwigii* as an emerging pathogen (Flores-Carrero et al., 2016). The isolation of these strains in food is a cause of concern especially when the origin is unknown.

Isolation of autochthonous bacteria at JKUAT during nightshade fermentation was necessary to help understand the diversity and their influence in the fermentation. As described in section 3.10.3, 32 LAB strains and 13 Enterobacteriaceae strains were isolated. Among the LAB isolated, *Lb. plantarum* was the most commonly isolated from 0-144 h (Fig.3.35). *Lb. plantarum* has been previously often isolated from different African fermented foods (Kostinek et al., 2005; Mathara et al., 2004; Oguntoyinbo et al., 2011) and its known to produce plantaracins (Cho et al., 2010) with acid tolerant and probiotic properties. *Pediococcus pentosaceus* was the second most frequently isolated between 48-144 h of fermentation *Ped. pentosaceus* is obligately

homofermentative, Gram-positive coccus-shaped, DL-lactate producing LAB (Franz et al., 2014) commonly used in fermentation of vegetables, dairy products, meat, fruit juice and silage (Dietrich, 1998). They are known to produce pediocins with a wide range of inhibitory activity against food pathogens (Cheun et al., 2000; Luchansky et al., 1992; Mattila-Sandholm et al., 1991) and are important in preparation of several commercial probiotic feeds (Yirga, 2015). *Lb. fermentum* was isolated at 24 h and 144 h it is obligately heterofermentative rod-shaped, producing CO_2 and DL-lactate from sugar metabolism. It occurs in diverse habitats, including the human gut, milk products, fermenting plant material and animal (Dellaglio et al., 2004). This species is considered a good probiotic candidate, due to its ability to withstand gastro-intestinal conditions (Jimenez et al., 2010). It was reported to have potential for the prevention of community-acquired infections (López-Huertas, 2015), modulation of immune system and production of antimicrobial compounds. Different species of *Enterococcus* were also isolated at 0-48 h. Three strains belonging to family *Leuconostocaceae* and the genus *Weissella* were isolated at 48 h and 72 h they included W. *confusa* and *W.oryzae*. Members of genus *Weissella* are Gram-positive, coccoid or rod-shaped morphology (Collins et al., 1993). They are obligately heterofermentative-producing CO_2 from sugar metabolism with either D, or DL-lactate (Fusco et al., 2015). They occur in a wide range of habitats, e.g., on the skin and in the milk and feces of animals, from saliva, breast milk, feces and vagina of humans, from plants and vegetables, as well as from a variety of fermented foods (Fusco et al., 2015). *W. oryzae* is associated with rice grain silage fermentation and has the potential for biotechnological applications as an effective inoculant for rice grains (Tanizawa et al., 2014). The study showed African nightshade leaves harbors diverse group of naturally occurring LAB.

Among the enterobacteria isolated, all stemmed from the spontaneous fermentation at 0-144 h. *Escherichia coli* was the most frequent isolates. *E. coli* is a common cause of diarrhoea related illness globally, is the most common cause of uncomplicated and complicated urinary tract infections, and a leading cause of bacteraemia and neonatal meningitis. Increasing antibiotic resistance among *E. coli* contributes to morbidity, mortality, and substantial health-care and societal costs associated with infection (Poolman, 2017). *E. coli* is a common indicator of faecal contamination; hence, isolation of this species from the control and especially from an unknown source could indicate heavy contamination of the sample and could pose a health risk.

In addition, *Klebsiella* like *E. coli* is also a member of the *Enterobacteriaceae* family commonly found in the environment especially, soil, vegetables and water. *K. oxytoca* is frequently

isolated from human clinical samples and is usually responsible for nosocomial infections. *Enterobacter* species are widely dispersed in nature and exist in a diverse range of environments such as soil, water, households; food processing plants, vegetation, and warm-blooded animals. *Enterobacter* are also increasingly recognized as potential human pathogens, especially as agents of nosocomial infections in the clinical environment. *Providencia* is a genus also within the Enterobacteriaceae family and is known to cause 'travelers' diarrhea. *P. rettgeri* is a primary cause of nosocomial urinary tract infection and has been isolated in wounds and intra-abdominal infections (Yoh et al., 2005). Hence, isolation of these bacteria in the spontaneous fermentation and especially with their origin unknown might also constitute a potential health risk.

There is limited information on the use of starter culture in sub-Saharan Africa (Holzapfel, 2002). However, the use of different molecular fingerprinting techniques to follow progress of starter cultures in the fermentation has been reported, especially RAPD-PCR and PFGE fingerprinting has been utilized to monitor the development of a mixed starter culture comprising of strains of species *Lb. plantarum* and *Lb. fermentum* in the fermentations of gari (Kostinek et al., 2008) and African kale (Oguntoyinbo et al, et al., 2016). The authors demonstrated that RAPD-PCR was an adequate method for studying the development of starter strains during fermentation. In our study, the suitability of RAPD as a fingerprint typing method to assess clonal relationships was confirmed. RAPD-PCR has been used successfully for strain typing however; PFGE is recommended as a confirmatory or rather more accurate method (Malathum et al 1998). The disadvantages of PFGE typing are that a lot of time is needed for DNA preparation and it requires the use of expensive consumables and specialized electrophoresis equipment. Hence, RAPD-PCR fingerprinting was used to type the isolates, followed by application of PFGE typing of selected representative isolates of different RAPD-PCR clusters. This ensured the confirmation of clonal relationships in a large collection of RAPD-PCR typed strains by PFGE.

The results showed that LAB dominated the fermentation with 90 *Lb. plantarum* strains isolated, 47 strains stemmed from the batch inoculated with *Lb. plantarum* BFE 5092 while 47 *Lb. fermentum* strains isolated, 46 strains originated from batch inoculated with *Lb. fermentum* BFE 6620. RAPD-PCR profiles of both *Lb. plantarum* and *Lb. fermentum* isolates showed that strains were grouped in different sub-clusters and that only few showed RAPD-PCR profiles similar to those of the starter strains. (Fig.3.37 & 3.40). Hence, the inoculated starter strains did not appear to have establish themselves as dominant LAB; however, they may have initiated the fermentation,

creating the conditions favourable to other lactobacilli or other LAB which were more capable of taking over the fermentation and establishing themselves as microorganisms dominating the fermentation. The LAB must have stemmed from the plant materials. On the other hand, PFGE for selected strains suggested that there could be clonal relatedness as the PFGE pulsotypes were very similar. However, only few strains (e.g. in the case for *Lb. plantarum*) could be investigated because of time constraint, and furthermore it is known that for some species PFGE with specific restriction enzymes may not successfully discriminate to the clonal level, which may have been the case in this study also. Therefore, RAPD-PCR could be considered as a more suitable method for monitoring the inoculated starter strain in this study.

The effects of fermentation on safety, nutritional quality and sensory attributes of nightshade leaves

The role of LAB starters on selected pathogenic bacteria was performed by co-inoculating the pathogens *L. monocytogenes* and *S.* Enteritidis together with the starter cultures *Lb. plantarum* BFE 5092 and *Lb. fermentum* BFE 6620. The results showed that the fermentations where pathogens were co-inoculated with starter cultures led to a fast and deep reduction of the pH within first 24 h below 4.0 and the pH stayed at this low level until 144 h of the fermentation, with the final pH reaching ca. 3.4 (Fig.3.23). The reduction of the pH was due to increased LAB counts reaching 1 x 10^9 cfu/ml within the first 24 h and remained at this highest count up to 144 h of the fermentation (Fig.3.25). Concomitant to this, *Listeria monocytogenes* was completely inhibited within 48 h with the pH < 3.6. *Salmonella* Enteritidis inhibition occurred after 144 h and at this point of the fermentation with starter bacteria, the pH was < 3.5 (Fig.3.23). The growth of enterobacteria was completely inhibited after 144 h of the fermentation. In the fermentation with starter strains, only, enterobacteria and yeast and moulds were diminished to below detectable levels within 48 h of the fermentation (Fig.3.24). It is clear that lowering the pH due LAB fermentation below 4.0 is vital to inhibit the growth of Gram-negative and pathogenic bacteria (Motarjemi & Nout, 1996). The use of starter cultures (when only initiating the fermentations) eliminated the pathogens completely to undetected levels, even with enrichment steps nothing could be recovered after fermentation.

Oguntoyinbo et al. (2016) showed that during African kale fermentation with the starters *Lb. plantarum* BFE 5092 and *Lb. fermentum* BFE 6620 that were co-inoculated with pathogenic

Listeria and *Salmonella*, that rapid growth of the starters and quick reduction of pH let to the inhibition of these pathogens within 72 h of the fermentation. Indeed, its known that *L. monocytogenes* and other food-borne pathogens are sensitive to 3-phenyllactic acid which is commonly produced by many LAB species, especially *Lb. plantarum*. This acid has a broad-spectrum activity (Liu et al., 2017). *Lb. plantarum* BFE 5092 also produces plantaracins EF, JK and N with ability to act as protective cultures in food preservation especially against *L. monocytogenes* (Cho et al., 2010).

In the control fermentation, i.e. inoculated with pathogens without starter bacteria as well as spontaneous fermentations, showed only a slow reduction of pH only reaching below 5.0 after 144 h of fermentation (Fig.3.25). The slow reduction of the pH, which was due to slow growth of LAB reaching only > 1 x 10^5 cfu/ml between 24-48 h after which they remained at this level up to 144 h (Fig.3.23 & 3.25). Therefore, due to delayed growth of LAB, a slow decrease in pH (remaining > 4.0) and a slow accumulation of lactic acid led to the growth of enterobacteria and the pathogens *L. monocytogenes and Salmonella* Enteritidis. These pathogens can also fermentation sugars leading to lactic acid production especially *L. monocytogenes* (Rees et al., 2017). This could explain the low pH of about 5.0 after 144 h of fermentation. Hence, fermentation may serve to improve food safety and inhibits the growth of diarrhoea-causing pathogens (Chelule et al., 2010; Holzapfel, 1997; Motarjemi & Nout, 1996).

The changes that occur during food processing such as fermentation are usually due to enzymatic activities by microorganisms and/or endogenous enzymes. These processes can cause significant changes that may affect sensory characteristics, microbial safety and the nutritional quality (Svanberg & Lorri, 1997) of the raw materials. The chemical analyses of fresh, fermented with starter culture, fermented without starters (control) and solar dried nightshade leaves showed fermentation with or without starter culture statistically significantly increased ash content by 48-72 % while solar drying did not affect ash contents (Fig.3.42). This could be because the addition of 3.0 % salt in the fermentation contributed to increased ash content after analysis. The addition of salt was necessary for inhibits growth of spoilage microorganisms, allowing salt-tolerant LAB to thrive, as well as for the enhancement of flavour and the microbiological stability of the final product (Bautista-Gallego et al., 2010; Hurtado et al., 2009; Peñas et al., 2017). This study was in agreement with that of Oguntoyinbo et al. (2016), who reported an increased ash content after addition of 2.5 % salt in fermentation of African kale. Salt induces plasmolysis in plant cells which

in turn releases mineral salts and nutrients from the vacuole and which enables for proper growth of LAB around the submerged product (Gardner et al., 2001; Rakin et al., 2007; Wouters et al., 2013). Determination of the ash content represents the total mineral content in foods; it is also important part of proximate analysis for nutritional evaluation (Marshall, 2010).

On the other hand, fermentation led to a reduction of protein content by 15-33 % (for the MRI based fermentation with or without starter culture inoculation) and about 36-38 % for JKUAT based fermentation with or without starter cultures These reductions, however, were not statistically significant (Fig.3.43) Solar drying did not have any impact on protein content (Fig.3.43). This result is in agreement with Mepba et al. (2007), who reported that sun drying of edible Nigerian leafy vegetables had no effect on protein content. It is hypothesized that pH decrease during fermentation can cause partial protein denaturation and other produced complexes (Czarnecka et al., 1998). Different studies show that fermentation could increase or decrease protein content, although the reduction appeared to depend on the type of fermentation and microorganisms involved (Granito et al., 2002; Khetarpaul & Chauhan, 1989; Mugendi et al., 2010). Increased protein catabolism by fermenting microorganisms may account for loss of protein and production and accumulation of ammonia, a by-product of metabolic deamination. Some strains of bacteria are known to possess deaminases (Khetarpaul & Chauhan, 1989).

The results also showed that fermentation decreased vitamins B_1, B_2 and C both in the starter inoculated and the control fermentations. The MRI based fermentation done with starter cultures showed a reduction in vitamin C content by 66 % after fermentation and a reduction by 81 % occurred in the case of the spontaneous fermentation. In JKUAT based fermentations with or without inoculated starter cultures vitamin C concentrations were reduced by ca. 75 %, while solar drying caused a statistically significant, massive loss of vitamin C of over 97 % (Fig.3.44). Our results furthermore showed that MRI based fermentations with or without starter cultures reduced vitamin B_I concentrations by 71-73 %, while JKUAT fermentations caused a 68-76 % decrease (Fig.3.46). However, MRI based fermentation led to reduction of vitamin B_2 by 30-36 % while JKUAT fermentation reduced vitamin B_2 by ca. 47 % (Fig.3.47). The loss of vitamin B_1 due to fermentation was statistically significant both for MRI and JKUAT fermentations with or without starter cultures. On the other hand MRI based fermentation, the loss of vitamin B_2 was not statistically significant while fermentation at JKUAT did have the impact on vitamin B_2 ($p < 0.05$) (Fig.3.47). This study was in agreement with Doblado et al. (2003), who recorded reduction in

thiamine (vitamin B_1) during controlled LAB and natural fermentation of *Vigna sinensis* flour and with Oguntoyinbo et al. (2016) who reported reduction of vitamin B_1 and B_2 in kale fermentation. Solar drying led to losses of vitamin B_1 and B_2 of 13-19 %, which, however, were not statistically significant ($p > 0.05$).

Vitamin C, B_1 and B_2 are water-soluble and this could explain why there was a decrease in these vitamins, since a submerged fermentation of nightshade leaves was used that could have led to leaching of these vitamins into the watery phase. Vitamin C is most sensitive to destruction when the sample is subjected to light, low relative humidity, storage at reduced temperatures for longer time and is easily oxidized in aqueous solutions, especially in the presence of oxygen, metal ions, alkaline pH and at high temperature (Lee & Kader, 2000). The loss may also have occurred because of the fact that leaves were washed in water before fermentation. Vitamin B_1 and B_2 on the other hand are stable during heat and oxygen exposure, but readily destroyed by UV rays and sunlight (Northrop-Clewes & Thurnham, 2012).

Even though fermentation with starter cultures caused a decrease in vitamin C, fermented leaves still contained about 257 mg/100 g (MRI) and 159 mg/100g (JKUAT) vitamin C content, to supplement the daily recommended intake. Traditional methods of vegetable preparation, involving washing with water and boiling for > 5 min, can result in vitamin C loss by 20-94 % (Lyimo et al., 1991; Nursal & Yücecan, 2000; Oboh, 2005; Rumm-Kreuter & Demmel, 1990; Suttikomin, 2002; Vinha et al., 2015). Oboh, (2005) reported that blanching of green leafy vegetables for 5 min caused a vitamin C reduction by 47.5-82.4 %. Despite this, 100 g of traditionally fermented nightshade leaves could still provide enough vitamin C to supplement the required daily intake of 60 mg for adults in African countries (FDA, 2013). Fermented nightshade leaves had > 1.0 mg/100 g of vitamin B_2 (riboflavin), which was within the recommended daily dietary intake of 1.3 mg for adult men and 1.1 mg for adult women (Northrop-Clewes & Thurnham, 2012). Although fermentation clearly leads to reduction of these vitamins, they are still present in appreciable concentration and also through fermentation the shelf life of food can be attained, hence vegetable fermentation should be encouraged.

Fermentation of plant material with or without the use of starter cultures at MRI reduced the vitamin E content by 10-19 %, although this decrease was not statistically significant. The JKUAT based fermentation and solar drying led to increased vitamin E content by 24-34 % and 13 %

respectively (Fig.3.45). According to the food and nutritional board of the U.S institute of Medicine, α-tocopherol should be used to estimate requirement and intake recommendations (Institute of Medicine, 2000). Vitamin E is fat/lipid-soluble with antioxidant capacity; the oxidative degradation of vitamin E is usually accelerated by light, heat, alkaline conditions and lipoxidase enzymes (Eitenmiller et al., 2008). Despite a slight decrease in vitamin E, the fermented leaves had enough vitamin E to supplement recommended daily intake of 15 mg for people > 14 years (Institute of Medicine, 2000). Hence, postharvest handling of foods can have a direct impact on increase or decrease of vitamin E. Due to relative instability of vitamin E, different studies have reported varying results of vitamin E from similar products (Eitenmiller & Lee, 2004; Eitenmiller et al., 2008).

Fermentation of nightshade with LAB starters led to the reduction or complete depletion of fructose and glucose (Fig.3.48 & 3.49). Spontaneous fermentation led to increased fructose by over 100 % (MRI) and 18 % (JKUAT), while solar drying caused 20 % increase (Fig.3.48). The increasing in monosaccharide and disaccharides after solar drying could be explained by the fact that during drying process water is removed from samples, concentrating the sugars and possibly also oligosaccharide/ polysaccharide might be broken down into monosaccharides and disaccharides (Priecina & Karklina, 2014). Even though on average there was an increase of sucrose in the fermentation with or without use of starter cultures both at MRI and JKUAT ($p > 0.05$), individual fermentations showed a decrease or compete depletion in two out of the three fermentation batches. This results are in line with our findings, that the use of starter culture led to rapid reduction of pH as well as accumulation of lactic acid due to faster growth of LAB. The decrease of fructose and glucose in nightshade fermentation with LAB starter cultures showed that these starters were able to hydrolyse oligosaccharides present and the products of their degradation (monosaccharides) using them for their metabolism (Czarnecka et al., 1998). The findings are in agreement with Oguntoyinbo et al. (2016) and Wafula e al. (2017) who reported that *Lb. plantarum* BFE 5092 and *Lb. fermentum* BFE 6620 possess genes involved in phosphoenolpyruvate and phosphotransferase systems for utilization of sugars such as sucrose, fructose, and glucose among others. The slow growth of LAB in the uninoculated batches led to slow metabolism of sugars and the fact that addition sugar was provided that could explain the increase.

The nightshade storage experiments showed that solar drying and storage of solar dried vegetables had a significant impact on vitamin C as discussed above. Storage of solar dried

vegetables did not have a significant impact on vitamin B_1, B_2, E and protein ($p.> 0.05$), indicating that this preservation technique does not lead to significant reductions in these health-related compounds. Furthermore, solar drying and storage of the dried vegetables at either 10 ^oC or 25 ^oC led to increase in glucose and sucrose contents ($p< 0.05$) as discussed above. On microbial contamination of the vegetables, however, solar drying does not lead to increased safety and quality of the product, rather the microbial quality deteriorates and safety may become compromised. This is because storage time and temperature seemed to promote microbial growth especially enterobacteria and yeast and moulds (Fig.3.51). Based on our results, fermentation is more suitable than solar drying, as it ensures product safety and quality as well as it improves sensory attributes, while solar drying cause massive loss of vitamin C without any guarantee of product safety and quality.

Based on the sensory attributes, 55 % women and 45 % men participated in the sensory panel, 80 % of the panellists said would recommend eating fermented vegetables, they cited colour, smell, taste and appearance as good (although the feel in the mouth was only satisfying), while 20 % declined to recommend eating of fermented vegetables, citing colour as moderate (mean score 5.3) and further said fermented vegetables are insufficient (mean score 3.0) as compared to fresh or unfermented (Tab.3.29) vegetables. These findings that 80% recommended eating the vegetables and found colour, smell, taste and appearance good are in agreement with Holzapfel (1997) who reported that fermentation of food with selected starter cultures improves sensory qualities such as taste, aroma, visual appearance, texture and consistency. The gender was important especially in Africa women play a big role in food preparation hence their opinion represents large segment of the women cooks in rural areas, which would be important for adoption of this technology.

5.0 Conclusion and recommendations

The results showed that all tested starter cultures in combination with a 3 % salt-sugar solution resulted in a fast and stable pH reduction. Hence, 3% salt-sugar solution was established as the optimal fermentation condition with *Lb. plantarum* BFE 5092 and *Lb. fermentum* BFE 6620 used as a starter cultures for nightshade fermentation. The use of starter *Lb. plantarum* BFE 5092 and *Lb. fermentum* BFE 6620, had a significant influence on the microbial composition in nightshade fermentation, this was noted by rapid and stable reduction of the pH and accumulation of lactic acid. Hence, the growths of spoilage pathogenic microorganisms were inhibited resulting to products with improved safety and quality.

The microbial ecological studies based on DGGE and high through put sequencing, showed the use of starter culture *Lb. plantarum* BFE 5092 and *Lb. fermentum* BFE 6620 had an impact on the biodiversity and dynamics of the background microbiota during fermentation of vegetable African nightshade. Spontaneous fermentation showed unreliable and unpredictable results, this was due to slow growth of LAB hence insufficient acidification of the product. The success of the inoculated starter cultures through RAPD and PFGE analysis showed that the starters may have initiated the fermentation but later, overtaken by indigenous LAB initially present on the plant materials.

Even though, fermentation led to reduction of water-soluble vitamins, the fermented product still contained a significant amount of vitamin B_1, B_2 and C enough to supplement the daily-recommended intake. Solar drying on the other hand had a huge impact on vitamin C. Additionally, solar drying coupled with storage did not guarantee the safety and quality of the product. Fermentation of African nightshade seems to improve the sensory attributes of colour, taste, smell and appearance. There was generally a moderate acceptability of the fermented nightshade leaves. Hence, with proper awareness fermentation might be an option for the local consumers for ensuring product with improved safety, quality and shelve-life.

In the final analysis, therefore, fermentation of vegetables in Africa should be given priority as a very suitable postharvest preservation technique. Based on our results, fermentation seems to be more suitable than solar drying, which is widely adopted in sub-Saharan Africa. Fermentation as a postharvest preservation technology is especially important as the African continent is endowed with rich variety of indigenous vegetables that rich in micronutrients whose uptake could contribute to a better health of the people. Technologies for the production of locally fermented vegetables

especially starter culture development should be explored and encouraged by local academic and research institutions, local communities and other stakeholders. Most importantly, African governments must commit themselves to the protection of its people by promoting and developing this technology for enhancing food safety, and availability.

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Annexes

Annex 1: PCR programmes

Annex 1A: RAPD-PCR program with M13

Cycle	PCR step	Temperature	Time
	Initial denaturation	94°C	2 min
40x	**Denaturation**	94°C	1 min
	Annealing	40°C	20 sec
	Elongation	72°C	2 min (0.6°C/sec)
	Store	4°C	3°C/sec

Annex 1B: PCR program for AtpD primers

Cycle	PCR step	Temperature	Time
	Initial denaturation	95°C	3 min
35x	**Denaturation**	95°C	1 min
	Annealing	50°C	1 min
	Elongation	72°C	1 min
	Final elongation	72°C	7 min

Annex 1C: PCR program for 16S rRNA primers

Cycle	PCR step	Temperature	Time
	Initial denaturation	94°C	2 min
		56°C	1 min
		72°C	2 min
32x	**Denaturation**	94°C	1 min
	Annealing	56°C	1 min
	Elongation	72°C	2 min
	Final elongation	94°C	1 min
		56°C	1 min
		72°C	6 min

Annex 1D: A touchdown PCR program for the amplification of 16S rRNA genes for DGGE

Cycle	PCR step	Temperature	Time
1X	**Initial denaturation**	94^{o}C	5 min
10X	**Denaturation**	94^{o}C	30 sec
	Annealing	62-52^{o}C	1 min

	Elongation	72°C	30 sec
22X	**Denaturation**	94°C	30 sec
	Annealing	56°C	1 min
	Elongation	72°C	30 sec
1X	**Final elongation**	72°C	7 min

Annex 1E: PCR program for 16S rRNA V3 and V4 region with Illumina high throughput sequencing

Cycle	**PCR step**	**Temperature**	**Time**
1X	**Initial denaturation**	95°C	1 min
35X	**Denaturation**	95°C	15 sec
	Annealing	50°C	15 sec
	Elongation	72°C	15 sec

Annex 1F: Program for Index PCR

Cycle	**PCR step**	**Temperature**	**Time**
1X	**Initial denaturation**	98°C	3 min
8X	**Denaturation**	98°C	30 sec
	Annealing	55°C	30 sec
	Elongation	72°C	15 sec
1X	**Final extension**	72°C	5 min

Annex 2: Solvents and laboratory chemicals used, incuding safety data information according to the Globally Harmonised System of Classification and Labelling of Chemicals (GHS)

Annex 2A: Solvents and laboratory chemicals used

Solvent/Chemical	**Producer**	**Acronym**	**Formula**	**CAS-no.**	**Amount**	**Pictograms**	**H and P Statements**
Methanol (HPLC-LC-MS grade)	Merck	MeOH	CH_4O	67-56-1	363 ml	 Danger	H225 H301 + H311 + H331 H370 P210 P240 P280 P302 + P352 P304 + P340 P308 + P310 P403 + P233
Ammonium acetate	Merck	Acetic acid, ammonium salt	CH_3COONH_4	631-61-8	57.8 g	-	-
Sodium acetate anhydrous	Merck	Acetic acid sodium salt	CH_3COONa	127-09-3	205 g	-	-
Taka-Diastase	Pfaltz & Bauer Inc.		-	9001-19-8	25 mg/ml	-	-
Riboflavin (99 %)	Merck	Vitamin B_2	$C_{17}H_{20}N_4O_6$	83-88-5	100 mg	-	-
Sodium hydroxide	Merck	Soda caustic	NaOH	1310-73-2	-	 Danger	H290 H314 P280 P301 + P330 + P331 P305 + P351 + P338 P308 + P310
Potassium hydroxide	Merck	Potash caustic	KOH	1310-58-3	-	 Danger	H290 H302 H314 P280 P301 + P330 + P331 P305 + P351 + P338 P308 + P310

Tetraethyl ammonium chloride	Merck	TMA	$(CH_3)_4NCl$	75-57-0	1 g	 Danger	H300 H311 H315 H370 H411 P273 P280 P302 + P352 P308 + P310
Acetonitrile (HPLC grade)	Merck	ACN	CH_3CN	75-05-8	-	 Danger	H225 H302 + H312 + H332 H319 P210 P240 P302 + P352 P305 + P351 + P338 P403 + P233
Sodium 1-heptane sulfonate LiChropur®	Merck	1-heptanesulfonic acid sodium salt	$C_7H_{15}NaO_3S$	22767-50-6	-	-	-
tert-butyl methyl ether Uvasol®	Merck	MTBE	$(CH_3)_3COCH_3$	1634-04-4	80 ml	 Danger	H225 H315 P210 P240 P302 + P352 P403 + P233
Butylhydroxytoluene	Merck	BHT	$C_{15}H_{24}O$	128-37-0	-	 Danger	H410 P273
1,2-phenylenediamine	Merck	1,2-diaminobenzene	$1,2\text{-}(NH_2)_2C_6H_4$	95-54-5	1.5 g	 Danger	H301 H312 + H332 H317 H319 H341 H351 H410 P273 P280

							P302 + P352 P305 + P351 + P338 P308 + P310
L (+) ascorbic acid	VWR chemicals	Vitamin C	$C_6H_8O_6$	50-81-7	100 mg	-	-
Thiamin chloride-HCl (99 %)	Merck	Vitamin B_1	$C_{12}H_{17}ClN_4OS * HCl$	67-03-8	100 mg	-	-
Acetic acid (100 %)	Merck	Ethanoic acid	CH_3COOH	64-19-7	-	 Danger	H226 H290 H314 P210 P280 P301+ P330 + P331 P305+P351 + P338 P308 + P310
Hydrochloric acid (37.0 % w/w)	Merck	Hydrogen chloride solution	HCl	7647-01-0	-	 Danger	H290 H314 H335 P280 P301 + P330 + P331 P305 + P351 + P338 P308 + P310
Acetone (99.8 %)	Merck	2-propanone	CH_3COCH_3	67-64-1	-	 Danger	H225 H319 H336 P210 P240 P305 + P351 + P338 P403 + P233
Sulphuric acid 98 % (N_2-free)	Merck	Sulfuric acid solution	H_2SO_4	7664-93-9	54.4 ml	 Danger	H290 H314 P280 P301 + P330 + P331 P305 + P351 + P338 P308 + P310
Kjeldahl tablets	Merck	Selenium reaction mixture	$Na_2S_2O_8/CuSO_2$	-	-	 Danger	H411 P273

Boric acid (99 %)	Merck	Orthoboric acid	H_3BO_3	10043-35-3	20 g	 Danger	H360FD P201 P308 + P313
Meta-phosphoric acid	Merck	m-phosphoric acid	HPO_3	37267-86-0	75 g	 Danger	H314 P280 P301 + P330 + P331 P305 + P351 + P338 P308 + P310
Ethanol (96 %)	Merck	EtOH	C_2H_5OH	64-17-5	-	 Danger	H225 H319 P210 P240 P305 + P351 + P338 P403 + P233
Denatured ethanol (99.8 %)	Roth	EtOH	C_2H_6O	64-17-5	-	 Danger	H225 H319 P210 P240 P305 + P351 + P338 P403 + P233
Fructose	Sigma-Aldrich	D (−)-fructose	$C_6H_{12}O_6$	57-48-7	200 mg	-	-
Glucose	Sigma-Aldrich	Dextrose	$C_6H_{12}O_6$	50-99-7	200 mg	-	-
Sucrose	Sigma-Aldrich	D (+)-saccharose	$C_{12}H_{22}O_{11}$	57-50-1	200 mg	-	-
Ammonium persulfate	Bio-Rad laboratories	APS	$(NH_4)_2S_2O_8$	7727-54-0	0.1 g	 Danger	H272 H302 H315 H317 H319 H334 H335 P210 P280 P284 P305 + P351 + P338 P342 + P311

Acrylamide/bis-acrylamide (37:5:1)	Bio-Rad laboratories	2-propenamide	CH_2=$CHCONH_2$	79-06-1	-	 Danger	H301 H312 H315 H317 H319 H340 H350 H361 H372 P201 P302 +P352 P260 P280 P305 + P351 + P338 P308 + P310
Formamide	Bio-Rad laboratories	Formic acid amide	CH_3NO	75-12-7	-	 Danger	H360D P281 P201 P308 + P313
Urea	Bio-Rad laboratories	Carbamide	CH_4N_2O	57-13-6	-	-	-
disodium ETDA	Roth	2Na	$C_{10}H_{14}N_2O_8$*2Na*2H$_2$O	6381-92-6	-	 Danger	H332 H373 P260 P314
Tris base 99.3 % (buffer grade)	Roth	Tris buffer	$C_4H_{11}NO_3$	77-86-1	-	 Warning	H315 H319 H335 P280 P302 + P352
TRIS-hydrochloride 99 % p.a	Roth	Tris-HCl	$C_4H_{11}NO_3$.HCl	1185-53-1	-	 Warning	H315 H319 H335 P280 P302 + P352

Chloroform	Merck	TCM	$CHCl_3$	67-66-3	-	 Danger	H302 H315 H319 H331 H351 H361d H372 P281 P302 + P352 P304 + P340 P305 + P351 + P338 P308 + P310
2-pentanol	Merck	Isopropanol	$CH_3CH(OH)CH_3$	67-63-0	-	 Danger	H225 H319 H336 P210 P240 P305 + P351 + P338 P403 + P233
Isoamyl alcohol	Merck	Iso-pentyl alcohol	$(CH_3)_2CHCH_2CH_2OH$	123-51-3	-	 Danger	H226 H332 H335 P210 P304 + P340
Guanidium thiocyanate	Merck	GTC	$H_2NC(NH)NH_2$ * HSCN	593-84-0	-	 Danger	H302 + H312 + H332 H314 H412 P273 P280 P301 + P330 + P331 P302 + P352 P305 + P351 + P338 P308 + P310
Mutanolysin	Sigma-Aldrich	N-acetyl muramidase	-	55466-22-3	-	-	-
RNAse	Sigma-Aldrich	RNase A	-	9001-99-4	10 mg	-	-
Lysozyme	Sigma-Aldrich	Muramidase	-	12650-88-3	100 mg	-	-
InCERT agarose	Lonza	-	-	-	-	-	-

Biozym Gold agarose	Biozyme	-	-	-	-	-	-
D(+)-glucose monohydrate	Merck	Dextrose	$C_6H_{12}O_6 * H_2O$	14431-43-7	-	-	-
Agarose LE	Genaxxon bioscience	-	-	9012-36-6	-	-	-
Ethidium bromide	Roth	EtBr	$C_{21}H_{20}BrN_3$	1239-45-8	3 µg	 Danger	H341 H302 H332 + H330 P261-P281-P311
Proteinase K (100 mg/ml)	Sigma-Aldrich	Tritirachium alkaline proteinase		39450-01-6	100 mg	 Danger	H315 H319 H335 P302 + P352 P304 + P340
Sodium chloride	Merck	NaCl	NaCl	7647-14-5	-	-	-
Deoxycholate	Sigma-Aldrich	Desoxycholic acid sodium salt	$C_{24}H_{39}NaO_4$	302-95-4	-	 Warning	H302 H335
SYBR Gold	Invitrogen	-	-	67-68-5	-	-	H227
L-tartaric acid	Merck	2,3-dihydroxybutanedioic acid	$C_4H_6O_6$	87-69-4	-	 Warning	H319 P305 + P351 + P338
Hydrogen peroxide 30 %	Merck	Perhydrol/H_2O_2	H_2O_2	7722-84-1	-	 Danger	H302 H318 H413 P273 P280 P305 + P351 + P338 P313
N,N,N',N'-tetramethylethylenediamine	Bio-Rad laboratories	TEMED	$(CH_3)_2NCH_2CH_2N(CH_3)_2$	110-18-9	-	 Danger	H225 H314 H302 + H332 P210 P240 P280

							P301 + P330 + P331 P303 + P361+P353 P305 + P351 + P338 P308 + P310 P403 + P233P403 + P233
Sodium dodecyl sulfate	Merck	SDS	$C_{12}H_{25}OSO_2ONa$	151-21-3	-	 Danger	H228 H302 H315 H318 H335 H412 P210 P273 P280 P302 + P352 P305 + P351 + P338 P313
N-Lauroylsarcosine sodium salt	Sigma-Aldrich	Sarcosyl	CH_3-$(CH_2)_{10}$-CO-N(CH_3)-CH_2-COONa	137-16-6	-	 Danger	H315 H317 H318 H330 P280 P302 + P352 P304 + P340 P305 + P351 + P338 P308 + P310
Ammonium dihydrogen phosphate	Merck	Ammonium biphosphate	$(NH_4)H_2PO_4$	7722-76-1	-	-	-
Potassium ferricyanide-(III)	Merck	Potassium hexacyanoferrate(III)	$K_3[Fe(CN)_6]$	13746-66-2	200 mg	-	-
Methyl red (sodium salt)	Merck	2-[(4-dimethylamino) phenylazo]benzoic acid sodium salt	$C_{15}H_{14}N_3NaO_2$	845-10-3	-	-	-
Methylene blue	Merck	Methylthioninium chloride	$C_{16}H_{18}ClN_3S$ * x H_2O (x=2-3)	200-515-2	-	 Warning	H302
Bromophenol blue	Sigma-Aldrich	Sodium Salt	$C_{19}H_9Br_4O_5SNa$	34725-61-6	-	-	-

sodium salt							
Xylene cyanol	Sigma-Aldrich	-	$C_{25}H_{27}N_2NaO_6S_2$	2650-17-1	-	-	-
Glycerol	Sigma-Aldrich	Glycerin	$CH_2OHCHOHCH_2OH$	56-81-5	-	-	-
Chloramphenicol	Roth	Chloromycetin	$C_{11}H_{12}Cl_2N_2O_5$	56-75-7	0.25 g	 Danger	H350 H351 H361d P201 P202 P280
Ampicillin	Roth	Ampicillin trihydrate	$C_{16}H_{18}N_3NaO_4S$	7177-48-2	1 g	 Danger	H317 H334 P280 P261 P302 + P352 P342 + P311
Erythromycin	Roth	Erythrocin	$C_{37}H_{67}NO_{13}$	114-07-8	0.5	-	-
Streptomycin	Roth	Streptomycin sulfate	$C_{42}H_{84}N_{14}O_{36}S_3$	3810-74-0	0.5 g	 Danger	H302 H361d P202 P280 P301 + P312 P308 + P313
Tetracycline	Roth	Tetracycline hydrochloride	$C_{22}H_{24}N_2O_8.HCl$	64-75-5	0.5 g	 Danger	H319 H361d P280 P305 + P351 + P338 P308 + P313
Vancomycin	Roth	Vancomycin hydrochloride	$C_{66}H_{75}Cl_2N_9O_{24}.HCl$	1404-93-9	0.25 g	 Warning	H317
MilliQ water	Sigma-Aldrich	Deionized water	H_2O	7732-18-5	-	-	-
*Asc*I Restriction Enzyme	New England biolabs	-	-	-	-	-	-
CutSmart buffer	New England biolabs	-	-	-	-	-	-
*Not*I-HF restriction	New England	-	-	-	-	-	-

enzyme	biolabs						
DNA size marker (0.1-10.0 kb)	New England biolabs	-	-	-	-	-	-
DNA size marker (100 bp/500 bp)	Bio-Rad laboratories	-	-	-	-	-	-

Annex 2B: Chemical mixtures used , incuding safety data information according to the Globally Harmonised System of Classification and Labelling of Chemicals (GHS)

Chemical mixture	**Producer**	**Substance**	**acronym**	**Formula**	**CAS-no.**	**Amount**	**Pictograms**	**H and P Statements**
TAE buffer	Bio-Rad laboratories	Tris base /Acetic Acid/EDTA buffer in distilled water	TAE	-	6381-92-6	-	 Warning	H319 P280 P264
Ringer solution	Merck	Ammonium chloride, calcium chloride-2 hydrate ,potassium chloride and sodium chloride in distilled water	-	-	10043-52-4/2125-02-9	-	 Warning	H319 P305 + P351 + P338
GES solution	-	Guanidium thiocyanate, ETDA, sarkosyl	GES	-	N.A	-	See Annex 2A for Guanidium thiocyanate, ETDA, sarkosyl	See Annex 2A for Guanidium thiocyanate, ETDA, sarkosyl
TERMLS	-	Lysozyme, sucrose, Tris-HCl EDTA, mutanolysin, RNAse	TERMLS	-	N.A	-	See Annex 2A for Lysozyme, sucrose, Tris-HCl EDTA, mutanolysin, RNAse	See Annex 2A for Lysozyme, sucrose, Tris-HCl EDTA, mutanolysin, RNAse
2 x gel loading buffer	Bio-Rad laboratories	2 % bromophenol blue, 2 % xylene cyanol, 70 % glycerol in distilled water	Loading dye	-	N.A	-	See Annex 2A for bromophenol blue, xylene cyanol and glycerol	See Annex 2A for bromophenol blue, xylene cyanol and glycerol
Eluent A	-	Methanol: bidistilled water	-	-	N.A	91:9 (v:v)	See Annex 2A for methanol	See Annex 2A for methanol

Eluent B	-	tert-methylbutylether: methanol: bidistilled water	-		N.A	80:18:2 (v:v:v)	See Annex 2-A for tert-methylbutylether and methanol	See Annex 2-A for tert-methylbutylether and methanol
Taschiro indicator	-	Methyl red and methylene blue	Indicator	-	N.A	-	See Annex 2A for Methyl red and methylene blue	See Annex 2A for Methyl red and methylene blue
Chloroform -2-pentanol	-	Chloroform: 2-pentanol	-	-	N.A	24:1 (v:v)	See Annex 2A for Chloroform and 2-pentanol	See Annex 2A for Chloroform and 2-pentanol
TE buffer	-	10 mM Tris-HCl, 1 mM EDTA	-	-	N.A	-	See Annex 2A for for Tris-HCl and EDTA	See Annex 2A for for Tris-HCl and EDTA

N.A: not available

Annex 3: Analytical equipment

Annex 3A: Equipment used for freeze-drying

Name of device	**Model Name**	**Manufacturer**
Freeze dryer	Christ alpha 1-4 LD plus	SciQuip The Scientific Equipment Specialists, Newtown Shropshire, UK
Freeze dryer	Christ alpha 2-4 LSC plus	Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany
Titanium knife mill	Grindomix GM200	Retsch GmbH, Haan Germany

Annex 3B: Equipment used for dry mass and ash

Name of device	**Model Name**	**Manufacturer**
Weighing balance	Precisa XT220A	Precisa balances AG, Zurich, CH
Crucibles	Nickel	Self-made
Desiccator	Duran group	Wertheim, Germany
oven	T6060	Heraeus instruments, Hanau Germany
Incinerator	EK20/30	Michaelis, Düsseldorf, Germany
Water bath	Stirrer	Variomag, Telesystem, RM
	Thermostat	Julabo, type ED

Annex 3C: Equipment used for vitamin B_1 and B_2 determination

Name of device	**Model Name**	**Manufacturer**
Autoclave	Sano clave (LA-S-3-20-MC5J)	Wolf, Geislingen, Germany
Ultrapure water system	LaboStar™ DI/ UV2	Siemens, Barsbüttel, Germany
Cooling water bath	Thermo- Haake	Haake, Karlsruhe, Germany
pH meter	PMX 3000	WTW GmbH, Weilheim, Germany

Annex 3D: Equipment used for vitamin C determination

Name of device	**Model Name**	**Manufacturer**
Weighing balance	Precisa XT220A	WTW GmbH, Weilheim, Germany
Microcentrifuge	Biofuge primo	Heraeus instruments, Hanau Germany
pH meter	PMX 3000	WTW GmbH, Weilheim, Germany

Annex 3E: Equipment used for vitamin E analysis

Name of device	Model Name	Manufacturer
Microcentrifuge	Biofuge primo	Heraeus instruments, Hanau Germany
Ultrasonicator	SONOREX DIGITEC	BANDELIN electronic GmbH & Co. KG, Berlin, Germany
Mixer	Vortex mixer	Corning Inc. New York, USA

Annex 3F: Equipment used for protein analysis

Name of device	Model Name	Manufacturer
Weighing balance	Precisa XT220A	Precisa balances AG, Zurich, CH
Heating device	B-414	Büchi Labortechnik AG, Postfach, Switzerland
Digestion unit	K-435	Büchi Labortechnik AG, Postfach, Switzerland
N_2-distillation unit	Kjel Flex-K-360	Büchi Labortechnik AG, Postfach, Switzerland
Titration unit	Schott titronic universal	Schott Instruments, Mainz, Germany

Annex 3G: Equipment used for analysis of soluble sugars

Name of device	Model Name	Manufacturer
Weighing balance	Precisa XT220A	Precisa balances AG, Zurich, CH
Water bath		Grant instruments, Cambridge shire, U.K
Cooling water bath	Thermo- Haake	Haake, Karlsruhe, Germany
Microcentrifuge	Biofuge primo	Heraeus instruments, Hanau Germany

Annex 3H: HPLC method parameters and system components

	HPLC method	System components	HPLC parameters
3H.1	HPLC FLD (Vitamin B_1/B_2)	Agilent 1100 series (Agilent Waldbronn Germany): Pump 1, binary (G1312A) Pump 2, isocratic (G1310A) Auto sampler (G1313A) Column heater (G1316A) Fluorescence detector (G1321A), Degasser (G1322A) Software: (Chemstation: A.08.03, July 2000)	**Column:** Phenomenex Luna® 3µm C18 (2) 100 A, LC column 150 x 3 mm (Phenomenex, Aschaffenburg, Germany) **Injection volume:** 20 µl **Column temperature:** 35 ^oC Auto sampler temperature: room temperature. **Mobile phase:** 20 % methanol and 80 % 10 mM $NH_4H_2PO_4$- buffer containing 1 g/l tetraethylammonium chloride and sodium heptane sulfonate at pH 3.5.

			Flow pump 1 (binary): 0.8 ml/min. **Flow pump 2** (isocratic**)**: Post column derivatization: 0.4 % K_2 (Fe (CN) $_6$) in 15 % NaOH: 0.2 ml/min. 12.00 min to 21.99 min. **Detector settings**: **FLD:** 0.00 min to11.99 min: Excitation 450 nm; emission 525 nm (vitamin B_2) 12.00 min to 22.00 min: Excitation 365 nm; emission 435 nm (vitamin B_1). **Recording rate:** 74 Hz **Time constant:** 14ms.
3H.2	HPLC FLD (Vitamin C)	Shimadzu system (Tokyo, Japan): Pump (2) (LC-10AT) Auto sampler injector (Lachrom l-2200, Merck, Darmstadt, Germany) Column thermostat (L-5025, Merck, Darmstadt, Germany), Tefzel capillary tube (1/16´ = 1.58 mm x 0.3 mm,20 m length loop) Fluorescence detector (RF-551) Software: LC 10 Class chromatographic software Installed system: Gateway 2000 computer	**Column:** Grom-Sil-NH_2 (250 mm x 4.6 mm, 5 µm); pre-column oxidation for simultaneous analysis of ascorbic and dehydroascorbic acids. **Injection volume:** 10 µl **Column temperature:** 20-25 ^oC **Mobile phase:** 70 % acetonitrile in 30 % 50 mM ammonium dihydrogen phosphate (NH_2 H_2 PO_4). Flow rate: 0.8 ml/min. **Post column derivatization:** 1,2-phenylenediamine, 2.5 M sodium acetate and diluted m-metaphosphoric acid/acetic acid (3 %/8 %); flow rate: 0.3 ml/min; retention time: 6.2 min. **Detector settings:** FLD: Excitation: 350 nm; emission: 430 nm.
3H.3	HPLC FLD (Vitamin E)	Nexera X2 (Shimadzu Duisburg, Germany): Pump (2) (LC-30AD, Nexera) Auto sampler (SIL-30AC, Nexera) Column heater (CTO-20AC, prominence) Degasser (DGU-20A5, prominence), Fluorescence detector (RF-20AXS, prominence) Communication bus module (CBM-20A,prominence) Diode array detector (SPD-M20A)	**Column:** Develosil RP Aqueous ; C30, 150 x 3 mm, 3 µm (Phenomenex, Aschaffenburg, Germany) **Injection volume:** 10 µl. **Column temperature:** 18 ^oC **Auto sampler temperature:** 4 ^oC **Gradient elution:** Methanol/water (91:9, v/v; eluent A) and tert-methyl-butylether/methanol/water (80:18:2; v/v/v; eluent B): 0-5 min 0 % B, 5-25 min 0-5 % B, 25-40 min 5 % B, 40-46 min 5-55 % B, 46-48 min 55-100 % B, 48-51 min 100 % B, 51-53 min 100-0 % B, 53-63 min 0 % B. Flow rate: 0.5 ml/min. **Detector settings**: **FLD:** Excitation: 295 nm; emission: 330 nm.

3H.4	HPLC RID (sugars)	Agilent 1100 series (Agilent Waldbronn Germany): Pump 1, binary (G1312A) Auto sampler (G1313A) Column heater (G1316A) Refractive index detector**,** Degasser (G1322A) (Chemstation: A.08.03, July 2000)	**Column:** Reprosil-polyamine-2; 250 mm x 4 mm, 5 µm (Dr. Maisch, Ammerbuch, Germany) **Injection volume:** 50µl; **Column temperature:** 35 ^{o}C. Auto sampler temperature: room temperature **Mobile phase:** 70 %:30 % acetonitrile/water (isocratic). Flow rate: 1.0 ml/min. Elution time: Fructose 5.8 min, glucose 6.9 min; while sucrose 8.8 min.

Annex 4: Consumables for nutrient analysis

Consumable (s)	**Manufacturer**
Pipette tips	VWR, Darmstadt, Germany
Falcon tubes (15 ml / 50 ml)	Greiner Bio-One GmbH, Frickenhausen
Falcon tubes (15 ml/50 ml)	Corning Inc. New York, USA
Prepleated filter paper Ø 18,5 cm, 595 ½	Whatman, Dassel, Germany
Pipet tips (2-5000 µl)	Eppendorf AG, Hamburg, Germany
Bulb pipettes 100 ml	Brand, Wertheim, Germany
HPLC vials (Brown)	VWR, Bruchsal, Germany
PTFE membrane filter (0.2µm,4 mm syringe filter)	Phenomenex, Aschaffenburg, Germany
10-1000 ml volumetric flasks	VWR, Bruchsal, Germany
100-1000 ml conical flasks	VWR, Bruchsal, Germany
Kjeldahl digestion flask (300 ml)	Büchi Labortechnik GmbH, Essen, Germany
Single use syringe (5 ml norm Ject)	Henke-Sass-Wolf, Tuttlingen, Germany
Membrane Filters 0.2µm (Spartan 30/0.2 RC)	Whatman, Dassel, Germany
HPLC vials (Brown	WICOM, Heppenheim, Germany

Annex 5: List of devices and equipment used for fermentation and molecular work

Name of device	**Model Name**	**Manufacturer**
DGGE apparatus	DCode Universal Mutation Detection System	Bio-Rad Laboratory GmbH, Munich, Germany

Gel documentation	ChemiDoc™ XRS +	Bio-Rad Laboratory GmbH, Munich, Germany
Gel chamber	40-1214	Peqlab Biotechnologie GmbH, Erlangen, Germany
pH-meters	pH 526	Scientific, Technical workshops GmbH, Wilhelm
	HI 2211pH/ ORP Meter	Hanna instruments, Japan
Photometer	SmartSpec™ Plus	Bio-Rad Laboratory GmbH, Munich, Germany
Power supply	Powerpac 300	Bio-Rad Laboratory GmbH, Munich
Thermocycler	Primus	PEQLAB GmbH, Erlangen, Germany
Gradient PCR thermocycler	Primus 96 advanced®	PEQLAB GmbH, Erlangen, Germany
Vacuum dryer	Concentrator 5301	Sales Eppendorf, Wesseling-Berzdorf
Weighing balance	Precisa 3100C	Pesa balances AG, Zurich, CH
Microcentrifuge	Biofuge ™ pico	Heraeus Instruments, Hanau Germany
Mega centrifuge	Heraeus ™ Megafuge ™ 1.0R	Thermo Fisher Scientific, Waltham, MA, USA
Universal centrifuge	Heraeus™ Multifuge™ X3R	Thermo Fisher Scientific, Waltham, MA, USA
Super speed Centrifuge	Sorvall RC 26 plus	Thermo Fisher Scientific, Waltham, MA, USA
Standard UV transilluminator	FluorChem 5500	Alpha Innotech Corporation, San Leandro, CA, USA
Gel electrophoresis chamber	GNA-200	Pharmacia Biotech Europe GmbH, Freiburg
Fluorometer	Qubit® 2.0	Life Technologies GmbH, Darmstadt
Stomacher (homogenizer)	400 circulator	Seward company Limited, UK
	Mixer	AES Chemunex GmbH, Bruchsal, Germany
Titration unit	Schott Titronic universal	Schott Instruments, Mainz, Germany
Incubator, 30°C	Heraeus B12	Thermo Electron LED, Langenselbold
Incubator, 41.5°C	B30	Memmert GmbH, Schwabach, Germany
Incubator, 37°C	KB 115	BINDER GmbH, Tuttlingen, Germany
Pipettes	Pipetman Classic ™ P1000, P200, P20, P2	Gilson Inc., Middleton, WI, USA
Bulb pipette 10 ml	Finnpipette, T40662/4500	ThermoLabsystems
Safety Cabinet	HA2472	Heraeus Instruments, Hanau Germany

Thermo Incubator	Thriller®	PEQLAB GmbH, Erlangen, Germany
Vortexer	Genius® 1, Touch Mixer	Scientific Industries Inc., Bohemia, NY, USA

Annex 6: Consumables for fermentation and molecular workAnnex

Consumable (s)	**Manufacturer**
Pipette tips	VWR, Darmstadt, Germany
Nunc ™ cryogenic vials, 1.8 mL	Thermo Fisher Scientific, Waltham, MA, USA
Eppendorf tubes (1.5 ml/2 ml)	Eppendorf AG, Hamburg, Germany
Fermentation pots with stones and lid 5 L	Nik. Schmitt & Sohn, Germany
Culture buckets 5 L	Turskys supermarket, Nairobi, Kenya
ERNESTO® salad spinner	Lidl Stiftung & Co. KG, Neckarsulm, Germany
UV-cuvettes	VWR, Bruchsal, Germany
Falcon tubes (15 ml/50 ml)	Greiner Bio-One GmbH, Frickenhausen
PCR tubes	Eppendorf AG, Hamburg, Germany
Petri dishes, sterile, Ø 90 mm	VWR, Bruchsal, Germany
qubit® assay tubes	Life Technologies GmbH, Darmstadt Germany
Sterile syringe	Whatman, Dassel, Germany
Pipet tips(0.2-1000 µl)	Eppendorf AG, Hamburg, Germany
Bulb pipettes tips10 ml	Brand, Wertheim, Germany
Acrodisc syringe filter 0.2µm ultipor nylon	PALL Life Sciences, MI, USA
Anaerobic jar, 2.5 l	Merck KGaA, Darmstadt, Germany
Anaerocult® A	Merck KGaA, Darmstadt, Germany
Anaerotest® test strips	Merck KGaA, Darmstadt, Germany
Disposable inoculation loops	VWR, Bruchsal, Germany

Annex 7: Enzyme kits and molecular biological kits

Kit name	**Uses**	**Manufacturer**
D-lactic acid (D-lactate) / L-lactic acid (L-lactate) UV test	Determination of D-lactic acid and L-lactic acid in foodstuffs and other materials	Boehringer Mannheim/R-Biopharm AG, Darmstadt, Germany
Reagents kit for DGGE	DGGE analysis	Bio-Rad Laboratory GmbH, Munich, Germany

Sucrose /D-glucose UV test	Determination of sucrose / D-glucose in foods and other materials	Boehringer Mannheim / R-Biopharm AG, Darmstadt
E.Z.N.A bacteria DNA	Bacteria DNA isolation	Omega bio-tek, USA
Qubit® dsDNA HS Assay	DNA concentration measurement (0.2 to 100 ng)	Life Technologies GmbH, Darmstadt, Germany
Allin ™ Hot Start Taq Mastermix, 2X	RAPD-PCR, PCR for sequencing (ATPD)	highQu GmbH, Kraichtal, Germany
Mag-Bind® RxnPure Plus	Purification of PCR products	Omega bio-tek Inc., Norcross, USA
Qiagen Genomic-tip 100/G kit	Bacteria DNA isolation	Qiagen, Manchester, United Kingdom
peqGOLD bacterial DNA kit	Bacteria DNA isolation	Peqlab, Erlangen, Germany
Illumina Nextera XT library prep kit	Libraries preparation for sequencing on the MiSeq	Illumina, San Diego, USA

Annex 8: List of working antibiotics for malt-glucose agar

Antibiotic	**Initial conc.**	**Final conc.**	**Preparation**
Ampicillin	100 mg/ml	100 µg/ml	1 g ampicillin was dissolved in 10 ml sterile distilled water and mixed thoroughly to dissolve, followed by sterilization through 0.2 µm membrane filter and was stored at 2-8 ^{o}C.
Chloramphenicol	25 mg/ml	25 µg/ml	0.25 g chloramphenicol was added in 10 ml 100 % absolute ethanol and properly mixed to dissolve followed by filtration through 0.2 µm membrane filter and was stored at 2-8 ^{o}C.
Erythromycin	50 mg/ml	50 µg/ml	0.5 g erythromycin was dissolved in 10 ml 100 % absolute ethanol followed by mixing and filtration through 0.2 µm membrane filter and was stored at 2-8 ^{o}C.
Streptomycin	50 mg/ml	50 µg/ml	0.5 g streptomycin was dissolved in 10 ml 70 % ethanol followed by mixing and filtration through 0.2 µm membrane filter and was stored at 2-8 ^{o}C.
Tetracycline	50 mg/ml	50 µg/ml	0.5 g tetracycline was dissolved in 10 ml sterile distilled water followed by mixing and filtration through 0.2 µm membrane filter and was stored at 2-8 ^{o}C.
Vancomycin	25 mg/ml	25 µg/ml	0.25 g vancomycin was added in 10 ml sterile distilled water and properly mixed to dissolve followed by filtration through 0.2 µm membrane filter and was stored at 2-8 ^{o}C.

Annex 9: Culture media

Annex 9A: List of culture media, suppliers, uses, composition and preparation as used at the MRI, Germany.

Name	Company	Uses	Composition (g/l)	Preparation
Malt glucose agar with antibiotics	Merck, Darmstadt, Germany	Enumeration of yeasts and moulds	Malt extract 17; D-glucose-monohydrate 5; and agar –agar 15	17 g of malt extract were mixed with 5 g of D-glucose monohydrate in 1 litre of distilled water, and then 15 g of agar-agar were added. The pH was adjusted to 7.0 with 5M NaOH; the medium autoclaved for 15 min at 121 ^{o}C for 15 min. After autoclaving, it was cooled to 45-50 ^{o}C followed by addition of 500 µl of ampicillin (100 µg/ml), chloramphenicol (25 µg/ml), erythromycin (50 µg/ml), streptomycin (50 µg/ml), tetracycline (50 µg/ml) and vancomycin (25 µg/ml) and dispensed on plates.
MRS Broth (Lactobacillus Broth, DE MAN, ROGOSA and SHARPE)	Merck, Darmstadt, Germany	Isolation and enrichment of all species of lactic acid bacteria	Peptone from casein 10.0; meat extract 8.0; yeast extract 4.0; D (+)-glucose 20.0; dipotassium hydrogen phosphate 2.0; Tween® 80 1.0; di-ammonium hydrogen citrate 2.0; sodium acetate 5.0; magnesium sulphate 0.2; manganese sulphate 0.04.	52.2 g MRS Broth were dissolved in 1 litre of distilled water and autoclaved for 15 min at 121 ^{o}C .
MRS Agar	Merck, Darmstadt, Germany	Enumeration of lactic acid bacteria	MRS broth with agar-agar 15.0	The medium was autoclaved for 15 min at 121 ^{o}C.
Ringer solution/tablets	Merck, Darmstadt	Diluent for preparing suspensions in bacteriological studies	Ammonium chloride 0.00525; sodium 0.005; Calcium chloride-2 hydrate 0.04; potassium chloride 0.00525; sodium chloride 1.125	Ready Ringer tablets were used to make a ¼ strength Ringer solution. One tablet was dissolved in 500 ml distilled water and then autoclaved for 15 min at 121 ^{o}C.
Standard I nutrient broth	Merck, Darmstadt, Germany	Enumeration, isolation and enrichment of	15.0 g peptones; 3.0 g yeast extract; 6.0 g sodium chloride	25 g were suspended in 1 litre distilled water and autoclaved for 15 min at

		fastidious aerobic bacteria	and 1.0 g D (+) glucose.	121 ^{o}C.
Standard I nutrient agar	Merck, Darmstadt, Germany	Cultivation and enumeration of fastidious aerobic bacteria	Standard I nutrient broth containing agar-agar 15.0	The medium was autoclaved for 15 min at 121 ^{o}C.
VRBD (violet red bile dextrose) agar	Merck, Darmstadt, Germany	Isolation and enumeration of all Enterobacteriaceae species in foodstuffs.	Peptone form meat 7.0 g; yeast extract 3.0 g; sodium chloride 5.0 g; D (+) glucose 10.0 g; bile salt mixture 1.5 g; neutral red 0.03 g; crystal violet 0.002 g; agar-agar 13.0 g.	39.5 g were suspended in 1liter distilled water. The medium was heated on the hot plate with constant stirring until completely dissolved. Then it was allowed to boil for not longer than 2 min at 90 ^{o}C.
Agar- agar 1	Carl Roth, Karlsruhe, Germany	Gelling agent	70 % agarose and approximately 30 % agaropectin	15 g per litre .

Annex 9B: Media used for challenge test in nightshade fermentations with selected pathogens

Name	**Company**	**Uses**	**Composition (g/L)**	**Preparation**
XLD (Xylose Lysine Deoxycholate) Agar	Biokar diagnostics, Beauvais Cedex, France	Isolation and differentiation of pathogenic Enterobacteriaceae especially of *Shigella* and *Salmonella* species	Yeast extract 3.0; L-lysine 5.0; lactose; 7.5; sucrose 5; xylose 3.5; sodium deoxycholate 2.5; sodium chloride 5.0; sodium thiosulfate; 6.8; ferric ammonium citrate 0.8; phenol red 80.0 mg; bacteriological agar 13.5	52.9 g of dehydrated medium were suspended in 1 litre of distilled water. It was heated to 90 ^{o}C with frequent agitation until the medium was completely dissolved. The medium was not autoclaved.
PALCAM Listeria Selective Agar Base	Merck, Darmstadt, Germany	Selective and differential medium for the detection and isolation of *Listeria monocytogenes*	Peptone 23.0; yeast extract 3.0; starch 1.0; sodium chloride 5.0; agar-agar 13.0; D (-) mannitol 10.0; ammonium iron (III) citrate 0.5; esculin 0.8; glucose 0.5; lithium chloride 15.0; phenol red 0.08.	Suspend 35.9 g in 500 ml of distilled water , autoclave 15 min at 121 ^{o}C). Dissolve the contents of 1 vial of PALCAM Listeria Selective Supplement (see below) in 1 ml sterile distilled water and add to the sterile medium cooled to 50 ^{o}C. If necessary rinse the vial with 1 ml of sterile distilled water . Mix well and pour plates.
Potato Dextrose	Merck,	Isolation and	Potato infusion 4.0	39 g were suspended in 1

Agar	Darmstadt, Germany	enumeration of yeasts and moulds from foodstuffs and other materials	(infusion from 200 g potatoes); D (+) glucose 20.0; agar-agar 15.0; pH 5.7,	litre distilled water heated to boil, and followed by autoclaving for 15 min at 121 ^{o}C. Sterile 10 % tartaric acid was added (14 ml/l) before dispensing on plates (final pH approx.3.5).
Agar- agar 1	Carl Roth, Karlsruhe, Germany	Gelling agent	70 % agarose and approximately 30 % agaropectin	15 g per litre .
Rappaport Vassiliadis soya broth (RVS)	Biokar diagnostics, Beauvais Cedex, France	Selective enrichment of *Salmonella* from food stuffs and other materials	Papaic digest of soybean meal 4.5; Sodium chloride 7.20; Potassium dihydrogen phosphate 1.26 ; di-potassium hydrogen phosphate 0.18; Magnesium chloride anhydrous 13.4; Malachite green oxalate 36.0 mg	26.6 g were dissolved in 1 litre of distilled water , the media was stirred to dissolve and dispensed in tubes followed by autoclaving at 115 ^{o}C for 15 min.
Buffered peptone water (BPW)	Biokar diagnostics, Beauvais Cedex, France	Preliminary, non-selective enrichment of pathogenic enterobacteria bacteria, from foodstuffs and other materials.	Peptone from casein 10.0; sodium chloride 5.0; disodium hydrogen phosphate dodecahydrate 9.0; potassium dihydrogen phosphate 1.5.	25.5 g were suspended in 1 litre distilled water, dispensed into suitable containers and autoclaved for 15 min at 121 ^{o}C.
Muller-Kauffmann Tetrathionate-Novobiocin Broth (MKTTn)	Biokar diagnostics, Beauvais Cedex, France	Selective enrichment of *Salmonella* from food and animal feed stuffs	Tryptone 8.6; meat extract 4.3; bile salts 4.78; sodium chloride 2.6; calcium carbonate 38.7; sodium thiosulfate anhydrous 30.45; brilliant green 9.6 mg	89.4 g of dehydrated medium were suspended in 1 litre of distilled or deionized water. It was slowly boiled with constant stirring. Boiling was continued for 2 min without autoclaving.
PALCAM Listeria Selective Supplement	Merck, Darmstadt, Germany	Inhibits the growth of bacteria in the selective cultivation of *Listeria monocytogenes*	Composition per (vial): Polymixin-B-sulphate 5.0 mg; ceftazidime 10.0 mg; acriflavine 2.5 mg.	The original lyophilisate vial was dissolved in about 1 ml of sterile distilled water. The vial content was mixed evenly into 500 ml of sterile medium base cooled to about 45 to 50 ^{o}C.
L-Palcam selective broth	Merck, Darmstadt,	Selective medium used for the differentiation and	Peptone 23.0: yeast extract 5.0; lithium chloride 10.0;	23.7 g were suspended in 500 ml of distilled water and autoclaved at 121 ^{o}C for

base	Germany	isolation of *Listeria monocytogenes* from milk and cheese, as well as in other food products, even highly contaminated.	esculin 0.8; ammonium iron-III-citrate 0.5; D (-) mannitol 5.0; soybean lecithin 1.0; tween 80 2.0; phenol 0.08.	15 min. Then 1 vial of the Palcam *Listeria* selective supplement contents was suspended in sterile distilled water and added to the basic broth, which had been cooled below 50 ^{o}C. Carefully, it was swirled to mix the selective supplement into the broth homogeneously.
L(+) tartaric acid	Merck, Darmstadt, Germany	Used in potato dextrose agar preparation to lower the pH of the medium thus inhibiting growth of bacteria	Not specified	10 % L (+) tartaric acid was prepared by dissolving 10 g L (+) tartaric acid in 100 ml of distilled water and was autoclaved at 121 ^{o}C for 15 min.
Brilliant green agar (Edel and Kampelmacher)	Biokar diagnostics, Beauvais Cedex, France	selective medium used to isolate *Salmonella* in food products and water, especially when the bacteria are present is small numbers	Tryptone 10.0; meat extract 5.0; yeast extract.3.0; lactose 10.0; sucrose 10.0; disodium phosphate 1.0; monosodium phosphate 0.6; phenol red 90.0 mg; brilliant green 5.0 mg; bacteriological agar.14.0	53.7 g of dehydrated media were suspended in 1 litre of distilled water. It was slowly brought to boil, with constant stirring until completely dissolved. Then dispensed in tubes or flasks. The medium was not autoclaved.
BPLS agar (Brilliant-green phenol-red lactose sucrose agar)	Merck, Darmstadt, Germany	Selective agar for isolation of *Salmonella*	Peptic 5.0; peptone from casein 5.0; yeast extract 3.0; sodium chloride 5.0; lactose 10.0; sucrose 10.0; phenol red 0.08; brilliant green 0.0125; agar-agar 13.0.	51 g were suspended in 1 litre of distilled water and autoclaved for 15 min at 121 ^{o}C.

Annex 9C: List of culture media, suppliers, uses, composition and preparation as used at JKUAT, Kenya

Name	Company	Uses	Composition (g/L)	Preparation
MRS agar	Himedia, Mumbai, India	Cultivation of all Lactic acid bacteria	Proteose peptone 10.0; beef extract 10.0; yeast extract 5.0; dextrose 20.0; polysorbate 80 1.0; ammonium citrate 2.0; sodium acetate 5.0; magnesium sulphate 0.1; manganese sulphate 0.05; dipotassium phosphate 2.0, agar; 12.0	67.15 g were suspended in 1 litre of distilled water and autoclaved for 15 min at 121 ^{o}C.
MRS broth	Himedia, Mumbai, India	Cultivation of all Lactic acid bacteria	Proteose peptone 10.0; beef extract 10.0; yeast extract 5.0; dextrose 20.0; polysorbate 80 1.0; ammonium citrate 2.0; sodium acetate 5.0; magnesium sulphate 0.1; manganese sulphate 0.05; dipotassium phosphate 2.0	55.15 g were dissolved in 1 litre of distilled water and autoclaved for 15 min at 121 ^{o}C.
VRBG (violet red bile glucose) agar	Himedia, Mumbai, India	Detection and enumeration of Enterobacteriaceae	Yeast extract 3.0; pancreatic digest of gelatin 7.0; bile salts 1.5; sodium chloride 5.0; glucose monohydrate 10.0; neutral red 0.03; crystal violet 0.002; agar-agar 15.0.	38.53 g of dehydrated medium were suspended in 1 litre distilled water. The medium was heated on the hot plate with constant stirring until completely dissolved. Then it was allowed to boil for not longer than 2 min at 90 ^{o}C.
Nutrient broth	Himedia, Mumbai, India	Sterility testing and cultivation of non-fastidious microorganisms	Peptic digest of animal tissue 5.0; beef extract 1.5; sodium chloride 5.0g and yeast extract 1.5	13 g were suspended in 1 litre distilled water and autoclaved for 15 min at 121 ^{o}C.
Potato dextrose agar	Himedia, Mumbai, India	Isolation and enumeration of yeasts and moulds from dairy and other food stuffs	Potatoes infusion from 200.0; dextrose 20.0; agar-agar 15.0.	39 g were suspended in 1 litre distilled water (pH 5.6), heated to boil, and followed by autoclaving for 15 min at 121 ^{o}C. After cooling to 45 ^{o}C, sterile 10 % tartaric acid (14 ml/l) was added before

				dispensing on plates. (Final pH approx.3.5).
L (+) tartaric acid	Sigma Aldrich, St. Louis, USA	Inhibits bacterial growth	Not specified	10 % L (+) tartaric acid was prepared by dissolving 10 g L (+) tartaric acid in 100 ml of distilled water followed by autoclaving at 121 ^{o}C for 15 min.
Agar-agar	Himedia, Mumbai, India	Gelling agent	70 % agarose and approximately 30 % agaropectin	15 g per litre.

Annex 10: Special assessment scheme for sensory tests with a 9-point scale of fermented African nightshade vegetable and answering guide

Q#	Attributes	- P O I N T S -								
		9	8	7	6	5	4	3	2	1
	Quality domain	**Excellent**	**Very good**	**Good**	**Satisfying**	**Moderate**	**Sufficient**	**Insufficient**	**Bad**	**Very bad**
1]	How would you describe the color of the vegetable									
2]	How do you find the appearance of the fermented vegetables									
3]	Rate the smell of the fermented vegetable									
4]	How do you find the taste of the fermented vegetable									
5]	How is the feeling of the vegetables in your mouth									
6]	How do you compare the fermented vegetable to the normally prepared indigenous vegetables in terms of colour, appearance, smell and taste									

7] Gender Male ◯ Female ◯

8] Have you ever heard of fermented vegetables YES ◯ ◯

9] Would you recommend the fermented vegetables to others ◯ ◯

NOTE

KINDLY TICK APPROPRIATELY AND CHOOSE ONLY ONE OPTION PER QUESTION

Questionnaire answering guide

Q#	Attributes	- P O I N T S -								
		9	8	7	6	5	4	3	2	1
	Quality domain	**Excellent**	**Very good**	**Good**	**Satisfying**	**Moderate**	**Sufficient**	**Insufficient**	**Bad**	**Very bad**
1]	How would you describe the color of the vegetable	Dark brown	grey	Very grey	slight discoloration	moderate discoloration	browning	yellowing	pale	black
2]	How do you find the appearance of the fermented vegetables	Under destroyed	No marks	Firm	Slightly damaged	Moderately damaged	Shrunk	Pasty	Slimy	Mucoid
3]	Rate the smell of the fermented vegetable	Aromatic	Balanced	Mild sour	Flat	off flavour	Pungent	Pickled	Rancid	Moldy
4]	How do you find the taste of the fermented vegetable	Aromatic	Balanced	Mild sour	Flat	too sour	Pungent	Too sour	Moldy	Metallic
5]	How is the feeling of the vegetables in your mouth	Firm	Smooth	Crisp	Slightly dry	Too stringy	Too soft	Hard	fibrous	Slimy
6]	How do you compare the fermented vegetable to the normally prepared indigenous vegetables	Far much Better	Much better	Slightly better	Better	Moderately better	No difference	Worse	Much worse	Not sure

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Dedication

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Declaration

I hereby declare on oath, that I have written this thesis by my own and have not used other than the acknowledged resources and aids. The submitted written version corresponds to the version on the electronic storage medium. I hereby declare that I have not previously applied or pursued for a doctorate (Ph.D. studies)

Signature..... Date .....

Eliud Nalianya Wafula