Alkaline fermentation of Bambara groundnut (*Vigna subterranean* L. *Verdc*.) as *dawadawa*-like

African food condiment

By

Akanni Gabriel Bidemi

Submitted in partial fulfilment of the requirements for the degree

PhD (Food Science)

in the

Department of Food Science

Faculty of Natural and Agricultural Sciences

University of Pretoria

November, 2017

DECLARATION

I, Gabriel Bidemi Akanni declare that the thesis, which I hereby submit for the degree PhD Food Science at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

November, 2017

ABSTRACT

Alkaline fermentation of Bambara groundnut (*Vigna subterranean* L. *Verdc*.) as *dawadawa*-like African food condiment

By

Akanni Gabriel Bidemi

Supervisors: Prof. E. M. Buys

Prof. H. L. de Kock

Degree: PhD Food Science

Dawadawa is an African condiment produced solely from the spontaneous alkaline fermentation of African locust beans but production from other legumes such as Bambara groundnut to produce *dawadawa*-type condiments has become of interest. *Dawadawa* is an integral part of the African diet due to its distinct aroma and flavour enhancing properties when added during cooking of soups or stews which are imparted by volatile compounds in the condiments. *Bacillus* species are known to dominate the alkaline fermentation of legumes in the production of *dawadawa*. *Bacillus* species isolated from spontaneously fermented *dawadawa* were identified using MALDI–TOF MS as *B. cereus* (35%), *B. licheniformis* (30%), *B. pumilus* (21%), *B. subtilis* (10%) and *B. amyloliquefaciens* (4%). Further molecular typing was performed using GTG_5 rep-PCR typing, 16S rRNA and *gyr*A gene sequencing.

Alkaline fermentation of Bambara groundnut using *B. subtilis* subsp. *subtilis* (strain SFBA3), *B. amyloliquefaciens* subsp. *plantarum* (strain SFBA2), *B. cereus* (strain PALB7) and *B. licheniformis* (strain OALB2) starter cultures was reported. Volatile compounds were isolated from the *dawadawa*-like condiments using headspace solid phase microextraction (SPME) and analysed by comprehensive gas chromatography coupled to time of flight mass spectrometry (GC × GC–TOFMS). Acids, aldehydes and alcohols accounted for over 70% of the volatile compounds produced in the *Bacillus* fermented samples.

B. subtilis subsp. *subtilis* SFBA3 produced the highest content of acids (5089.88 µg kg^{-1}), while the highest content of aldehydes (2811.16 µg kg^{-1}) and alcohols (1255.58 µg kg^{-1}) was detected with *B. cereus* PALB7 and *B. licheniformis* OALB2, respectively. Sulphur–containing compounds concentration (84.44 µg kg^{-1}) was highest for *B. amyloliquefaciens* SFBA2. The highest concentrations of 2–methyl butanoic acid and 3-methyl butanoic acid, indicative of typical *dawadawa* aroma, were produced by *B. subtilis* subsp. *subtilis* SFBA3. The sensory properties of *dawadawa*-like condiments produced using *B. subtilis* subsp. *subtilis* (strain SFBA3), *B. amyloliquefaciens* subsp. *plantarum* (strain SFBA2), *B. licheniformis* (strain OALB2) and *B. pumilus* (strain PALB2) for fermentation of Bambara groundnut were evaluated in terms of aroma, flavour, aftertaste and colour. A trained descriptive sensory panel evaluated the sensory characteristics of the uncooked paste and cooked broths using 3 appearance, 9 aroma and 18 flavour descriptors. The intensities of ammoniacal, pungent, chocolate/cocoa, rancid and *dawadawa* aromas differed significantly amongst the condiments made using the different *Bacillus* starter cultures. Strain SFBA3 had the highest intensity of pungent and *dawadawa* aroma, while ammoniacal aroma were more pronounced in strains SFBA2 and PALB7. Strain OALB2 had the lowest intensity of *dawadawa* aroma. The production of *dawadawa*–like African condiments using *Bacillus* strains starter cultures from the alkaline fermentation of Bambara groundnut was achieved. The production of affordable *dawadawa*–like condiments from Bambara groundnut using *Bacillus* starter cultures identified with polyphasic identification methods would be beneficial in terms of product quality and flavour consistency in the small to medium scale food industry in Africa.

DEDICATION

I dedicate this work to my late mother, Grace Olubisi Alarape Akanni – The love and sacrifices you have made for me with your fervent prayers has made this dream turn into reality. To my late father, Ganiyu Bamidele Akanni, you laid the foundations on which I strive more earnestly to build on. To my dear daughter, Ayooluwatofunmi Chimamanda Akanni, for the joy you give me through the darkest moments. To God, my ever dependable and reliable source of help.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1.0   INTRODUCTION AND PROBLEM STATEMENT

Traditional African food condiments are products of alkaline fermentation of legumes which has been carried out for centuries in West and Central Africa. These condiments impact flavour and enhance meatiness in soups, sauces and other prepared dishes (Beaumont, 2002). They also contribute significantly to the intake of dietary proteins, essential amino acids, fatty acids and B vitamins particularly riboflavin (Campbell-Platt, 1980; Odunfa, 1983; Parkouda *et al*., 2009). The alkaline fermentation process is usually spontaneous; carried out with rudimental equipment and hygiene is questionable. The natural microbial diversity comprising of both beneficial and spoilage microorganisms are often found in these condiments. This results in products with inconsistent sensory and flavour quality. Furthermore, these African condiments lack an appealing image because they are sold without proper packaging in the open market (Ogunshe *et al*., 2013). Several indigenous African legumes are used for the production African condiments such as African locust bean (*Parkia biglobosa*) for the production of *dawadawa* (Campbell–Platt, 1980), African oil bean (*Pentaclethra macrophylla*) for the production of *ugba* (Odunfa and Oyewole, 1986), melon seed (*Citrullus vulgaris*) for production of *ogiri* (Odunfa, 1981) and Bambara groundnut (*Vigna subterranean* L *Verdc.*) for production of a *dawadawa*–like condiment (Barimalaa *et al*., 1994). However, there is limited use of Bambara groundnut despite its relatively high crude protein (16–24%) and carbohydrate (50–60%) contents which is quite similar to that reported for cowpea and pigeon pea (Heller *et al*., 1997; Brough *et al*., 1993; Eltayeb *et al*., 2011).

During the alkaline fermentation of legumes to produce African food condiments, *Bacillus* species have been highlighted as the dominant microorganisms (Odunfa, 1988; Parkouda *et al*., 2009). The major role of *Bacillus* species during fermentation of these legumes involves production of proteases for hydrolyzing proteins to peptides, amino acids and releasing ammonia, thereby creating an alkaline pH which aids the inhibition of spoilage microorganisms (Parkouda *et al*., 2009). *Bacillus* species isolated from *dawadawa* made from African locust bean include *B. amyloliquefaciens*, *B. atrophaeus*, *B. badius*, *B. cereus*, *B. firmus*, *B. fumus*, *B. licheniformis*, *B. megaterium*, *B. mojavensis*, *B. mycoides*, *B. pumilus*, *B. subtilis*, *B. sphaericus*

and *B. thuringiensis* (Amoa-Awua *et al*., 2006; Azokpota *et al*., 2007; Ouoba *et al*., 2004; Parkouda *et al*., 2009). Although members of the *Bacillus* genus are known to dominate the total microbial flora, there are conflicting reports in literature about the dominating *Bacillus* species. The methods of identification of these *Bacillus* species have been predominantly based on phenotypic characteristics of the microorganisms such as the morphology; and biochemical tests which give less accurate result unlike genotypic identification. Reports on the genotypic identification of the *Bacillus* species in African food condiments using molecular techniques highlighted members of the *B. subtilis* group complex as the dominant species (Adewunmi *et al*., 2013; Ouoba *et al.*, 2004; Oguntoyinbo *et al*., 2010). However, the use of commercial starter cultures does not find widespread application in the alkaline fermentation of foods. Currently, the only *Bacillus* strain as commercial starter culture is *B. subtilis* var. *natto* for the Japanese 'natto' production (Kubo *et al*., 2011).

African food condiments, particularly *dawadawa* are characterized by pungent or ammoniacal flavour, which is the main criterion for assessing product quality and consumer acceptability (Allagheny *et al*., 1996; Azokpota *et al*., 2008). Volatile compounds that contribute to flavour in African condiments such as pyrazines, aldehydes, ketones, esters, alcohols and acids were identified in *dawadawa* produced from African locust bean and soybeans (Azokpota *et al*., 2008; Ouoba *et al*., 2005). However, volatile compounds that characterize the *dawadawa*–like African food condiment produced from the alkaline fermentation of Bambara groundnuts have never been reported.

The application of molecular typed *Bacillus* starter cultures for the production of *dawadawa*–like African food condiments could improve food safety and product consistency in terms of volatile compounds production and sensory properties of the product. Therefore, this study will endeavour to characterise *Bacillus* starter cultures for the *dawadawa*–like African condiments production and their interactions in terms of volatile compounds production and sensory properties during alkaline fermentation of Bambara groundnut.

2.0   LITERATURE REVIEW

2.1   Legume–based alkaline fermented food condiments of the world

Legume-based alkaline fermented food condiments are commonly found in parts of Asia and Africa, such as Japanese *Natto*, Thailand *Thua-nao*, Chinese *Dou-shi*, Indian *Kinema*, Nigerian *Dawadawa* or *Iru*, Ghanaian *Kpalugu*, Burkina Faso *Soumbala* and Beninese *Sonru* to mention a few (Parkouda *et al*., 2009; Odunfa, 1988; Kubo *et al*., 2011). Alkaline-fermentation is defined as a fermentation process during which the pH of the substrate increases to alkaline values which may be as high as pH 9–11 (Omafuvbe *et al*., 2002; Sarkar and Tamang, 1995). The increase in pH is due to degradation of proteins from the raw material into peptides, amino acids and ammonia (Kiers *et al*., 2000) or due to alkali-treatment during production (Wang and Fung, 1996).

Legume-based alkaline fermented food condiments are important part of diets of the indigenous people because of their sensory attributes and high nutritional value (Dakwa *et al*., 2005). They serve as nutritional supplements to the traditional diets of a majority of people in Asian and African countries, who rely largely on starchy staples such as cereals, cassava, yam and plantain that are rich in calories but poor in protein and other nutrients (Achi, 2005; Dakwa *et al*., 2005). In most parts of Asia, these alkaline fermented food condiments serve as an integral part of the diet and they are mainly produced from soya bean (*Glycine max*) (Wang and Fung, 1996; Parkouda *et al*., 2009; Kubo *et al*., 2011). In West Africa, these condiments serve as a source of protein (usually as a low-cost meat substitute), aroma and flavour enhancer in African dishes; indigenous legumes generally rich in protein are used for their production (Odunfa, 1988; Omafuvbe *et al*., 2004; Parkouda *et al*., 2009). Legumes are generally rich in protein (20–40%) but also contain other anti-nutritional compounds such as phytic acid, tannins and trypsin inhibitor (Obizoba and Egbuna, 1992; Oyeleke *et al*., 2012). Fermentation of legumes has been reported to reduce these anti-nutritional compounds in addition to improving the protein content and digestibility (Ouoba *et al*., 2007).

2.2   Production of legume-based alkaline fermented food condiments from Africa

The production of alkaline fermented African food condiments is usually from wild legume seeds; it is usually a traditional family art done in households and fermentation is spontaneous, comprising of microbial diversity indigenous to the production site (Odunfa, 1981; Ogbadu and Okagbue, 1988). In most cases, there is a combination of both beneficial and spoilage microorganisms found in the condiments. Furthermore, the process is often carried out with rudimental equipment which are not hygienic (Ogunshe *et al*., 2013). The legume-based substrate and the production process differ from one country to another; however, there are similarities in the production steps and most importantly in the fermentation. *Dawadawa* is a traditional African food condiment usually produced from African locust bean.

2.2.1   Substrates for the production of *dawadawa* African food condiment

Dawadawa, a traditional alkaline-fermented food condiment is prepared from legumes including seeds from African locust bean which is a wild tree as well as various cultivated plant seeds (Ouoba *et al*., 2004; Terlabie *et al*., 2006). The basic attribute of the substrate is their high protein or carbohydrate contents. Legume-based substrates mostly employed are African locust bean, soya bean and Bambara groundnut.

2.2.1.1 African locust bean (*Parkia biglobosa*)

African locust bean (*Parkia biglobosa*) is a perennial leguminous tree which belongs to the family *Leguminosae* and subfamily *Mimosoideae* (Campbell–Platt, 1980; Pelig-Ba, 2009). Other common names are *dawadawa* in Ghana; *soumbala* in Burkina Faso; and *iru* in Benin and Nigeria. The distribution of the Parkia tree is in the savannah regions of West Africa stretching from Gambia in the west to Cameroon in the east. The African locust beans from the *Parkia* tree mature in the dry season in February and March, providing valuable food in the middle of the traditional "hungry season" before the new harvest (Campbell–Platt, 1980). The sole use of African locust bean is for *dawadawa* condiment production; being the major substrate for the production of the condiment, however; its availability is dwindling over the past years. The name *dawadawa* or other traditional names such as *iru* and *soumbala* are synonymous to African locust bean in various regions of West Africa (Odunfa, 1988; Ouoba *et al*., 2007).

4

The composition of African locust bean has not been extensively reviewed, proximate composition of dried seeds indicated carbohydrate (13–42%), crude protein (17 – 45%), fat (7–43%), crude fibre (3–9%), ash (3.3–15%), moisture (11–13%) and organic matter (9%) (Campbell–Platt, 1980; Ibrahim and Antai, 1986; Pelig–Ba, 2009). The African locust bean and *dawadawa* are particularly useful sources of protein to the poorer sections of the community. African locust beans were found to provide the second cheapest source of protein (cost per 100 grams' protein) after groundnuts (Campbell–Platt, 1980). Parkia beans are deficient in the sulphur-containing amino acids. The amino acid pattern of fermented *Parkia* beans are generally similar to that of raw beans, with a small decrease in essential sulphur-containing amino acids and large decrease in the non-essential aspartic and glutamic acids. Fermentation has been reported to increase the riboflavin content of African locust bean seeds.

2.2.1.2 Soya bean (*Glycine max* L.)

Soybean (*Glycine max* L.) is a leguminous plant of the family *Leguminosae*. It typically grows in tropical, subtropical and temperate climates (Kolapo, 2011). It is cultivated throughout East and South Asia, Africa and America (Kolapo, 2011). Soybean seeds contain an average amount of 36.5–41% protein on a dry weight basis (Medic *et al*., 2014). Soybean proteins are well-balanced in the essential amino acids; have a high lysine content but are slightly deficient in sulphur containing amino acids (methionine and cysteine) and threonine (Bau *et al*., 1997; Medic *et al*., 2014). Globally, soybean is one of the largest sources of vegetable seed oil (Kayembe and Van Rensburg, 2013). The seeds lipid content accounts for 8.1–24% (Medic *et al*., 2014). Soybeans are high in polyunsaturated fatty acids (PUFA) (85% of the lipid fraction), and contain no cholesterol (Kolapo, 2011). The most abundant fatty acid is linoleic acid, followed by oleic, palmitic, linolenic, and stearic acids (Medic *et al*., 2014). Due to their high protein and lipid content, soybeans contain only moderate amounts of carbohydrates (29–35%) (Medic *et al*., 2014).

Structural carbohydrates in soybeans include cell-wall polysaccharides (cellulose, hemicellulose, and pectins), while non-structural carbohydrates involve starch and different mono-, di-, and oligosaccharides. The most abundant soluble sugars in soybeans are sucrose, raffinose and stachyose. In general,

approximately half of the total carbohydrates in soybean seeds are structural carbohydrates, and the other half are nonstructural (Medic *et al*., 2014). Soybeans contain around 9.3% dietary fibers; they are very rich in hemicellulose and cellulose, but exhibit low levels of lignin (Bau *et al*., 1997). Vitamins present in the seed include thiamin, niacin, riboflavin, cholin, vitamins E and K (Kolapo, 2011), and ascorbic acid (Kayembe and Van Rensburg, 2013). Soybeans are also a source of calcium, iron, zinc, copper and manganese (Bau *et al*., 1997). The anti-nutritional factors in soybeans include α-galactosides, trypsin inhibitors, phytic acid, lectins (haemagglutinins) and lipase inhibitors (Medic *et al*., 2014). However, consumption of soybeans has been linked with many health benefits. The proportion of potassium to sodium (3:1–11:1) is believed to prevent high blood sugar level and help to keep blood sugar levels under control in diabetic patients. Soy protein can also help diabetic patients in preventing kidney diseases and improving the cholesterol profile. Furthermore, soybean isoflavones have been associated with lower prevalence of coronary heart disease through controlling cholesterol, blood pressure, vascular function and direct effects on the cells of the artery wall. They are also related to reduction of bone loss that typically occurs after menopause, and can help women with low bone mineral content prevent hip fractures in post-menopausal years. Additionally, in areas of the world with regular soybean consumption, lower rates of colon cancer, as well as some other cancers, including breast cancer, are observed (Kolapo, 2011).

The nutrient composition of soybean has influenced its history, the ancient Chinese evolved methods for its preparations such as soy curd, cheese, sauce, paste used to flavour and enrich their staple diet of cereals. They also used the sprouts of the seeds as a vegetable, while some evidence also exists that methods of expressing the oil were evolved about the 4th century A.D. (Aykroyd and Doughty, 1982). Soybeans have also been used extensively in fermentation applications, including soy sauce, *natto* (whole soybean product), soy pastes and fermented *tofu*.

In Africa, soybeans are used in many household preparations, as they are considered an inexpensive source of dietary protein, mineral, and vitamin for both rural and urban dwellers. The various uses include *dawadawa* production, fortification of cassava based *gari* and *tapioca*, preparation of weaning food, soups

and porridges (Omafuvbe *et al*., 2000; Obatolu, 2002). Furthermore, soymilk and its derived products (soy-coconut milk based yoghurt, soy-cow milk based yoghurt, soy-corn milk) are becoming more and more popular due to the health benefits credited to soybeans (Kolapo and Oladimeji, 2008; Kolapo and Sanni, 2009; Kolapo, 2011).

2.2.1.3 Bambara groundnut (*Vigna subterranea* L. *Verdc*.)

Bambara groundnut originated from the African continent and has been cultivated in tropical Africa for centuries. The common name actually appears to be derived from a tribe, the Bambara, a district on the upper Niger near Timbuctoo. Nowadays, Bambara groundnut is also found in parts of South America, Asia and Oceania (Bamshaiye *et al*., 2011; Hillocks *et al*., 2012). Bambara groundnut belongs to the family of *Fabaceae* and sub family of *Faboidea*, and its botanical name is *Voandzeia subterranea* (L.) *thousars*, synonym of *Vigna subterranea* (Bamshaiye *et al*., 2011). Bambara groundnut is considered the third most important food legume in Africa after groundnuts (*Arachis hypogea*) and cowpeas (*Vigna unguiculata*) (Hillocks *et al*., 2012). It is considered a 'safer' crop because it has the ability to grow in soils too poor to support the growth of other legumes, it is drought-tolerant, and resistant to pests and diseases (Bamshaiye *et al*., 2011; Brough and Azam-Ali, 1992). Nevertheless, it is still regarded as a poor man's food and given less value because it is seen as a snack or food supplement, rather than a cash crop (Bamshaiye *et al*., 2011).

Bambara groundnut is a pulse legume with high nutritional value. The carbohydrate (50–61.3%), protein (16–21%) and lipid (6%) contents are relatively equilibrated and give Bambara groundnut a gross energy value greater than those of common pulses such as cowpea, lentil and pigeon pea (Yusuf *et al*., 2008). Its protein is rich in total essential amino acids (32.7% of protein content) with lysine the most predominant (average value of 10.3%) and appreciable amounts of leucine, phenylalanine, histidine and valine (Minka and Bruneteau, 2000). Bambara groundnut has higher content of methionine compared to other legumes such as cowpea and lentil (Murevanhema and Jideani, 2013). The lipid content of Bambara seeds accounts for 6–9.7% (Adebowale *et al*., 2011; Yusuf *et al*., 2008). The PUFA linoleic (44%) and linolenic (21%) and the saturated palmitic acid (30%) were the most predominant fatty acids while stearic acid was present

in small quantities (Minka and Bruneteau, 2000). This legume is a good source of calcium, potassium, magnesium, phosphorus and iron (Amarteifio *et al*., 2006). Red seeds contain almost twice as much iron as the cream seeds, and their consumption could be useful in areas where iron deficiency is a problem (Bamshaiye *et al*., 2011). Bambara groundnut may be consumed fresh or roasted for the immature seeds while the fully matured ripe seeds demand extended periods of soaking and boiling to render them edible (Bamshaiye *et al*., 2011; Brough and Azam-Ali, 1992).

In many African countries this legume is used in the preparation of numerous traditional recipes, including cakes, steamed balls, relishes and sauces. Roasted, pulverized seeds of Bambara are incorporated into soups. Many times Bambara groundnut based foods are accompanied by cassava or corn preparations. Bambara flour could be a potential alternative for the fortification of traditional weaning foods in Africa. Other food applications of Bambara groundnut involve milk production (a modified version of extraction of cowpea milk) and fermented products (Bamshaiye *et al*., 2011; Brough and Azam-Ali, 1992; Hillocks *et al*., 2012). The Bambara groundnut husks have long been used as an animal feed while in some African countries the leaves have been attributed medicinal properties (Bamshaiye *et al*., 2011).

2.3   Production process of *dawadawa* **African food condiment**

The African food condiment *dawadawa* is the most prominent in West African countries, with the fermentation of African locust bean (*Parkia biglobosa*) as substrate (Azokpota *et al*., 2008; Odunfa, 1988; Parkouda *et al*., 2009). African locust bean is a perennial deciduous wild tree seed which is often laborious to process (Alabi *et al*., 2005). The local names for *dawadawa* in different geographical regions includes: *irú* (Yoruba tribes of the western Nigerian), *dawadawa*/*dàdáwà* (Hausa tribes of the northern Nigeria), *kpalugu* (Ghana), *kinda* (Sierra Leone), *netetou* (Gambia and Senegal), *soumbala*/*soumbara* (Burkina Faso including many francophone West African countries) and *sonru* (Benin Republic) (Campbell–Platt, 1980; Ogunshe*et al*., 2013). *Dawadawa* production is based on traditional knowledge and experience. There are therefore differences in the procedures employed in different areas and localities (Odunfa, 1988). Generally, the process is uncontrolled and there are no national standards for the product

in the different countries. The duration of fermentation, other processing parameters and to some extent the microbial species involved vary, leading to variations in product quality (Sanni, 1993; Sanni *et al*., 2000).

The production process of other alkaline fermented African food condiments from other substrates is similar to that of *dawadawa*, whilst a longer or shorter fermentation time is applied (Odunfa, 1988; Azokpota *et al*., 2008; Parkouda *et al*., 2009). The production process of *dawadawa* could serve as a production process model for African food condiment production.

Pre-fermentation processing of dawadawa:

The raw African locust bean seeds are harvested with pods from wild *dawadawa* trees in the savannah, followed by removal of the seeds from the pods, and then washed in water. The seeds are continuously boiled for 12 hours. Boiled seeds are dehulled by hand-peeling or peeling with a mortar and pestle (Odunfa, 1988).

Fermentation:

Various incubation materials are employed locally for the production of *dawadawa* or related products from African locust beans. Frequently used are banana leaves with a clean calabash as holding container or jute bags with a basket as holding container (Antai and Ibrahim 1986; Odunfa, 1988). Fermentation is usually natural uninoculated fermentation carried out at ambient temperature for 3–5 days.

Post-fermentation and packaging:

After fermentation, sodium chloride is usually added to *dawadawa* as a preservative, then moulded into balls, sun-dried and sold in the open market.

Dawadawa–like condiments have been produced from other substrates such as soybean and there is a report on the attempted use of Bambara groundnut (Dakwa *et al*., 2005; Amadi *et al*., 1999). The process of production is more or less the same as that from African locust bean but with few modifications. Unlike the the long initial cooking time associated with African locust bean during *dawadawa* production; however, the initial cooking is eliminated with production from soybean or Bambara groundnut as substrates.

Seeds are soaked in water until soft; then dehulled by-hand, this is followed by cooking times of 12 h or1

5 min for soybean or Bambara groundnut, respectively (Omafuvbe *et al*., 2000; Barimalaa *et al*., 1994).

The flowchart for the production of *dawadawa* or *iru* from the seeds of African locust bean (Figure 2.1) is

 typical of fermentation of most fermented legumes or vegetable protein from Africa used as condiments.

2.4   Microorganisms associated with African food condiments

In the traditional method of alkaline fermented African food condiments production, the microorganisms

associated with the various stages of fermentation are mainly bacteria but the occurrence of yeasts species

have been noted in some condiments. The following bacteria have been identified during the fermentation

of various leguminous substrates: *Bacillus*, *Pseudomonas*, *Micrococcus*, *Lactobacillus*, *Streptococcus*,

Leuconostoc and *Staphylococcus* (Ouoba *et al*., 2004; Odunfa, 1988; Omafuvbe *et al*., 2004; Parkouda *et*

al., 2009). The isolates are mesophiles with optimum growth temperature range of 30-50 °C and are capable

of utilizing various carbohydrates as carbon sources (Parkouda *et al*., 2009). Most of the microorganisms

found are due to contamination from various sources such as the equipment used and from food handlers,

since there was no direct inoculation with microorganisms.

This brings about inconsistency in the product quality from different producers and geographical zones.

However, the predominant bacteria isolated from fermenting and fermented seeds were composed of

various species of *Bacillus* (Table 2.1) constituting over 95% of the total microbial population density

(Azokpota *et al*., 2008; Parkuoda *et al*., 2009). The dominance of *Bacillus* species could be attributed to

their ability to produce heat-resistant spores, thus surviving the several heating steps in the alkaline

fermented African food condiments production process (Ouoba *et al*., 2007). Table 2.1 summarizes the

Bacillus species, substrates, product name and the geographical region of African food condiments

produced by alkaline fermentation.

African locust bean seeds

↓

Sorting and Cleaning

↓

First cooking: Boiled for 12 h or more

(Addition of nail / wood ash to quicken boiling of the cotyledons)

↓

Dehulling (hands / mortar and pestle)

↓

Washing / rinsing with water

↓

Second cooking: Re–boiled for 1-2 h

(Addition of "kaun" to soften the cotyledons)

↓

Draining through a local sieve / woven basket / plastic or metallic sieve

↓

Wrapped in banana leaves or jute bags whilst hot

↓

Spreading in fermenting calabashes

↓

Calabash trays stacked together and covered with woven baskets and wrapped with jute bags / cloth

↓

Fermentation (2-5 days) at room temperature

↓

Dawawawa or Irú

Figure 2.1: Schematic flow chart of the traditional production process of an African food condiment *dawadawa* (Adapted from Odunfa, 1988).

2.5   *Bacillus* **species in African food condiments**

Bacillus species are aerobic endorespore forming gram-positive rods. The genus was created in 1872 by F. Cohn who changed the name of Ehrenberg's 1935 *Vibrio subtilis* to *Bacillus subtilis* (Harwood, 1989). *Bacillus subtilis* is the type species of the genus. Representatives of this genus are widely distributed in the air, soil and water. Some *Bacillus* strains are able to tolerate extreme conditions such as high and low pH as well as high and low temperatures.

The *Bacillus* found in spontaneous alkaline fermentation of African food condiments comprises species such as *B. subtilis*, *B. licheniformis*, *B. megaterium*, *B. amyloliquefaciens*, *B. firmus*, *B. pumilus*, *B. cereus* (Amadi *et al*., 1999; Ouoba *et al*., 2004; Dakwa *et al*., 2005). It has been reported that during fermentation *Bacillus* species produces amylase, galactanase, galactosidase, glucosidase and fructofuranosidase which are capable of degrading the polysaccharides and oligosaccharides (stachyose, raffinose, sucrose, verbascose, galactamannan and arabinogalactan) in the legume-based substrates (Kiers *et al*., 2000; Omafuvbe *et al*., 2000; Terlabie *et al*., 2006). The *Bacillus* species are proteolytic and thus quickly hydrolyze the proteins in the beans giving amino acids which are further metabolized, giving rise to changes in pH value from about 6.50 to 8.50 and the development of strong ammonical odour (Gernah *et al*., 2007; Sarkar *et al*., 1994). Not all of the *Bacillus* species involved in the alkaline fermentation are generally regarded as safe (GRAS) organisms.

Table 2.1: Traditional Alkaline fermented legumes or vegetable proteinaceous seeds and the main microorganisms involved in the production of African food condiments and related products

Substrate/Raw material	Product	Country	Microorganisms	Sensory properties	References
African locust bean (*Parkia biglobosa)*	*Dawadawa*	Nigeria, Ghana	*B. subtilis, B. pumilus, B. licheniformis, B. firmus, B. atrophaeus, B. amyloliquefaciens, B. mojavensis, B. megaterium, B. brevis, B. polymyxa, Lysininbacillus sphaericus, Leuconostoc* spp., *Staphylococcus* spp., *Pseudomonas aeruginosa*	Alkaline, sticky	Amoa-Awua *et al.*, 2006; Meerak *et al.*, 2008, Jideani and Okeke, 1991; Odunfa, 1981, Odunfa and Oyewole, 1986; Ogbadu and Okagbue, 1988; Omafuvbe *et al.*, 2004,
	Iru, Afitin and *Sonru*	Nigeria, Benin	*B. subtilis, B. pumilus, B. licheniformis, B. megaterium, B. firmus, B. atrophaeus, B. amyloliquefaciens, B. mojavensis, B. cereus, Lysininbacillus sphaericus, S. saprophyticus*	Alkaline, sticky	Meerak *et al.*, 2008; Azokpota *et al.*, 2008
	Soumbala	Burkina Faso	*B. pumilus, B. atrophaeus, B. amyloliquefaciens, B. mojavensis, Lysininbacillus sphaericus, B. subtilis, B. thuringiensis, B. licheniformis, B. cereus, B. badius, B. firmus, B. megaterium, B. mycoides, B. sphaericus, Peanibacillus alvei, Peanibacillus larvae, Brevibacillus laterosporus, Brevibacillus borstelensis*	Alkaline, sticky	Ouoba *et al.*, 2004; Sarkar *et al.*, 2002
	Kinda	Sierra Leone	*B. pumilus, B. licheniformis, B. subtilis, B. atrophaeus, B. amyloliquefaciens, B. mojavensis, Lysininbacillus sphaericus*	Alkaline, sticky	Meerak *et al.*, 2008

Table 2.1: Continued

Substrate/Raw material	Product	Country	Microorganisms	Sensory properties	References
African locust bean (*Parkia biglobosa)*	*Netetou*	Senegal	*B. lichenformis*, *B. coagulans*, *B. subtilis*, *B. pumilus*, *Staphylococuss* spp., *Micrococcus* spp.		Ouoba *et al*., 2004
Soya bean (*Glycine max* (L.) Merr.)	Soy–dawadawa	Ghana	*B. subtilis*, *B. pumilus*, *B. licheniformis*, *B. cereus*, *B. firmus*		Dakwa *et al*., 2005
	Soya dawadawa	Nigeria	*B. subtilis*, *B. licheniformis*, *B. circulans*, *B. pumilus*, *B. megaterium*, *Staphylococcus saprophyticus*, *S. epidermidis*, *Micrococcus luteus*, *Pseudomonas aeruginosa*		Dike and Odunfa, 2003; Jideani and Okeke, 1991; Omafuvbe *et al*., 2002
Bambara groundnut (*Vigna subterranea* (L.) *Verdc*.)	*Dawadawa* –type product	Nigeria	*B. subtilis*, *B. licheniformis*		Amadi *et al*., 1999; Barimalaa *et al*., 1994, Fadahunsi and Olubunmi, 2010
Mesquite (*Prosopis africana*)	*Okpehe*	Nigeria	*B. subtilis*, *B. licheniformis*, *B. pumilus*, *B. megaterium*, *B. cereus*, *S. epidermidis*, *Micrococcus luteus*, *Escherichia coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Lactobacillus* spp., *Proteus* spp., *Pseudomonas* spp., *Enterococcus* spp., *Staphylococcus* spp., *Micrococcus* spp.		Achi, 1992; Ogunshe *et al*., 2007; Oguntoyinbo *et al*., 2007; Oguntoyinbo and Oni, 2004, Oguntoyinbo *et al*., 2010
African oil bean (*Pentaclethra macrophylla* Benth.)	*Ugba*	Nigeria	*B. subtilis*, *B. pumilus*, *B. licheniformis*, *B. brevis*, *B. megaterium*, *B. polymyxa*, *B. coagulans*, *B. macerans*, *B. cereus*, *Lactobacillus spp.*, *Micrococcus spp.*,		Isu and Abu, 2000; Isu and Ofuya, 2000; Mbajunwa *et al*., 1998; Sanni *et al*., 2000, Sanni *et al*., 2002, Ahaotu *et al*., 2013

Table 2.1: Continued

Substrate/Raw material	Product	Country	Microorganisms	Sensory properties	References
			Pseudomonas chlororaphis, Micrococcus roseus, S. saprophyticus, S. aureus		
Melon seeds, castor oil seeds, pumpkin bean, sesame	*Ogiri/Ogili*	West, East and Central Africa	*B. subtilis, B. pumilus, B. licheniformis, B. megaterium, B. firmus, B. polymyxa, Pediococcus* spp., *Staphylococcus* spp., *S. saprophyticus, Lactobacillus plantarum, Pseudomonas aeruginosa*		Odunfa and Oyewole, 1986; Jideani and Okeke, 1991; Omafuvbe *et al*., 2004; Sanni *et al*., 2000; Sanni and Ogbonna, 1991
African yam bean (*Sphenostylis stenocarpa*)	*Owoh*	Nigeria	*B. licheniformis, B. pumilus, B. subtilis, Staphylococcus* spp.		Ogbonna et al., 2001
Roselle (*Hibiscus sabdariffa*)	*Bikalga*	Burkina Faso	*B. subtilis, B. licheniformis, B. megaterium, B. pumilus, B. cereus, B. badius, Brevibacillus bortelensis, B. sphaericus, B. fusiformis and Staphylococcus* spp.		Ouoba *et al*., 2008; Bengaly, 2001
	Mbuja	Cameroon	*B. licheniformis, B. polymyxa, B. laterosporus, B. cereus, B. circulans, B. subtilis, B. pumilus, B. brevis*		Mohamadou *et al*., 2013
Sickle pod leaves (*Senna obtusifolia*)	*Kawal*	Sudan	*B. subtilis, Propionibacterium* spp., *Lactobacillus plantarum, S. sciuri, yeasts*	Alkaline, strong flavoured, dried ball	Dirar, 1984; Dirar*et al*., 1985

Table 2.1: Continued

Substrate/Raw material	Product	Country	Microorganisms	Sensory properties	References
Baobab seed (*Adansonia digitata* L.)	*Maari*	Burkina Faso	*B. subtilis*, *B. licheniformis*, *B. cereus*, *B. pumilus*, *B. coagulans*, *B. megaterium*, *B. endophyticus*, *B. circulans*, *Paenibacillus polymyxa*, *Lysinibacillus sphaericus*, *Lysinibacillus fusiformis*, *Brevibacillus borstelensis*, *S. sciuri*, *S. gallinarium*, *S. hominis*, *Aerococcus viridans*		Parkouda *et al.*, 2010; Thorsen *et al.*, 2015, Kaboré *et al.*, 2013

B. cereus produces three recognized heat labile diarrheal toxins; the three component protein complexes non-hemolytic enterotoxin (*Nhe* A, B, C), hemolysin BL (*Hbl* A, D, C) and the single protein cytotoxin *Cyt*K. However, the spread of virulence characteristics, particularly the enterotoxin among other *Bacillus* species in this group has been reported (Rowan *et al*., 2001). Virulence genes such as the haemolysin genes *Hbl* and *Nhe* were found in some strains of *B. licheniformis*, *B. amyloliquefaciens*, *B. circulans*, *B. pasteurii* and *B. thuringiensis* subsp. *kurstaki* (Phelps and McKillip, 2002; Rowan *et al*., 2001). Therefore, distribution of virulence genes among the strains of *Bacillus* needs to be properly investigated so that only non-pathogenic strains are selected as starter cultures for the fermentation processes. The knowledge of enterotoxin production potential will aid production of safe and high quality fermented vegetable protein in West Africa (Oguntoyinbo and Sanni, 2007).

In Japanese *natto* production, the fermentation is usually inoculated with selected pure cultures of *Bacillus subtilis* subsp. *natto* (Kubo *et al*., 2011), but in other countries, the fermentations use traditional methods and rely on natural contamination. In most cases, however, the major bacteria involved are strains of *B. subtilis* (Aderibigbe and Odunfa, 1990; Sarkar *et al*., 1994). Until the early 20th century, Japanese *natto* was produced by wrapping boiled soybeans in rice straws, inhabited by *B. subtilis* (*natto*). Currently, *natto* is widely produced industrially using *B. subtilis* subsp. *natto* as starter culture (Kiuchi and Watanabe, 2004). This has been the only well characterised microorganism for the commercial production of legume-based food condiments worldwide. Therefore, the quest to develop starter cultures for the alkaline fermented African food condiments. Attempted research by various authors to develop starter cultures for the individual African food condiments has identified *B. subtilis* as the predominant specie using phenotypic method only (Omafuvbe *et al*., 2000; Sanni *et al*., 2002; Amoa-Awua *et al*., 2006). Research using genotypic identification methods were reported to a lesser extent (Ouoba *et al.* 2004; Oguntoyinbo *et al*., 2010; Adewunmi *et al*., 2013). The occurrence of *B. subtilis* species as the predominant microorganism in *dawadawa* has been shown; however, reports on the distinct intra-species differences in terms of their metabolic traits is still limited. For example, notable differences were reported in the extracellular enzyme production ability of seven *B. subtilis* isolates from fermented African locust bean (Aderibigbe and Odunfa, 1990).

2.6   Identification and taxonomic characteristics of *Bacillus* species

The identification of *Bacillus* species and related genera in alkaline fermented African food condiments have traditionally been on the basis of phenotypic properties, structure, and physiology (Odunfa, 1981). The use of only these characteristics resulted often in misidentification of the *Bacillus* species (Ouoba *et al*., 2004). Currently, studies on identification of the microorganisms involved in these fermentations are based on polyphasic approach that includes molecular biological methods in combination with classical microbiological and phenotypic methods (Daffonchio *et al*., 1998; Parkouda *et al*., 2010; Thorsen *et al*., 2011). Polyphasic approach has allowed accurate and more reliable identification of microorganisms involved in alkaline fermentations. The general scheme for identification includes isolation, phenotypic characterization, genotypic grouping, selection of representative of each genotypic group, and genotypic typing.

2.6.1   Phenotypic identification

A preliminary analysis in *Bacillus* identification often involves one or more phenotypic methods based on morphology, growth parameters, physiological and biochemical profiles. Morphological characteristics include shape, size, surface characteristics, pigmentation, Gram–staining, sporulation characteristics, and motility. *Bacillus* and related genera are generally Gram positive, spore-forming, catalase-positive microorganisms, cultivable in several microbiological media. Endospore formation, found in the group of *Bacillus* assumed the most important survival strategy peculiar to the genera when in adverse environment (Moir, 2006). The biochemical tests use specific growth media, nutrients, chemicals, or growth conditions to elicit observable or measurable biochemical responses from the *Bacillus* and related genera, thereby enabling their characterization and further identification. Recognized tests include phenol red carbohydrate fermentation, catalase production, oxidase test, oxidation-fermentation, methyl red test, Voges–Proskauer reaction, nitrate reduction, starch hydrolysis, tryptophan hydrolysis, hydrogen sulphide production, citrate utilization, and litmus milk reactions.

The API 50 CHB system, *Microgen™ Bacillus ID* is one of the numerous miniaturized and automated commercial systems are available with well-defined quality control procedures that allow rapid characterization of *Bacillus* (Logan and Berkeley, 1984). Phenotypic characteristics formerly considered suitable for the typing of individual

strains within a species, however, it has been recognized for a long time that the genus *Bacillus* is phenotypically heterogeneous (Claus and Berkeley, 1986; Priest, 1993). Misidentification of some bacteria reported in some studies indicates that the identification of bacteria based on the phenotypic properties alone may not always be suitable (Towner and Cockayne, 1993; Ouoba *et al*., 2004).

2.6.2   Molecular identification

Molecular techniques such as polymerase chain reaction (PCR)-based, DNA fingerprinting and gene sequencing methods may be applied as tools for either species identification or differentiation of strains. The major advantages of these genotyping methods are based on their discriminatory power and their universal applicability. With molecular typing methods, closely related strains with similar phenotypic features may consistently be distinguished. Methods that have been used in the identification and genotypic characterization of *Bacillus* and related genera include internal transcribed spacer PCR (ITS-PCR) (Liu *et al*., 1997); random amplification polymorphic DNA-PCR (RAPD-PCR) (Daffonchio *et al*., 1998); repetitive sequence-based PCR (rep-PCR) (Da Silva *et al*., 1999); pulsed field gel electrophoresis (PFGE) (Yamada *et al*., 1999); restriction fragment length polymorphism (RFLP) analysis of rRNA operons as well as sequencing of 16S rRNA, *gyr*A, *gyr*B, and *rpo*B genes (Chun and Bae, 2000; Herman and Heyndrickx, 2000; Mendo *et al*., 2000; Thorsen *et al*., 2011). The 16S rRNA gene based taxonomy is a clear way forward for bacterial identification (Woese, 1987). Reclassification of several species of *Bacillus* based on 16S rRNA sequence alignment have been achieved such as the successful identification of *B. subtilis* and *B. pumilus* in several investigations (Wang *et al*., 2007). However, sequencing of the 16S rRNA gene for identification of *Bacillus* shows limitations in differentiating closely related species, therefore, used for basic classification for species delineation. Analysis based on pair wise alignment of 16S rRNA gene sequences showed limited variation in these closely related species of *B. subtilis* group (e.g. *B. subtilis* and *B. amyloliquefaciens* showed more than 99% similarities), which prevented the resolution of strains and species relationship. It was not possible to distinguish *B. subtilis* from *B. licheniformis* or *B. cereus* from *B. thuringiensis* by16S rRNA sequencing (Azokpota *et al*., 2007; Parkouda *et al*., 2010). Both groups have been reported too closely related that they could not easily be distinguished by sequencing the 16S rRNA (Daffonchio *et al*., 1998).

The RFLP analysis of rRNA operons has been reported to discriminate the species in the genus *Bacillus* except closely related members of *B. cereus* group (*B. cereus*, *B. thuringiensis* and *B. mycoides*) and the *B. subtilis* group (*B. subtilis*, *B. amyloliquefaciens* and *B. licheniformis*) (Daffonchio *et al*., 1998). It is very difficult to differentiate these closely related members because of very high sequence homology in the ribosomal operons. The 16S-23S rRNA gene internal transcribed spacer (ITS)-RFLP analysis also not differentiated *B. subtilis, B. amyloliquefaciens* and *B. licheniformis* (Daffonchio *et al*., 1998). Chun and Bae (2000) demonstrated that the different strains of *Bacillus* could show almost identical 16S rRNA sequences, but significantly low *gyr*A nucleotide (NT) similarities. They concluded that the method for the amplification and sequencing of partial *gyr*A genes may be useful for the rapid identification of *B. subtilis* and allied taxa, especially organisms in these taxa, which cannot be differentiated by using conventional phenotypic tests and 16S rRNA analysis (Chun and Bae, 2000). Additionally, sequencing of other genes, such as *rpo*B and *gyr*B were reported better discriminatory for improved identification and differentiation of closely related *Bacillus* species (De Clerck and De Vos, 2004). Nowadays taxonomy based on multi– locus sequence typing (MLST) of housekeeping genes have been reported as a promising tool for differentiating closely related *Bacillus* species.

2.7   Volatile compounds of *dawadawa* African fermented condiments

The widespread use of African condiments is due to their peculiar odour, pleasant taste and nutritional qualities (Owens *et al*., 1997; Azokpota *et al*., 2010; Ouoba *et al*., 2005). The formation of volatile compounds in alkaline fermentation has been attributed to the metabolic activities of *Bacillus* species during fermentation (Ouoba *et al*., 2005; Beaumont, 2002; Omafuvbe *et al*., 2000). The major role of *Bacillus* species involves hydrolyzing proteins to peptides, amino acids and releasing ammonia thereby creating an alkaline pH, which aids the inhibition of spoilage microorganisms (Allagheny *et al*., 1996; Parkouda *et al*., 2009). Conversely, proteolytic activities of microorganisms on legume proteins and utilization of free amino acids during fermentation lead to the formation of ammonia that gives characteristic pungent or ammoniacal flavour of these condiments (Azokpota *et al*., 2008; Leejeerajumnean *et al*., 2001; Owens *et al*., 1997). According to Beaumont (2002), the amino acid content of

dawadawa, in particular glutamate contributes to the flavour enhancement, as well as peptides and aroma volatile constituents to be responsible for the flavour of the product.

The pyrazines, aldehydes, ketones, esters, alcohols, acids, alkanes, alkenes, benzenes, phenols, sulphurs and furans groups have been identified in dawadawa produced from African locust bean and soybeans (Azokpota *et al*., 2008; Ouoba *et al*., 2005; Dakwa *et al*., 2005; Azokpota *et al.*, 2010; Onyenekwe *et al*., 2012). However, volatile compounds that characterize *dawadawa*–like African food condiments produced from the controlled alkaline fermentation of Bambara groundnuts have not been reported. A summary of volatile compounds formed in African fermented condiments can be found in Table 2.2.

Azokpota *et al.* (2010) identified 2,5–dimethylpyrazine, tetramethylpyrazine, 3–methylbutanal, 2–decanone, 3,5–dimethylphenylmethanol, ethyl linoleate and chlorobenzene as major volatile compounds in *dawadawa* made from the controlled fermentation of African locust bean using *B. subtilis* starter cultures. Amines and pyridines were additional compounds found in soumbala (Ouoba *et al*., 2005). The heat treatment during the production contributes to the flavour by formation of pyrazines originating from reactions between sugars and amino acids (Ouoba *et al*., 2005; Owen *et al*., 1997). High production of acetoin, 2,5–dimethylpyrazine and trimethylpyrazine were reported during *B. subtilis* fermentation of soybean seed (Owen *et al.*, 1997). The pre-treatment steps of soybeans, either boiling or roasting before fermentation, affects the volatile aroma profile of soy-*dawadawa* with increase in the levels of 3–methyl butan–1–ol, 2-methyl–1–propanol, benzaldehyde, 5–methyl–2–phenyl–2–hexenal, 3–methylbutyl pentanoate, hexadecanoic acid, trimethyl pyrazine, and tetracosane in the soy-*dawadawa* (Dakwa *et al*., 2005). Ouoba *et al*. (2005) reported quantitative differences in the volatile compounds profile of soumbala produced by pure or mixed cultures of *B. subtilis* and *B. pumilus*, as well as the spontaneously fermented condiment. Volatile compounds production during alkaline fermentation is *Bacillus* strain dependent. It was observed in fermented African locust bean seeds that the volatile compounds varied qualitatively and quantitatively between two *B. subtilis* strains used as starter cultures (Ouoba *et al*., 2005). Thus, the profiling of volatile compounds production during controlled microbial alkaline fermentation of legumes is one of the steps which could give information for the future selection of starter cultures (Azokpota *et al*., 2010).

Analytical methods for volatile compounds analysis in African food condiments

The methods that have been employed for volatile compounds analysis of fermented African locust bean (*dawadawa*) were microscale steam distillation low-density solvent extraction, mainly the Likens–Nikerson method (Ouoba *et al*., 2005; Azokpota *et al*., 2008, Azokpota *et al*., 2010) and purge-and-trap onto Tenax (Owens *et al*., 1997) extraction for fermented soybean (soy-*dawadawa*), followed by gas chromatography – mass spectrometry (GC-MS) analysis. The Likens–Nickerson distillation extraction is considered to be acceptable as *dawadawa* is boiled during the preparation of soups. Distillation is employed for volatile compounds extraction, especially when heat-labile compounds need to be extracted. However, the main drawback is the possible formation of artefacts due to the long-term influence of high temperature on heat–labile compounds.

Solid phase microextraction (SPME) is widely used for the solvent free extraction of food and beverages (Junior *et al*., 2011; Mahattanatawee and Rouseff, 2014). Solvent free analyte enrichment provides aroma extracts that are more representative of food aroma when compared to those obtained by solvent extraction (Naudé and Rohwer, 2013). Headspace SPME involves sampling of the vapour that is directly above the food matrix and by concentrating the headspace with an adsorbent. This method has several advantages over classical distillation techniques, it is generally quicker, highly reproducible and yields ''true" aroma profiles, as artefact formation is minimised (Kataoka *et al*., 2000).

2.8   Sensory attributes of *dawadawa* condiments

There is a dearth of knowledge on the descriptive sensory evaluation of *dawadawa* condiments and other African condiments in general. *Dawadawa* is known for its characteristic pungent and ammoniacal aroma. The fact that the condiment has an objectionable odour or not (usually due to the levels of ammonia present) will determine its consumer preference (Allagheny *et al*., 1996). Sensory attributes of *dawadawa* is crucial for the consumer's acceptance of the finished product. For instance, Azokpota *et al*., (2010) highlighted smell as the major criterion for *dawadawa* purchase; condiments with a strong odour or too weak odour are often rejected by some traditional consumers. Though information on the descriptive sensory analysis of *dawadawa* condiments is limited, an informal screening of *dawadawa* indicated that characteristics such as ''cheese", ''smoke", ''meaty" and ''fatty/rancid"

were associated with the taste (Beaumont, 2002). Also, other reports on the sensory analysis of *dawadawa*; indicated four (4) broad descriptors for the condiments which are odour, flavour, texture and colour attributes (Azokpota *et al*., 2010; Ouoba *et al*., 2005). The flavour of food products is generally related to the volatile compounds present in the product. The relationship between the sensory attributes and volatile compounds responsible for flavour in *dawadawa* has not been established. It would be of value to highlight distinct sensory properties of *dawadawa* and link the volatile compounds which are the major drivers of its flavour. The necessity of modernization of traditional fermentation of making *dawadawa* by use of well-selected starter cultures and constant fermentation temperature and time would encourage the understanding of the *dawadawa* flavours and its descriptive sensory analysis.

Table 2.2: Volatile compounds in traditional African food condiments from alkaline fermented legumes or vegetable proteinaceous seeds

Product	Substrate	Country	Fermentation condition	Volatile compounds (groups)	References
Soumbala	African locust bean	Burkina Faso	Controlled: *B. subtilis*, *B.pumilus*	Pyrazines, aldehydes, ketones, esters, acids, alcohols, alkanes, amines, pyridines, phenols, sulphur compounds and furan	Ouoba *et al*., 2005
Afitin, *iru* and *sonru*	African locust bean	Benin	Controlled: *B. subtilis*	Pyrazines,aldehydes, ketones, esters, acids, alcohols and sulphur compounds	Azokpota *et al*., 2010
Afitin, *iru* and *sonru*	African locust bean	Benin	Spontaneous fermentation	Pyrazines, aldehydes, ketones, esters, acids, alcohols, alkenes, phenols, sulphur compounds, furan, and benzenes	Azokpota *et al*., 2008
Soy-*dawadawa*	soybeans	Ghana	Spontaneous fermentation: *Bacillus*, Lactic acid bacteria and yeast	Pyrazines, aldehydes, ketones, esters, acids, alcohols, alkanes, alkenes, sulphur compounds and aromatic compounds	Dakwa *et al*., 2005
Ogiri	Castor Oil Bean	Nigeria	Controlled: *B. subtilis*	Ketone, esters, acids, alcohol and furan	Ojinnaka and Ojimelukwe, 2013
Ogiri, *Daddawa*	Melonseed, African locust bean and soybean	Nigeria	Spontaneous fermentation	Pyrazine, aldehydes, alcohols, ketones, esters, alkanoic acids, alkanes and alkenes	Onyenekwe *et al*., 2012
Daddawa	Soybean	Nigeria	Controlled: *B. subtilis*	Pyrazines, aldehydes, alcohols,ketones, aliphatic acids and esters, furans, sulphur compounds and aromatic compounds	Owens *et al*., 1997
Ugba	African Oil Bean	Nigeria	Controlled: *B. subtilis* and *B. megaterium*	Pyrazine, aldehydes, alcohols, ketones, esters, alkanes and alkenes	Nwokeleme and Ugwuanyi, 2015

2.9   Conclusions

Traditional alkaline fermented food condiment such as *dawadawa* is an integral part of the diet in West and Central Africa. The processing and consumption of traditional fermented *dawadawa* is associated with safety challenges because production is by uncontrolled or spontaneous fermentation. *Bacillus* species have been highlighted as the predominant microorganism responsible for *dawadawa* condiments production and could serve as potential starter cultures for the controlled fermentation. There have been some discrepancies in the phenotypic identification of *Bacillus* starter cultures; however, this study endeavours to address the diversity of genotypically closely related *Bacillus* strains in terms of their phenotypic characteristics, volatile compounds and sensory properties during alkaline fermentation. The substrate for *dawadawa* production has solely been African locust bean but its availability is dwindling. Bambara groundnut which is an underutilised indigenous legume holds potential as a suitable replacement due to its availability and nutritional value. However, information on the use of Bambara groundnut for *dawadawa* production has been limited. The sensory properties of *dawadawa* condiments have not been fully described. Therefore, this study will focus on the production of *dawadawa* from Bambara groundnut using *Bacillus* starter cultures under controlled alkaline fermentations. In addition, the volatile compounds and sensory properties of the *dawadawa*-type condiments will be evaluated.

3.0   HYPOTHESES AND OBJECTIVES

3.1   Hypotheses

The microbial diversity of legume–based African condiments (*dawadawa*) consists of similar bacterial genera but genetically diverse *Bacillus* species predominate the fermentations. Different African condiments from legumes will be characterised by genetically diverse *Bacillus* species variants (strains). Strain diversity abound in the *B. subtilis* group complex, intra-species diversity is evident between *B. subtilis* subsp. *subtilis* 168 and *B. subtilis* subsp. *natto* (Kubo *et al*., 2011; Kunst *et al*., 1997). *B. subtilis* subsp. *natto* is used for alkaline fermentation of soybean into *natto* with mucilage and volatile compounds production (Kiuchi and Watanabe, 2004), however, ability to ferment is absent in the *B. subtilis* subsp. *subtilis* 168.

Individual *Bacillus* strains responsible for the alkaline fermentation of Bambara groundnut into *dawadawa*-like African condiments will possess unique metabolic properties leading to formation of distinct flavour compounds. The production of *natto* and *dawadawa* from soybeans fermentation showed differences in the major volatile compounds composition which ranges from ketones, acids and pyrazines for *natto* while aldehydes, pyrazines, alcohols and ketones are produced in *dawadawa* (Owens *et al*., 2005; Ouoba *et al*., 2005). The *natto* production was by fermentation with commercial strain of *Bacillus subtilis* subsp. *natto* while *dawadawa* was by *B. subtilis* isolate. Ouoba *et al*. (2005) reported that volatile compounds of fermented African locust bean seeds vary qualitatively and quantitatively according to strain of *B. subtilis* involved in the fermentation.

3.2   Objectives

To investigate the predominance and genetic diversity of *Bacillus* species from various African condiments with both phenotypic and genotypic methods in order to highlight the unique characteristics of individual *Bacillus* starter cultures during the alkaline fermentation of Bambara groundnut into African food condiments.

To characterise the volatile compounds produced by different *Bacillus* species during alkaline fermentation of Bambara groundnuts into *dawadawa*–like African condiment thus elucidating the metabolic characteristics of each *Bacillus* strain with the potential to develop a novel condiment.

To describe the sensory and flavour properties of the *dawadawa*–like African condiments produced by using the selected *Bacillus* species starter cultures with the aim of understanding the commercial potential of the condiments produced from different starter cultures.

4.0   RESEARCH

4.1   Diversity and functionality of *Bacillus* species associated with alkaline fermentation of

Bambara groundnut (*Vigna subterranean* L. *Verdc*) into *dawadawa*–like African condiment

4.1.1   Abstract

The diversity of *Bacillus* species in *dawadawa* was investigated; aiding potential starter cultures selection for alkaline fermentation of Bambara groundnut into *dawadawa*–like condiments based on their genotypic and volatile compound profiles. *Bacillus* species ($n = 71$) isolated from spontaneously fermented *dawadawa* were identified using matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI–TOFMS) as *B. cereus* (35%), *B. licheniformis* (30%), *B. pumilus* (21%), *B. subtilis* (10%) and *B. amyloliquefaciens* (4%). Further molecular typing was performed using GTG_5 rep-PCR typing, 16S rRNA and *gyr*A gene sequencing. The *gyr*A gene sequence analysis exhibited the highest species discriminatory power with *B. subtilis* subsp. *subtilis*, *B. amyloliquefaciens* subsp. *plantarum*, *B. pumilus* and *B. licheniformis* as the distinct clusters. Representative strains from each cluster were then used as starter cultures for the production of *dawadawa* from Bambara groundnut. Volatile compounds analysis using headspace solid phase microextraction (SPME) and comprehensive gas chromatography coupled to time of flight mass spectrometry (GC × GC–TOFMS) identified distinct chemical profiles produced by each of the four strains. Volatile compounds produced by *B. subtilis* subsp. *subtilis* (strain SFBA3) were categorized by dimethyl disulphide, methanethiol and nonanal while *B. amyloliquefaciens* subsp. *plantarum* (strain SFBA2) produced acetic acid and hexadecanoic acid. *B. cereus* (strain PALB7) produced 2,5–dimethyl pyrazine and 2–butanone which were not detected in the other condiments. Hexanal was the main compound produced by *B. licheniformis* (strain OALB7).

4.1.2 Introduction

Dawadawa is an African condiment produced solely from the spontaneous alkaline fermentation of African locust beans (*Parkia biglobosa*) (Campbell–Platt, 1980; Odunfa, 1988) and occasionally from soybeans (*Glycine max*) to produce soy *dawadawa* or Bambara groundnut (*Vigna subterranean* L. *Verdc*.) which is an underutilized African legume for *dawadawa*–like condiments production (Barimalaa *et al*., 1994; Fadahunsi and Olubunmi, 2010; Omafuvbe *et al*., 2000). *Dawadawa* is an integral part of the African diet due to its distinct aroma and flavour enhancing properties when added during cooking of soups or stews which are imparted by volatile compounds in the condiments (Odunfa, 1988; Omafuvbe *et al*., 2000). *Bacillus* species are known to dominate the alkaline fermentation of legumes in the production of various traditional foods and condiments in Asia and Africa. These fermented condiments include Indian *kinema*, Chinese *doushi*, Thai *thua nao*, Japanese *natto* and West African *dawadawa*/*soumbala* (Azokpota *et al*., 2008; Beaumont, 2002; Parkouda *et al*., 2009). The major role of *Bacillus* species in such condiments involves production of proteases for hydrolyzing proteins to peptides and amino acids and releasing ammonia, thereby creating an alkaline pH which aids the inhibition of spoilage microorganisms (Parkouda *et al*., 2009). *Bacillus* species isolated from *dawadawa* made from African locust bean include *B. amyloliquefaciens*, *B. atrophaeus*, *B. badius*, *B. cereus*, *B. firmus*, *B. fumus*, *B. licheniformis*, *B. megaterium*, *B. mojavensis*, *B. mycoides*, *B. pumilus*, *B. subtilis*, *B. sphaericus* and *B. thuringiensis* (Amoa-Awua *et al*., 2006; Azokpota *et al*., 2007; Ouoba *et al*., 2004; Parkouda *et al*., 2009). The use of starter cultures does not find widespread application in the traditional fermentation of food in Africa.

In the past decade, several works were dedicated to the characterization of the *Bacillus* species of these fermented products (Azokpota *et al.,* 2007; Oguntoyinbo *et al*., 2011; Ouoba *et al*., 2004). However, *Bacillus* strains elucidating desirable biochemical changes in traditional *dawadawa* have not yet been accurately identified. Unlike the application of *Bacillus subtilis* var *natto* in the production of the Japanesse *natto*, the use of indigenous commercial starter cultures is not available for *dawadawa* production. Limitations of the phenotypic identification methods to accurately differentiate individual *Bacillus* species and complexity in the use of simply16S rDNA PCR characterization to fully distinguish closely related species within these *Bacillus* groups has imposed constraints on the successful identification of potential starter cultures for alkaline

fermentation and production of African food condiments (Chun and Bae, 2000; Oguntoyinbo *et al*., 2011; Wang *et al*., 2007).

The use of polyphasic classification methods such as the repetitive sequence-based PCR (Rep–PCR), DNA sequencing of the 16S rRNA gene in combination with core protein-coding genes (*gyr*A gene for instance), to accompany conventional phenotypic tests are important tools useful to identify and differentiate genetic diversity between closely related *Bacillus* species (Chun and Bae, 2000; Parkouda *et al*., 2009; Rooney *et al*., 2009). Matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) has been highlighted as an effective method used to rapidly identify whole microbial cells growing on a solid medium (Balážová *et al*., 2014; Pavlovic *et al*., 2013; Šedo and Zdráhal, 2016). The profiling of volatile compounds production during controlled microbial alkaline fermentation of legumes is one of the steps which could give information for the future selection of starter cultures (Azokpota *et al*., 2008). Pyrazines, aldehydes, ketones, esters, alcohols, acids, alkanes, alkenes, benzenes, phenols, sulphur compounds and furans are chemical groups identified in *dawadawa* produced from other legumes (African locust bean and soybeans) but no report from Bambara groundnut (Azokpota *et al*., 2008; Azokpota *et al*., 2007).

Therefore, the main objective of this study was to classify the genetic diversity of *Bacillus* species involved in alkaline fermentation of Bambara groundnut using polyphasic identification methods and evaluating volatile compounds that would aid in the development of novel *dawadawa*–like condiment.

4.1.3   Materials and Methods

4.1.3.1 Sources of traditional *dawadawa*

Dawadawa condiment in form of sun-dried balls that was produced from African locust bean were purchased during January – March, 2015; from open–markets in Ibadan, Lagos and Akure in Nigeria and also from Accra, Ghana. Three samples each were collected from these different locations and stored in air tight cooler boxes before analysis. The spontaneous fermentation of Bambara groundnut to produce *dawadawa*–like condiment was carried out on a laboratory scale.

4.1.3.2 Microbiological and biochemical analyses

Ten grams (10 g) each condiment were suspended in 90 ml of 0.1% peptone buffer water (Merck, Darmstadt, Germany) and homogenized in a stomacher (Lab Blender, Model 400, Art Medical Instrument (Pty) Ltd, Johannesburg, South Africa). For spore-forming bacteria, 10 mL of the primary dilution was heated at 80 °C for 10 min, then 50μL of each dilution was incubated on Luria Bertani agar (LB) (Merck) at 37 °C for 48 h. A total of 71 spore-forming isolates obtained were stored in LB broth (Merck) cryopreserved at − 70 °C for further analysis. The pH of the *dawadawa* condiments were also determined using a pH meter (Model pH 211; Hanna Instruments, Woonsocket, RI, USA).

Phenotypic characterizations of spore forming isolates were examined for cell morphology, motility and presence of endospores by microscopy. Gram stain determination was performed using the KOH method, a portion of bacteria colony were mixed with a drop of 3% KOH on a glass slide for 1 min, colonies with stringy mixture when lifted with a loop are gram negative. Catalase production, hydrolysis of starch, growth at different pH values, temperatures and NaCl concentrations, Voges-Proskauer and methyl red test, nitrate reduction, citrate utilisation, propionate utilisation, and fermentation of sugars were determined according to prescribed methods (Leboffe and Pierce, 2012). Proteolytic activity was determined on plate count skim milk agar with 0.1% skim milk powder at 10 °C for 7 days. Presumptive aerobic spore-forming bacteria colonies were purified and further identified using PCR analysis and MALDI–TOF MS.

4.1.3.3 MALDI-TOF MS confirmation of the presumptive spore-forming bacteria isolates

Purified bacterial cultures isolated from the selective media were transferred, in duplicate, directly to the MALDI–TOF MS steel polished target plate (Bruker, Bremen, Germany) and overlaid with the α-cyano-4-hydroxy-cinnamic acid matrix (Bruker) (Pavlovic *et al*., 2013). The target plate was subsequently analyzed using microflex LT MALDI–TOF MS (Bruker) in conjunction with Biotyper automation software and library (Bruker). A score-oriented dendrogram was generated based on crosswise minimum spanning tree (MSP) matching.

4.1.3.4 Genotypic characterization

Bacillus isolates were grown overnight in LB broth at 37 °C and centrifuged at 10,000 x *g*. DNA was extracted by resuspending bacterial pellets in 0.2 ml lysis buffer [10 mmol^{-1}Tris–HCl (pH 8.0), 1 mmol^{-1} EDTA, 1% Triton X-100] (Sigma-Aldrich, Steinheim, Germany) and incubated for 30 min at 95 °C with agitation. The tubes were then cooled to 4°C and centrifuged for 10 min at 12,000 x *g*; the supernatant was used directly for amplification (Goldenberger *et al.*, 1995).

(GTG)$_5$-Rep–PCR fingerprinting

The 20 μL reaction consisted of 10 μL, 2 x PCR Master Mix (Kapa Biosystems, Boston, MA, USA) containing KapaTaq DNA polymerase (0.05 U/μl, 1.25 U per 25 μl), reaction buffer with Mg^{2+} and 0.4 mM of each dNTP with loading dye, 0.8 μM (GTG)$_5$ primer (Versalovic *et al.*, 1994), 2 μL DNA template and 4% dimethylsulfoxide (Sigma-Aldrich, St Louis, USA). Amplification was carried out as follows: initial denaturation at 95 °C for 5 min; 35 cycles of 95 °C for 1 min, 52 °C for 1 min and

72 °C for 3 min; and a final elongation step at 72 °C for 10 min.

16S rRNA and gyrA gene amplification and sequencing

A total of 24 isolates were selected based on the Rep-PCR clustering for sequencing of the 16S rRNA gene. Partial 16S rRNA gene amplification using forward primer fD1 (5´-AGA GTT TGATCC TGG CTC AG-3´) and reverse primer rD1 (5´-AAG GAG GTG ATC CAG CCG CA-3´) (Weisburg *et al.*, 1991). The PCR was carried with initial denaturation at 95 °C for 5 min; 35 cycles of 95 °C for 2 min; 42 °C for 30 s and 72 °C for 4 min; and a final elongation step at 72 °C for 10 min. The PCR products were confirmed by electrophoresis and visualized under UV light with a Gel Doc system (Bio-Rad, Hercules, CA, USA). Grouping of the rep– PCR fingerprints was performed using GELCOMPAR II version 5.10 software (Applied Maths, Sint–Martens– Latem, Belgium) based on the Dice similarity coefficient and the UPGMA algorithm to obtain a dendogram.

4.1.3.5 Phylogenetic analysis

The amplified PCR products were purified with a QIAquick PCR purification kit (Qiagen, Valencia, CA, USA), bidirectional 16S rRNA sequencing was performed in similar reaction conditions to those outlined above. Sequencing reactions were conducted using the ABI BigDye version 3.0 sequencing kit (Applied Biosystems, Waltham, MA, USA) following the manufacturer's suggested protocol but at 25% of the recommended volume. Reaction products were purified via ethanol precipitation and run on an ABI 3730 genetic analyzer (Applied Biosystems). Sequence chromatograms were analysed with the software FinchTV version 1.4.0 (Geospiza, Incorporation, Seattle, WA, USA) and aligned using BioEdit Sequence Alignment Edition (Ibis Therapeutics, Carlsbad, CA, USA). The obtained sequences were subjected to BLAST for comparing sequence homologies in the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/). Representative isolates tentatively identified as *B. subtilis* group members (based on 16S rRNA gene sequencing) were selected for sequencing of the *gyr*A genes as described by Chun and Bae (2000). The resultant partial 16S rRNA and *gyr*A gene sequences were assembled and aligned online using MAFFT version 7 multiple sequence alignment program (http://mafft.cbrc.jp/alignment/software/). The Molecular Evolutionary Genetics Analysis (MEGA) v 5.2 program (Tamura *et al.*, 2011) was used to calculate evolutionary distances and to infer trees based on neighbour-joining (NJ) and Jukes and Cantor-model (Jukes and Cantor, 1969; Saitou and Nei, 1987). The reliability of internal branches was assessed from 1000 bootstrap pseudoreplicates.

4.1.3.6 Production of *dawadawa* from Bambara groundnut using starter cultures

Fermentation of Bambara groundnut

The fermentation and production of *dawadawa*–like condiments from Bambara groundnut was carried out as previously described by Barimalaa *et al*. (1994). Briefly, 40 g of seeds were steeped in 200 ml distilled water at 24°C for 24 h, after which the seeds were dehulled manually using a mortar and pestle. Cotyledons recovered were boiled in distilled water at 100 °C for 15 min. The cooked cotyledons were then drained with a sieve (1 mm pore size). Fifty (50) grams of the cooked cotyledons were placed in sterile 50mm x 70mm zip lock perforated polythene bags (Apak Packaging, Johannesburg, South Africa). The *B. subtilis* subsp. *subtilis* SFBA3, *B. amyloliquefaciens* subsp. *plantarum* SFBA2, *B. cereus* PALB7 and *B. licheniformis* OALB2 strains

were then used to inoculate the Bambara groundnut to obtain final inoculum level of 4 X 10^4 cfu/g. Fermentation was carried out at 30 °C for 120 h. A control fermentation was conducted without any inoculation. After fermentation, 10 g of fermenting Bambara groundnut was aseptically placed in stomacher bag and 90 ml of sterile distilled water was added and homogenized in a stomacher, 1 ml of this 10^{-1} dilution was removed and further diluted in a ten–fold dilution series.

4.1.3.7 Determination of volatile compounds produced by *Bacillus* species

Chemical standards

Five grams of *dawadawa* were spiked with 6.25 µg 1,8-cineole (eucalyptol) analytical reference standard (Sigma-Aldrich (Pty) Ltd. Kempton Park, South Africa). The headspace sampling of the spiked sample was extracted as described below and analysed with GCxGC-TOF MS. For linear retention index determination *n*-alkanes (C_8–C_{28}) were used (Merck, Pretoria, South Africa).

Gas chromatography - time of flight mass spectrometry (GC x GC–TOF MS)

Headspace samplings were done with solid phase microextraction (SPME) device fitted with a 2–50/30µm DVB/Carboxen/PDMS StableFlex fibre (Supelco, Sigma-Aldrich (Pty) Ltd. Kempton Park, South Africa). The fibre was exposed to the headspace above the sample for 20 min. After extraction the SPME device was removed from the vial and desorbed in the injection port of a GC x GC-TOF MS. Compound separation was done using a LECO Pegasus 4D GC x GC–TOF MS with an Agilent 7890 GC (LECO Africa (Pty) Ltd., Kempton Park, South Africa). The system included a secondary oven and a dual stage modulator. Nitrogen gas was used for the hot jets and nitrogen gas cooled with liquid nitrogen was used for the cold jets. The carrier gas, helium, was of ultra-high purity grade (Afrox, Gauteng, South Africa) and was set at a flow rate of 1.4 ml min^{-1} in the constant flow mode. The capillary column set consisted of an apolar Rxi-5SilMS 30 m x 0.25 mm ID x 0.25 µm df (Restek, Bellefonte, PA, USA) as the primary column and a high temperature midpolar Rxi–17Sil MS 0.97 m x 0.25 mm ID x 0.25 µm df (Restek, Bellefonte, PA, USA) as the secondary column. Identification of compounds in the samples was done by comparison of mass spectra to that of the National Institute of Standards and Technology (NIST14) library and by experimental linear retention indices (RI_{exp}). Compounds reported had

a spectral match quality of ≥ 80%. Semi-quantification of the compounds was performed by using the internal standard method of quantification (Naudé *et al*., 2016).

4.1.4 Results and Discussion

4.1.4.1 Isolation and phenotypic identification of spore-forming bacteria

The total aerobic counts ranged from 9.04 ± 0.14 $\log_{10}$ CFU/g to 9.58 ± 0.19 $\log_{10}$ CFU/g. Sporeformers counts ranged from 8.0 ± 0.1 $\log_{10}$ CFU/g to 8.3 ± 0.2 $\log_{10}$ CFU/g (Table 4.1). All spore-forming isolates from spontaneous fermentation of African locust bean and Bambara groundnut *dawadawa* were characterized as Gram positive, catalase positive, endospore forming and rod shaped based on the phenotypic characteristics. The predominant *Bacillus* isolate was *B. cereus*, followed by *B. pumilus*, *B. licheniformis* and *B. subtilis* in decreasing order. The *B. amyloliquefaciens* was identified only in the Bambara groundnut *dawadawa*–like condiment. The *B. cereus* had the highest number of isolates, however; the combined population of *B. pumilus*, *B. licheniformis* and *B. subtilis* which are members of the *B. subtilis* group outnumbered the *B. cereus* population. The presence of *B. amyloliquefaciens* in the Bambara groundnut condiment alone may be attributed to the composition of the substrate which has a higher carbohydrate content (63.5%) compared to African locust bean (49%) (Campbell–Platt, 1980; Heller *et al*., 1997). Studies on African food condiments such as *soumbala*, *sonru*/*Iru*, *bikalga* and *mbuja* have reported the occurrence of *Bacillus* species, with *B. subtilis* group (*B. subtilis*, *B. licheniformis*, *B. pumilus* and *B. amyloliquefaciens*) often dominating the fermentation process (Azokpota *et al*., 2007; Mohamadou *et al.,* 2007; Ouoba *et al*., 2008). The incidence of *B. cereus* highlighted its importance as part of the microflora of alkaline fermentation as with other food condiments based on alkaline fermented seed.

Table 4.1: The average count of *Bacillus* species associated with spontaneous fermentation of African locust bean and Bambara groundnut in the production of *dawadawa* ($n = 3$)

Samples		AFL_{Accra}	AFL_{Ibadan}	AFL_{Lagos}	AFL_{Akrure}	BGN
pH		7.9	8.2	8.5	8	7.9
Log_{10} cfu/g	Total Aerobic count	9.06 ± 0.13	9.23 ± 0.07	9.04 ± 0.14	9.58 ± 0.19	9.07 ± 0.09
	Sporeformers	8.29 ± 0.04	8.33 ± 0.16	8.04 ± 0.12	8.38 ± 0.11	8.03 ± 0.17

AFL – African locust bean; BGN – Bambara groundnut

4.1.4.2 Isolates identified by MALDI–TOF Mass Spectrometry

MALDI–TOFMS analysis identified all the aerobic spore forming bacteria isolates as *Bacillus* species. Overall, two distinct cluster groups were identified namely *B. cereus* and *B. subtilis* based on 85% distance level and higher (Figure 4.1). African locust bean *dawadawa* condiments had a higher incidence of *B. cereus* than Bambara groundnut *dawadawa*. The mass spectra fingerprinting deduced revealed a clear interspecies divergence in the *B. subtilis* groups; however, *B. cereus* species was the only specie identified in its group. Of the 71 *Bacillus* isolates from both *dawadawa* types, *B. cereus* accounted for 25 isolates while *B. subtilis* group accounted for 46 isolates which comprised members; *B. lichenformis* (9 isolates), *B. pumilus* (12 isolates), *B. amyloliquefaciens* (12 isolates) and *B. subtilis* (13 isolates), respectively. MALDI TOF MS an emerging proteomics based rapid identification system, identified greater divergence with more species identified in the *B. subtilis* group than the phenotypic test. However, the only limitation was that of identifying those closely related *Bacillus* on subspecies level. Similar discrepancies in identifying closely related species were reported by other authors (Pavlovic *et al.,* 2013; Zeller-Péronnet *et al*., 2013); and this has been attributed to the lack of adequate databases for strain comparison or inadequate method optimization for the MALDI–TOF MS. Classification using MALDI–TOF MS analysis for several closely related bacteria can be achieved by using additional sample preparation steps (Balážová *et al*., 2014; Šedo and Zdráhal, 2016).
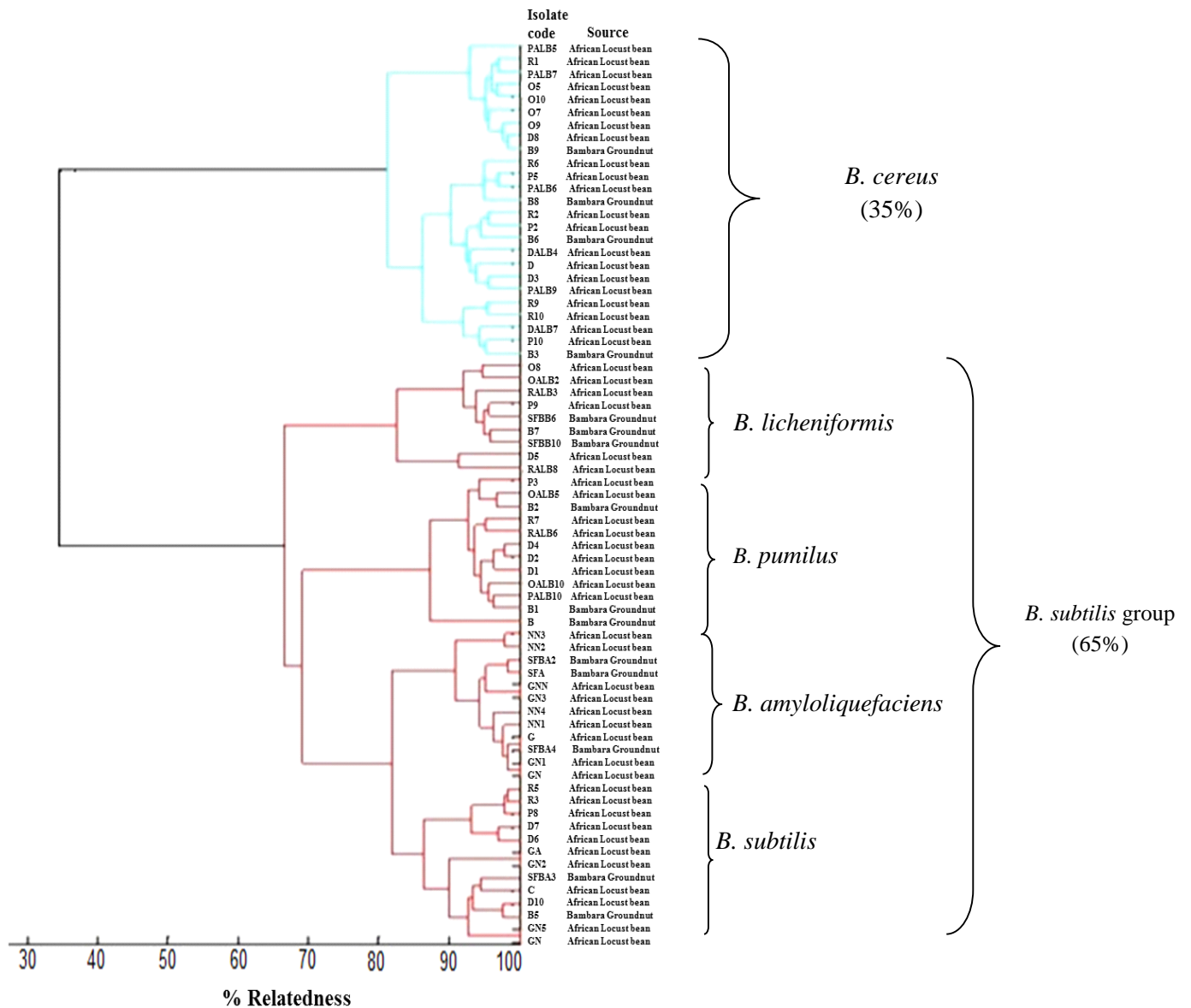
Figure 4.1: Score-oriented dendrogram of MALDI-TOF Mass Spectrometry profiles showing genetic relationships between *Bacillus* spp. isolated from *dawadawa* African condiments produced from spontaneous alkaline fermentation of African locust bean or Bambara groundnut. The vertical line represents clusters of isolates that showed 80% strain similarity which was taken as the threshold for closely related isolates.

4.1.4.3 Genotypic identification

GTG$_5$ rep-PCR and 16S rDNA sequence identity

The isolated *Bacillus* species were grouped by GTG$_5$ rep-PCR fingerprinting analysis into five clusters (Figure 4.2). The clusters confirmed intra-species diversity within the *B. subtilis* group. Representative isolates from the individual clusters were picked for 16S rRNA gene sequencing and identities of isolates were confirmed by blasting it against the available databases. The isolates belonging to GTG$_5$rep-PCR cluster 1A (Figure 4.2) were found to belong to the *B. amyloliquefaciens* by 16S rRNA gene sequencing (99.8–100% similarity ratio to EzTaxon deposited sequences) while groups 1B belong to *B. pumilus* by 16S rRNA gene sequencing (97.8–100% similarity ratio). Cluster 2, the largest, which comprised of 12 isolates were confirmed with 16S rRNA gene sequencing (85–100% similarity ratio) as *B. licheniformis*. Clusters 3 and 4 represent the *B. subtilis* which by 16S rRNA gene sequencing had 99.8–100% similarity ratio. Cluster 5 is the *B. cereus* by 16S rRNA gene sequencing with 97.8–100% similarity ratio. The phylogenetic tree based on the 16S rDNA sequences showed 5 groups (Figure 4.3). Isolate SFBA 3 clustered with the *B. subtilis* subsp*subtilis* strain 168 and *B. subtilis* subsp *natto* strain MBS04-6, the latter a commercial strain for *natto* fermentation. Isolate SFBA 2 clustered with *B. amyloliquefaciens* strains. Five strains each segregated into the *B. licheniformis* and *B. pumilus* strains respectively. A total of six strains segregated with the *B. cereus* strain which formed a distinct cluster on the phylogenetic tree (Figure 4.3); however, a number of the isolates were previously identified as *B. pumilus* with phenotypic tests and proteomics (Figure 4.2). GTG$_5$ rep-PCR has been shown to be a useful technique in the subtyping of *Bacillus* species (Da Silva *et al*., 1999; Herman and Heyndrickx, 2000; Porwal *et al*., 2009). However, protein coding genes such as *gyr*A used in this study exhibited much higher genetic variation for the classification of closely related taxa within the *B. subtilis* group. The large degree of variation in the individual group fingerprints suggests substantial intra-species genetic diversity may exist and highlights the very high resolution of 16S rDNA and *gyr*A gene sequencing.
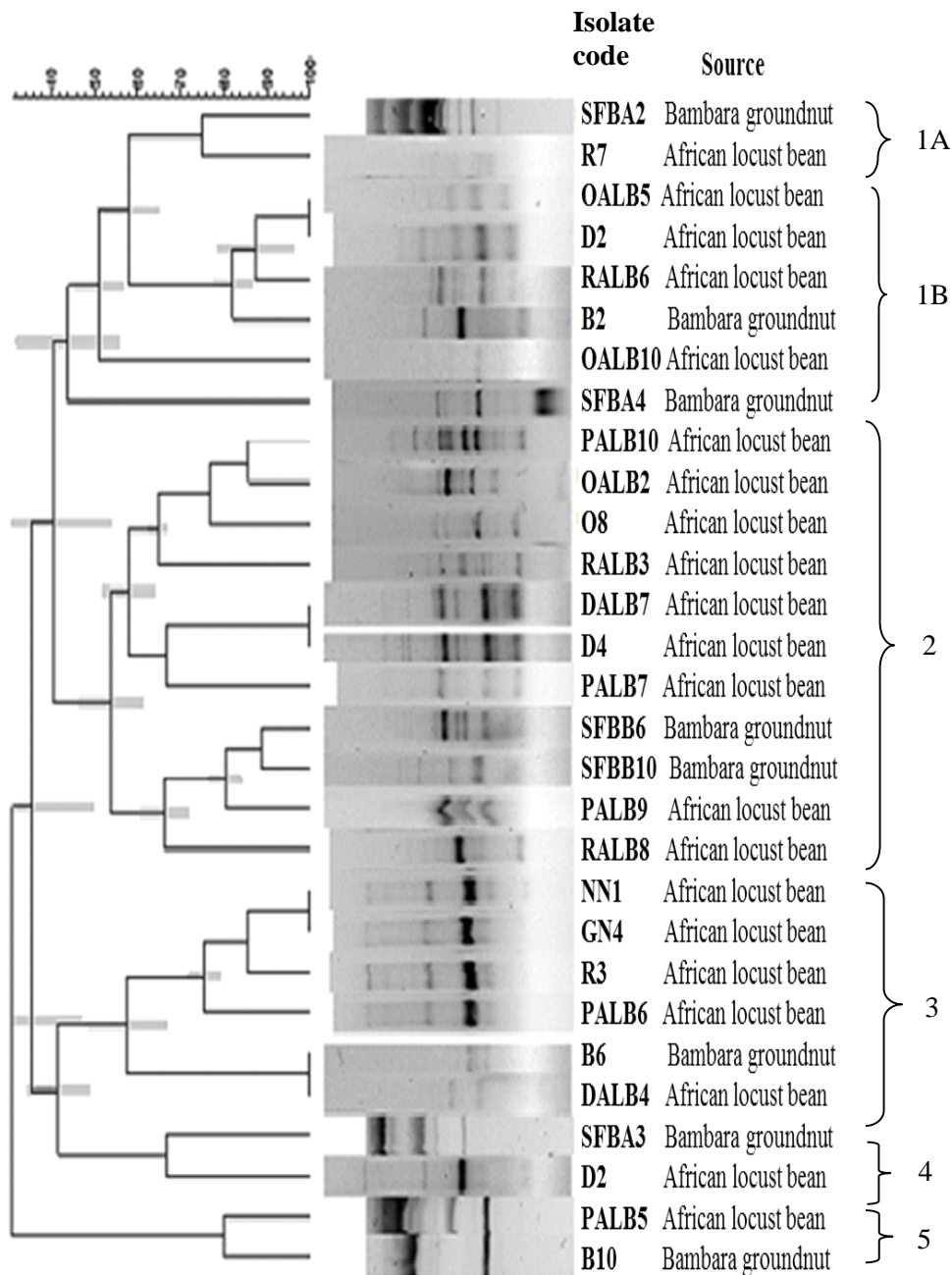
Figure 4.2: Dendrogram based on Dice coefficient of similarity with the Unweighted Pair Group Method with Arithmetic averages (UPGMA) of GTG$_5$ rep-PCR fingerprint patterns of species in the *Bacillus subtilis* group sampled from spontaneous alkaline fermentation of African locust bean and Bambara groundnut. Presumptive *Bacillus* species isolates in cluster 1A are *B. amyloliquefaciens*, 1B are *B. pumilus*, 2 are *B. lichenformis*, 3 and 4 are *B. subtilis* and 5 are *B. cereus.*
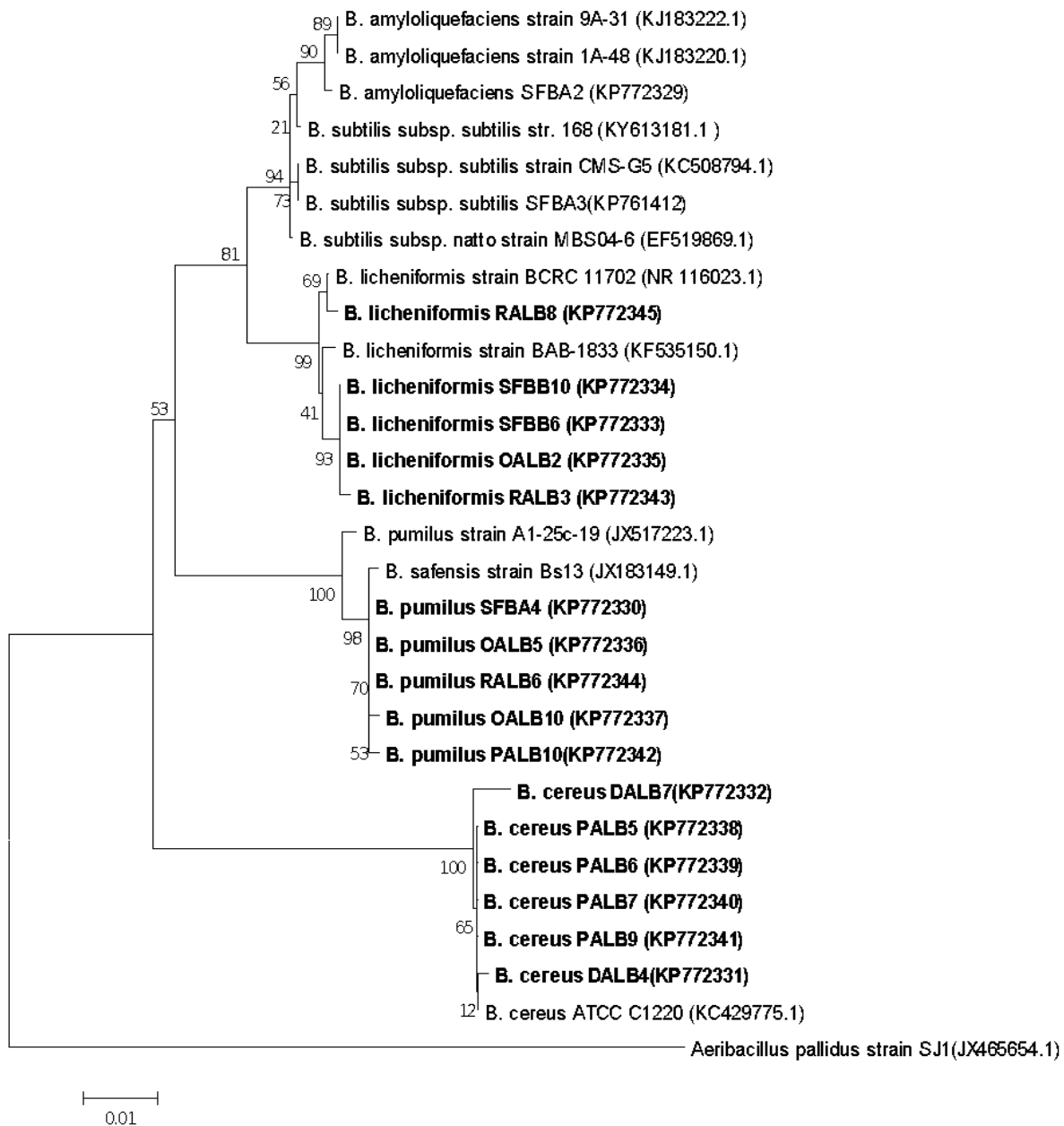
Figure 4.3: Phylogenetic relationship of *Bacillus* strains from the spontaneous alkaline fermentation of African locust bean and Bambara groundnut inferred from the alignment of the 16S rRNA gene sequences. Bootstrap values (expressed as percentages of 1000 replications) are shown at branch points; > 50% were considered significant. Because the ML tree was very similar to the NJ tree, only the latter is shown here. Tree was rooted with *Aeribacilus pallidus* strain SJ1. *Bacillus* species isolated in the study represented in bold

GyrA sequence identity

Further classification using *gyr*A genes sequencing with a total of 15 isolates selected, a representation of each group identified by 16S rRNA sequence, were used to differentiate closely related species of the *B. subtilis* group (99–100% similarity ratio to GenBank sequences). The *gyr*A gene differentiated isolates SFBA 3 and SFBA 4 as *B. subtilis* subsp. *subtilis* and *B. amyloliquefaciens* subsp. *plantarum* respectively which could not be identified with the phenotypic, proteomics and 16S rDNA sequence methods. The *B. licheniformis* strains identity were all confirmed with *gyr*A sequence, however, there were no positive *gyr*A sequences for *B. pumilus* isolate in this study (Figure 4.4).

The *B. subtilis* subsp. *natto* type strain MS04-6 which is a commercial starter culture for Japanese *natto* alkaline fermentation shared 90.2% *gyr*A sequence similarity with the *B. subtilis* subsp. *subtilis* strain SFBA3 which was isolated from the spontaneous fermentation of Bambara groundnut. This suggests the potential of this strain as a commercial starter culture for *dawadawa* production. *B. amyloliquefaciens* subsp. *plantarum* identified from the spontaneous fermentation of Bambara groundnut has rarely been reported in alkaline fermentation. Previous authors only recorded the isolation of *B. lichenformis* as the sole organism responsible for fermentation of Bambara groundnut (Amadi *et al*., 1999; Barimalaa *et al*., 1994; Fadahunsi and Olubunmi 2010). However, findings in this study revealed other *Bacillus* species present such as *B. subtilis* subsp. *subtilis* and *B. amyloliquefaciens* subsp. *plantarum*. This work not only establishes the genetic relationship between *Bacillus* species from alkaline fermentation of African locust bean and Bambara groundnut but with the *Bacillus* strain from the Japanesse *natto*.

4.1.4.4 Microbial growth on Bambara groundnut

The study identified two major groups of *Bacillus* species complex; the *B. subtilis* group (*B. subtilis, B. amyloliquefaciens* and *B. licheniformis*) and *B. cereus* group were identified from the spontaneous fermentation of African locust bean and Bambara groundnut.
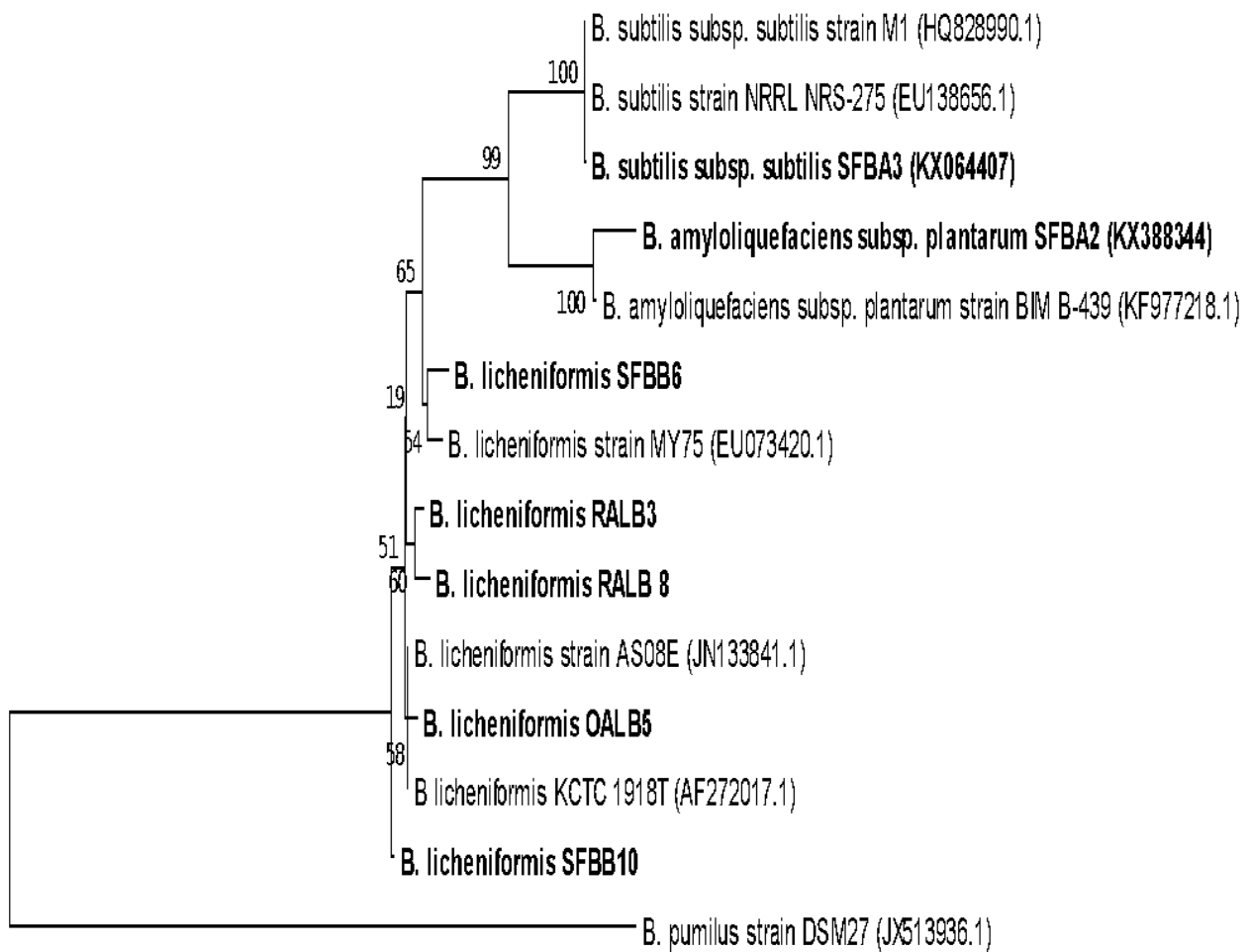
Figure 4.4: Phylogenetic relationship of *Bacillus* strains from the spontaneous alkaline fermentation of African locust bean and Bambara groundnut inferred from the alignment of the *gyr*A gene sequences. Bootstrap values (expressed as percentages of 1000 replications) are shown at branch points; > 50% were considered significant. Because the ML tree was very similar to the NJ tree, only the latter is shown here. Tree was rooted with *B pumilus* strain DSM27. *Bacillus* species isolated in the study represented in bold.

Two strains *B. subtilis* subsp. *subtilis* SFBA3, *B. amyloliquefaciens* subsp. *plantarum* SFBA2 isolated from spontaneous fermentation of Bambara groundnut while *B. licheniformis* OALB2 and *B. cereus* PALB7 strains isolated from African locust bean were evaluated as potential starter cultures for the controlled fermentation of Bambara groundnut into *dawadawa*–like condiment. The microbial cultivation of *Bacillus* species on Bambara groundnut showed a maximum specific growth rate of 0.13, 0.14, 0.13 and 0.10 h^{-1} for *B. cereus* PALB7, *B. amyloliquefaciens* subsp. *plantarum* SFBA2, *B. subtilis* subsp. *subtilis* SFBA3 and *B. licheniformis* respectively. Both *B. cereus* PALB7 and control had a rapid growth throughout the fermentation as indicated by an increase in spore-forming bacteria counts with a concomitant pH increase from 6.90 to 8.20 for *B. cereus* PALB7 at 48 h. The *B. amyloliquefaciens* subsp. *plantarum* SFBA2 and *B. licheniformis* OALB2 strains had the highest growth at 72 h while *B. subtilis* subsp. *subtilis* SFBA3 at 96 h. The highest alkaline pH recorded for all of the *Bacillus* species was pH 8.5 at 96 h. The controlled fermentation with *Bacillus* strains starter culture highlighted *B. cereus* PALB7 to have the highest growth and alkaline pH at the short time of fermentation of 48 h. The *B. cereus* PALB7 was chosen as potential starter culture because *B. cereus* is a common natural microflora of spontaneously fermented African locust bean and Bambara groundnut (Odunfa, 1988; Oguntoyinbo and Oni, 2004; Okanlawon *et al*., 2010). *B. cereus* has been used as starter culture for African locust bean fermentation into *dawadawa* with satisfactory product quality (Okanlawon *et al*., 2010). Though *B. cereus* is known to cause mild food poisoning due to the production of up to three enterotoxins and one emetic toxin (Stenfors Arnesen *et al*., 2008); however, it should be noted that not all strains of *B. cereus* carry enterotoxin genes and certain strains are used as probiotics in feed and food (Cutting, 2011).

4.1.4.5 Volatile compounds production

Aldehydes and acids were generally the major groups of compound formed by all the *Bacillus* strains (Table 4.2). *B. licheniformis* OALB7 produced the highest number (58) and level (15 030 ng/g) of compounds. The profile of OALB7 was dominated by aldehydes, in particular hexanal at 4 710 ng/g. Hexanal is generally responsible for a green odour (Zhao *et al.,* 2011). Esters were produced by *B. licheniformis* OALB7 with decanoic acid methyl ester described as having a fermented odour, wine-like, fruity, floral, oily. Esters have been known to constitute a major volatile compound in various African fermented condiments (Beaumont,

2002; Leejeerajumnean *et al*., 2001). The esters are presumably the consequence of chemical reactions between microbial acidic and alcoholic metabolites (Leejeerajumnean *et al*., 2001). A trace amount (2 ng/g) of 2,5–dimethyl pyrazine was present, while sulphur and phenol compounds were not detected (Table 4.2).

B. amyloliquefaciens subsp. *plantarum* SFBA2 produced the second highest number (36) and level (11 952 ng/g) of compounds (Table 4.2). SFBA2 was characterised by organic acids, notably acetic acid (3 460 ng/g) and *n*-hexadecanoic acid (2 160 ng/g). The level of phenols was 700 ng/g, dominated mainly by phenol (530 ng/g). Dimethyl trisulphide and dimethyl disulphide were present at levels 70 ng/g and 610 ng/g, respectively. Dimethyl trisulphide and dimethyl disulphide showed meaty and vegetal aromas, respectively. Concentrations of aldehydes (470 ng/g) and ketones (150 ng/g) were the second lowest, while pyrazines and esters were not detected for this product. *B. cereus* PALB7 produced 34 compounds which was the second lowest number of compounds for the four *dawadawa*–like condiments. *B. cereus* PALB7 produced the second lowest level of total compounds at 6 572 ng/g (Table 4.2). *B. subtilis* subsp. *subtilis* SFBA3 produced the lowest number (27) and quantitatively the lowest level (2 398 ng/g) of compounds. This product was characterised by the high level of sulphur compounds (1 110 ng/g) with methanethiol and dimethyl disulphide detected. No pyrazines were detected. *B. cereus* PALB7 was characterised by ketones, alcohols and 2,5–dimethyl pyrazine. Esters were not detected for *B. cereus* PALB7. Of the four *dawadawa*–like condiments, 2,5–dimethyl pyrazine was produced only by *B. cereus* PALB7 (30 ng/g). 2–Butanone (methyl ethyl ketone) was the major ketone (1 380 ng/g) present in *B. cereus* PALB7 and was not detected in the other three samples (Table 4.2). The major volatile compound groups reported for traditional *dawadawa* made from African locust bean were pyrazines, aldehydes, alkenes, ketones, alcohols, esters and benzene derivatives. In contrast, aldehydes, acids and ketones dominated *dawadawa*–like condiments from Bambara groundnuts. Pyrazines have been found in highest concentrations in *dawadawa* from African locust bean (Azokpota *et al*., 2008). Only *B. cereus* PALB7 produced 2,5–dimethyl pyrazine in relation to the traditional condiments.

Table 4.2: Relative concentrations of volatile compounds in the headspace of *dawadawa*–like condiment produced from *Bacillus* fermented Bambara groundnut using GC x GC-TOF MS

Compounds	Relative concentration ng/g				
Aldehydes	**OALB2**	**PALB7**	**SFBA2**	**SFBA3**[*]	**RI$_{exp}$**[a]
Acetaldehyde	ND[b]	300	ND	50	379
Propanal, 2-methyl-	40	ND	ND	ND	386
Butanal	40	ND	ND	ND	618
Hexanal	4710	50	ND	ND	791
Heptanal	480	ND	ND	ND	886
2-Heptenal, (E)-	140	ND	ND	ND	891
Benzaldehyde	250	570	230	90	953
Octanal	360	ND	ND	ND	1014
Benzeneacetaldehyde	240	ND	90	ND	1062
2-Octenal, (E)-	310	ND	ND	ND	1062
Nonanal	950	150	140	260	1108
2-Nonenal, (E)-	60	ND	ND	ND	1162
Decanal	90	ND	ND	ND	1204
2,4-Nonadienal, (E,E)-	30	ND	ND	ND	1217
4-Oxononanal	310	ND	ND	ND	1244
2-Decenal, (Z)-	220	ND	ND	ND	1263
Undecanal	70	ND	ND	ND	1309
2,4-Decadienal, (E,E)-	680	ND	ND	ND	1320
2-Undecenal	200	ND	ND	ND	1364
Dodecanal	110	20	ND	ND	1406
Pentadecanal-	ND	ND	ND	20	1713
Hexadecanal	30	40	20	ND	1713
Total ng/g	**9 310**	**1 140**	**470**	**420**	
Acids					
Acetic acid	280	70	3460	ND	737
Acetic acid, methoxy-	350	50	ND	ND	738
Acetic acid, methoxy-, anhydride	100	ND	ND	ND	740
Propanoic acid, 2-methyl-	ND	170	310	ND	791
Butanoic acid, 3-methyl-	ND	1270	860	ND	828
Phosphonic acid, (p-hydroxyphenyl)-	50	ND	ND	ND	895

Compounds	Relative concentration ng/g				
Phosphonic acid, (p-hydroxyphenyl)-	50	ND	ND	ND	895
5-Oxotetrahydrofuran-2-carboxylic acid	30	ND	ND	ND	1360
Undecanoic acid	ND	ND	30	ND	1795
Phthalic acid, cyclohexylisohexyl ester	10	ND	4	ND	1847
n-Hexadecanoic acid	990	240	2160	90	1955
9,12-Octadecadienoic acid (Z,Z)-	590	130	1360	80	2128
Octadecanoic acid	ND	ND	140	ND	2157
Cyclopentaneundecanoic acid	60	ND	ND	ND	2257
Oleic Acid	50	ND	710	ND	2484
17-Octadecynoic acid	ND	60	ND	90	2648
Total ng/g	**2 510**	**2 000**	**9 040**	**250**	
Ketones					
2-Butanone	ND	1380	ND	ND	398
1-Octen-3-one	110	ND	ND	ND	915
2,3-Octanedione	260	ND	ND	ND	924
2(3H)-Furanone, 5-ethyldihydro-	10	ND	ND	ND	1028
5,9-Undecadien-2-one, 6,10-dimethyl-, (Z)-	20	ND	ND	ND	1445
2-Decanone	70	40	20	ND	1495
Benzophenone	ND	10	ND	10	1631
2-Dodecanone	ND	40	100	70	1897
ç Dodecalactone	ND	ND	30	40	2099
Total ng/g	**470**	**1 470**	**150**	**110**	
Esters					
Hexadecanoic acid, methyl ester	ND	ND	ND	10	1921
Decanoic acid, methyl ester	20	ND	ND	ND	1921
Benzoic acid, 2-hydroxy-, phenylmethyl ester	2	ND	ND	ND	1874
11,14-Eicosadienoic acid, methyl ester	110	ND	ND	ND	2162
Total ng/g	**130**	**ND**	**ND**	**10**	
Pyrazine					
Pyrazine, 2,5-dimethyl-	ND	30	ND	ND	861
Total ng/g	**2**	**30**	**ND**	**ND**	

Table 4.1: Continued

Compounds	Relative concentration ng/g				
Alcohols					
Ethanol	ND	710	ND	ND	369
1-Butanol, 2-methyl-	570	ND	ND	ND	530
Cyclobutanol, 2-ethyl-	ND	110	ND	ND	642
1-Octen-3-ol	120	ND	ND	ND	919
1-Hexanol, 2-ethyl-	40	ND	ND	ND	993
Ethanol, 2-phenoxy-	50	50	ND	ND	1223
trans-2-Undecen-1-ol	ND	ND	ND	30	1409
n-Tridecan-1-ol	ND	10	ND	ND	1490
2-Tridecen-1-ol, (E)-	40	ND	ND	20	1510
10-Undecyn-1-ol	ND	110	ND	ND	1660
8-Dodecenol	ND	ND	ND	10	1667
(Z)6,(Z)9-Pentadecadien-1-ol	ND	ND	10	ND	1863
1-Dodecanol	ND	20	ND	ND	1879
Total ng/g	**820**	**1 010**	**10**	**60**	
Nitrogen-containing compounds					
dl-Alanine	ND	ND	ND	60	719
1,2-Propanediamine	ND	290	ND	ND	721
Alanine	ND	ND	460	ND	726
Hydroxyurea	ND	ND	ND	90	731
2-Ethynyl pyridine	10	ND	ND	ND	894
1H-Pyrrole-2,5-dione	60	ND	ND	ND	1010
2,5-Pyrrolidinedione	10	ND	ND	ND	1154
Pyridine, 1-acetyl-1,2,3,4-tetrahydro-	10	ND	ND	ND	1192
Indole	20	ND	ND	ND	1297
Morpholine, 4-octadecyl-	ND	ND	30	ND	1884
Total ng/g	**110**	**290**	**490**	**150**	
Sulphur compounds					
Methanethiol	ND	ND	3	110	734
Propanesulphonylacetonitrile	ND	ND	ND	210	736
Disulphide, dimethyl	ND	ND	610	770	776
Dimethyl trisulphide	ND	ND	70	20	885

Table 4.1: Continued

Compounds	Relative concentration ng/g				
Benzoyl isothiocyanate	ND	30	ND	ND	1653
1,3-Benzenediol, monobenzoate	ND	10	ND	ND	1776
Benzene, [(methylsulphinyl)methyl]-	ND	1	ND	ND	1874
Total ng/g	**ND**	**40**	**680**	**1 110**	
Alkanes					
Octane, 3,5-dimethyl-	30	ND	ND	ND	1002
2,3,5-Trioxabicyclo[2.1.0]pentane, 1,4-bis(phenylmethyl)-	ND	ND	60	ND	1056
Octane, 1,1'-oxybis-	ND	110	50	60	1660
Undecane	10	ND	ND	ND	1699
Total ng/g	**40**	**110**	**110**	**60**	
Phenols					
Phenol	ND	ND	530	70	1000
2,6-Bis(1,1-dimethylethyl)-4-(1-oxopropyl)phenol	ND	40	50	50	1619
9,12-Octadecadien-1-ol, (Z,Z)-	ND	ND	40	ND	2055
Phenol, 4,4'-(1-methylethylidene)bis-	ND	ND	80	30	2171
Total ng/g	**ND**	**40**	**700**	**150**	
Alkenes					
3-Tetradecene, (E)-	ND	ND	10	ND	1490
1,E-11,Z-13-Octadecatriene	ND	ND	60	ND	1660
1,6,11-Dodecatriene, (Z)-	ND	ND	ND	50	1660
1,11-Dodecadiene	ND	30	20	ND	1667
1,12-Tridecadiene	ND	ND	30	ND	1866
1-Nonadecene	ND	ND	70	ND	1889
5-Octadecene, (E)-	ND	ND	10	ND	1953
1-Docosene	290	ND	ND	ND	2314
Total ng/g	**290**	**30**	**190**	**50**	

Table 4.1: Continued

Compounds	Relative concentration ng/g				
Furans					
Furan, 2-pentyl-	1090	70	20	30	896
2-n-Octylfuran	20	ND	ND	ND	1293
Total ng/g	**1 110**	**70**	**20**	**30**	
Total ng/g (all classes)	**15 030**	**6 572**	**11 952**	**2 398**	
Number of compounds produced		**58**	**34**	**36**	**27**

[a]RI$_{exp}$:Experimental Retention Index on a Rxi-5SilMS x Rxi-17SilMS column system. [b]ND: not detected.
[*]SFBA3, *B. subtilis* subsp. *subtilis*; PALB7, *B. cereus*; OALB2, *B. licheniformis* and SFBA2, *B. amyloliquefaciens* subsp. *plantarum*.

4.1.5   Conclusions

The present work identified the mixed-culture of *Bacillus* species involved in spontaneous fermentation of African locust bean and Bambara groundnut with members of the *B. subtilis* group being the main species. The polyphasic approach for identifying these *Bacillus* species was successful in classifying closely related members of the *B. subtilis* group. Volatile compounds production differed amongst *Bacillus* strains investigated. Regarding the results obtained from the present work, the investigated *B. subtilis* subsp. *subtilis* SFBA3, *B. amyloliquefaciens* subsp. *plantarum* SFBA2, *B. cereus* PALB7 and *B. licheniformis* OALB2 were highlighted as potential starter cultures for the alkaline fermentation of Bambara groundnut into *dawadawa*-type condiment.

4.2 Characterisation of volatile compounds in a *dawadawa*–like African food condiment produced from Bambara groundnut (*Vigna subterranean* (L.**) *Verdc*) using *Bacillus* species starter cultures**

4.2.1 Abstract

The volatile compounds of a *dawadawa*–like African food condiment produced from the alkaline fermentation of Bambara groundnut (*Vigna subterranea* (L.) Verdc.) using *Bacillus* starter cultures have never been reported. Volatile compounds were isolated from *dawadawa*–like condiments using headspace solid phase microextraction (SPME) and analysed by comprehensive gas chromatography coupled to time of flight mass spectrometry (GC × GC–TOFMS). Acids, aldehydes and alcohols accounted for over 70% volatile compounds produced in the *Bacillus* fermented samples. *B. subtilis* subsp. *subtilis* SFBA3 produced the highest content of acids (5089.88 µg kg^{-1}), while the highest content of aldehydes (2811.16 µg kg^{-1}) and alcohols (1255.58 µg kg^{-1}) was detected with *B. cereus* PALB7 and *B. licheniformis* OALB2, respectively. Sulphur-containing compounds concentration (84.44 µg kg^{-1}) was highest for *B. amyloliquefaciens* SFBA2. Maximum 2-methyl butanoic acid and 3-methyl butanoic acid concentrations, indicative of typical *dawadawa* aroma, were produced by *B. subtilis* subsp. *subtilis* SFBA3.

4.2.2 Introduction

Dawadawa is a traditional condiment manufactured by fermenting the African locust bean (*Parkia biglobosa*). The condiment is a culinary product used to impart flavour and enhance meatiness in soups, sauces and other prepared dishes (Beaumont, 2002). In the western and central African savannah regions, *dawadawa* is a delicacy considered as one of the most important food condiments. *Dawadawa* is generally produced from the spontaneous alkaline fermentation of the African locust bean with *Bacillus* species as the dominant microorganisms. However, there has been occasional production of *dawadawa* from soybeans (*Glycine max*) to manufacture soy-*dawadawa* or to a lesser extent use of Bambara groundnuts (*Vigna subterranean* L. *Verdc*.). Bambara groundnut is an underutilized African legume with potential for production of *dawadawa*–like condiments (Omafuvbe *et al*., 2000; Barimalaa *et al*., 1994; Fadahunsi and Olubunmi, 2010). The major role of *Bacillus* species in such condiments involves hydrolyzing proteins to peptides, amino acids and releasing ammonia thereby creating an alkaline pH which aids the inhibition of spoilage microorganisms (Parkouda *et al*., 2009). The pungent ammoniacal flavour characteristic of these condiments is produced from proteolytic activities of microorganisms on legume proteins and utilization of free amino acids during fermentation; consequently, ammonia is formed (Allagheny *et al*., 1996; Azokpota *et al*., 2008; Leejeerajumnean *et al*., 2001; Owens *et al*., 1997).

The profiling of volatile compounds production during controlled microbial alkaline fermentation of legumes is one of the steps which could give information for the future selection of starter cultures (Azokpota *et al*., 2010). Pyrazines, aldehydes, ketones, esters, alcohols, acids, alkanes, alkenes, benzenes, phenols, sulphurs and furans groups were identified in *dawadawa* produced from African locust bean and soybeans (Azokpota *et al*., 2008; Ouoba *et al*., 2005). However, volatile compounds that characterize *dawadawa*–like African food condiment produced from the controlled alkaline fermentation of Bambara groundnuts have not been reported previously. The methods that have been employed to isolate volatile compounds from fermented African locust bean (*dawadawa*) were microscale steam distillation low-density solvent extraction, mainly the Likens–Nikerson method (Ouoba *et al*., 2005; Azokpota *et al*., 2008, Azokpota *et al*., 2010) and purge-and-trap onto Tenax (Owens *et al*., 1997) extraction for fermented soybean (soy-*dawadawa*), followed by gas

chromatography – mass spectrometry (GC-MS) analysis. The distillation method is employed for volatile compounds extraction, especially when heat-labile compounds need to be extracted. However, the main drawback is the possible formation of artefacts due to the long-term influence of high temperature on heat-labile compounds. Solid phase microextraction (SPME) is widely used for the solvent free extraction of food and beverages (Junior *et al*., 2011; Mahattanatawee and Rouseff, 2014; Xu *et al*., 2017; Gao *et al*., 2016). Solvent free analyte enrichment provides aroma extracts that are more representative of food aroma when compared to those obtained by solvent extraction (Naudé and Rohwer, 2013). Headspace SPME involves sampling of the vapour that is directly above the food matrix and by concentrating the headspace with an adsorbent. This method has several advantages over classical distillation techniques, it is generally quicker, highly reproducible and yields ''true'' aroma profiles, as artefact formation is minimised (Kataoka *et al*., 2000).

The purpose of this study was to determine and compare the profiles of volatile compounds produced during alkaline fermentation of Bambara groundnut into *dawadawa*–like condiments by four (4) *Bacillus* starter cultures using headspace SPME and comprehensive gas chromatography with time-of-flight mass spectrometry (GC x GC-TOFMS). The characterization of volatile compounds of *dawadawa*–like condiments from alkaline fermented Bambara groundnuts are reported here for the first time.

4.2.3   Materials and methods

4.2.3.1 *Bacillus* starter culture

Starter cultures *B. subtilis* subsp. *subtilis* SFBA3, *B. amyloliquefaciens* subsp. *plantarum* SFBA2, *B. cereus* PALB7 and *B. licheniformis* OALB2 isolated and characterized from previous work (Chapter 4.1) were used to inoculate Bambara groundnuts sourced from Zimbabwe, Southern Africa, by Triotrade (Silverton, South Africa).

4.2.3.2 Fermentation of Bambara groundnut

Dawadawa–like condiments produced from Bambara groundnut was prepared according to the process described by Barimalaa *et al*. (1994) with slight modifications. Forty grams of seeds were steeped in 100 ml distilled water at 24 °C for 24 h, after which the seeds were dehulled manually using a mortar and pestle.

Cotyledons recovered were boiled in distilled water at 100 °C for 15 min. The cooked cotyledons were then drained with a sieve (1 mm pore size). Fifty (50) grams of the cooked cotyledons were placed in 50mm x 70mm zip lock perforated polythene bags (Apak Packaging, Johannesburg, South Africa) before inoculation with a starter culture. The inoculum was thoroughly mixed with the substrate using a sterile spatula. The samples were incubated at 30 °C for 120 h.

4.2.3.3 Headspace sampling with solid phase microextraction (SPME)

Five grams each of the alkaline fermented Bambara groundnut *dawadawa*–like condiments were placed in 24 ml glass vials. The vials were sealed with tin foil and a screw cap with a centre hole of 3.2 mm radius lined with a Teflon® septum (Separations, Randburg, South Africa). Samples were vortexed for 50 s before immersing them in a water bath at 50 °C for 15 min to equilibrate. The extractions were done with a SPME device fitted with a 2–50/30µm DVB/Carboxen/PDMS StableFlex fibre (Supelco, Sigma-Aldrich (Pty) Ltd. Kempton Park, South Africa). The fibre was exposed to the headspace above the sample for 20 min. After extraction the SPME device was removed from the vial and desorbed in the injection port of a GC x GC–TOFMS as described below. The fibre was conditioned between extractions by heating it in a GC injection port (split flow mode 50:1) for 20 min at 250 °C.

4.2.3.4 Chemical standards

Five grams of *dawadawa*-type condiments were spiked with 6.25 µg 1,8–cineole (eucalyptol) analytical reference standard (Sigma-Aldrich (Pty) Ltd. Kempton Park, South Africa). The headspace sampling of the spiked sample was extracted as described above and analysed with GCxGC–TOFMS. For linear retention index determination n–alkanes (C_8–C_{28}) were used (Merck, Pretoria, South Africa).

4.2.3.5 Comprehensive gas chromatography–time of flight mass spectrometry (GC x GC–TOFMS)

Compound separation was done using a LECO Pegasus 4D GC x GC-TOFMS with an Agilent 7890 GC (LECO Africa (Pty) Ltd., Kempton Park, South Africa). The system included a secondary oven and a dual stage modulator. Nitrogen gas was used for the hot jets and nitrogen gas cooled with liquid nitrogen was used for the cold jets. The carrier gas, helium, was of ultra-high purity grade (Afrox, Gauteng, South Africa) and was set at

a flow rate of 1.4 ml min^{-1} in the constant flow mode. The capillary column set consisted of an apolar Rxi-5SilMS 30 m x 0.25 mm ID x 0.25 μm df (Restek, Bellefonte, PA, USA) as the primary column and a high temperature midpolar Rxi–17Sil MS 0.97 m x 0.25 mm ID x 0.25 μm df (Restek, Bellefonte, PA, USA) as the secondary column. The SPME fibre was desorbed for 5 min in a SPME inlet liner (Supelco, Sigma-Aldrich (Pty) Ltd. Kempton Park, South Africa) of a GC inlet at 230 °C. The GC inlet was operated in the splitless mode with a splitless time of 54 s. The GC primary oven temperature programme was 35 °C (3 min) at 8 °C min^{-1} to 280 °C (5 min). The secondary oven was programmed identical to the primary oven, but offset by +5 °C. The modulator temperature offset was 20 °C. The modulation period was 2 s with a hot pulse time of 0.4 s. The MS transfer line temperature was set at 280 °C and the ion source temperature was set at 200 °C. The electron energy was 70 eV in the electron impact ionization mode (EI+), the data acquisition rate was 100 spectra s^{-1}, the mass acquisition range was 35–500 Da and the detector voltage was set at 1815 V. Tentative identification of compounds in the samples was done by comparison of mass spectra to that of the National Institute of Standards and Technology (NIST14) library. Compounds reported had a spectral match quality of ≥80%. Experimental linear retention indices (RI$_{exp}$) were calculated. Semi-quantification of the compounds was performed by using the internal standard method of quantification.

4.2.4 Statistical analysis

Statistical analysis was performed using XLSTAT 2014 software (AddinSoft™ SARL, Paris, France). The interaction effects of different *Bacillus* species starter cultures and volatile compounds formed were determined using analysis of variance (ANOVA) by the Modelling data option. Significant differences between means were determined using Fisher least significant difference test (LSD) at 5% probability level ($p < 0.05$). Principal component analysis (PCA) was conducted to show a visual interpretation of differences among *Bacillus* species starter cultures and volatile compounds formed using a vector distance plot.

4.2.5 Results and Discussion

The microbial characteristics of the controlled fermented condiments showed that *Bacillus* starter cultures reached from about 4×10^4 cfu g^{-1} in the inoculated sterile cooked cotyledons to about 6×10^8 cfu g^{-1} at 120 h in cotyledons with *B. cereus* PALB7, *B. amyloliquefaciens* subsp. *plantarum* SFBA2, *B. subtilis* subsp. *subtilis*

54

SFBA3 or *B. licheniformis* OALB2. The spontaneously fermented Bambara groundnut control had an initial *Bacillus* count of 2 x 10^3 cfu g^{-1} to attain a final count of 3×10^8 cfu g^{-1}. The control produced a condiment with a mixed microbial population comprising of *Bacillus* species and pathogenic microbes such as *Staphylococcus*, *Enterobacteriacae*, yeasts and moulds which contaminated the final product (data not shown). In this study, as inocula of pure cultures of *Bacillus* have been used during controlled fermentation, no contaminated microbial population was seen. However, due to the level of microbial contamination in the control sample, it was not further analyzed for volatile compounds. Both *B. cereus* PALB7 and the control had rapid growth throughout the fermentation as indicated by an increase in spore-forming bacteria counts with a concomitant pH increase from 6.90 to 8.20 for *B. cereus* PALB7 at 48 h. *B. amyloliquefaciens* subsp. *plantarum* SFBA2 and *B. licheniformis* OALB2 had the highest growth at 72 h while *B. subtilis* subsp. *subtilis* SFBA3 at 96 h. The highest alkaline pH recorded for all of the *Bacillus* species was pH 8.5 at 96 h. The final pH values in all the condiments are in the range of the pH 8 - 9 which generally characterize alkaline fermented products.

A total of 131volatile compounds were tentatively identified in the four *dawadawa*–like samples (Table 4.3), with 91, 57, 57 and 69 compounds in samples inoculated with *Bacillus* species SFBA3, PALB7, OALB2 and SFBA2 respectively (Table 4.3). These volatile compounds included aldehydes, acids, ketones, esters, pyrazines, alcohols, nitrogen-containing compounds, sulphur-containing compounds and furans. Additionally, alkanes (60), alkenes (8) and benzene-containing compounds (31) were also detected. Quantitatively, the most abundant volatile compounds were the organic acids (maximum total concentration 5089.88 µg kg^{-1} in *B. subtilis* subsp. *subtilis* SFBA3 sample) followed by the aldehydes (maximum total concentration of 2811.16 µg kg^{-1} in *B. cereus* PALB7 sample); alcohols and ketones (maximum total concentration 1255.58 µg kg^{-1} and 889.05 µg kg^{-1}, respectively in *B. licheniformis* OALB2). Comparatively, *B. amyloliquefaciens* SFBA2 had the highest concentration of sulphur-containing compounds (84.44 µg kg^{-1}). Benzaldehyde and hexanal were prominent compounds produced by all the *Bacillus* strains. Likewise ethanol, benzyl alcohol and 1–octen–3–ol were produced by all the *Bacillus* strains. 1–octen–3–ol is described as an important contributor to aroma with mushroom and fermented-like odour, the compound is considered to be a product of the oxidation of linoleic acid or other polyunsaturated fatty acid (Pham *et al*., 2008).

In *B. subtilis* subsp. *subtilis* SFBA3 inoculated samples, 3–methyl butanoic acid (1653.02 µg kg^{-1}) and acetic acid (657 µg kg^{-1}) were the organic acids produced at the highest levels. Acetic acid imparts a sour, vinegar note (Zhang *et al*., 2014; Zhao *et al*., 2011), while 3–methyl butanoic acid is described as acidic, sour, pungent, fruity, stinky, ripe fatty and fruity notes (Park *et al*., 2013). This strain produced the highest concentration of esters. Esters have been known to constitute a major volatile compound in African fermented condiments. The esters are presumably the consequence of chemical reactions between microbial acidic and alcoholic metabolites (Leejeerajumnean *et al*., 2001). The profile of the condiment from *B. cereus* PALB7 was dominated by aldehydes, in particular benzaldehyde and hexanal, at 2710.59 µg kg^{-1} and 53.54 µg kg^{-1}, respectively (Table 4.3). Benzaldehyde is generally responsible for a pleasant, sweet, aromatic note, while hexanal gives a green odour (Zhao *et al*., 2011; Mahattanatawee and Rouseff, 2014; Zhang *et al*., 2014). *B. cereus PALB7* produced the highest level of nitrogen-containing compounds. The condiment fermented with *B. licheniformis* OALB2 had the highest level of ketones at 889.05 µg kg^{-1} and was dominated by acetoin (875.39 µg kg^{-1}), usually characterized as having a butter-like aroma (Owens *et al*., 1997). However, all *Bacillus* strains produced acetophenone which is characterized by sweet and floral odours (Jirovetz *et al*., 2002). The condiment fermented with *B. licheniformis* OALB2 had a profile characterized with overall high levels of ketones, pyrazines and alcohols. Tetramethyl pyrazine, trimethyl pyrazine and 2, 5-dimethyl pyrazine were produced in relatively high concentrations by all of the *Bacillus* starter cultures. Pyrazines are typical aroma components of heated food to which they give a characteristic roasted or nutty flavour. Metabolic activities of microorganisms generally generate various precursors such as amino acids, monosaccharides and ammonia needed for the formation of pyrazines, while pyrazines are formed by accompanied non-enzymatic step such as heating (Owens *et al*., 1997).

In terms of alcohol, 2,3–butanediol was produced at the highest level by OALB2, with an odour description of fruity, creamy and buttery (Park *et al*., 2013). *B. amyloliquefaciens* SFBA2 produced the highest concentration of sulphur containing compounds. Sulphur-containing compounds, such as dimethyl disulphide, are reported to have strong pungent odours in *dawadawa* and they have a great influence on overall product aroma. SFBA2 produced the highest level of dimethyl disulphide (40.45 µg kg^{-1}). Dimethyl disulphide is described as onion, sulphurous, pungent (Chin *et al*., 2011) and sulphury, cabbage-like (Mahattanatawee and Rouseff, 2014).

Compounds such as hexanal, 2–pentylfuran, 1–hexanol and 1–octen–3–ol, produced by all the *Bacillus* strains are recognized as contributing to the green and beany aroma of cooked beans (Sugawara *et al.*, 1985). Despite some similarities, the aroma profiles of the different samples of *dawadawa*–like condiments were qualitatively and quantitatively different (Table 4.3).

The PCA on data from the aroma analysis of fermented samples showed that 82% of the variation could be explained by two principal components (P1 vs P2). A bi-plot showing scores and loadings shows the distinct compounds that differentiated each *Bacillus* group (Figure 4.5). PC1 explaining 49% of variation, separated the condiment fermented with SFBA3 in the first quadrant on the right from the other three condiments on the left. The condiment fermented with SFBA3 was characterized by higher concentrations of 2–methyl–butanoic acid, 3–methyl–butanoic acid, 2–methyl–propanoic acid, acetic acid, 3–methyl–butanoic acid ethyl ester, 2–methyl–propanoic acid ethyl ester, 3–methyl–1–butanol and benzyl alcohol (Figure 4.5; Table 4.3). The OALB2 condiment was associated with higher concentrations of acetoin, pyrazine, methyl pyrazine, trimethyl pyrazine and tetramethyl pyrazine, and 2,3–butanediol. PC2 explained the additional 33% variation separating the condiment with SFBA2 or PALB7 in the second quadrant from the condiments fermented with OALB2 in the third quadrant of the plot. Condiments fermented with the two strains, PALB7 and SFBA2, grouped together. The condiment fermented with PALB7 was characterized with higher concentrations of benzaldehyde and hexanal, while SFBA2 had higher concentrations of 2-methyl butanamine and indole.

The major volatile compound groups reported for traditional *dawadawa* made from African locust bean were pyrazines, aldehydes, alkenes, ketones, alcohols, esters and benzene derivatives. Pyrazines have been found in highest concentrations in *dawadawa* from African locust bean (Ouoba *et al.*, 2005; Azokpota *et al.*, 2008; Azokpota *et al.*, 2010, Onyenekwe *et al.*, 2012). In contrast, aldehydes, acids and alcohols dominated *dawadawa*–like condiments from Bambara groundnuts. Pyrazine levels in *dawadawa*–like condiments from Bambara groundnut (338 µg kg^{-1}) are considerably lower than that of African locust bean *dawadawa* (> 448000 µg kg^{-1}) (Ouoba *et al.*, 2005; Azokpota *et al.*, 2008; Azokpota *et al.*, 2010). The low levels of pyrazines found in this study were probably due to the mild conditions of the headspace solid phase microextraction (SPME) method used. Other authors used the Likens–Nickerson simultaneous distillation–extraction method that could

possibly influence pyrazine formation due to the higher heating step requirements. Heating protein–rich food matrices has been reported to influence the formation of pyrazines (Owens *et al*., 1997).

There has been no study reporting on the interrelationship of volatile compounds and *dawadawa* aroma/flavour in the literature. Beaumont (2002) reported on studies conducted in support of a US patent for flavourant composition prepared by fermentation (Heyland *et al*., 1995) suggesting a correlation between *dawadawa* aroma and the presence of 2-methyl butanoic acid and 3-methyl butanoic acid in the finished fermented protein base. Both 2,3–butanediol and 2–methyl butanoic acid are produced from branched-chained amino acids such as valine, leucine, and isoleucine during fermentation via catabolism processes, including oxidation and transamination (Park *et al*., 2013). It is noteworthy that 2–methyl butanoic acid and 3–methyl butanoic acid with their accompanying esters, typically characteristic of *dawadawa* aroma, were produced by all the *Bacillus* starter cultures.

Table 4.3: Volatile compounds in the headspace of *dawadawa*–like condiment produced from *Bacillus* fermented Bambara groundnut using GC x GC-TOFMS

	Compounds	RI$_{exp}$*	Mean concentration (μg kg^{-1} of product by dried weight basis) n=2			
			SFBA3[†]	PALB7	OALB2	SFBA2
	Aldehydes (12)					
1	Butanal, 3-methyl-	<800	14.60 [b¶] ± 4.49	14.03 [b] ± 2.10	49.23 [a] ± 28.80	37.92 [a] ± 13.96
2	Hexanal	<800	40.61 [a] ± 1.12	53.54 [a] ± 20.98	52.71 [a] ± 48.79	38.59 [a] ± 0.76
3	3-Furaldehyde	<800	27.04 [a] ± 1.52	2.19 [b] ± 0.15	53.75 [a] ± 70.13	6.21 [b] ± 0.22
4	Heptanal	<800	30.17 [a] ± 14.29	3.15 [b] ± 0.44	2.80 [b] ± 2.41	3.40 [b] ± 0.08
5	2-Heptenal, (Z)-	871	2.83 [a] ± 0.81	ND	ND	ND
6	2-Heptenal, (E)-	873	ND	0.80[a] ± 0.25	ND	ND
7	Benzaldehyde	878	1205.46 [ab] ± 178.10	2710.59 [a] ± 311.50	1012.78[b] ± 332.98	1782.82[ab] ± 219.07
8	Octanal	945	4.61 [a] ± 1.15	5.12 [a] ± 1.03	3.50 [a] ± 2.60	5.72 [a] ± 0.71
9	Benzeneacetaldehyde	1009	7.63 [a] ± 3.58	0.84[b] ± 0.50	2.05 [ab] ± 0.29	3.45 [ab] ± 0.84
10	Benzaldehyde, 2-hydroxy-	1014	ND	ND	ND	5.90 [a] ± 2.01
11	Nonanal	1096	16.21 [a] ± 5.67	17.52 [a] ± 0.56	15.98 [a] ± 8.62	22.10 [a] ± 1.66
12	Decanal	1237	1.43 [a] ± 0.74	3.40 [a] ± 0.63	3.94 [a] ± 2.55	5.19 [a] ± 0.03
	Total		**1350.60**	**2811.16**	**1196.75**	**1911.29**
	Acids (16)					
13	Methyl isobutyrate	<800	7.92[a] ± 5.24	ND	ND	ND

Table 4.3: Continued

	Compounds	RI_{exp}*	Mean concentration (μg kg^{-1} of product by dried weight basis) n=2			
			SFBA3†	PALB7	OALB2	SFBA2
14	Acetic acid	<800	657.94[a] ± 264.38	ND	11.40[b] ± 13.46	ND
15	Propanoic acid	<800	51.97[a] ± 6.71	ND	ND	ND
16	Methyl isovalerate	<800	66.99[a] ± 4.76	ND	ND	ND
17	Propanoic acid, 2-methyl-	<800	606.83[a] ± 41.42	31.61[b] ± 4.38	23.35[b] ± 4.49	14.97[b] ± 7.91
18	Butanoic acid	<800	14.42[a] ± 6.76	ND	ND	ND
19	Butanoic acid, 3-methyl-	<800	1653.02[a] ± 299.15	42.50[b] ± 13.40	191.94[b] ± 55.89	14.58[b] ± 6.00
20	Butanoic acid, 2-methyl-	806	1649.37[a] ± 355.21	22.99[ab] ± 3.75	39.32[ab] ± 3.45	11.29[b] ± 2.09
21	4-Methyl-2-oxovaleric acid	849	8.19[a] ± 0.34	ND	ND	ND
22	L-Lactic acid	899	ND	ND	6.18[a] ± 0.21	ND
23	Isobutyl isovalerate	950	0.48[a] ± 0.19	ND	ND	ND
24	Acetic acid, anhydride	1175	246.80[a] ± 263.08	ND	211.24[a] ± 34.19	ND
25	Isobornyl formate	1343	ND	0.88[a] ± 0.25	1.34[a] ± 1.05	0.62[a] ± 0.09
26	4-tert-Butylcyclohexyl acetate	1345	ND	1.10[a] ± 0.05	ND	1.09[ab] ± 0.33
27	Pentanoic acid	1933	0.86[a] ± 0.71	ND	ND	ND
28	Acetic acid, methoxy-	1940	4.57[a] ± 4.74	ND	119.59[b] ± 168.46	ND
	Total		**5089.88**	**99.08**	**604.37**	**42.55**

Table 4.3: Continued

	Compounds	RIexp*	Mean concentration (µg kg⁻¹ of product by dried weight basis) n=2			
			SFBA3[†]	PALB7	OALB2	SFBA2
	Ketones (21)					
29	2,3-Dihydroindole-2-one, 5-methoxy-1,3-dimethyl-3-(dimethylamino) methyl	<800	ND	ND	ND	50.11 a ± 14.41
30	2-Butanone	<800	1.44[a] ± 0.09	ND	ND	7.88[a] ± 0.70
31	Acetoin	<800	120.52[b] ± 37.04	ND	875.39[a] ± 87.74	ND
32	2-Pentanone, 4-hydroxy-4-methyl-	<800	10.50[a] ± 4.83	1.57[b] ± 0.77	ND	1.37[b] ± 0.84
33	2-Heptanone	<800	ND	1.25[a] ± 0.39	ND	ND
34	3-(Methylthio)-2-butanone	<800	ND	ND	0.69[a] ± 0.48	0.07[b] ± 0.02
35	Butyrolactone	820	0.35[a] ± 0.08	ND	ND	ND
36	2-Heptanone, 4-methyl-	840	0.48[a] ± 0.27	ND	ND	0.12[b] ± 0.02
37	2(3H)-Furanone, dihydro-3-methyl-	873	0.34[a] ± 0.10	ND	ND	ND
38	5-Hepten-2-one, 6-methyl-	916	2.78[a] ± 0.28	2.42[a] ± 0.21	1.88[a] ± 0.11	3.18[a] ± 1.10
39	3-Octanone	919	0.57[a] ± 0.16	ND	1.12[a] ± 1.04	ND
40	2-Octanone	926	3.81[a] ± 4.01	ND	0.66[a] ± 0.70	ND
41	2(3H)-Furanone, 5-ethyldihydro-	1029	5.98[a] ± 0.01	ND	ND	ND
42	Acetophenone	1041	2.84[a] ± 0.35	3.37[a] ± 0.11	3.51[a] ± 0.12	4.04[a] ± 0.22
43	2-Nonanone	1079	ND	ND	ND	0.60[a] ± 0.00

Table 4.3: Continued

	Compounds	RIexp*	Mean concentration (μg kg^{-1} of product by dried weight basis) n=2			
			SFBA3[†]	PALB7	OALB2	SFBA2
44	Bicyclo[2.2.1]heptan-2-one, 1,7,7-trimethyl-, (1S)-	1158	4.41 [ab] ± 0.48	4.13 [ab] ± 0.44	5.80 [a] ± 1.69	3.24 [b] ± 0.38
45	2-Decanone	1175	ND	1.05 [a] ± 0.15	ND	1.92 [a] ± 0.93
46	1,2-Propanedione, 1-phenyl-	1185	0.52[a] ± 0.06	ND	ND	ND
47	2-Dodecanone	1347	0.20[a] ± 0.07	ND	ND	0.50 [a] ± 0.04
48	2-Tridecanone	1427	ND	ND	ND	0.32 [a] ± 0.10
	Total		**34.22**	**13.79**	**889.05**	**73.34**
	Esters (17)					
49	Propanoic acid, ethyl ester	<800	8.07 [a] ± 9.55	ND	ND	ND
50	Propanoic acid, 2-methyl-, ethyl ester	<800	24.86 [a] ± 1.83	ND	ND	ND
51	Butanoic acid, 2-methyl-, ethyl ester	<800	36.79 [a] ± 0.84	2.31 [b] ± 0.84	ND	1.24 [b] ± 0.54
52	Butanoic acid, 3-methyl-, ethyl ester	<800	75.01 [a] ± 4.09	0.91 [b] ± 0.84	ND	2.19 [ab] ± 0.52
53	Butanoic acid, 2-methyl-, propyl ester	854	0.49 [a] ± 0.18	ND	ND	ND
54	Butanoic acid, 3-methyl-, propyl ester	861	0.41 [a] ± 0.08	ND	ND	ND

Table 4.3: Continued

	Compounds	RIexp*	Mean concentration (µg kg^{-1} of product by dried weight basis) n=2			
			SFBA3[†]	PALB7	OALB2	SFBA2
55	Pentanoic acid, 4-methyl-, ethyl ester	885	1.04 [a] ± 0.36	ND	ND	ND
56	Pentanoic acid, 4-methyl-, methyl ester	935	1.14 [a] ± 0.03	ND	ND	ND
57	Butanoic acid, 2methyl, 2methyl propyl ester	945	0.94 [a] ± 0.08	ND	ND	ND
58	Propanoic acid, 2-methyl, 3-methylbutyl ester	959	6.91[a] ± 2.29	ND	ND	ND
59	Butanoic acid, 2-methyl-, 3-methylbutyl ester	1088	3.82 [a] ± 0.01	ND	ND	0.26 [b] ± 0.01
60	Butanoic acid, 3-methyl-, 3-methylbutyl ester	1099	1.98 [a] ± 0.35	ND	ND	ND
61	Acetic acid, phenyl methyl ester	1180	2.07 [a] ± 0.07	ND	ND	ND
62	Propanoic acid, 2-methyl-, phenylmethyl ester	1353	2.09 [a] ± 0.09	ND	ND	ND
63	Propanoic acid, 2-methyl-, 3-hydroxy-2,2,4-trimethylpentyl ester	1446	ND	1.52 [a] ± 0.21	1.84 [a] ± 2.07	0.64 [b] ± 0.18
64	Butanoic acid, 3-methyl-, phenylmethyl ester	1463	0.95 [a] ± 0.31	ND	ND	ND

Table 4.3: Continued

	Compounds	RIexp*	Mean concentration (µg kg^{-1} of product by dried weight basis) n=2			
			SFBA3[†]	PALB7	OALB2	SFBA2
65	2,2,4-Trimethyl-1,3-pentanediol diisobutyrate	1686	ND	2.14 [a] ± 0.79	ND	ND
	Total		**166.58**	**4.74**	**1.84**	**4.33**
	Pyrazines (7)					
66	Pyrazine	<800	ND	ND	4.67 [a] ± 3.57	ND
67	Pyrazine, methyl-	<800	6.51 [b] ± 0.92	7.79 [b] ± 2.75	14.96 [a] ± 4.66	7.20 [b] ± 0.92
68	Pyrazine, 2,5-dimethyl-	808	12.39 [b] ± 0.67	54.97 [a] ± 12.46	30.67 [ab] ± 3.52	35.64 [ab] ± 2.24
69	Pyrazine, trimethyl-	950	8.82 [b] ± 0.04	12.85 [b] ± 2.81	89.13 [a] ± 35.29	17.62 [b] ± 2.06
70	Pyrazine, 3-ethyl-2,5-dimethyl-	1060	ND	2.37 [b] ± 1.54	ND	4.17 [a] ± 1.40
71	Pyrazine, tetramethyl-	1072	7.18 [b] ± 0.25	3.34 [b] ± 1.22	195.51 [a] ± 85.77	5.41 [b] ± 1.12
72	2,3,5-Trimethyl-6-ethylpyrazine	1171	ND	ND	3.21 [a] ± 1.87	ND
	Total		**34.90**	**81.31**	**338.15**	**70.04**
	Alcohols (31)					
73	Ethanol	<800	190.7 [a] ± 7.34	279.58 [a] ± 79.67	144.40 [a] ± 10.64	211.93 [a] ± 27.35
74	1-Butanol, 3-methyl-	<800	135.25 [a] ± 23.89	41.98 [b] ± 0.54	ND [c]	58.84 [b] ± 82.81
75	1-Pentanol	<800	ND	ND	87.68 [a] ± 102.68	1.65 [a] ± 1.46
76	1-Butanol, 2-methyl-	<800	ND	ND	ND	51.20 [a] ± 67.63

Table 4.3: Continued

	Compounds	RIexp*	Mean concentration (µg kg^{-1} of product by dried weight basis) n=2			
			SFBA3[†]	PALB7	OALB2	SFBA2
77	Isopropyl Alcohol	<800	ND	ND	25.24 [a] ± 32.92	ND
78	4-Penten-1-ol, 2-methyl-	<800	ND	ND	55.60 [a] ± 19.49	ND
79	5-Hexyn-3-ol	<800	2.37 [a] ± 1.45	ND	ND	ND
80	DL-2,3-Butanediol	<800	ND	ND	19.05 [a] ± 24.27	ND
81	Ethanol, 2-(methylthio)-	<800	1.56 [b] ± 0.52	ND	3.10 [a] ± 0.51	ND
82	1-Hexanol	<800	ND	10.58 [a] ± 2.45	ND	11.52 [a] ± 0.44
83	3-Furanmethanol	<800	6.14 [a] ± 1.59	ND	ND	ND
84	2,3-Butanediol	<800	ND	ND	682.75 [a] ± 59.56	ND
85	Ethanol, 2-butoxy-	<800	ND	2.37[b] ± 0.3	8.62[a] ± 7.0	1.34[b] ± 0.4
86	Ethanol, 2-butoxy-	<800	ND	2.37 [a] ± 0.28	10.67 [a] ± 8.49	1.34 [a] ± 0.42
87	4-Heptanol, 3,5-dimethyl-	858	6.04 [a] ± 1.01	ND	8.66 [a] ± 6.61	ND
88	2,3-Butanediol, [S-(R*,R*)]-	871	ND	ND	39.33 [a] ± 28.03	ND
89	2-Heptanol, 5-methyl-	887	ND	0.74 [a] ± 0.54	ND	ND
90	1-Butanol, 3-methyl-, propanoate	892	1.79 [a] ± 0.32	ND	ND	ND
91	1-Heptanol	899	1.27 [a] ± 0.20	ND	ND	ND
92	1-Octen-3-ol	912	2.63 [a] ± 0.05	2.55 [a] ± 0.72	3.23 [a] ± 1.07	3.23 [a] ± 0.39
93	1-Hexanol, 2-ethyl-	986	ND	12.32 [a] ± 15.11	22.21 [a] ± 7.01	26.76 [a] ± 1.22
94	Benzyl alcohol	1000	348.97 [a] ± 8.68	85.51 [b] ± 16.54	76.56 [b] ± 24.40	234.36 [a] ± 14.87
95	7-Octen-2-ol, 2,6-dimethyl-	1050	ND	3.05 [a] ± 0.98	ND	ND

Table 4.3: Continued

	Compounds	RIexp*	Mean concentration (µg kg^{-1} of product by dried weight basis) n=2			
			SFBA3[†]	PALB7	OALB2	SFBA2
96	Phenylethyl Alcohol	1113	26.61 [b] ± 0.45	5.68 [c] ± 5.21	65.15 [a] ± 25.33	14.73 [bc] ± 3.42
97	Levomenthol	1201	0.43 [b] ± 0.05	0.74 [a] ± 0.02	0.85 [a] ± 0.78 [b]	ND
98	1,6-Octadien-3-ol,3,7-dimethyl-, formate	1293	1.00 [a] ± 0.19	ND	ND	0.98 [a] ± 0.32
99	1-Octanol, 2-butyl-	1369	1.14 [a] ± 0.16	ND	0.64 [a] ± 0.20	1.51 [a] ± 0.15
100	n-Tridecan-1-ol	1379	0.82 [a] ± 0.33	ND	ND	ND
101	2,2,4-Trimethyl-1,3-pentanediol diisobutyrate	1686	0.29 [a] ± 0.02	ND	1.84 [a] ± 2.10	0.84 [a] ± 0.27
102	Ethanol, 2-nitro-	2012	7.61 [a] ± 6.12	ND	ND	ND
	Total		**734.65**	**447.47**	**1255.58**	**619.74**
	Nitrogen-containing compounds (14)					
103	N-Dimethylaminomethyl-tert.-butyl-isopropylphosphine	<800	145.18 [b] ± 3.98	483.22 [a] ± 4.41	ND	ND
104	2-Butanamine, 2-methyl-	<800	ND	ND	ND	9.29 [a] ± 5.47
105	Dimethylamine	<800	7.01 [a] ± 9.62	ND	ND	ND
106	Urea	<800	3.34 [a] ± 1.86	ND	ND	ND
107	Guanidine carbonate	<800	4.96[a] ± 0.61	ND	ND	ND

Table 4.3: Continued

	Compounds	RIexp*	Mean concentration (µg kg^{-1} of product by dried weight basis) n=2			
			SFBA3[†]	PALB7	OALB2	SFBA2
108	Methylamine, N,N-dimethyl-	<800	8.36 [b] ± 11.62	ND	ND	35.28 [a] ± 15.90
109	Oxime-, methoxy-phenyl-	811	ND	43.32 [a] ± 5.05	ND	ND
110	Oxime-, methoxy-phenyl-	858	19.68[a] ± 5.63	ND	212.54[a] ± 250.12	103.94[a] ± 86.47
111	Dimethylamine	876	ND	ND	0.35 [a] ± 0.06	ND
112	Benzonitrile	914	2.44 [b] ± 0.07	6.38 [a] ± 0.67	ND	4.73 [b] ± 0.62
113	N,N'-Dibenzylidene ethylenediamine	1117	ND	ND	ND	0.50 [a] ± 0.30
114	Aziridine, 2-phenyl-	1317	1.31[a] ± 0.83	ND	ND	ND
115	Indole	1353	ND	ND	ND	36.94 [a] ± 1.93
116	1-Butanamine, 3-methyl-N-(2-phenylethylidene)-	1453	ND	1.90 [a] ± 0.38	ND	ND
	Total		**192.28**	**534.82**	**212.89**	**190.68**
	Sulphur compounds (5)					
117	Methanethiol	<800	0.62 [b] ± 0.10	30.96 [a] ± 42.42	30.77 [a] ± 10.92	28.68 [a] ± 3.40
118	Disulphide, dimethyl	<800	30.28 [a] ± 2.22	7.78[b] ± 0.49	ND	40.45 [a] ± 14.58
119	Methional	<800	0.49 [b] ± 0.06	ND	ND	1.12[a]± 0.31
120	Benzo[b]thiophene	1221	0.41 [b] ± 0.09	0.59 [a] ± 0.01	ND	0.67 [a] ± 0.14
121	2-Phenyl-1-phenylsulfonylaziridine	1295	ND	2.49 [b] ± 0.93	ND	13.52 [a] ± 17.77
	Total		**31.80**	**41.82**	**30.77**	**84.44**

Table 4.3: Continued

	Compounds	RIexp*	Mean concentration (µg kg^{-1} of product by dried weight basis) n=2			
			SFBA3[†]	PALB7	OALB2	SFBA2
	Sesquiterpenes (3)					
122	α-Copaene	1453	1.16[a] ± 0.08	1.80[a] ± 0.30	1.46[a] ± 0.63	1.56[a] ± 0.03
123	Aromandendrene	1552	ND	ND	0.71[a] ± 0.19	1.04[a] ± 0.08
124	trans-Calamenene	1619	ND	0.22[a] ± 0.02	ND	0.28[a] ± 0.00
	Total		**1.16**	**2.02**	**2.17**	**2.88**
	Monoterpenes (5)					
125	ß-Pinene	901	ND	ND	0.75[a] ± 0.21	0.33[a] ± 0.00
126	ß-Myrcene	923	0.06[a] ± 0.00	0.15[a] ± 0.00	ND	0.10[a] ± 0.06
127	Limonene	983	28.04[a] ± 1.46	24.34[a] ± 3.67	ND	26.26[a] ± 0.62
128	*o*-Cymene	1027	2.10[b] ± 0.88	6.60[ab] ± 7.05	17.31[a] ± 4.49	2.36[b] ± 1.41
129	(+)-2-Carene	1070	ND	0.79[a] ± 0.36	ND	ND
	Total		**30.20**	**31.88**	**18.06**	**29.05**
	Furans (2)					
130	Furfural	<800	0.76[a] ± 0.16	ND	ND	ND
131	Furan, 2-pentyl-	923	2.36[a] ± 0.18	2.84[a] ± 0.62	3.50[a] ± 0.48	2.44[a] ± 0.21
	Total		**3.12**	**2.84**	**3.50**	**2.44**

	Compounds	RIexp*	Mean concentration (µg kg^{-1} of product by dried weight basis) n=2			
			SFBA3[†]	PALB7	OALB2	SFBA2
	Alkanes (60)					
132	Pentane, 2-methyl-	<800	0.12 [a¶] ± 0.04	ND	ND	ND
133	Pentane, 3-methyl-	<800	0.32 [a] ± 0.16	ND	ND	ND
134	Heptane, 4-methyl-	<800	ND	ND	ND	0.45 [a] ± 0.07
135	Octane	<800	1.21 [a] ± 0.50	ND	ND	1.06 [a] ± 0.21
136	Heptane, 2,4-dimethyl-	<800	3.34 [a] ± 0.33	2.53 [a] ± 1.57	ND	ND
137	Octane, 4-methyl-	<800	4.26 [a] ± 1.48	1.14 [b] ± 0.02	ND	3.84 [a] ± 2.67
138	Nonane	<800	1.43 [a] ± 0.70	1.38 [a] ± 0.37	2.86 [a] ± 0.24	1.16 [a] ± 0.15
139	Nonane, 4-methyl-	878	ND	2.28 [a] ± 0.25	ND	ND
140	Nonane, 2-methyl-	885	ND	ND	3.43 [a] ± 2.01	1.00 [b] ± 0.4
141	Nonane, 3-methyl-	894	5.79 [a] ± 0.32	ND	ND	ND
142	Decane, 2,6,7-trimethyl-	895	ND	ND	ND	2.18 [a] ± 0.54
143	Cyclohexane, 1-methyl-2-propyl-	914	2.29 [a] ± 1.63	3.11 [a] ± 3.44	2.43 [a] ± 1.05	ND
144	Decane	940	7.17 [a] ± 0.53	7.85 [a] ± 1.93	13.39 [a] ± 0.04	4.79 [a] ± 0.47
145	Nonane, 2,6-dimethyl-	957	5.52 [a] ± 2.95	ND	4.47 [a] ± 2.14	5.17 [a] ± 0.40
146	7-Oxabicyclo[2.2.1]heptane, 1-methyl-4-(1-methylethyl)-	964	2.42 [a] ± 0.08	1.30 [ab] ± 0.12	1.34 [ab] ± 0.22	0.92 [b] ± 0.23
147	Octane, 6-ethyl-2-methyl-	986	ND	ND	ND	0.60 [a] ± 0.21
148	Indane	991[‡]	0.96 [a] ± 0.05	ND	1.61 [a] ± 0.37	1.49 [a] ± 0.19

Table 4.3: Continued

	Compounds	RIexp*	Mean concentration (μg kg^{-1} of product by dried weight basis) n=2			
			SFBA3[†]	PALB7	OALB2	SFBA2
149	Cyclohexane, butyl-	991[‡]	ND	0.53 [b] ± 0.24	1.15 [a] ± 0.36	0.32 [b] ± 0.30
150	Hexadecane	993[‡]	1.92 [a] ± 0.60	3.17 [a] ± 1.31	2.35 [a] ± 1.94	3.57 [a] ± 1.21
151	Dodecane, 2,7,10-trimethyl-	993[‡]	ND	0.90 [a] ± 1.00	ND	ND
152	Cyclopentane, pentyl-	995	0.72 [a] ± 0.11	0.82 [a] ± 0.10	0.72 [a] ± 0.40	ND
153	Nonane, 3,7-dimethyl-	1000	ND	ND	0.97 [a] ± 0.28	ND
154	Tridecane, 6-methyl-	1012	ND	0.24 [a] ± 0.15	ND	ND
155	Decane, 4-methyl-	1031	3.58 [a] ± 1.07	2.35 [a] ± 0.23	2.10 [a] ± 0.74	ND [c]
156	Decane, 2-methyl-	1038	1.58 [a] ± 0.19	1.27 [a] ± 0.29	2.34 [a] ± 0.41	0.88 [a] ± 0.03
157	Decane, 3-methyl-	1048	1.89 [a] ± 0.71	1.43 [a] ± 0.44	1.85 [a] ± 0.37	1.14 [a] ± 0.45
158	Cyclooctane, 1,4-dimethyl-, cis-	1055	7.46 [b] ± 0.60	4.68 [b] ± 4.25	ND	21.83 [a] ± 4.84
159	1-Nonylcycloheptane	1077	2.58 [a] ± 0.43	ND	ND	ND
160	Octane, 2,3,6,7-tetramethyl-	1079	2.28 [b] ± 0.33	ND	ND	2.90 [a] ± 1.07
161	Undecane	1091	28.11 [b] ± 1.01	26.51 [b] ± 4.01	42.16 [a] ± 11.27	28.86 [b] ± 2.21
162	Undecane, 4-methyl-	1110	4.96 [a] ± 1.08	0.73 [b] ± 0.47	1.05 [b] ± 0.05	3.62 [a] ± 0.40
163	Decane, 3,7-dimethyl-	1127	1.89 [a] ± 0.13	1.37 [a] ± 0.20	3.28 [a] ± 1.09	1.42 [a] ± 0.50
164	Undecane, 3-methyl-	1139	1.77 [a] ± 1.37	2.62 [a] ± 1.29	3.79 [a] ± 1.69	1.92 [a] ± 0.85
165	Cyclohexane, pentyl-	1142	ND	ND	1.87 [a] ± 0.30	0.71 [a] ± 0.11
166	Cyclopentane, butyl-	1146	0.65 [a] ± 0.92	ND	ND	ND
167	Hexane, 4-ethyl-2-methyl-	1165	ND	0.82 [a] ± 0.13	ND	ND

Table 4.3: Continued

	Compounds	RIexp*	Mean concentration (µg kg^{-1} of product by dried weight basis) n=2			
			SFBA3[†]	PALB7	OALB2	SFBA2
168	Undecane, 2-methyl-	1180	1.16[a] ± 0.13	1.38[a] ± 0.12	1.48[a] ± 0.04	1.11[a] ± 0.38
169	Dodecane	1228	5.23[a] ± 0.75	7.94[a] ± 0.76	8.08[a] ± 1.51	5.56[a] ± 0.85
170	Undecane, 2,6-dimethyl-	1245	1.74[b] ± 0.84	5.18[a] ± 1.35	7.81[a] ± 6.68	6.78[a] ± 5.65
171	Heptadecane, 2,6,10,14-tetramethyl-	1257	ND	1.53[a] ± 1.41	ND	1.04[a] ± 0.39
172	Dodecane, 4,6-dimethyl-	1281	ND	ND	ND	2.00[a] ± 0.30
173	Cyclohexane, hexyl-	1283	ND	0.78[a] ± 0.01	0.78[a] ± 0.12	ND
174	Nonane, 4,5-dimethyl-	1297	1.39[b] ± 0.04	ND	3.12[a] ± 2.09	1.19[b] ± 0.01
175	Dodecane, 4-methyl-	1304	0.35[a] ± 0.06	ND	0.43[a] ± 0.11	0.73[a] ± 0.45
176	Undecane, 2,10-dimethyl-	1312	0.63[a] ± 0.75	ND	ND	ND
177	Undecane, 5-methyl-	1322	1.33[a] ± 0.07	ND	ND	ND
178	Tridecane	1358	4.13[a] ± 0.03	7.00[a] ± 0.49	6.26[a] ± 1.47	5.99[a] ± 1.13
179	Decane, 2,6,8-trimethyl-	1383	2.84[a] ± 0.12	5.07[a] ± 2.07	4.54[a] ± 0.63	5.97[a] ± 0.31
180	Heptane, 2,2,4,6,6-pentamethyl-	1388	0.38 ± 0.06[a]	ND	ND	ND
181	Dodecane, 2,7,10-trimethyl-	1396	ND	ND	ND	1.32[a] ± 0.35
182	Undecane, 4,7-dimethyl-	1405	ND	ND	2.66[a] ± 1.77	ND
183	Tridecane, 4-methyl-	1427	0.30[b] ± 0.15	1.19[b] ± 0.41	ND	3.25[a] ± 3.68
184	Tridecane, 2-methyl-	1434	ND	ND	ND	3.97[a] ± 3.67
185	Tridecane, 3-methyl-	1444	0.86[a] ± 0.32	1.27[a] ± 0.39	1.73[a] ± 1.60	1.53[a] ± 0.37

Table 4.3: Continued

	Compounds	RIexp*	Mean concentration (µg kg^{-1} of product by dried weight basis) n=2			
			SFBA3[†]	PALB7	OALB2	SFBA2
186	Dodecane, 2,6,10-trimethyl-	1448	1.05[a] ± 0.76	2.83[a] ± 1.98	1.08[a] ± 0.27	1.03[a] ± 0.23
187	Tetradecane	1477	2.65[a] ± 0.44	4.60[a] ± 0.41	2.85[a] ± 1.09	3.58[a] ± 0.83
188	Cyclohexane, octyl-	1535	ND	0.43[a] ± 0.08	ND	ND
189	Decane, 2,9-dimethyl-	1578	ND	ND	ND	0.30[a] ± 0.04
190	Pentadecane	1590	ND	1.51[a] ± 0.27	ND	ND
	Total		**119.14**	**107.77**	**133.98**	**136.22**
	Alkenes (8)					
191	2,4-Dimethyl-1-heptene	<800	19.01[a] ± 4.41	ND	ND	ND
192	2,4-Dimethyl-1-heptene	<800	ND	ND	11.24[a] ± 7.06	9.52[a] ± 0.27
193	1,3,5-Cycloheptatriene, 7,7-dimethyl-	813	ND	0.63[a] ± 0.19	ND	ND
194	1-Octene, 3,7-dimethyl-	1063	6.48[a] ± 0.65	ND	ND	ND
195	(E)-1-Phenyl-1-butene	1158	3.18[a] ± 3.03	1.49[a] ± 0.31	1.57[a] ± 0.34	1.20[a] ± 0.39
196	2,2-Dimethylindene, 2,3-dihydro-	1144	ND	ND	0.24[a] ± 0.04	ND
197	1-Undecene, 7-methyl-	1369	ND	1.85[a] ± 1.01	ND	ND
198	Dicyclopentadiene	1719	ND	0.49[a] ± 0.04	ND	0.47[a] ± 0.07
	Total		**28.67**	**4.46**	**13.04**	**11.19**

	Compounds	RIexp*	Mean concentration (μg kg^{-1} of product by dried weight basis) n=2			
			SFBA3[†]	PALB7	OALB2	SFBA2
	Ether (1)					
199	Oxirane, (methoxymethyl)-	<800	4.30 [a] ± 1.77	ND	ND	ND
	Total		**4.30**	**0**	**0**	**0**
	Polyaromatic compounds (8)					
200	Bicyclo[4.2.0]octa-1,3,5-triene	<800	27.88 [a] ± 1.29	ND	ND	ND
201	(E)-4,8-Dimethylnona-1,3,7-triene	1110	ND	ND	ND	0.78 [a] ± 0.06
202	1-Methyldecahydro naphthalene	1113	ND	1.28 [a] ± 0.97	1.69 [a] ± 0.96	ND
203	Naphthalene, decahydro-, trans-	1029	ND	0.56 [a] ± 0.18	ND	ND
204	1H-Indene, 2,3-dihydro-4-methyl-	1146	ND	1.98 [a] ± 2.19	ND	ND
205	Naphthalene, 1,2,3,4-tetrahydro-	1177	0.99 [a] ± 0.17	0.97 [a] ± 0.24	1.39 [a] ± 0.27	1.05 [a] ± 0.17
206	Naphthalene	1211	2.89 [a] ± 0.01	3.89 [a] ± 0.26	3.71 [a] ± 0.56	4.06 [a] ± 0.28
207	Naphthalene, 1-methyl-	1358	ND	0.51 [a] ± 0.08	ND	ND
	Total		**31.76**	**9.19**	**6.79**	**5.89**
	Aromatic compounds (31)					
208	Ethylbenzene	<800	11.02 [a] ± 2.53	ND	ND	4.72 [b] ± 0.67
209	Benzene, 1,3-dimethyl-	<800	42.35 [ab] ± 6.56	25.02 [b] ± 2.13	53.31 [a] ± 11.48	ND
210	Benzene, (1-methylethyl)-	813	1.04 [a] ± 0.73	ND	1.31 [a] ± 0.26	0.87 [a] ± 0.57
211	Benzene, propyl-	863	3.00 [a] ± 0.21	2.18 [a] ± 0.11	4.11 [a] ± 1.45	2.09 [a] ± 0.68

Table 4.3: Continued

	Compounds	RIexp*	Mean concentration (μg kg^{-1} of product by dried weight basis) n=2			
			SFBA3[†]	PALB7	OALB2	SFBA2
212	Benzene, 1-ethyl-3-methyl-	876	4.91[ab] ± 0.29	5.44[ab] ± 0.83	8.21[a] ± 0.85	3.93[b] ± 1.33
213	Benzene, 1-ethyl-4-methyl-	880	6.59[a] ± 5.35	ND	ND	3.84[a] ± 0.51
214	Benzene, 1-ethyl-2-methyl-	901	ND	5.06[a] ± 0.84	8.63[a] ± 4.79	4.54[a] ± 0.45
215	Benzonitrile	914	ND	ND	3.20[a] ± 1.31	ND
216	Benzene, 1,2,3-trimethyl-	928	15.81[a] ± 3.75	20.38[a] ± 0.23	23.37[a] ± 11.98	20.11[a] ± 0.82
217	Benzene, (1-methylpropyl)-	952	1.07[a] ± 0.07	ND	1.94[a] ± 0.14	ND
218	Benzene, 1,4-diethyl-	1012	2.21[a] ± 0.38	ND	ND	ND
219	Benzene, 1-methyl-3-propyl-	1017	5.82[a] ± 4.30	ND	4.79[a] ± 1.14	7.85[a] ± 8.93
220	Benzene, 2-ethyl-1,4-dimethyl-	1027	4.40[a] ± 3.03	4.68[a] ± 0.33	ND	ND
221	Benzene, 1-methyl-4-propyl-	1038	2.43[b] ± 0.22	4.66[a] ± 1.27	ND	ND
222	Benzene, 1-ethyl-2,4-dimethyl-	1058	ND	3.35[a] ± 0.18	ND	ND
223	Benzene, 1-methyl-4-(2-propenyl)-	1060	ND	ND	ND	1.77[a] ± 1.14
224	Benzene, 1-methyl-3-(1-methylethyl)-	1061	6.23[a] ± 1.25	1.84[a] ± 0.14	7.89[a] ± 3.24	5.36[a] ± 1.33
225	Benzene, (2-methyl-2-propenyl)-	1063	ND	0.52[a] ± 0.42	ND	ND
226	Benzene, 4-ethyl-1,2-dimethyl-	1076	ND	ND	ND	3.70[a] ± 2.25
227	Benzene, 1,2,3,4-tetramethyl-	1117	4.03[a] ± 1.88	4.20[a] ± 1.15	ND	3.44[a] ± 0.77
228	Benzene, 1,3-diethyl-5-methyl-	1149	2.98[a] ± 0.67	3.98[a] ± 0.47	4.38[a] ± 1.08	3.79[a] ± 1.71
229	Benzene, 1-methyl-4-butyl	1163	ND	0.79[a] ± 0.01	ND	0.58[a] ± 0.19

	Compounds	RIexp*	Mean concentration (µg kg^{-1} of product by dried weight basis) n=2			
			SFBA3[†]	PALB7	OALB2	SFBA2
230	Benzene, pentyl-	1171	ND	0.22 [a] ± 0.15	1.06 [a] ± 0.04	0.22 [a] ± 0.02
231	Benzene, (1,1-dimethylpropyl)-	1173	1.92 [ab] ± 0.72	1.57 [b] ± 0.79	3.27 [a] ± 3.36	1.72 [ab] ± 0.08
232	Benzene, 1-methyl-4-(1-methylpropyl)-	1180	2.35 [a] ± 0.84	2.71 [a] ± 0.62	2.91 [a] ± 0.62	1.31 [a] ± 1.71
233	Benzene, 1,4-diethyl-2-methyl-	1182	ND	ND	1.68 [a] ± 1.03	ND
234	Benzo[b]thiophene	1221	ND	ND	0.59 [a] ± 0.05	ND
235	Benzene, 1-ethyl-4-(1-methylethyl)-	1223	ND	1.74 [a] ± 0.20	ND	ND
236	Benzene, 1,3-bis(1,1-dimethylethyl)-	1293	0.97 [a] ± 0.12	1.69 [a] ± 0.36	1.48 [a] ± 0.60	1.16 [a] ± 0.03
237	Benzene, 3-cyclohexen-1-yl-	1401	0.22 [a] ± 0.06	0.29 [a] ± 0.01	0.22 [a] ± 0.05	0.24 [a] ± 0.01
	Total		**119.35**	**90.32**	**132.35**	**71.26**
	Phenols (1)					
238	Phenol	916	18.18 [ab] ± 1.37	2.97 [b] ± 0.94	19.32 [ab] ± 4.40	71.86 [a] ± 12.75
	Total		**18.18**	**2.97**	**19.32**	**71.86**

[†]SFBA3, *B. subtilis* subsp. *subtilis*; PALB7, *B. cereus*; OALB2, *B. licheniformis* and SFBA2, *B. amyloliquefaciens* subsp. *plantarum*;

*RI$_{exp}$: Experimental Retention Index on an Rxi-5SilMS x Rxi-17SilMS column system using C$_8$-C$_{28}$ as external references.

ND: Not detected.

[¶]Different letters in the same row indicate significant differences (P < 0.05).

[‡]Co-elution of 2 compounds (1D) but separated by GC x GC on the 2D retention times

The odour activity values (OAVs) are presented in Table 4.4. OAVs give an indication of the odour potency of a single odourant in a food itself, based on its odour threshold in the respective food matrix (Van Gemert and Netternbreijer, 1977; Park *et al*., 2013). The OAVs for prominent volatile compounds in *dawadawa*–like condiments were calculated by dividing the measured concentrations with odour thresholds obtained from the literature (Van Gemert and Netternbreijer, 1977). Of all the volatile compounds detected, only those displaying OAVs greater than one, which are hexanal, benzaldehyde, benzeneacetaldehyde, nonanal, heptanal, decanal, 3–methylbutanoic acid, 2–methylbutanoic acid and dimethyl disulphide were deemed to contribute to overall *dawadawa* aroma. The OAVs for 3–methyl butanoic acid and 2–methyl butanoic acid, the compounds reported as the main indication of typical *dawadawa* aroma (Beaumont, 2002), were highest for SFBA3 with values of 14 and 16 respectively (Table 4.4). However, the OAVs for these compounds were less than one for the other condiments, except for OALB2 which had an OAV of 2 for 3-methyl butanoic acid. The aldehydes, hexanal, benzaldehyde, nonanal and decanal, characterised the *Bacillus* fermented condiments with OAVs greater than one recorded for all. Dimethyl disulphide was not detected in OALB2, but high OAVs were recorded for the other three condiments. *Dawadawa* aroma has not been fully characterised, yet the volatile compounds reported here are only an indication of several compounds that may be contributing to the perceived pungent, ammoniacal and putrid aroma, however; more work is required in identifying correlations of specific volatile compounds that classify *dawadawa* aroma in particular.
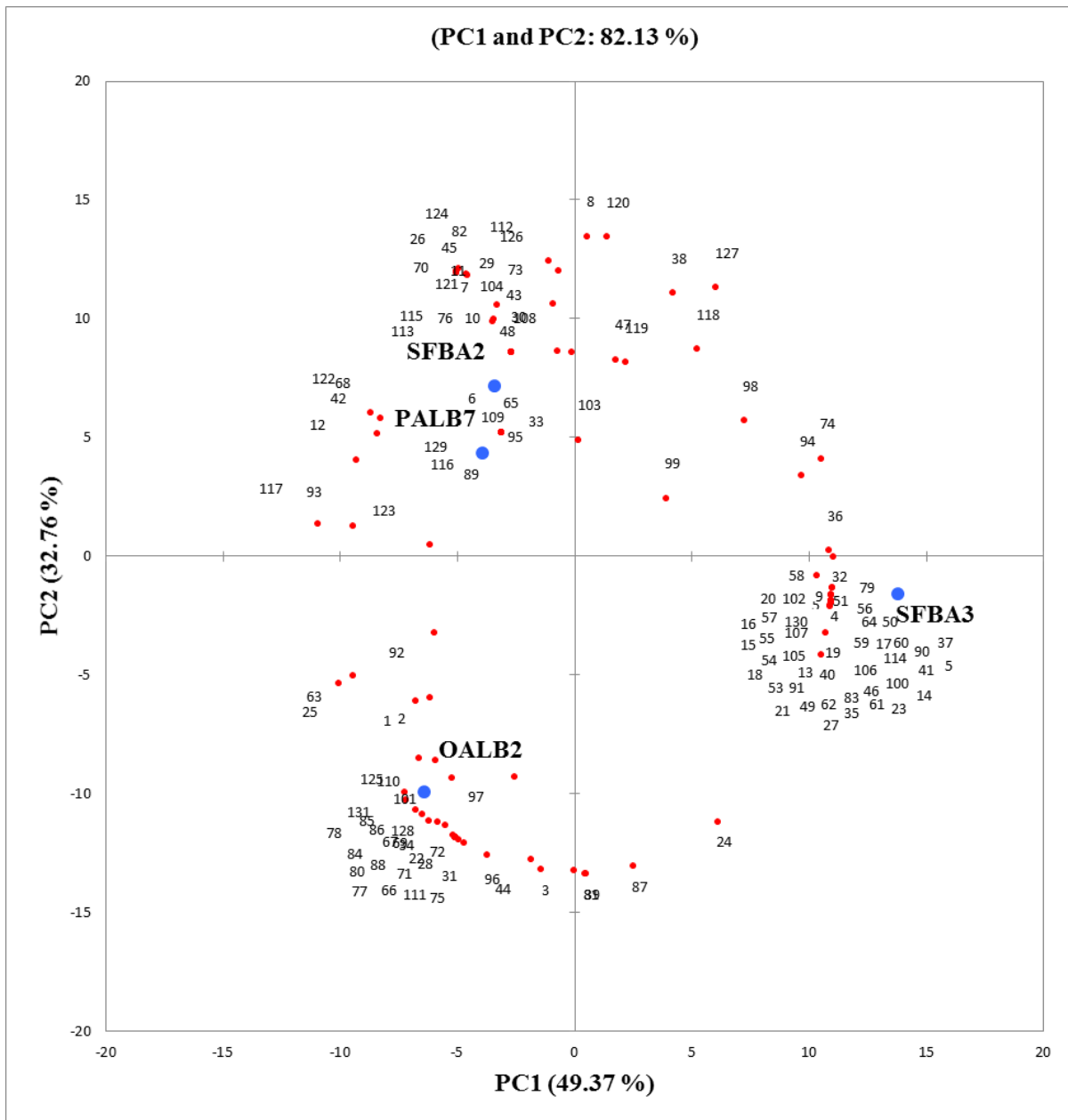
Figure 4.5: Principal component loadings and scores of concentrations of volatile compounds in *dawadawa*–like condiments produced from alkaline fermentation of Bambara groundnut. PALB7, with *B. cereus* PALB7; SFBA2, with *B. amyloliquefaciens* subp. *plantarum* SFBA2; OALB2, with *B. licheniformis* OALB2 and SFBA3, with *B. subtilis* subsp. *subtilis* SFBA3 (Compounds 1–131, see Table 4.3)

Table 4.4: Odour thresholds and odour activity values (OAVs) of volatile compounds in *dawadawa*–like condiments

	ODT	Concentration μg kg^{-1}				OAV			
		SFBA3	PALB7	OALB2	SFBA2	SFBA3	PALB7	OALB2	SFBA2
Hexanal	4.5	40.6	53.5	52.7	38.6	9	12	12	9
Benzaldehyde	350	1205.5	2710.6	1012.8	1782.8	3	8	3	5
Benzeneacetaldehyde	4	7.6	0.8	2.1	3.5	2	<1	1	1
Nonanal	1	16.2	17.5	16.0	22.1	16	18	16	22
Heptanal	3	30.2	3.2	2.8	3.4	10	1	1	1
Decanal	0.1	1.4	3.4	3.9	5.2	14	34	39	52
Butanoic acid, 3-methyl-	120	1653.0	42.5	191.9	14.6	14	<1	2	<1
Butanoic acid, 2-methyl-	100	1649.4	23.0	39.3	11.3	16	<1	<1	<1
Acetoin	800	120.5	0.0	875.4	0.0	<1	<1	1	<1
Dimethyl disulphide	0.33	30.3	7.8	0.0	40.5	92	24	0	123

*Data taken from van Gemert and Netternbreijer (1977) and Park *et al*., (2013).
¶Odour-activity values (OAV) were calculated by dividing the concentrations by the respective odour threshold (ODT)

4.2.6.   Conclusions

In this study, the effect of different *Bacillus* starter cultures on the volatile flavour compounds of a *dawadawa*–like condiment from Bambara groundnut was investigated. Headspace SPME and GCxGC–TOFMS analysis of the volatile profiles indicate that distinct chemical profiles were observed for each of the four *Bacillus* strains. Differences in the levels of volatile compounds in condiments produced by *Bacillus* starter cultures were observed. Both the concentrations of components and also the proportions of compounds in the condiments from different starter cultures differed significantly. Aldehydes, acids and ketones were identified as key volatile compounds. All *Bacillus* strains were able to produce 2– and 3–methylbutanoic acid in considerable amounts, *B. subtilis* subsp. *subtilis* SFBA3 fermentation was indicative of *dawadawa* aroma with the production of high levels of 2–methyl butanoic acid and 3–methyl butanoic acid in the finished fermented products but low levels were observed in the products manufactured using the other strains. This may suggest that this *Bacillus* strain has potential as a commercial starter culture for alkaline fermentation and production of *dawadawa* from Bambara groundnuts.

**4.3	Sensory quality of *dawadawa*–like African food condiments produced from alkaline fermentation of an underutilized legume: Bambara groundnut (*Vigna subterranea* L. *Verdc*)**

**4.3.1	Abstract**

Dawadawa is a West African traditional food condiment. It is a product of alkaline fermentation of legumes with characteristic pungent and ammoniacal flavour. It usually serves as flavour enhancer in soups or as a low-cost meat substitute. It is typically made from African locust bean (*Parkia biglobosa*) but production from other legumes for example Bambara groundnut (*Vigna subterranean* L. *Verdc*) has become of interest. The sensory properties of *dawadawa*-like condiments produced using *Bacillus subtilis* subsp. *subtilis* (strain SFBA3), *Bacillus amyloliquefaciens* subsp.   *plantarum*   (strain   SFBA2),   *B. licheniformis* (strain OALB2) and *B. cereus* (strain PALB7) for fermentation of Bambara groundnut were evaluated. The *Bacillus* fermented condiments and a control (a commercial bouillon cube) were evaluated in terms of aroma, overall aroma, flavour, aftertaste and colour. A trained descriptive sensory panel evaluated the sensory characteristics of the uncooked paste and cooked broths using 3 appearance, 9 aroma and 18 flavour descriptors. The intensities of ammoniacal, pungent, chocolate/cocoa, rancid and *dawadawa* aromas differed significantly amongst the condiments made using the different *Bacillus* starter cultures. Strain SFBA3 had the highest intensity of pungent and *dawadawa* aroma, while ammoniacal aroma were more pronounced in strains SFBA2 and PALB7. Strain OALB2 had the lowest intensity of *dawadawa* aroma. The intensity of beany and *dawadawa* flavours differed in the broths for the different condiment treatments. *B. cereus* PALB7 had the highest intensity of *dawadawa* flavour, *dawadawa* aftertaste, sweet aftertaste, overall flavour and overall aroma in the cooked broths. The control broth had higher intensities of meaty and umami flavours than the *Bacillus* fermented condiments. This research contributes to the understanding of sensory properties of *dawadawa*–like condiments from Bambara groundnut. *B. cereus* PALB7 was the starter culture providing the most intense *dawadawa*–like flavour profile.

4.3.2 Introduction

Traditional alkaline fermented African food condiments such as *dawadawa* are an integral part of the West and Central African diet due to their distinct aroma and flavour enhancing properties when added to soups and sauces (Odunfa, 1988; Omafuvbe *et al.*, 2000). *Dawadawa* is produced solely from the spontaneous alkaline fermentation (pH 7–9) of African locust beans (*Parkia biglobosa*) with *Bacillus* species highlighted as the predominant microbial species responsible (Campbell–Platt, 1980; Odunfa, 1988). Several West African countries have various names for the condiment which include; *dawadawa*, *iru* (Nigeria, Ghana), *soumbala* (Burkina Faso), *netetou* (Senegal, Mali), *sonru*, *iru* and *afitin* (Benin) (Azokpota *et al.*, 2007; Ouoba *et al.*, 2004). It is one of the most prominent condiments in the Savannah region of West and Central Africa where the African locust bean tree grows abundantly (Teklehaimanot, 2004). Bambara groundnut is cultivated and readily available; unlike African locust bean trees that grow in the wild requiring long hours to hunt for seeds that are only produced seasonally (Dakwa *et al.*, 2005).

Currently, other legumes, Bambara groundnut for instance, are being promoted in some West African countries as alternatives for making *dawadawa*. The choice of substrate for fermentation could play an important role on the product quality in terms of sensory properties and overall appeal. In *dawadawa* from African locust bean the following species have been found *B. amyloliquefaciens*, *B. atrophaeus*, *B. badius*, *B. cereus*, *B. firmus*, *B. fumus*, *B. licheniformis*, *B. megaterium*, *B. mojavensis*, *B. mycoides*, *B. pumilus*, *B. subtilis*, *B. sphaericus* and *B. thuringiensis* (Parkouda *et al.*, 2009; Ouoba *et al.*, 2004; Azokpota *et al.*, 2007). Previous studies have highlighted potential *Bacillus* starter cultures, which are members of the *B. subtilis* group that are capable of alkaline fermentation of Bambara groundnut (Chapter 4.1). The acids, aldehydes ketones and alcohols were the predominant volatile compounds produced by these *Bacillus* species during the production of *dawadawa*–like condiments from Bambara groundnut (Chapter 4.2).

Bambara groundnut (*Vigna subterraenea* L. *Verdc*) is an underutilized legume in Africa; which is usually classified as a poor man's food (Swanevelder, 1998). It is ranked as the third most important grain legume after groundnut (*Arachis hypogaea* L.) and cowpea (*Vigna unguiculata*) (Afoakwa *et al*., 2007). It has advantages over other legumes for its ability to grow in poor soils and superior drought tolerance. Widely grown in various parts of Africa, Bambara groundnut is a potential crop for combating malnutrition on the continent. Owing to its nutritional benefits, interest has shifted to other potential uses of Bambara groundnut such as milk, protein–rich composite meals and condiments (Poulter and Caygill, 1980; Barimalaa *et al*., 1994; Barimalaa *et al*., 2005). Microbial alkaline fermentation of legumes into African food condiments, *dawadawa* for example, have been highlighted to increase its protein levels and improve digestibility (Odunfa, 1988; Oboh, 2006).

However, there is still a dearth of knowledge on the descriptive sensory evaluation of *dawadawa* condiments and other African condiments in general. The sensory characteristics of alkaline fermented condiments made from Bambara groundnut have not been reported. The objectives of this study was to determine the effect of *Bacillus* strains on the sensory properties of the *dawadawa*–like condiments produced from alkaline fermented Bambara groundnut.

4.3.3 Materials and methods

4.3.3.1 *Dawadawa*–like condiments

Alkaline fermentation of Bambara groundnut using *B. subtilis* subsp. *subtilis* SFBA3, *B. amyloliquefaciens* subsp. *plantarum* SFBA2, *B. cereus* PALB7 and *B. licheniformis* OALB7 starter cultures previously isolated and characterised were used to manufacture 4 *dawadawa*–like condiments in triplicate on a laboratory-scale as described in section 4.1.3.6.

4.3.3.2 Descriptive Sensory evaluation

Preparation of condiments for evaluation

There were five (5) treatments, which were the 4 *dawadawa*–like condiments and a popular commercial bouillon cubed paste product used in West Africa served as a control. Condiments were presented to the sensory panellists in two forms, as an uncooked paste and as a cooked broth. The uncooked pastes (10 g portions) were presented for evaluation of appearance and aroma in 50 ml clear glass McCartney bottles with aluminium screw caps with a silicon rubber liner. For preparation of broths, 50 g of each paste were added to 450 ml of boiling water in stainless steel pots (2.5 L, 18–10 Edelstahl, Rostfrei, Prochef, San Diego, CA, USA) while stirring to have a homogenous broth. The broth was simmered for 15 min on low heat on a 2000W single plate stove (STA001, ANVIL, Germiston, South Africa). The broths (15 ml portions) were served in glass ramekins covered with aluminium foil and kept warm on a Salton warming tray (Salton House, Manchester, UK) at ± 50 ºC.

Sensory panel

Panellists, made up of persons from West African origin who are familiar with traditional *dawadawa* made from African locust bean, were screened for sensory acuity in terms of primary tastes – sweet, salt, sour, bitter and umami. A panel of thirteen evaluators were trained for 9 h following the generic descriptive analysis method described by Einstein (1990). During the training, each panellist described the differences between the five cooked and uncooked condiment samples during three sessions. Descriptive terms and scale anchors were developed, defined and agreed upon for evaluation (Table 4.5).

Descriptive analysis of uncooked and cooked condiments

The sensory evaluation of the condiments was conducted in a sensory evaluation laboratory with individual booths equipped with computers for direct data entry using Compusense five® Release 5.6 (Compusense, Guelph, Canada). Panellists evaluated all samples in triplicate over 3 days, with one session per day. Each panellist received five samples of the uncooked condiments on a white tray to evaluate the aroma and

appearance. After a 10 min break, in order to avoid fatigue, the cooked condiment broths were evaluated. Each panellist received the broths on a white tray, with five stainless steel teaspoons, a serviette and a foam polystyrene disposable cup filled with filtered tap water for rinsing the mouth before and between tasting the samples. Additionally, sliced raw carrots were supplied as palate cleanser. The order of sample presentation followed the Williams design. The panellists evaluated the cooked broths in terms of aroma, appearance, flavour and aftertaste using 30 descriptive terms (Table 4.5). Aroma was evaluated using short sniffs immediately after unscrewing the capped McCartney bottles or after removing the foil covers. A spoonful of the broth was sipped into the mouth to evaluate flavour. After swallowing, the panellists evaluated aftertaste. Structured line scales with ten demarcated points was used to measure the intensity of each attribute for a given sample. The minimum value was 0, denoting not perceived. The maximum point was 9, denoting strongly perceived.

4.3.4   Statistical analyses

The main and interaction effects of *Bacillus* strains and sample type (uncooked or cooked) was determined using analysis of variance (ANOVA) by the modelling data option (XLSTAT 2014 by AddinSoft™ SARL, Paris, France). Fisher least significant difference test (LSD) at 5% probability level ($p < 0.05$) was used to separate means. Principal component analysis (PCA) was conducted to simplify the multivariate description and allow for a visual interpretation of differences among condiments manufactured on three different occasions.

4.3.5   Results and Discussions

In the uncooked *dawadawa*–like condiments, 6 aroma and 2 appearance attributes described significant differences among the condiments (Table 4.6). The aroma attributes were pungent, *dawadawa* aroma, ammoniacal, chocolatey/cocoa, rancid and overall aroma intensities. The aroma of the control was much less pungent and the *dawadawa* aroma, characteristic ammoniacal aroma as well as rancid aroma were much less intense compared to the *dawadawa* condiments. The condiments based on SFBA3, SFBA2 and

OALB2 were generally more pungent with higher intensity of ammoniacal aroma compared to PALB7. The intensity of *dawadawa* aroma was significantly lower for OALB2 compared to SFBA3. Chocolatey/cocoa aroma was significantly higher in the control in contrast to the *Bacillus* fermented condiments. SFBA3 ranked highest for rancid aroma. In terms of overall aroma intensities, all the condiments were rated above 5 with the SFBA3 fermented condiment having significantly higher overall aroma compared to the condiment manufactured with PALB7 (Table 4.6) and the control not different to the *dawadawa*–like condiments. Condiments fermented with OALB2 and SFBA3 starter cultures were significantly browner, compared to condiments with more cream coloured SFBA2 and PALB7 and on par with the commercial product (control). Traditional African locust bean *dawadawa* made by spontaneous fermentation have been characterised as a dark brown coloured condiment with ammoniacal or pungent aroma (Beaumont, 2002; Odunfa, 1988). Ammonia is produced majorly during the metabolic activity of the bacteria in proteolysis of the legume proteins and utilisation of the released amino acids with a concomitant rise in pH (Allagheny *et al*., 1996; Owens *et al*., 1997).

Brown colour (browning) is generally associated with Maillard reactions resulting from degradation of amino acids and sugars during the heating process contributing to flavour and aroma generating reactions; this has characterised legume based fermented foods as well (Leejeerajumnean *et al*., 2001, Ouoba *et al*., 2005). Browning could be influenced by microbial enzymes, mainly the activities of carbohydrate-cleaving enzyme (β-glucosidase and α-amylase) which are precursors of reducing sugars and carbohydrate derivatives, while secondly, protease with its hydrolysis activity such as conversion of proteins to amino acids and small peptides, can also act as reactants to initiate the Maillard reaction. Wittanalai *et al*. (2012) reported increased browning in *Kapi*; a fermented soybean product from Thailand after fermentation with *B. subtilis* and *B. amyloliquefaciens* strains. The strains OALB2 and SFBA3 potentially contributed more microbial enzymes hydrolysing carbohydrates and proteins in Bambara groundnut, leading to more Maillard browning of the condiments. Amongst the cooked *dawadawa*–like and control broths, 6 aroma,

85

12 flavour and 2 appearance descriptors (Table 4.7) differentiated the boths. The broths differed significantly for all the aroma attributes except pungent, nutty and fishy. The pungent or ammoniacal aroma of *dawadawa* has been noted to mellow down with cooking (Beaumont, 2002); this was observed in this study as a 2-3 fold reduction in intensities of these attributes in the cooked *dawadawa*–like broths (Table 4.7) compared with the uncooked. *Dawadawa* aroma intensity was significantly different for all the *Bacillus* fermented cooked condiments with PALB7 having the highest intensity and the control the lowest. The intensity of ammoniacal, beany, chocolatey/cocoa and rancid flavours were not significantly different amongst the *Bacillus* fermented condiments but all were significantly more intense in comparison with the control boullion. In terms of flavour, the control was more salty, umami, sweet and meaty flavoured than the *Bacillus* fermented condiments. Broths SFBA2, PALB7 were more metallic than OALB2 and SFBA3 broths. *Dawadawa* flavour intensities have been linked to varying concentrations of 3–methyl butanoic acid in fermented African locust bean (Beaumont, 2002; Owens *et al*., 1997).

Table 4.5: Lexicon used to describe sensory characteristics *dawadawa*–like condiments made from alkaline fermentation of Bambara groundnut by *Bacillus* species

Attribute	Definition
Aroma (perceived orthonasally by opening the screw cap cover of bottle and sniffing the content)	
Overall Aroma	Intensity of aroma perceived by the olfactory sense
Pungent	Physically penetrating sensation in the nasal cavity. Sharp, irritant.
Dawadawa	The smell associated with traditional West African *dawadawa* condiment from fermentation of African locust bean (*Parkia biglobosa*)
Ammoniacal	Pungent, stale urine
Nutty	The non-specific nutlike flavours that are characteristic of several different nuts, e.g. peanuts, hazelnuts, pecans, almonds
Beany	Aromatics associated with cooked legumes, beans, peas, peanuts and soybean
Fishy	Smell of raw not fresh fish
Chocolatey/Cocoa	The characteristic aromatic of roasted cocoa beans
Rancid	The flavour associated with sour milk
Flavour (perceived during consumption of the products including retronasally perceived aromatics, basic tastes and trigeminal nerve interaction effects)	
Overall flavour	Intensity of flavour (pleasant or unpleasant) perceived in the palate during mastication
Salty	Salty a fundamental taste sensation of which sodium chloride is typical
Umami	Flat, salty and brothy flavour of a monosodium glutamate solution, a basic taste
Soy	Characteristic soybean odour strong in soymilk made from raw soybeans hydrated in cold water

Attribute	Definition
Metallic	Flavour of metal in the mouth
Meaty	The aromatic reminiscent of cooked red meat
Dawadawa	The flavour associated with traditional West African *dawadawa* condiment, a product from fermentation of African locust bean (*Parkia biglobosa*)
Peanut	The flavours that are characteristic of peanut butter spread
Sweet	Fundamental taste sensation of which sucrose is typical
Sour	Fundamental taste sensation of which lactic acid and citric acid are typical
Bitter	Fundamental taste sensation of which caffeine or quinine are typical
Aftertaste	
Overall aftertaste	Intensity of the flavour sensation which occurs after the elimination of the product and which differs from the sensations perceived whilst the product was in the mouth
Bitter aftertaste	Intensity of a lingering bitter taste
Beany aftertaste	Intensity of aftertaste associated with cooked legumes
Umami aftertaste	Umami flavour in the mouth after the condiment has been swallowed.
Dawadawa aftertaste	*Dawadawa* flavour in the mouth after the condiment has been swallowed.
Nutty aftertaste	Nutty flavour in the mouth after the condiment has been swallowed.
Sweet aftertaste	Intensity of a lingering sweet taste
Appearance	
Brown colour	Dark colour between red and yellow. Intensity of brown colour of *dawadawa*-like condiment
Cream colour	Intensity of cream colour of *dawadawa*-like condiment
Speckles	Mark with a large number of small spots or patches of colour

Amongst the *Bacillus* fermented *dawadawa*–like broths, SFBA2 had a significantly lower *dawadawa* flavour intensity correlating to the low levels of 3-methyl butanoic acid production reported for this strain (Chapter 4.2). The PALB7 condiment was significantly more bitter than the other broths. The bitterness could be associated with a higher presence of bitter peptides. Bitter peptides are associated with fermented foods such as cheese, soy sauce and *miso* caused by the hydrolytic activities of microbial proteases on the proteins in these foods (Maehashia and Huang, 2009).

The control had a significantly lower beany aftertaste but more intense umami, sweet and overall aftertaste than the *Bacillus* fermented broths. Sugawara (1985) reported that beany and green aroma in *natto* (Japanese food made from soybeans fermented with *Bacillus subtilis* var. *natto*) was attributed to the presence of hexanal and 1–octen-3–ol. These compounds were also identified in these *dawadawa*–like condiments (Chapter 4.2). Sweet aftertaste was significantly higher for the control. There was no significant difference for the umami and overall aftertaste among the *Bacillus* fermented broths, however, SFBA2 again had a lower *dawadawa* aftertaste. The control and OALB2 broths were browner than the other broths while SFBA2 and PALB7 were least brown.

Both the control and OALB2 broths had significantly lower cream colour than SFBA2, PALB7 and SFBA3 condiment broths. The PALB7 had the highest intensity of *dawadawa* aroma, overall aroma, overall flavour, *dawadawa* aftertaste and sweet aftertaste all of which makes it the condiment with characteristic properties probably most similar to the traditional *dawadawa*. Strain SFBA 3 is the closest to PALB7 in terms of intensities for *dawadawa*, *dawadawa* aftertaste and overall flavour followed by SFAB2.

Table 4.6: Sensory profiles of uncooked *dawadawa*–like condiments from alkaline fermentation of Bambara groundnut with four different *Bacillus* species starter cultures and a commercial bouillon as control

Descriptors	ANOVA	Control$_{(s)}$	*Bacillus* species starter cultures			
			SFBA2$_{(s)}$	PALB7$_{(s)}$	OALB2$_{(s)}$	SFBA3$_{(s)}$
Aroma						
Pungent	***	$1.6^d \pm 1.6$	$6.0^a \pm 2.3$	$4.3^c \pm 3.0$	$5.0^{bc} \pm 2.7$	$5.3^{ab} \pm 2.3$
Dawadawa	***	$0.7^c \pm 1.2$	$5.3^{ab} \pm 2.5$	$5.2^{ab} \pm 2.6$	$4.8^b \pm 2.5$	$5.9^a \pm 2.5$
Ammoniacal	***	$0.6^d \pm 0.7$	$4.9^a \pm 2.6$	$3.3^c \pm 2.8$	$3.7^{bc} \pm 2.8$	$4.2^{ab} \pm 2.3$
Nutty	NS	$1.9^a \pm 2.6$	$2.0^a \pm 2.3$	$2.4^a \pm 2.5$	$1.8^a \pm 1.9$	$2.2^a \pm 2.5$
Beany	NS	$1.2^b \pm 2.3$	$2.1^a \pm 2.6$	$2.4^a \pm 2.3$	$1.9^{ab} \pm 2.1$	$2.0^{ab} \pm 2.0$
Fishy	NS	$1.9^a \pm 2.5$	$2.2^a \pm 2.6$	$1.8^a \pm 2.0$	$2.5^a \pm 2.3$	$2.5^a \pm 2.6$
Chocolatey/cocoa	***	$2.8^a \pm 2.9$	$0.8^b \pm 1.2$	$1.0^b \pm 1.5$	$1.3^b \pm 1.7$	$1.0^b \pm 1.5$
Rancid	***	$0.6^c \pm 0.9$	$2.3^b \pm 2.6$	$2.0^b \pm 2.2$	$2.0^b \pm 2.3$	$3.5^a \pm 3.1$
Overall aroma	***	$6.0^{bc} \pm 2.4$	$6.7^{ab} \pm 1.6$	$5.7^c \pm 2.0$	$6.3^{abc} \pm 2.0$	$6.9^a \pm 1.7$
Appearance						
Brown colour	***	$7.4^a \pm 1.4$	$3.7^c \pm 1.8$	$3.6^c \pm 1.8$	$7.3^a \pm 1.8$	$6.1^b \pm 1.7$
Cream colour	***	$0.8^d \pm 1.3$	$4.7^a \pm 2.9$	$4.9^a \pm 2.8$	$1.7^c \pm 2.2$	$2.7^b \pm 2.3$
Speckles	*	$1.6^{ab} \pm 1.7$	$2.1^a \pm 2.3$	$1.4^b \pm 1.5$	$1.5^{ab} \pm 2.0$	$1.3^b \pm 1.5$

$_{(s)}$denotes uncooked solids; ***P value < 0.001;**P-value < 0.01, *P-value < 0.05, NS not significant, for a specific sensory property, mean values with different letters differ significantly (P < 0.05)
0–10; 0 is least intensity of attribute and 10 is high intensity

Table 4.7: Sensory profiles of cooked broths from *dawadawa*–like condiments from alkaline fermentation of Bambara groundnut with four different *Bacillus* species starter cultures and a commercial bouillon as control

Descriptors	ANOVA	Control	*Bacillus* species starter cultures			
		Control$_{(br)}$	SFBA2$_{(br)}$	PALB7$_{(br)}$	OALB2$_{(br)}$	SFBA3$_{(br)}$
Aroma						
Pungent	NS	1.5^{b} ±1.7	2.3^{a} ±2.3	2.1^{a}±1.9	2.1^{a} ±2.1	2.0^{ab} ±1.8
Dawadawa	***	0.8^{c} ±1.3	3.2^{b} ±2.4	4.2^{a} ±2.3	3.5^{ab} ±2.4	3.5^{ab} ±2.4
Ammoniacal	*	0.6^{b} ±1.2	1.4^{a}±1.6	1.5^{a} ±1.9	1.3^{a} ±1.6	1.2^{a} ±1.5
Nutty	NS	1.7^{a} ±2.4	1.7^{a} ±2.2	1.8^{a} ±2.1	1.4^{a} ±1.8	1.6^{a} ±2.2
Beany	**	1.2^{b} ±2.4	2.3^{a} ±2.3	2.9^{a} ±2.7	2.3^{a} ±2.3	2.6^{a} ±2.4
Fishy	NS	1.7^{ab} ±2.2	1.5^{ab} ±2.1	2.1^{a} ±2.2	1.2^{b} ±1.4	1.8^{ab} ±1.9
Chocolatey/cocoa	***	1.7^{a} ±2.3	0.6^{b} ±0.8	0.7^{b} ±1.0	0.7^{b} ±1.3	0.6^{b} ±0.8
Rancid	*	0.4^{b} ±0.6	1.2^{a} ±2.0	0.9^{a} ±1.3	0.8^{ab} ±1.1	0.9^{a} ±1.4
Overall aroma	***	5.7^{a} ±2.0	4.1^{c}±2.3	5.5^{ab} ±1.7	4.8^{bc} ±1.9	4.6^{c} ±1.8
Flavour						
Salty	***	5.5^{a} ±2.6	0.8^{b} ±1.2	1.3^{b} ±1.4	1.1^{b} ±1.7	1.0^{b} ±1.4
Umami	***	3.4^{a} ±3.3	1.1^{b} ±1.4	1.6^{b} ±2.1	1.4^{b} ±1.9	1.4^{b} ±1.7
Soy	NS	1.7^{a} ±2.6	1.2^{a}±1.7	1.6^{a}±2.1	1.4^{a} ±2.1	1.3^{a} ±1.7
Metallic	*	1.6^{ab} ±2.2	2.0^{a} ±2.7	1.9^{a} ±2.5	1.6^{ab} ±2.3	1.1^{b} ±1.4
Meaty	***	4.2^{a}±3.1	0.7^{b} ±1.0	1.4^{b} ±1.8	1.3^{b} ±2.0	0.9^{b}±1.2
Dawadawa	***	0.9^{c}±1.7	2.8^{b} ±2.5	4.0^{a} ±2.4	3.7^{a} ±2.7	3.6^{ab} ±2.7
Peanut	NS	1.0^{b} ±1.8	1.2^{ab} ±1.9	1.7^{a} ±2.4	1.3^{ab} ±1.8	1.5^{ab} ±2.1
Sweet	***	4.0^{a} ±2.7	0.9^{b} ±1.2	1.1^{b} ±1.4	1.0^{b}±1.4	1.0^{b} ±1.5
Sour	NS	1.3^{a} ±2.1	0.7^{b} ±1.0	0.9^{ab} ±1.5	0.8^{ab} ±1.1	0.8^{ab} ±1.2
Bitter	NS	0.7^{b} ±1.5	1.0^{ab} ±1.4	1.3^{a} ±1.7	1.0^{ab} ±1.7	1.0^{ab} ±1.5
Overall flavour	***	7.0^{a} ±1.6	3.2^{c} ±2.4	4.8^{b} ±1.9	4.5^{b} ±2.1	4.3^{b} ±2.1
Aftertaste						
Bitter aftertaste	NS	1.0^{a} ±1.8	1.2^{a} ±1.9	1.5^{a} ±1.8	1.4^{a} ±1.9	1.2^{a} ±1.6
Beany aftertaste	*	0.7^{b}±1.6	1.6^{a} ±2.3	2.3^{a} ±2.6	1.7^{a} ±2.0	1.7^{a} ±2.0

Table 4.7: Continued

Descriptors	ANOVA	Control	*Bacillus* species starter cultures			
		Control$_{(br)}$	SFBA2$_{(br)}$	PALB7$_{(br)}$	OALB2$_{(br)}$	SFBA3$_{(br)}$
Umami aftertaste	***	2.8^a ±2.9	0.8^b ±1.4	1.3^b ±1.7	1.3^b ±2.0	1.0^b ±1.4
Dawadawa aftertaste	***	1.0^c ±1.9	2.2^b ±2.1	3.8^a ±2.5	3.5^a ±2.5	3.4^a ±2.5
Nutty aftertaste	NS	1.1^b ±1.9	1.3^{ab} ±1.9	1.9^a ±2.6	1.7^{ab} ±2.3	1.5^{ab} ±2.0
Sweet aftertaste	***	3.6^a ±2.4	0.5^c ±0.7	1.3^b ±2.1	1.1^{bc} ±1.6	0.9^{bc} ±1.6
Overall aftertaste	***	5.8^a ±2.0	3.4^b ±2.3	4.1^b ±2.3	3.6^b ±2.5	3.6^b ±2.4
Appearance						
Brown colour	***	5.9^a ±2.4	1.0^d ±1.9	1.5^{cd}±1.6	4.5^b ±2.3	2.0^c ±1.8
Cream colour	***	1.5^c ±2.2	4.7^a ±3.0	5.1^a ±2.5	2.9^b ±2.2	5.4^a±2.1
Speckles	NS	1.2^a ±2.0	0.5^b ±0.7	0.7^{ab} ±1.1	1.1^a ±1.6	1.2^a ±2.0

$_{(br)}$ denotes cooked broth; ***P value < 0.001;**P-value < 0.01, *P-value < 0.05, NS not significant; For a specific sensory property, mean values with different letters differ significantly (P < 0.05)
0–10; 0 is least intensity of descriptor and 10 is intensity

The OALB2 strain had the least intense overall aroma, overall flavour, *dawadawa* aftertaste and sweet aftertaste suggesting it may be the least appropriate starter culture for the alkaline fermentation of Bambara groundnut into *dawadawa*–like condiments. By PCA, the first two PCs accounted for 82% of the total variation in the significant sensory properties of the condiments (Figure 4.6). PC1 explaining 74% of variation, separated the condiments according to differences in aroma, flavour and colour, with the PALB7, SFBA3 and SFBA2 fermented condiments to the left, while the OALB2 condiment is positioned in the centre and the control more to the right. The *Bacillus* fermented condiments were identified as more intense in terms of pungent, *dawadawa* and ammoniacal aroma descriptors. The PALB7, SFBA3 SFBA2 and OALB2 condiments were identified as more intense in terms of aroma, flavour and aftertaste descriptors for *dawadawa*, ammoniacal, bitter, rancid, beany, and cream colour compared to the control (Figure 4.6).
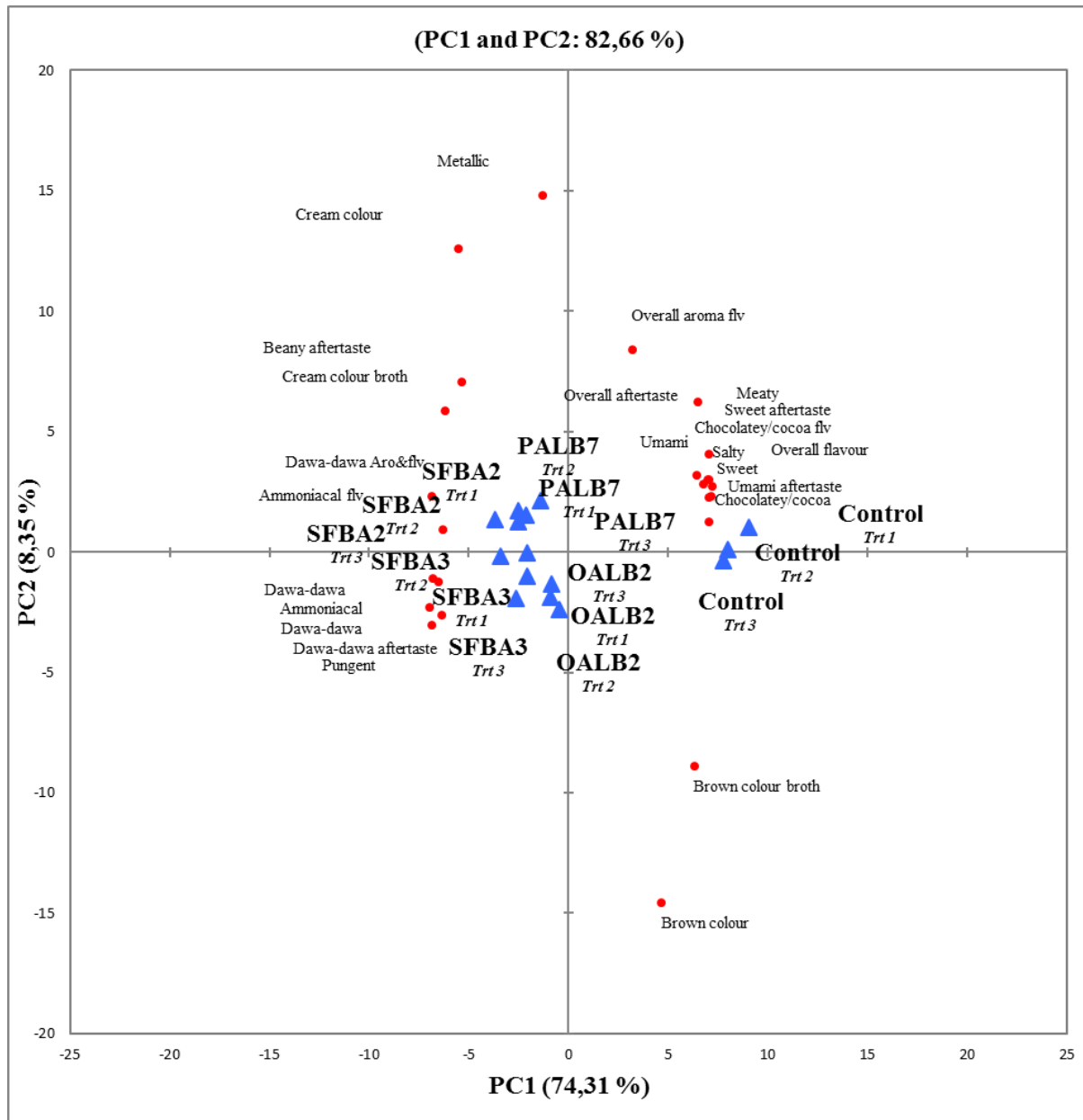
(PC1 and PC2: 82,66 %)

Figure 4.6: PCA of sensory properties of condiments prepared from alkaline fermentation of Bambara groundnut using different *Bacillus* starter cultures and a commercial bouillon as control: Plot of PC1 and PC2 scores of sensory properties in terms of in terms of taste, flavour, aroma and visual attributes. Trt 1, 2 and 3 represents the replicates of each *Bacillus* fermented Bambara groundnut.

The control on the right of the plot showed intense umami, chocolatey/cocoa (aroma, flavour and aftertaste), meaty, sweet, salty and more brown colour. The chocolatey/cocoa aroma attribute on the right of the plot shows the control was generally perceived as completely different from the fermented condiments. Condiments to the left of the plot were more cream coloured while the colour of the condiments towards the right darker brown in colour. PC2 explained the additional 8% variation separating the condiments according to differences in *dawadawa* aroma, ammoniacal aroma, overall aroma, overall flavour, umami and brown colour. The cream coloured condiments are positioned at the top and brown coloured condiments towards the bottom. On PC2, most attributes were found in the top part of the plot meaning these attributes are the major descriptors of these condiments (Figure 4.6).

The intensity of ammoniacal, pungent, chocolate/cocoa, rancid and *dawadawa*–like aromas were significantly different among the condiments. The intensity of beany and *dawadawa* flavours differed in the broths for the different *Bacillus* starter cultures. There is a strong positive correlation between pungent, ammoniacal and *dawadawa* for both the uncooked pastes and cooked broths with SFBA2 having the highest intensity of pungent (Tables 4.6 and 4.7). It was reported that ammoniacal or pungent aroma are the main aroma descriptor for *dawadawa* condiments from alkaline fermented African locust bean (Odunfa, 1988).

The level of ammonia odour determines the acceptability to consumers of *dawadawa* from African locust bean (Allagheny *et al*., 1996). There are levels where the ammonia aroma might be objectionable depending on consumers' preference. However, the threshold level of ammonia before it becomes unpleasant or objectionable has never been reported.

4.3.6 Conclusion

The sensory properties of *Bacillus* alkaline fermented Bambara groundnut into *dawadawa*–like condiments suggests these condiments were most probably comparable to traditional *dawadawa*. A lexicon that describe the *dawadawa*–like condiments were reported for the first time. Significant sensory differences were apparent between condiments made using the different *Bacillus* starter cultures. It is recommended that the selective use of *B. subtilis* subsp. *subtilis* (strain SFBA3), *B. amyloliquefaciens* subsp. p*lantarum* (strain SFBA2) and *B. cereus* (strain PALB7) starter cultures could produce acceptable *dawadawa*–like condiments made with Bambara groundnut.

5.0   GENERAL DISCUSSION

This general discussion will focus on a critical review of the methodologies applied in this research; discuss the findings of the effects of molecular typed *Bacillus* strains as starter cultures for alkaline fermentation of Bambara groundnut and volatile compounds formed in the *dawadawa*–like condiments associated with the strains. In addition, the sensory properties of the *dawadawa*–like condiments produced will be discussed. Lastly, future research ideas to explore the potential of *Bacillus* starter cultures for *dawadawa*–like condiments are proposed.

5.1   Methodological considerations

The presumptive identification of the *Bacillus* species associated with *dawadawa* condiments made from spontaneous fermentation of the traditional substrate African locust bean and Bambara groundnut were determined as a starting position for potential starter culture selection. Phenotypic characterization such as cellular morphology, Gram staining, catalase reaction and endospore formation testing of the *Bacillus* isolates were performed (Claus and Berkeley, 1986). Phenotypic characteristics were formerly considered suitable for the typing of individual strains within a species, however, it has been recognized for a long time that the genus *Bacillus* is phenotypically heterogeneous (Claus and Berkeley, 1986; Priest *et al.*, 1988).

The tentative *Bacillus* isolates were then identified using Matrix Assisted Laser Desorption/Ionization Time–of–Flight Mass Spectrometry (MALDI-TOF MS). The dendrogram was constructed in order to determine the genetic diversity among the dominant *Bacillus* species in the *dawadawa*. The comparison was done in order to firstly differentiate the *B. cereus* and *B. subtilis* complexes, furthermore, to delineate the diverse species of the *B. subtilis* complex present in the fermentation. In this study, *Bacillus* species were fully differentiated by MALDI-TOF MS, however, there were constraints to *Bacillus* identification to subspecies or strain level. Similar discrepancies in identifying closely related species were reported by other authors (Pavlovic *et al.*, 2013; Zeller–Péronnet *et al.*, 2013). Lack of adequate databases for strain

comparison or inadequate method optimization steps for the MALDI–TOF MS have been proposed as the cause of the observed limitation (Balážová *et al*., 2014; Šedo and Zdráhal, 2016).

Genotypic GTG$_5$ PCR fingerprinting of the selected isolates of the *B. subtilis* group showed varied genetic diversity with clustering into 5 groups. The essence of the fingerprints was to show underpinned genetic variations between the species of the *B. subtilis* group (Da Silva *et al*., 1999; Herman and Heyndrickx, 2000). The genetic variations were confirmed with 16S rDNA sequences and phylogenetic analysis. *Bacillus subtilis* complex are known to be genetically diverse and the limitation of 16S rDNA was the inability to differentiate closely related species in the *B. subtilis* group (Rooney *et al*., 2008; Kubo *et al*., 2011). However, this limitation was overcome by the sequencing of the protein coding DNA gyrase subunit A (*gyr*A) gene (Chun and Bae, 2000). The *gyr*A gene was efficient for both species and subspecie differentiation in the *B. subtilis* group especially for *B. subtilis* subsp. *subtilis* and *B. amyloliquefaciens* subsp. *plantarum*. Ideally, sequencing of other protein coding genes such as RNA polymerase subunit B (*rpo*B), phosphoribosylaminoimidazolecarboxamide formyltransferase (*pur*H), DNA polymerase III sub-unit alpha (*pol*C) and heat-shock protein (*gro*EL) (Rooney *et al*., 2008) would have been appropriate for *Bacillus* strain typing, however, *gyr*A sequencing showed high differentiating power for the selected species of the *B. subtilis* group. The combination of MALDI TOF MS and *gyr*A gene sequencing for the *B. subtilis* group species exhibited the differentiating power needed for potential *Bacillus* starter culture selection. Bambara groundnut fermentation into *dawadawa*–like condiments using *Bacillus* species starter cultures was carried out on a pilot scale. Soaking of Bambara groundnut seeds at 24 °C for 24 h in a water bath was to limit microbial enzyme activity produced by microorganisms naturally present in the seeds during soaking. Soaking of legumes for more than 24 h has been reported to lead to microbial enzyme activation and fermentation (Kayitesi *et al*., 2013). Cooking of dehulled cotyledons for 15 min in boiling water was carried out to prevent the disintegration of the cotyledons at a longer cooking time as also recommended by other authors (Barimalaa *et al*., 1994). Although survival of sporeformers could be possible in the boiled

cotyledons, sterilisation of the cotyledons in the autoclave at either 110 °C or 121 °C for 10 min resulted in the disintegration of the cotyledons. Wrapping of boiled cotyledons was performed in perforated polyethylene bags as opposed to the use of banana leaves in the natural fermentations. This was done in order to minimise contamination and the perforations on the bags were to ensure oxygen transfer for the microorganisms during fermentation. The highest alkaline final pH recorded for all of the *Bacillus* species starter cultures was pH 8.5 at 96 h. This is in corroboration with the final pH 7.5–9 range of *Bacillus* species alkaline fermented from Africa and Asia (Parkouda *et al*., 2009).

Volatile compounds extracted from *dawadawa*–like condiments were analysed using headspace solid phase microextraction (SPME) while the microscale steam distillation extraction (Likens–Nikerson method) was used for volatile compounds in *dawadawa* from African locust bean by previous authors (Ouoba *et al*., 2005; Azokpota *et al*., 2008; Azokpota *et al*., 2010). Headspace SPME method was used as a non-destructive, non-invasive, sampling technique to collect volatiles concentrated in the headspace that yields ''true" aroma profiles unlike the Likens–Nikerson distillation method in which artefact formation is noticeable in the GC–MS data (Kataoka *et al*., 2000). A comprehensive two–dimensional gas chromatography time-of-flight mass spectrometry (GC × GC–TOFMS) used in this study is reputed for high-resolution detection of co-eluting compounds (Chin *et al*., 2011). The volatile compounds profile of the *dawadawa*–like condiment in this study was more comprehensive than that reported by previous authors; this is in correlation with the improved volatile compounds identification with GC × GC TOFMS analysis in other food samples (Whitener *et al*., 2016; Göğüş *et al*., 2011). The volatile compounds identification from the MS spectra was based on the NIST library database and semi–quantification was by the use of eucalyptol internal standard. This was because it would have been extremely expensive to get pure analytical reference standards for all the volatile compounds identified.

Descriptive sensory analysis of *dawadawa*–like condiments was performed to characterize attributes that describe the condiments. Lexicons to uniquely describe the *dawadawa* condiments were developed. The

descriptive analysis not only investigated the sensory attributes of the *Bacillus* fermented condiments in dry and wet prepared forms, it also compared these condiments with a commercial boullion cube as reference standard. Comparison with traditional *dawadawa* from African locust bean would have been of value, however, South African custom regulations (where the research was conducted) prohibits African locust bean seed importation from West Africa which is the geographical location of most African locust bean trees. Some authors evaluated *dawadawa* condiments made from fermented African locust bean or soybean in tomato stew only or accompanied with a rice meal (Ouoba *et al*., 2005; Terlabie *et al*., 2006). However, our evaluation of *dawadawa*–like condiments as a cooked broth (i.e. dissolved in boiling water) was to give the actual attributes of the condiments without the influence of other meal ingredients or natural flavour enhancing substances such as salts and lipids found in stews.

The sensory panel for the evaluation of *dawadawa* consisted of persons from West Africa. This is because people from South Africa (where the research was conducted) are not familiar with the condiments since it has not been part of their regular diet. Furthermore, the panellists were to use their memory of the sensory properties of traditional *dawadawa* to evaluate *dawadawa*–like condiments made from Bambara groundnut. The descriptive sensory analysis was conducted with limited sensory training and experience because of practical time constraints and because the target group (West Africans) were expected to use prior knowledge of the product to judge.

5.2   Potential of *dawadawa*–like African food condiment production from Bambara groundnut using *Bacillus* starter cultures and transfer of technology to local communities (Commercialization)
The feasibility studies of alkaline fermentation of Bambara groundnut into *dawadawa*–like condiment using molecular typed *Bacillus* species starter cultures was indicative of potential use of the substrate. Bambara groundnut is a more available substrate for production than African locust bean both generally in Africa and in South Africa where this research was conducted. Although it differed considerably in composition to the latter. The availability of the Bambara groundnut seeds is an added advantage to the producers of

dawadawa because it saves time used in hunting for African locust bean in the wild. Compositional differences highlighted are higher crude protein (26%–47%) and lipids (31%–43%) contents in African locust bean compared to Bambara groundnut which had crude protein (16%–22%) and lipids (6.0–9.7%) content, respectively (Campbell-Platt, 1980; Brough and Azam-Ali, 1992; Adebowale *et al*., 2011; Yusuf *et al*., 2008). These could be the reasons for variations in the final product in terms of sensory quality. In addition, the protein content of the substrate would be a major determination of the extent to which proteolytic activity would be carried out by the *Bacillus* species. Notable differences have been found in *dawadawa* made from African locust bean and soybean, with the latter having shorter shelf-life and less appeal to consumers (Kolapo *at al*., 2008). The effect of substrate pre-conditioning, for example, roasting of soybean before fermentation gave a condiment that was similar to the traditional condiment made from African locust bean (Dakwa *et al*., 2005). In chapter 2, it was established that *B. cereus* PALB7, *B. amyloliquefaciens* subsp. *plantarum* SFBA2, *B. subtilis* subsp. *subtilis* SFBA3 and *B. licheniformis* OALB2 have the ability to grow on Bambara groundnut. The diversity of genotypically closely related *Bacillus* strains was investigated with relations to their phenotypic traits and metabolic characteristics. The ability of the *Bacillus* strains to utilize complex carbohydrates was investigated in the phenotypic tests; however, ability to utilize complex sugars in the Bambara groundnut substrate was not further studied. The *B. cereus* PALB7 strain in the controlled fermentation was highlighted to have the highest growth rate and alkaline pH at the short time of fermentation of 48 h which could be attributed to its ability to utilise complex sugars and protein in the substrate better than the other strains. Studies of complex sugars utilisation in Bambara groundnut by the individual typed *Bacillus* species starter cultures would be valuable. The close phylogenetic affiliation of *B. subtilis* sub *subtilis* SFBA3 with the Japanesse *natto* strain *B. subtilis* subsp. *natto* MBS04-6 could infer that there are inherent genetic attributes in the SFBA3 strain to produce condiments which would have high nutritional and sensory quality similar to *natto* produced from strain MBS04-6.

The volatile compounds identified in the *dawadawa*–like condiments in this study were in correlation with that obtained in traditional *dawadawa* from African locust bean (Azokpota *et al*., 2010; Ouoba *et al*., 2005). Aldehydes, acids, alcohols and ketones were the key volatile compounds produced by all the molecular typed *Bacillus* starter cultures (Table 4.2.1). Volatile compounds produced at high concentrations by the four *Bacillus* starter cultures were benzaldehyde, hexanal, 2–methylbutanoic acid, 3–methylbutanoic acid, ethanol, benzyl alcohol, 1 octen–3–ol, acetophenone, tetramethylpyrazine, trimethylpyrazine and 2,5–dimethylpyrazine. The volatile compounds profile of *dawadawa*–like condiments from this study differed from the traditional *dawadawa* from African locust bean most significantly in terms of the low level of pyrazines reported in this study (Ouoba *et al*., 2005; Azokpota *et al*., 2008; Azokpota *et al*., 2010, Onyenekwe *et al*., 2012). Pyrazines are typical aroma components of heated food to which they give a characteristic roasted or nutty flavour (Owens *et al*., 1997). Metabolic activities of microorganisms on protein dense substrates generally generate various precursors such as amino acids, monosaccharides and ammonia needed for the formation of pyrazines, then pyrazine formation is by non-enzymatic reaction such as heating (Owens *et al*., 1997). The level of pyrazines in *dawadawa*–like condiments from Bambara groundnut could possibly be increased by roasting of the seeds as a pre-conditioning step before dehulling, cooking and fermentation. Amongst the *Bacillus* starter cultures significant differences in volatile compounds profiled ensued; distinguishingly, *B*. *subtilis* subsp. *subtilis* SFBA3 produced acetic acid which imparts a sour, vinegar note (Zhang *et al*., 2014; Zhao *et al*., 2011); 3-methyl butanoic acid is described as acidic, sour, pungent, fruity, stinky, ripe fatty and fruity notes (Park *et al*., 2013) and diverse esters. Esters have been known to constitute a major group of volatile compounds in African fermented condiments. The esters are presumably the consequence of chemical reactions between microbial acidic and alcoholic metabolites (Leejeerajumnean *et al*., 2001). *B*. *cereus* PALB7 produced benzaldehyde which is generally responsible for a pleasant, sweet, aromatic note; and hexanal which gives a green odour (Mahattanatawee *et al*., 2014; Zhang *et al*., 2014; Zhao *et al*., 2011). *B*. *licheniformis* OALB2 was dominated by acetoin usually characterized as having a butter-like aroma (Owens *et al*., 1997). *B*. *amyloliquefaciens* SFBA2

produced dimethyl disulphide, reported to have strong pungent odours in traditional *dawadawa* and they have a great influence on overall product aroma intensity.

Although, there have been no formal report on the descriptive sensory analysis of the traditional *dawadawa* condiment, the general perception has been a dark brown coloured condiment with ammoniacal or pungent aroma (Beaumont, 2002; Odunfa, 1988). Ammonia is produced majorly during the metabolic activity of the bacteria in proteolysis of the legume proteins and utilisation of the released amino acids with a concomitant rise in pH (Allagheny *et al*., 1996; Owens *et al*., 1997). The descriptive sensory analysis of *dawadawa*–like condiments from Bambara groundnut gives a baseline for describing attributes pertinent to consumer perception and acceptance of *dawadawa* in general. In this study, both *B. subtilis* subsp. *subtilis* SFBA3 and *B. cereus* PALB7 were highlighted to have the most intense aroma, flavour and aftertaste rating for *dawadawa* (Table 4.3.2). This is due to appreciable levels of 2-methylbutanoic acid 3-methylbutanoic acid produced by both *Bacillus* strains. Beaumont (2002) reported on studies conducted in support of a US patent for flavourant composition prepared by fermentation (Heyland *et al*., 1995) suggesting a correlation between *dawadawa* aroma and the presence of 2-methyl butanoic acid and 3-methyl butanoic acid in the finished fermented protein base. The 2- methylbutanoic acid is produced from branched-chained amino acids such as valine, leucine, and isoleucine during fermentation via catabolism processes, including oxidation and transamination (Park *et al*., 2013). It is noteworthy that varying levels of 2-methylbutanoic acid and 3-methylbutanoic acid with their accompanying esters were produced by all the *Bacillus* starter cultures. *B. amyloliquefaciens* SFBA2 was typical of the pungent and ammoniacal aroma which could be attributed to the high levels of dimethyl disulphide produced. The *B. licheniformis* OALB2 had a mellowed down overall flavour of *dawadawa*. This suggests the limited ability of the strain on either the substrate utilisation or fermentation potential. The *dawadawa* aroma from *Bacillus* fermented legumes has not been fully characterised, yet the volatile compounds reported here is only an indication of several compounds that may be contributing to the perceived pungent, ammoniacal and putrid aroma. Generally, it is known

that volatile compounds identified by instrumental analysis are not necessarily drivers for perceived aroma or flavour (McGorrin, 2002). Therefore, due to the complexity of volatile compounds and flavour perception, this study endeavours to link both based on suggested literature. More work is required in identifying correlations of specific volatile compounds that classify *dawadawa* aroma in particular.

Based on the findings in this study, it was observed that the use of *Bacillus* species starter cultures produced *dawadawa* condiments in which the growth of pathogenic microorganisms was inhibited and the quality of the final product improved. It would be recommended to select *B. subtilis* subsp. *subtilis* SFBA3 and *B. amyloliquefaciens* subsp. *plantarum* SFBA2 as potential starter cultures for use by local entrepreneurs or industries involved in the production of *dawadawa* from Bambara groundnut on a medium to large scale. The *B. cereus* PALB7 strain had a shorter fermentation time and sensory quality, it should be noted that *B. cereus* does not have the generally regarded as safe (GRAS) status, although not all *B. cereus* strains possess toxigenic genes (Stenfors Arnesen *et al*., 2008). However, *B. cereus* had been selected as starter culture for African locust bean fermentation into *dawadawa* with satisfactory product quality (Okanlawon *et al*., 2010). The greater availability of Bambara groundnut over African locust bean would be a great advantage for setting up a profit driven and cost effective *dawadawa* processing factory.

5.3   Future research

This study identified potential starter cultures which are from the *B. subtilis* group namely *B. subtilis* subsp. *subtilis* SFBA3, *B. amyloliquefaciens* SFBA2 and *B. licheniformis* OALB2 while *B. cereus* PALB7 from the *B. cereus* group. Starter cultures are selected on the basis of their genetic diversity into the two major groups *B. subtilis* and *B. cereus* groups. The test for the presence of toxigenic genes in these strains must be ascertained, some of these *Bacillus* species have been implicated in food poisoning mostly *B. cereus* and *B. licheniformis*. To ensure food safety of the *dawadawa*–like condiments produced using these *Bacillus* strains, it would be important to test that the strains do not possess toxigenic traits and production of toxins is absent.

The use of Bambara groundnut as a suitable substrate for *dawadawa*–like condiments was established, however, the effect of further pre-processing steps such as roasting of the seed before fermentation as was reported for soybean (Dakwa *et al*., 2005) needs to be investigated to improve flavour and taste of the *dawadawa*–like condiments. *Dawadawa* condiment is solely made from African locust bean as it is known in West and Central Africa. It would be of value to compare the sensory analysis of *dawadawa*–like condiments made from Bambara groundnut with the traditional *dawadawa* from African locust bean as reference standard.

The analysis of volatile compounds using GC × GC TOFMS could be used as benchmark for the volatile compounds from *dawadawa* and it could be compared with other *dawadawa* condiments; this can find applications in food quality and safety to ascertain if the condiment is of the right quality. This can be used in predicting food flavour and off-flavours in *dawadawa* condiments. The metabolic activities of the *Bacillus* starter cultures on the Bambara groundnut and the resultant metabolic products need to be investigated. The use of high-throughput techniques such as phenotypic microarrays to study the metabolic pathways of the *Bacillus* strains and the metabolites produced during alkaline fermentation would be of value.

6.0   CONCLUSIONS AND RECOMMENDATIONS

This study showed that certain *Bacillus* species associated with the traditional African food condiment *dawadawa* possess desirable fermentation and volatile compounds production attributes, hence become potential starter cultures. The *Bacillus* species that were characterized in this study were isolated from naturally fermented *dawadawa* condiments and are likely to be safe choice as starter cultures. The selected *Bacillus* species would improve food safety and quality of the *dawadawa* condiments. The use of Bambara groundnut as an alternative substrate to African locust bean eliminates the cumbersome search for African locust bean in the wild; since Bambara groundnut widely cultivated in Africa and it is readily available. Bambara groundnut as substrate reduces the extended cooking time of 12 h or more for first cooking and 1-2 h of second cooking with African locust bean, thus, saving time and economic resources.

This study highlighted the volatile compounds produced by the *Bacillus* species from the alkaline fermentation of Bambara groundnut and its implication on the sensory quality of the *dawadawa*–like condiments. The volatile compounds of the *dawadawa*–like condiments from Bambara groundnut are reported for the first time in this study. The profiles of the volatile compounds produced are comparable to that from the traditional *dawadawa* from African locust bean. However, the identification of the most suitable *Bacillus* strains for commercial production of *dawadawa* from both Bambara groundnut and African locust bean would be of value.

The value of this research lies in the fact that it is the first report of a formal scientifically developed description of the sensory properties of the *dawadawa* condiments from Bambara groundnut under controlled fermentation with *Bacillus* starter cultures. The lexicon and methodology developed can serve as the baseline for comparison with traditional *dawadawa* from African locust bean and for studies on commercial optimisation of *dawadawa* flavour.

Finally, application of the potential *Bacillus* starter cultures for the household and medium scale production of *dawadawa* is very limited. Therefore, the commercial use of these *Bacillus* species characterised in this study could be encouraged amongst local producers of *dawadawa* to improve product safety. The *Bacillus* strains could be developed into freeze dried starter culture similar to the *B. subtilis* var. *subtilis* of the Japanesse *natto*.

7.0   REFERENCES

Adebowale, Y. A., Schwarzenbolz, U. and Henle, T. (2011). Protein isolates from Bambara groundnut (*Voandzeia subterranean* L.): Chemical characterization and functional properties. *International Journal of Food Properties*, 14, 758–775.

Aderibigbe, E.Y. and Odunfa, S.A. (1990). Growth and extra cellular enzyme production by strains of *Bacillus* species isolated from fermenting African locust bean, *iru*. *Journal of Applied Bacteriology*, 69, 662–671.

Adewunmi, G.A., Oguntoyinbo, F.A., Keisam, S., Romi, W. and Jeyaram, K. (2013). Combination of culture-independent and culture dependent molecular methods for the determination of bacterial community of *iru*, a fermented *Parkia biglobosa* seeds. *Frontiers in Microbiology*, 3, 1–9.

Achi, O.K. (1992). Microorganisms associated with natural fermentation of *Prosopis africana* seeds for the production of *okpiye*. *Plant Foods for Human Nutrition*, 42, 297–304.

Achi, O.K. (2005) Traditional fermented protein condiments in Nigeria. *African Journal of Biotechnology*, 4, 1612–1621.

Afoakwa, E. O., Budu, A. S., and Merson, A. B. (2007). Application of response surface methodology for optimizing the pre-processing conditions of Bambara groundnut (*Voandzeian subterranea*) during canning. *International Journal of Food Engineering*, 2, 1–19.

Ahaotu, I., Anyogu, A.,Njoku, O.H., Odu N.N., Sutherland, J.P. and Ouoba L.I.I. (2013). Molecular identification and safety of *Bacillus* species involved in the fermentation of African oil beans (*Pentaclethra macrophylla* Benth) for production of Ugba. *International Journal of Food Microbiology*, 162, 95–104.

Alabi, D.A., Akinsulire, O.R. and Sanyaolu, M.A. (2005). Qualitative determination of chemical and nutritional composition of *Parkia biglobosa* (Jacq.) Benth. *African Journal of Biotechnology*, 4, 812–815.

Allagheny, N., Obanu, Z.A., Campbell-Platt, G. and Owens, J.D. (1996). Control of ammonia formation during *Bacillus subtilis* fermentation of legumes. *International Journal of Food Microbiology*, *29*, 321–333.

Amadi, E.N., Barimalaa, I.S. and Omosigho, J. (1999). Influence of temperature on the fermentation of Bambara groundnut (*Vigna subterranea*) to produce a *dawadawa*-type product. *Plant Foods for Human Nutrition*, *54*, 13–20.

Amarteifio, J.O., Tibe O. and Njogu R.M. (2006). The mineral composition of Bambara groundnut (*Vigna subterranea* (L) Verdc) grown in Southern Africa. *African Journal of Biotechnology*, 5, 2408–2411.

Amoa-Awua, W.K., Terlabie, N.N., and Sakyi-Dawson, E. (2006). Screening of 42 *Bacillus* isolates for ability to ferment soybeans into *dawadawa*. *International Journal of Food Microbiology*, 106, 343–347.

Antai, S.P. and Ibrahim M.H. (1986). Microorganisms associated with African locust bean (*Parkia filicoidea* Welw) fermentation for *dawadawa* production. *Journal of Applied Microbiology*, 61, 145–148.

Aykroyd, W. R. and Doughty, J. (1982). Legumes in human nutrition. Food and Agriculture Organization of the United Nations. *FAO Food Nutrition Papers*, *20*, 1–152.

Azokpota, P., Møller, P.L., Houghouigan, J.D. and Jakobson, M. (2007). Biodiversity of predominant *Bacillus* isolated from *afitin*, *iru* and *sonru* at different fermentation time. *International Journal of Biological and Chemical Sciences*, 1, 211–222.

Azokpota, P., Hounhouigan, J.D., Annan, N.T., Nago, M.C. and Jakobsen M. (2008). Diversity of volatile compounds of afitin, iru and sonru, three fermented food condiments from Benin. *World Journal of Microbiology and Biotechnology*, *24*, 879–885.

Azokpota, P., Hounhouigan, J.D., Annan, N.T., Odjo, T., Nagoa, M.C. and Jakobsen M. (2010). Volatile compounds profile and sensory evaluation of Beninese condiments produced by inocula of *Bacillus subtilis*. *Journal of the Science of Food and Agriculture*, *90*, 438–444.

Balážová, T., Šedo, O., Štefanić, P., Mandić-Mulec, I., Vos, M. and Zdráhal, Z. (2014). Improvement in *Staphylococcus* and *Bacillus* strain differentiation by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry profiling by using microwave-assisted enzymatic digestion. *Rapid Communications in Mass Spectrometry*, 28, 1855–1861.

Bamshaiye, O. M., Adegbola, J. A. and Bamishaiye, E. I. (2011). Bambara groundnut: an under-utilized nut in Africa. *Advances in Agricultural Biotechnology*, 1, 60–72.

Barimalaa, I.S., Achinewhu, S.C., Yibatama, I. and Amadi, E.N. (1994). Studies on the solid substrate fermentation of Bambara groundnut (*Vigna subterranea* (L) Verdc). *Journal of the Science of Food and Agriculture*, 66, 443–446.

Barimalaa, I.S., Agoha, G., Oboh, C.A. and Kiin-Kabari, D.B. (2005). Studies on Bambara groundnut flour performance in *Okpa* preparation. *Journal of the Science of Food and Agriculture*, 85, 413–417.

Bau, H., Villaume, C., Nicolas, J.-P. and Mejean, L. (1997). Effect of germination on chemical composition, biochemical constituents and antinutritional factors of soya bean (*Glycine max*) seeds. *Journal of the Science of Food and Agriculture*, 73, 1–9.

Beaumont, M. (2002). Flavouring composition prepared by fermentation with *Bacillus* spp. *International Journal of Food Microbiology*, **75**,189–196.

Brough, S.H. and Azam-Ali, S.N. (1992). The effect of soil moisture on proximate composition of Bambara groundnut (*Vigna subterranea* (L.) Verdc). *Journal of the Science of Food and Agriculture*, 60, 197–203.

Brough S.H., Azam-Ali, S.N. and Taylor A.J. (1993). The potential of Bambara groundnut (*Vigna subterranea*) in vegetable milk production and basic protein functionality systems. *Food Chemistry*, 47, 277–283.

Campbell–Platt, G. (1980). African locust bean (*Parkia* species) and its West African fermented food product, *dawadawa*. *Ecology of Food and Nutrition*, 9, 123–132.

Chin, S.-T., Eyres, G.T. and Marriott P.J. (2011). Identification of potent odourants in wine and brewed coffee using gas chromatography–olfactometry and comprehensive two-dimensional gas chromatography. *Journal of Chromatography A*, *1218*, 7487–7498.

Chun, J. and Bae, K.S. (2000). Phylogenetic analysis of *Bacillus subtilis* and related taxa based on partial *gyr*A gene sequences. *Antonie van Leeuwenhoek*, 78, 123–127.

Claus, D. and Berkeley, R.C.W. (1986). Genus *Bacillus* Cohn 1872. In: Sneath PHA, Mair NS, Sharpe ME, and Holt JG (eds), *Bergey's Manual of Systematic Bacteriology*, Vol. 2. Williams and Wilkins, Baltimore, Maryland, pp 1105–1140.

Cutting, S.M. (2011). *Bacillus* probiotics. *Food Microbiology*, 28, 214–220.

Daffonchio, D., Borin, S., Frova, G., Manachini, P.L. and Sorlini, C. (1998). PCR fingerprinting of whole genomes: the spacers between the 16S and 23S rRNA genes and of intergenic tRNA gene regions reveal a different intraspecific genomic variability of *Bacillus cereus* and *Bacillus licheniformis*. *International Journal of System Bacteriology*, 48, 107–116.

Dakwa, S., Sakyi-Dawson, E., Diako, C., Annan, N.T. and Amoa-Awua, W.K. (2005). Effect of boiling and roasting on the fermentation of soybeans into soy–*dawadawa*. *International Journal of Food Microbiology*, 104, 69–82.

Da Silva, K.R.A., Rabinovitch, L. and Seldin, L. (1999). Phenotypic and genetic diversity among *Bacillus sphaericus* strains isolated in Brazil, potentially useful as biological control agents against mosquito larvae. *Research Microbiology*, 150, 153–160.

De Clerck, E. and De Vos, P. (2004). Genotypic diversity among *Bacillus licheniformis* strain from various sources. *FEMS Microbiology Letters*, 231, 91–98.

Dike, E.N. and Odunfa, S.A. (2003). Microbiological and biochemical evaluation of fermented soyabean product – soy*dawadawa*. *Journal of Food Science and* Technology, 40, 606–610.

Dirar, H. A. (1984). Kawal, meat substitute from fermented *Cassia obtusifolia* leaves. *Economic Botany*, 38, 342–349.

Dirar, H. A., Harper, D.B., and Collins, M.A. (1985). Biochemical and microbiological studies on Kawal, a meat substitute derived by fermentation of *Cassia obtusifolia* leaves. *Journal of the Science of Food and Agriculture*, 36, 881–892.

Einstein, M.A. (1991). Descriptive Techniques and their Hybridization. In: Lawless, H.T., Klein, B.P. (Eds.), Sensory Science Theory and Applications in Foods. Marcel Dekker, New York, pp. 317–338.

Eltayeb, A. R. S., Ali, A. O., Abou-arab, A. A., and Abu-Salem, F. M. (2011). Chemical composition and functional properties of flour and protein isolate extracted from Bambara groundnut (*Vigna subterranean*). *African Journal of Food Science*, 5, 82–90.

Fadahunsi, I.F. and Olubunmi, P.D. (2010). Microbiological and enzymatic studies during the development of an 'Iru' (a local Nigerian indigenous fermented condiment) like condiment from Bambara Nut [*Voandzeiasubterranea* (L) Thours]. *Malaysian Journal of Microbiology*, 6, 123–126.

111

Gao, P., Wang, W., Jiang, Q., Xu, Y. and Xia, W. (2016). Effect of autochthonous starter cultures on the volatile flavour compounds of Chinese traditional fermented fish (*Suan yu*). *International Journal of Food Science and Technology*, 51, 1630–1637.

Gernah, D.I., Inyang, C.U. and Ezeora, N.L. (2007). Incubation and fermentation of African locust beans (*Parkiabiglobosa*) in production of *dawadawa*. *Journal of Food Processing and Preservation*, 31, 227–239.

Goldenberger, D., Perschil, I., Ritzler, M. and Altwegg, M. (1995). A simple "universal" DNA extraction procedure using SDS and proteinase K is compatible with direct PCR amplification. *PCR Methods and Applications*, 4, 368–370.

Göğüş, F., Lewis A.C. and Özel, M.Z. (2011). Analysis of black mulberry volatiles using GCxGC–TOF/MS. *International Journal of Food Properties*, 14, 29–36.

Harwood, C.R. (1989). *Bacillus. Plenum press*. New York p 29–38.

Heller, J., Begemann, F. and Mushonga, J. (1997). Bambara groundnut (*Vignasubterranea* (L.) Verdc.) Promoting the conservation and use of underutilised and neglected crops. 9. Proceedings of the Workshop on Conservation and Improvement of Bambara Groundnut (*Vigna subterranean* (L.) Verdc.). Institute of Plant Genetics and Crop Plant Research, Gatersleben/Department of Research and Specialist Services, Harare/Interna-tional Plant Genetic Resources Institute, Rome, Italy, pp 102–118. https://hortintl.cals.ncsu.edu/sites/default/files/articles/bambara_groundnut.pdf. [Accessed 21 May 2015]

Herman, L. and Heyndrickx, M. (2000). The presence of intragenically located REP–like elements in *Bacillus sporothermodurans* is sufficient for REP–PCR typing. *Research in Microbiology*, 151, 255–261.

Hillocks, R.J., Bennett, C. and Mponda, O. M. (2012). Bambara nut: A review of utilization, market potential and crop improvement. *African Crop Science Journal*, 20, 1–16.

Heyland, S., Dac, T.H., Hose, H. and Wood, R.D. (1995). Flavorant composition prepared by fermentation. *United States Patent* 5,476,773.

Isu, N.R. and Ofuya, C.O. (2000). Improvement of the traditional processing and fermentation of African oil beans (*Pentaclethramacrophylla*Benthram) into a food snack ugba. *International Journal of Food Microbiology*, 59, 235–239.

Isu, N.R. and Abu, G.O. (2000). An evaluation of the effect of *Bacillus* cells and *Bacillus* spores in association with cowpea granules as starter cultures for the fermentation of African oil bean (*Pentaclethra macrophylla* Bentham) to ugba. *Plant Foods for Human Nutrition*, 55, 127–138.

Jideani, I.A.O. and Okeke, C.R. (1991). Comparative study of microorganisms and sensory attributes of condiments from the fermentation of different seeds. *Plant Foods for Human Nutrition*, 41, 27–34.

Jirovetz, L., Smith, D. and Buchbauer, G. (2002). Aroma compound analysis of *Eruca sativa* (Brassicaceae) SPME headspace leaf samples using GC, GC-MS, and olfactometry. *Journal of Agricultural and Food Chemistry*, *50*, 4643–4646.

Jukes T.H. and Cantor C.R. (1969). Evolution of protein molecules. In: Munro HN (ed) Mammalian Prote in Metabolism. Academic Press, New York, p 21–132.

Junior, S.B., de Marchi Tavares de Melo, A., Zini, C.A. and Godoy, H.T. (2011). Optimization of the extraction conditions of the volatile compounds from chili peppers by headspace solid phase micro-extraction. *Journal of Chromatography A*, *1218*, 3345–3350.

Kaboré, D., Nielsen, D.S., Sawadogo-Lingani, H., Diawara, B., Dicko, M.H., Jakobsen, M. and Thorsen, L. (2013). Inhibition of *Bacillus cereus* growth by bacteriocin producing *Bacillus subtilis* isolated from fermented baobab seeds (*maari*) is substrate dependent. *International Journal of Food Microbiology*, 162, 114–119.

Kataoka, H., Lord, H. and Pawliszyn, J. (2000). Application of solid-phase microextraction in food analysis: Review. *Journal of Chromatography A*, *880*, 35–62.

Kayembe, N. C. and Van Rensburg, C. J. (2013). Germination as a processing technique for soybeans in small-scale farming. *South African Journal of Animal Science*, 43, 167–173.

Kayitesi, E., Duodu, K.G., Minnaar A. and de Kock H.L. (2013). Effect of micronisation of pre–conditioned cowpeas on cooking time and sensory properties of cooked cowpeas. *Journal of the Science of Food and Agriculture*, 93, 838–845.

Kiers, J.L., VanLaeken, A.E.A., Rombouts, F.M. and Nout, M.J.R (2000). *In vitro* digestibility of *Bacillus* fermented soya bean. *International Journal of Food Microbiology*, 60, 163–169.

Kiuchi, K. and Watanabe, S. (2004). Industrialization of Japanese *natto*. In: Steinkraus, K.H. (Ed.), Industrialization of indigenousfermented foods -Revised and Expanded, 2nd (ed), Marcel Dekker, New York, pp. 193–246.

Kolapo, A. L. and Oladimeji, G. R. (2008). Production and quality evaluation of Soy-corn milk. *Journal of Applied Biosciences*, 1, 40–45.

Kolapo, A. L. and Sanni, M. O. (2009). A comparative evaluation of the macronutrient and micronutrient profiles of soybean-fortified gari and tapioca. *Food and Nutrition Bulletin*, 30, 90–94.

Kolapo, A. L. (2011). Soybean : Africa's Potential Cinderella Food Crop. In: Prof.Tzi-Bun Ng (Ed.), Soybean – Biochemistry, Chemistry and Physiology. ISBN: 978-953-307-219-7. InTech. http://www.intechopen.com/books/soybean-biochemistry-chemistry-and-physiology/soybean-africa-potentialcinderella-food-crop [Accessed: May23, 2017].

Kubo, Y., Rooney, A.P., Tsukakoshi, Y., Nakaqawa, R., Hasegawa, H. and Kimura, K. (2011). Phylogenetic analysis of *Bacillus subtilis* strains applicable to *natto* (fermented soybean) production. *Applied and Environmental Microbiology*, 77, 6463–6469.

Kunst, F., Ogasawara, N., Moszer, I., Albertini, A. M., Alloni, G., Azevedo, V., Bertero, M. G., Bessieres, P., Bolotin, A., Borchert, S., Borriss, R., Boursier, L., Brans, A., Braun, M., Brignell, S. C., Bron, S., Brouillet, S., Bruschi, C. V., Caldwell, B., Capuano, V., Carter, N. M., Choi, S. K., Codani, J. J., Connerton, I. F. and Danchin, A. (1997). The complete genome sequence of the Gram–positive bacterium *Bacillus subtilis*. *Nature* 390, 249–256.

Leboffe, M.J. and Pierce, B.E. (2012). Microbiology: laboratory theory and application, Morton Publishing Company, Englewood, Colorado

Leejeerajumnean, A., Duckham, S.C., Owens, J.D. and Ames J.M. (2001). Volatile compounds in *Bacillus*–fermented soybeans. *Journal of the Science of Food and Agriculture*, 81, 525–529.

Liu, P.Y.F., Ke, S.C. and Chen, S.L. (1997). Use of pulsed-fields gel electrophoresis to investigate a pseudo-outbreak of *Bacillus cereus* in a pediatric unit. *Journal of Clinical Microbiology*, 35, 1533–1535.

Logan, N.A. and Berkeley, R.C.W. (1984). Identification of *Bacillus* Strains Using the API System. *Journal of General Microbiology*, 130, 1871–1882.

Maehashia, K. and Huang L. (2009) Review: Bitter peptides and bitter taste receptors. *Cellular and Molecular Life Sciences*, 66, 1661–1671.

Mahattanatawee, K. and Rouseff R.L. (2014). Comparison of aroma active and sulfur volatiles in three fragrant rice cultivars using GC–Olfactometry and GC–PFPD. *Food Chemistry*, 154, 1–6.

Mbajunwa, O.K., Akingbala, J.O., Mulongoy, K. and Oguntimein, G. (1998). Starter culture evaluation for the production of ugba from African oil bean seed *Pentaclethramacrophylla*. *Journal of the Science of Food and Agriculture,* 77, 127–132.

McGorrin, R.G. (2002). Character impact compounds: Flavors and off-flavors in foods: In Marsili, R. (Ed.), Flavor, fragrance and odor analysis, Marcel Dekker Inc., New York , pp. 375–413.

Medic, J., Atkinson, C. and Hurburgh, C. (2014). Current Knowledge in Soybean Composition. *Journal of the American Oil Chemists' Society*, 91, 363–384.

Meerak, J., Yukphan, P., Miyashita, M., Sato, H., Nakagawa, Y., and Tahara, Y. (2008). Phylogeny of *γ*-polyglutamic acid-producing *Bacillus* strains isolated from a fermented locust bean product manufactured in West Africa. *The Journal of General and Applied Microbiology*, 54, 159–166.

Mendo, S.A.L.V., Henriques, S.I., Correria, A.C.M. and Duarte, J.M.C. (2000). Genetic characterisation of new thermo–tolerant *Bacillus licheniformis* strain. *Current Microbiology*, 40, 137–139.

Minka, S.R. and Bruneteau, M. (2000). Partial chemical composition of Bambara pea [*Vigna subterranea* (L.) Verde]. *Food Chemistry*, 68, 273–276.

Mohamadou, B., Mbofung, C.M. and Barbier, G. (2013). Genotypic and phenotypic diversity among *Bacillus* species isolated from *Mbuja*, a Cameroonian traditional fermented condiment. *African Journal of Biotechnology*, 12, 1335–1343.

Mohamadou, B.A., Mbofung, C.M.F. and Thouvenot, D. (2007). Functional potential of a product from traditional biotechnology antioxidant and probiotic potential of Mbuja, produced by fermentation of *Hibiscus sabdariffa* seeds in Cameroon. *Journal of Food Technology*, 5, 164–168.

Moir, A. (2006). How do spores germinate? *Journal of Applied Microbiology*, 101, 1–5.

Murevanhema, Y. Y. and Jideani, V. A. (2013). Potential of Bambara groundnut (*Vigna subterranean* (L.) Verdc) milk as a probiotic beverage-a review. *Critical Reviews in Food Science and Nutrition*, 53, 954–967.

Naudé, Y. and Rohwer, E.R. (2013).Investigating the coffee flavour in South African Pinotage wine using novel offline olfactometry and comprehensive gas chromatography with time of flight mass spectrometry. *Journal of Chromatography A*, *1271*, 176–180.

Naudé, Y., Makuwa, R. and Maharaj, V. (2016). Investigating volatile compounds in the vapour phase of (1) a hot water infusion of rhizomes, and of (2) rhizomes of *Siphonochilus aethiopicus* using head space solid phase microextraction and gas chromatography with time of flight mass spectrometry. *South African Journal of Botany*, 106, 144–148.

Nwokeleme, C.O. and Ugwuanyi, J.O. (2015). Evolution of volatile flavour compounds during fermentation of African oil bean (*Pentaclethra macrophylla* Benth) Seeds for ''Ugba'' Production. *International Journal of Food Science*, 2015, 1–8.

Oboh, G. (2006). Nutrient and antinutrient composition of condiments produced from some fermented underutilized legumes. *Journal of Food Biochemistry* 30, 579–588.

Obatolu, V. A. (2002). Nutrient and sensory qualities of extruded malted or unmalted millet/soybean mixture. *Food Chemistry*, 76, 129–133.

Obizoba, I.C. and Egbuna, H.I. (1992). Effect of germination and fermentation on the nutritional quality of Bambara nut (*Voandzeia subterranea* L. Thouars) and its product (milk). *Plant Foods for Human Nutrition*, 42, 13–23.

Odunfa, S.A. (1981). Micro-organisms associated with the fermentation of African locust bean (*Parkia filicoidea*) during iru fermentation. *Journal of Plant Food*, 3, 245–250.

Odunfa, S.A. (1983). Carbohydrate changes in fermenting Locust Bean (*Parkia filicoidea*) during Iru preparation. *QualitasPlantarum/ Plant Foods for Human Nutrition*, 32, 3–10.

Odunfa, S. A. and Oyewole, O. B. (1986), Identification of *Bacillus* species from 'iru', a fermented African locust bean product. *Journal of Basic Microbiology*, 26, 101–108.

Odunfa, S.A. (1988). African fermented foods: from art to science. *MIRCEN Journal*, 4, 259–273.

Ogbadu, L. and Okagbue, R.N. (1988). Bacterial fermentation of soya bean for daddawa production. *Journal of Applied Bacteriology*, 65, 353–356.

Ogbonna D.N., Sokari T.G. and Achinewhu S.C. (2001). Development of an owoh-type product from African yam beans (*Sphenostylis stenocarpa*) (Hoechst (ex. A. Rich) Harms) by solid substrate fermentation. *Plant Foods for Human Nutrition*, 56, 183-194.

Ogunshe, A.A.O., Omotosho, M.O. and Ayansina, A.D.V. (2007). Microbial studies and biochemical characteristics of controlled fermented *Afiyo*– a Nigeria fermented food condiment from *Prosopis africana* (Guill and Perr) Taub. *Pakistan Journal of Nutrition*, 6, 620–627.

Ogunshe, A.O., Fawole, O.A., Adewale, O.A., and Olaoye, S.O. (2013). Critical Hazard Points of Indigenous Food Fermentation Processing of Irú, the Most–Popular Nigerian Fermented Food–Seasoning Condiment. *Internet Journal of Food Safety*, 15, 1–6.

Oguntoyinbo, F.A. and Oni, O.M. (2004). Incidence and characterization of *Bacillus cereus* isolated from traditional fermented meals in Nigeria. *Journal of Food Protection*, 67, 2805–2808.

Oguntoyinbo, F.A. and Sanni, A.I. (2007). Determination of toxigenic potentials of *Bacillus* strains isolated from Okpehe, a Nigerian fermented condiment. *World Journal of Microbiology and Biotechnology*, 23, 65–70.

Oguntoyinbo, F.A., Huch, M., Schillinger, U., Holzapfel, W.H., Sanni, A.I. and Franz, C.M.A.P. (2010). Diversity of *Bacillus* species isolated from Okpehe, a traditional termented soup condiment from Nigeria. *Journal of Food Protection*, 73, 870–878.

Oguntoyinbo, F.A., Tourlomousis, P., Gasson, M.J. and Narbad, A. (2011). Analysis of bacterial communities of traditional fermented West African cereal foods using culture independent methods. *International Journal of Food Microbiology*, 145, 205–210.

Ojinnaka, M.C. and Ojimelukwe, P.C. (2013). Study of the volatile compounds and amino acid profile in *Bacillus* fermented castor oil bean condiment. *Journal of Food Research*, 2, 191–203.

Okanlawon, B.M., Ogunbanwo, S.T. and Okunlola, A.O. (2010). Growth of *Bacillus cereus* isolated from some traditional condiments under different regimens. *African Journal of Biotechnology*, 8, 2129–2135.

Omafuvbe, B.O., Shonukan, O.O. and Abiose, S.H. (2000). Microbiological and biochemical changes in the traditional fermentation of soybean for 'soy-daddawa'—Nigerian food condiment. *Food Microbiology*, 17, 469–474.

Omafuvbe, B.O., Abiose, S.H. and Shonukan, O.O. (2002). Fermentation of soybean (*Glycine max*) for soy–*daddawa* production by starter cultures of *Bacillus*. *Food Microbiology*, 19, 561–566.

Omafuvbe, B.O., Falade, O.S., Osuntogun, B.A. and Adewusi, S.R.A. (2004). Chemical and biochemical changes in African locust bean (*Parkia biglobosa*) and melon (*Citrullus vulgaris*) seeds during fermentation to condiments. *Pakistan Journal of Nutrition*, 3, 140–145.

Onyenekwe, P.C., Odeh, C. and Nweze, C.C. (2012). Volatile constituents of ogiri, soybean daddawa and locust bean daddawa three fermented Nigerian food flavour enhancers. *Electronic Journal of Environmental, Agricultural and Food Chemistry*, *11*, 115–221.

Ouoba, L.I.I., Diawara, B., Amoa-Awua, W.K., Traoré, A.S. and Møller, P.L. (2004). Genotyping of starter cultures of *Bacillus subtilis* and *Bacillus pumilus* for fermentation of African locust bean (*Parkia biglobosa*) to produce Soumbala. *International Journal of Food Microbiology*, 90, 197–205.

Ouoba, L.I.I., Diawara, B., Annan, N.T., Poll, L. and Jakobsen, M. (2005). Volatile compounds of Soumbala, a fermented African locust bean (*Parkia biglobosa*) food condiment. *Journal of Applied Microbiology*, *99*, 1413–1421.

Ouoba, L.I.I., Diawara, B., Christensen, T., Mikkelsen, J.D., and Jakobsen, M. (2007). Degradation of polysaccharides and nondigestible oligosaccharides by *Bacillus subtilis* and *Bacillus pumilus* isolated from soumbala, a fermented African locust bean *Parkia biglobosa*, food Condiment. *European Food Research and Technology*, 224, 689–694.

Ouoba, L.I.I., Parkouda, C., Diawara, B., Scotti, C. and Varnam, A.H. (2008). Identification of *Bacillus* spp. from Bikalga, fermented seeds of *Hibiscus sabdariffa*: phenotypic and genotypic characterization. *Journal of Applied Microbiology*, 104, 122–131.

Owens, J.D., Allagheny, N., Kipping, G. and Ames J.M. (1997). Formation of volatile compounds during *Bacillus subtilis* fermentation of soya beans. *Journal of the Science of Food and Agriculture*, *74*, 132–140.

Oyeleke, G.O., Afolabi, O., and Ishola, A.D. (2012). Some quality characteristics and carbohydrate fractions of Bambara groundnut (*Vigna subterranea* L.) Seed Flour. IOSR *Journal of Applied Chemistry*, 2, 16–19.

Park, H.-J., Lee, S.M., Song, S.H. and Kim, Y.-S. (2013). Characterization of volatile components in Makgeolli, a traditional Korean rice wine, with or without pasteurization, during Storage. *Molecules*, 18, 5317–5325.

Parkouda, C., Nielsen, D.S., Azokpota, P., Ouoba, L.I.I., Amoa-Awua, W.K., Thorsen, L., Hounhouigan, J.D., Jensen, J.S., Tano-Debrah, K. and Diawara, B. (2009). The microbiology of alkaline-fermentation of indigenous seeds used as food condiments in Africa and Asia. *Critical Reviews in Microbiology*, 35, 139–156.

Parkouda, C., Thorsen, L., Compaore, C.S., Nielsen, D.S., Tano-Debrah, K., Jensen, J.S., Diawara, B. and Jakobsen, M. (2010). Microorganisms associated with Maari, a Baobab seed fermented product. *International Journal of Food Microbiology*, 142, 292–301.

Pavlovic, M., Huber, I., Konrad, R. and Busch, U. (2013). Application of MALDI-TOF MS for the Identification of Food Borne Bacteria. *The Open Microbiology Journal*, 7, 135–141.

Pelig–Ba, K.B. (2009). Effect of ash, KOH and millet on the fermentation of *Parkia biglobosa* seed to form a condiment. *Pakistan Journal of Nutrition*, 8, 1548–1554.

Pham, A.J., Schilling, M.W., Mikel, W.B., Williams, J.B., Martin, J.M. and Coggins, P.C. (2008). Relationships between sensory descriptors, consumer acceptability and volatile flavour compounds of American dry-cured ham. *Meat Science*, 80, 728–737.

Phelps, R.J. and McKillip, J.L. (2002). Enterotoxin production in natural Isolates of *Bacillaceae* outside the *Bacillus cereus* group. *Applied and Environmental Microbiology*, 68, 3147–3151.

Porwal, S., Lal, S., Cheema, S. and Kalia, V.C. (2009). Phylogeny in aid of the present and novel microbial lineages: diversity in *Bacillus*. *PLoS One*, 4, e4438.

Poulter, N.H. and Caygill, J.C. (1980). Vegetable milk processing and rehydration characteristics of Bambara groundnut [*Voandzeia subterranea* (L.) thouars]. *Journal of the Science of Food and Agriculture*, 31, 1158–1163.

Priest, F.G. (1993) *Bacillus subtilis* and other gram-positive bacteria. In: Sonenshein A, Hoch J and LosickR(eds), *Systematics and ecology of Bacillus*. American Society for Microbiology, Washington D.C.,pp 3–6.

Rooney, A.P., Price, N.P., Ehrhardt, C., Swezey, J.L. and Bannan, J.D. (2009). Phylogeny and molecular taxonomy of the *Bacillus subtilis* species complex and description of *Bacillus subtilis* subsp. *inaquosorum* subsp. nov. *International Journal of Systematic and Evolutionary Microbiology*, 59, 2429–2436.

Rowan, N.J., Deans, K., Anderson, J.G., Hunter, I.S. and Chaitong, T. (2001). Putative virulence factor expression by clinical and food isolates of *Bacillus* spp. after growth in reconstituted infant milk formulae. *Applied and Environmental Microbiology*, 67, 3871–3881.

Sanni, A.I. and Ogbonna, D.N. (1991). The production of owoh, a Nigerian fermented seasoning agent from cotton seed *Gossypium hirsutum* L. *Food Microbiology*, 8, 223–229.

Sanni, A.I. (1993). The need for process optimization of African fermented foods and beverages. *International Journal of Food Microbiology*, 18, 85–95.

Sanni, A.I., Ayernor, G.S., Sakyi-Dawson, E. and Sefa-Dedeh, S. (2000). Aerobic spore-forming bacteria and chemical composition of some Nigerian fermented soup condiments. *Plant Foods for Human Nutrition*, 55, 111–118.

Sanni, A.I., Onilude, A.A., Fadahunsi, I.F., Ogunbanwo, S.T. and Afolabi, R.O. (2002). Selection of starter cultures for the production of *ugba*, a fermented soup condiment. *European Food Research and Technology*, 215, 176–180.

Saitou, N. and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, 4, 406–425.

Sarkar, P.K., Tamang, J.P., Cook, P.E., and Owens, J.D. (1994). Kinema – a traditional soya beans fermented food: proximate composition and microflora. *Food Microbiology*, 11, 47–55.

Sarkar, P.K. and Tamang, J.P. (1995). Changes in the microbial profile and proximate composition during natural and controlled fermentations of soybeans to produce kinema. *Food Microbiology*, 12, 317–325.

Sarkar, P.K., Hasenack, B. and Nout, M.J.R. (2002). Diversity and functionality of *Bacillus* and related genera isolated from spontaneously fermented soya bean (Indian *kinema*) and locust beans (African *soumbala*). *International Journal of Food Microbiology*, 77, 175–186.

Šedo, O. and Zdráhal, Z. (2016). Modulation of the discriminatory power of MALDI–TOF MS profiling for distinguishing between closely related bacterial strains. In: Demirev P and Sandrin TR (eds) Applications of Mass Spectrometry in Microbiology. Springer, Switzerland, pp 185–202.

Stenfors Arnesen, L.P., Fagerlund, A. and Granum, P.E. (2008). From soil to gut: *Bacillus cereus* and its food poisoning toxins. *FEMS Microbiology Reviews*, 32, 579–606.

Sugawara, E., Ito, T., Odagiri, S., Kubota, K. and Kobayashi, A. (1985). Comparison of compositions of odor components of natto and cooked soybeans. *Agricultural and Biological Chemistry*, *49*, 311–317.

Swanevelder, J.C. (1998). Bambara food for Africa (*Vigna subterranean*–Bambara groundnut). National department of Agriculture. ARC-Grain Crops Institute, South Africa.
http://www.nda.agric.za/docs/brochures/bambara.pdf . [Accessed 24 April 2014]

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution*, 28, 2731–2739.

Teklehaimanot, Z. (2004). Exploiting the potential of indigenous agroforestry trees: *Parkia biglobosa* and *Vitellaria paradoxa* in sub-Saharan Africa.In: Nair PKR, Rao MR, Buck LE (eds), *New Vistas in Agroforestry: A Compendium for the 1st World Congress of Afroforestry*. Kluwer Academic Publishers, Boston, pp 207–220.

Terlabie, N.N., Sakyi-Dawson, E. and Amoa-Awua, W.K. (2006). The comparative ability of four isolates of *Bacillus subtilis* to ferment soybeans into *dawadawa*. *International Journal of Food Microbiology*, 106, 145–152.

Thorsen, L., Azokpota, P., Munk Hansen, B., HvillumRønsbo, M., Fog Nielsen, K., Hounhouigan, D.J. and Jakobsen, M. (2011). Formation of cereulide and enterotoxinsby *Bacillus cereus* in fermented African locust beans. *Food Microbiology*, 28, 1441–1447.

Thorsen, L., Kando, C.K., Sawadogo, H., Larsen, N., Diawara, B., Ouédraogo, G.A., Hendriksen, N.B and Jespersen, L. (2015). Characteristics and phylogeny of *Bacillus cereus* strains isolated from Maari, a traditional West African food condiment. *International Journal of Food Microbiology*, 196, 70–78.

Towner, K.J. and Cockayne, A. (1993). Molecular Methods for Microbial Identification and Typing. 1st Edition. Chapman and Hall, London.

Van Gemert, L.J. and Netternbreijer, A.H. (1977). *Compilation of Odour Threshold Values in Air and Water*. Zeist, The Netherlands: TNO Central Institute for Nutrition and Food Research.

Versalovic, J., Schneider, M., De Bruijn, F.J. and Lupski, J.R. (1994). Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods in Molecular and Cellular Biology*, 5, 25–40.

Wang, J. and Fung, D.Y. (1996). Alkaline-fermented foods: a review with emphasis on pidan fermentation. *Critical Reviews in Microbiology,* 22, 101–138.

Wang, L.T., Lee, F.L., Tai, C.J. and Kasai, H. (2007). Comparison of *gyr*B gene sequences, 16S rRNA gene sequences and DNA–DNA hybridization in the *Bacillus subtilis* group. *International Journal of Systematic and Evolutionary Microbiology*, 57, 1846–1850.

Weisburg W.G., Barns S.M., Pelletier D.A., and Lane D.J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology*, 173, 697–703.

Wittanalai, S., Deming R.L. and Rakariyatham N. (2012). Characterization of soybean *Kapi* during fermentation with *Bacillus* species. *Food Biotechnology*, 26, 199–217.

Whitener, M.E.B., Stanstrup J., Panzeri, V., Carlin, S, Divol, B, Du Toit, M. and Vrhovsek, U. (2016). Untangling the wine metabolome by combining untargeted SPME–GCxGC–TOF–MS and sensory analysis to profile Sauvignon blanc co-fermented with seven different yeasts. *Metabolomics,* 12:53.

Woese, C. R. (1987). Bacterial evolution. *Microbiological Reviews*, 51, 221–271.

Xu, Y., Limwachiranon, J., Li, L., Ru, Q. and Luo, Z. (2017). Characterisation of volatile compounds of farmed soft-shelled turtle (*Pelodiscussinensis*) by solid-phase microextraction and the influence of matrix pH on the release of volatiles. *International Journal of Food Science and Technology*, 52, 275–281.

Yamada, S., Okashi, E., Agata, N. and Ventkateswaran, K. (1999). Cloning and nucleotide sequence analysis of *gyrB* of *Bacillus cereus*, *B. thuringiensis*, *B. mycoides*and *B. anthracis* and their application to the detection of *B. cereus* in rice. *Applied Environmental Microbiology*, 65, 1483–1490.

Yusuf, A., Ayedun, H. and Sanni, L. (2008) Chemical composition and functional properties of raw and roasted Nigerian benniseed (*Sesam umindicum*) and Bambara groundnut (*Vigna subterranean*). *Food Chemistry*, 111, 277–282.

Zeller-Péronnet, V., Brockmann, E., Pavlovic, M., Timke, M., Busch U. and Huber, I. (2013). Potential of MALDI-TOF MS for strain discrimination within the species *Leuconostoc mesenteroides* and *Leuconostoc pseudomesenteroides*. *Journal für Verbraucherschutz und Lebensmittelsicherheit*, 803, 205–214.

Zhang, Y., Fraatz, M.A., Horlamus, F., Quitmann H. and Zorn, H. (2014). Identification of potent odorants in a novel nonalcoholic beverage produced by fermentation of wort with shiitake (*Lentinula edodes*). *Journal of Agricultural and Food Chemistry*, *62*, 4195–4203.

Zhao, J., Dai, X., Liu, X., Zhang, H., Tang, J. and Chen, W. (2011). Comparison of aroma compounds in naturally fermented and inoculated Chinese soybean pastes by GC–MS and GC–Olfactometry analysis. *Food Control*, *22*, 1008–1013.

8.0 PUBLICATIONS AND CONFERENCE PRESENTATIONS BASED ON THIS RESEARCH

Publications

Gabriel B. Akanni, Yvette Naudé, Henriëtte L. de Kock and Elna M. Buys (2017). Diversity and functionality of *Bacillus* species associated with alkaline fermentation of Bambara groundnut (*Vigna subterranean* L. *Verdc*.) into *dawadawa*-type African condiment. *European Food Research and Technology* (Accepted).

Gabriel B. Akanni, Henriëtte L. de Kock, Yvette Naudé and Elna M. Buys (2017). Volatile compounds produced by *Bacillus* species alkaline fermentation of Bambara groundnut (*Vigna subterranean* (L.) *Verdc*.) into a *dawadaw*a - type African food condiment using headspace solid-phase microectraction and GC x GC-TOFMS. *International Journal of Food Properties* (Submitted).

Conferences

Gabriel Akanni, Henriette de Kock and Elna Buys 2016. The Microbiological Quality of Alkaline fermented Bambara Groundnut into '*dawadawa*'-type African Food Condiments using *Bacillus* species Starter Cultures. FOODMICRO 2016 – 25TH international ICFMH conference, July 19 – 22, 2016: University College Dublin, Ireland.

Gabriel Akanni, Elna Buys and Henriette de Kock 2016. Sensory characteristics and related flavour compound profiles of '*dawadawa*'-type African food condiments using *Bacillus* species starter cultures. 14TH Annual South African Association of the Flavour and Fragrance Industry (SAAFFI) 1-Day Seminar and Workshop, 3 March, Bytes Conference Centre, Midrand, South Africa

Gabriel Akanni, Elna Buys and Henriette de Kock 2015. Sensory characteristics and related flavour compound profiles of alkaline fermented African food condiments using *Bacillus* species starter cultures. 1ST AfroSense Conference, 23 – 26 November, STIAS, Stellenbosch University, South Africa

Gabriel Akanni, Henriette de Kock, Amanda Minnaar and Elna Buys 2015. Molecular characterization of *Bacillus* species and flavour compound profile of alkaline fermented African food condiments. 21ST SAAFoST BIENNIAL INTERNATIONAL CONGRESS & EXHIBITION 7 - 9 September, Marine Parade, Durban, South Africa

Gabriel Akanni, Henriette de Kock, Amanda Minnaar and Elna Buys 2015. The Microbial Diversity and Characterization of *Bacillus* Species for the Enhanced Fermentation of Bambara Groundnut in the Production of African Food Condiments. Annual meeting of International Association of Food Protection, July 25 - 28. Portland, OR, USA.

Gabriel Akanni, Henriette de Kock, Amanda Minnaar and Elna Buys 2014. Microbial diversity and characterisation of *Bacillus* species for the improved fermentation of Bambara groundnut in the production of African food condiments. FOOD MICRO 2014 Conference, 1st – 4th September, Nantes, France

Gabriel Akanni, Henriette de Kock, Amanda Minnaar and Elna Buys 2013. Diversity and characterisation of *Bacillus* species for the improved alkaline fermentation of Bambara groundnut in the production of African food condiments. 20TH SAAFoST BIENNIAL INTERNATIONAL CONGRESS & EXHIBITION 7 - 9 October, CSIR Convention Centre, Pretoria, South Africa