MOLECULAR IDENTIFICATION AND SAFETY ASSESSMENT OF MICROORGANISMS IN *OKPEYE* SOLD IN OWERRI AND ONITSHA MARKETS IN SOUTH- EASTERN NIGERIA

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CERTIFICATION

This is to certify that this project work "**MOLECULAR IDENTIFICATION AND SAFETY ASSESSMENT OF MICROOGANISMS IN *OKPEYE* SOLD IN OWERRI AND ONITSHA MARKETS IN SOUTH-EASTERN NIGERIA**" was carried out by **AGUNWAH IJEOMA MAUREEN** with Registration number **20154998688** is a student of the Department of Food Science and Technology, Federal University of Technology, Owerri.

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DEDICATION

To the covenant keeping God, I remain in awe of You forever.

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Table of Contents

CERTIFICATION i

DEDICATION ii

ACKNOWLEDGEMENTS iv

ABSTRACT **Error! Bookmark not defined.**

CHAPTER ONE 1

INTRODUCTION 1

1.1 Background of the study 1

1.2 Statement of problem 4

1.3 Objectives of the study 5

1.4 Justification of the study 6

1.5 Scope of study 6

CHAPTER TWO 8

LITERATURE REVIEW 8

2.1 Legumes 8

2.2 Traditional processing technologies of legumes 10

2.2.1 Soaking 10

2.2.2 Cooking 11

2.2.3 Dehulling 12

2. 2.4 Fermentation 13

2.3 Effects of fermentation on nutrients and minerals of legumes 15

2.3.1 Carbohydrates 15

2.3.2 Protein 15

2.3.3 Lipids 17

2.3.4 Minerals **Error! Bookmark not defined.**

2.3.5 Phytochemicals 18

2.4 Starter cultures 19

2.4.1 Traditional fermentation and starter cultures 19

2.4.2 Commercial fermentation and starter cultures 20

2.4.3: The use of starter culture in fermented foods 21

2.4.4: Traditional Approaches 22

2.4.5: Inoculation to Improve Process Control **Error! Bookmark not defined.**

2.4.6: Starter Cultures as Inoculants of Fermentation Processes 22

2.4.7: Defined Starter Cultures as Inoculants of Fermentation Processes 22

2.4.8: Selection of Starter Cultures and Their Application in Small-Scale Fermentations **Error! Bookmark not defined.**

2.4.9: Selection Criteria for Starter Culture Development 22

2.4.10: Technical Considerations Technical **Error! Bookmark not defined.**

2.4.11: Antagonism **Error! Bookmark not defined.**

2.4.12: Bacteriocins **Error! Bookmark not defined.**

2.5 Food condiments 24

2.5.1: Fermented condiments 25

2.6 Processing of some fermented food condiments 26

2.6.1 *Ogiri* 26

2.6.2 *Dawadawa/Iru* 27

2.6.3 *Ugba* 27

2.6.4 *Owoh* 28

2.6.5: *Bikalga* 29

2.7 *Prosopis africana* 29

2.7.1 Uses of *Prosopis africana* 31

2.8: African indigenous fermented foods. 35

2.8.1: Microbial hazards in indigenous fermented foods 39

2.8.2: Safety of microorganisms in African fermented foods 42

2.8.2: Microbiology of African fermented condiments 44
2.9 Nutritional properties of fermented condiments 46
2.10: Toxins in fermented foods 52
2.10.1: Bacterial Toxins 54
2.10.2: Other Toxic Materials in fermented foods 55
2.10.3: Detection Methods 56
2.11: Sensory Evaluation Methods 60
2.12: Types of Sensory Evaluation Methods 61
2.12.1: Discriminative Tests 61
2.12.2: Triangular Test 61
2.12.3: Duo-Trio Tests 62
2.12.4: Paired Comparison Tests 62
2.12.5: Descriptive Tests 62
2.12.6: Free Choice Profile 63
2.12.7: Quantitative Descriptive Analysis 63
2.12.8: Flavour Profile Analysis 63
2.12.9: Texture profile analysis 64
2.12.10: Time Intensity Analysis 64
2.12.11: Affective/ Consumer Acceptance Tests 64
2.12.12: Preference Ranking 65
2.12.13: Hedonic Rating Scale 65
2.12.14: Some of the Requirements for Sensory Evaluation 65
2.13 Summary of literature review 66
CHAPTER THREE 68
MATERIALS AND METHODS 68
3.1 MATERIALS 68
3.1.1 PROCUREMENT OF RAW MATERIALS 68

3.1.2 EQUIPMENT AND CHEMICAL REAGENTS 68

3.2 METHODS 68

3.2.1 Isolation of bacteria from commercial *okpeye* samples 68

3.3 Phenotypic Identification of the *Okpeye Commercial S*amples 69

3.3.1 Gram staining 69

3.3.2 Catalase test 70

3.3.3 Oxidase test 70

3.4 GENOTYPIC IDENTIFICATION 70

3.4.1 Extraction of DNA 70

3.4.2 Rep- PCR fingerprinting of the Isolates 71

3.4.3 16S rRNA gene amplification (PCR) 72

3.4.4 Purification of 16s PCR products. 73

3.4.5 Sequencing of *gyrB* and *rpoB* genes 73

3.5 Safety Assessment of some microorganisms isolated from commercial *okpeye* samples 74

3.5.1 Haemolytic Activity on Blood Agar 74

3.5.2 Enterotoxin (*Nhe, Hbl*), Cytotoxin (*CytK, entFM*) and emetic toxin (*ces*) production by *B. cereus* strains 74

3.5.3 Antibiotic susceptibility test 75

3.6: BATCH PRODUCTION OF *OKPEYE* FROM *P. AFRICANA* SEEDS 78

3.6.1 INOCULUM PREPARATION **Error! Bookmark not defined.**

3.6.2 Production of *okpeye* using the starter cultures **Error! Bookmark not defined.**

3.7 MICROBIOLOGICAL ANALYSIS 82

3.8: CHEMICAL ANALYSIS 82

3.8.1 pH and Temperature Determination 82

3.8.2: Total titratable acidity determination 82

3.9: PROXIMATE ANALYSIS 83

3.9.1: Moisture determination 83

3.9.2: Crude protein determination by micro Kjeldahl method. 83

3.9.3: Ash determination 84

3.9.4: Fat Determination 84

3.9.5: Crude fibre determination 85

3.9.6: Carbohydrate determination 85

3.10: PHYTOCHEMICAL ANALYSIS 85

3.10.1: Alkaloids determination 85

3.10.2: Phytate determination 86

3.10.3: Tannin determination 86

3.10.4: Flavonoid determination 87

3.10.5: Cyanogenic glycosides determination 87

3.10.6: Saponin determination 88

3.10.7: Oxalate determination 88

3.10.8: Total phenol determination 89

3.11: SENSORY EVALUATION 90

3.12: STATISTICAL ANALYSIS 91

CHAPTER FOUR 92

RESULTS AND DISCUSSION 92

4.1 Results 92

4.2 DISCUSSION 121

4.2.1: Bacterial counts in *okpeye* from some local markets in South Eastern Nigeria 121

4.2.2: Phenotypic characteristics of *okpeye* commercial samples from Anambra and Imo State 122

4.2.3: Origin and identification of microorganisms from commercial samples of *okpeye* 122

4.2.4: Identification of microorganisms from *okpeye* using 16s rRNA sequencing and *gyrB/ rpoB* sequencing for closely related species. 125

4.2.5: Analysis of toxin genes from *Bacillus cereus* isolated from *okpeye* 127

4.2.6: Haemolytic activities of some microorganisms isolated from *okpeye* 129

4.2.7: Antibiotic susceptibility of some microorganisms isolated from *okpeye* 130

4.2.8: Bacterial count obtained on NA and MHA during fermentation using starter cultures 133

4.2.9: Effect of Fermentation and starter cultures on the pH, Temperature and TTA on the Fermenting Mash of *okpeye* 135

4.2.10: Effects of Starter Cultures on the Proximate Composition of *Okpeye* 138

4.2.11: Qualitative and Quantitative Analysis of the Phytochemical Properties of *Okpeye* fermented using Starter Cultures 141

4.2.12: Sensory results of okpeye fermented using starter cultures 144

CHAPTER FIVE 147

CONCLUSION AND RECOMMENDATION 147

5.1 CONCLUSION 147

5.2 RECOMMENDATIONS 149

5.3 CONTRIBUTION TO KNOWLEDGE 150

REFERENCES 152

Appendix 199

LIST OF TABLES

Table 2.1: Predominant microorganisms associated with some African fermented condiments 58

Table 2.1: Predominant microorganisms associated with some African fermented condiments (Cont'd). 59

Table 2.1: Predominant microorganisms associated with some African fermented condiments (Cont'd). 60

Table 2.1: Predominant microorganisms associated with some African fermented condiments (Cont'd). 61

Table 2.2: Some Bacterial contamination of indigenous fermented foods from Africa 70

Table 2.2: Some Bacterial contamination of indigenous fermented foods from Africa (Cont'd). 71

Table 2.2: Some Bacterial contamination of indigenous fermented foods from Africa (Cont'd). 72

Table 3.1: Primers used in this study 89

Table 3.1: Primers used in this study (Cont'd). 90

Table 4.1: Origin and identification of microorganisms from *okpeye* 105

Table 4.1: Origin and identification of microorganisms from *okpeye* (Cont'd). 106

Table 4.1: (Contd.). Origin and identification of microorganisms from *okpeye* 107

Table 4.1: Origin and identification of microorganisms from *okpeye* (Cont'd). 108

Table 4.2: Identification of microorganisms from *okpeye* using 16s rRNA sequencing and *gyrB/ rpoB*sequencing for closely related species. 109

Table 4.2: Identification of microorganisms from *okpeye* using 16s rRNAsequencing and *gyrB/ rpoB*sequencing for closely related species (Cont'd). 110

Table 4.2: Identification of microorganisms from *okpeye* using 16s rRNA sequencing and *gyrB/ rpoB*sequencing for closely related species (Cont'd). 111

Table 4.3: PCR analysis of toxin genes from *Bacillus cereus* isolated from *okpeye* 112

Table 4.4: Haemolytic activities of some microorganisms isolated from *okpeye* 113

Table 4.4: Haemolytic activities of some microorganisms isolated from *okpeye* (Cont'd). 114

Table 4.5: Antibiotic susceptibility of some microorganisms isolated from *okpeye* 115

Table 4.5: Antibiotic susceptibility of some microorganisms isolated from *okpeye* (Cont'd) 116

Table 4.6: Effect of Fermentation on the pH, Temperature and TTA on the Fermenting Mash of *okpeye* from 0- 168 hours 120

Table 4.7: Effect of Starter Culture on the pH, Temperature and TTA on the fermenting mash 121
Table 4.8: Effects of Starter Cultures on the Proximate Composition of *Okpeye* 122

Table 4.9: Qualitative Analysis of the Phytochemical Properties of *Okpeye* fermented using Starter Cultures 123

Table 4.10: Quantitative Analysis of the Phytochemical Properties of *Okpeye* fermented using Starter Cultures 124

Table 4.11: Sensory evaluation of *okpeye* fermented using starter cultures 125

LIST OF FIGURES

Fig 2.1: *Prosopis Africana* tree 42

Fig 2.2: Seeds of *Prosopis africana pulp* 42

Fig 2.3: Seeds of *Prosopis africana*

Fig 3.1: Spontaneous fermentation of *okpeye* *92*

Fig 3.2: Production of *okpeye* using starter cultures 94

Figure 4.1. Bacterial counts in *okpeye* from some markets in South Eastern Nigeria 104

Figure 4.2: Bacterial count obtained on nutrient agar during fermentation using starter cultures 118

Figure 4.3: Bacterial count obtained on Mueller-hinton agar during fermentation using starter cultures 119

LIST OF PLATES

Plate 1a: Colonies isolated from *okpeye* commercial samples (ORM) on Tryptone soy agar 210

Plate 1b: Colonies isolated from *okpeye* commercial samples (AOM) on Tryptone soy agar 211

Plate 2: Colonies isolated from *okpeye* commercial samples (AOM) on MacConkey agar 212

Plate 3: Colonies isolated from *okpeye* commercial samples (AOM) on Mannitol salt agar 213

Plate 4: Colonies isolated from *okpeye* commercial samples (OWB) on MacConkey agar 214

Plate 5: Colonies isolated from *okpeye* commercial samples (OEM) on Mannitol salt agar 215

Plate 6: Colonies isolated from *okpeye* commercial samples (OWB) on Mannitol salt agar 216

Plate 7: Colonies isolated from *okpeye* commercial samples (AOM) on MacConkey agar 217

Plate 8: Colonies isolated from *okpeye* commercial samples (AOM) mannitol salt agar 218

Plate 9: Non- haemolytic activity of O27 isolate on blood agar 230

Plate 10: Haemolytic activity of A11 isolate on blood agar 231

Plate 11: Haemolytic activity of O12 isolate on blood agar 232

Plate 12: Antibiotic susceptibility test on some isolates 233

Plate 13: Antibiotic susceptibility test on some isolates 234

Plate 14: Antibiotic susceptibility test on some isolates 235

Plate 15: Antibiotic susceptibility test on some isolates 236

Plate 16: Antibiotic susceptibility test on some isolates 237

LIST OF PICTURES

Picture 1: DNA bands of all Imo isolates on gel electrophoresis 227

Picture 2: DNA bands of all Anambra Isolates on gel electrophoresis 228

Picture 3: Electrophoresis band of isolates for 16s sequencing 229

Picture 4: *Prosopis africana* cooked seeds 255

Picture 5: Dehulled *Prosopis africana* seeds 256

Picture 6: *Prosopis africana* seeds innoculated with starter cultures 257

Picture 7: fermenting seeds of *Prosopis africana* 258

Picture 8: pounding of fermented seeds of *Prosopis africana* *259*

Picture 9: Moulded *okpeye* fermented with starter cultures 260

Picture 10: Dried *okpeye* fermented with starter cultures 261

ABSTRACT

Okpeye an indigenous fermented condiment produced from *Prosopis africana* seeds was assessed to evaluate the microbilogical quality of the condiment. Eighteen (18) commercial samples of *okpeye* were purchased from six markets in two cities in South Eastern Nigeria. Fifty-nine (59) bacteria colonies were isolated and identified at the species level by phenotyping and sequencing of the 16S rRNA, *gyrB* and *rpoB* genes. *Bacillus* (47.4%) and *Staphylococcus* (42.3%) were the predominant bacterial species in *okpeye*. Overall, *B. amyloliquefaciens* and *S. simulans* were the most frequently occurring bacteria and were present in all samples. In addition, *B. cereus* was isolated in samples obtained from all markets. Other bacterial species included *B. velezensis, Oceanobacillus caeni*, *S. cohnii, Escherichia fergusonni* and *Vagacoccus lutrae.* The *B. cereus* isolates (10) were screened for the presence of 8 enterotoxin genes (*hblA*, *hblC*, *hblD*, *nheA*, *nheB*, *nheC*, *cytK*, *entFM*) and one emetic gene (*cesB*). The non-haemolytic enterotoxin (*nhe ABC*) and haemolytic enterotoxin (*hbl ABD*) complexes were present in 70% and 50% of *B. cereus* respectively. The positive rate of *cytK* and *entFM* genes was 70%, while the *cesB* gene was 30%. The haemolysis test revealed twenty-three isolates out of the twenty-eight isolates tested showing full haemolytic activity on blood agar. Two isolates (O22 and A22) showed partial haemolysis while three (O11, O15 and A12) showed no haemolysis. Antibiotic susceptibility assessment showed that most of the isolates were susceptible to gentamicin, tetracycline, streptomycin, and erythromycin but resistant to ciprofloxacin and vancomycin. These findings highlight the need for further controls to reduce contamination with potential pathogenic bacteria in indigenous fermented condiments such as *okpey*e. Two isolates that gave the best safety assessment results were used as starter cultures for the fermentation of *Prosopis africana* seeds. The bacterial load of seeds fermented with the starter cultures was slightly higher than the spontaneously fermented seeds with a value range of (7.7- 9.4 log10 Cfu/g). The seeds fermented with mixed cultures of *B. licheniformis* and *B. amyloliquefaciens* had the highest pH (9.14), temperature (39.96^{o}C) and titratable acidity (TTA) (1.03%) after 168 hours of fermentation. The protein, fat, ash and carbohydrate content were higher in *okpeye* fermented with starter cultures (B, C and D) than that of the commercial samples (E). The spontaneously fermented sample (A) had the highest protein content (27.80%) than all the other samples. The phytochemical composition showed that *okpeye* fermented with mixed cultures of *B. licheniformis* and *B. amyloliquefaciens* had the highest oxalates (11.52%), saponins (0.05%), phytates (549.75 mg/100g), alkaloids (13.03%) and flavonoids (14.20%) than the commercial and spontaneously fermented samples. The spontaneously fermented samples had the highest tannin (13.29 mg/100g) and total phenol (330.36 mg/g) than all the other samples. The commercial sample had the least phytochemical nutrients except for phytate (58.53 mg/100g) and cyanide (1.60 mg/g). The sensory analysis showed that soup cooked with *okpeye* fermented with mixed cultures of *B. licheniformis* and *B. amyloliquefaciens* had the best results in aroma (7.27), flavour (7.40), appearance (7.30), after taste (7.17) and overall acceptability (7.31). The sensory results showed that *okpeye* fermented with mixed cultures of *B. licheniformis* and *B. amyloliquefaciens* was well accepted by consumers. Based on this acceptability as well as the nutritional composition and phytochemical composition, mixed cultures of *B. licheniformis* and *B. amyloliquefaciens* c be recommended in the fermentation of *P. africana* seeds to produce safe and good quality okpeye.

Key words: *Okpeye*, 16srRNA sequencing, Starter culture, Antibiotics, Enterotoxin genes, Haemolysis, *Bacillus, gyrB, rpoB* genes.

CHAPTER ONE

INTRODUCTION

1.1 Background of the study

Traditional fermented food is an important part of the cultural heritage and diet that has been widely consumed since ancient times (Kavitakea, Kandasamaya, Devib & Shettya, 2017). They are produced through alkaline fermentation or lactic acid bacteria, yeasts or mixtures of both. These foods make a valuable contribution to the continent's effort to achieve its sustainable development goals focused on food security, poverty alleviation and gender equality (Anyogu, Olukorede, Anumudu, Onyeaka, Areo, Obadina, Odimba, & Nwaiwu, 2021; Franz, Huch, Mathara, Abriouel, Benomar, Reid, Galvez & Holzapfel, 2014). It has been shown that fermentation enhances the nutritional, healthy, organoleptic and preservative properties of food as well as increases income sources. It also increases food availability and food diversity, reduces post-harvest losses, helps sustain food sovereignty and thus plays an important role in developing economies (Adesulu & Awojobi, 2014).

Fermented condiments are products derived primarily from the fermentation of legumes and oilseeds by microorganisms such as *iru* from African locust bean, *ogiri* from melon seeds, *dawadawa* from African locust bean*, soumbala* from Locust bean, *ugba* from African oil bean seed, *owoh* from cotton seeds and *okpeye* from mesquite seeds (Olasupo, Odunfa & Obayori, 2010; Okechukwu, Ewelike, Ukaoma, Emejulu & Azuwike, 2012).

Okpeye is a traditional fermented soup condiment produced from *Prosopis africana* seeds. According to Oguntoyinbo, Sanni, Franzs & Holzapfel, (2007) it used as a flavouring agent for sauces, soups and other dishes. In addition to its primary role as flavour enhancer, it also serves

as a tasty and low-cost protein source among the people of Eastern and Mid-Western Nigeria (Okechukwu et al., 2012).

The microbial community in fermented condiments is diverse with notable diversity observed among different samples. Culture-based methods like biochemical and phenotypic-based identification of fermented condiments have been extensively investigated by some authors (Isu & Ofuya, 2000; Sanni, Onilude, Fadahunsi, Ogubanwo, & Afolabi, 2002; Ogueke & Aririatu, 2004). These methods present several limitations, such as being time-consuming, laborious and not being able to detect non-culturable populations in complex ecosystems with dominant populations. Therefore, a more comprehensive understanding of microbial composition in fermented food products is needed to detect microorganisms in low numbers.

In the last decade, culture-independent approaches which include polymerase chain reaction (PCR)-based amplification and gene sequencing of 16S rRNA genes are increasingly being used to study the predominant bacterial populations of African fermented condiments such as *ogiri* (Ademola, Adeyemi, Ezeokoli, Ayeni, Obadina, Somorin, Omemu, Adeleke, Nwangburuka, Oluwafemi, Oyewole & Ezekiel, 2018), *ugba* (Ahaotu, Anyogu, Njoku, Odu, Sutherland & Ouoba, 2013), *bikalga* (Ouoba,Thorsen & Varnam, 2008), *soumbala* (Ouoba, Diawara, Amoa-Awua, Traore & Moller, 2004; OuobaVouidibo-Mbozo, Anyogu, Obioha, Sutherland, Jespersen, & Ghoddusi, 2019) and *maari* (Parkaouda, Thorsen, Compaor´e, Nielsen, Tano-Debrah, Jensen, Diawara & Jakobsen, 2010). However, such has not been done or reported for *okpeye*.

The uncontrolled nature of the production process of most fermented foods including *okpeye* and the use of unhygienic practices for food preparation and storage compromises the microbiological quality and safety of many traditional fermented foods (Anyogu et al., 2021). It is imperative to explore and utilize microbial starter cultures to assist in improving the traditional processes of most of our indigenous fermented condiments. Since the use of starter

cultures has generally been recognized as one of the significant ways of ensuring product consistency, optimizing production processes, guaranteeing product quality of the fermented products to a reasonable extent and eliminating the problem of foodborne pathogens (Eman, 2009). To enable the selection of a starter culture, safety criteria such as toxin assessment, haemolytic activities and antibiotic susceptibility should be screened. Moreover, it is important to know the exact identity of the bacteria found in *okpeye* and to characterize and distinguish them at the subspecies level (Kabore, Gagnon, Roy, Sawadogo-Lingani, Diawara, & LaPointe, 2018). It has equally been established that the selection of suitable bacteria for controlled fermentation, industrial application, probiotics, or as a potential starter culture for the enhancement process are strain dependent (Adesulu-Dahunsi, Sanni, & Jeyaram, 2017).

Recently, studies utilizing molecular-based methods for identification have highlighted toxigenic *B. cereus* is one of the predominant species in West African-based condiments. Ahaotu et al., (2013) reported that in *ugba,* Adewumi, Oguntoyinbo, Singh, Keisam, Romi & Jeyaram (2014) did the same in *iru*, likewise, Mahammadou, Mbofung & Barbier (2013) reported so in *mbuja* and this organism poses a great risk to the health of consumers. Consequently, foodborne pathogenic bacteriaare routinely isolated from similar plant-based fermented condiments available for commercial sale giving rise to food safety and public health concerns (Adekoya et al., 2019; Owusu-Kwarteng, Parkouda, Adewumi, Ouoba & Jespersen, 2020).

Thus far, only a few studies have investigated the microbiological quality and safety of *okpeye* available for retail sale. Fowoyo (2017) enumerated and identified microorganisms from eight commercial samples of *okpeye* using standard methods. *Bacillus* species as well as pathogenic microorganisms like *Staphylococcus aureus* and *Escherichia coli* were identified. In Oguntoyibo, Huch, Cho, Schillinger, Holzapfel, Sanni & Franz, (2010), *Bacillus* species was identified and found to be dominant using Randomly Amplified Polymorphic DNA (RAPD-

PCR) and 16s rRNA sequencing. Although the exact number of *Bacillus cereus* was not determined in the study, the species was found to harbour enterotoxigenic genes using multiple PCR inferring the ability of the microorganism to cause food poisoning. Ademola et al., (2018) also analyzed the bacterial community of retailed *iru* and *ogiri* as well as during the different processing stages during production using a phylogenetic method of the 16srRNA gene. *Enterococcus faecium, Proteus mirabalis* and *Staphylococcus* spp were identified and known to be pathogenic microorganisms. Thus, this study aims to investigate the microbial diversity in commercial *okpeye* samples available for retail sale in some South Eastern parts of Nigeria using the genotypic method. The safety assessment of some attributes of the condiment will be investigated and the most likely safe identified microorganism isolated from the commercial sample will be used as a starter culture for fermentation of *Prosopis africana* seeds for guaranteed safety.

1.2 Statement of problem

Indigenous fermented condiments have been widely consumed in Nigeria and used in many dishes as flavour enhancers. However, the spontaneous nature and unhygienic measures during and after processing predispose these condiments to contamination by pathogenic microorganisms.

The occurrence of these undesirable gram-negative and gram-positive pathogenic bacteria has led to concern for food safety and the health of the consuming public. Potential spore formers such as *Bacillus cereus* are of great concern because of their capacity to survive different processing conditions including heat treatment.

In addition, *B. cereus* is recognized as an opportunistic human pathogen capable of causing two types of food poisoning; namely emetic food poisoning resulting from the production of heat-stable toxin, cereulide which results in vomiting 5–6 h after ingestion. The second and the

type is the diarrheal type which results from the production of various enterotoxins in the small intestine of the host causing abdominal pain and diarrhea 8 to 16 hours after consumption.

Using phenotypic methods of identification that has been the norm in the last two decades would not give an in-depth knowledge of the detailed microbial community in *okpeye* condiments especially the microbial populations that occur in low numbers.

In addition, it is obvious that starter cultures are not normally used in the fermentation process and therefore variations in the quality and stability of this product is observed. It is imperative to use molecular tools to investigate the microbial diversity, especially the predominant microorganisms of commercial *okpeye* sold and consumed in South-Eastern Nigeria and to evaluate how safe they are for the health purposes of the consumers.

1.3 Objectives of the study

The main objective of the study was to carry out the molecular identification and safety assessment of microorganism in *okpeye* sold in Owerri and Onitsha markets in South-Eastern Nigeria.

The specific objectives include were for:

i. Enumeration and isolation of the bacteria in commercial samples using different agars
ii. Investigation of the phenotypic characteristics of the all the isolates.
iii. DNA purification of the isolates and 16s sequencing.
iv. Identification based on 16s sequencing to genus and species level including *gyrB* and *rpoB* sequencing.
v. Safety assessment of microorganisms present in the *okpeye* isolates.
vi. Carrying out fermentation of *okpeye* from the predominant and safe microorganisms identified as starter cultures.
vii. Determination of some fermentation parameters during the fermentation period.

viii. Proximate, phytochemical and sensory evaluation of the condiment produced using starter culture.

1.4 Justification of the study

Okpeye is an indigenous fermented condiment normally used to enhance soup flavour and dishes. The complexity of the micro-biodiversity of indigenous fermented condiments makes it difficult to identify and characterize the relevant functional microorganisms using single conventional methods. However, developed molecular tools have given an alternative to conventional methods because genome sequence is independent of phenotypic characteristics and varies among species.

A combined understanding of the bacterial diversity and the identification of the predominant microorganism that ferments this condiment would be very useful in ascertaining its safety to the consuming public. The knowledge that the major fermenting microorganisms are microbiologically safe which includes their inability to produce toxins in the condiment and being susceptible to different antibiotics would be very useful.

Furthermore, the use of ascertained safe microorganisms as starter cultures during fermentation would guarantee consistency, improve product safety, enhance product quality and commercialization of our indigenous condiments. This study would also provide robust data for further work to be done on the technological characteristics, the importance of the non-pathogenic organisms and their roles in the fermentation process for better food quality and safety management.

1.5 Scope of the study

Eighteen commercial (18) samples were purchased from three retailers in six different markets in Imo (Relief, World Bank and Ekeukwu Owerri) and Anambra (Ose, Bridgehead and Ochanga) States, South Eastern Nigeria. The 18 samples were compressed into six samples and

ten-fold dilutions of suitable dilutions spread on Tryptone soya agar (TSA), Mannitol salt phenol-red agar and MacConkey agar in duplicates and plates incubated. Morphological characteristics of colonies on all the plates were examined and the number of colonies forming units (CFU) was recorded. The dominant colonies were re-isolated and purified by streaking several times on TSA, then the stock cultures maintained in beads and stored at −20 °C for further analyses. Phenotypic identification was recorded including colony morphology, Gram stain, catalase and oxidase test to identify the organismsand the chromosomal DNA of each isolate was extracted using InstaGene™ Matrix.

The total DNA isolated from each of the 59 isolates was fingerprinted using Repetitive element PCR (rep-PCR), sequencing and sent for sequencing. Safety assessmenttest carried out washaemolysis on blood agar, PCR detection of enterotoxigenic genes and antibiotic susceptibility test. After the safety assessment test, the isolates determined to be non-pathogenic and free from toxins was selected and used as starter cultures to initiate and carry out controlled fermentation of the condiment. During the fermentation period, some fermentation parameters were monitored out and an enumeration of the fermenting organisms done. After fermentation, some analysis was carried out on the condiments including proximate, phytochemical and sensory analysis.

CHAPTER TWO

LITERATURE REVIEW

2.1 Legumes

Legumes are broad diversity of crops that are included in flowering plants producing seeds in pods that are often cultured for food and feeds. Legumes ranked as 3rd largest family of flowering plants having more than 19500 species and over 750 genera (Lewis, Schrire, Mackinder & Lock, 2016). The term "legume" has been mainly derived from Latin word *legumen* which means the seeds that are harvested in pods. In some regions, legumes are considered pulses, pea, or member of bean family which consist of oilseeds such as soybeans, peanuts, alfafa, clover, mesquite, and pulses, including the dry grains of peas, chickpeas, lentils, peas, beans, and lupins. These are cultivated throughout the world for their seeds, harvested and marketed as primary products. Some important legume include chickpeas (*Cicer arietinum*), pigeon pea (*Cajanus cajan*), lentil (*Lens culinaris*), mung bean (*Vigna radiata*), soybeans (*Glycine max*), winged bean (*Psophocarpus tetragonoloba*), cowpea (*Vigna unguiculata*), pea (*Pisum sativum)*, groundnut (*Arachis hypogaea*), and black gram (*Vigna mungo*) and some of the most important legumes in the world are peas, beans, peanuts, soybeans, and chickpeas (Reyes-Moreno, Okamura-Esparza, Armienta-Rodelo, Gomez-Garza & Milán-Carrillo, 2000). The edible seeds of legumes play an important role in diet providing essential nutrients with medium to high calorie value. These are inexpensive, valuable and easily available sources of good proteins, and are most commonly consumed in South Asian regions including Pakistan, India, and Bangladesh (Hayat Ahmad, Masud, Ahmed & Bashir, 2014) and there is growing interest in expanding domestic consumption, due to increased awareness of their health benefits.

Legumes have a special place in the diet of humans because they contain nearly 2 to 3 times more protein than cereals, Cowpeas, for example, contain about 25% protein (Annor, Sakyi-

Dawson & Ssaalia, 2010). Legumes are also excellent sources of complex carbohydrates and have been reported as beneficial for cardiovascular diseases and diabetes by some researchers probably due to the large amounts of water-soluble fiber and a large content of phenolics (Enujiugha, 2010). Legumes are also a good source of vitamins (thiamine, riboflavin, niacin, vitamin B6, and folic acid) and certain minerals like calcium, iron, copper, zinc, potassium and magnesium. These are also an excellent source of polyunsaturated fatty acids (linoleic and linolenic acids). Indeed, several studies suggest that increased consumption of legumes may protect against diseases such as cancer, diabetes, osteoporosis, and cardiovascular diseases, among others (Tharanathan & Mahadevamma, 2003). Legumes further offer a practical avenue for diet diversification as consumers look for a greater balance between plant and animal food sources. With growing concerns about the impact of agricultural practices on the environment, the addition of legumes in crop rotation cycles could have beneficial impacts as they could fix nitrogen in soils, thereby reducing the need for chemical fertilizers. Factors that have limited their consumption in North America include the longer time required for their preparation, the possible gastrointestinal (GI) discomfort due to the presence of indigestible carbohydrates that ferment in the GI tract causing gas and bloating, and their typical beany flavor.

Extensive research in breeding, food quality, and processing has helped to overcome some of these limitations, increasing the acceptability of legumes in the North American diet and facilitating their use in food formulation (Martín-Cabrejas, Aguilera & Pedrosa, 2009). Although legumes are rich in proteins, the quality of their protein is not nutritionally adequate. This is because they lack sulphur-containing amino acids such as methionine and cysteine. These limiting amino acids are, however, complemented by the use of legume cereal blends in diets. Cereals, being rich in sulphur-containing amino acids, complement the legume proteins, hence improving the quality of the protein. Other factors such as low protein digestibility, the presence of antinutritional factors such as trypsin inhibitors, lectins, phytates, polyphenols, and

flatulence factors make some legume seeds underutilized (Ragab, Kijora, Ati & Danier, 2010). Most of these antinutritional factors could, however, be reduced or eliminated by various processing techniques. The loss of nutrients occurs during food preparation and processing; however, the processor should limit these losses to enhance the nutritional quality of food. Different processing techniques are required to inactivate or remove antinutritional factors, thus enhancing the nutritional quality of legumes. The chemical and physical methods of processing legumes include soaking, boiling, microwave cooking, autoclaving, extrusion cooking, irradiation, fermentation and germination. Sometimes a single processing treatment is not effective against antinutrients therefore combination of two or more methods is used.

2.2 Traditional processing technologies of legumes

The traditional processing of legumes is labour intensive and is mostly done by women, especially in developing countries in Asia and Africa. The major traditional techniques used in the processing of legumes are soaking, boiling/cooking, dehulling, and fermentation, among others.

2.2.1 Soaking

Legumes are primarily soaked in water and/or salt solutions (0.25–1%) to soften the cotyledon, which then hastens cooking. This process involves adding water and/or salt solution to the legumes and discarding the water after some time or cooking with the soak water. Soaking allows the water to disperse in the protein fraction and starch granules which facilitate the protein denaturation and starch gelatinization, which softens the texture of beans (Siddiq & Uebersax, 2012). Different soaking times have also been reported, but in most cases, the soaking is done overnight. Soaking of legumes could be done in either warm water or water at ambient temperature. Besides its primary role of shortening the cooking times of legumes, soaking has been reported to significantly reduce the phytate and phytic acid contents of

legumes (Toledo & Canniatti-Brazaca, 2008; Kumar, Sinha, Makkar & Becker., 2010). This was observed when legumes were not cooked with the soaking water. The flatulence factors in legumes are also reduced by soaking, as a result of the leaching out of stachyose and raffinose. Soaking also increases the protein digestibility of legumes as confirmed in chickpeas, lentils, and different types of legumes (Martín-Cabrejas et al., 2009; Arif, Ahmad, Masud, Khalid, hayat, Siddique & Ali, 2012).). Soaking further results in the reduction of the mineral contents of legumes, due to the loss in the soaking water, especially when the water is discarded; however, their bioavailability is increased after soaking (Martín-Cabrejas et al., 2009). The increase in the bioavailability of minerals might be attributed to the reduction in antinutritional factors during soaking.

2.2.2 Cooking

The cooking of legumes has been practiced for years. It is one of the most common processing techniques applied to legumes, and involves boiling the legume seeds in water till they are soft. Traditionally, determination of the required softness of cooked legumes is done by pressing the legumes with the thumb. The cooking methods might greatly affect the nutrient contents of the food. The best method of increasing the nutritional properties of beans is by soaking the beans in a salt solution and then cooking the soaked beans with fresh water. This makes the legume soften and tenderize, as the beans contain some complex sugars that are indigestible by enzymes that result in gastric issues this problem could be solved by soaking the legume seeds before cooking. Several changes occur during the cooking of legumes apart from softening, there is gelatinization of starch, denaturation of proteins, and browning of the seeds (Enujiugha, 2010).

Many researchers have shown that heat processing improved the absorption and digestibility of iron (Wang, Wu & Shyu, 2014). This provides both nutritional and health benefits which is the reason health professionals encourage the greater consumption of beans (Messina, 2014).

Besides reducing the antinutritive factors in legumes, cooking reduces the amounts of stachyose and raffinose. The longer cooking times required have been an obstacle to legume use. To reduce the legume cooking times, potash has been used traditionally to help soften the legume cotyledons. Sodium bicarbonate, trisodium phosphate, and ammonium carbonates have also been exploited to reduce the cooking times of legumes.

2.2.3 Dehulling

Dehulling is the removal of the outer coat of food legumes or cereals. It is one of the basic processing and common step in legume processing. Dehulling of most condiments is normally done traditionally with mortar and pestle or by rubbing between fingers which makes the process labor-intensive, inefficient, unhygienic and time-consuming (Martín-Cabrejas et al., 2009). In most homes in Nigeria, it is manually done by rubbing off the seed coat by hand after soaking for a few minutes. The dehulling treatment applied to grain legumes could be either dry or wet treatment. Both treatments are aimed at loosening the seed coat.

The dry method involves treating the grain with a small amount of oil and then drying for 2 to 3 days under the sun. The oil penetrates through the husk to the cotyledon and releases its binding under mild heat during drying. The dry dehulling treatment has the disadvantage of high dehulling losses due to breakage and powdering. It has been demonstrated that there are marked differences in the dehulling efficiency of legumes. For example, soybeans (*Glycine max*), faba beans (*Vicia faba equine L.*) and field peas (*Pisum sativum L.*) have better-dehulling efficiencies (about 70%) compared to the others. These dehulling efficiencies were attributed to the resistance of seed splitting during dehulling and also to the fact that the seed coat of these legumes is loosely bound to their cotyledons.

2. 2.4 Fermentation

Fermentation is one of the oldest methods of food preservation known to man (Omafuvbe, Falade, Osuntogun & Adewusi, 2004) and the oldest preservation technology in the world. This has been known and practiced by the human race since prehistoric times, long before the scientific underlying principles were understood. Fermentation of food (natural or controlled) is a process that has been in existence for a long time and was traditionally carried out by our ancestors. Fermentation was also defined by Akiko & William (2004) as the chemical transformation of organic substances into smaller compounds by the action of enzymes, and complex organic catalysts, which are produced by microorganisms such as molds, yeasts, or bacteria. Enzymes work by hydrolysis which is a process of chemical decomposition in which a compound is split into others by reacting with water from a smaller compound. A protein molecule is broken down by enzyme protease into polypeptides and peptides, and then into numerous amino acids, which are easily digested by the body.

Carbohydrates are also acted upon by enzyme amylase which reduces starches and complex sugars to simple sugars. Fat molecules are hydrolyzed by the enzyme lipase into simpler free fatty acids. In a broader sense, it is a process in which microorganisms produce chemical changes in organic substrates through the action of enzymes produced by these organisms. Fermentation is also defined by Walker, Adhous, Drummond, smith, nimmo, Arnott & Satsangi (2004) as the slow decomposition process of organic substances induced by micro-organisms, or by complex nitrogenous substances (enzymes) of plant or animal origin. It could be described as a biochemical change, which is brought about by the anaerobic or partially anaerobic oxidation of carbohydrates by either micro-organisms or enzymes. This is distinct from putrefaction, which is the degradation of protein materials (FAO, 2003). Fermentation thus leads to flavor enhancement, and complex molecules reduction (oligosaccharides and

proteins) but reduces the shelf life of the seeds and exposes the product to post-fermentation contamination (Oguntoyinbo et al., 2007).

Fermentation is also used to enhance the bioavailability of nutrients from different crops and also improve its organoleptic properties as well as extend the shelf life (Chaves- Lopez, Serio, Grande-Tovar, Cuervo-Mulet, Delgado-Ospina & Paparella, 2014). It makes food safe by inhibiting the growth of pathogenic bacteria due to the antimicrobial activity of lactic acid but detoxifies aflatoxin. With these desirable benefits, fermentation has been considered an effective way to reduce the risk of mineral deficiency among populations, especially in developing countries where unrefined cereals and /or pulses are highly consumed. It is also associated with the proliferation of microorganisms such as yeast and molds that might cause food safety concerns, reduction in provitamin A and antioxidant carotenoids as well as loss of vitamins and minerals (Ortiz- Ferruzi, 2017). Post-processing techniques proposed for condiment production in Africa include drying and salting of the final product (Achi, 2005; Eman, 2009).

However, while these methods could increase shelf life considerably, it is characterized by inherent disadvantages such as loss of volatile compounds and vitamins. However, studies have shown that fermentation drastically reduces anti-nutritional factors in many fermented legumes-based foods (Khan, Khan & Dullah, 2012; Okorie & Olasupo, 2014). It is well known that these foods contain naturally occurring toxins and anti-nutritional compounds. The removal of anti-nutrients from Nigerian fermented food is an important step in ensuring toxicological safety and quality. Fermentation plays a significant role in the detoxification of substrates; for instance, the removal of toxins during *kawal* production, through the fermentation of the leaves of *Cassia obtusitfolia* in Sudan has been shown to improve safety quality and acceptability (Egwim, Amanabo & Yahaya, 2013; Taylor & Duodu, 2015). Most of the legumes and oil seeds used for the production of condiments are inedible in their

unfermented state because they suffer from one drawback or the other. For instance, legumes are a particularly rich source of natural toxicants, including proteinase inhibitors, amylase inhibitors, metal chelates, flatus factors, hemagglutinins, saponins, cyanogens, lathyrogens, tannins, allergens, acetylenicfurans and isoflavonoid phytoalexins (Issoufou, Guo- Wei, Tidjani, Jin & Yong-Hui, 2013; Oguntoyinbo, 2014).

2.3 Effects of fermentation on legumes

2.3.1 Carbohydrates

The major carbohydrate in legumes is starch which provides the most calories in developing countries (Chaves- Lopez et al, 2014). Fermentation activates starch- hydrolyzing enzymes such as α amylase and maltase which degrade starch into maltodextrins and simple sugars respectively (Osman, 2011). Studies have shown an increase in glucose during the early stages of fermentation due to the starch-hydrolyzing effect of activated maltase on α amylase. The glucose released during fermentation is a preferred substrate for microorganisms fermenting the food and could partly explain the decrease in total carbohydrates after 24 hours of fermentation. When both glucose and fructose were present during the fermentation of some legumes and cereals, most of the microorganism's preferred glucose to fructose as a source of energy since the level of fructose remained the same.

2.3.2 Protein

The effect of fermentation on proteins has yielded inconsistent results likely due to different experimental designs, study durations, and variation in the initial protein or amino acid profile of foods. Several studies had reported an increase (Pranoto*et a*l., 2013), while others observed a decrease (Osman, 2011) in protein and/or some amino acids upon fermentation. It appears that most of these effects might not reflect actual changes but relative changes due to the loss of dry matter as a result of microorganisms hydrolyzing and metabolizing carbohydrates and

fats as a source of energy. While an increase in protein may partly be attributed to the loss of dry matter during fermentation, bacterial fermentation is known to increase lysine content in fermented grains. This increase might partly be due to the degradation of complex proteins by microorganisms thereby releasing peptides and amino acids (Pranoto et al., 2013). However, it is reported that fermenting microorganisms also uses amino acid which could lower the protein content and quality of some fermented food (Osman, 2011; Pranoto et al., 2013). Fermentation increases the digestibility of plant proteins (Alka, Neelam & Shruti, 2012). Plant protein has poor digestibility relative to animal protein. Poor protein digestibility may cause gastrointestinal upset which might result in fecal excretion of protein. Hence, increased protein digestibility could reduce the levels of undigested proteins which can potentially cause food allergies due to poor absorption in the gut combination of fermentation with other processing methods has more advantages. For example, fermentation followed by cooking was effective in increasing the digestibility of grain protein, bringing it nearly to the same level as meat and also partial pre-digestion of grain proteins by bacteria during fermentation (Day &Morawicki, 2018).

There is also a reduction in tannins, oxalate, phytic acid, and carbohydrates which could be complex with proteins and hence limit accessibility by digestive enzymes (Hassan, Yusuf, Adebolu & Onifade, 2015). More improvement in protein digestibility by fermentation is due to the partial breakdown of complex storage protein into more soluble forms. When comparing the effect of *Lactobacillus plantarum* and natural fermentation for 36 hr on protein digestibility of sorghum flours using in vitro models, protein digestibility was increased by 92% and 47% using *L. plantarum* and natural fermentation, respectively. This increase was attributed to increased proteolytic enzymes in *L. plantarum* that could not only degrade tannins which complex with proteins but also break down complex proteins thereby liberating more peptides and amino acids. Doudu, Taylor, belton & Hamaker (2003) have previously reported that *L.*

plantarum possesses tannase that can cleave the protein–tannin complex thereby liberating proteins. Unfortunately, fermenting microflora could also utilize amino acids and proteins during fermentation resulting in loss of amino acid and proteins. It therefore, remains the optimum conditions for fermentation that could lead to maximum protein digestibility with minimal loss of protein.

2.3.3 Lipids

The significant lipolysis of legumes yields predominantly oleic, linoleic and linolenic acids (Ouoba, Cantor, Diawara, Traore, & Jakobsen, 2003). Free fatty acids, particularly oleic, linoleic and linolenic acids were associated with non-specific antitryptic activity and as such, extensive hydrolysis could diminish nutritional quality. Although oils constitute up to 40% of the legumes used in food fermentations, extensive lipolysis does not take place. Reports of low levels of lipase activity in *Parkia biglobosa* during *dawadawa* production as well as low levels of lipase in melon seed fermentation have been reported. Similarly, minimal participation of lipase in *Pentachletera macrophylla* during *ugba* production has been reported also.

Low lipase activity in some fermented foods has been considered desirable because of problems with objectionable taste and the development of rancidity. However, there are reports of the beneficial effects of lipase in the development of characteristic flavours and aromas (Ouoba, Diawara, Annan, Poll & Jakobsen, 2005). These results appear contradictory and require further study. An interesting area would involve the effects of change in lipid quantity and quality on the organoleptic characteristics of fermented condiment since there have been reports that aldehydes resulting from oxidation of lipids cause off-flavour and odour problems. However, organic solvent treatment of fermented condiments from locust bean, melon seed and soya bean on the objectionable odour, did not show any appreciation of the odour (Okorie & Olasupo, 2014). Not much can be said about the flavouring components of fermented

vegetable proteins used as condiments as no research has been carried out on this aspect. However, there is no doubt that amines, peptides and glutamic acid all contribute to this flavour.

2.3.4 Phytochemicals

For a long period, the importance of phytochemicals (phytonutrients) to human nutrition and health was not well known. Phytochemicals are important plant secondary metabolic products produced in phenylpropanoid biosynthesis and shikimate pathways during the growth of plants (Zhang, Xu, Xia, Jiang & Yang, 2015). During growth, L-phenylalanine, under the effect of phenylalanine ammonia lyase (PAL) catalyzation, changes into cinnamic acid. From then on, many phenolic components such as caffeic acid, ferulic among others are synthesized. These could later be converted into tannins, flavonoids, lignins, and other compounds. Advances in research have revealed the importance of these phytonutrients to human health under their antioxidant properties (Zhang et al., 2015), cholesterol-lowering effect (Golzarand, Mirmiran, Bahadoran, Alamdari & Azizi, 2014), and reduction in the production of pro-inflammatory cytokines and immune suppressive cells (Lesinski, Reville, Mace, Young, Ahn-Jarvis, Thomas-Ahner & Clinton, 2015).

Fermentation has a significant effect on phytochemicals that are both beneficial and adverse. Fermentation of high carotenoid biofortified maize resulted in a significant loss of carotenoids (Ortiz, Nkhata, Buechler, Rocheford & Ferruzzi, 2017) depending on the duration of the fermentation process (Ortiz et al., 2017). Wang et al., (2014) investigated the effect of fermentation on antioxidant profiles of four cereals using *Bacillus subtilis* and *L. plantarum*. There was a significant increase in the total phenolic acid and total flavonoid contents with the greatest increase in samples with starter culture. Dordevic, Marinkovic & Dimitrijevic-Brankovic (2010) demonstrated that *Lactobacillus rhamnosus* was more effective than *Saccharomyces cerevisiae* in releasing total phenolics during fermentation of cereals. During fermentation, microorganisms break down cereal grain matrices leading to the release of bound

phytochemicals (Dordevic et al., 2010). *L. plantarum* and *B. subtilis* have been previously reported to possess β-glucosidase that could cleave glucoside bonds between phytochemicals and sugars thereby releasing phytochemicals (Kuo, Cheng, Wu, Huang & Lee, 2006). Thus, the ability of fermentation to increase the antioxidant properties of foods can be explored as a cost-effective way to reduce oxidative stress within the body after consuming such foods.

2.4 Starter cultures

A starter culture is a preparation of microbiological cultures that performs or initiates fermentation. Starters usually consist of a cultivation medium, such as grains, seeds, or nutrient liquids that have been well colonized by the microorganisms used for the fermentation (Bachmann, Pronk, Kleerebezem & Tuesink, 2015). Initially, starter cultures needed to be prepared just before use. Today, they might be frozen and lyophilized or dried and prepared on an industrial scale and the choice of starter culture is usually dependent upon the substrate or raw material being fermented. A starter culture might consist of bacteria, molds, yeasts, or a combination thereof. Lactic acid bacteria are of critical importance in fermentation. Desirable properties of starter cultures include rapid acidification, predictable fermentation processes, desirable sensory characteristics (including taste, texture, aroma, and consistency), and the reduction of harmful microbiota (Katz, 2012).

2.4.1 Traditional fermentation and starter cultures

Fermentation could occur either through traditional methods or industrial production. Early methods of fermentation occurred in small batches and involved spontaneous fermentation from microorganisms present in raw goods (i.e., vegetables, fruits, milk, meat, and grains) under the proper conditions (Cogan, Beresford, Steele, Broadbent, Shah, & Ustunol, 2007). The outcome and taste of the fermented product in traditional fermentation are dependent on the quality and range of microbiota contained in the natural starter culture. For other

fermentation processes, inoculation is necessary and a small sample of a previously fermented product is saved and used as inoculate for new raw material (Guillo & Giudicci, 2009). This is referred to as back slopping and this method is still used today in the making of some artisanal cheeses, kombucha, sourdough, and sauerkraut.

2.4.2 Commercial fermentation and starter cultures

While methods of traditional fermentation are still being used in various cultures today, the majority of fermented goods are now produced through large-scale industrial techniques. With the discovery of microorganisms, and recent insight into their genetics, metabolism, and interaction with raw material have allowed for the ability to improve products and the process of fermentation by using specifically designed and isolated starter cultures (Holzapfel, 2002). Bread, alcohol, and vinegar were among the first to be 'produced' from starter cultures in the nineteenth century, leading to the industrialization of the process, then dairy and meat fermentation followed a century later. Today, manufacturers of fermented foods could either use highly concentrated ready-to-use starter cultures or propagate the starter culture in factory production (Katz, 2012). The decision to use one starter culture method over another depends on various factors including economic value, the number of products produced, the degree of automated fermentation, and the level of microbiology expertise. Variations in commercial starter cultures include direct vat inoculates, frozen or freeze-dried cultures, or starter cultures formulated with stabilizer and carrier genes (Katz, 2012). Lactic acid bacteria constitute the majority of the volume and value of modern starter cultures. A commercial culture starter with direct inoculation results in the highest level of safety and yield flexibility (Ng, Yeung & Tong, 2011).

2.4.3: The use of starter culture in fermented foods

The use of starter cultures for the production of alkaline fermented foods including seasoning agents have been making progress in the last two decades. Modern researches on fermented foods have begun to adopt new approaches that focus on understanding the profile and the role of associated microorganisms in alkaline fermentations. Many different techniques have been adopted to study the diversity of micro flora of fermented foods and their possible roles. This might be grouped into two: cultural/physiological methods and molecular methods (Temmerman, Hiys & Swings, 2004). Molecular techniques are of great importance in studying the microbial profiles, succession and functionality in traditional fermented foods. Functional genomics is a useful tool in improving traditional process as this enables comparisons of traits of microorganisms involve in food fermentation and enables selection of organisms with desirable traits as potential starter cultures.

The microorganisms used as starter cultures in food processing are selected based on food substrate, with the objective of achieving objective and reproducible bio-modification. In the efforts towards commercialization and upgrading of African alkaline fermented foods to industrial level, different species of microorganism have been studied and screened (Achi, 2005).

Pure cultures of *B. subtilis var.* natto is used in the commercial preparation of Japanese natto (Kiuchi, 2004). Species of *B. subtilis* have been studied and demonstrated as potential starters for *soumbala* (Ouoba, Cantor, Diawara, Traore & Jakobsen, 2003; Ouoba et al., 2004). Similarly, strains of *Bacillus* species have been screened and suggested as starters by researchers from Nigeria. These include *B. subtilis* B12, for *ugba* (Sanni et al., 2002) and *B. subtilis* for *okpeye* (Oguntoyinbo, 2014). The use of *B. subtilis* fpdp2, *B. subtilis* 24BP2 for *soy dawadawa* production has been demonstrated (Amoa-Awua, Terlabie, Sakyi-Dawson, 2006; Terlabie, Sakyi-Dawson & Amoa-Awua, 2006).

Despite successful applications and demonstrated beneficial roles of various starter cultures in food fermentations, their use in commercial traditional food productions is still limited and a subject of controversy. However, the prospect for commercializing the production of starter cultures for use in production of traditional foods and seasoning agents look promising.

2.4.4 Starter Cultures as Inoculants of Fermentation Processes

In the fermented food sector, "Appropriate" starter cultures are widely used as inoculants, from household to industrial, low-income and low-middle-income economies (Holzapel, 1997). These starter cultures are generally produced using a back-slopping process that uses samples from a previous batch of a fermented product as inoculants. The inoculation belt used in traditional fermentations in West Africa serves as a carrier of undefined fermenting microorganisms, and is one example of an appropriate starter culture (Holzapel, 1997). It usually comprises of a woven fiber or mat or a piece of wood or woven sponge, saturated with a previous fermentation batch's "high"-quality product. To act as an inoculant, it is immersed in a new batch.

The inoculation belt is used for the production of native fermented porridges, *uji* and *mawe*, as well as for the production of Ghanaian beer *pito*. Another example of an "appropriate" starter culture produced by back slopping is *iru*. This starter culture is produced from concentrated fermented *dawadawa* (a result of fermented legumes), mixed with unfermented legumes, vegetables such as pepper, and cereals such as ground maize. It is preserved in a dried form and is used as an inoculant in *dawadawa* fermentations in West Africa (Holzapel, 1997).

2.4.5: Selection Criteria for Starter Culture Development

Spontaneous food fermentations are neither predictable nor controllable because the microbial flora of the environment cannot be determined. Therefore, single-and mixed-strain cultures

must be tested on the pilot scale before being used in small-scale operations. According to Holzapfel (1997), the introduction of starter cultures in traditional small-scale fermentation should take into account considerations for improving the processing conditions and quality of the product by rapid accelerated metabolic activities (acidification or alcohol production, improved and more predictable fermentation processes, desirable sensory attributes, improved safety and reduced hygienic and toxicological risks.

The primary screening is based primarily on the evaluation of candidate strains' ability to withstand the pressure conditions imposed by fermentation processes (Gilberto, Dão, Ana, Susan, Luiz, Antonio & Carlos, 2019). At the beginning of the process, microbial cells are impaired by osmotic pressure due to the high concentration of solutes in the fermentation medium, such as grape sugar, hydrolyzed malt or sugar cane, milk matrix proteins and lipids and vegetable sodium (Yousef & Courtney, 2003). Cell exposition to such hypertonic conditions leads to an efflux of water from the cell, decreasing both turgor pressure and water availability (Bauer & Pretorious, 2000). It causes main microbial metabolism enzymes to be inactivated, in addition to increasing toxic ion concentration and eventually cell death. Nonetheless, through natural selection it is possible to distinguish strains that are well suited to these stressful environments (Gilberto et al., 2019). These resistant strains respond to osmotic stress by accumulating specific solutes, such as potassium ions, amino acids such as (glutamine, glutamate, proline, y-aminobutyrate, and glycinebetaine) and sugar (sucrose, trehalose, and glucosyl glycerol), which decrease the activity of water within the cytoplasm and increase both cell volume and turgor near their prestress values.

When the cell adapts to the new environment and fermentation begins, other stressors become relevant, such as the accumulation of organic acids and alcohols, changes in temperature, and acidifies the environment ((Yousef & Courtney, 2003). Rising temperature and/or decreasing pH are probably the most common environmental factors that starter cultures have to deal with

during fermentation processes. Commercial starter cultures usually come from either food substrates or the processes in which they are applied. Environmental conditions, back-slopping, adaptation and repeated use of similar utensils might help select the microbial communities typical of the fermentation process. The selection of appropriate starter strains should take into account their interactions in mixed cultures, taking into account the behavior of these strains under defined conditions and within the food substrate. Other factors to be considered include competitive behaviour, viability and survival, antagonism against pathogens and spoilage microbes, the rate of acid or alcohol production, organoleptic changes, primary metabolites of fermentation, degradation of antinutritive factors, detoxification and probiotic features ((Holzapel, 1997).

2.5 Food condiments

The term condiment comes from the Latin word *condimentum*, meaning "spice, seasoning or sauce" and is from the Latin *condere*, meaning "preserve, pickle, season". Food condiments or spices are strong-smelling, sharp-tasting substances usually used to improve or enhance the flavour of food (Odebunmi, Oluwaniyi & Bashiru*.,* 2009). Other researchers defined spice as dried seeds, fruits, roots, bark, leaves, or vegetative substances used in small quantities as food additives for flavour, colour, or as preservatives. They are usually of vegetable source such as mustard, ginger, garlic, coriander, locust bean, etc. FAO, (2004) reported that spices in food minimize the rate of rancidity, and improve colour and flavour intensity of food and food products. Many condiments are either packed in packets or containers for example ketchup particularly when served with take-out food meals. Others are used during cooking to add flavour or texture to the food such as *okpeye* and *iru*.

Condiments were known in some parts of the world like Ancient Rome, Ancient China, Ancient Greece and Ancient India to improve the taste of spoiling foods before food

preservation methods were discovered, pungent strong-smelling spices and condiments were used to make the food more acceptable to the mind or feelings.

2.5.1: Fermented condiments

Fermented foods are foods produced or preserved by the action of microorganisms and the results are interesting flavours, textures, and smells of foods. Fermented foods are also those foods that have been subjected to the action of micro-organisms or enzymes so that desirable biochemical changes cause significant modification to the food.

The use of indigenous fermented vegetable proteins as seasonings and flavour enhancers in Nigeria is very popular, especially among rural dwellers. These fermented condiments bear different names according to the country or region from which they are produced. African locust bean tree (*Parkia biglobosa*), for instance, is one of the most common plants whose seeds are used as protein source condiment after fermentation. It is consumed by various socio-ethnic groups in the West African sub-region, and it bears different names across the region. It is popularly known as *afitin/sonru/iru*in Benin (Azokpota, Hounhouigan, Annan, Nago & Jakobsen, 2008; Azokpota, Hounhouigan & Nago, 2006), *iru/dawadawa* in Nigeria (Ajayi, Akinrinde & Akinwunmi, 2015; Daramola, Fasominu, Oje & Makanju, 2009), *soumbala* in Burkina Faso (Ouoba et al., 2003) and *netetu* in Senegal (Ndir, Lognay, Wathelet, Cornelius, Marlier & Thonart, 2000).

Even within a country, the names of these condiments vary from one part to another. The origin of such names, however, could be attributed to the region or area of manufacture of the condiment, the type of legume or oil seed used and the spelling according to the region or area. In Nigeria, for instance, the Yorubas of Southwestern Nigeria locally call fermented condiments *iru*, the Hausas of the Northern part call it *dawadawa* and the Ibos of the Eastern part call it *ogiri* (Olasupo, 2006). *Owoh*, on the other hand, is a popular name for fermented condiments among the Urhobos and Itsekiris in the Niger Delta region, while the Igala and

Idoma people of the Middle Belt region call it *okpeye* (Olasupo et al., 2010). The traditionally fermented foods in Nigeria are sub-divided into:

- ✓ Roots and tubers (*garri*, *lafun* and *fufu*)
- ✓ Cereals (*ogi*)
- ✓ Legumes (*dawadawa* and *iru*)
- ✓ Milk (local cheeses)
- ✓ Beverages (palm wine and *pito*)

2.6 Processing of some fermented food enhancers

2.6.1 *Ogiri*

Ogiri is a popular African fermented seasoning, traditionally prepared by the fermentation of castor oil seeds (*Ricinus communis*). Depending on locality, season and availability it might also be obtained by fermenting melon seeds (*Citrullus vulgaris)* and fluted pumpkin seeds (*Telfairia occidentalis*). *Ogiri* is used in flavouring many traditional soups. It is regarded as an indispensable seasoning in the preparation of specialized soups which are highly cherished and extensively consumed by the Igbo ethnic group in South-Eastern Nigeria. Like many indigenous fermented products, the production of *ogiri* is still done traditionally and the process might vary between cultures. The production process includes the shelled seeds of castor oil wrapped in blanched banana leaves and boiled for about 8 hours until the seeds are properly cooked. The wrapped seeds are then placed near the fireplace to ferment for 4–6 days depending on the intensity of the fire.

On completion of this stage, the fermented seeds which are now sticky and strong smelling are ground on a grinding stone or mortar into a fine paste which is divided into small portions and packaged in blanched banana leaves. (Ibeabuchi, Olawuni, Iheagwara, Ojukwu & Ofoedu,

2014). They could be packed and placed near the fireplace or in a warm place to ferment further for 1–2 days. At this stage, the fermented condiment is ready for use or sale and it has a characteristic strong pungent flavour. *Bacillus* strains mainly *B. subtilis and B. licheniformis* have been reported as the predominant fermenting microorganisms (Enujiugha, 2009).

2.6.2 *Dawadawa/Iru*

This is one of the most important food condiments in Nigeria and many countries of West and Central Africa. *Dawadawa* or *iru* is made from seeds of African locust-bean (*Parkia biglobosa*) seed, a leguminous tree found in the Savannah region of Africa, Southeast Asia and South America. *Dawadawa* is produced traditionally by boiling the African locust beans for twelve hours in excess water until they are very soft to allow for hand dehulling after which the separated cotyledon is boiled for another two hours to soften it. The cotyledon is then wrapped with enough banana leaves (*Musa saplentum*) and packed with cover to ferment at room temperature (Okpara & Ugwuanyi, 2017).

After fermentation, it is normally sun-dried and moulded into round balls or flattened cakes. Due to its high protein content, it has great potential as a key protein source and basic ingredient for food supplements. An improved process for industrial production of *dawadawa* involves dehulling African locust bean with ball (disc) mill, cooking in a pressure retort for one hour inoculating with *Bacillus subtilis* culture, drying the fermented beans and milling into a powder.

2.6.3 *Ugba*

Ugba is a Nigerian-based condiment prepared by fermentation of seeds of the African oil bean (*Pentaclathra macrophylla*). It is also known as *ukpaka* by the Igbos in the South eastern part of Nigeria where it is most popular. *Ugba* is consumed as a delicacy, and appetizer, or used as a flavouring agent in various traditional dishes. Prepared in different ways, *ugba* is an important

food product for various traditional ceremonies (Ugwuanyi, 2016). The production, like other traditional processes, is still carried out in various homes on a small scale under uncontrolled conditions resulting in products that are non-uniform in quality. The basic procedures involve boiling oil bean seeds for 12 hours or more, removing the seed coat, and slicing the cotyledons into thin slices. The slices are then soaked in water overnight, washed thoroughly, and wrapped with fresh leaves for fermentation to take place.

Fermentation is usually done at ambient temperature and the duration varies depending on the intended use. Fermentation can last as short as 3 days or up to 5 days. Microbiological and biochemical changes that take place during the traditional process have been studied extensively. A diverse group of microorganisms were reported to participate in the traditional fermentation of African oil bean, with *Bacillus cereus* dominating the process (Ahaotu et al., 2013).

2.6.4 *Owoh*

Owoh is another fermented condiment mainly used as a seasoning in mid-Western Nigeria. The raw seeds are toxic and inedible. The traditional process involves boiling cotton seeds until they are properly cooked and become tender. The seed coats are removed manually. The cotyledons are then washed, wrapped in banana leaves and boiled again for 1–2 h. The wraps are removed from the water and placed in calabashes or earthen pots, and then covered with jute sacks and placed in a warm location (often beside the fireplace) to ferment

Fermentation is done at ambient temperature for 2–3 days. At the end of the fermentation, the mash is ground and moulded into balls. The product might be used at this point, but preferably it is sun-dried to extend the shelf life and also to develop a more desirable aroma. The major fermentative organisms are reported to be Bacillus species including *B. subtilis, B. licheniformis* and *B. pumilus.* (Ezekiel, Ogunshe & Jegede, 2015).

2.6.5: *Bikalga*

One of the most common condiments in Burkina Faso is *Bikalga*, also known by different ethnic names in different parts of African. It is known as *dawadawa-botso* in Niger Republic, *datou* in Mali, *furundu* in Sudan and *mbuja* in Cameroon. It is a product of *Hibiscus sabdariffa L*, alkaline fermentation commonly referred to as Roselle or sorrel (Parkouda, Nielsen, Azokpota, Ouoba, AmoaAwua, Thorsen, Hounhouigan, Jensen, Tano-Debrah & Diawara, 2009). *Bikalga* is used to flavour most dishes and is a good source of protein (22 - 30 per cent), lipids, carbohydrates, essential amino acids and fatty acids in the diet (Yagoub, Mohamed, Ahmed & El Tinay, 2004). *Bikalga* production includes seed washing, overnight cooking (12 - 24 hours) with the addition of liquid ash as a softening and alkalizing agent, followed by 3 - 4 days of fermentation, crushing, moulding, overnight steaming and finally sun drying.

The predominant organisms involved in the fermentation of *Hibiscus sabdariffa* to *Bikalga* are *Bacillus spp*. notably *B. subtilis subsp. subtilis and B. licheniformis*. This condiment is also steeped in lukewarm water during the preparation of food for a few minutes and steep water is used to make stews, soups, sauces among other foods (Parkouda et al., 2009).

2.7 *Prosopis africana*

Prosopis are pods bearing trees as seen in figure 2.1 or shrubs that occur in arid and semiarid zones of America, India, Africa and Asia. *Prosopis* consists of about 45 species of leguminous spiny trees and shrubs growing widely in subtropical and tropical regions of America, Africa and southwest Asia (Geesing, 2011). *Prosopis Africana* is the only *Prosopis* native to intertropical Africa, occurring from Senegal to Cameroon to Ethiopia throughout the Sudanian and Guinean ecozones, reaching the border of the Sahelian ecozones in the north (Le Houerou, 2011). It is present in Savannah, wooded grassland and riverine forests and also grows in a variety of soil types from sandy to clay soils. The trees of *Prosopis africana* are common in

the middle belt and Northern parts of Nigeria respectively (Ajiboye, 2009). Its young leaves and shoots are fodder that is highly sought after towards the end of dry season.

2.7.1: Description and Distribution

Prosopis africana is a perennial plant which reaches 4-20 m in height and has an open crown and slightly rounded buttresses. The bark is very dark, and scaly and has orange to reddish brown-white streaks. Its flowers are greenish white to yellow with glabrous petals, free-standing stamens and fragrant. The pods are dark brown, cylindrical, thick and hard, shiny with woody walls (Weber, Larwanou, Abasse & Kalinganire, 2008). The pods are usually compartmented with about ten loose, rattling seeds per pod with a thin, inter-marginal line around.

In selection of seeds, mature pods ar ground are ground in stone mills and clean seeds obtained by sieving and flotation. Fresh non-dried seed lots do not require a pretreatment. Pretreatment is often required to overcome physical dormancy in dried seeds. In planting, spacings ranging from 6x6m to 13x13 m have been used in triangular formations. Closer spacings produces faster canopy closure but appears to have little effect on forage yield of the mature forest. In their natural environment, pods are eaten and the seeds distributed by animals. The conditions experienced in the digestive tract assist germination. This process could be simulated by soaking the seeds in 0.5 M HCl for 24 hours.

The germination is aided by scarification using a scalpel, knife or file, makes the seed coat permeable to water, and hence facilitates germination. Alternatively boil seed and leave them to cool in the water for 24 hours. Seeds that are treated with 95% tetraoxosulphate vi (H_2SO4) for 5 minutes show increased levels of germination. Once seed-coat dormancy is broken, germination usually occurs within a week at 25^o C (+/-5^o C). In a germination test germination was observed on day 5 at 30ºC, day 6 at 25ºC and day 8 at 20ºC. Seed germination reaches

80% by day 8 at 25ºC, which is the optimum germination temperature for *P. africana*. Growth is fairly slow and therefore, seedling protection is recommended.

The pods are usually picked directly from the tree by shaking or beating fruit bearing branches. The period of time within which the pods could be collected is relatively short. Extraction of Prosopis seed (Fig 2.3) is generally difficult because the seeds are imbedded in a pulpy mesocarp (Fig 2.2) within a hard dry pod. Grinding mills have been used to remove the outer dry pod. The pods are then soaked in a 0.1 M solution of hydrochloric acid for 24 hours. The pods could then be washed in water for 1 hour and sun dried. The dried mass could then be pounded to separate the seeds from the coating. An alternative method is to allow the pods to dry for several days, followed by immersion in boiling water and allowing them to cool as they soak for 24 hours, after which the fruits are easily opened. With effort the seeds could be cleaned by hand. In its natural habitat, *Prosopis africana* flowers just before the rainy season while its seeds mature between February and March.

2.7.2 Uses of *Prosopis africana*

a) **Food**- in many areas, the fermented seeds are used as flavour enhancers such as *okpeye.*
b) **Fodder**- young leaves and shoots are used as fodders, branches are frequently broken off or lopped and cattle eat the pods.
c) **Fuels** - the wood has a high calorific value of about 1720 joules/kg and produces excellent charcoal and firewood.
d) **Timber-** in Senegal, it is preferred for art and craft work while in Ghana, it is used for pestles, mortars, mallets, cudgets and furniture construction of railway lines, and boat building.
e) **Gum or resin** – *Prosopis africana* yields a gum tannin or dyestuff. The roots and barks contain tannin which gives a reddish tint to the leather.

f) **Medicine**- the leaves are used for the treatment of headaches and toothache. The leaves and bark are combined and used to treat rheumatism. Remedies for skin diseases, fever and eye washes are obtained from the bark. The roots are also used to treat gonorrhea, tooth and stomach ache, dysentery and bronchitis. The bark could also be used for wound dressing lotions and as a source of potash for soap making. In Mali, the leaves, bark, twigs and roots are used to treat and relieve bronchitis, dermatitis, tooth decay, dysentery, malaria and stomach cramps. In Ghana, the boiled roots serve as a poultice for sore throat, root decoction for toothache, and bark as a dressing or lotion for wounds and cuts. The pod's ashes are also a source of potash for soap making.

g) **Services-** the trees of *Prosopis africana* helps in soil conservation suitable for shades in dry areas, have the potential to improve soil fertility by fixing atmospheric nitrogen, provide a useful mulch for the soil and are suitable as an avenue tree. It also has great potential for parkland agro-forestry systems and for improved agroforestry technologies in the Sahel, where it grows well in valleys and rocky soils.

Fig 2.1: ***Prosopis africana*** **tree**

Source: www.westafricanplants.senckerberg.

Fig 2.2: *Prosopis africana* pulp
Source: www.westafricanplants.senckerberg.

Fig 2.3: Seeds of *Prosopis africana*

2.8: Indigenous fermented foods.

Accurate identification and characterization of the microbiota of indigenous fermented foods (IFFs) is a critical first step for the selection of multi-functional starter cultures. Starter cultures are required for the controlled and large-scale production of IFFs with improved quality and safety attributes (Ahaotu, Anyogu, Obioha, Aririatu, Ibekwe, Oranusi, Sutherland & Ouoba et al., 2017; Edema & Sanni, 2008; Soro-Yao, Brou, Amani, Thonart & Dje et al., 2014).

Previously, the beneficial effects of fermented foods were focused mainly on preservation and desirable organoleptic characteristics. Nowadays, a better understanding of themicroorganisms involved in fermentation processes has drawn more attention to the various health benefits associated with IFFs. An important aspect of the production process of alkaline fermented condiments such as *iru*, *soumbala* and *bikalga* produced from these protein-rich substrates is the long cooking time, and this heating process might select for spore formers that are more heat resistant (Ouoba, Diawara, Christensen, Mikkelsen & Jakobsen, 2007; Parkouda et al., 2009). In addition, the degradation of proteins during fermentation by *Bacillus* spp., most significantly, *B. subtilis*, *B. pumilus*, and *B. licheniformis*, leads to the accumulation of peptides and ammonia. This leads to an increase in pH, which also favours the proliferation of *Bacillus* spp. (Ouoba et al., 2008).

Understanding the microbes involved in African IFF production would help in the design of starter cultures. However, culture-independent methods rely solely on DNA, so do not allow for the direct selection of microbial starters. Depending on the method used, DNA may be extracted from live and dead cells (Mukisa et al., 2012; Diaz et al., 2019). These limitations may be overcome by using both culture-dependent and independent methods (Adewunmi, Oguntoyinbo, Keisam, Romi & Jeyaram, 2013; Schoustra, Kasase, Toarta, Kassen & Poulain, 2013). Despite these recent developments, there remain significant knowledge gaps concerning the microbiota of IFFs from Africa.

2.8.1: Molecular identification techniques of microorganisms in fermented foods

Taxonomic information of an unknown microbe is highly essential to establish its biodiversity, relationship among other organisms in the ecosystem and its functional aspects (Gevers et al., 2005). Thus, proper isolation and identification is mandatory before deducing the novel characteristic of any microbial isolate. The prevalent conventional techniques are not sufficient to provide a complete draft for microbial taxonomy as these conventional techniques describe only shape, colour, size, staining properties, motility, host-range, pathogenicity and assimilation of carbon sources (Prakash et al., 2007). They are also time consuming as well as dependent upon many environmental factors (Rastogi & Sani, 2011). A comprehensive approach is required to furnish the information and subsequent derivation of a microbial lineage such as DNA amplification and sequencing which include (16S rRNA, *gyrB, rpoA, rpoB, rpoC, rpoD*), whole genome sequencing and are better due to their fast reactions, high specificity and less chance of error.

Despite the progress and development of genome-based techniques, no individual technique can be relied upon solely as a source of taxonomic information due to their protection strategy — viable but not culturable (VBNC) state (Rompre et al., 2002) and culture independent techniques targeting a single gene (such as 16S rRNA gene) also cannot provide adequate resolution for proper microbial identification (Koser et al., 2012). Therefore, the use of a polyphasic approach involving a combination of molecular biology techniques and conventional microbiological methods is necessary to obtain a better understanding of microbial diversity.

2.8.1.1: DNA amplification and sequencing: the gene sequence analyses discriminate several strains of microorganisms in a more precise manner rather than the conventional practices. As in the case of 80% of isolates, a close match occurs within a described species and 10% represent a new species within a genus and the remaining 10% might represent novel taxa

which can only be deduced through the sequence-based identification procedures in contrast to the conventional practices (Drancourt et al., 2000). The reasons behind the use of 16S rRNA gene to be utilised for identification purpose include the occurrence of the gene in all organisms performing the same function, the gene sequence is conserved sufficiently containing conserved, variable and hyper-variable regions, and around 1500 bp of sequence size which is relatively easy to sequence and large enough to contain sufficient information for identification and analysis of phylogeny (Clarridge, 2004). A major advantage of this method is the rapid and accurate identification of species.

2.8.1.2: *rpoa, rpob, rpoc* and *rpod* gene sequencing: The most common entity among ribosomal RNA encoding genes is their universality and *rpoB* encodes the β-subunit of the bacterial RNA polymerase Some recent developments regarding the *rpoB* based identification system includes the identification of four hyper-variable zones along the gene sequences, that is two zones on the *rpoB* gene of 350 and 450 base pairs (bp) in size and two more flanking spacers at both the ends of the same gene, i.e. *rpoL–rpoB,* 301–310 bp, and *rpoB–rpoC*, 86–177 bp (Gundi et al., 2009). Hence, species-specific primers have been designed from the hyper-variable regions among the gene for rapid identification of any bacterial species and has been shown to be more discriminative than the 16S rRNA gene. Similarly, *rpoB* gene is found to exist as a single copy in the bacterial genome which limits the nonspecific amplification and chances of experimental errors. Also, *rpoA* gene sequences that code for the α-subunit of RNA polymerase have been shown to be useful for many bacterial species like *Enterococcus* spp. and lactic acid bacteria (Naser et al., 2005). *rpoC*gene is also taken into account for the identification of many species of bacteria, it encodes for beta subunits of DNA dependent RNA polymerase and is organized in an operon. The conserved regions of *rpoD* sequences have been employed widely for the identification procedures of many *Actinobacteria species* as well as *E. coli*.

***2.8.1.3: gyrA and gyrB* sequencing**: DNA gyrase is encoded by both *gyrB* and *gyrA* which belongs to the single gene family. The presence of highly conserved motifs in these gene sequences provides a useful tool for the designing of universal primers for the study of bacterial identification and diversity. As higher genetic variation is observed among the protein coding genes, they can be used for the identification and classification of closely related taxa. Variation of *gyrA* gene sequences has been found to discriminate among *B. subtilis* group whereas *gyrB* is useful to discriminate among members of *B. cereus* group. The greatest advantage of using *gyrB* sequences in identification practices is that, the average base substitution rate of 16S rRNA gene is 1% per 50 million years, whereas, the rate is estimated to be 0.7–0.8% per one million years in the case of *gyrB* (Chun & Bae, 2000). Hence, those species having completely identical 16S rDNA sequence could be differentiated using *gyrB* gene sequences in addition to the 16S rDNA sequence data.

2.8.1.4: PCR based finger printing techniques: There are various PCR fingerprinting techniques available for the amplification of polymorphic DNA through specific selection of primer annealing sites. These molecular typing methods of bacteria are extremely reliable, rapid and highly discriminative as well as reproducible. The fundamentals behind these techniques are the use of PCR with primer sets that are complimentary to the highly conserved, naturally occurring, repetitive DNA sequences present in multiple copies in distinct intergenic positions throughout the genome of most Gram-negative and Gram-positive bacteria. In comparison to REP-PCR, ERIC-PCR is highly sensitive and useful in detecting microorganisms from any environment. BOX-PCR is the superior to all the techniques creating distinct fingerprinting patterns, however, ERIC and REP-PCR are the methods which are primarily used for genotyping (Frye & Healy, 2006). Most importantly, arbitrarily primed polymerase chain reaction (AP-PCR) can amplify fragments of DNA from any genome varying the size distribution of amplified fragments among species. Thus, the closely related taxa

possess similar fragment distributions, while that of the distantly related taxa are more divergent, hence, providing considerable phylogenetic information (Healy et al., 2005).

2.8.1.5: Whole genome sequencing: Whole genome sequencing (WGS) also known as full genome sequencing, complete genome sequencing, or entire genome sequencing, is the process of determining or sequencing of the entire genome or DNA sequence of an organism and shows a complete microbial profile of the community (Illumina, 2013). It has recently emerged as a new tool, and offers great potential in the way we investigate, assess and manage microbiological food safety issues and illnesses. It allows the identification and characterization of micro-organisms with a level of precision not previously possible, therefore potentially minimizing much of the uncertainty which impacts the ability to respond and manage microbiological food safety issues effectively and efficiently. Applying WGS as a new method has important applications in food safety, particularly for foodborne disease surveillance and outbreak investigations (Ronholm et al., 2017). This, in addition to its rapidly declining costs, increases the attractiveness of incorporating such a tool in food safety management.

2.8.2: Microbial hazards in indigenous fermented foods

Globally, food safety remains a significant challenge. The World Health Organization (WHO) estimates that as many as 1 in 10 people fall ill, and more than 120,000 children under 5 die each year after consuming unsafe food. Africa bears a high burden of the global incidence of foodborne illness with an estimated annual morbidity of 90 million (WHO, 2015; WHO 2017). Microbial hazards, including foodborne pathogens and their toxins, are primary aetiological agents of foodborne disease (FBD) and a growing public health issue. Fermented foods are generally considered safe. Fermenting organisms, especially LAB, produce a range of antimicrobial compounds such as organic acids, ethanol, bacteriocins and hydrogen peroxide,

which are antagonistic to the growth and survival of foodborne pathogens (Adinsi, Mestres, Akissoe, Viera-Dalode, Anihouvi, Durand & Hounhouigan, 2017; Mpofu, Linnemann, Nout, Zwietering, Smid & den Besten, 2016).

Cason et al. (2020) reported the decline of pathogenic and spoilage organisms during cereal fermentation for sesotho production. Similar observations were made by Karamoko, Djeni, N'guessan, Bouatenin & Dje (2012), who noted a 4 – 7 log reduction in faecal coliform counts within a 24 h period in fermenting palm wine. When investigating the microbiological quality of milk products in Tanzania, Schoder, Maichin, Lema & Laffa (2013) detected *Salmonella and Escherichia coli* in raw milk but not in fermented milk. In addition, Oguntoyinbo & Narbad (2015) isolated bacteriocin producing *Lactobacillus plantarum* strains from *kunu* and *ogi* that showed 300 antimicrobial activity against *Salmonella enterica*. However, indigenous practices for food production are often based on spontaneous fermentation that is chance inoculation or the use of back slopping where utensils from a previous fermentation are reused.

Limited knowledge and utilisation of Hazard Analysis and Critical Control Points (HACCP) and good manufacturing processes (GMP) by farmers, food producers and handlers could lead to production and processing occurring under unhygienic conditions. These factors lead to variation in the microbial profile of IFFs, and consequently, the presence of spoilage and pathogenic bacteria in these foods could be ruled out (Oguntoyinbo, 2014; Olasupo et al., 2016). An evaluation of the microbiological quality of water used for processing, fermenting broth, and the fermented cassava product, lafun, identified microbial hazards such as coliforms, including *Salmonella* spp. and *Staphylococcus* spp. (Lateef & Ojo, 2015). Potentially pathogenic bacteria have been found in utensils used for the fermentation process (Gran, Mutukumira, Wetlesen & Narvhus, 2002; Jans et al., 2017); however, only a few studies investigate the complete production chain to identify the source of contamination (Ademola et al., 2018; Thorsen et al., 2015).

Adedeji et al. (2017) reported similarities in the microbial profiles of potentially pathogenic bacteria isolated from two fermented condiments from the same producer at the retail level. It is important to note that these bacteria were not present in the raw materials, suggesting unhygienic processing and handling. This underscores the need for further research to identify contamination sources to support the management of food safety hazards. The microbiological safety of fermented vegetable proteins usually dominated by *Bacillus spp*. and *Staphylococcus* spp. requires consideration, as acid production, a potent antimicrobial attribute in lactic fermented IFFs, is not present (Ahaotu et al., 2013; Ouoba, Vouidibo-Mbozo, Anyogu, Obioha, Sutherland, Jespersen & Ghodddusi, 2019). *Bacillus cereus* and *Staphylococcus aureus* can produce toxins in food during their growth and have been identified in these products (Oranusi et al., 2015). *Bacillus* spp. are the dominant organisms involved in the fermentation of oil bean seeds; however, the presence of *Bacillus cereus* is routinely reported (Ahaotu et al., 2013; Ouoba et al., 2008; Parkouda et al., 2009; Thorsen et al., 2015). Ahaotu et al. (2013) isolated *Bacillus cereus* capable of producing enterotoxins in *ugba* under fermentation conditions.

A similar observation was made by Ouoba et al. (2008) when investigating *B. cereus* involved in locust bean fermentation for soumbala production. However, these are heat-labile toxins that should be denatured with adequate cooking. Thorsen et al. (2015) detected the heat-stable, emetic type toxin-producing *B. cereus* strains in fermented baobab seeds, which is of concern. The metabolic activities of some microorganisms involved in the fermentation process provide antagonistic conditions to the growth and survival of foodborne pathogens. However, inappropriate handling and the use of unsanitary packaging material can introduce microbial hazards post processing (Adinsi et al., 2017; Mpofu, Linnemann, Nout, Zweitering & Smid, den- Bensten, 2016; Schoder et al., 2013). The occurrence of potentially pathogenic bacteria at the retail level across all food categories highlights the potential risks to public health associated with IFFs (Odom, Udensi & Nwanekezi, 2012; Owusu-Kwarteng et al., 2018). Of

particular concern is the isolation of pathogenic bacteria from cereal-based and dairy fermented products, some of which are used as weaning or complementary foods (Adekoya et al., 2019; Samet Bali, Felfoul, Lajnaf, Attia & Ayadi, 2016). The presence of these organisms in ready-to-eat products suggests that IFFs might serve as vehicles of pathogenic bacteria. Therefore, the safety of these foods should not be taken for granted or assumed. The use of next-generation sequencing methods has provided more insight in identifying microbial hazards in IFFs, highlighting a role for metagenomic approaches as food safety tools (Walsh et al., 2017).

2.8.3: Safety of microorganisms in African fermented foods

The fermentative process, which is considered a crucial phase in the production of fermented foods, is spontaneous or achieved by back-slopping, a process that affects product quality and safety. However, the spontaneous nature of the fermentation processes and poor control measures during and after processing predispose African fermented foods to a diversity of microorganisms. Thus, it is possible to find mixed populations of microorganisms including the technologically important, as well as microbial pathogens with negative implications for food safety and public health. The introduction of pathogenic organisms could occur at different stages of the value chain through raw materials, the processing environment, and food handlers. An evaluation of the microbiological quality of water used for processing, and fermenting broth has identified microbial hazards such as coliforms, including *Salmonella spp*. and *Staphylococcus* spp. (Lateef & Ojo, 2015). Potentially pathogenic bacteria have also been found in utensils used for the fermentation process (Gran et al., 2002; Jans et al., 2017);

Furthermore, several pathogenic bacteria and molds, and their toxins have been identified in African fermented seed condiments. Specifically, pathogenic bacteria such as *B. cereus*, *Staphylococcus* spp., *Eschericia coli*, *Salmonella* spp., *Shigella* spp., and *Clostridium* spp., as well as mycotoxigenic molds such as *Aspergilus flavus* and *A. parasiticus*, have been isolated

from African alkaline fermented seed condiments, giving rise to food safety and public health concerns (Adekoya *et al.,* 2019; Parkouda et al., 2009).

The occurrence of *B. cereus* in West African alkaline fermented food condiments is prevalent and of considerable concern for food safety and the health of the consuming public. *B. cereus* strains have been isolated in high numbers in most fermented condiments in Nigeria and Africa generally. Ahaotu et al., (2013) isolated *B. cereus* from *ugba* a Nigerian fermented condiment. Ouoba et al., (2008) reported the presence of *B. cereus* from *soumbala* and *bikalga*. Also, in *okpeye*, *B. cereus* was isolated, found to be toxigenic, and was capable of producing toxins in mixed starter culture fermentation for *okpeye* production (Oguntoyinbo et al., 2007).

Another group of microorganisms that are commonly found in West African fermented seed condiments, with food safety and public health concerns, are members of the genus *Staphylococcus* and other potentially pathogenic species. Adedeji et al., (2017) reported the presence of *S. sciuri subsp. sciuri* and *Alcaligenes faecalis, B. anthracis, Proteus mirabilis* although their toxin production profiles were not determined. The presence of *Acinetobacter baumanii, Clostridium sartagofum, E. casseliflavus, Comamonas testosteronii* and *Aeromonas* species were reported in *ugba* (Okorie, Olasupo, Anike, Elemo & Isikhuemehen et al., 2017). Adekoya et al., (2019) also reported the presence of high numbers of members of the *Enterobacteriaceae* family in *ogiri*, *ugba* and *iru*, with *Sphingomonas paucimobilis* and *E. coli* being the dominant Gram-negative bacterial species detected. The author also reported toxigenic mold like *A. flavus* producing which produced mycotoxins from the condiments.

Furthermore, Ouoba et al., (2019) reported the presence of coagulase-negative and positive *Staphylococcus* species in alkaline fermented foods *soumbala* and bikalga using Rep-PCR/PFGE. The species reported include *S. epidermidis, S. pasteuri, S. condimenti, S. piscifermentans* and *S. simulans* from *soumbala* as well as *S. aureus, S. cohnii, S.*

*haemolyticus*and *M. caseolyticus* from *bikalga.* All these preceding reports raise genuine concerns about the microbiological safety of fermented seed condiments and pose major challenges to the development and consumer acceptance of indigenous fermented condiments.

2.8.4: Microbiology of African fermented condiments

The microbiota of any fermenting food matrix is a function of the hygienic status of the production environment, the utensils, the raw material used and the handlers. The traditional fermentation method employed in the processing of most fermented African condiments is by chance inoculation (Ogueke, Anosike & Owuamanam, 2015; Sanni et al., 2002).

In a pioneer study by Odunfa (1981), colony morphology, and microscopic examination, including cultural and biochemical characterization were used to identify the microorganisms associated with *iru.* Similarly, bacterial isolates in *daddawa* using cultural characteristics and biochemical tests have been done. The microorganisms associated with the natural fermentation of mesquite seeds [*Prosopis africana* (Tuab)] for the production of *okpiye/okpeye* were also determined by microscopic parameters, cultural morphology and sugar fermentation profiles (Achi 1992).

Recently, the availability of diverse culture-dependent molecular typing methods and culture-independent molecular assessment technologies, based on analysis of DNA or RNA has led to an understanding of the microbial composition and quantification, biodiversity and distribution of these fermented food condiments (Table 2.1). For example, Adedeji et al (2017) and Ademola et al (2018) used the OTU of 16S rRNA gene sequencing to identify and characterize major fermenting microorganisms found in *iru* and *ogiri* (melon seed [*Citrullus vulgaris*] fermented condiment in Nigeria).

Also, in *soy-daddawa*, a Nigerian fermented soybean condiment, PCR- DGGE analysis of the 16S rRNA gene was used to identify the dominant *Bacillus* species occurring throughout the fermentation of *soy-daddawa* (Ezeokoli et al., 2016).

Microbial interactions occurring in spontaneously fermented seeds in Africa are also generally accompanied bya mixed population of aerobic endospore forming bacteria (AEB), particularly the genera *Bacillus, Brevibacillus, Paenibacillus* and *Lysinibacillus*. Other microorganisms that have been identified, along with the major *Bacillus* spp. dominating African seed condiments fermentation include members of the genera *Stapylococcus, Micrococcus, Proteus, Pseudomonas, Brevibacterium, Aerococcus*, lactic acid bacteria, yeasts and molds (Dakwa, Sakyi-Dawsom, Diako, Annan & Amoa-Awua, 2005; Jeff-Agboola, 2007; Ouoba, Nyanga-Koumou, Parkouda, Sawadogo, Kobawila, Keleke, Diawara, Louembe & Sutherland, 2010; Okorie & Olasupo 2013;). *Staphylococcus vitulinus* and *S. saprophyticus* was detected and identified in *iru* for the first time based on PCR-DGGE analysis of 16S rRNA gene and as a culture-independent molecular approach, among the major bacterial species where they represent about 63% of the bacterial population Adewumi et al., 2013; Adewumi, 2016;). Indeed, *Staphylococci* have been previously thought to be the second dominant bacterial flora after *Bacillus* species or co-dominate with *bacilli* in various indigenous African fermented condiments, using traditional culture-dependent techniques, involving phenotypic, biochemical and molecular characterization (Ogbonna, Sokari & Achinewhu, 2001; Parkouda et al., 2010; Chadare, Jonkman, Wolkers-Rooijackers, Nout, Hounhouigan & Zweitering, 2011; Jeyaram, Romi, Singh, Adewumi, Basanti & Oguntoyinbo, 2011; Okorie & Olasupo 2013)

For the succession of microbial species involved in the alkaline fermentation of seed condiments, *Bacillus* species are often reported to dominate throughout the fermentation period while LAB are often detected at the later stages of the fermentation processes. It is glaring from

these reports that spontaneous fermentation of indigenous and African condiments involves a complex microbial community, their interactions, and metabolic activity greatly influence the quality of these fermented condiments.

2.9 Nutritional properties of fermented flavour enhancers

Fermentation has generally been observed to improve the nutritional qualities and safety of fermented food products (Anukam & Reid, 2009; Chung, Mee, Se & Sang, 2010). Due to the proteinaceous nature of the plant raw materials used for the processing of these condiments, the natural fermentation of these raw materials is generally characterized by extensive hydrolysis of the proteins into peptides, amino acids and ammonia, resulting in pH increasing to about 8–10 in the final product (Leejeerajumnean, Duckham, Owens & Ames, 2001; Omafuvbe et al., 2004).

Proximate analysis of most fermented vegetable proteins of African origin has shown that these condiments are rich sources of protein, essential amino acids, vitamins and minerals. These components have been found to increase during fermentation of these condiments (Chung et al., 2010; Tofalo, Schirone, Perpetuini, Angelozzi, Suzzi & Corsetti, 2012). Other important biochemical activities such as the degradation of oligosaccharides to simple sugars have also been reported (Omafuvbe, Shonukan & Abiose, 2000; Ouoba, et al., 2007). During the fermentation process, *Bacillus spp.* degrade plant proteins into peptides and amino acids and the free amino acids are also utilized by these *Bacillus* as carbon and nitrogen sources, to produce ammonia/ammonium hydroxide, resulting in high pH values and the associated typical odour of these fermented condiments (Amoa-Awua et al., 2014; Ouoba, Diawara, Annan, Poll & Jakobsen, 2005; Parkouda et al., 2009).

Oil constitutes a major component of the legumes and oil seeds but lipolytic activities are minimal during the production of most African fermented food condiments. Many reports

confirm that vitamin levels are higher in fermented vegetable protein foods than in raw materials especially for riboflavin, thiamine, niacin, vitamin C and folic acid (Chung et al., 2010). Degradation of anti-nutritive factors, improvements in digestibility and increase in concentrations of vitamins, minerals, and essential amino acids have also been reported for fermented seed condiments (Makanjuola & Ajayi 2012; Okechukwu et al., 2012; Parkouda et al., 2009). Also, an increase in the concentration of essential amino acids, particularly, methionine, phenylalanine, leucine, isoleucine and lysine during alkaline fermentation of African locust bean has been reported (Ouoba et al., 2003).

Flavour enhancers made from proteins may be a good source of certain B vitamins but they are found to be deficient in ascorbate and some fat-soluble vitamins which are lost during fermentation. It is evident that most fermented food condiments of African origin are good sources of essential nutrients and could be used to produce complementary food supplements and macronutrients in fermented legumes and therefore enhance food quality.

Table 2.1: Predominant microorganisms associated with some African fermented flavour enhancers

Fermented condiment	Raw material/ substrate	Country	Predominant microorganisms	Method of identification	Reference
Ugba	African oil bean seeds	Nigeria	*B. cereus, Lysinibacillusxylanilyticus, B. clausii B. licheniformis, B. subtilis, B. safensis*	ITS-PCR, Rep-PCR for clustering, 16S rRNA, *gyrB, rpoB* gene sequencing,	Ahaotu et al., (2013)
Bikalga	Roselle seeds	Burkina faso	*B. subtilis, B. licheniformis.*	API50, ITS-PCR, rep-PCR and DNA sequencing	Ouoba et al., (2008).
Okpehe	*Prosopis africana*seeds	Nigeria	*Bacillis subtilis, B. amyloliquefaciens, B. cereus, B. licheniformis*	RAPD-PCR, ARDRA fingerprinting, 16S rRNA gene sequencing	Oguntoyinbo et al., (2007)
Maari	Baobab seeds	Burkina faso	*B. subtilis, S. sciuri, Ent. faecium*	API, rep-PCR (GTG)5-fingerprinting) and 16S rRNA gene sequencing.	Parkouda et al., (2010)

Table 2.1: Predominant microorganisms associated with some African fermented flavour enhancers (Cont'd)

Fermented condiment	Raw material/ substrate	Country	Predominant microorganisms	Method of identification	Reference
Iru	*Parkia biglobosa seed*	Nigeria	*B. subtilis, B. amyloliquefaciens, B. cereus, B. licheniformis, B. pumilus and Brevibacillusformosus*	Phenotypic tests, ARDRA, ITS-PCR, ITS-PCR-RFLP, RAPD-PCR, PCR-DGGE, 16S rRNA gene sequencing.	Adewumi et al., (2013); Adewumi et al., (2014).
Ntobambodi	Cassava leaves	Republic of Congo	*B. safensis, B. pumilus*	ITS-PCR, Rep-PCR for clustering, 16S rRNA, *gyrB, rpoB*gene sequencing.	VouidibioMbozo, Kobawilaa, Anyogu, Awamaria, Louembe & Sutherland (2017)
Soumbala	Locust bean	Burkina faso	*B. subtilis, B. pumilus, Staphylococcus simulans*	TS-PCR RFLP, 16S rRNA gene sequencing.	Ouoba et al., (2004); Ouoba et al., (2019)
Ikpiru	*Hibiscus sabdariffa*	Benin	*B. subtilis, B. cereus, B. amyloliquefaciens, B. licheniformis, B. safensis, B. altitudinis, B. aryabhattai, B. flexus, B. circulans*	Phenotypic tests, rep-PCR, M13-PCR, 16S rRNA, *gyrA*and *gyrB*genes sequencing.	Agbobatinkpo et al., (2013)

Table 2.1: Predominant microorganisms associated with some African fermented flavour enhancers (Cont'd)

Fermented condiment	Raw material/ substrate	Country	Predominant microorganisms	Method of identification	Reference
Ogiri	Melon/Castor oil seeds	Nigeria	*B. safensis, B. siamensis, B. altitudinis, B. encimensis*	16S rRNA gene sequencing.	Ademola et al., (2018)
Dawadawa	Soybeans	Nigeria	*B. amyloliquefaciens* *B. licheniformis, B. pumilus, B. subtilis, S. epidermidis, S. pseudintermedius*	High throughput sequencing 16S rRNA gene sequencing	Ezeokoli et al., (2018)
Mbuja	*Hibiscus sabdariffa*	Cameroon	*B. subtilis, B. megaterium, B. amyloliquefaciens, B. pumilus and B. cereus.*	Phenotypic tests, 16S rRNA and *gyrB* genes sequencing	Mohamadou et al., (2013).
Dawadawa	Bambara groundnut	Ghana	*B. subtilis subsp. subtilis, B. amyloliquefaciens subsp. plantarum, B. pumilus and B. licheniformis*	MALDI-TOF MS, 16S rRNA and *gyrA*genes sequencing	Akanni, De Kock, Naude & Buys (2018)
Afitin and *sonru*	*Parkia biglobosa*seed	Benin	*B. subtilis, B. licheniformis, B. cereus,*	ITS-PCR-RFLP, 16S rRNA gene sequencing.	Azokpota, Moller, Houghouigan & Jakobsen, (2007)

Table 2.1: Predominant microorganisms associated with some African fermented flavour enhancers (Cont'd)

Fermented condiment	Raw material/ substrate	Country	Predominant microorganisms	Method of identification	Reference
Owoh	cotton seeds (*Gossypium hirsutum*).	Nigeria	*B. subtilis, B. licheniformis, B. pumilus.*	Phenotypic tests	Sanni & Ogbonna (1991); Ezekiel et al., (2015).
Soumbara	*Parkia biglobosa seed*	C^ote d'Ivoire	*B. subtilis, B. velezensis, B. pumilis,*	16S rRNA genes sequencing, RFLP analysis	Adjoumani, Ouattara, Germain & Zeze (2019)

Source: Owusu-kwarteng et al., 2020

2.10: Toxins in fermented foods

All fungal toxins are collectively called mycotoxins and several mycotoxins (aflatoxins, ochratoxin A, zearalenone, deoxynivalenol, and citrinin) have been reported to be present in fermented foods (FF). The fungal toxins in South African traditionally brewed beers were investigated. The grains, barley, and maize used for the beer production, locally brewed and commercial alcohol beverages, were screened for the mycotoxin producer, and it was discovered that the grains contain *Aspergillus* spp*., Penicillium* spp*., Mucor* spp*., and Rhizopus* spp*.,* and some commercial beverages contained aflatoxins while some local beverages contained ochratoxin A and zearalenone (Odhav & Naicker, 2002).

Attieke, a traditional Ivorian fermented food, can be produced using mold-covered cassava inoculum. The inoculum and *attieke* samples were subjected to screening of mycotoxins. The results suggested that ochratoxin (0.2 µg/kg) and deoxynivalenol were present in the sample in trace amounts, and the biological testing study revealed that the consumption of *attieke* is safe (Kastner, Kandler, Hotz, Bleisch, Lacroix & Meile, 2010). The Nigerian food samples (maize, millet, Sorghum, sesame, fermented cassava, and flakes) were screened for ochratoxin A contamination. About 98% of the samples contained ochratoxin A, and more than 74% of samples contained > 5 µg of toxin/kg of the sample, which is regarded as unsafe to human health as per the European Union standard (Makun, Adeniran, Mailafiya, Ayanda, Mudashiru, Ojukwu, Jagaba, Usman & Salihu, 2013).

The traditional Nigerian condiments *iru* and *ogiri* and their raw materials, locust bean and melon seeds, respectively, were studied for mycotoxins and microbial contaminations. Pathogens such as *Bacillus anthracis, Staphylococcus sciuri subsp. sciuri, Alcaligenes faecalis, and Proteus mirabilis* were found in many of the samples, and about 25% and 23.5% of pathogens detected in the samples belonged to *Bacillus, and Staphylococcus* spp. family, respectively. The results suggest that some of the Nigerian raw materials and condiments are

severely contaminated with deadly pathogens. Aflatoxin was found in ogiri and melon while there were no toxins in other samples. The level of mycotoxin was low in the studied food samples (Adedeji, Ezeokoli, Ezekiel, Obadina, Somorin, Sulyok, Adeleke, Warth, Nwangburuka & Omemu, 2017).

In another study, Southwest Nigerian fermented food samples (maize gruel, sorghum gruel, locust bean, melon seeds, and African oil bean seeds) were found to contain mycotoxins. About 82% of tested samples had mycotoxin contamination. Fumonisin B1 was found to be dominantly present in sorghum gruel samples, where African oil bean seeds had aflatoxin B1 (3–36 μg/kg), and sterigmatocystin. One of the samples was found with multi-mycotoxin contamination. The studies claimed that people were not aware of toxins, their consequences, and the unhygienic practices (Adekoya, Obadina, Olorunfemi, Akande, Landschoot, De Saeger & Njobeh, 2017). Aflatoxin is a potent carcinogen and is an immunotoxic and hepatotoxic compound (Kensler, Roebuck, Wogan & Groopman, 2011; Roze, Hong & Linz, 2013). *Meju*, a fermented soybean sample, was screened for fungal contamination. *Meju* prepared with ethanolic extracts of *Nelumbo nucifera, Allium sativum, and Ginkgo biloba* was studied. The results showed that *Meju* samples contained about ten fungal species, which came under the genera *Agaricaceae, Mucor, Penicillium, Aspergillus, and Paecilomyces. Meju* prepared without plant extracts contained aflatoxin B1 and the ochratoxin A producing fungus, *Aspergillus ruber* (Shukla, Park, Park, Kim, Park, Dubey, Jeon, Khang & Kim, 2017). Fermented milk products are predominantly contaminated with aflatoxins. The fermentation and storage temperature, storage time, acidity, pH, heat processes, aflatoxin concentration, and strain used for the fermentation process are the major factors that influence the free aflatoxins in fermented milk products (Arab, Sohrabvandi, Mortazavian, Mohammadi & Rezaei Tavirani, 2012).

2.10.1: Bacterial Toxins

Bacillus spp. *is*olated from *Soumbala* (fermented *Parkia biglobosa* seeds) and *Bikalga* (fermented *Hibiscus sabdariffa* seeds) have shown enterotoxin production. *B. cereus* isolates are hemolysis positive and produce enterotoxins in both laboratory media and in fermented products (Ouoba et al., 2008). Recently, Agbobatinkpo, Thorsen, Nielsen, Azokpota, Akissoe, Hounhouigan & Jakobsen (2013) reported the prevalence of anerobic-spore forming *Bacillus spp*. in fermented *Hibiscus sabdariffa* seeds. They reported that among isolates, *B. subtilis, B. cereus, B. amyloliquefaciens, B. licheniformis, B. safensis, B. altitudinis, B. aryabhattai, B. flexus, and B. circulans* were predominant species (Table 2.2).

Yellow-water is a food flavor-enhancer, a by-product of Chinese traditional brewing liquor. Among the 50 samples, 17 samples contained toxigenic *B. cereus* contamination. More than 90% of the isolates were positive for at least two genes of the toxin coding genes. The study suggested that the microbiological quality of yellow-water needs improvement (Lin, Cai, Song, Yaun, Li, Zhang, Xing & Xiang, 2015). Gergoush (a fermented Sudanese bread snack) is made by several fermentation processes using different starter cultures.

A series of screenings on *Bacillus spp*. predominance in the starter cultures used to produce gergoush revealed that *B. cereus sensulato* (40–68%), *B. licheniformis* (16–27%), *B. subtilis* (8–32%), and *B. sonorensis* (4–20%) are the common contaminants. However, no viable cells were found after the baking process, so the study proved that the gergoush was microbiologically safe (Thorsen, Abdelgadir, Ronsbo, Abban, Hamad, Nielsen & Jakobsen, 2011). About eighty-seven *B. cereus s*ensulato strains were isolated from representative Korean fermented soybean products (Doenjang, ssamjang, kochujang, and cho-kochujang). The strains were positive for the enterotoxin coding genes in PCR analysis and were resistant to most of the β-lactam antibiotics and could potentially cause diarrheal diseases (Yim, Kim, Chon, Kim, Kim, Choi, Choi & Seo, 2015).

Adedeji et al. (2017) screened the Nigerian traditional condiments (*iru and ogiri*) for toxic compounds and microbial contamination. The result showed that the samples of *iru and ogiri*were deeply contaminated with enterotoxigenic microbes such as *Alcaligenes faecalis, Proteus mirabilis, Staphylococcus sciuri* subsp. *sciuri, and Bacillus anthracis*. The study claimed that the people who prepare the FF in the south-western Nigerian region are unaware of hygienic practices (Adedeji et al., 2017). *Clostridium botulinum* is the causative agent of botulism, a paralytic disease caused by bacterial neurotoxins and several foodborne botulism cases have been reported (Fu & Wang, 2008; Rebagliati, Philippi, Tornese, Paiva, Rossi & Troncoso, 2009).

2.10.2: Other Toxic Materials in fermented foods

Biogenic amines (BA) are another major toxic entity in fermented foods. Fish, fish products, and fermented foods have been detected with a high amount of BA, and this causes serious health issues to consumers. The presence and level of BA in food material is an indicator of microbial activity (microbial decarboxylation) in stored or processed foods. Histamine, putrescine, tyramine, cadaverine, and β-phenylethylamine are the common BA in FF, specifically, histamine is accountable for several food poisonings and outbreaks. Unhygienic manufacturing and storage practices are the reason for histamine poisoning in fish products. Several *Lactobacillus spp., Bacillus* spp*., Bacillus subtilis strains, Staphylococcus* spp*., Streptococcus* spp*., and Enterococci* spp. can produce BA in FF such as sausages, wine, natto, miso, and douchi (Doeun, Davaatseren & chung, 2017). The source, factors affecting the formation, toxicological properties, detection, and prevention of BA, and BA content in foods have been reported previously (Doeun et al., 2017; Spano, Russo, Lonvaud-Funel, Lucas, Alexandre, Grandvalet, Coton, Coton, Barnavon & Bach, 2010; Madejsika, Michalski, Pawul-Gruba & Oseket, 2018). Some of the plant toxins such as cyanogenic glycosides have been reported in fermented foods (fermented bamboo shoots). The traditional methods of cooking

effectively reduced the cyanide toxins from several bamboo shoot-based foods soidon, soibum, and soijin (Devi, Chakma &Yenkokpam, 2017).

2.10.3: Detection Methods

Several conventional detection methods are available for detecting the presence of pathogens and toxins in FF. Culture-dependent methods (growing the microbes on laboratory media and characterization), biochemical profiles, microscopic observations, and PCR were commonly used for microbial detection. Immunology-based methods such as ELISA, Limulus amoebocyte lysate, rabbit pyrogen test, and chromatography techniques were employed for the detection of toxins in FF. Recently, many biosensors have been in use to detect the contamination in food processing and storage. Different bioreceptors (such as enzymes, antigen/antibody, phages, and nucleic acids) were used in the biosensors to improve the efficiency and accuracy. Optical, electrochemical, nanomaterial-based sensors exist to detect the toxic substances in foods.

A detailed review of biosensors used for the detection of pathogens and toxins has been reported recently (Alahi & Mukhopaddhyay, 2017). A PCR-based detection of endosymbionts of *Rhizopus microsporus*, which is commonly used to produce tempe and sufu, has been reported. A quick PCR validation (ketosynthase gene) of the starter cultures used to produce soybean fermented product could be a safe way to detect the toxicogenic symbiotic bacteria (Rohn, Scherlach, Mobius, Partida- Martinez & Christain- Hertweck, 2010).

Moller, Gunvig & Bertram (2010) proved that NMR (Nuclear Magnetic Resonance) relaxometry is a reliable and propitious technique for monitoring the microbial safety in fermented meat production. Shiga toxin producing *E. coli* (STEC) strains could be detected by PCR. The primers of *rpoB, eaeA, stx1, and stx2* genes were used for the promising detection of STEC in meat and dairy samples.

Table 2.2: Some Bacterial contamination of indigenous fermented foods from Africa

Country	Food product	Pathogens	Analytical method	References
Republic of Benin	*Lanhouin*	*B. cereus*	Convectional	Anihouvi, Ayernor, Hounhouigan & Sakyi-Dawson (2006) Thorsen, Kando, Sawadogo, Larsen, Diawara, Ouédraogo, Hendriksen & Jespersen (2015)
Republic of Benin	*Gowe*	*E. coli*	Convectional	Adinsi et al. (2017)
Bukinafaso	*Bikalga, Soumbala*	*B. cereus*	Convectional and PCR	Thorsen & Varnam (2008)
Bukinafaso	*Maari*	*B. cereus*	Convectional and PCR	Thorsen et al. (2015)
Bukinafaso	Fermented milk	*S. aureus, coliforms*	Convectional	Ciss´e, Muandze-Nzambe, Somda, Sawadogo Drabo, Tapsoba, Zongo, Traoré & Savadogo (2019)
Cote d'Ivoire	*Attieke*	*B. cereus* *S. aureus* *Klebsiella* spp. *Citrobacter* spp.	Convectional	Kouam´e, Djeni, N' guessan & Dje (2013)
Cote d'Ivoire, Kenya, Somalia	Fermented milk	*S. aureus*	Conventional and PCR	Jans et al. (2017)
Egypt	*Karish* *Ras*	*E. coli*	Conventional and PCR	Ombarak, Hinenoya, Awasthi, Iguchi, Shima, Elbagory & Yamasaki (2016)
Egypt	*Kareish*	*B. cereus*	Conventional and serology	Sadek, Fathi & Salem et al. (2006)
Ethiopia	*Kocho*	*Acinetobacter spp.*	Conventional and PCR	Birmeta, Bakeeva & Passoth (2019)

Table 2.2:Some Bacterial contamination of indigenous fermented foods from Africa (Contd')

Country	Food product	Pathogens	Analytical method	References
Ethiopia	Fermented milk	*Escherichia* spp., *Shigella* spp., *Klebsiella* spp.	Conventional and PCR	Fall, Tounkara, Diop, Thiaw &Thornart (2017)
Ghana	*Nunu*	*L. monocytogenes*	Conventional and PCR	Owusu-Kwarteng, Wuni, Akabanda & Jespersen (2018)
Ghana	*Nunu*	*E. coli,* *K. pneumoniae*	PCR	Walsh et al. (2017)
Morocco	*Lben,* *Jben*	*E. coli 0157:H7*	Conventional and serology	Benkerroum, (2013)
Nigeria	*Lafun*	*E. coli, S. aureus, Salmonella typhimurium*	Conventional	(Lateef & Ojo, 2015)
Nigeria	*Kindirmo*	*S. aureus,* *E. coli*	Conventional	Dikko, Malik &Egena (2011
Nigeria	*Kindirmo, Manshanu*	*L. monocytogenes*	Conventional and PCR	Usman, Kwaga, Kabir & Olonitola (2016)
Nigeria	*Iru*	*B. cereus group*	PCR	Adedeji, Ezeokoli, Ezekiel, Obadina, Somorin, Sulyok, Adeleke, Warth, Nwangburuka & Omemu (2017)
Nigeria	*Ogiri igbo*	*E. coli,* *S. aureus*	Conventional	Oranusi, Okereke, Braide & Okorondu (2015)
Nigeria	*Burukutu*	*S. aureus* *E. coli*	Conventional	Alo, Eze & Eda (2012)
Nigeria	*Wara*	*S. aureus, E. coli* *B. cereus* *Salmonella* spp	Conventional	Omemu, Obadina, Taiwo & Obuotor (2014)

Table 2.2: (Contd). Some Bacterial contamination of indigenous fermented foods from Africa

Country	Food product	Pathogens	Analytical method	References
Nigeria	*Ugba*	*B. cereus*	Conventional and PCR	Ahaotu et al. (2013)
Nigeria	*Iru*	*B. cereus group*	PCR	Ademola et al. (2018)
Nigeria	*Ogi* *Ogiri*	*B. cereus* *E. coli*	PCR	Adekoya, Njobeh, Obadina, Chilaka, Okoth, De Boevre, & De Saeger (2019)
Rwanda	*Ikigage*	*Coliforms*	Conventional	Lyumugabe, Kamaliza, Bajyana &Thonart (2010)
Senegal	*Guedj*	*Salmonella* spp., *Staphylococcus* spp.	Conventional	Fall et al. (2017)
South Africa	*Mahewu* *Umquobothi* *Ogiri*	*B. cereus* *B. cereus* *E. coli*	PCR	Adekoya et al. (2019)
Tunisia	*Rayeb*	*S. aureus* *Coliforms*	Conventional	Samet-Bali, Felfoul, Lajnaf, Attia & Ayadi (2016)
Uganda	*Bongo*	*Staphylococcus* spp., *Enterobactericeae*	Conventional	Mukisa, Ssendagala & Byakika et al. (2020)
Uganda	*Obushera*	*Coliforms* *Staphylococus* spp.	Conventional	Byakika, Mukisa, Byaruhanga, Male & Muyanja (2019)
Zimbabwe	Naturally sour milk	*E. coli*	Conventional	Gran, Mutukumira, Wetlesen & Narvhus (2002)

2.11: Sensory Evaluation Methods

Sensory evaluation measures the reaction to stimuli resulting from the consumption of a food or food product (Meligaard, Civille & Carr, 2014; Stone, 2012). It is concerned with providing answers to questions about product quality and existing competitor products as well as questions that are most often asked by persons concerned with technical, development, research and production; not only that but also factory managers, quality assurance managers and marketing managers (Mason & Nottingham, 2002). Normally, the personal preference and powers of perception involve the use of sense organs (Singham, Birwal & Yadav, 2015) and the right answers to sensory questions are extremely important for the existence of any food product tested as they determine its acceptability and provide effective decisions for future product success in the market (De Pelsmaeker, Gellynck, Delbaere, Declercq & Dewettinck, 2015).

Sensory evaluation helps in eliminating or controlling sources of unwanted error, which could either be through control of the environment, proper experimental design and sampling of human subjects to test the products. When conducting sensory analysis on food quality, it is imperative to know that there is possibility of getting wrong answers, which in turn could risk the competition of a particular food product in the market. Wrong information on food product could lead to miscommunication, improper decisions, wasted consumer research, and much more wasted time, effort and material costs (Singham et al., 2015). Panel members form an important tool of sensory analysis. Its value depends on the objectivity, precision and reproducibility of their judgments. Panelists need to be carefully screened, trained, calibrated and validated (Singh- Ackbaradi & Maharaj, 2014). Training enhances individual's understanding of sensory attributes and hence enable them provide accurate, valid, consistent, and standardized sensory measurements that could be reproduced. Each panelist must detect, recognize, and agree upon the exact connotation of each descriptive term and also be guided to

ensure consistence in their judgments by using reference standards that demonstrate variation in specific descriptive terms (Fuller, 2011).

Panelists are given test samples (food product) and should state their preferences. The word "preferred" could mean most acceptable, tastes best, looks best, would buy and the like (Singh-Ackbaradi *et al*., 2014). Consumer responses from the product are used to modify the product. The new food product could again be tested and where possible or necessary, modified further. Sensory analysis can therefore play a fundamental role in the management of product quality in the food industry (Mason & Nottingham, 2002).

2.12: Types of Sensory Evaluation Methods

2.12.1: Discriminative Tests

These tests are designed to determine if a difference exists between food products while the Panelists should be knowledgeable about the product in question for easy choice. Each member is required to make a choice among the given food products. Discriminative tests at some point may be used for different purposes (such as determining sample differences/ similarities and or quantity of degree of difference/similarities (Stone, 2012).

2.12.2: Triangular Test

In this test, normally three samples are involved when determining the overall difference between two products. Out of the three samples, two are similar and one is dissimilar. The samples must be coded with individual three-digit numbers. The taster is required to select the sample which is different from others. In these tests the chance of choosing the required sample correctly is greater. It is recommended that no more than six samples be evaluated at one testing session because the method is liable to fatigue of panelists. The tests require fewer tasters, at least 4-8 tasters are considered enough to carry single testing (Valentin, Pecher, Nguyen, Chambers & Abdi, 2012; Lawless & Heymann, 2010).

2.12.3: Duo-Trio Tests

This determines whether or not a sensory difference exists between two samples. There is always a reference sample and two test samples; of the two test samples, one sample is identical to the reference, and the other one is the test sample (Lee & Kim, 2008). The panel members are asked to identify the sample that is similar to reference sample. Duo-trio tests are sometimes used instead of triangle tests to compare unknown differences between samples however, they are considered less efficient than triangle tests. In this method, at least 7-10 evaluators are recommended (Lawless & Heymann, 2010; Purcell, 2017).

2.12.4: Paired Comparison Tests

These are applied when a difference in chemical composition of the sample which requires a sensory assessment is well known. Two differently coded samples are presented to each panelist at the same time and the task is to choose the sample that is perceived higher in the specified sensory attribute (Yang & May, 2017). Tasters are asked to judge the samples by comparing them without needing to rate the magnitude of the difference, for example, “are the two samples identical or different?” or “which of the two samples sugary?”. Compared to triangular test, paired comparison test is less tedious and frequently used for strongly flavored or complex products. At least 7-10 panelists like in the duo-trio are recommended in this test.

2.12.5: Descriptive Tests

In these tests, sensory attributes of products are characterized in order of their appearances and relative intensities are assigned. These provide more detailed profiles of a product by identifying the different characteristics within the product and quantifying them. Descriptive tests are more comprehensive and sophisticated as compared to discriminative tests (Pimentel, Gomes da Cruz & Deliza, 2015). They provide the basis for mapping product similarities and variances and determining those sensory characteristics that are important to acceptance. It is

normally performed by 6 to 15 meticulously selected and trained panelists. They are trained to evaluate products similar to how any instrument would give a reading. Descriptive tests include Free Choice Profile (FCP), Quantitative Descriptive Analysis (QDA), Flavor Profile Analysis (FPA), Texture Profile Analysis (TPF) and Time Intensity Analysis (TIA) (Meligaard et al., 2014).

2.12.6: Free Choice Profile (FCP)

In this method, there is no prior training of the panelists, each judge decides his/her own list of attributes to label the product. The judge should constantly be trained and the response computerized, then a time-intensity curve obtained for the determined attribute. Analysis of variance is used to analyze three parameters from the curve, namely maximum intensity, the point at which maximum is reached, and the first point at which no more perception occurs (Cruz, Cadena & Walker, 2010).

2.12.7: Quantitative Descriptive Analysis (QDA)

This consists of progressive survey of sensory terms for a product generated by a trained sensory panelist using nontechnical language. Trained judges normally reach a consensus on the relative discrepancies between the samples (Stone & Sidel, 2004). QDA and FCP have the same purpose of determining the intensities of all product attributes and also defining the complete sensory profile.

2.12.8: Flavour Profile Analysis

This is useful for identifying sample taste and odour. It is a technique that provides a written record of noticeable aroma of a product, flavour and aftertaste components. Panelists characterize individual aroma and flavour in the order perceived and assign a constant rating scale. Normally 4-6 panelists are suggested. They independently examine the product and

record their impression in terms of aroma, flavour and aftertaste. Finally, report is presented to a panel leader in an open discussion (Curren, Snyder, Abraham & Suffet, 2010).

2.12.9: Texture profile analysis (TPA)

This has been widely applied to test solid and semisolid food products. Usually, it involves a panel of 6-9 members. Textural attributes and other evaluation procedures are established unanimously by panel members before carrying out the evaluation of the products in question (Mochizuki, 2005; Rosenthal, 2010). Texture profile analysis is convenient for rapid evaluation of food texture which is normally measured only by humans. In some experiments, liquid samples that could keep their shape but flow under gravity are poured into a cup and subjected to uniaxial compression. Then the parameters obtained from uniaxial compression are then discussed without considering the physical meaning of these parameters namely hardness, cohesiveness and adhesiveness (Rosenthal, 2010).

2.12.10: Time Intensity Analysis (TIA)

This is used to estimate the change in intensity of a determined characteristic with time. It has the main role of determining the intensity of any descriptor term in a product with time. TIA and FCP are among the descriptive sensory tests mostly used in scientific studies and by the food companies (Cruz et al., 2010).

2.12.11: Affective/ Consumer Acceptance Tests

Affective methods are also called subjective methods and very useful for evaluating food acceptability or preference (which product is liked or preferred). Normally large number of respondents is required (50-150 panelists considered adequate). Panelists are not trained but selected based on previous use of product, economic social level and geographical area (Meligaard, Civille & Carr et al., 2014).

2.12.12: Preference Ranking

In this technique, three or more samples are rank-ordered with one sample being preferred over the other. This type of test supply information about people's likes and dislikes of a product and determine how various samples differ based on a single distinguishing attribute. In consumer analysis, the panelists are asked to rank the coded samples according to their preference (Hein, Jaeger, Tom & Delahunty, 2008).

2.12.13: Hedonic Rating Scale

This is among of the widely used sensory evaluation methods that measure consumers' level of liking of food products (Lawless & Heymann, 2010). In practice there are 9-point Hedonic scale, 7-point Hedonic scale and 5-point Hedonic scale. The 9-point Hedonic scale range from "like extremely'' to "dislike extremely''. Practically, not fewer than five points are recommended (Stone, 2012). The panelists can be trained or semi trained and usually between ten to fifty people.

2.12.14: Some of the Requirements for Sensory Evaluation

There should be clear objective for conducting sensory analysis, appropriate area for preparation of food samples to be tasted, test procedures which should be properly implemented in a way that reduce risks to health of participants, good laboratory, proper method of sample presentation, sensory panel members suitable for evaluation (If training is needed, they must be trained), utensils / glass wares suitable for different foods to be tested, sophisticated sensory booths with controlled temperature (20^{o}C – 22^{o}C) and relative humidity at 40 ± 5%, suitable lighting in booths, right coding of samples (usually 3 digit coding is preferred) and special software for statistical analysis of sensory data.(Kemp, Hollowood & Hort, 2013). Sensory evaluation is very crucial in the new product development. When properly implemented with controls, careful screening of panelists, effective training as well as proper

statistical interpretation, it could provide many benefits and form a basis on which accurate decisions can be made relating to the food product in question (Drake, 2007).

2.13 Summary of literature review

Fermented foods abound in the native African cuisine either as main course meals, beverages, or food condiments, and in most cases, constitute the main source of nutrition for the rural dwellers. Protein-rich plant seeds are fermented into condiments in Nigeria and Africa as a whole and they constitute an important component of the peoples' diets. These condiments are popular across the for their sensory characteristics and high nutritional value, where they serve as flavouring in soups and stews, and form a significant source of low-cost protein in the predominantly starchy staple region.

The fermentative process, which is considered as a crucial phase in the production of fermented foods, is spontaneous or achieved by back-slopping, a process that affects product quality and safety. Consequently, a huge diversity of microorganisms has been detected associated with the fermentation of West African traditional fermented seed condiments. Thus, it is possible to find a heterogeneous population of microbiota, including the technologically important, as well toxigenic microorganisms in the spontaneously fermented condiments with negative implications for food safety and public health. In addition, the natural fermentation process, heavy handling, improper transportation, and poor packaging increase the risks of exposure to varieties of microorganisms, some of which could be pathogenic.

For the past few decades, the identification of microorganisms in fermented food have been based on phenotypic approach with its inherent shortcomings, especially its inability to isolate and identify viable, but unculturable, microorganisms. Unculturable, yet viable, microorganisms are known to be in most food matrix which are usually in low numbers. Recently, there has been a boom in the identification of these microorganisms using molecular

based techniques which include polymerase chain reaction (PCR)-based amplification, amplicon sequencing, high throughput (HTS), sequencing and gene sequencing of 16S rRNA genes. Using these molecular techniques have helped to identify major and minor microbial population, diversity and succession in fermented food condiments. It has also helped to investigate their individual roles during fermentation; Many are either contaminants or pathogens, serving minor/major roles in fermentation.

Pathogenic microorganisms such as *B. cereus, Staphylococcus* spp*., E. coli, Salmonella* spp*., Shigella* spp*., and Clostridium* spp. have been isolated from fermented condiments, giving rise to food safety and public health concerns. The occurrence of *B. cereus*, an opportunistic human pathogen which causes food poisoning is prevalent and of considerable concern for food safety and health of the consuming public. This organism has been isolated in high numbers from several fermented seed condiments including *ogiri, okpeye, ugba, sonru, iru, afitin, dawadawa* and *bikalga*. It is therefore imperative to investigate the safety of these condiments sold in commercial market.

Furthermore, the use of microbial starters, their improvement, development, and standardization have been driving forces in transforming traditional food fermentations in developing countries from an art to a science. The use of well- defined safe starter cultures during fermentation of these condiments would guarantee consistency, improve product safety and enhance product quality of our indigenous condiments.

CHAPTER THREE

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 PROCUREMENT OF RAW MATERIALS

The 18 *okpeye* samples (250 g) were purchased from three retailers in six different markets in Imo (Relief, World Bank and Ekeukwu Owerri) and Anambra (Ose, Bridgehead and Ochanga) States, South Eastern Nigeria. The three samples from each market were grouped together to form six samples altogether.

3.1.2 EQUIPMENT AND CHEMICAL REAGENTS

The chemical reagents used for this research were of analytical grade. The Analyses were done at the laboratories of the Department of Food Science and Technology, Federal University of Technology, Owerri and Biomedical Science laboratory, University of West London, United Kingdom.

3.2 METHODS

3.2.1 Isolation of bacteria from commercial *okpeye* samples

The 18 samples were compressed into six samples (6) according to the six different markets. Bacteria were isolated from six (6) commercial samples of *okpeye* bought at different markets in Owerri and Onitsha in Imo and Anambra States respectively. Ten (10 g) grams of each sample were measured and put into a stomacher bag and 90ml of maximum recovery diluents were added to it to make up 100 ml aliquots. 1ml were homogenized for 1–2 min in 9 ml maximum recovery diluents (MRD; Oxoid CM0733, Basingstoke, UK). Ten-fold dilutions were prepared in MRD and 0.1 ml of suitable dilutions were spread on Tryptone soya agar (TSA; Oxoid CM0733), Mannitol salt phenol-red agar (MSA: Millipore 1.05404.0500) and MacConkey agar (MAC: Oxoid CMO0007) in duplicates. The TSA plates were incubated at

27 °C for 24 h to enumerate the population of aerobic mesophiles, MSA plates were incubated at 37 °C for 24-48h for the enumeration of *Staphylococcus* and MAC plates also incubated at 37°C for 24-48h to enumerate *Enterobacteriaceae* (Plates 9-14). Morphological characteristics of colonies on all the plates were examined and the number of colonies forming units (CFU) was recorded (Appendix i). Representative dominant colonies were isolated and purified by streaking several times on TSA. Stock cultures were maintained in beads and stored at −20 °C for further analyses.

3.3 Phenotypic identification of the *okpeye commercial s*amples

Phenotypic identification methods help to provide useful but preliminary information about a microorganism before identifying the bacterium using molecular techniques. These include colony morphology, Gram staining, catalase and oxidase test (Appendix ii).

3.3.1 Gram staining

A loop-full of distilled water was placed on a slide and a minimum amount of colony was smeared on the slide mixing it thoroughly with the water allowing it to dry completely. Heat fix was done by passing the slide quickly through the Bunsen flame three or four times. The slide was placed on the rack over the staining vessel and covered with crystal violet, allowed to stay for one minute and gently rinsed with running water. Then the smear was covered with iodine and left for one minute and rinsed with water. Holding the slide at an angle with forceps, drops of ethanol was added on the smearand quickly and rinsed with water immediately. The smear was covered with the counterstain safranin, left for 30 seconds to one minute, rinsed with water and gently blot-dried with a paper towel. The slide was allowed to air dry and examined microscopically using some oil for the oil immersion lens.

3.3.2 Catalase test

According to the manufacture's instructions, a loop full of inoculum was placed on a glass slide, and carefully a drop of hydrogen peroxide (H_2O_2) was placed on the colony. Immediate formation of bubbles indicated a positive result while few or no bubbles indicated a negative result.

3.3.3 Oxidase test

Following the manufacturer's instructions, a strip was taken, and a medium swipe of each isolate was done and left on the bench. A positive result gave a blue/purple coloration within a few seconds. A negative result did not show discoloration but remained unchanged.

3.4 GENOTYPIC IDENTIFICATION

3.4.1 Extraction of DNA

The stock cultures that were maintained on beads and stored at −20 °C from (section 3.2.1) were restreaked on Tryptone Soya Agar TSA; Oxoid CM0733)) and incubated at 37 °C for 18-24 h to get distinct colonies. Chromosomal DNA of each isolate was extracted using InstaGene™ Matrix (Bio-Rad 732–6030, Hemel Hempstead, UK) according to manufacturer's instructions. 1ml of autoclaved high-purity water was added into sterile Eppendorf tubes and 2 to 3 of pure colonies taken carefully with a sterile loop and transferred into the tube mixing it thoroughly. The tubes were centrifuged for 2 minutes at 12000 rpm (Microstar 30 centrifuge, VWR) to obtain microbial cell pellets. The supernatant was decanted using sterile pippete tips leaving the microbial pellets at the bottom of the tubes. The microbial pellets were thoroughly mixed with 100 µl InstaGene mix and transferred to a heating block at 56°C for 30 minutes (Ouoba et al., 2008). The tubes were vortexed for 10 seconds and transferred back to the heating block at 100°C for 8 minutes. The tubes were vortexed again for 10 seconds, and transferred to a centrifuge at 12000 rpm for 3 minutes. 60 µl of the DNA was then transferred to a new

sterile Eppendorf tube and stored at −20 °C until required. The DNA concentration and purity (ng/uL) were estimated by using the Nanophotometer (IMPLEN) recording the DNA concentration, A/230/260 and A/230/260 concentrations respectively.

Electrophoresis: the DNA fragments were separated by applying 5 µl of each PCR product with 2 µl of loading buffer to 1.8% agarose gel (Sigma Life Science, SLCB 1897). DNA molecular marker (Direct Load TM Wide Range DNA Marker; Sigma) was included as standard. The gel was run in tris-borate-EDTA (1x TBE; Sigma T4415) buffer for 1h 30 min at 120 V, stained with 20 µl Sybersafe in 200 ml distilled water solution for 30 minutes. Bands were revealed with ultraviolet light and photographed using a Gboxsyngene (geneflow) (See appendix iii).

3.4.2 Rep- PCR fingerprinting of the isolates

The total DNA isolated from each of the 61 isolates was fingerprinted using Repetitive element PCR (rep-PCR) before identification by sequencing the 16S rRNA gene (Anyogu et al., 2014) using the GTG5 primer as described by Ouoba et al. (2008). Amplification was carried out in 25µl of reaction mixture containing 2 µl of DNA template, 2.50µl of 10xPCR buffer without $MgCl_2$ (10 U Applied Biosystems N808-0161), 4.00µl of dNTP (1.25 mMol)), 2.00 µl of $MgCl_2$ (25 mmol), 4.00 µl of primer GTG5 (5GTG GTG GTGGTGGTG- 5 pmol μl^{-1})1), 0.25 µl of Taq polymerase (5 U; Applied Biosystems N808-0161) and 10.25 µl of autoclaved high purity water (Sigma W4502). Amplification consisted of 30 PCR cycles in a thermocycler (Eppendorf). The cycling program was as follows: initial denaturation at 94°C for 4 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 45°C for 1 min and elongation at 65°C for 8 min. The PCR ended with a final extension at 65°C for 16 min, and the amplified product cooled at 4°C. The DNA fragments were separated by applying 10µl of each PCR product with 2 µl of loading buffer to 1.8% agarose gel (Sigma Life Science, SLCB 1897). DNA molecular marker (Direct Load TM Wide Range DNA Marker; Sigma) was

included as standard. The gel was run in tris-borate-EDTA (10 X TBE; Sigma T4415) buffer for 2 h at 120 V, stained with 20 µlSybersafe in 200 ml distilled water solution for 30 minutes. Bands were revealed with ultraviolet light and photographed using a Gboxsyngene (geneflow). (Plates 9 and 10 in appendix).

3.4.3 16S rRNA gene amplification (PCR)

An almost complete portion (full length 1560 bp) of the 16S rRNA gene for the 61 isolates whose DNA was extracted as described in section 3.4.1 was amplified using the forward primer 27F and either of the reverse primers 1100R/1492R using the methods as described by Ouoba et al., (2008) for the combination (27F/1100R) (27F/1492R). 50 µL volumes of the PCR products were amplified by mixing 1 µL each of extracted DNA with 5 µL of 10X PCR buffer (10 U Applied Biosystems), 5 µL of DNTP (1.25 mmol -1; thermos scientific), 0.5 µL of primer 27F, 0.5 µL of primer 1100R/1492R, 0.2 µL of AmpliTaq polymerase (Applied Biosystems) and 37.75 µL of sterile high purity water. 0.2 µl of AmpliTaq polymerase (Applied Biosystems) and 37.75 µl of sterile high-purity water. The amplification was achieved by 35 PCR cycles with the following procedure: first denaturation at 95°C for 5 min, then 35 cycles at 94°C for 1 min, annealing at 55°C for 1 min and elongation at 72°C for 1 min. The final extension was carried out at 72°C for 5 min and the product cooled at 4°C.

The PCR was confirmed by applying 5µl of each PCR product mixed with 2 µl of loading buffer to 1.8% solidified agarose gel (Sigma Life Science). DNA molecular marker (Direct Load TM Wide Range DNA Marker; Sigma) was included as standard. The gel was run in tris-borate-EDTA (10 X TBE; Sigma T4415) buffer for 1h at 120 V, stained with 20 µl Sybersafe in 200 ml distilled water solution for 30 minutes. Bands were revealed with ultraviolet light and photographed using a Gboxsyngene (geneflow). (Plate 17 in appendix).

3.4.4 Purification of 16s PCR products

The PCR product was purified using a QIAquick PCR Purification kit (Qiagen GmbH, Hilden, Germany) as instruction was stated inside the kit. An equal volume of membrane binding solution was added to the same volume of labeled PCR product. The solution was added to the tubes with the column from the kit and centrifuged at 16,000 rpm for 1 minute. The liquid at the bottom of the tubes was discarded and transferred to the columns with new labeled tubes. The columns were washed with 700 ml column wash to remove further unwanted compounds still stuck in the columns, centrifuged at 16,000 rpm for 1min and the flow through discarded. This process was repeated and centrifuged at 16000 rpm for 5 minutes. The tubes were spined for any extra liquid to be expelled and left for 30minutes for the ethanol to evaporate completely. New Eppendorf tubes were labeled and the columns were transferred to them, 15 µl of nuclease clear water was added to the columns to elute the DNA in each of the columns. The tubes were incubated at room temperature for 5 minutes, and centrifuged at 16,000 rpm for 1 minute. The columns were finally discarded as the DNA was now inside the tubes. All The DNA was stored at 4°C and sent for sequencing. Sequences were compared to those present in the Gene bank and Ezibiocloud database (Yoon *et al.*, 2017).

3.4.5 Sequencing of *gyrB* and *rpoB* genes

Bacterial identification was first carried out by amplification and partial sequencing of the 16S rRNA gene as described above. In the case where closely related species could not be separated by 16S rRNA gene sequencing, sequencing of *gyrB* and *rpoB*genes was carried out. For the *gyrB* gene, the reaction mixture described by Thorsen et al. (2011) was applied with the following PCR conditions: 94°C for 2 min, then 30 cycles at 94°C for 1 min, 66°C for 1 min and 72°C for 2 min. The final extension was carried out at 72°C for 7 min and the products cooled to 4°C. For the amplification of the *rpoB* gene, the method described by Anyogu et al. (2014) was used along with the following conditions: 94°C for 2 min followed by 40 cycles of

94°C for 30 s, 51°C for 45 s, 68°C for 50 s, and a final extension of 68°C for 90 s. Electrophoresis was used to check the PCR products and positive amplicons were purified as described previously.

3.5 Safety assessment of some microorganisms isolated from commercial *okpeye* samples

3.5.1 Haemolytic activity on blood agar

Twenty-eight (28) isolates of *Bacillus* and non-*Bacillus* including each representative isolates from the rep-PCR fingerprinting were screened for their haemolytic activity on blood agar as described by (Kavitha, Raja & Perumal, 2018). Columbia agar base (Oxoid CM003) was autoclaved at 121°C for 15 min and Sheep blood (7%) added after cooling to 50°C before distribution into Petri dishes. The isolates were streaked on the agar plates and incubated at 37°C for 48 h. Haemolysis was noted by the emergence of a zone of clearing around the colonies. Isolates without any clearing around the colonies were noted as non-haemolytic. (Plates 18-20 in appendix).

3.5.2 Enterotoxin (*Nhe, Hbl*), Cytotoxin (*CytK, entFM*) and emetic toxin (*ces*) production by *B. cereus* strains

For the detection of *nheA, nheB, nheC, hblA, cytK, entFM* and *Ces* using PCR, the reaction mixture was carried out as described by Guinebretière & Broussolle, (2002). The reaction mixture was a total volume of 25 µL comprising 1 µl of DNA, 2.5 µl of PCR buffer II (10·; Applied Biosystems), 2.5 µl of dNTP (2.5 mmol l–1; Thermo Scientific), 1.0 µl of primer 1(10 mmol; Applied Biosystems), 1.0 µl of primer 2 (10 mmol; Applied Biosystems), 0.25 µl of DNA polymerase (Applied Biosystems) and 14.40 µl of sterile high purity water. The reaction was performed under the following conditions: first denaturation step at 95°C for 3 min, then 35 cycles of denaturation at 94°C for 30 seconds, and annealing was done at different temperatures for the different genes (Table 3.1). The extension was at 72°C for 90 sec and a final extension at 72°C for 5 min. Positive PCR product amplicons were confirmed using

agarose gel electrophoresis as previously described. A known *Bacillus cereus* (B13) was used as a control during the PCR reaction mixture (Ouoba et al., 2008). During electrophoresis, a mixture without a DNA template served as a general control to detect positive and negative reactions.

3.5.3 Antibiotic susceptibility test

Antibiotic susceptibility of twenty-eight (28) isolates of *Bacillus* and non-*Bacillus* including each representative isolate from the rep-PCR fingerprinting was evaluated by using the disk diffusion method as recommended by EUCAST (2019). Mueller-Hinton agar was prepared according to the manufacturer's instructions, autoclaved and poured onto agar plates. A bacterial suspension with an Optical Density (OD) of Mc Farland Standard 0.5 (BioMérieux) was applied to the surface of Mueller Hinton (CM000x, Oxoid) agar plates by streaking back and forth very close together as you move across and down the plate using a cotton swab. The plates were turned and streaked again in a perpendicular direction to ensure an even distribution of the inoculum. Using a marker, the plate was divided into four parts and labeled accordingly. Using tweezers, antimicrobial susceptibility test discs (Oxoid) with concentrations were placed on the center of the divided plates; Ampicillin (10 µg), Penicillin G (10 µg), Tetracycline (30 µg), Gentamicin (10 µg), Streptomycin (25 µg), Erythromycin (15 µg), Ciprofloxacin (5 µg), Vancomycin (30 µg), chloramphenicol (30 µg) and Kanamycin (30 µg). The plates were placed upside down in an incubator and incubated at 37^{o}C for 24-48 h. After incubation, a clear area around the disc was observed for the isolates susceptible to antibiotics (Plates 1 and 2). The zones of diameter in mm were measured, and the antibiotics breakpoints of S (susceptible) and R (resistance) and I (intermediate) were recorded (Appendix iv) according to Clinical and Laboratory Standards Institute (CLSI, 2012) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2019).

Table 3.1: Primers used in this study

Target[a]	Primer	Sequence (5' – 3')	Annealing temperature	Amplicon size (bp)	Reference
16S RNA	fd1	AGAGTTTGATCCTGCCTCAG	55	1463	Lane (1991)
	1492R	CGGTTACCTTGTTACGACTT			Weisburg et al., (1991)
gyrB	GyrB-F	GAAGTCATCATGACCGTTCTGCAYGCNGGNGGNAARTTYGA	66	900	Thorsen et al., (2011)
	GyrB-R	AGCAGGGTACGGATGTGCGAGCCRTCNACRTCNGCRTCNGTCAT			
gyrB	Up1-F	GAAGTCATCATGACCGTTCTGCAYGCNGGNGGNAARTTYGA	66	920	Yamamoto & Harayana (1995)
	Up2- R	AGCAGGGTACGGATGTGCGAGCCRTCNACRTCNGCRTCNGTCAT			
nheA	NheA-F	TACGCTAAGGAGGGGCA	56	480	Hansen & Hendriksen (2001)
	NheA-R	GTTTTTATTGCTTCATCGGCT			
nheB	NheB-F	CTATCAGCACTTATGGCAG	54	754	
	NheB-R	ACTCCTAGCGGTGTTCC			
nheC	NheC-F	CGGTAGTGATTGCTGGG	54	564	
	NheC-R	CAGCATTCGTACTTGCCAA			

Table 3.1: Primers used in this study (Cont'd)

Target[a]	Primer	Sequence (5' – 3')	Annealing temperature	Amplicon size (bp)	Reference
hblA	HBLA1	GTGCAGATGTTGATGCCGAT	56	301	Fagerlund et al., (2004)
	HBLA2	ATGCCACTGCGTGGACATAT			
hblC	L2A	AATGGTCATCGGAACTCTAT		731	
	L2B	CTCGCTGTTCTGCTGTTAAT			
hblD	L1A	AATCAAGAGCTGTCACGAAT		411	
	L1B	CACCAATTGACCATGCTAAT			
cytK	CK-F-1859	ACAGATATCGG(GT)CAAAATGC	54	809	Guinebretière et al., (2002)
	CK-R-2668	TCCAACCCAGTT(AT)(GC)CAGTTC			
entFM	EntFM-F	ATGAAAAAAGTAATTTGCAGG	60	1269	Asano et al., (1997)
	EntFM-R	TTAGTATGCTTTTGTGTAACC			
cesB	Ces-F	GGTGACACATTATCATATAAGGTG	58	1271	Ehling-Schulz *et al.*, (2005)
	Ces-R	GTAAGCGAACCTGTCTGTAACAACA			

3.6: PRODUCTION OF *OKPEYE* FROM *P. africana* SEEDS

3.6.1: Batch production of *Prosopis africana* seeds

The method of Ogunshe et al. (2007) with some modifications was used for the batch production of *Prosopis africana* seeds for *okpeye* production (Figure 2.3). Three thousand grams (3000g) of seeds were purchased from Obollor Afor market in Nsukka, sorted, washed and soaked for four hours. The seeds were boiled in a pressure pot for 2 hours until the cotyledons separated from the seed coats. The seeds were allowed to cool and the seed coats were removed manually by pressing between fingertips. (Figure 3.1). The cotyledons separated from the coats were rinsed in water and cooked for another 30minutes until the water dried up. The cotyledons were allowed to cool to ambient temperature before dividing them into different portions for further use (Plates 3 and 4).

3.6.2 Inoculum preparation

Bacillus spp. originally identified and isolated from commercial samples of *okpeye* stored in beads from section 3.2.1 were streaked repeatedly on Nutrient agar (NA) plates and incubated at 37°C for 24 hours. The selection of *Bacillus strains* used as starter cultures for the fermentation of *P. africana* seeds was based on phenotypic and genotypic characterization of the isolates as well as their non-haemolytic and sensitivity to many antibiotics tested in this study. From the NA plates containing *Bacillus strains* incubated for 24 hours at 37°C, the surface growth of the distinct colonies was transferred into test tubes. Serial dilutions were made to obtain an inoculum of 10^4–10^6 cells ml $^{-1}$comparable to turbidity of 0.5 MacFarland standard.

3.6.3 Production of *okpeye* mash and inoculation of the mash using the starter cultures

From the cotyledons prepared in section 3.6.1, four parallel fermentation batches were prepared as follows: batches 1, 2 and 3 of 500 g each were autoclaved for 20 minutes at 121°C to ensure

the sterility of the cotyledons and aseptically transferred into three different plastic sieves wiped down with 70% ethanol. Batch 4 also containing 500 g was not autoclaved nor inoculated with any microorganism (spontaneous fermentation). It was transferred into a plastic sieve, lined, covered with *okpeye* leaves and kept for natural fermentation to take place. Four mililitres (4 ml) of the single and mixed starter organisms were inoculated to each of the three batches (BL, BA and BL and BA) and covered with foil wiped down with ethanol. These batches were carefully and aseptically wrapped in a sterilized net and kept to ferment for 168 h. Samples were collected at 0 h and every 24 hours for analysis till the end of the fermentation. At the end of the fermentation (168 h) and after drying, the different types of *okpeye* produced were: sample A- spontaneously fermenting *okpeye,* B - *okpeye* fermented with mixed starter cultures of *Bacillus licheniformis* and *Bacillus amyloliquefaciens*, C- *okpeye* fermented with a single starter culture of *Bacillus amyloliquefaciens*, D- *okpeye* fermented with a single starter culture of *Bacillus licheniformis.* (Plates 3-8).

Fig 3.1: Spontaneous fermentation of *okpeye*

Source: Ogunshe et al., 2007

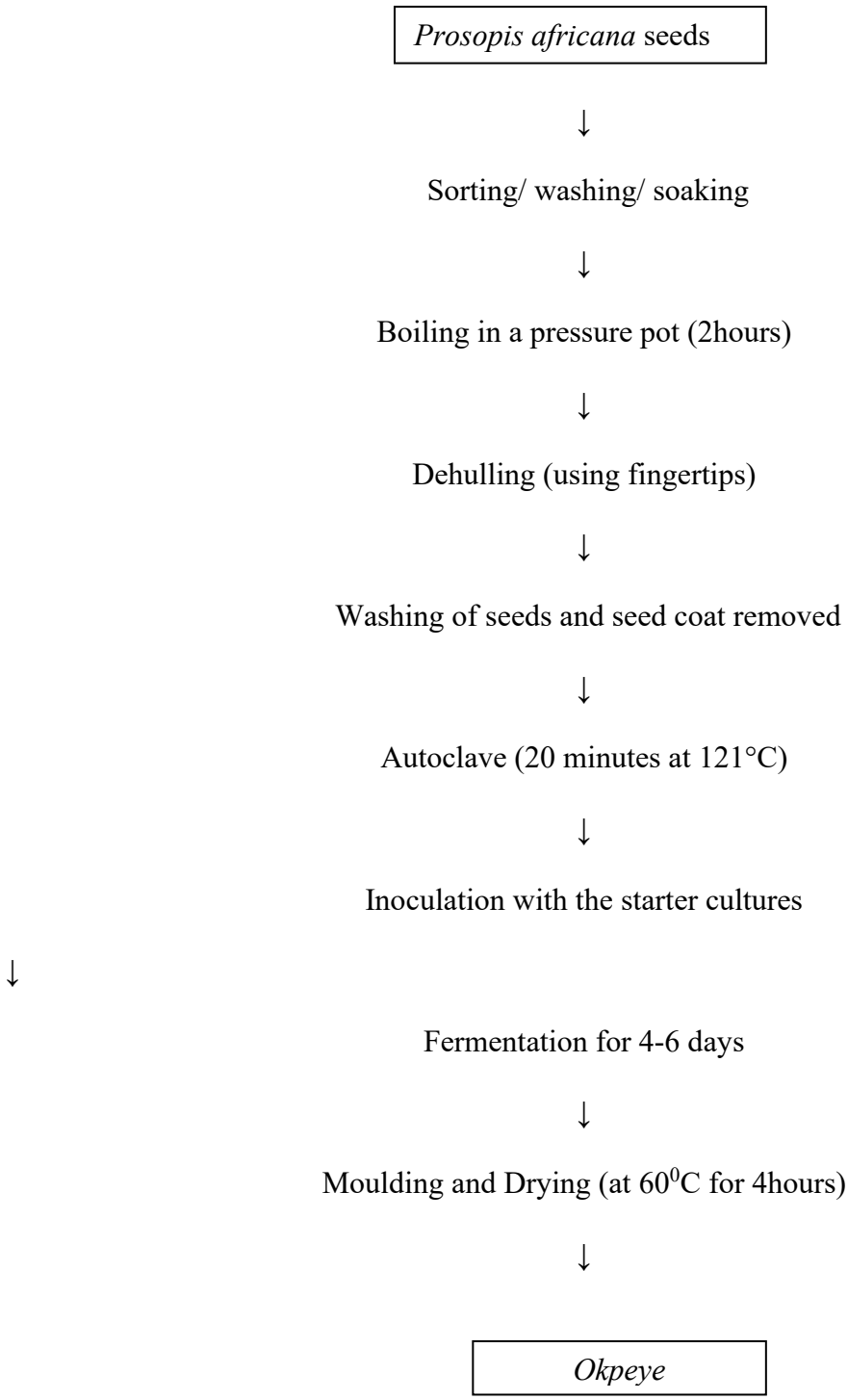


Fig 3.2: Production of *okpeye* using starter cultures

Source: Ogunshe et al., 2007

3.7 MICROBIOLOGICAL ANALYSIS

The total bacterial count of the fermenting mash of *Prosopis africana* seeds was monitored. At 24-hour intervals, 10 g of each sample was homogenized in 90 ml of sterile autoclaved water. Ten-fold serial dilutions were prepared as described in section 3.12 and microorganisms were enumerated on NA (Nutrient agar 10^6 dilution) and MHA (Mueller-Hinton agar 10^4) plates respectively after 24- 48 hours incubation at 37°C (Appendix v).

3.8: CHEMICAL ANALYSIS

3.8.1 pH and temperature determination

The pH and temperature of all the fermenting mash were determined in triplicates every 24 hours of fermentation using pH meter (Jenway 3505) that had been standardized with pH 4 and pH 7 buffers. Five grams (5 g) of the samples were mixed uniformly with 50 ml distilled water in a beaker. The digital display observed after inserting the glass electrode into the solution was recorded as the pH and then temperature settings as well to take the temperature after it has stabilized (Appendix vi).

3.8.2: Total titratable acidity determination

Total titratable aciditywas done according to the method as described by (AOAC, 2005). Two (2) g of ground sample was mixed with 20 ml distilled water in a 250 ml beaker. The mixture was filtered on Whatman filter paper (No 1) and the filtrate was collected in a conical flask. Two (2) drops of 1% phenolphthalein indicator were added to 10 ml of the filtrate and titrated against 0.1 N NaOH solution to a faint pink colour. The average titre value was recorded and the titratable acidity was calculated as the percentage of Acetic acid in the sample (Appendix vi).

3.9: PROXIMATE ANALYSIS

The moisture, crude protein, fat content, ash, and fibre composition were determined by the method according to AOAC (2005) while the carbohydrate content was calculated by difference method.

3.9.1: Moisture determination

The samples were mixed thoroughly and the moisture content was determined by weighing 2 g of the sample into a glass petri dish, which had been previously dried and weighed. The dish was placed inside the oven for 4 hours at 60^0C. the samples were finally dried to a constant weight, cooled and weighed.

$$\% \text{ Moisture} = \frac{\text{Difference in weight X 100}}{\text{Weight of sample used}} \qquad \text{equation 3.1}$$

3.9.2: Crude protein determination by micro Kjeldahl method.

A quantity of 0.5 g of the sample was added 10ml of concentrated tetraoxosulphate vi acid and 1 g of the catalyst mixture (potassium sulphate, copper sulphate and selenium powder) was added. The mixture was heated cautiously on a digestion rack under a fume hood for a few minutes until frothing ceased and then the heat was increased to digest for 1 hour. The mixture was allowed to cool and made up to a known volume of distilled water (100 ml). The digested sample was transferred into a distillation apparatus (micro Kjeldhal distillation apparatus) and distilled. 10 ml of the distillate was titrated with 0.1ml HCL to the end point of first pink colour.

$$\% \text{ N} = \frac{\text{(a-b) x 0.01 x 14.0057 x c x 100}}{\text{d x e}} \qquad \text{equ 3.2}$$

a = titre value for the sample

b = titre value for the blank

c = Volume to which digest is made up of distilled water

d = Aliquot taken for distillation

e = Weight of dried sample (mg)

% Crude protein = % N X conversion factor (6.25).

3.9.3: Ash determination

Two grams (2 g) of the sample was weighed into a porcelain crucible and placed in a muffle furnace preheated to 600^0C. This temperature was held for 2 hours until the product turns to ash and then the crucible was transferred directly to the desiccator, cooled and weighed immediately.

$$\%\text{Ash} = \frac{\text{Difference in weight X 100}}{\text{Weight of sample used}} \quad \text{equ 3.3}$$

3.9.4: Fat Determination

Two fifty mililitre (250 ml) of clean boiling flask was dried in the oven at 105^0C for 30 minutes. Two grams (2 g) of the sample was transferred into the flask and 300 ml of petroleum ether was added. The thimble was plugged and the extraction thimble was covered with cotton wool and the Soxhlet apparatus was assembled. The source of heat (electrothermal heating mantle) was adjusted so that the ether boiled gently and was left to siphon/reflux for 6 hours. The flask was detached (which now contains all the oil) and then filtered through Whatman No 1 filter paper into a weighed beaker, washing the paper finally with a small portion of hot fresh ether. The solvent was evaporated at100^0C and the beaker was washed and dried in the oven to get the empty weight

$$\%\text{Fat} = \frac{\text{Difference in weight X 100}}{\text{Weight of sample used}} \quad \text{equ 3.4}$$

3.9.5: Crude fibre determination

Two grams (2 g) of the sample was defatted with petroleum ether, boiled under reflux for 30minutes with 200 ml of a solution containing 1.25 g of H_2SO_4 per 100 ml of solution. The solution was filtered with Whatman No 1 filter paper, washed three times with hot distilled water, dried and carefully transferred the residue into a quick-fit flask. The residue was then transferred to a beaker and boiled for 30 minutes with 200ml of a solution containing 1.25 g of carbonate-free sodium hydroxide per 100 ml. The residue was filtered and transferred into a crucible, dried in the oven and weighed. Then the sample was ashed at 550^0C in a muffle furnace, cooled in a desiccator and the dried weight was taken.

$$\%\text{Crude fibre} = \frac{\text{loss in weight X 100}}{\text{Weight after incineration}} \quad \text{equ 3.5}$$

3.9.6: Carbohydrate determination

The carbohydrate content was determined using difference method. Thus, carbohydrate was calculated as

100 - (%Fats + %Ash + %Moisture + %Protein + % Crude Fibre) = % Carbohydrate

3.10: PHYTOCHEMICAL ANALYSIS

3.10.1: Alkaloids determination

The total content of alkaloids was determined according to the method described by Biradar & Racheti (2013). Five gram (5 g) of each sample was added to 50 ml of a solution containing 10% acetic acid in ethanol and mildly stirred for 48 hours. After filtration, the extracts were concentrated to one-quarter of the original volume and 2 ml of 3% tetraoxosulphate (vi) acid and 8 ml of water were added to reach pH 2.5. This solution was transferred to a separator funnel where 10 ml of petroleum ether: diethyl ether (1:1) solution was added. The bottom phase was collected and added to concentrated ammonium hydroxide solution until

precipitation was complete (pH 8.0). The whole solution was allowed to settle and the precipitated phase was collected and washed again with ammonium hydroxide and chloroform. The precipitated phase was then dried first with sodium sulphate and then completely dried and weighed to estimate the percentage of alkaloids.

$$\text{Percentage alkaloid} = \frac{W3 - W2 \times 100}{W1} \quad \text{equ 3.6}$$

Where; W1 = weight of sample

W2 = weight of empty flask

W3 = weight of flask and residue

3.10.2: Phytate determination

Phytic acid was determined using a method as reported by Biradar &Racheti (2013). Four grams (4 g) of ground sample wassoaked in 100 ml of 2% hydrochloric acid (HCl) for 3 hours and then filtered through two layers of filter paper. Then 25ml of the filtrate was placed in a 250 ml conical flask and 5 ml of 0.3% ammonium thiocyanate (NH_4SCN) solutionwas added as an indicator. 53.5 ml of distilled water was then added to reach the proper acidity. The mixture was titrated against ferric chloride ($FeCl_3$) solution, which contains about 0.00195 g of iron per ml of $FeCl_3$ solution untila brownish-yellow colour was obtained. The result was multiplied by the factor 1.95 to obtain phytate result was multiplied by factor 3.55 to convert to phytate value in mg/100 g.

3.10.3: Tannin determination

Thirty mililitre (30 ml) of petroleum ether was added to 5.0 g of the already crushed sample in a conical flask and corked for 24 hours. It was then filtered and allowed to stand for 15 minutes to evaporate the petroleum ether. It was reextracted by soaking in 50 ml of 10% acetic acid in ethanol for 4 hours. The sample was then filtered and the filtrate was collected. Twenty- five

mililitres (25 ml) of ammonium hydroxide (NH_4OH) was added to the filtrate to precipitate the solution. It was then heated to remove some ammonium hydroxide (NH_4OH) still in solution. The remaining volume of the solution after heating was noted and 5 ml of this solution was taken and 20 ml of ethanol was added to it and then titrated with 0.1M sodium hydroxide (NaOH) using phenolphthalein as an indicator until the pink endpoint was reached. Tannins content was then calculated and expressed in mg/100g (Aremu, Olaofe& Akintayo*et al*., 2006).

$$\text{Soluble tannins (mg/100g)} = \frac{\text{C (mg) X extract volume (m)}}{\text{10 X Aliquot (ml) X W1 (g)}} \qquad \text{equ 3.7}$$

3.10.4: Flavonoid determination

Two grammes (2 g) of the sample was weighed and extracted with 100 cm^3 of 80% methanol at room temperature. The mixture was then filtered through filter paper into a 250 cm^3 beaker and the filtrate was transferred into a water bath and allowed to evaporate to dryness and weighed. The % flavonoid was calculated. (Krishnaiah, Devi, Bono & Sarbattly et al., 2009).

$$\text{Percentage flavonoid} = \frac{W3 - W2 \text{ X } 100}{W1} \qquad \text{equ 3.8}$$

Where W_3 = weight of beaker and residue

W_2 = weight of empty beaker

W_1 =weight of sample

3.10.5: Cyanogenic glycosides determination

Cyanogenic glycoside was determined by titration method according to AOAC (2006). Ten grams (10 g) of ground sample was transferred into a Kjeldahl flask and 200 ml water was added and allowed to stand for 2 to 4 hours. After steam distillation, 150 to 160 ml of distillate was added to NaOH solution and diluted to a definite volume (250 ml). To 100 ml distillate, 8

ml of 6M NH_4OH and 2 ml 5% KI solution was added and titrated with 0.02M $AgNO_3$ until an end point was reached.

3.10.6: Saponin determination

Saponin was determined according to the method described by (Obadoni & Ochuko, 2001). Ten mililitre (10 ml) of 20% aqueous ethanol was added to 5 g of the mashed sample, the mixture was put in a shaker hot water bath for 4 hours at about 55^0C. The mixture was filtered and the residue was reextracted using 20% ethanol. The concentrate was transferred into 250 mL separating funnel and 20 mL of diethyl ether was added and shaken together. The aqueous layer was recovered while the ether was later discarded. The purification process was repeated. 60 ml of n-butanol was added and washed twice with 10 mL of 5% aqueous NaCl.

$$\% \text{ Saponin} = \frac{\text{Initial weight} - \text{final weight of the sample} \times 100}{\text{Initial weight}} \quad \text{equ 3.9}$$

3.10.7: Oxalate determination

This method for oxalate determination according to AOAC (2005), comprises digestion, oxalate precipitation and permanganate titration. For digestion two (2) grammes of the sample was suspended in 190 mL of distilled water in a 250 mL volumetric flask, followed by the addition of 10 mL of 6M HCL and the suspension was digested at 100^0 C for 1 hour. The solution was cooled and then made up to 250 mL mark before filtration. For oxalate precipitation, duplicate portions of the filtrate were measured into breakers and four drops of methyl red indicator were added. Then NH_4OH solution was added (dropwise) until the test solution changed from pink to faint yellow colour (pH 4.0 - 4.5). Each portion was then heated to 90^0C, cooled and filtered to remove precipitate containing ferrous ion. The filtrate was again

heated to 90^0C and 10 mL of 5% $CaCl_2$ solution was added with constant stirring. The solution was then heated and left overnight at 25^0C, after which it was centrifuged at 2500 rpm for 5minutes. The supernatant was decanted, and the precipitate completely dissolved in 10 mL of 20% (v/v) H_2SO_4 solution

Permanganate titration

The total filtrate resulting from the digestion of 2 g of sample was made up to 300 mL. Aliquots of 125 mL of the filtrate were heated until near boiling and then titrated against 0.05M standardized $KMnO_4$ solutions to a faint pink colour which persisted for 30 seconds.

The percentage oxalate was calculated using the formula:

$$\frac{T \times (Vme)\ (Df) \times 100}{(ME) \times (MF)} \qquad \text{equ 3.10}$$

Where T is titre of $KMnO_4$ (ml),

Vme is the volume-mass equivalent

Df is the Dilution factor

Vt/A Where Vt is the total volume of filtrate (300ml)

A is the aliquot used i.e., 250ml

ME is the molar equivalent of $KMnO_4$ in oxalate and

Mf is the mass of the sample used

3.10.8: Total phenol determination

Five grams (5 g) sample was boiled with 50 ml of ether for the extraction of the phenolic component for 15 min. Five (5) milliliters of the extract was pipetted out into a 50 ml flask, then 10 ml of distilled water was added. Two (2) ml of NH_4OH solution and 5 ml of concentrated amyl alcohol were also added. The samples were made up to mark and left to react for 30 minutes for colour development. This was read at 505 nm. The concentration of

the sample was determined by using the standard of gallic acid equivalent (GAE). The formula below was used for the calculation.

$$\frac{Cs}{As} = \frac{Cstd}{Astd} \qquad \text{equ. 3.11}$$

Where: -

Cs = concentration of sample

Cstd = concentration of standard

As = Absorbance of sample

Astd = Absorbance of standard

S

3.11: SENSORY EVALUATION

The sensory acceptability of the broth cooked with *okpeye* produced using starter cultures (samples B, C and D), spontaneously fermented *okpeye* (sample A) and commercial *okpeye* from an Owerri market used as control (sample E) was assessed using the nine-point hedonic scale where 9 is liked extremely and 1 is dislike extremely (Iwe, 2002). The sensory analysis was done using thirty (30) panelists from the Department of Food Science and Technology, Owerri familiar with the taste of *okpeye* (Appendix vii). They were given five sets of broth with five different samples of *okpeye* to evaluate the broth based on the aroma, flavour, appearance, after taste and overall acceptability. Sample A was broth cooked with spontaneously fermented *okpeye,* sample B was broth prepared with *okpeye* fermented with mixed starter cultures of *Bacillus licheniformis* and *Bacillus amyloliquefaciens*, sample C was broth prepared with *okpeye* fermented with single starter culture of *Bacillus amyloliquefaciens*, sample D was broth prepared with *okpeye* fermented with single starter culture of *Bacillus*

licheniformis and sample E was broth prepared with commercial *okpeye* and served as the control (Appendix viii).

3.12: STATISTICAL ANALYSIS

Each experiment was performed in triplicates. The data obtained were analyzed using Analysis of Variance (ANOVA). Statistical Product for Service solutions (SPSS) version 26 was used for statistical analysis of data obtained. Means were separated using the DUNCAN method ($p< 0.05$).

3.13: EXPERIMENTAL DESIGN

The experimental design is a 2- factor factorial design with two main factors: fermentation time with levels (0, 24, 48, 72, 96, 120, 144,168 hours) and starter cultures (*Bacillus licheniformis* and *Bacillus amyloliquefaciens* singly and in combination). The response variables include the chemical parameters, proximate composition, phytochemical properties and sensory parameters (Appendix ix).

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Results

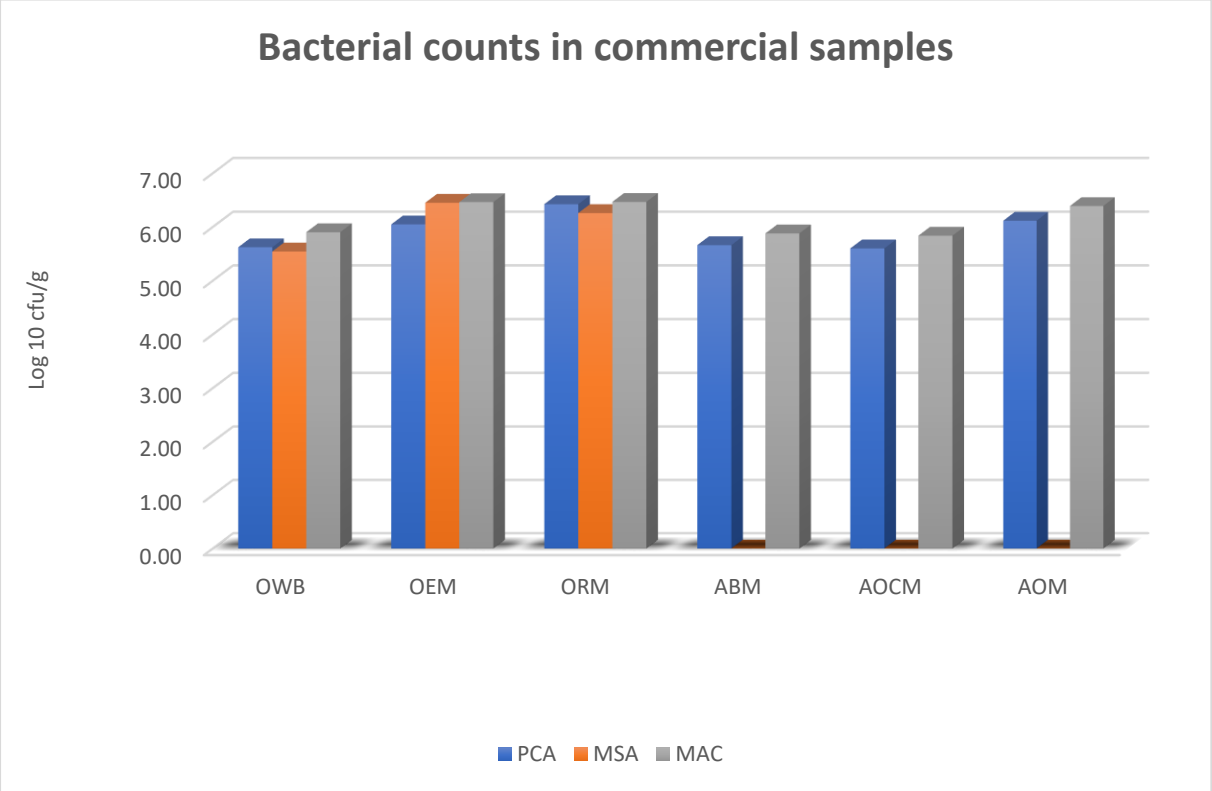


Figure 4.1.1. Bacterial counts in commercial samples of *okpeye* from some markets in South Eastern Nigeria

Key; (OWB; Owerri World Bank Market, OEM; Owerri Ekeukwu Market, ORM; Owerr Relief Market, ABM; Anambra Bridgehead market, AOCM; Anambra Ochanga Market, AOM; Anambra Ose market). aerobic mesophile counts on plate count agar (PCA), *Staphylococcus* counts on Mannitol Salt agar (MSA) and Enterobacteriaceae counts on MacConkey agar (McC).

Aerobic mesophile counts on PCA ranged between 10^5 to 10^6 CFU/g in samples from all markets, *Staphylococci* were present in *okpeye* samples collected in all the markets ranging from 10^5 to 10^6 CFU/g while bacteria isolated on MacConkey agar were detected in all *okpeye* samples at levels ranging from 10^5 to 10^6 CFU/g.

Table 4.1.1: Origin and identification of microorganisms from *okpeye*

Location	Isolates	16s sequencing	% Similarity	Length of sequence
OWB	OW 3	*Bacillus tequilensis*	99.77	895
OWB	OW 4	*Heyndricxiaoleronia*	99.05	697
OWB	OW 5	*Shigella flexneri*	97.56	900
OWB	OW 6	*Bacillus tequilensis/ cabrialesii*	95.72	934
OWB	OW 7	*Bacillus tequilensis/ cabrialesii*	96.54	926
OWB	OW 8	*Bacillus atrophaeus/velezensis*	95.84	950
OWB	OW 9	*Bacillus atrophaeus/velezensis*	94.96	939
OWB	OW 10	*Escherichia fergusonni*	99.65	888
OWB	OW 11	*Bacillus licheniformis*	98.84	958
OWB	OW 12	*Oceanobacilluscaeni*	94.35	743
OWB	OW 13	*Oceanobacilluscaeni*	98.17	893
OWB	OW 14	*Bacillus tequilensis/ cabrialesii*	97.08/ 97.08	686
OWB	OW 15	*Bacillus amyloliquefaciens/ siamensis*	99.9	647
OWB	OW 16	*Paenibacillusbarcinonensis*	99.00	706
OWB	OW 17	*Lynsinibacillusxylanilyticus*	99.08	900
OWB	OW 18	*Staphylococcus simulans*	99.31	894
OWB	OW 19	*Bacillus cereus/ paramycoides/paranthracis*	99.82	602

Table 4.1.1: Origin and identification of microorganisms from *okpeye* (Cont'd.)

Location	Isolates	16s sequencing	% Similarity	Length of sequence
OEM	OW 20	*Bacillus cereus/ paramycoides/paranthracis*	99.82	602
OEM	OW 21	*Bacillus cereus/ paramycoides/paranthracis*	98.99	894
OEM	OW 22	*Bacillus cereus/ paramycoides/paranthracis*	98.99	894
OEM	OW 23	*Bacillus tequilensis/ cabrialesii/ maquosorum*	95.75	954
OEM	OW 24	*Bacillus licheniformis/ haynesii*	96.82/96.71	900
ORM	OW 25	*Bacillus velezensis*	99.88	893
ORM	OW 26	*Staphylococcus nepalensis*	98.33	900
ORM	OW 27	*Bacillus amyloliquefaciens*	99.65	895
ORM	OW 28	*Staphylococcus nepalensis*	99.68	660
ORM	OW 29	*Bacillus amyloliquefaciens*	99.88	893
ORM	OW 30	*Staphylococcus nepalensis*	98.33	900
ORM	OW 31	*Bacillus siamensis*	99.77	904
ORM	OW 32	*Staphylococcus ureilyticus/ cohnii*	95.65/ 95.54	932
ORM	OW 33	*Staphylococcus nepalensis*	96.52	890
ORM	OW 34	*Bacillus cereus/paramycoides*	96.07/96.04	804

Table 4.1.1: Origin and identification of microorganisms from *okpeye* (Cont'd.)

Location	Isolates	16s sequencing	% Similarity	Length of sequence
ORM	OW 35	*Bacillus gaemokensis/anthracis*	94.67	926
ORM	OW 36	*Staphylococcus nepalensis*	96.52	890
AOCM	A 1	*Bacillus cereus/paramycoides*	94.67	937
AOCM	A 2	*Lysinibacillusxylanilyticus*	95.80	894
AOCM	A 3	*Bacillus cereus*	99.48	616
AOCM	A4	*Vagacoccuslutrae*	99.54	904
AOCM	A 5	*Staphylococcus cohnii*	99.88	894
AOCM	A 6	*Staphylococcus nepalensis*	99.54	893
AOCM	A 7	*Staphylococcus simulans*	98.99	899
AOCM	A 8	*Staphylococcus simulans*	99.77	892
AOCM	A 9	*Staphylococcus simulans*	99.88	896
AOM	A 10	*Staphylococcus simulans*	99.66	899
AOM	A 11	*Bacillus cereus/paramycoides*	99.88/ 99.88	897
AOM	A 12	*Vagococcuscarniphilus*	99.4	538
AOM	A 13	*Staphylococcus cohnii*	99.77	890
AOM	A 14	*Staphylococcus simulans*	99.88	906
AOM	A 15	*Bacillus amyloliquefaciens*	99.54	900
AOM	A 16	*Bacillus amyloliquefaciens*	99.65	886

Table 4.1.1: Origin and identification of microorganisms from *okpeye* (Cont'd.)

Location	Isolates	16s sequencing	% Similarity	Length of sequence
ABM	A 17	*Bacillus cereus/ toyonensis*	100.00	683
ABM	A 18	*Staphylococcus simulans*	99.77	888
ABM	A 19	*Staphylococcus simulans*	98.96	956
ABM	A 20	*Staphylococcus cohnii*	99.77	889
ABM	A 21	*Bacillus cereus/paramycoides*	99.54	895
ABM	A 22	*Staphylococcus simulans*	98.95	956
ABM	A 23	*Staphylococcus simulans*	99.88	888
ABM	A 24	*Staphylococcus simulans*	99.88	945
ABM	A 25	*Bacillus amyloliquefaciens*	99.77	886

Sample collection sites in Owerri (OWB; Owerri World Bank Market, OEM; Owerri Ekeukwu Market, ORM; Owerri Relief Market) and Anambra (ABM; Anambra Bridgehead market, AOCM; Anambra Ochanga Market, AOM; Anambra Ose market)

Out of the 61 isolates, OWB 1 and OWB 2 were not viable during DNA isolation. Of the 59 isolates, one of the predominant groups was identified as *Bacillus* (47.4%), the second predominant group (42.3%) was *Staphylococcus* species while the other bacterial species (10.3%) was identified based on sequence similarity to reference strains in the EzBiocloud database.

Table 4.1.2: Identification of microorganisms from *okpeye* using 16s rRNA sequencing and *gyrB/ rpoB* sequencing for closely related species

Location[a]	Isolate code	Identification 16S rRNA gene sequencing	Identification *gyrB*/*rpoB* sequencing
OWB	OW3	*Bacillus tequilensis*	
OWB	OW4	*Heyndrickxiaoleronia*	
OWB	OW5	*Shigella flexneri*	
OWB	OW6	*B. cabrialesii/tequilensis*	*B. tequilensis*
OWB	OW7	*B. cabrialesii/tequilensis*	
OWB	OW8	*B. atrophaeus/velezensis*	
OWB	OW9	*B. atrophaeus/velezensis*	*B. velezensis*
OWB	OW10	*Escherichia fergusonni*	
OWB	OW11	*B. licheniformis*	
OWB	OW12	*Oceanobacilluscaeni*	
OWB	OW13	*O. caeni*	
OWB	OW14	*B. tequilensis/ cabrialesii*	
OWB	OW15	*B. amyloliquefaciens/ siamensis*	*B. amyloliquifaciens*
OWB	OW16	*Paenibacillusbarcinonensis*	
OWB	OW17	*Lynsinibacillusxylanilyticus*	
OWB	OW18	*Staphylococcus simulans*	
OWB	OW19	*B. cereus/ paramycoides/paranthracis*	*B. cereus*
OEM	OW20	*B. cereus/ paramycoides/paranthracis*	*B. cereus*
OEM	OW21	*B. cereus/ paramycoides/paranthracis*	*B. cereus*

Table 4.1.2: Identification of microorganisms from *okpeye* using 16s rRNA sequencing and *gyrB/ rpoB*sequencing for closely related species (Cont'd.)

Location[a]	Isolate code	Identification 16S rRNA gene sequencing	Identification *gyrB*/*rpoB* sequencing
OEM	OW22	*B. cereus/ paramycoides/paranthracis*	*B. cereus*
OEM	OW23	*B. tequilensis/ cabrialesii/ maquosorum*	
OEM	OW24	*B. licheniformis/ haynesii*	*B. licheniformis*
ORM	OW25	*B. velezensis*	
ORM	OW26	*S. nepalensis*	
ORM	OW27	*B. amyloliquefaciens*	
ORM	OW28	*S. nepalensis*	
ORM	OW29	*B. amyloliquifaciens*	
ORM	OW30	*S. nepalensis*	
ORM	OW31	*B. siamensis*	
ORM	OW32	*S. cohnii/ureilyticus*	*S. cohnii*
ORM	OW33	*S. nepalensis*	
ORM	OW34	*B. cereus/paramycoides*	*B. cereus*
ORM	OW35	*B. gaemokensis/ anthracis*	*B. anthracis*
ORM	OW36	*S. nepalensis*	
AOCM	A1	*B. cereus/paramycoides*	*B. cereus*
AOCM	A2	*Lysinibacillusxylanilyticus*	
AOCM	A3	*B. cereus*	
AOCM	A4	*Vagacoccuslutrae*	
AOCM	A5	*S. cohnii*	
AOCM	A6	*S. nepalensis*	

Table 4.1.2: Identification of microorganisms from *okpeye* using 16s rRNA sequencing and *gyrB/ rpoB* sequencing for closely related species (Cont'd.)

Location	Isolate code	Identification 16S rRNA gene sequencing	Identification *gyrB*/*rpoB* sequencing
AOCM	A7	*S. simulans*	
AOCM	A8	*S. simulans*	
AOCM	A9	*S. simulans*	
AOM	A10	*S. simulans*	
AOM	A11	*B. cereus/paramycoides*	*B. cereus*
AOM	A12	*V. carniphilus*	
AOM	A13	*S. cohnii*	
AOM	A14	*S. simulans*	
AOM	A15	*B. amyloliquefaciens*	
AOM	A16	*B. amyloliquefaciens*	
ABM	A17	*B. cereus/ toyonensis*	*B. cereus*
ABM	A18	*S. simulans*	
ABM	A19	*S. simulans*	
ABM	A20	*S. cohnii*	
ABM	A21	*B. cereus/paramycoides*	*B. cereus*
ABM	A22	*S. simulans*	
ABM	A23	*S. simulans*	
ABM	A24	*S. simulans*	
ABM	A25	*B. amyloliquefaciens*	

[a]Sample collection sites in Owerri (OWB; Owerri World Bank Market, OEM; Owerri Ekeukwu Market, ORM; Owerri Relief Market) and Anambra (ABM; Anambra Bridgehead market, AOCM; Anambra Ochanga Market, AOM; Anambra Ose market)

Further identification was carried out by sequencing of *gyrB* and *rpoB* genes of some organisms with close percentage similarities.

Table 4.1.3: PCR analysis of toxin genes from *Bacillus cereus* isolated from *okpeye*

Isolate code	Microorganisms	*nheA*	*nheB*	*nheC*	*hblA*	*hblC*	*hblD*	*entFM*	*cytK*	*cesB*
O19	*B. cereus*	+	+	-	+	+	+	+	+	+
O20	*B. cereus*	+	+	-	+	+	+	+	+	+
O21	*B. cereus*	+	+	+	+	+	+	+	+	+
O22	*B. cereus*	+	+	+	+	+	+	+	+	-
O34	*B. cereus*	+	+	+	+	+	+	+	+	-
A1	*B. cereus*	+	-	+	-	-	-	-	-	-
A3	*B. cereus*	+	-	-	-	-	-	-	+	-
A11	*B. cereus*	+	+	+	+	+	+	+	+	-
A17	*B. cereus*	+	-	+	+	+	+	-	-	-
A21	*B. cereus*	+	+	+	-	-	-	+	-	-
B13[b]	Control	+	+	+	+	+	+	+	+	-

+: positive, -: negative [b:] positive control (Ouoba *et al*., 2008). Enterotoxin genes (*nheA, nheB, nheC, hblA, hblC, hblD, cytK, entFM*) and emetic toxin (*cesB*).

All *B. cereus* isolates identified in this study tested positive for one or more of the enterotoxin genes including haemolytic and non-haemolytic complex.

Table 4.1.4: Haemolytic activities of some microorganisms isolated from *okpeye*

Isolate Code	Isolate	Haemolysis
O6	*B. tequilensis*	+
O7	*B. tequilensis*	+
O8	*B. velezensis*	+
O9	*B. velezensis*	+
O11	*B. licheniformis*	-
O14	*B. tequilensis*	+
O15	*B. amyloliquefaciens*	-
O22	*B. cereus*	-+
O23	*B. tequilensis*	+
O24	*B. licheniformis*	+
O27	*B. amyloliquefaciens*	+
O31	*B siamensis*	+
O35	*B. anthracis*	+
A11	*B. cereus*	+
A16	*B. amyloliquefaciens*	+
O26	*S. nepalensis*	+
O30	*S. nepalensis*	+
O32	*S. cohnii*	+
A7	*S. simulans*	+

Table 4.1.4: Haemolytic activities of some microorganisms isolated from *okpeye* (Cont'd.)

Isolate Code	Isolate	Haemolysis
A13	*S. cohnii*	+
A22	*S. simulans*	-+
O4	*H. oleronia*	+
O12	*O. caeni*	+
O13	*O. caeni*	+
O17	*L. xylanilyticus*	+
A2	*L. xylanilyticus*	+
A4	*V. lutrae*	+
A12	*V. carniphilus*	-

(+) = Haemolysis, (-) = No Haemolysis, -+ (partial Haemolysis).

From the twenty-eight isolates tested, twenty- three (82.1%) showed full haemolytic activity on blood agar. Two isolates (O22 and A22) showed partial haemolysis while three (O11, O15 and A12) were the only ones that showed no haemolysis.

Table 4.1.5: Antibiotic susceptibility of some microorganisms isolated from *okpeye*

Isolate Code	Isolate	Antibiotic resistance profile[a]									
		Am	**P**	**T**	**G**	**S**	**Ci**	**E**	**Ch**	**K**	**V**
O6	*B. tequilensis*	S	S	S	S	S	R	S	S	S	S
O7	*B. tequilensis*	S	S	S	S	S	R	S	S	S	S
O8	*B. velezensis*	S	S	S	S	S	R	S	S	S	S
O9	*B. velezensis*	S	S	R	S	S	R	S	S	S	S
O11	*B. licheniformis*	R	S	S	S	S	R	S	R	S	S
O14	*B. tequilensis*	R	R	S	S	S	R	S	I	R	R
O15	*B. amyloliquefaciens*	S	S	S	S	S	R	S	S	S	S
O22	*B. cereus*	R	R	S	S	S	R	S	S	S	R
O23	*B. tequilensis*	S	S	S	S	S	R	S	S	S	S
O24	*B. licheniformis*	R	R	S	S	S	R	S	R	R	S
O27	*B. amyloliquefaciens*	R	R	S	S	S	R	R	R	R	R
O31	*B siamensis*	S	S	R	S	S	R	S	S	S	S
O35	*B. anthracis*	R	R	S	S	S	R	R	S	S	R
A11	*B. cereus*	R	R	R	S	S	R	S	S	S	R

Table 4.1.5: Antibiotic susceptibility of some microorganisms isolated from *okpeye* (Cont'd.)

Isolate Code	Isolate	Antibiotic resistance profile[a]									
		Am	**P**	**T**	**G**	**S**	**Ci**	**E**	**Ch**	**K**	**V**
A16	*B. amyloliquefaciens*	S	S	S	S	S	R	S	S	S	S
O26	*S. nepalensis*	S	S	S	S	S	R	R	S	I	R
O30	*S. nepalensis*	S	S	S	S	S	R	S	S	S	R
O32	*S. cohnii*	S	S	S	S	R	R	S	S	S	R
A7	*S. simulans*	S	S	S	S	R	S	S	S	S	I
A13	*S. cohnii*	I	R	S	S	R	R	R	S	R	R
A22	*S. simulans*	R	R	S	S	S	R	S	S	S	I
O4	*H. oleronia*	R	R	S	S	S	R	S	R	R	R
O12	*O. caeni*	S	S	S	S	S	R	S	S	S	S
O13	*O. caeni*	S	S	S	S	S	R	S	S	S	S
O17	*L. xylanilyticus*	S	S	S	S	S	R	R	S	S	S
A2	*L. xylanilyticus*	S	S	S	S	R	S	S	S	R	I
A4	*V. lutrae*	I	R	R	S	S	R	I	S	R	R
A12	*V. carniphilus*	R	R	R	S	R	R	R	R	R	R

[a]Am= Ampicillin, P= Penicillin G, T= Tetracycline, G= Gentamicin, S= Streptomycin, Ci= Ciproflaxin, E= Erythromycin, Ch= Chloramphenicol, K= Kanamycin, V= Vancomycin. **S**= susceptible, **I** intermediate, **R** resistant. The results were translated and zones of diameter were compared with both CLSI (2012) and EUCAST (2019) standard zone diameter breakpoint

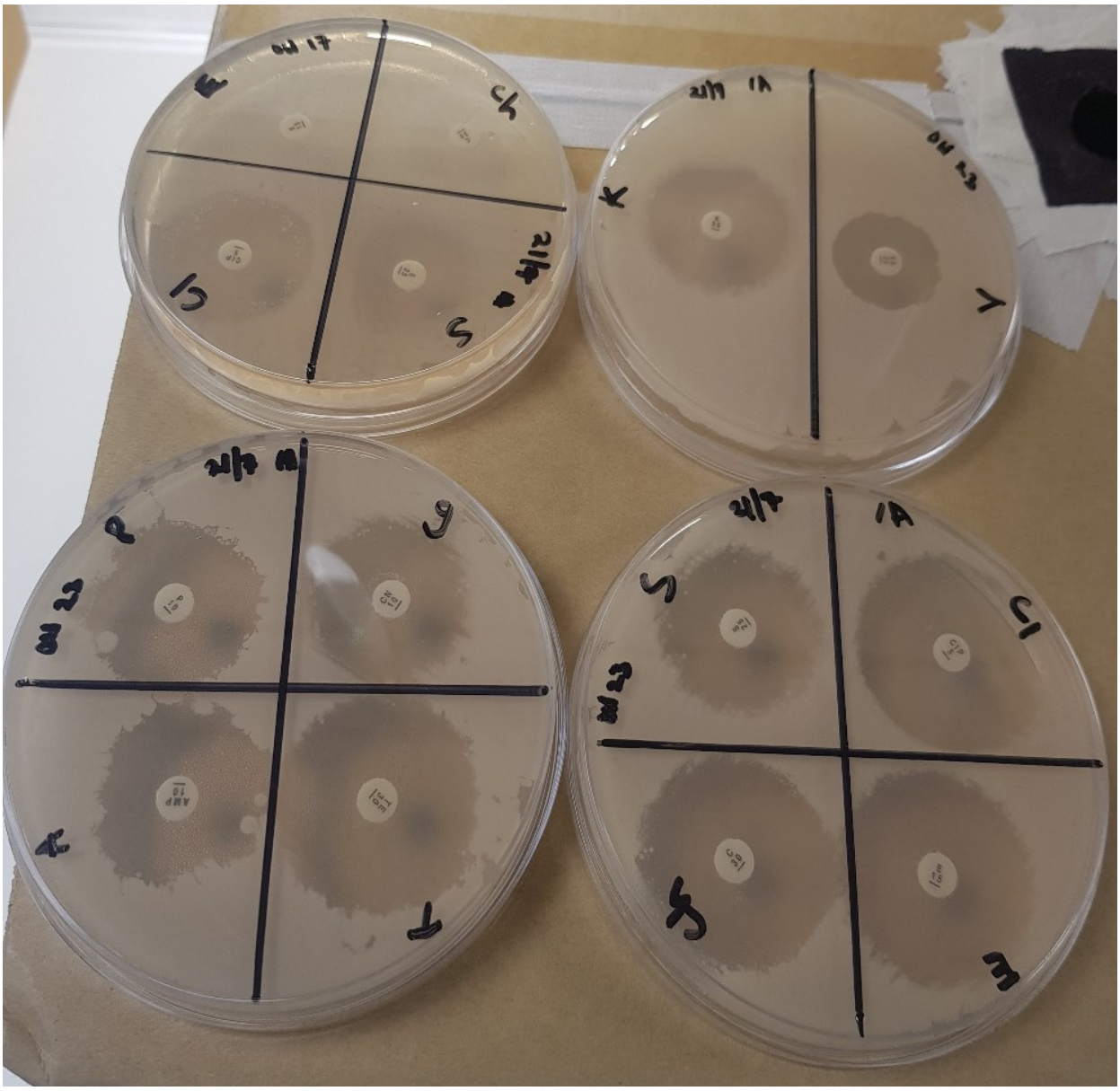


Plate 1: Antibiotic susceptibility test on some isolates

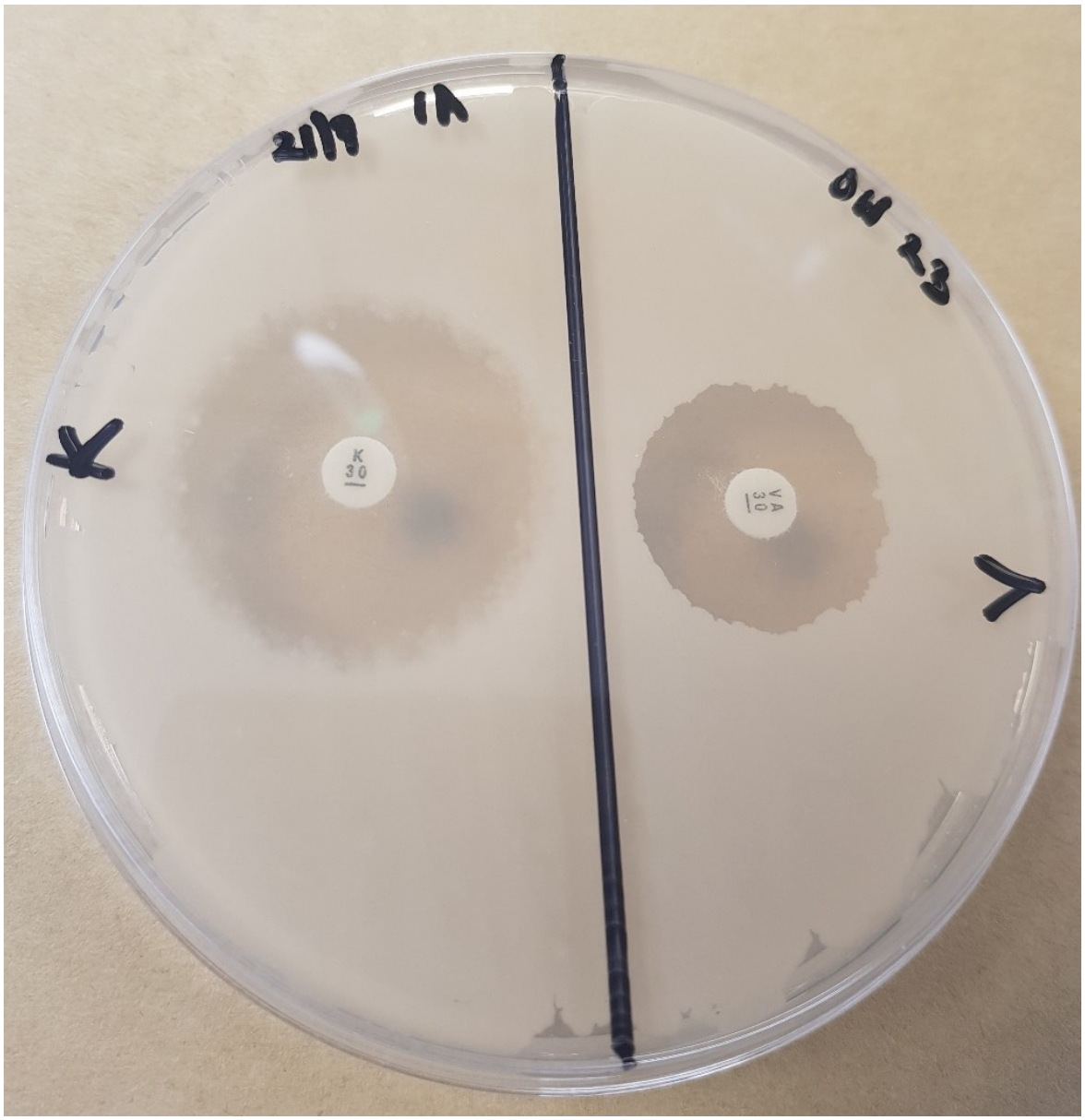
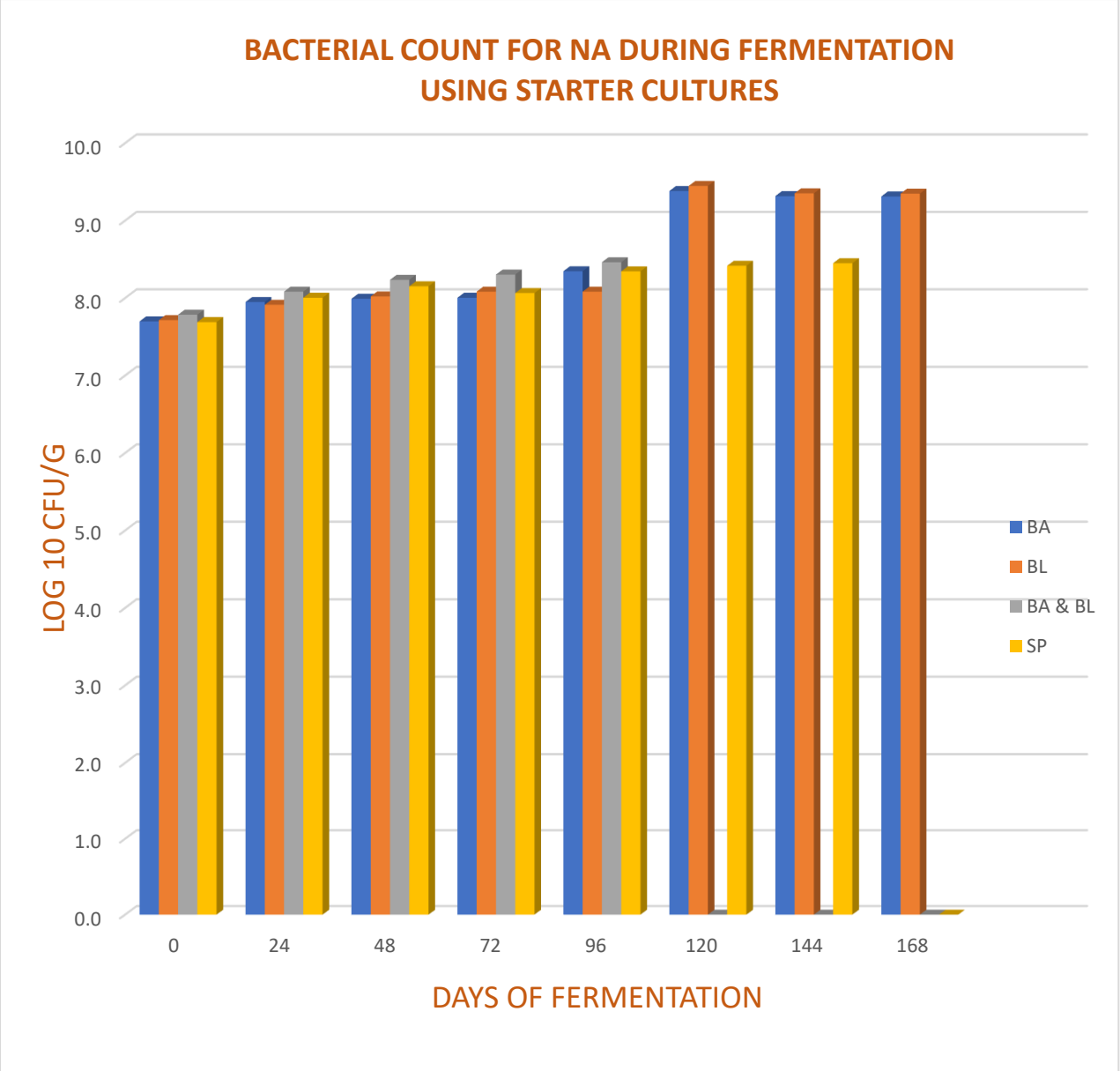


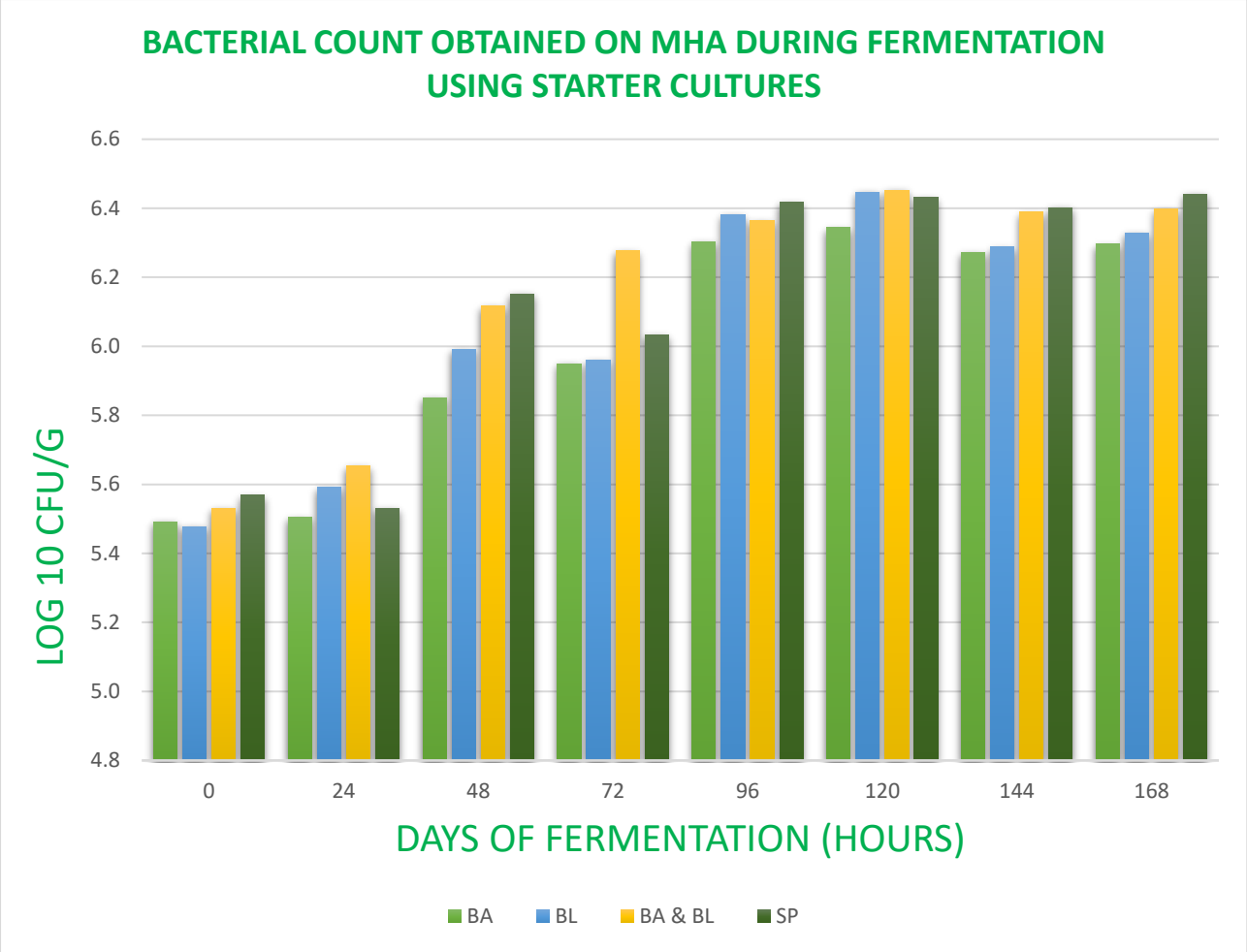
Plate 2: Antibiotic susceptibility test on some isolates



NA: Nutrient agar, BA- *Bacillus amyloliquefaciens;* Bl- *Bacillus licheniformis;* Ba & Bl- *Bacillus amyloliquefaciens and Bacillus licheniformis*, SP- Spontaneous fermentation.

Figure 4.1.2: **Bacterial count obtained on NA during fermentation using starter cultures**

At the onset of the fermentation (t= 0 h), the bacterial counts ranged from 7.7 to 7.8 Log CFU/g and the end of the fermentation (t= 168 h), there was an increase in bacterial load observed in all the samples with values ranging from 6.4 to 9.4 Log CFU/g.



MHA: Mueller-Hinton agar, BA- *Bacillus amyloliquefaciens;* Bl- *Bacillus licheniformis;* Ba & Bl- *Bacillus amyloliquefaciens and Bacillus licheniformis*, SP- Spontaneous fermentation.

Figure 4.1.3: Bacterial count obtained on MHA during fermentation using starter cultures

At the onset of the fermentation (t= 0 h), the bacterial counts ranged from 5.5 to 5.6 Log CFU/g and at the end of the fermentation (t= 168 h), there was an increase in bacterial load observed in all the samples with values ranging from 6.4 to 9.4 Log CFU/g.

Bacillus licheniformis and *Bacillus amyloliquefaciens* was chosen as starter cultures because they were isolated from the commercial samples of *okpeye* and also showed better results in the safety assessment test than other microorganisms isolated from the commercial samples.

Table 4.1.6: Effect of fermentation on the pH, temperature and TTA of the fermenting mash of *okpeye* from 0- 168 hours

FERMENTATION TIME (HOURS)	TEMPERATURE (°C)	pH	TTA (%)
0	$34.80^{f} \pm 0.60$	$6.76^{f} \pm 0.40$	$0.47^{e} \pm 0.12$
24	$37.86^{e} \pm 0.71$	$7.42^{e} \pm 0.30$	$0.56^{d} \pm 0.13$
48	$40.53^{ab} \pm 0.81$	$9.00^{c} \pm 0.39$	$0.94^{c} \pm 0.17$
72	$41.32^{a} \pm 1.99$	$9.58^{b} \pm 0.95$	$1.01^{bc} \pm 0.15$
96	$40.07^{bc} \pm 1.44$	$10.28^{a} \pm 1.16$	$1.18^{a} \pm 0.24$
120	$39.35^{cd} \pm 2.48$	$8.93^{cd} \pm 0.35$	$1.18^{a} \pm 0.24$
144	$37.75^{e} \pm 2.04$	$8.48^{d} \pm 0.41$	$1.08^{b} \pm 0.23$
168	$38.52^{de} \pm 1.40$	$8.49^{d} \pm 0.41$	$1.01^{bc} \pm 0.24$

Values are means with standard deviation. Mean values in the same column followed by different superscripts are significantly ($P < 0.05$) different.

The pH of all the samples of the fermenting *okpeye* monitored at 24-hour intervals throughout fermentation was within the range of 6.76 to 10. 28. The temperature ranged from 34.80 to 41.32°C while TTA ranged from 0.47 to 1.18. The lowest pH, temperature and TTA were recorded at (t= 0 h) while the highest values for pH were recorded at (t= 96 h), temperature at (t= 72 h) and TTA at (t= 96- 120 h).

Table 4.1.7: Effect of starter culture on the pH, temperature and TTA on the fermenting mash

MICRO-ORGANISM	TEMPERATURE (°C)	pH	TTA (%)
SP	$39.16^{b} \pm 1.99$	$8.48^{b} \pm 0.95$	$0.65^{c} \pm 0.15$
BA & BL	$39.96^{a} \pm 0.81$	$9.14^{a} \pm 0.39$	$1.03^{a} \pm 0.17$
BA	$38.05^{c} \pm 0.60$	$8.28^{b} \pm 0.40$	$1.08^{a} \pm 0.12$
BL	$37.93^{c} \pm 0.71$	$8.56^{b} \pm 0.30$	$0.95^{b} \pm 0.13$

Values are means with standard deviation. Mean values in the same column followed by different superscripts are significantly ($P < 0.05$) different.

Key: SP- spontaneously fermenting*okpeye,* BA & BL- *okpeye* fermenting with mixed starter cultures of *Bacillus licheniformis* and *Bacillus amyloliquefaciens*, B- *okpeye* fermenting with a single starter culture of *Bacillus amyloliquefaciens*, BL- *okpeye* fermenting with a single starter culture of *Bacillus licheniformis*.

Table 4.1.7 shows the effect of the starter culture on the pH, temperature and TTA of the fermenting *okpeye* mash. There was a significant ($P < 0.05$) difference between the pH of the different *okpeye* produced in the single and mixed starter cultures.

Plate 3: *Prosopis africana* cooked seeds

Plate 4: Dehulled *Prosopis africana* innoculated with starter cultures

Plate 5: fermenting seeds of *Prosopis Africana*

Plate 6: Pounding of fermented seeds of *Prosopis africana*

Plate 7: Moulded *okpeye* fermented with starter cultures

Plate 8: Dried *okpeye* fermented with starter cultures

Table 4.1.8: Effects of starter cultures on the proximate composition (%) of *okpeye*

Samples	Protein Content (%)	Fat Content (%)	Ash content (%)	Fibre Content (%)	Moisture Content (%)	Carbohydrate/NFE Content (%)
A	$27.80^{a} \pm 0.04$	$5.76^{a} \pm 0.04$	4.85a±0.03	$13.28^{b} \pm 0.11$	42.45c±0.31	5.86d±0.26
B	$26.70^{b \pm} 0.09$	$5.26^{bc} \pm 0.05$	4.71a±0.05	$12.51^{bc} \pm 0.12$	43.12bc±0.47	8.00b±0.85
C	$26.21^{bc \pm} 0.18$	$5.50^{ab} \pm 0.76$	4.30b±0.02	$12.73^{bc} \pm 0.21$	43.48b±0.25	7.78bc±0.19
D	$25.41^{d} \pm 0.48$	$5.15^{c} \pm 0.45$	4.36b±0.04	$12.09^{c} \pm 0.04$	43.29b±0.41	9.70a±0.15
E	$25.73^{cd \pm} 0.70$	$1.69^{e} \pm 0.31$	4.21b±0.37	$15.37^{a} \pm 1.00$	45.99a±0.64	6.90c±0.86

Values are means with standard deviation. Mean values in the same column followed by different superscripts are significantly ($P < 0.05$) different.

Key: A- spontaneously fermented *okpeye,* B- *okpeye* fermented with mixed starter cultures of *Bacillus licheniformis* and *Bacillus amyloliquefaciens*, C- *okpeye* fermented with single starter culture of *Bacillus amyloliquefaciens*, D- *okpeye* fermented with single starter culture of *Bacillus licheniformis*, E- control/ commercial *okpeye.*

Results from Table 4.1.8 shows the proximate composition (%) of the different samples of *okpeye.* The moisture content ranged from $42.45^{c} \pm 0.31$ to $45.99^{a} \pm 0.64$ %, the protein content from 25.41 to 27.80%, fat content from 1.69 to 5.76%, ash content from 4.21 to 4.85%, fibre content from 12.09 to 15.37% and carbohydrate content from 5.86 to 9.70%.

Table 4.1.9: Qualitative phytochemical properties of *okpeye* fermented using starter cultures

Samples	Oxalate (%)	Saponins (%)	Tannins (mg/100g)	Cyanide (mg/100g)	Phytate (mg/100g)	Alkaloids (%)	Total Phenol GAE mg/g	Flavonoids (%)
A	++	+	++	+	+++	++	+++	++
B	++	+	++	+	+++	++	+++	++
C	++	+	++	+	+++	++	++	++
D	++	+	++	+	+++	++	++	++
E	++	+	++	+	++	++	+++	++

Key: + trace; ++ present; +++ abundant Key: A- spontaneously fermented *okpeye,* B- *okpeye* fermented with mixed starter cultures of *Bacillus licheniformis* and *Bacillus amyloliquefaciens*, C- *okpeye* fermented with single starter culture of *Bacillus amyloliquefaciens*, D- *okpeye* fermented with single starter culture of *Bacillus licheniformis*, E- control/ commercial *okpeye.*

The qualitative phytochemical properties of the fermented samples of *okpeye* using starter cultures showed considerable presence of all phytochemicals tested except for saponins and cyanide which were in low quantities (Table 4.1.9).

Table 4.1.10: Quantitative analysis of the phytochemical properties of *okpeye* fermented using starter cultures

Samples	Oxalate (%)	Saponins (%)	Tannins (mg/100g)	Cyanide (mg/100g)	Phytate (mg/100g)	Alkaloids (%)	Total Phenol GAE mg/100g	Flavonoids (%)
A	7.54^{b}±0.14	0.03^{b}±0.04	13.29^{a}±0.01	0.17^{d}±0.02	451.92^{b}±2.21	12.69^{b}±0.23	330.36^{a}±0.20	12.37^{b}±0.01
B	11.52^{a}±0.91	0.05^{a}±0.03	11.45^{b}±0.50	0.35^{b}±0.04	549.75^{a}±0.577	13.03^{a}±0.03	154.58^{b}±0.40	14.20^{a}±0.20
C	11.06^{a}±0.01	0.02^{c}±0.02	8.25^{c}±0.29	0.26^{c}±0.12	400.41^{c}±0.12	11.67^{c}±0.15	55.02^{d}±0.55	11.27^{c}±0.06
D	6.51^{c}±0.76	0.01^{d}±0.01	6.57^{d}±0.17	0.33^{b}±0.02	225.10^{d}±1.01	10.46^{d}±0.20	33.29^{e}±0.13	11.60^{c}±0.26
E	3.00^{d}±0.65	0.01^{d}±0.01	6.40^{d}±0.16	1.60^{a}±0.04	58.53^{e}±7.85	3.18^{e}±0.24	120.46^{c}±1002	4.49^{d}±0.48

Values are means with standard deviation. Mean values in the same column followed by different superscripts are significantly ($P < 0.05$) different. Key: A- spontaneously fermented *okpeye,* B- *okpeye* fermented with mixed starter cultures of *Bacillus licheniformis* and *Bacillus amyloliquefaciens*, C- *okpeye* fermented with single starter culture of *Bacillus amyloliquefaciens*, D- *okpeye* fermented with single starter culture of *Bacillus licheniformis*, E- control/ commercial *okpeye.*

The quantitative phytochemical properties also wereanalysed and revealed the following values presented in Table 4.1.10. The oxalates ranged from 3.00 to 11.52%, saponin values ranged from 0.01 to 0.05%, tannins ranged from 6.40 to 13.29 mg/100g, cyanides ranged from 0.17 to 1.60%, phytate values ranged from 58.53 to 549. 75 mg/100g, alkaloids ranged from 3.18 to 13.03%, total phenols ranged from 33.00 to 330.36 mg/100g and flavonoids ranged from 4.49 to 14.20%.

Table 4.1.11: Sensory scores of *okpeye* fermented using starter cultures

SAMPLES	SENSORY PARAMETERS				
	AROMA	**FLAVOUR**	**APPEARANCE**	**AFTER-TASTE**	**OVERALL ACCEPTABILITY**
A	6.73 ^{b}±1.20	6.70 ^{b}±1.18	6.97 ^{b}±1.30	6.67 ^{a}±1.45	6.79 ^{b}±0.98
B	7.27 ^{a}±1.08	7.40 ^{a}±1.04	7.30^{a}±1.32	7.17 ^{a}±0.99	7.31 ^{a}±0.85
C	7.07 ^{ab}±1.02	7.03 ^{ab}±1.00	6.80 ^{b}±1.13	6.83 ^{a}±0.91	6.93 ^{b}±0.71
D	7.10 ^{ab}±1.54	7.03 ^{ab}±1.27	6.86 ^{b}±1.30	7.07 ^{a}±1.41	7.03 ^{ab}±1.06
E	6.61 ^{b}±1.15	6.94 ^{ab}±1.15	6.65 ^{b}±1.17	6.77 ^{a}±1.21	6.77 ^{b}±0.90

Values are means with standard deviation of 30 panelists. Mean values in the same column followed by different superscripts are significantly ($P < 0.05$) different.

Key: A- spontaneously fermented *okpeye,* B- *okpeye* fermented with mixed starter cultures of *Bacillus licheniformis* and *Bacillus amyloliquefaciens*, C- *okpeye* fermented with single starter culture of *Bacillus amyloliquefaciens*, D- *okpeye* fermented with single starter culture of *Bacillus licheniformis*, E- control/ commercial *okpeye.*

The sensory scores of broths prepared with *okpeye* condiment fermented with starter culture showed a favourable response from the panelist in all the attributes tested: aroma, flavour and appearance, after-taste and overall acceptability.

4.2 DISCUSSION

4.2.1: Bacterial counts in *okpeye* from some local markets in south eastern Nigeria

The microbiological quality and safety of commercial *okpeye*sold in some markets was investigated. The commercial samples of *okpeye* collected from six different markets in Anambra and Imo states respectively showed similar patterns in microbial counts with a few exceptions. (Fig. 4.1.1). Aerobic mesophile counts on PCA ranged between 10^5 to 10^6 CFU/g in samples from all markets. S*taphylococci* were present in *okpeye* samples collected in the three markets in Imo State (OWB, OEM, ORM). Also, in these samples, numbers ranged from 10^5 to 10^6 CFU/g. No *Staphylococci* were isolated from any of the Anambra state samples. Enterobacteriaceae was isolated on MacConkey agar were detected in all *okpeye* samples at levels ranging from 10^5 to 10^6 CFU/g.

In this study, bacterial counts were relatively high (6 – 7 $\log_{10}$ CFU/g) for aerobic mesophiles in samples obtained from all markets. These results agreed with several reports where total aerobic counts of up to 10 $\log_{10}$ CFU/g have been observed in indigenous fermented condiments such as *ugba*, *daddawa*, and *maari* (Kabore, Thorsen, Sandris Nielsen, Berner, Sawadogo-Lingani, Diawara, Dicko & Jakobsen, 2012; Ahaotu et al., 2013; Akanni et al., 2018). *Staphylococci* count in *okpeye* also ranged from 6 – 7 $\log_{10}$ CFU/g in all samples collectedand this corroborates the report by Ouoba et al. (2019) where *Staphylococci* count ranged between 6 – 13 $\log_{10}$ CFU/g in *bikalga*, *soumbala* and *ntobambodi*. High aerobic counts in food products at the retail level typically indicate the neglect of good hygiene and sanitary measures during production, and handling (Sadek, Abdel-Rahman, Azab, Darwesh & Hassan, 2018). However, environmental factors such as the increase in pH during the fermentation of protein-rich raw materials for condiment production would also influence the microbial population. The increase in pH during fermentation would select microorganisms that can tolerate alkaline conditions (Ouoba et al., 2019).

4.2.2: Phenotypic characteristics of *okpeye* commercial samples from Anambra and Imo State

From the phenotypic investigation carried out, the majority group was bacteria that exhibited features as expected for *Bacillus* isolates, most were Gram-positive, catalase-positive and oxidase-negative rod-shaped and endospore formers. (Appendix I). The minor group was Gram-negative, catalase positive/negative in grape-like clusters typical of *Staphylococcus* species. The combination of both culture-dependent and culture-independent methods revealed bacterial genera such as *Bacillus* and *Staphylococcus*, which have been previously reported by culture-based phenotypic studies (Ezeokoli et al., 2016; Ouoba et al., 2019).

4.2.3: Origin and identification of microorganisms from commercial samples of *okpeye*

The complexity of the micro-biodiversity of indigenous fermented condiments makes it difficult to identify and characterize the relevant functional microorganisms using single conventional methods (phenotypic characterization). However, developed molecular tools have given an alternative to conventional methods because genome sequence is independent of phenotypic characteristics and varies among species. Thus, PCR analysis and 16S rRNA gene sequencing have become very useful for the identification of microorganisms from various sources due to their simplicity, speed and reliability (Unban, Kochasee, Shetty & Khanongnuch, 2020).

The 16S rRNA genes of the predominant isolates from all the market samples were sequenced based on the sequence similarity to reference strains in the EzBiocloud database. The microorganisms were identified to genus and species level and revealed similarity rates ranging from 90.01 to 100% of all the isolates (Table 4.1.1). Of the 59 isolates, the principal groups were identified as *Bacillus* (47.4%) and are as follows; *B. cereus* (16.94%), *B. amyloliquefaciens* (10.17%), *B. tequilensis/ cabrialesii* (8.46%), *B. velezensis* (5.08%), *B. licheniformis* (3.39%), *O. caeni* (3.39%), *L. xylanilyticus* (3.39%), *P. barcinonensis* (1.69), *B.*

anthracis (1.69%) and *B. siamensis* (1.69%). It has been noted that B. *subtilis*, *B. pumilus, B. clausii, B. licheniformis, B. vallismortis, B. mojavensis, B. lentus, B. coagulans, B. fusiformis, B. atrophaeus,* and *B. amyloliquefaciens* was reliably identified using a 16S rRNA gene sequencing (EFSA, 2007).

The second predominant group (42.3%) comprising *Staphylococcus* species were identified as follows; *S. simulans* (16.94%), *S. nepalensis* (10.17%) and *S. ureilyticus/ cohnii* (6.78%). Other bacterial species identified included *O. caeni* (3.39%), *P. barcinonensi*s, *V. lutrae* (3.39%) (1.69), *L. xylanilyticus* (3.39%), *S. flexneri* (1.69%) and *E. fergusonni* (1.69%).

The microbial profile of the *okpeye* samples showed differences according to the market and city. In general, it was observed that *Staphylococcus* species were dominant in samples from Anambra State while *Bacillus* species were most prevalent in samples from Imo State (Table 4.1.1). *B. licheniformis*, *B. tequilensis and B. velezensis* were specific to samples collected in Owerri and none were isolated from any of the markets in Anambra. *S. simulans* were more predominant in Anambra Ochanja and Brigde head markets than in Anambra Ose market but none was found in any Owerri market. However, *S. nepalensis* was present only in Owerri relief and Anambra Ochanja market. However, there were also similarities across markets in both cities. For example, *B. cereus* and *B. amyloliquefaciens* were isolated from all the samples in Owerri and Anambra markets. (Table 4.1.1).

Okpeye, like other traditional fermented food condiments, has a wide microbial community revealing species and subspecies of bacteria within a locality and between the different localities where samples were taken. The isolation and enumeration of commercial samples collected from different sources showed that *Bacillus* species was most predominant as the endospore of these bacilli are believed to be associated with the cotyledons of these seeds from the onset of the fermentation process and has been found to steadily increase with an increase

in the fermentation period during the production of this condiment (Ogueke & Aririatu, 2004; Oguntoyinbo et al., 2007). Irrespective of raw material (legumes/ oil bean seeds) or country of production, most of these investigations, have reported the dominance of *Bacillus* spp during the fermentation of African indigenous condiments (Adedeji et al., 2017; Adewumi et al., 2013; Agbobatinkpo et al., 2013; Amoa-Awua et al., 2006; Azokpota et al., 2007; Ezeokoli et al., 2016; Meerak, Yukphan, Miyashita, Sato, Nakagawa &Tahara, 2008; Oguntoyinbo et al., 2010; Ouoba et al., 2008; Ouoba et al., 2017; Parkouda et al., 2009; Parkouda et al., 2010). Also, the dominance of *Bacillus* spp. in these foods has been attributed to their ability to degrade plant proteins into peptides and amino acids (Odunfa, 1985; Ouoba et al., 2003). In addition, the long cooking times used to soften the seeds before fermentation will be selective for spore formers such as *Bacillus* (Ouoba et al., 2003).

Other non-*Bacillus* and *Staphylococcus* species identified in this study also corroborates several secondary microorganisms including pathogenic bacteria and their toxins which have been identified in West African fermented seed condiments, especially pathogenic microorganisms. These pathogenic microorganisms are *Bacillus cereus*, *Staphylococcus* spp., *Escherichia coli*, *Salmonella* spp, *Shigella* spp and *Clostridium* spp. as well as mycotoxigenic molds such as *Aspergillus flavus* and *Aspergillus parasiticus* have been isolated from West African alkaline fermented seed condiments, giving rise to food safety and public health concerns (Adekoya *et al.*, 2019; Parkouda et al., 2009). However, it was also reported the presence of the species *Heyndricxia oleronia* (1.69%) in *okpeye* microbial ecology for the first time which is not associated with fermented condiments and could be attributed to the constant evolution of these microorganisms.

4.2.4: Identification of microorganisms from *okpeye* using 16s rRNA sequencing and *gyrB/ rpoB* sequencing for closely related species

Although 16S rRNA gene sequencing is considered an accurate identification tool, most of the organisms identified displayed a high percentage similarity to almost two different types of species in GenBank databases (Ezibiocloud). These observations could be explained by the limit of the technique used for microbial identification of organisms and changes in the DNA profile of the microorganisms. Previous studies have shown that this approach alone does not allow precise differences between *Bacillus subtilis* and *Bacillus amyloliquefaciens* (Porwal, Lal, Cheema & kalia, 2009; Sumpavapol, Tongyonk, Tanasupawat, Chokesajjawatee, Luxananil & visessanguan, 2010) and between *Bacillus cereus* and *Bacillus thuringiensis* (Chang, Shangkuan, Lin & Liu, 2003; Bhandari, Ahmod, Shah & Gupta., 2013) due to the high similarity observed in these species. Thus, *gyrB* and *rpoB*gene sequencing have been reported to be more informative and discriminatory for the identification of *Bacillus* species, subspecies and strains (Chun & Bae 2000; Chen & Tsen 2002; Lefevre, Racedo, Denayrolles & Ripert, 2017).

In this study, further identification was done and confirmed by sequencing *gyrB* and *rpoB* genes to differentiate closely related species with close similarity among some *Bacillus cereus*sensulato group and *Staphylococcus* group. This helped to identify the ten (10) isolates specifically as *Bacillus cereus* and one (1) as *Bacillus anthracis* as opposed to the initial identification of the isolates which revealed them as *B. cereus/ paramycoides/paranthracis* with 98.99 percent similarity. However, five (5) isolates out of *Bacillus* and *Staphylococcus* group could not be identified even with further sequencing and could be attributed to the sequencing pattern or inconsistency/changes in the DNA (Table 4.2.1).

In a surprising turn in this investigation, *Bacillus subtilis* which is well documented as the most predominant *Bacillus* species in most alkaline fermentation was not identified in this research.

This contrasts with this study where *B. amyloliquefaciens* and *B. cereus* were the most prevalent. Previous reports have highlighted *B. subtilis* as the most predominant *Bacillus* species in *okpeye* (Fowoye, 2017; Ogunshe et al., 2007). However, these studies relied on phenotyping alone for identification. Oguntoyinbo et al. (2007) reported that 50% of the *Bacillus* species isolated from *okpeye* was *Bacillus subtilis* even though *Bacillus subtilis subsp. spizizenii, Bacillus subtilis subsp. subtilis, B. amyloliquefaciens, B. licheniformis and B. firmus* were grouped in the same cluster II. Similar reports on the genotypic identification of the *Bacillus* species in African food condiments using molecular techniques also highlighted members of the *B. subtilis* group complex as the dominant species (Adewunmi et al., 2013; Ouoba et al., 2004; Oguntoyinbo et al., 2010).

The predominant *Bacillus* species found were *B. cereus* and *B. amyloliquefaciens* and this variation could be in the method of sequencing and primers used or could be a result of evolution and changes in the DNA of these microorganisms. There was a similar report on the dominance of *B. cereus* during the fermentation of *ugba* [*Pentaclethra macrophylla* (Benth using the genotypic method by Ahaotu et al. (2013). Many authors have also reported the presence of *B. cereus* strains in high numbers from several West African fermented seed condiments including *sonru, iru and afitin* (Azokpota et al., 2006; Azokpota et al., 2007; Thorsen et al., 2010), *ogiri* and *dawadawa* (Okanlawon, Ogunbanwo & Okunlola., 2010) and *bikalga* (Ouoba et al., 2008).

The second major group (*Staphylococcus*) encountered in this investigation could be attributed to possible contamination from leaf wrappings, fermentation materials, source of water and personal hygiene of the handlers although possible questions could arise in finding out if these species contribute during fermentation of *okpeye*. They have often been isolated from alkaline fermented foods as secondary microorganisms, but a clear indication of their role in fermentation is not established (Ouoba et al., 2019). Nevertheless, it could be suggested that

the presence of *Staphylococcus* species contributes to some biochemical activities such as those related to the degradation of proteins and lipids that some species can perform during meat fermentation to produce sausages (Casaburi, Monaco, Cavella, Toldra, Ercolini & Villani, 2008; Milicevic, Danilovic, Zdolec, Kozachinski, Dobranic & Savic, 2014). The microbial profile associated with the commercial *okpeye* samples will be influenced by bacterial sources and processing conditions along the value chain. Bacterial species associated with the raw material, ingredients, processing utensils, environment, and the processors would vary by locality (Anyogu et al., 2021; Iwuoha & Eke, 1996). The differences observed in this study may be attributed to these variations and highlight the need for further studies that characterize bacterial species along the processing chain.

4.2.5: Analysis of toxin genes from *Bacillus cereus* isolated from *okpeye*

All *B. cereus* isolates identified in this study were screened for the detection of toxin-producing genes. Five out of the ten (10) *B. cereus* tested possessed all genes encoding the production of non-haemolytic enterotoxin complex (*nheA*, *nheB*, *nheC*) as shown in Table 4.1.3. However, all *B. cereus* tested positive for the presence of *nheA*, while *nheB* and *nheC* were amplified in only seven of these isolates. Seven *B. cereus* were positive for the presence of all three genes encoding haemolysin BL complex (*hblA*, *hblC*, *hblD*). Similarly, 70% of the *B. cereus* isolates possessed the diarrhoeal toxins *cytK* and *entFM* although this did not always correspond with the presence of genes encoding the other enterotoxin genes. The emetic toxin (*cesB*) was detected in three *B. cereus* isolates. Only one *B. cereus* (O21), isolated from the *okpeye* samples purchased from Ekeukwu market in Owerri (OEM), tested positive for all toxin genes screened (Table 4.1. 3). However, three isolates (O22, O34, A11) possessed all toxin genes screened except for the emetic toxin (*Ces*). The toxin genes *nheA, nheB, hblA, hblC, hblD*, *cytK* and *entFM* were detected in all *B. cereus* isolated from the Owerri samples.

The predominance of *B. cereus* in retailed *okpeye* from this study calls for concern for the health of consumers. The predominance of *B. cereus* in this study is similar to observations in *ugba* reported by Ahaotu et al. (2013). There are other reports of the presence of *B. cereus* in indigenous fermented condiments available for retail sale (Adekoya *et al*., 2019; Ademola et al., 2018). This suggests that *B. cereus* is frequently associated with these foods. It has been stated that the presence of *B. cereus* in most traditional fermented condiments could be a potential source of food poisoning and therefore a public health concern (Adjoumani et al., 2019). All ten *B. cereus* isolates in this study had at least one gene or gene complex associated with the production of an enterotoxin or an emetic toxin (Table 4.1.3). In particular, genes encoding the non-haemolytic (*Nhe*) toxin complex (*nheA*, *nheB*, *nheC*) appears to be quite widespread in *B.* cereus from indigenous fermented condiments.

All ten are positive for *NheA* genes (100%), *NheB* genes (70%), *NheC* genes (70%) as well as *HblA* genes (70%) which is an indication that the isolates are virulent and possible causes of foodborne diseases. A high percentage (100%) of *B. cereus* harbour and express *Nhe* genes encoding non-haemolytic enterotoxin, as reported by Guinebretiere & Broussolle (2002) for *B. cereus* isolates from vegetables. Ahaotu et al. (2013) also reported that *B. cereus* isolated from *ugba* expressed the genes to produce enterotoxins and all the isolates (100%) had the *Nhe (NheA, NheB, NheC)* and *CytK*genes and one isolate exhibited the *HBl* genes (*HblA, HblB, HblD*) in addition to the others. In this study, genes encoding the enterotoxins *CytK* and Enterotoxin FM were also detected in *B. cereus*. While potential cereulide producers have previously been isolated from African fermented food condiments (Thorsen et al., 2015), to the best of our knowledge, this study is the first to report this observation in *okpeye*. Ouoba et al. (2008) also reported similar results from *Soumbala* and *Bikalga* (alkaline fermented condiments from Burkina Faso, West Africa), in which 100% of the isolates exhibited all three *Nhe* genes, 67% the *CytK* gene and 22% the *Hbl* genes and that the occurrence of *Nhe* and *Cytk*

genes seems to be more frequent than *Hbl* genes in *B. cereus* from such origins. Also, a study by Guinebretière, Velge, Couvert, Carlin & Debuyser, (2010) on the ability of *B. cereus* group strains to cause food poisoning revealed that *Nhe*genes are a constant part of the *B. cereus* group strains while the frequency of *Hbl* and *CytK* genes varies according to the phylogenetic group.

Furthermore, Owusu- Kwarteng, Akabanda, Neilsen, Tano-Debrah, Glover & Jespersen, (2017) also reported that *B. cereus* sensu lato isolated from cattle grazing soils and dairy products in Ghana exhibited the presence of enterotoxigenic genes (*hblA, hblC, hblD, nheA, nheB, nheC, cytK and entFM*), and one emetic gene (*ces*) using PCR screening. In addition, emetic toxin-producing genes have previously been detected at different low rates (1.5 to 17.2%) in *B. cereus* strains isolated from various food sources (Chaves, Cavados & Vivoni, 2012; Naranjo, Denayer, Botteldoorn, Delbrassinne, Veys, Waegenaere, Sirtaine, Driesen, Sipido & Mahillon, 2011) and the different prevalence rates could be attributed to the differences in properties in the food (Zuberovic, Troger, Granelli & Hellenas, 2014).

4.2.6: Haemolytic activities of some microorganisms isolated from *okpeye*

In addition to toxin production from the *B. cereus* isolates, haemolytic activity is an important determinant in assessing the virulence potential of bacteria. Most of the isolates tested displayed haemolytic activity on blood agar (Table 4.1.4). Beta (β)-haemolysis is considered harmful while alpha (α) and gamma (γ) haemolysis are considered safe (Jeon, Lee, Yang, Kim & Pak, 2017). Out of twenty- eight (28) isolates tested for haemolytic activity in this study, 23 among which *Bacillus* species make up 69% of the tested isolates displayed complete haemolysis (β) which is an indication that these organisms could lyse blood cells. This is similar to the results in Ouoba et al. (2008) in which 29 out of 41 *Bacillus* isolates from *Soumbala* and *Bikalga* showed haemolytic activity on blood agar. The results also indicate that the hemolytic factor of these isolates will decrease the amount of hemoglobin available as an

iron source for the host (Şeker, 2010). The presence of potentially pathogenic bacteria, and virulence factors provides further evidence that fermented condiments such as *okpeye* might be considered vehicles for the transmission of microbial hazards.

Only three (3) were γ-haemolytic on sheep blood agar plates (*Bacillus amyloliquefaciens*, *Bacillus licheniformis* and *Vagococcus carniphilus.*) which signified an absence of virulence activities and might not cause food borne illness. Some strains of *B. clausii, B. coagulans, B. licheniformis* and *B. subtilis* have been generally recognised as safe (GRAS) and known to be part of gut microflora (Cutting 2011; Ilinskaya, Ulyanova, Yarullina & Gataullin, 2017). The results are also in line with report of Dabire, Somda, Somda, Compaoré, Mogmenga, Ezeogu, Traoré & Ugwuanyi (2022) where some *Bacillus* strains showed γ-haemolysis on sheep blood agar plates. The ability of these *Bacillus* strains to show non- haemolysis on plates is an indication that they might not lyse blood cells and would be a useful criterion for selecting them as starter cultures.

4.2.7: Antibiotic susceptibility of some microorganisms isolated from *okpeye*

Effective antibiotic therapy is the main treatment for bacterial infections; Therefore, the rise of antibiotic-resistant infections is a major threat to public health (Nwobodo, Ugwu, Anie, Al-Quqaili, Ikem, Chigozie & Saki, 2022). Food remains an important route for the transmission of bacteria to consumers. In indigenous fermented foods, bacteria are often consumed in large quantities. Hence, antibiotic susceptibility is a very important criterion for assessing the safety of bacteria of food interest (Nwagu, Ugwuodo, Onwosi, Inyima, Uchendu & Akpuru, 2020). Indeed, bacterial antibiotic resistance is due to either (i) intrinsic properties (natural phenotypic traits) or (ii) the acquisition of resistance genes through mobile genetic elements, such as plasmids and transposons, or the mutation of indigenous genes (Sharma, Kumar, Goswami, Sangwan & Singh, 2014).

Twenty-eight isolates were tested for antimicrobial susceptibility with ten antibiotics: including ampicillin, penicillin, tetracycline, gentamicin, streptomycin, ciprofloxacin, erythromycin, chloramphenicol, kanamycin, and vancomycin. All the strains showed diversity in their susceptibility and resistance to the ten (10) antibiotics tested irrespective of the locality of the samples (Table 4.1.5). The microorganisms isolated from the Owerri market samples were susceptible to most of the antibiotics tested compared to isolates from *okpeye* samples purchased from Anambra. *B. tequilensis* (O6, O7, O23) which was susceptible to almost all antibiotics tested except for tetracycline and ciprofloxacin. Also, *B. amyloliquefaciens* O27, was multi-drug resistant to the antibiotics except for ampicillin, penicillin and ciprofloxacin while A16 identified from Anambra market was susceptible to the rest of the antibiotics. *S. cohnii* A13 and *V. carniphilus* A12 from the Anambra market were also multi-drug resistant to most of the antibiotics.

Interestingly, the most effective antibiotic was Gentamicin (100%) while all the isolates were resistant to Ciproflaxin except two isolates from the Anambra market (A2 and A7). 75% of *Bacillus* isolates tested were also most susceptible to Penicillin G, Tetracycline, Streptomycin, Erythromycin, Chloramphenicol and Kanamycin. This is similar to results that *Bacillus* species isolated from *soumbala* (fermented *Parkia biglobosa* seeds), *iru*(fermented African locust bean) and *mbuja* (fermented *Hibiscus sabdafiffa* fermented seeds) were all susceptible to Penicillin, Gentamicin, Erythromycin, levoflaxacin, ciprofloxacin, ofloxacin, vancomycin, streptomycin, azithromycin, rifampicin, ampiclox and Chloramphenicol (Mohammodu, gwenaelle, Carl & Georges, 2014; Nwagu et al., 2020; Olanbiwonimu, Esho, osinupebi, Awotundun & Fasiku, 2022; Dabire et al., 2022). Similar results have also been reported for susceptibility of *Bacillus* species to several antibiotics (Compaoré et al., 2013; Thankappan, Ramesh, Ramkumar, Natarajaseenivasan& Anbarasu, 2015; Kavitha et al., 2018). The most

predominant *Bacillus* species identified in this study *Bacillus cereus* exhibited 50% susceptibility and resistance while *Bacillus amyloliquifaciens* exhibited 90% susceptibility.

Owusu- Kwarteng et al. (2017) studies have also shown that *B. cereus* isolated from soil, milk and milk products and *daddawa* (fermented *Parkia biglobosa* seeds) were susceptible to Tetracycline, Erythromycin, Chloramphenicol, Gentamicin, amoxil, rifampicin, ampliclox and Vancomycin. In addition, multidrug resistance was observed in some isolates including *B. cereus*, the indiscriminate use of antibiotics in the medical, veterinary, and agricultural sectors contributes to selective pressure for antibiotic-resistant bacteria in the environment. These results support urgent calls on the need for large-scale surveillance studies to investigate the prevalence of antibiotic-resistant bacteria in indigenous fermented foods. However, *Bacillus amyloliquefaciens* which exhibited 90% susceptibility to the antibiotics tested is an indication that this strain is safer and could be considered as a starter culture for safety purposes.

4.2.7.1: Selection of starter culture for fermentation of *Prosopis africana* seeds into *okpeye*

The results obtained in this study demonstrated the safety issue associated with foods prepared by traditional, uncontrolled fermentation. The most predominant *Bacillus* identified was *B. amyloliquefaciens* and *B. cereus* which was present in all the markets. There is no possibility of using *B. cereus* as a starter culture for the production of *okpeye* as most of the *B. cereus* group investigated in this study possessed all genes encoding the production of haemolytic and non- haemolytic enterotoxin tested. There is a necessity to select non - *B. cereus* starter cultures for controlled fermentation of *okpeye* from the identified *Bacillus* species which would provide a safe product with increased nutritional and hygienic quality as well as improved stability of the condiment. Overall, the *Bacillus* identified that had the best results from the safety assessment conducted on all the identified microbial communities was *B. licheniformis* and *B. amyloliquefaciens*. Both microorganisms gave negative result for haemolytic activity (Table 4.1.4) and also had 80-90% susceptibility to the anibiotics tested (Table 4.1.5).

B. licheniformis was not predominant but have been reported to be associated for most fermented condiments while *B. amyloliquefaciens* was one of the predominant reported in the study and also associated with most fermented condiments. These two *Bacillus* species *B. licheniformis* and *B. amyloliquefaciens* were finally chosen and used as starter culture singly and in combination to carry out the fermentation of *Prosopis africana* into *okpeye*.

4.2.8: Bacterial count obtained on NA and MHA during fermentation using starter cultures

The *B. licheniformis* and *B. amyloliquefaciens* used as starter cultures have been reported in this study to be associated with *okpeye* and was isolated from the commercial *okpeye*

Fig 4.2.2 and Fig 4.2.3 show the growth of the different starter cultures inoculated in single, mixed and spontaneous fermentation in the cooked dehulled *Prosopis africana* seeds. At the onset of the fermentation (t= 0 h), the bacterial counts ranged from 5.5 to 5.6 Log CFU/g for the MHA agar and 7.7 to 7.8 Log CFU/g for NA. At the end of the fermentation (t= 168 h), there was an increase in bacterial load observed in all the samples with values ranging from 6.4 to 9.4 Log CFU/g. The starter cultures showed different abilities to grow in the *Prosopis Africana* seeds. A single starter of *Bacillus amyloliquefaciens* had a slightly lower count than the single starter of *Bacillus licheniformis* for both agars. The mixed starter cultures of *Bacillus licheniformis* and *Bacillus amyloliquefaciens* had the highest count for both agar and were too numerous to count on the plates (TNTC). However, the spontaneously fermented *Prosopis africana* seeds showed an increase in bacteria from 5.6 to 8.5 Log CFU/g all through the fermentation period.

The increase of microbial loads during the production of *okpeye* is an indication that the starter cultures used in the study can use *Prosopis africana* cooked cotyledons as a substrate for their growth. However, the fermentation capacity varied among the strains. The low count in both the single, mixed and the spontaneous fermentation at 24 hours could be attributed to the fact

that the *Bacillus* inoculum is still getting acclimatized to the new environment and the substrate. As the pH and temperature increased with an increase in fermentation time, *B. licheniformis* and *B. amyloliquefaciens* got more adapted to their environment and multiplied at their maximum rate with t= 120 - 168 hours having the highest bacterial count. Thus, the longer the fermentation time the higher the bacterial load. In addition, the high microbial loads observed with the single and mixed starter of *B. licheniformis* and *B. amyloliquefaciens* could be due to the fact that these strains were previously isolated from the commercial samples of *okpeye* and are therefore more able to use this substrate for their growth. Indeed, the autochthonous character of these starters gives them a better implantation during the fermentation process (Fessard, 2017).

The *Bacillus* loads (7.70 - 9.40 log CFU/g) found in the samples inoculated with starter cultures *B. licheniformis* and *B. amyloliquefaciens* were close to those of Ahonoukoun (2014) who had the highest microbial load of 9.5 log CFU/g during the controlled fermentation of *Afitin* with *B. subtilis* starter cultures. Ahaotu, Njoku, Elmore & Maduka, (2022) also recorded that during the fermentation of African oil bean seed slices inoculated with *Pseudomonas fluorescens* and *B. licheniformis*, microbial count increased from 3.0×10^6 to 1.5×10^9 CFU/g and 7.0×10^6 to 4.0×10^9 CFU/g respectively for single starters while microbial load of 7.0×10^6 to 1.72×10^{10} CFU/g was recorded for the sample inoculated with mixed culture. Furthermore, the increase in microbial load during the fermentation of *okpeye* with these starter cultures demonstrates the fermentation capacity of these starter cultures. A similar range of bacterial counts has also been reported previously (Amoa-Awua, Awusi, Owusu, Appiah, Ofori, Thorsen & Jespersen, 2014; Ajayi, Akinrinde & Akinwunmi, 2015; Guissou, Parkouda, Anais, Korotimi, Obulbiga & Savadogo, 2020).

4.2.9: Effect of Fermentation and starter cultures on the pH, Temperature and TTA on the Fermenting Mash of *okpeye*

The pH of all the samples of the fermenting *okpeye* monitored at 24-hour intervals throughout fermentation was within the range of 6.76 to 10. 28. The lowest pH, temperature and TTA were recorded at (t= 0 h) while the highest values were recorded at (t= 96 h) except for TTA which was highest at (t= 120 h). These values gradually decreased as fermentation ended (Table 4.1.6). Table 4.1.6 shows the effect of the starter culture on the pH, temperature and TTA of the fermenting *okpeye* mash. There was a significant ($p< 0.05$) difference between the pH of the different *okpeye* produced in the single and mixed starter cultures. The sample with *Bacillus amyloliquefaciens* starter culture had the lowest pH (8.28± 0.40), followed by the spontaneous fermentation (8.48± 0.95) and the sample with *Bacillus licheniformis* (8.56± 0.30). The sample with the mixed starter culture had the highest pH at 9.14. The temperature of the fermenting mash ranged from 34.80 to 41.32 throughout the days of fermentation. The highest temperature was recorded with the sample with mixed starters (39.96± 0.81) followed by the spontaneously fermented sample (39.16± 1.99). The samples with the single starters had the lowest temperature and there was no significant ($p< 0.05$) difference between them. The total titrable acidity ranged from 0.47 to 1.18 as the fermentation progressed. The spontaneous fermentation (sample A) had the lowest TTA (0.65± 0.15) while the sample with mixed starter culture had the highest TTA (1.08± 0.12).

During the fermentation process, the pH of all the samples gradually increased from an acid state to an alkaline state (6.76- 10.28) with the highest pH recorded between 72-96 hours. It is an indication of alkaline fermentation process. There was no significant ($p< 0.05$) difference in the pH of the samples A and B but there was a significant ($p< 0.05$) difference between A, B and C, D. This result is in agreement with those recorded for similar African fermented condiments by other authors (Azokpota et al., 2006; Akabanda et al., 2018; Mohammadou,

Mbofung, Mounier & Coton, 2018; Ibrahim, Dandare, Sa'adat, Adamu, Fatima & Shinkafi, 2018). This alkaline pH is due to the proteolytic activity of the fermenting microorganisms, which degrade proteins and release ammonia in the medium (Mohammadou et al., 2018). The observed increase in pH during fermentation time could be attributed to the ability of *Bacillus* species to hydrolyze protein into amino acids and ammonia. Also, the proteinaceous nature of the plant raw materials used for the processing of these natural food condiments is generally characterized by extensive hydrolysis of the proteins into peptides, amino acids and ammonia, resulting in pH increasing to about 8–10 in the final product (Leejeerajumnean *et al.,* 2001; Omafuvbe et al., 2004). Oguntoyinbo et al*,* (2007) reported an increase in the pH of 6.0- 9.0 during the production of *okpeye i*n batches using a mixture of *Bacillus* and *Enterococcus* strains. Reports from Ahaotu et al. (2022) revealed a similar pH range during the fermentation of *ugba* using *Bacillus* strains as a starter culture.

During the fermentation, the TTA of the fermenting mash increased from (0.47- 1.18), with samples with starter cultures having higher TTA than the sample without starter. The higher TTA observed with the samples with starter cultures could be attributed to the high metabolic activities of the *Bacillus* strains leading to the change of acid-producing microorganisms during fermentation (Xia, Lui, Tang, Lei, Meng & Liu, 2022). An increase in TTA values could also result from the amylolytic, hydrolytic and lipolytic activities of the fermenting organisms that breakdown fats and carbohydrates (Poopoola, Jolaoso, Afolabi & Akintokun, 2007). TTA is a basic indicator of the food fermentation process and it has been debated that the higher the TTA, the stronger the fermentation flavours. It can affect the accumulation of metabolites and the growth of microorganisms, thereby changing the quality and flavour of food (Wu, Tian, Lui, Shi, Tao & Wu, 2018).

In this study, as fermentation also progressed, the temperature increased from 34ºC at 0hours to 41ºC at 72 hours (Table 4.1.7). The relationship between fermenting activities of the

microorganisms and temperature could be observed by the gradual increase in temperature especially with samples with the mixed starter cultures of *B. licheniformis* and *B. amyloliquefaciens.* The optimum growth temperature of *Bacillus licheniformis* and *Bacillus amyloliquefaciens* used as starters are between 35 and 50ºC and would also contribute to the increase in temperature during the fermentation. Gberikon & Agbulu (2015) reported an increase in temperature of 35ºC to 50ºC during the fermentation of *Glycine max* seeds with mixed starter cultures of *Bacillus* strains. It was observed that the fermenting mash of *Prosopis africana* seeds with mixed and single starters fermented within 120 hours at a temperature of 41^0C as shown by the softness and stickiness of the mash and its characteristics of pungent smell.

The mash of spontaneous fermentation without starters was still strong and non-sticky at 120 hours and was allowed to ferment till 168 hours which corresponds with the time it takes the local producers to complete the fermentation of this condiment. This could be attributed to the fact that starters are known to fasten the production process of fermentation and the increase in temperature is due to the increase in metabolic activities during which heat is being dissipated (Odunfa, 1984). There was also a gradual drop in temperature and pH as the fermentation process was being completed due to reduced metabolic activities as seen in Table 4.1.7. Thus, it could be deduced that the fermentation got to its peak at 72 hours as indicated by the peak temperature of fermentation (41ºC). The rest of the fermentation was sustained by the already produced enzymes (Ogueke, Anosike & Owuamanam, 2015).

4.2.10: Effects of Starter Cultures on the Proximate Composition (%) of *Okpeye*

Results from Table 4.1.8 show the proximate composition of the different samples of *okpeye* from the starter cultures. The nutritional composition of *okpeye* produced using starter cultures showed varied results in all samples. There was a significant ($p< 0.05$) difference between the samples with the starter cultures and the spontaneous and commercial samples (Table 4.1.8) Generally, samples (B, C and D) with single and mixed starters had protein, fat, ash, and carbohydrate content almost at par with the sample without starter and the commercial sample used as control. According to Bukar & Balarabe (2019), similar variations in the values in proximate composition using different densities of *B. subtilis* as a starter in the fermentation of *daddawa* against samples without a starter were recorded. Gberikon, Agbulu &Yaji, (2015) also reported nutritional values of natural fermentation of this condiment viz, crude protein (24.70 - 30.79%), crude lipid (10.01 -11.03%), fibre (5.01 - 7.07%) and carbohydrates (26.01 - 28.17%).

The moisture content of fermented *okpeye* with starter cultures ranged between 43.12±0.47 to 43.48±0.25%, the spontaneous fermentation was 42.45±0.31% whereas the control/ commercial sample showed 45.99±0.64%. The moisture content for all the samples ranged from 42.25% – to 43.48% and were comparable to the control/commercial which had a moisture content of 45.99%. Oguntoyinbo et al., (2001) recorded moisture contents of 40.70 - 60.0% in *okpeye* samples from market sources. Similar results in the study of Omafuvbe et al (2004) indicated that the moisture content of the processed African locust bean and melon seeds ranged between 51.9-56.7% and 43.0-44.1%.

There was no significant ($p> 0.05$) difference between the ash content of the *okpeye* using single starter cultures of *Bacillus licheniformis* and *Bacillus amyloliquefaciens* (4.30±0.02 and 4.36±0.04%). The spontaneous fermentation had the highest ash content 4.85±0.03% followed by *okpeye* with mixed starter culture with a value of 4.71±0.05%. The commercial sample had

the lowest ash content at 4.21±0.37%. The ash content of all the samples was in the same range (4.21 -4.85%) and the low ash content after fermentation can be attributed to the loss of some minerals during the fermentation processes (Oluwaniyi & Bazambo, 2016). This value has a direct relationship with the high level of phytate in this study since phytates have been implicated in the 'hard-to-cook' phenomenon of legumes and could also bind some essential minerals (Farinde, Olanipekun & Olasupo, 2018).

The protein content varied between 25.73 to 27.80% with the *okpeye* fermented with a single starter culture of *Bacillus licheniformis* having the lowest protein content of 25.41± 0.48% compared with *okpeye* with mixed starter culture which had a higher protein content than the single starter cultures. The protein content of the samples produced with the starter cultures was slightly higher than the commercial samples and might be due to the proteolytic activity of the fermenting strains (Mohammadou et al., 2018). This might have resulted from using mixed culture of the isolates (*B. licheniformis* and *B. amyloliquefaciens*) which were given an equal chance to act on the substrate and the accelerated rates that accompany the growth of microorganisms in mixed cultures. For example, a higher growth rate was observed when *B. subtilis* was co-cultured with *Staphylococcus epidermidis* compared to when it was cultured alone (Olanbiwoninu & Odunfa, 2018). The result obtained in this study is consistent with the findings of Balogun, Oyeyiola & Kolawole, (2017) who reported high crude protein content when mixed culture of *Bacillus subtilis and Bacillus licheniformis* were used to ferment *Prosopis Africana* seeds. The higher protein with the starters is also similar to those of the results recorded during the production of *iru* using *B. subtilis* as a starter culture (Aderibigbe, Omodara & Afolabi, 2018). The higher protein content could also be due to the higher metabolic activity of the starters leading to the breakdown of the substrates with the release of more proteinous materials.

For the crude fat content, it ranged from 1.69 to 5.76% with the commercial samples having the lowest fat content 1.69± 0.31%. *Okpeye* with the starter cultures had similar fat content from 5.15± 0.45 to 5.50± 0.76%. The highest fat content was recorded with the spontaneous fermented *okpeye* at 5.76± 0.04%. The fat content of the samples with starters in this study was higher than the commercial sample but could be considered low (5.15- 5.50%). The decrease in fat content might be because of some lipolytic enzymes released during fermentation, thus hydrolyzing fat to glycerol and fatty acid. This low-fat content might be considered desirable since high amounts of fatty acids in foods could cause rancidity thereby making the food taste sour (Fowoyo, 2017).

Regarding the fibre content, the commercial sample had the highest fibre value of 15.37±1.00% while the lowest value was *okpeye* fermented with a single starter culture of *Bacillus licheniformis* (12.09± 0.04). There was also a significant ($p< 0.05$) difference between the fibre content of all the *okpeye* samples. The commercial sample had higher values in fiber content than the samples with starters which could be a result of other materials added to the condiment to increase its quantity to make more profit.

For the carbohydrate content, the *okpeye* fermented with a single starter culture of *Bacillus licheniformis* had the highest carbohydrate value 9.70±0.15% while the spontaneous and commercial samples gave the lowest carbohydrate value (5.86±0.26 and 6.90±0.86). This study was not in agreement with Gberikon, Agbulu &Yaji, (2015) who reported carbohydrate content of (26.01 - 28.17%) in the natural fermentation of *okpeye*. The carbohydarate content in this study had a value of (5.86 -9.70%) including samples with starters, without starters and commercial samples. There are reports in literature where the predominance of carbohydrates in fermented leguminous seeds is highlighted (Olasupo et al., 2016; Fowoyo, 2017; Ajatta, Olaoye & Enujiugha, 2018). The reduced carbohydrate in all the *okpeye* samples might be associated with its transformation and utilisation by fermenting microorganisms and also the

starters have not been able to reduce the carbohydrates into sugars easily utilizable by the fermenting organisms during fermentation effectively.

4.2.11: Qualitative and Quantitative composition of the Phytochemical Properties of *Okpeye* samples

Okpeye being of legume origin is known to contain antinutrients which are compounds that significantly reduce the nutritional value of legumes. They are also known to adversely affect the bioavailability and utilization of nutrients, resulting in neurological disorders and even death (Olagunja, Ezekiel, Ogunshe, Oyeyinka & Ijabadenyi, 2018). Anti-nutritional screening of the samples revealed the presence of alkaloids, flavonoids, phenols, phytates, oxalates, and tannins while saponins and cyanide were in trace levels in all samples analyzed (Table 4.1.9).

The quantitative phytochemical properties were analysed and revealed the following values presented in Table 4.1.10. The oxalates ranged from 3.00 to 11.52%, with sample the commercial sample having the lowest value of 3.00±0.65% and *okpeye* fermented with mixed starter cultures of *Bacillus licheniformis* and *Bacillus amyloliquefaciens* sample having the highest value of 11.52±0.91%. There was a significant ($p< 0.05$) difference between the oxalate content of all the *okpeye* samples.

The saponin values ranged from 0.01 to 0.05% with *okpeye* fermented with mixed starter cultures of *Bacillus licheniformis* and *Bacillus amyloliquefaciens* having the highest value of 0.05±0.03% with slight variations between the other samples. The tannins ranged from 6.40 to 13.29 mg/100g with sample commercial sample having the lowest value of 6.40±0.16 mg/100g and the spontaneously fermented sample having the highest value of 13.29±0.01mg/100g. There was a significant ($p< 0.05$) difference between the tannin content of all the *okpeye* samples.

The cyanides ranged from 0.17 to 1.60% with the spontaneous sample having the lowest value 0.17±0.02% while the commercial sample had the highest value 1.60±0.04%. The phytate values ranged from 58.53 to 549. 75 mg/100g with the commercial sample having the lowest value of 58.53±7.85 mg/100g and the sample with *okpeye* fermented with mixed starter cultures of *Bacillus licheniformis* and *Bacillus amyloliquefaciens* having the highest value of 549.75^{a}±0.577 mg/100g.

Alkaloids ranged from 3.18 to 13.03% with sample commercial sample having the lowest value of 3.18±0.24% and the sample with *okpeye* fermented with mixed starter cultures of *Bacillus licheniformis* and *Bacillus amyloliquefaciens* having the highest value of B having the highest value 13.03±0.03%. The total phenols ranged from 33.00 to 330.36 mg/100g with *okpeye* fermented with a single starter culture of *Bacillus licheniformis* having the lowest phenol content 33.29±0.13 mg/100g) and the spontaneous sample having the highest phenol content 330.36±0.20 mg/100g). There was a significant ($p< 0.05$) difference between the total phenol content of all the *okpeye* samples. The flavonoids ranged from 4.49 to 14.20% with the commercial sample having the lowest and *okpeye* fermented with mixed starter cultures of *Bacillus licheniformis* and *Bacillus amyloliquefaciens*, having the highest value.

The total phenols and flavonoids gave higher values in the sample with starters compared tothe commercial samples (Table 4.1.10). Phytochemicals such as (phenols and flavonoids) have for long been known to protect body cells by mopping up reactive oxygen species, scavenging free radicals, and chelating metal catalysts, thereby preventing diseases linked to oxidative stress as well as demonstrating antimicrobial properties (Alia, Horcajo, Bravo & Goya, 2003; Oboh & Akindahunsi, 2004; Zhang et al., 2015; Mace, Hansen & Rupasinghe, 2017). Higher levels of phenol in the sample with starters in this study are similar to the reports given during the fermentation of *iru* with *Bacillus subtilis* (Atere, Oyetayo & Akinyosoye, 2019; Omodara & Aderibigbe, 2014).

The alkaloids content of the samples with starters gave a higher value (> 41%) than the commercial sample. Alkaloids have been known to possess anti-diarrheal and anti-dysenteric properties. Saponin which has been implicated in causing bloating and also reducing protein digestibility due to the formation of sparingly digestible saponin-protein complexes (Das, Banerjee, Chakraborty, Pakhira & Shrivastava, 2012), was found to be trace (< 0.1) in all the samples. The cyanogenic glycoside composition of the samples with starter was higher than the commercial sample. This is acceptable since cyanide is known to be toxic and fermentation has been known to reduce the cyanide contents in legumes.

There was a significant ($P < 0.05$) higher level of phytates in the samples with starter than in the commercial samples. Phytate and phytic acid have been known to have a strong binding affinity to dietary minerals, calcium, zinc, and iron, inhibiting their absorption (Schlemmer, Frolich, Prieto & Grases, 2009) and thus its degradation during fermentation could increase the availability of some minerals (Senapathi, Kumari, Raj, Prajapati, Sandhu, Angmo, Taweechotipatr, Chandel, Pinto, Tanasupawat, Sharma, Thorat & Bhalla, 2015). The result obtained in this study is consistent with the findings of Balogun, Ahmed, Akintayo, Aruna, Omovbude& Shittu, (2021) during the controlled fermentation of pigeon pea condiment using *Bacillus* and *Lactobacillus* species. Tannins and oxalates values recorded in the *okpeye* samples with starter cultures were higher than the commercial sample and were significantly ($p < 0.05$) different among the single and mixed starters. Tannins are among the components that could help to reduce the risk factors associated with suffering from cardiovascular diseases and some forms of cancer (Pizzi, 2019). The high levels of tannins in this study are also similar to reports given by Akpata, Ani & Nwodo (2023) on phytonutrients and anti-nutrient composition of aqueous extract of fermented seeds of *Prosopis africana*. These phytochemicals (tannin, flavonoids, alkaloids and phenols) present in higher values in the samples with starter cultures

than the commercial sample is an indication that *Prosopis africana* fermented with *B. licheniformis* and *B. amyloliquefaciens* could still serve as a good source of phyto-nutrients.

4.2.12: Sensory scores of *okpeye* fermented using starter cultures

The organoleptic properties of locally produced fermented condiments are a major quality attribute and significantly impact consumer approval due to their distinctive aroma and flavour (Ugwuanyi, 2016). Also, the proteolytic process is a key step that influences the taste profile of fermented food condiments which involves the degradation of protein to yield free amino acids. Studies have also indicated that the aroma, flavour and taste imparted on soups and sauces cooked using fermented condiments are as a result of volatile compounds like aldehydes, organic acids, ketones and phenols (Onyenekwe, Odeh & Nweze, 2012; Ojinnaka & Ojimelukwe, 2013). These organic compounds are usually released during microbial fermentation which involves the degradation of lipids and amino acids (Nwokeleme & Ugwuanyi, 2015; Ahaotu et al., 2022).

In this study, the aroma of all the broth ranged from (6.61 to 7.27), flavour ranged from (6.70 to 7.40), appearance ranged from (6.65 to 7.30), aftertaste ranged (6.67 to 7.17) and the overall acceptability ranged from (6.77 to 7.31). The broth cooked using *okpeye* fermented with mixed starter cultures of *Bacillus licheniformis* and *Bacillus amyloliquefaciens* gave better values from 7.27±1.08 to 7.40±1.04 while the commercial sample had the lowest value of 6.61±1.15 to 6.94±1.15 (Table 4.12).

For the aroma, all five broths cooked using *okpeye* fermented with starter cultures of *B. licheniformis* and *B. amyloliquefaciens* singly and in combination ranged from 7.10 to 7.30 and this improvement in aroma could be attributed to some aromatic substances released by the fermenting microorganisms (Ouoba et al., 2005). For the aftertaste of the broth which ranged from (6.83 to 7.17), there was no significant ($p < 0.05$) difference for aftertaste between all the

samples. It is known that proteolytic process is a key step that influences the taste profile of fermented food condiments which involves the degradation of protein to yield free amino acids (Alkanni et al., 2007).

For the appearance of the all the broth, the values ranged from 6.68 to 7.30, the improved appearance of the soups could be as a result of browning reactions generally associated with Maillard reactions resulting from degradation of amino acids and sugars during the fermentation process. This process also contributes to flavour and aroma generating reactions and has characterised legume based fermented foods as well (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.

The flavour values of broth with *okpeye* fermented with starter which ranged from 7.03 to 7.40 had a higher value than the soup cooked with *okpeye* fermented spontaneously as well as thecommercal *okpeye.* This could be due to the release of amines, peptides and some glutamic acids during the fermentation process (Dakwa et al., 2005). For the overall acceptability, broth cooked using *okpeye* fermented with mixed starter cultures of *Bacillus licheniformis* and *Bacillus amyloliquefaciens* had the best acceptability (7.31±0.85) while the commercial sample had the lowest value among the samples (6.77±0.90).

Generally, there was a significant ($p < 0.05$) difference between broth prepared from all the *okpeye* fermented with starter culture, the spontaneous and the commercial *okpeye* in terms of aroma, flavour, appearance, after taste and overall acceptability (Table 4.1.11). The panelist's ratings of all broth prepared with *okpeye* fermented with starter cultures were higher than the

commercial sample in all the attributes with a mean value of 6.77. These higher values could be attributed to the fact that the starter cultures facilitated faster metabolism during fermentation period and retained most of the sensory attributes. The result obtained from this study is interesting since consumers rarely prefer a new food product to an already existing familiar one (Akintayo, Hashim, Adereti, Balogun, Bolarinwa, Abiodun, Dauda, Solaja & Alabi, 2020).

Broth cooked using *okpeye* from the mixed starter culture gave a significantly ($p < 0.05$) better result in terms of aroma, flavour, appearance aftertaste and overall acceptability than the sample from spontaneous fermentation and commercial *okpeye.* The preference in all the attributes tested for in the soup prepared might be due to the use of starter culture given it a unique taste, flavour, apperance and aroma in all the *okpeye* fermented with starter cultures (Adamu, Farouq, magashi & Ibrahim, 2019). This results also agreed with the report of Balogun et al. (2017) that products from a controlled fermentation are superior in quality to those from the natural wild processes. The results from the sensory scores are an indication that *B. licheniformis* and *B. amyloliquefaciens* would produce safe *okpeye* and as well give acceptable sensory qualities comparable and even better than commercial *okpeye.*

CHAPTER FIVE

CONCLUSION AND RECOMMENDATION

5.1 CONCLUSION

This study investigated the molecular identification of the microbial community associated with *okpeye* available for retail. From the phenotypic identification, aerobic mesophile and *Staphylococci* were detected from all the markets in Anambra and Imo state at levels ranging from 10^5 to 10^7 CFU/g showing the predominance of *Bacillus spp.* in the commercial samples collected from all six markets.

The 16S rRNA sequencing of the 59 isolates from the commercial samples based on sequence similarity to reference strains in the EzBiocloud database the predominant groups as *Bacillus with B. amyloliquefaciens* (10.1%) and *B. cereus* (16.9%) having the highest number. The second predominant group was *Staphylococcus* species with *S. simulans* (16.94%) and *S. nepalensis* (10.17%) having the highest number. Other bacterial species which include *O. caeni*, *P. barcinonensis*, *V. lutrae*, *L. xylanilyticus*, *S. flexneri* and *E. fergusonni* were also identified in low numbers.

In general, it was observed that *Staphylococcus* species were dominant in samples from Anambra while *Bacillus* species were most prevalent in samples from Imo State. Also, most of the microorganisms identified are considered pathogenic, especially *B. cereus* which was one of the predominant *Bacillus spp* and it cuts across all the markets.

All ten *B. cereus* isolates in this study had at least one gene or gene complex associated with the production of an enterotoxin or an emetic toxin. All *B. cereus* tested positive for the presence of *nheA*, while *nheB* and *nheC* were detected in only seven of the isolates. Seven *B. cereus* were positive for the presence of all three genes encoding haemolysin BL complex

(*hblA*, *hblC*, *hblD*). Similarly, seven of the *B. cereus* isolates possessed the diarrhoeal toxins *cytK* and *entFM* while the emetic toxin (*cesB*) was detected in three *B. cereus* isolates.

Twenty- three isolates out of the twenty-eight isolates tested showed full haemolytic activity on blood agar. Two isolates (O22 and A22) showed partial haemolysis while three (O11, O15 and A12) were the only ones that showed no haemolysis. In addition, twenty-eight isolates tested for antimicrobial susceptibility with these ten antibiotics: ampicillin, penicillin, tetracycline, gentamicin, streptomycin, ciprofloxacin, erythromycin, chloramphenicol, kanamycin, and vancomycin revealed that most of the isolates were susceptible to the antibiotics irrespective of the locality of the samples.

Overall, the sensitivity and resistance of *B. licheniformis* and *B. amyloliquefaciens* to the different classes of antibiotics suggests that these strains might not carry antibiotic resistance genes that could be transferred to pathogenic microorganisms. Also, the results from this study are important for the selection of potential starter cultures for the production of *okpeye* to deliver a pathogen-free product.

The effect of the starter cultures on the pH, temperature and total titrable acidity of the fermenting samples were comparable with those of the spontaneous fermentation indicating the positivity of using starter cultures in the fermentation of indigenous fermented foods.

The proximate composition (%) of the different samples of *okpeye* from the starter cultures revealed slightly higher protein content, fat content, and carbohydrate content than the commercial samples and spontaneously fermented samples while the ash, fibre and moisture content were also comparable to the commercial and spontaneously fermented condiments. Overall, sample B with a mixed starter of *B. licheniformis* and *B. amyloliquefaciens* culture had higher nutritional values among the samples. These results are beneficial as they would

still provide better nutritional properties as well as guarantee a safe product with uniform quality.

The phytochemical analysis of *okpeye* samples with starter cultures (B, C and D) revealed higher amounts of Phyto nutrients including oxalates, saponin, tannins, alkaloids, phenols, phytates and flavonoids than the commercial and spontaneously fermented condiment except for cyanide. While most of these phytonutrients are beneficial, the high level of phytate is however not favourable as phytates are known to decrease the availability of minerals.

The sensory analysis of the broths cooked using *okpeye* samples with starter cultures faired favourably when compared with the spontaneously fermented and commercial samples. Overall, sample B with mixed starter of *B. licheniformis* and *B. amyloliquefaciens* culture had the best sensory quality.

This study has provided adequate data on the diverse bacterial populations associated with commercial *okpeye* in different markets of South Eastern Nigeria. It further revealed that *B. licheniformis* and *B. amyloliquefaciens* might be the choice starter culture for *okpeye* condiment as they gave nutritional and Phyto nutrients comparable to the commercial and spontaneously fermented samples.

5.2 RECOMMENDATIONS

From the identification of the microbial community in *okpeye* from this study, it is important to use including 16S rRNA sequencing and other advanced molecular tool to accurately identify the microbial community in indigenous fermented foods including both beneficial and non- beneficial microorganisms.

Practical steps to improve the processing of *okpeye* should be encouraged which include educating local processors on the improvement of personal hygiene and safety measures during and after processing to enhance food safety which include; use of gloves when in direct contact

with the processing intermediate or final product and proper cleaning of their local processing equipment.

The successful development of the starter culture from this study indicates that strains with negative haemolysis and sensitivity to a maximum number of antibiotics should be encouraged while toxigenic and antibiotic-resistant strains should be discouraged.

There is also a need to develop stable *B. licheniformis* and *B. amyloliquefaciens* starters in forms that illiterate and semi-literate local processors could reproducibly utilize; and to bring these starters to play roles in the local industry.

These selected safe *Bacillus* strains, *B. licheniformis* and *B. amyloliquefaciens* developed from this study need to be investigated further to find out their technological properties and if they can be carriers of probiotic-starter cultures for the development of novel technological processes for the production of therapeutic and health-promoting functional fermented foods.

Having developed starter cultures from this study, it is recommended that the volatile components of *okpeye* produced from these selected safe *Bacillus* strains, *B. licheniformis* and *B. amyloliquefaciens* should be analysed to find out the specific compounds responsible for the aroma and flavour of the condiment.

Further investigation of these *Bacillus* strains as vehicles for probiotic delivery would be useful for both human and animal food/feed formulations.

5.3 CONTRIBUTION TO KNOWLEDGE

From this study, it was evident that the microbial profile of retail *okpeye* sold in some markets in the South East in Nigeria showed the dominance of *Staphylococcus* species in Anambra state markets and the dominance of *Bacillus* species in Imo state markets.

The microorganism *Heyndricxia oleronia* was identified in *okpeye* microbial ecology for the first time using 16S rRNA sequencing which has not been associated with any fermented condiments before nor reported before.

For the first time it is being reported that the most predominant *Bacillus* strains identified from commercial samples of *okpeye* was *B. amyloliquefaciens* and *B. cereus* as opposed to *B. subtilis* that has been previously reported by many authors for this condiment.

From this study, *B. cereus* was identified in all markets from both states and for the first-time genes encoding the emetic toxin (*CesB*) was detected in commercial *okpeye.*

From this study, *B. licheniformis* and *B. amyloliquefaciens* do not carry haemolytic genes (not able to lyse blood cells) and was also susceptible (70 -90%) to majority of the antibiotics that was used in the study making them suitable for use as starter cultures. This is being reported for the first time.

For the first time the use of starter culture preparations containing *B. licheniformis* and *B. amyloliquefaciens was* used to successfully ferment dehulled *Prosopis africana* seeds for production of *okpeye* and the fermentation reached its peak at 72 to 96 hours.

The study also revealed that there is paucity of information using a combination of the starter cultures gave higher proximate and the sensory value than the commercial *okpeye*. The study will form a baseline for further researches.

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Appendix I

Total microbial counts on different agar for *okpeye* commercial samples from different markets in Anambra and Imo state

Sample names	Sample codes	Media types	Dilution factor	No of colonies	Colony forming units (cfu/g)
Owerri world bank market	OWB	TSA	10^{-3}	42	4.2×10^5
		MSA	10^{-3}	35	3.5×10^5
		MAC	10^{-3}	80	8.0×10^5
Owerri Ekeukwu market	OEM	TSA	10^{-3}	112	1.12×10^6
		MSA	10^{-3}	283	2.83×10^6
		MAC	10^{-3}	291	2.91×10^6
	ORM	TSA	10^{-3}	266	2.66×10^6

Owerri Relief market		MSA	10^{-3}	182	1.82×10^6
		MAC	10^{-3}	292	2.93×10^6
Anambra Ose Market	AOM	TSA	10^{-3}	131	1.31×10^6
		MSA	10^{-3}	TNTC	TNTC
		MAC	10^{-3}	246	2.46×10^6
Anambra Bridge head market	ABM	TSA	10^{-3}	46	4.6×10^5
		MSA	10^{-3}	TNTC	TNTC
		MAC	10^{-3}	76	7.6×10^5
Anambra Ochanja Market	AOCM	TSA	10^{-3}	40	4.0×10^5
		MSA	10^{-3}	TNTC	TNTC
		MAC	10^{-3}	69	6.9×10^5

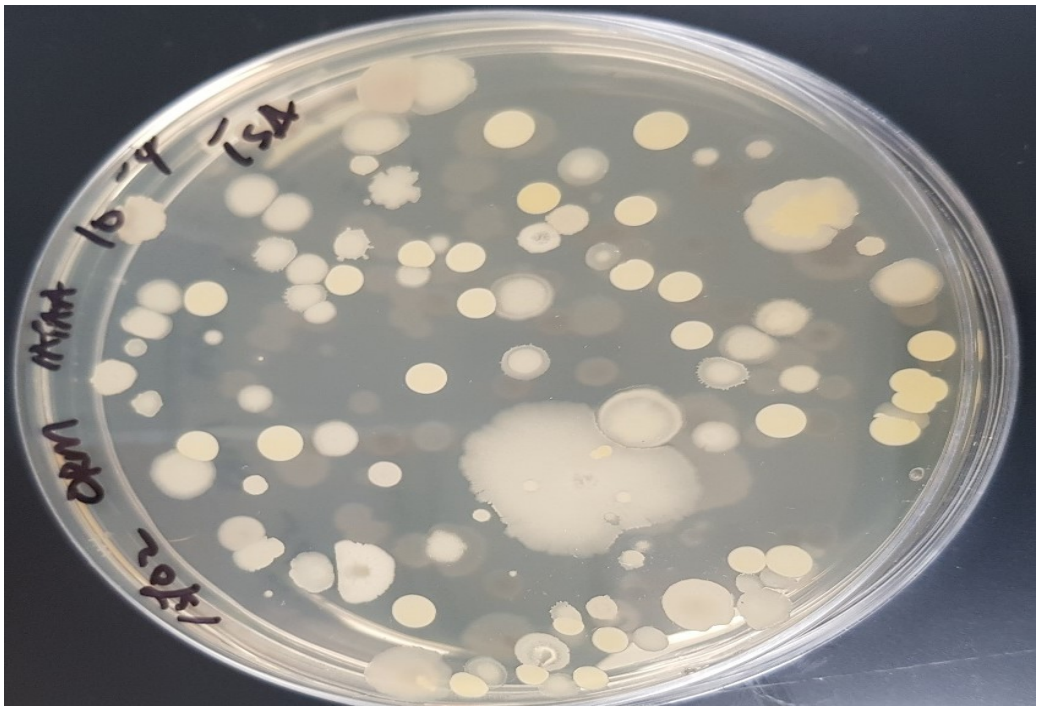


Plate 9: Colonies isolated from *okpeye* commercial samples (ORM) on Tryptone soy agar

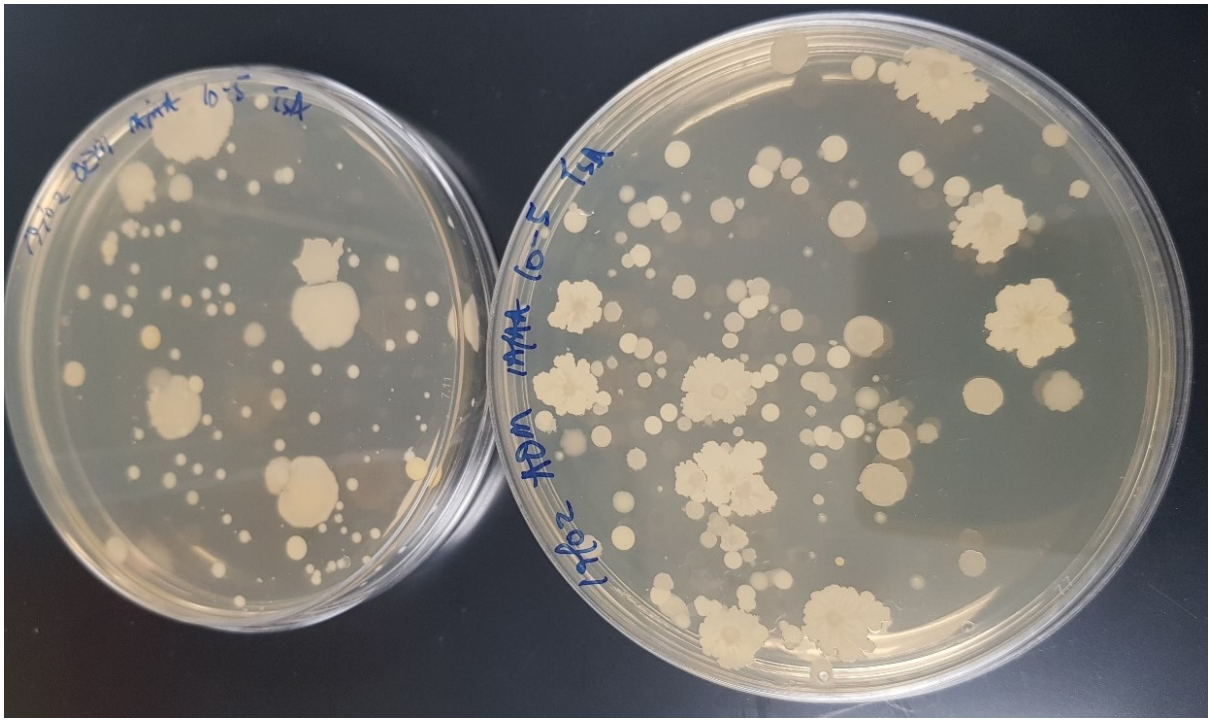


Plate 10: Colonies isolated from *okpeye* commercial samples (AOM) on Tryptone soy agar

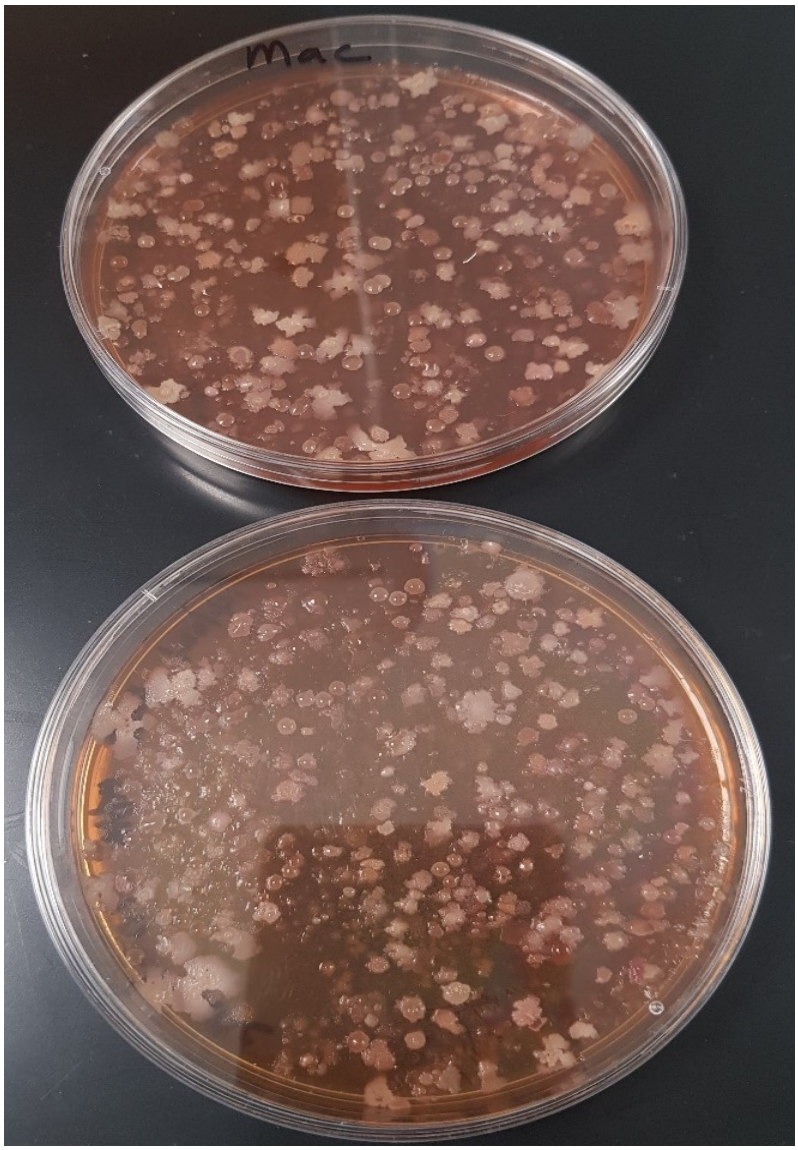


Plate 11: Colonies isolated from *okpeye* commercial samples (AOM) on MacConkey agar

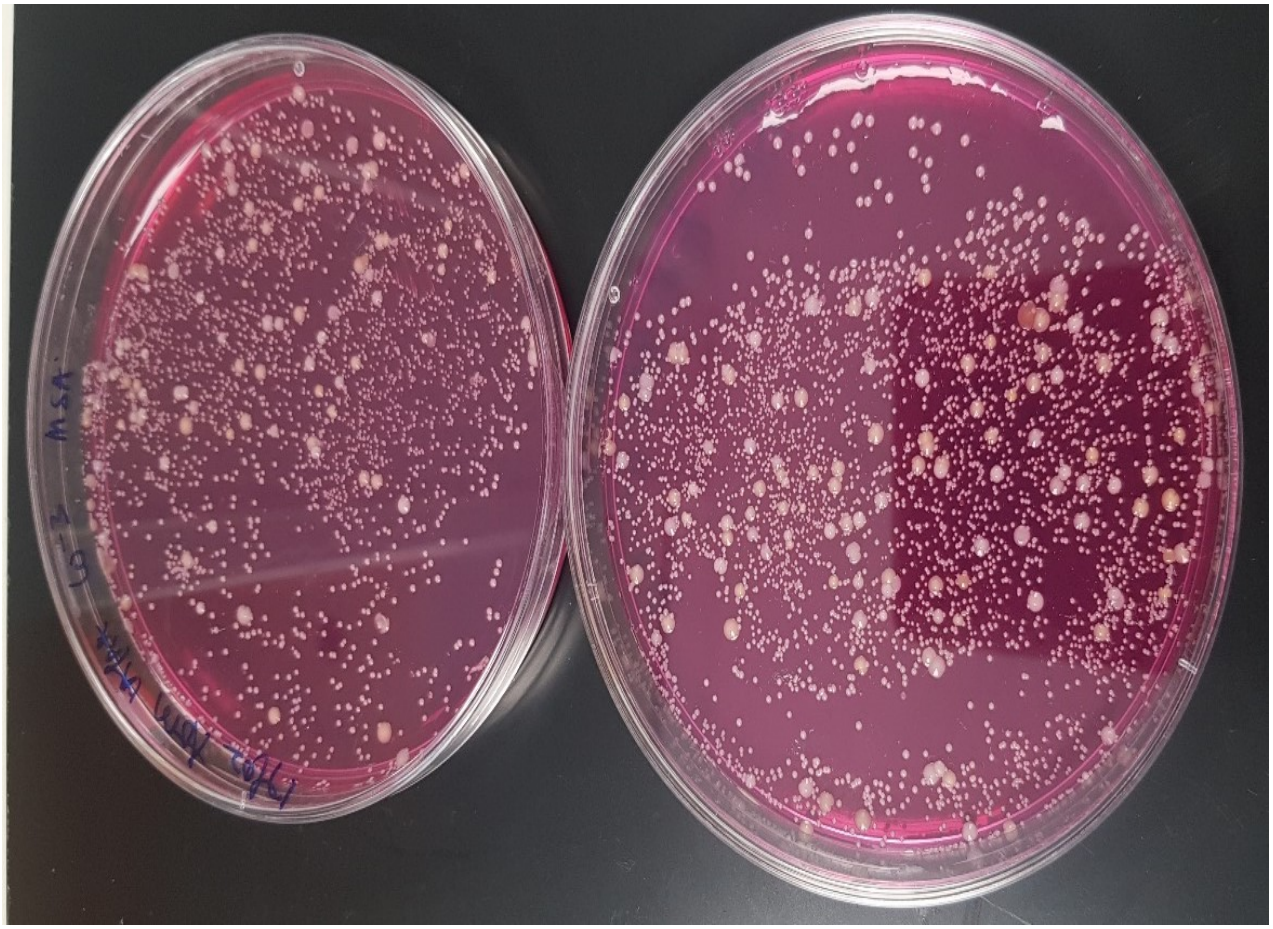


Plate 12: Colonies isolated from *okpeye* commercial samples (AOM) on Mannitol salt agar

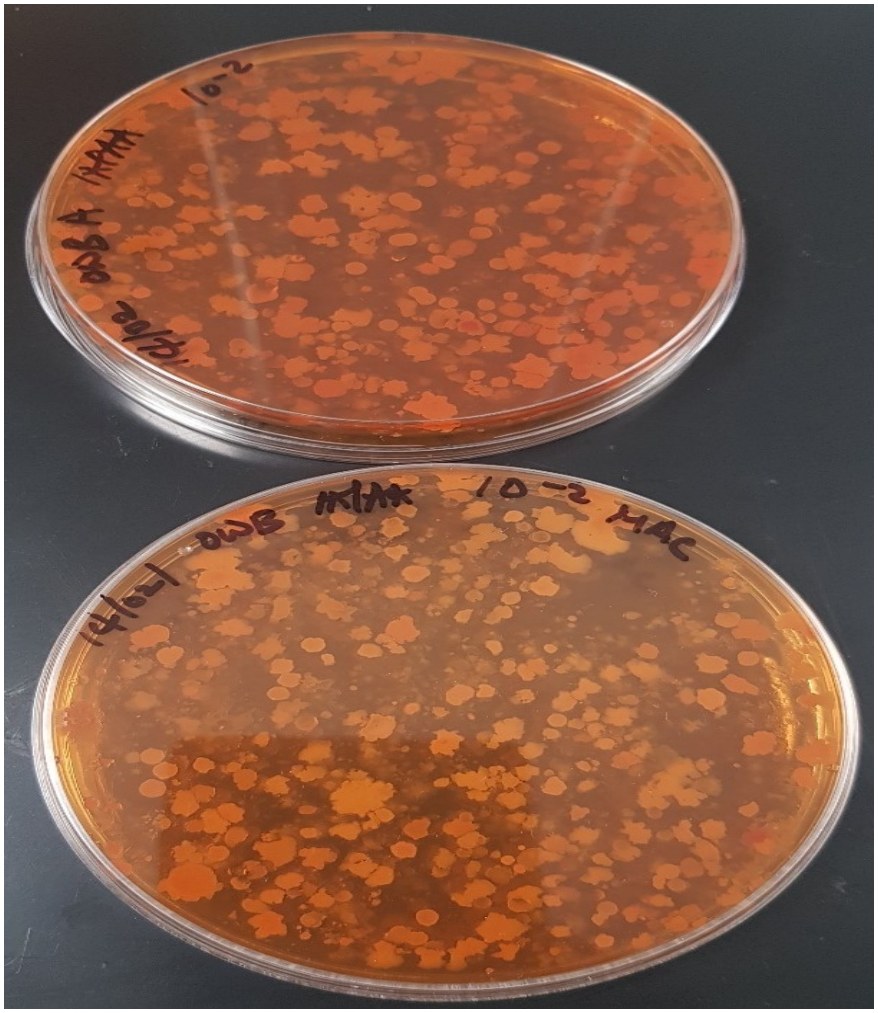


Plate 13: colonies isolated from *okpeye* commercial samples (OWB) on MacConkey agar

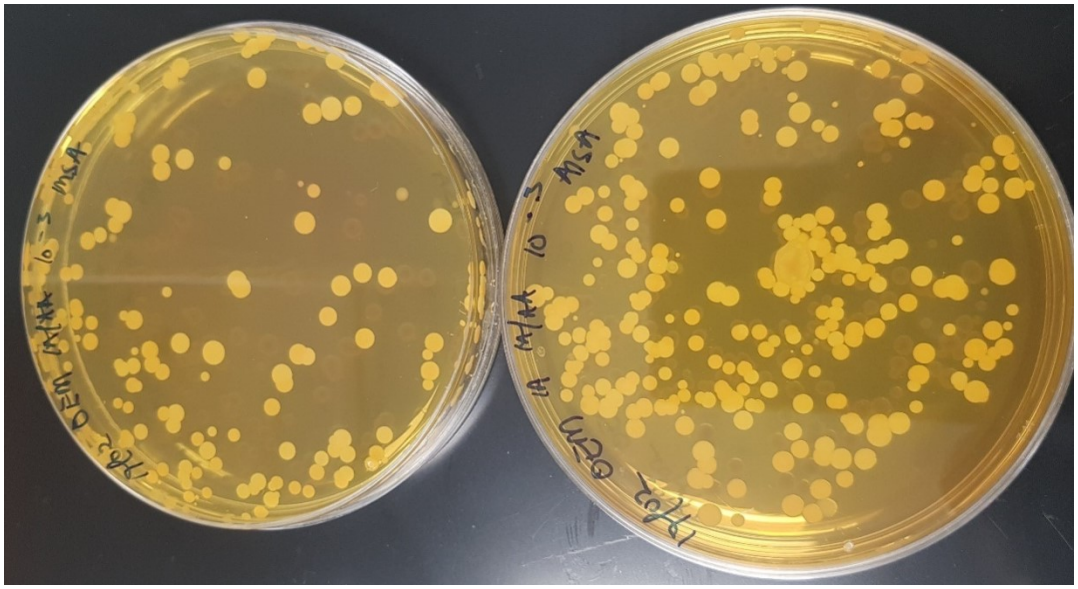


Plate 14: Colonies isolated from *okpeye* commercial samples (OEM) on Mannitol salt agar

Appendix II

Phenotypic characteristics of *okpeye* commercial samples from Anambra and Imo State

Sample codes	Agar isolated	Colony morphology/ characteristics	Microscopic characteristics	Catalase reaction	Oxidase reaction
OWR 1	TSA	Moderate irregular whitish colonies	Gram-positive rods in short chains	+	-
OWR 2	TSA	Small mucoid white colonies	Gram-positive rods in chains	+	-
OWR 3	TSA	Rough dry irregular white colonies	Gram-positive rods in single and short chains	+	-
OWR 4	TSA	Tiny smooth creamy colony	Gram-positive rods in chains with central spores	+	+
OWR 5	TSA	Large dull flat colony	Gram-positive rods in singles with spores	+	-
OWR 6	TSA	Small slimy white colony	Gram-positive rods in singles with spores	+	-

OWR 7	TSA	Large dull flat creamy colony	Gram-positive rods in chains with central spores	+	-
OWR 8	TSA	Large irregular creamy colony	Gram-positive rods in short chains	+	-
OWR 9	TSA	Moderate irregular white colony	Gram-positive rods in singles	+	-
OWR 10	TSA	Small dull brownish colonies	Gram-positive rods in singles	+	+
OWR 11	MSA	Small irregular yellow colonies	Gram-positive rods in singles	+	-

Phenotypic characteristics of *okpeye* commercial samples from Anambra and Imo State (Cont'd.).

Sample codes	Agar isolated	Colony morphology/ characteristics	Microscopic characteristics	Catalase reaction	Oxidase reaction
OWR 12	MSA	Moderate flat rough colonies	Gram-positive rods in singles	+	+
OWR 13	MAC	Small rough cream colonies	Gram-positive rods in chains with central spores	+	+
OWR 14	MAC	Large rough raised filamentous colonies	Small gram-negative rods in singles and short chains	+	-
OWR 15	MAC	Moderate raised rough colony	Gram-positive rods in chains with central spores	+	-
OWR 16	MAC	Small raised mucoid colony	Slender gram-negative rods in singles and short chains	+	+

OWR 17	TSA	Small smooth yellowish colony	Gram-negative rods in short chains with central spores	+	+
OWR 18	TSA	Small transparent pinkish colony	Short gram-positive rods in singles	+	+
OWR 19	TSA	Large irregular rough edges colonies	Gram-positive rods in singles and short chains	-	+
OWR 20	TSA	Smooth irregular creamy colony	Short gram-negative rods in singles and chains	+	-
OWR 21	TSA	Large shiny rough colonies	Gram-positive rods in single and short chains	+	-
OWR 22	TSA	Moderate rough raised white colonies	Short gram-positive rods in pairs	+	-

Phenotypic characteristics of *okpeye* commercial samples from Anambra and Imo State (Cont'd.)

Sample codes	Agar isolated	Colony morphology/ characteristics	Microscopic characteristics	Catalase reaction	Oxidase reaction
OWR 23	MAC	Small flat creamy colonies	Short gram-positive rods in chains	+	-
OWR 24	MSA	Moderate mucoid cream colonies	Short gram-positive rods in pairs	+	+
OWR 25	TSA	Small irregularly raised cream colonies	Gram-positive rods in chains with central spores	+	+
OWR 26	TSA	Small smooth brownish colonies	Gram-positive rods in singles and pairs	+	+
OWR 27	TSA	Moderate raised mucoid colonies	Gram-positive rods in singles with central spores	+	+
OWR 28	TSA	Tiny smooth yellow colonies	Gram-positive in cocci in clusters,	+	-

			few in tetrads and pairs		
OWR 29	TSA	Small creamy mucoid colony	Short gram-positive rods in chains with central spores	+	-
OWR 30	TSA	Small smooth yellow colonies	Gram-positive cocci in clusters and few in pairs	+	-
OWR 31	TSA	Large mucoid yellow colonies	Gram-positive rods in chains with central spores	-	-
OWR 32	TSA	Tiny smooth deep yellow colonies	Gram-positive cocci predominantly in tetrads, few in pairs	+	-
OWR 33	TSA	Large irregularly raised white colony	Gram-positive cocci in clusters and few in pairs	+	-

Phenotypic characteristics of *okpeye* commercial samples from Anambra and Imo State (Cont'd.)

Sample codes	Agar isolated	Colony morphology/ characteristics	Microscopic characteristics	Catalase reaction	Oxidase reaction
OWR 33	TSA	Large irregularly raised white colony	Gram-positive cocci in clusters and few in pairs	+	-
OWR 34	TSA	Small smooth cream colonies	Gram-positive rods in singles and in chains	-	-
OWR 35	TSA	Small mucoid cream colonies	Gram-positive rods in singles	+	-
OWR 36	MSA	Small cream mucoid colony	Gram-positive cocci in clusters and few in pairs	+	-
ANM 1	TSA	Moderate rough orange colonies	Short gram-positive rods in singles and chains	+	-
ANM 2	TSA	Moderate transparent white colonies	Gram-positive rods in singles	+	+

ANM 3	TSA	Small creamy colonies	Gram-positive rods in pairs and in chains	+	-
ANM 4	TSA	Moderate transparent yellow colonies	Gram-positive cocci in clusters and few in pairs	-	-
ANM 5	TSA	Large irregular creamy colonies	Gram-positive cocci in clusters and few in pairs	+	-
ANM 6	MSA	Small smooth mucoid colonies	Gram-positive cocci predominantly in tetrads and few in pairs	+	-
ANM 7	MSA	Moderate smooth cream colonies	Gram-positive rods predominantly in tetrads and few in pairs	+	-

Phenotypic characteristics of *okpeye* commercial samples from Anambra and Imo State (Cont'd.)

Sample codes	Agar isolated	Colony morphology/ characteristics	Microscopic characteristics	Catalase reaction	Oxidase reaction
ANM 8	MAC	Small smooth creamy colonies	Gram-positive cocci in pairs and few in clusters	+	-
ANM 9	MAC	Small smooth white colonies	Gram-positive cocci in pairs and clusters	+	-
ANM 10	TSA	Tiny smooth white colonies	Gram-positive cocci in pairs	+	-
ANM 11	TSA	Small smooth cream colonies	Small gram-positive rods in chains and pairs	+	-
ANM 12	TSA	Tiny dull irregular cream colony	Short gram-positive rods in chains with central spores	+	-

ANM 13	MSA	Small smooth yellow mucoid colonies	Gram-positive cocci in pairs	+	-
ANM 14	MSA	Tiny smooth white colonies	Gram-positive cocci in clusters	+	-
ANM 15	MAC	Small rough raised colonies	Gram-positive rods in singles and chains	+	-
ANM 16	MAC	Moderate raised mucoid colonies	Gram-positive rods with central spores	+	-
ANM 17	TSA	Moderate irregular white colonies	Small gram-negative rods in singles and pairs	+	-
ANM 18	TSA	Small smooth creamy colonies	Gram-positive cocci in pairs, few in clusters	+	-
ANM 19	TSA	Smooth creamy colonies	Gram-positive cocci in clusters	+	-

Phenotypic characteristics of *okpeye* commercial samples from Anambra and Imo State (Cont'd.)

Sample codes	Agar isolated	Colony morphology/ characteristics	Microscopic characteristics	Catalase reaction	Oxidase reaction
ANM 20	TSA	Small creamy colonies	Gram-positive cocci in clusters, few in pairs	+	-
ANM 21	TSA	Moderate smooth colonies	Small gram-positive rods in singles and short chains	+	-

ANM 22	TSA	Small irregular cream colonies	Gram-positive rods in pairs	+	-
ANM 23	MSA	Small creamy colonies	Gram-positive cocci in clusters, few in pairs	+	-
ANM 24	MSA	Small smooth creamy colonies	Gram-positive cocci in clusters, few in tetrads	+	-
ANM 25	MAC	Moderate rough raised mucoid colonies	Short gram-positive cocci predominantly in tetrads, few in pairs	+	-

Appendix III

DNA concentration for all isolates from commercial samples of *okpeye*

S/N	ISOLATES	DNA CONCENTRATION (ul)	A/260/280	A/260/230	GEL CONCENTRATION
1	O3	54.600	1.826	2.328	1.103
2	O4	26.950	1.827	2.156	0.536
3	O5	34.900	1.732	1.876	0.701
4	O6	32.900	1.833	2.123	0.663
5	O7	28.250	1.812	1.794	0.577
6	O8	23.400	1.779	1.690	0.476
7	O9	47.750	1.775	2.226	0.970
8	O10	32.350	1.777	2.114	0.658
9	O11	31.150	1.816	1.740	0.634
10	O12	38.950	1.866	2.094	0.778

11	O13	36.450	1.864	1.744	0.725
12	O14	51.950	1.893	1.782	1.040
13	O15	49.700	1.897	2.054	1.000
14	O16	42.250	1.881	2.105	0.909
15	O17	28.400	1.832	1.775	0.569
16	O18	53.450	1.895	2.025	1.080
17	O19	36.100	1.861	1.703	0.725
18	O20	28.450	1.841	1.616	0.573
19	O21	34.800	1.866	1.776	0.697
20	O22	41.450	1.897	1.731	0.841
21	O23	47.000	1.918	1.561	0.957
22	O24	43.050	1.922	1.809	0.873
23	O25	37.200	1.908	1.793	0.753
24	O26	40.900	1.826	2.317	0.865
25	O27	58.500	1.854	2.393	0.643
25	O28	29.400	1.826	2.170	0.697
27	O29	41.400	1.844	2.306	0.854
28	O30	53.150	1.855	2.361	0.877
29	O31	56.200	1.825	2.213	0.945
30	O32	66.050	1.861	2.221	0.734

DNA concentration for all isolates from commercial samples of *okpeye* (Cont'd.)

S/N	ISOLATES	DNA CONCENTRATION	A/260/280	A/260/230	GEL CONCENTRATION
31	O33	50.232	1.897	2.088	0.996
32	O34	18.880	1.918	1.701	0.454
33	035	30.400	1.831	1.876	0.606
34	036	29.00	1.865	1.940	0.581
35	A1	49.600	1.863	2.000	0.990
36	A2	35.250	1.850	2.086	0.705
37	A3	51.800	1.904	2.086	0.705
38	A4	44.500	1.904	1.616	1.047
39	A5	55.500	1.800	1.654	0.954
40	A6	16.500	1.935	2.023	0.754

41	A7	40.400	1.875	2.143	0.807
42	A8	46.600	1.864	2.026	0.935
43	A9	38.700	1.870	1.990	0.776
44	A10	22.000	1.781	2.353	0.432
45	A11	37.500	1.870	2.026	0.935
46	A12	34.300	1.845	2.120	0.463
47	A13	23.050	1.773	1.945	0.461
48	A14	13.350	1.638	1.467	0,654
49	A15	35.700	1.821	2.082	0.753
50	A16	35.400	1.821	2.010	0823
51	A17	33.600	1.811	2.203	0.673
52	A18	51.670	1.899	1.764	0.673
53	A19	14.450	1.661	2.221	1.644
54	A20	23.500	1.873	1.829	0.465
55	A21	47.000	1.845	2.350	0.958
55	A22	29.000	1.865	1.940	0.581
57	A23	21.950	1.799	2.217	0.441
58	A24	24.000	1.811	1.690	0.488
59	A25	30.250	1.667	1.340	0.754

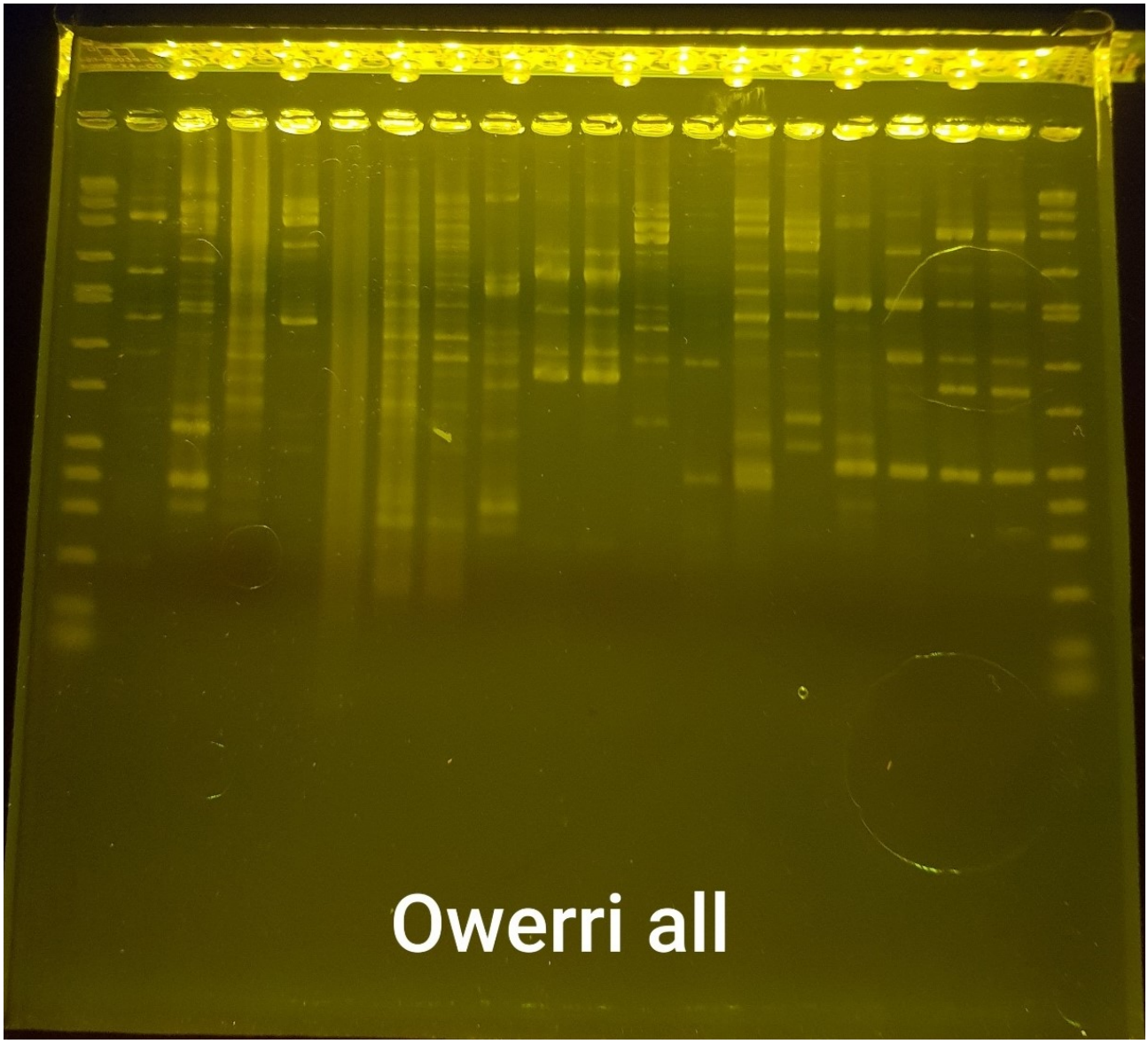


Plate 15: DNA bands of all Imo isolates on gel electrophoresis

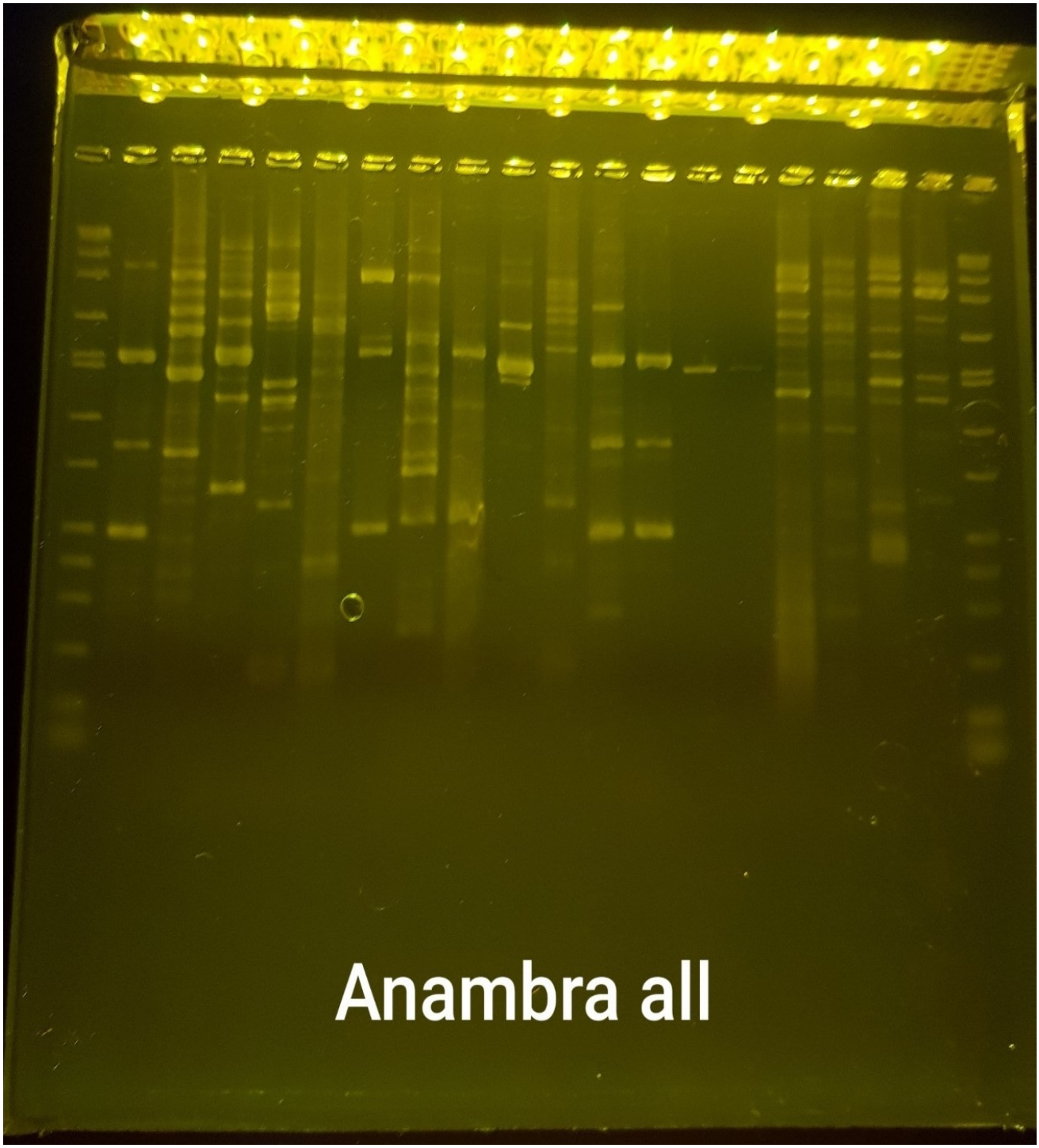
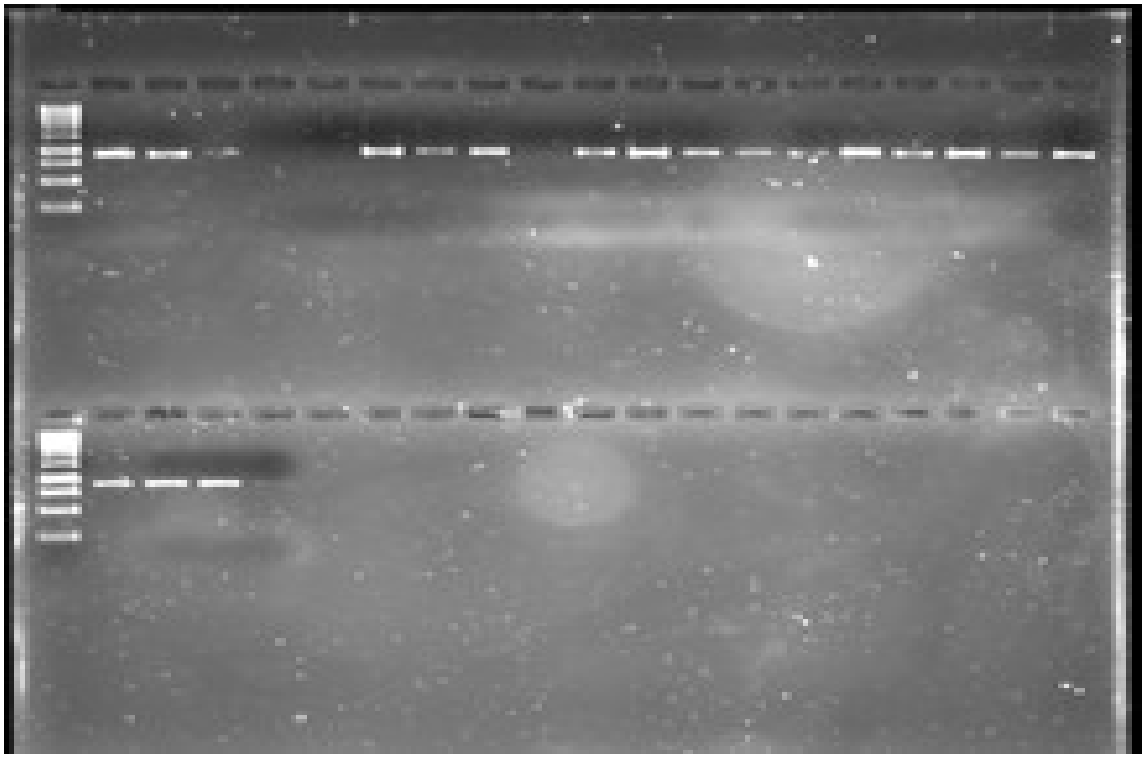


Plate 16: DNA bands of all Anambra Isolates on gel electrophoresis



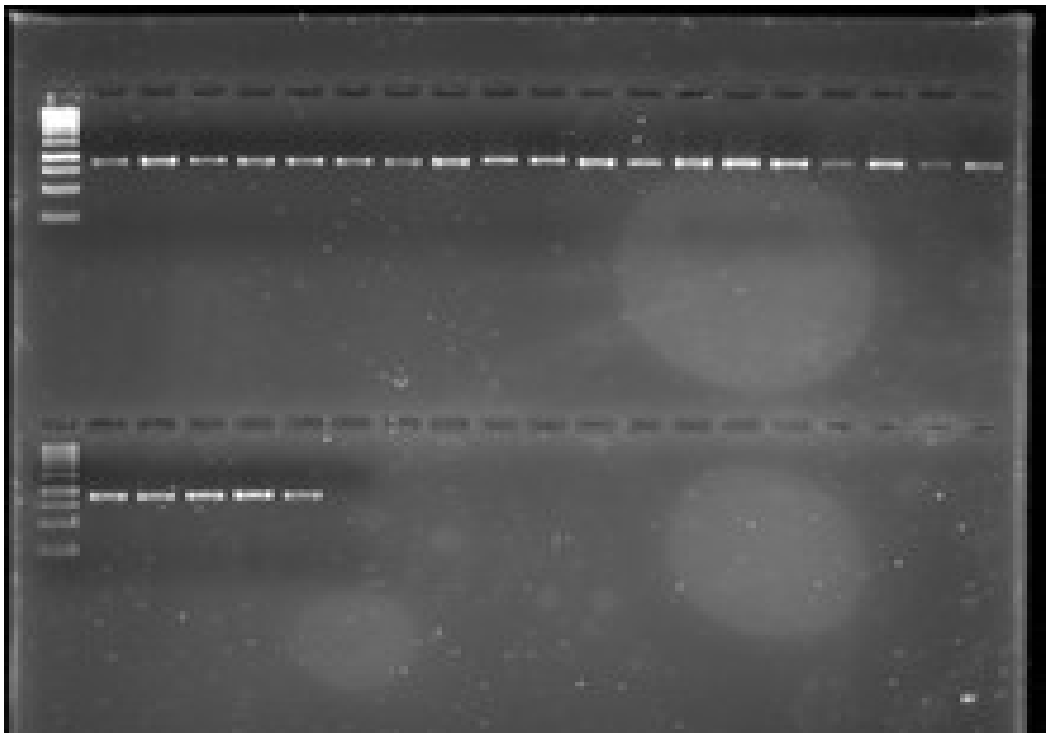


Plate 17: Electrophoresis band of isolates for 16s sequencing

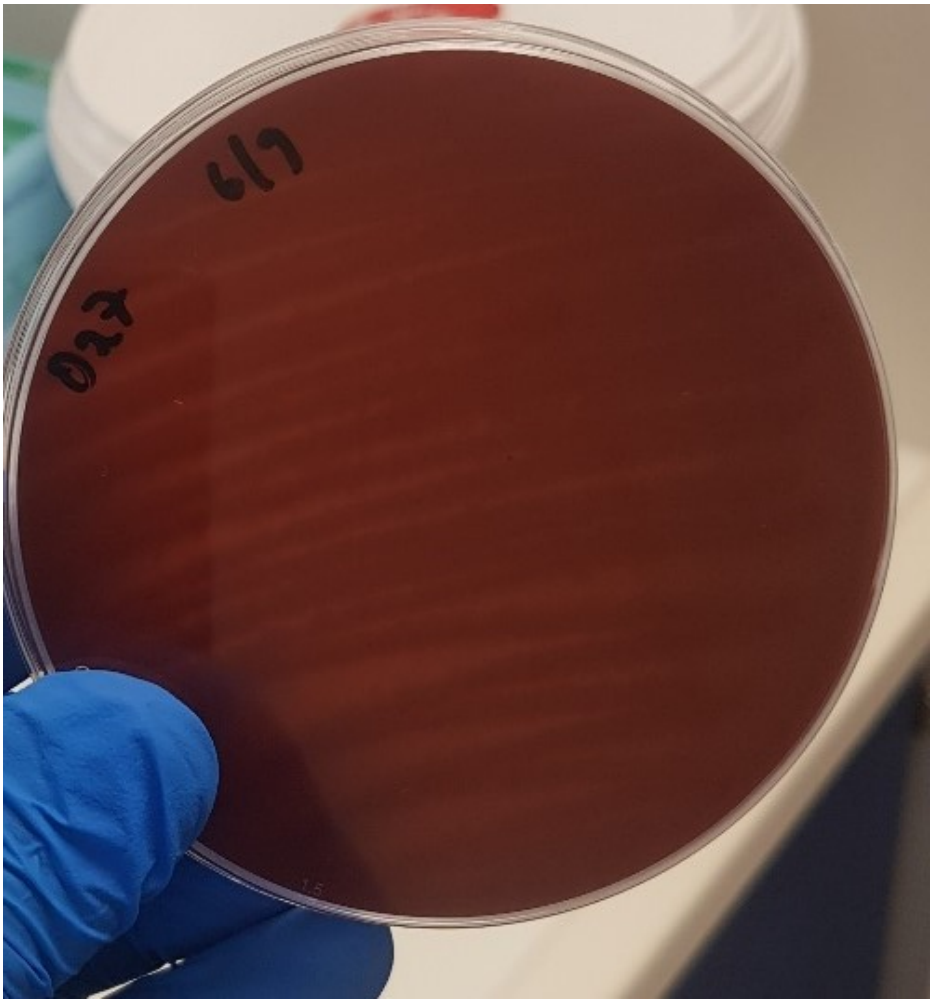


Plate 18: Non- haemolytic activity of O27 isolate on blood agar

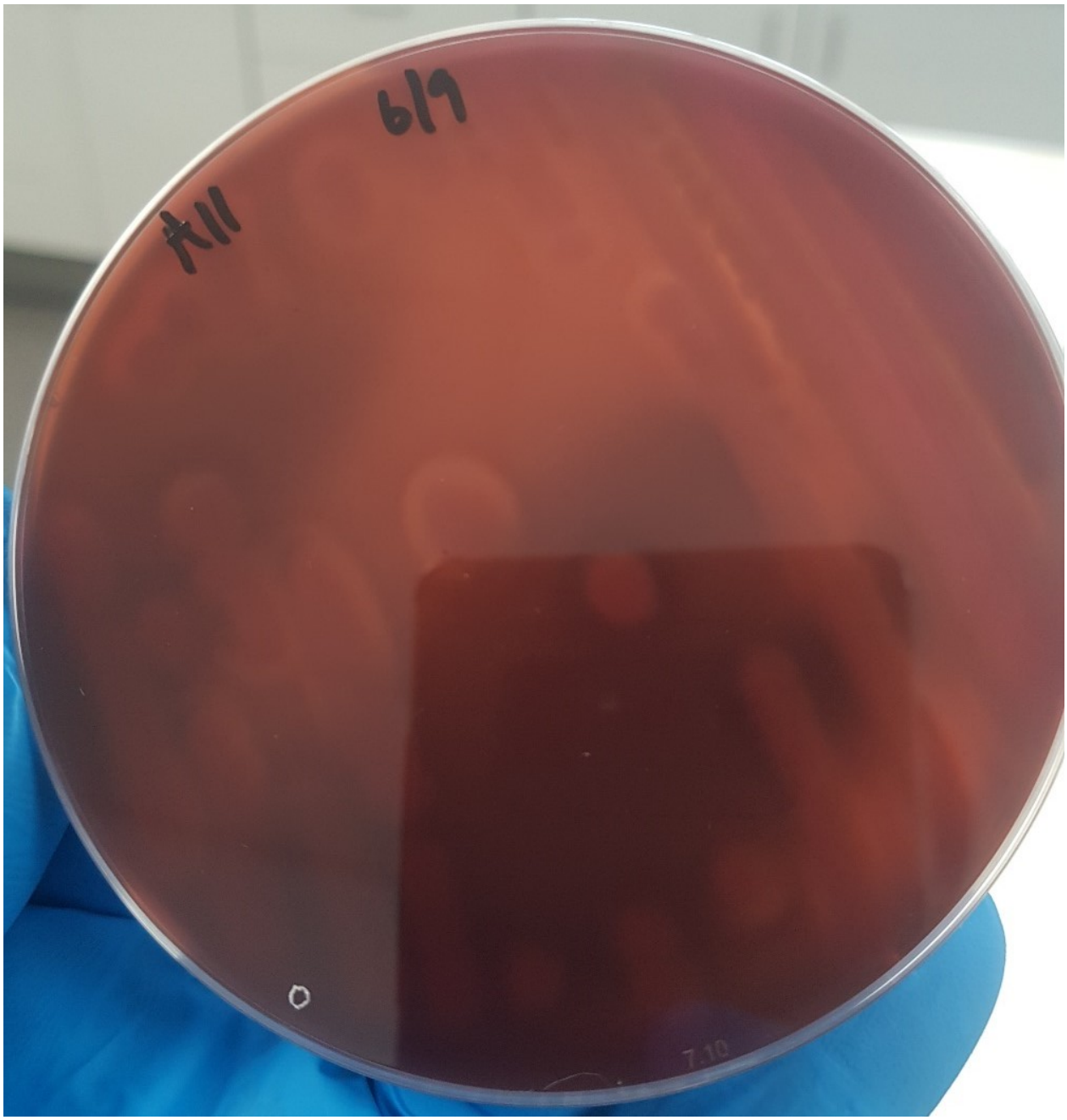


Plate 19: Haemolytic activity of A11 isolate on blood agar

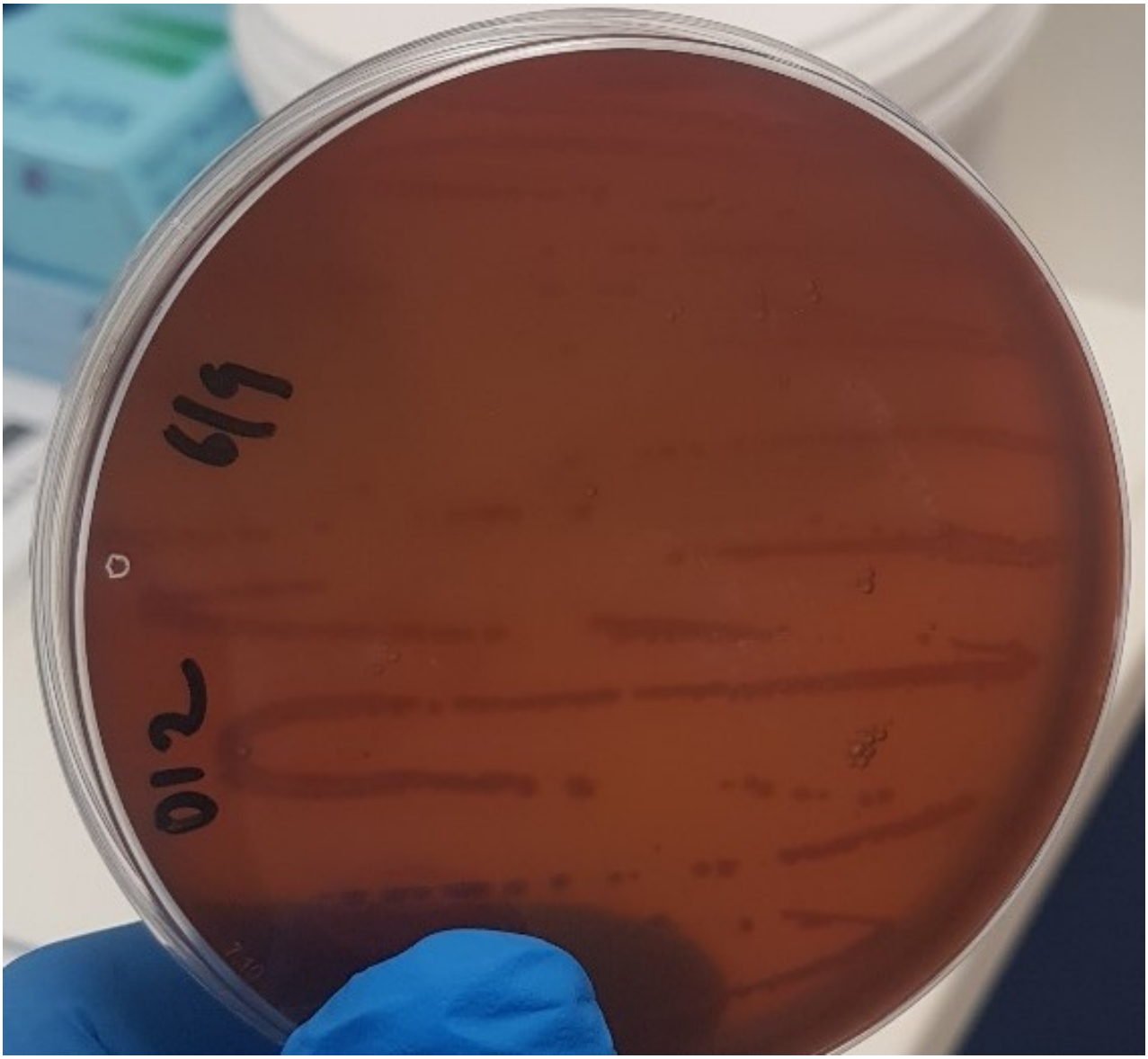


Plate 20: Haemolytic activity of O12 isolate on blood agar

Appendix IV

Zones of inhibition of some isolates tested to different antibiotics

S/N	ISOLATES	ANTIBIOTICS	ZONES OF INHIBITION
1	O13	AM	3.5cm = 35mm
		P	3.6cm = 36mm
		T	3.1cm =31mm
		G	2.4cm = 24mm
		S	1.9cm = 19mm
		CI	3.1cm = 31mm
		E	2.6cm = 26mm
		CH	2.7cm = 27mm
		K	2.3cm = 23mm
		V	2.1cm = 21mm
2	O27	AM	0.6cm = 6mm
		P	0.6cm = 6mm
		T	3.5cm = 35cm
		G	2.4cm = 24mm
		S	1.6cm = 16mm
		CI	3.6cm = 36mm
		E	0
		CH	1.5cm = 15mm
		K	0.8cm = 8mm
		V	0.4cm = 4mmS
3	O22	AM	0
		P	0
		T	2.1cm = 21mm

		G	2.2cm = 22mm
		S	1.9cm = 19mm
		CI	2.6cm = 26mm
		E	2.5cm = 25mm
		CH	2.0cm = 20mm
		K	1.8cm = 18mm
		V	1.6cm = 16mm
4	O12	AM	3.8cm = 38mm
		P	4.1cm = 41mm
		T	3.9cm = 39mm
		G	3.6cm = 36mm
		S	3.0cm = 30mm
		CI	3.3cm = 33mm
		E	3.9cm = 39mm
		CH	3.2cm = 32mm
		K	3.6cm = 36mm
		V	3.1cm = 31mm
5	A11	AM	0.4cm = 4mm
		P	0
		T	0.4cm = 4mm
		G	2.2cm = 22mm
		S	1.6cm = 16mm
		CI	2.5cm = 25mm
		E	1.6cm = 16mm
		CH	2.3cm = 23mm
		K	1.9cm = 19mm
		V	1.5cm = 15mm

Zones of inhibition of some isolates tested to different ntibiotics (Cont'd.)

S/N	ISOLATES	ANTIBIOTICS	ZONES OF INHIBITION
6	A16	AM	1.9cm = 19mm
		P	2.4cm = 24mm
		T	2.5cm = 25mm
		G	2.2cm = 22mm
		S	1.5cm = 15mm
		CI	2.8cm = 28mm
		E	2.9cm = 29mm
		CH	2.8cm = 28mm
		K	2.4cm = 24mm
		V	2.0cm = 20mm
7	A2	AM	4.1cm = 41mm
		P	3.9cm = 39mm
		T	3.8cm = 38mm
		G	3.5cm = 35mm
		S	0
		CI	1.5cm = 15mm
		E	2.4cm = 24mm
		CH	2.0cm = 20mm
		K	1.0cm =10mm
		V	1.5cm = 15mm
8	A7	AM	3.6cm = 36mm
		P	4.2cm = 42mm
		T	2.8cm = 28mm
		G	2.5cm = 25mm
		S	2.0cm = 20mm

		CI	3.0cm = 30mm
		E	2.7cm = 27mm
		CH	2.5cm = 25mm
		K	2.4cm = 24mm
		V	1.5cm = 15mm
9	O32	AM	3.4cm = 34mm
		P	3.1cm = 31mm
		T	2.8cm = 28mm
		G	2.5cm = 25mm
		S	1.4cm = 14mm
		CI	2.4cm = 24mm
		E	2.6cm = 26mm
		CH	2.3cm = 23mm
		K	2.5cm = 25mm
		V	1.6cm = 16mm
10	O4	AM	1.2cm = 12mm
		P	1.0cm = 10mm
		T	2.9cm = 29mm
		G	2.5cm = 25mm
		S	2.5cm = 25mm
		CI	3.8cm = 38mm
		E	2.0cm = 20mm
		CH	1.6cm = 16mm
		K	0.8cm = 8mm
		V	0.8cm = 8mm

Zones of inhibition of some isolates tested to different antibiotics (Cont'd.)

S/N	ISOLATES	ANTIBIOTICS	ZONES OF INHIBITION
11	A12	AM	0
		P	0
		T	0
		G	1.8cm = 18mm
		S	0.8cm = 8mm
		CI	1.9cm = 19mm
		E	0
		CH	0
		K	1.6cm = 16mm
		V	0
12	A13	AM	1.4cm = 14mm
		P	1.2cm = 12mm
		T	2.6cm = 26mm
		G	2.5cm = 25mm
		S	1.9cm = 19mm
		CI	2.5cm = 25mm
		E	1.2cm = 12mm
		CH	2.1cm = 21mm
		K	1.2cm = 12mm
		V	1.0cm = 10mm
13	O30	AM	3.6cm = 36mm
		P	3.5cm = 35mm
		T	2.8cm = 28mm
		G	2.6cm = 26mm
		S	2.1cm = 21mm

		CI	2.6cm = 26mm
		E	1.0cm = 10mm
		CH	2.0cm = 20mm
		K	2.1cm = 21mm
		V	1.6cm = 16mm
14	A4	AM	0.7cm = 14mm
		P	1.0cm = 10mm
		T	Clear zone
		G	2.6cm = 26mm
		S	2.2cm = 22mm
		CI	Clear zone
		E	1.8cm = 18mm
		CH	2.8cm = 28mm
		K	1.0cm = 10mm
		V	0.6cm = 6mm
15	A22	AM	0.9cm = 9mm
		P	0.8cm = 8mm
		T	2.0cm = 20mm
		G	2.2cm = 22mm
		S	2.3cm = 23mm
		CI	2.5cm = 25mm
		E	1.7cm = 17mm
		CH	2.0cm = 20mm
		K	2.0cm = 20mm
		V	1.5cm = 15mm

Zones of inhibition of some isolates tested to different antibiotics (Cont'd.)

S/N	ISOLATES	ANTIBIOTICS	ZONES OF INHIBITION
16	O31	AM	2.9cm = 29mm
		P	2.6cm = 26mm
		T	1.3cm = 13mm
		G	2.0cm = 20mm
		S	1.7cm = 17mm
		CI	2.9cm = 29mm
		E	2.8cm = 28mm
		CH	2.6cm = 26mm
		K	2.2cm = 22mm
		V	2.0cm = 20mm
17	O35	AM	0.8mm = 8mm
		P	0.6mm = 6mm
		T	2.0cm = 20mm
		G	2.4cm = 24mm
		S	1.7cm = 17mm
		CI	2.2cm = 22mm
		E	1.2cm = 12mm
		CH	1.9cm = 19mm
		K	2.5cm = 25mm
		V	1.2cm = 12mm
18	O26	AM	2.9cm = 29mm
		P	3.3cm = 33mm
		T	2.0cm = 20mm
		G	2.4cm = 24mm
		S	1.9cm = 19mm

		CI	2.5cm = 25mm
		E	0.9cm = 9mm
		CH	2.2cm = 22mm
		K	0.7cm = 7mm
		V	1.0cm = 10mm
19	O6	AM	2.4cm = 24mm
		P	2.2cm = 22mm
		T	2.0cm = 20cm
		G	2.6cm = 26mm
		S	3.3cm = 33mm
		CI	2.9cm = 29mm
		E	2.5cm = 25mm
		CH	2.7cm = 27mm
		K	2.2cm = 22mm
		V	2.4cm = 24mm
20	O7	AM	2.1cm = 21mm
		P	2.7cm = 27mm
		T	2.9cm = 29mm
		G	2.3cm = 23mm
		S	2.2cm = 23mm
		CI	2.6cm = 26mm
		E	2.5cm = 25mm
		CH	2.4cm = 24mm
		K	2.7cm = 27mm
		V	2.1cm = 21mm

Zones of inhibition of some isolates tested to different antibiotics

S/N	ISOLATES	ANTIBIOTICS	ZONES OF INHIBITION
21	O8	AM	2.9cm = 29mm
		P	2.3cm = 23mm
		T	2.2cm = 22mm
		G	1.9cm = 19mm
		S	1.6cm = 16mm
		CI	2.4cm = 24mm
		E	2.6cm = 26mm
		CH	2,7cm = 27mm
		K	2.1cm = 21mm
		V	1.8cm = 18mm
22	O9	AM	2.1cm = 21mm
		P	1.9cm = 19mm
		T	1.7cm = 17mm
		G	2.2cm = 22mm
		S	1.8cm = 18mm
		CI	2.0cm = 20mm
		E	2.7cm = 27mm
		CH	2.7cm = 27mm
		K	2.3cm = 23mm
		V	1.7cm = 17mm
23	O11	AM	1.0cm = 10mm
		P	1.7cm = 17mm
		T	2.3cm = 23mm
		G	2.2cm = 22mm
		S	1.8cm = 18mm

		CI	2.0cm = 20mm
		E	2.3cm = 23mm
		CH	1.0cm = 10mm
		K	2.4cm = 24mm
		V	1.8cm = 18mm
24	O14	AM	0.8cm = 8mm
		P	1.2cm = 12mm
		T	2.2cm = 22mm
		G	2.1cm = 21mm
		S	1.8cm =18mm
		CI	2.6cm = 26mm
		E	2.6cm = 26mm
		CH	1.6cm = 16mm
		K	1.2cm = 12mm
		V	1.0cm = 10mm
25	O15	AM	3.0cm = 30mm
		P	3.1cm = 31mm
		T	1.9cm = 19mm
		G	2.0cm = 20mm
		S	1.7cm = 17mm
		CI	3.1cm = 31mm
		E	2.8cm = 28mm
		CH	2.4cm = 24mm
		K	2.4cm = 24mm
		V	1.9cm = 19mm

Zones of inhibition of some isolates tested to different antibiotics (Cont'd.)

S/N	ISOLATES	ANTIBIOTICS	ZONES OF INHIBITION
26	O17	AM	3.2cm = 32mm
		P	3.0cm = 30mm
		T	2.6cm = 26mm
		G	2.3cm = 23mm
		S	2.0cm = 20mm
		CI	1.6cm = 16mm
		E	1.0cm = 10mm
		CH	2.5cm = 25mm
		K	2.5cm = 25cm
		V	1.9cm = 19mm
27	O23	AM	2.3cm = 23mm
		P	2.4cm = 24mm
		T	2.9cm = 29mm
		G	2.4cm = 24mm
		S	2.3cm = 23mm
		CI	3.0cm = 30mm
		E	2.8cm = 28mm
		CH	2.6cm = 26mm
		K	2.6cm = 26mm
		V	2.0cm = 26mm
28	O24	AM	1.0cm = 10mm
		P	1.0cm = 10mm
		T	3.1cm = 31mm
		G	2.1cm = 21mm
		S	1.5 cm = 15 mm

	CI	3.5 cm = 35 mm
	E	No zone
	CH	1.3 cm = 13 mm
	K	1.0 cm = 10 mm
	V	1.8 cm = 18 mm

Appendix V

Total Bacterial Count (CFU/g) of *okpeye* Inoculated with Starter Cultures during Fermentation and Spontaneously fermented *okpeye*

Fermentation time (Hours)	Microorganisms	NA/ no of colonies	MHA/ no of colonies	Colony forming units (cfu/g)	Colony forming units (cfu/g
		10^6	10^4	NA	MHA
0	BA	50	31	5.0×10^8	3.1×10^6
	BL	52	30	5.2×10^8	3.0×10^6
	BA & BL	61	34	6.1×10^8	3.4×10^6
	SP	49	37	4.9×10^8	3.7×10^6
24	BA	89	32	8.9×10^8	3.2×10^6
	BL	82	39	8.2×10^8	3.9×10^6
	BA & BL	121	45	1.21×10^9	4.5×10^6
	SP	101	34	1.01×10^9	3.4×10^6
48	BA	98	71	9.8×10^8	7.1×10^6
	BL	105	98	1.05×10^9	9.8×10^6
	BA & BL	172	131	1.72×10^9	1.31×10^7
	SP	142	121	1.42×10^9	1.42×10^7
72	BA	101	89	1.01×10^9	8.9×10^6
	BL	121	91	1.21×10^9	9.1×10^6
	BA & BL	201	189	2.01×10^9	1.89×10^7
	SP	116	108	1.16×10^9	1.08×10^7
96	BA	222	202	2.22×10^9	2.02×10^7
	BL	253	241	2.53×10^9	2.41×10^7
	BA & BL	290	231	2.90×10^9	2.31×10^7
	SP	221	261	2.21×10^9	2.61×10^7

Total Bacterial Count (CFU/g) of *okpeye* Inoculated with Starter Cultures during Fermentation and Spontaneously fermented *okpeye* (cont'd)

Fermentation time (Hours)	Microorganisms	NA/ no of colonies	MHA/ no of colonies	Colony forming units (cfu/g)	Colony forming units (cfu/g
		10^6	10^4	NA	MHA
120	BA	241	221	2.41×10^9	2.21×10^7
	BL	280	261	2.80×10^9	2.61×10^7
	BA & BL	TNTC	283	TNTC	2.83×10^7
	SP	261	270	2.61×10^9	2.70×10^7
144	BA	207	187	2.07×10^9	1.87×10^7
	BL	225	194	2.25×10^9	1.94×10^9
	BA & BL	TNTC	245	TNTC	2.45×10^9
	SP	282	252	2.82×10^9	2.52×10^7
168	BA	205	198	2.05×10^9	1.98×10^9
	BL	223	213	2.23×10^9	2.13×10^9
	BA & BL	TNTC	250	TNTC	2.50×10^9
	SP	TNTC	276	TNTC	2.76×10^7

Appendix VI

Fermentation preliminary studies

Fermentation time (hours)	Microorganisms	pH	TEMP (^{0}C)	TTA (%)
0	BA	6.50, 6.00, 7.00	34.70, 34.00, 34.50	0.520, 0.640, 0.486
	BL			
	BA & BL	6.50, 7.20, 7.50	35.00, 35.00, 35.40	0.540, 0.550, 0.639
	SP			
		6.80, 6.50, 7.00	34.00, 34.00, 35.00	0.450, 0.468, 0.495
		6.70, 6.50, 6.95	35.00, 35.00, 36.00	0.270, 0.288, 0.320
24	BA	7.20, 7.25, 7.50	37.70, 37.50, 37.80	0.621, 0.648, 0.603
	BL			
	BA &BL	7.00, 7.90, 7.50	36.90, 37.00, 36.80	0.630,0.657, 0.711
	SP			
		7.50, 7.00, 7.80	38.50, 38.55, 38.00	0.468, 0.657, 0.621
		7.20, 7.40, 7.80	38.20, 39.00, 38.40	0.315, 0. 378, 0.405
48	BA	8.80, 9.00, 8.50	39.80, 39.45, 39. 70	1.050, 1.161, 1.080
	BL			
	BA &BL	8. 90, 8.50, 8.70	40.50, 40.00, 40.70	0.900, 0.820, 0.945

	SP	9.70, 9.50, 9.00	41.70, 41.50, 41.90	1.026, 1.107, 1.062
		9.00, 9.50, 8.90	40.60, 40.50, 40.00	0.675, 0711, 0.738
72	BA	9.20, 9.50, 9.00	40.00, 40.50, 40.00	1.161, 1.220, 1.260
	BL			
	BA & BL	8.95, 8.50, 9.00	41.20, 41.00, 41.10	0.927, 0.999, 0.900
	SP			
		11.10, 11.00, 11.20	41.00, 41.15, 41.00	1.008, 1.044, 1.080
		9.00, 9.50, 9.00	40.70, 40.75, 47.50	0.765, 0.846, 0.945
96	BA	9.30, 9.50, 9.20	39.00, 39.50, 39.00	1.490, 1.420, 1.398
	BL			
	BA& BL	11.20, 11.00, 11.00	41.00, 40.00, 41.00	1.125, 1.150, 1.161
	SP			
		11.60, 11.90, 11.50	42.30, 41.50, 42.00	1.323, 1.360, 1.180
		9.20, 9.00, 9.00	38.85, 38.70, 38.00	0.828, 0.783, 0.882
120	BA	8.90, 8.50, 9.00	40.40, 40.00, 40.50	1.314, 1.386, 1.332
	BL			
	BA & BL	8.50, 8.50, 8.80	35.60, 35.50, 35.00	1.180, 1.240, 1.161

	SP	9.10, 9.00, 9.75,	41.70, 42.00, 40.00	1.404, 1.359, 1.341
		9.00, 9.10, 9.00	40.60, 40.50, 40.50	0.747, 0.819, 0.873
144	BA	8.00, 8.20, 7.80	35.00, 36.50, 36.00	1.180, 1.305, 1.269
	BL			
	BA & BL	8.50, 8.60, 8.50	36.00, 37.00, 36.50	1.134, 1.008, 1.08
	SP			
		8.70, 9.00, 9.20	40.60, 41.00, 40.50	1.215, 1.305, 1.251
		8.60, 8.00, 8.60	38.90, 38.00, 37.00	0.738, 0.774, 0.657
168	BA	8.00, 8.70, 8.20	37.50, 37.00, 37.20	1.125, 1.179, 1.080
	BL			
	BA & BL	8.00, 8.20, 8.50	37.40, 37.20, 37.50	1.035,1.134, 1.143
	SP			
		9.00, 8.00, 8.50	40.00, 41.20, 40.00	1.125, 1.215, 1.215
		9.10, 9.00, 8.70	39.20,39.00, 39.00	0.675, 0.585, 0.621

Key: SP- spontaneously fermenting *okpeye,* BA & BL- *okpeye* fermenting with mixed starter cultures of *Bacillus licheniformis* and *Bacillus amyloliquefaciens*, B- *okpeye* fermenting with a single starter culture of *Bacillus amyloliquefaciens*, BL- *okpeye* fermenting with a single starter culture of *Bacillus licheniformis*.

Appendix VII

SENSORY ANALYSIS TEMPLATE

NAME: …… **DATE**: ……

Please taste these samples of soups and indicate how much you like or dislike each sample by ticking the appropriate category under sample codes (A – E).

NOTE: please take water after tasting each sample

Category	**Like extremely (9)**	**Like very much (8)**	**Like moderately (7)**	**Like slightly (6)**	**Neither like nor dislike (5)**	**Dislike slightly (4)**	**Dislike moderately (3)**	**Dislike very much (2)**	**Dislike extremely (1)**
Aroma									
A									
B									
C									
D									
E									
Flavour									
A									
B									
C									
D									
E									
Appearance									
A									
B									
C									

D									
E									
After taste									
A									
B									
C									
D									
E									
Overall acceptability									
A									
B									
C									
D									
E									

Key: SP- spontaneously fermenting *okpeye,* BA & BL- *okpeye* fermenting with mixed starter cultures of *Bacillus licheniformis* and *Bacillus amyloliquefaciens*, B- *okpeye* fermenting with a single starter culture of *Bacillus amyloliquefaciens*, BL- *okpeye* fermenting with a single starter culture of *Bacillus licheniformis*.

Appendix VIII:

Sensory scores for the fermentation of sample using starter cultures.

PANELIST	AROMA		FLAVOUR		APPERANCE		AFTER TASTE		OVERALL ACCEPTABILITY	
1	A	7	A	7	A	8	A	9	A	7.8
	B	8	B	9	B	9	B	6	B	8
	C	7	C	7	C	7	C	7	C	7
	D	9	D	9	D	9	D	9	D	9
	E	5	E	6	E	6	E	5	E	5.5
2	A	6	A	7	A	7	A	6	A	6.5
	B	7	B	6	B	6	B	7	B	6.5
	C	7	C	7	C	6	C	7	C	6.7
	D	6	D	6	D	7	D	7	D	6.5
	E	7	E	7	E	6	E	6	E	6.5
3	A	6	A	7	A	3	A	8	A	6
	B	7	B	7	B	3	B	8	B	6.3
	C	7	C	8	C	3	C	6	C	6
	D	8	D	8	D	3	D	9	D	7
	E	7	E	8	E	3	E	9	E	6.8
4	A	4	A	6	A	7	A	7	A	6
	B	4	B	5	B	5	B	6	B	5

	C	5	C	6	C	6	C	6	C	5.8
	D	4	D	4	D	4	D	4	D	4
	E	7	E	7	E	7	E	7	E	7
5	A	7	A	6	A	7	A	6	A	6.5
	B	7	B	7	B	7	B	7	B	7
	C	8	C	8	C	7	C	8	C	7.5
	D	7	D	7	D	7	D	7	D	7
	E	7	E	8	E	6	E	7	E	7
6	A	8	A	8	A	9	A	8	A	8.3
	B	7	B	7	B	7	B	7	B	7
	C	6	C	6	C	7	C	7	C	6.5
	D	9	D	9	D	8	D	7	D	8.3
	E	6	E	6	E	7	E	7	E	6.5
7	A	7	A	6	A	7	A	7	A	6.8
	B	6	B	6	B	7	B	6	B	6.3
	C	8	C	5	C	6	C	6	C	6.3
	D	4	D	8	D	7	D	8	D	6.8
	E	4	E	4	E	5	E	6	E	4.8
	E	7	E	6	E	4	E	5	E	5.5
8	A	6	A	7	A	7	A	7	A	6.8

	B	7	B	8	B	7	B	7	B	7.3
	C	8	C	6	C	6	C	8	C	7
	D	8	D	6	D	7	D	6	D	6.8
	E	8	E	8	E	7	E	9	E	8
9	A	7	A	7	A	6	A	7	A	6.8
	B	5	B	7	B	6	B	4	B	5.5
	C	6	C	6	C	6	C	5	C	5.8
	D	5	D	6	D	6	D	5	D	5.5
	E	8	E	6	E	6	E	6	E	6.5
10	A	7	A	7	A	8	A	8	A	7.5
	B	7	B	7	B	8	B	8	B	7.5
	C	8	C	8	C	8	C	8	C	8
	D	8	D	8	D	8	D	7	D	7.8
	E	8	E	9	E	8	E	6	E	7.8
11	A	8	A	8	A	8	A	8	A	8
	B	7	B	7	B	7	B	7	B	7
	C	8	C	6	C	6	C	6	C	6.5
	D	5	D	5	D	5	D	5	D	5
	E	7	E	6	E	7	E	8	E	7
12	A	9	A	9	A	8	A	7	A	8.3

	B	8	B	8	B	8	B	7	B	7.8
	C	7	C	7	C	8	C	6	C	7
	D	8	D	7	D	7	D	7	D	7.3
	E	6	E	5	E	7	E	5	E	5.8
13	A	7	A	7	A	6	A	7	A	6.8
	B	7	B	6	B	5	B	7	B	6.3
	C	6	C	6	C	6	C	6	C	6
	D	8	D	8	D	7	D	8	D	7.8
	E	5	E	4	E	6	E	4	E	4.8
14	A	7	A	8	A	6	A	7	A	7
	B	7	B	7	B	7	B	6	B	6.8
	C	8	C	7	C	8	C	8	C	7.8
	D	8	D	9	D	8	D	8	D	8.3
	E	8	E	8	E	8	E	6	E	7.5
15	A	7	A	8	A	8	A	7	A	7.5
	B	8	B	6	B	6	B	8	B	7
	C	9	C	8	C	7	C	9	C	8
	D	7	D	8	D	8	D	9	D	8
	E	8	E	7	E	9	E	9	E	8.3
16	A	7	A	8	A	9	A	6	A	7.5

	B	8	B	7	B	8	B	7	B	7.5
	C	9	C	8	C	8	C	9	C	8.5
	D	7	D	7	D	8	D	8	D	7.5
	E	8	E	9	E	8	E	78	E	8
17	A	7	A	7	A	8	A	8	A	7.5
	B	8	B	8	B	8	B	8	B	7.8
	C	6	C	7	C	7	C	7	C	6.8
	D	7	D	6	D	6	D	7	D	6.5
	E	6	E	7	E	6	E	6	E	6.3
18	A	8	A	9	A	8	A	8	A	8.3
	B	7	B	8	B	7	B	8	B	7.5
	C	4	C	7	C	7	C	8	C	6.5
	D	4	D	9	D	8	D	8	D	7.3
	E	9	E	8	E	8	E	8	E	8.3
19	A	9	A	8	A	7	A	9	A	8.3
	B	7	B	7	B	6	B	6	B	6.5
	C	6	C	6	C	7	C	7	C	6.5
	D	6	D	7	D	7	D	6	D	6.5
	E	6	E	6	E	6	E	6	E	6
20	A	7	A	8	A	8	A	8	A	7.8

	B	8	B	7	B	8	B	7	B	7.5
	C	6	C	6	C	7	C	6	C	6.3
	D	6	D	6	D	6	D	6	D	6
	E	6	E	7	E	6	E	6	E	6.3
21	A	6	A	6	A	7	A	6	A	6.3
	B	7	B	6	B	7	B	6	B	6.5
	C	8	C	8	C	7	C	7	C	7.5
	D	8	D	8	D	7	D	7	D	7.5
	E	6	E	5	E	7	E	3	E	5.3
22	A	7	A	6	A	8	A	8	A	7.3
	B	5	B	5	B	7	B	6	B	5.8
	C	6	C	7	C	6	C	5	C	6
	D	7	D	5	D	5	D	7	D	6
	E	6	E	6	E	7	E	6	E	6.3
23	A	8	A	8	A	8	A	6	A	7.5
	B	8	B	8	B	8	B	6	B	7.5
	C	6	C	6	C	6	C	6	C	6
	D	7	D	7	D	7	D	6	D	6.8
	E	6	E	6	E	6	E	6	E	6
24	A	8	A	7	A	6	A	6	A	6.8

	B	7	B	7	B	5	B	7	B	6.5
	C	7	C	8	C	6	C	8	C	7.3
	D	8	D	9	D	7	D	7	D	8.3
	E	9	E	8	E	7	E	7	E	7.8
25	A	9	A	8	A	8	A	8	A	8.3
	B	8	B	7	B	7	B	7	B	7.3
	C	7	C	5	C	6	C	5	C	5.8
	D	6	D	7	D	6	D	4	D	5.8
	E	5	E	6	E	7	E	7	E	6.3
26	A	8	A	9	A	9	A	9	A	8.8
	B	6	B	7	B	7	B	8	B	7
	C	6	C	8	C	7	C	8	C	7.3
	D	7	D	8	D	6	D	9	D	7.5
	E	6	E	8	E	8	E	8	E	7.5
27	A	9	A	9	A	9	A	9	A	9
	B	9	B	9	B	8	B	8	B	8.5
	C	8	C	7	C	8	C	8	C	7.8
	D	7	D	7	D	7	D	7	D	7
	E	6	E	6	E	7	E	6	E	6.3
28	A	7	A	7	A	8	A	6	A	7

	B	6	B	6	B	7	B	7	B	6.5
	C	9	C	9	C	9	C	8	C	8.8
	D	6	D	6	D	7	D	6	D	6.3
	E	6	E	6	E	7	E	6	E	6.3
29	A	6	A	7	A	8	A	6	A	6.8
	B	8	B	9	B	8	B	7	B	8
	C	9	C	5	C	8	C	5	C	6.8
	D	7	D	7	D	9	D	7	D	7.5
	E	7	E	7	E	9	E	8	E	7.8
30	A	8	A	7	A	7	A	7	A	7.3
	B	6	B	7	B	7	B	6	B	6.5
	C	8	C	8	C	7	C	8	C	7.8
	D	6	D	7	D	7	D	6	D	6.5
	E	7	E	7	E	7	E	7	E	7

Appendix IX

Anova table for proximate, phytochemical and sensory evaluation of *okpeye* fermented with starter cultures

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Aroma	Between Groups	9.029	4	2.257	1.546	.192
	Within Groups	211.644	145	1.460		
	Total	220.673	149			
Flavour	Between Groups	7.637	4	1.909	1.462	.217
	Within Groups	189.303	145	1.306		
	Total	196.940	149			
Appearance	Between Groups	7.262	4	1.815	1.172	.326
	Within Groups	224.612	145	1.549		
	Total	231.873	149			
After taste	Between Groups	5.219	4	1.305	.891	.471
	Within Groups	212.281	145	1.464		
	Total	217.500	149			
Overall acceptability	Between Groups	5.779	4	1.445	1.749	.142
	Within Groups	119.761	145	.826		
	Total	125.540	149			

Multiple Comparisons

Dependent Variable		(I) Samples	(J) Samples	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
Aroma	Tamhane	A	B	-.533	.295	.546	-1.39	.33
			C	-.333	.287	.944	-1.17	.50
			D	-.370	.361	.975	-1.42	.68
			E	.120	.301	1.000	-.75	1.00
		B	A	.533	.295	.546	-.33	1.39
			C	.200	.271	.998	-.59	.99
			D	.163	.348	1.000	-.86	1.18
			E	.654	.285	.227	-.18	1.48

	C	A	.333	.287	.944	-.50	1.17
		B	-.200	.271	.998	-.99	.59
		D	-.037	.341	1.000	-1.04	.96
		E	.454	.277	.676	-.35	1.26
	D	A	.370	.361	.975	-.68	1.42
		B	-.163	.348	1.000	-1.18	.86
		C	.037	.341	1.000	-.96	1.04
		E	.491	.353	.845	-.54	1.52
	E	A	-.120	.301	1.000	-1.00	.75
		B	-.654	.285	.227	-1.48	.18
		C	-.454	.277	.676	-1.26	.35
		D	-.491	.353	.845	-1.52	.54

Flavour	Tamhane	A	B	-.700	.287	.164	-1.53	.13
			C	-.333	.282	.938	-1.16	.49
			D	-.334	.319	.971	-1.26	.59
			E	-.235	.306	.997	-1.12	.65
		B	A	.700	.287	.164	-.13	1.53
			C	.367	.263	.842	-.40	1.13
			D	.366	.302	.928	-.52	1.25
			E	.465	.288	.696	-.37	1.30
		C	A	.333	.282	.938	-.49	1.16
			B	-.367	.263	.842	-1.13	.40
			D	-.001	.298	1.000	-.87	.87
			E	.098	.284	1.000	-.73	.92

		D	A	.334	.319	.971	-.59	1.26
			B	-.366	.302	.928	-1.25	.52
			C	.001	.298	1.000	-.87	.87
			E	.099	.320	1.000	-.83	1.03
		E	A	.235	.306	.997	-.65	1.12
			B	-.465	.288	.696	-1.30	.37
			C	-.098	.284	1.000	-.92	.73
			D	-.099	.320	1.000	-1.03	.83
Appearance	Tamhane	A	B	-.333	.338	.981	-1.32	.65
			C	.167	.314	1.000	-.75	1.08
			D	.105	.339	1.000	-.88	1.09
			E	.322	.317	.977	-.60	1.24

	B	A	.333	.338	.981	-.65	1.32
		C	.500	.316	.720	-.42	1.42
		D	.438	.341	.898	-.56	1.43
		E	.655	.319	.368	-.27	1.58
	C	A	-.167	.314	1.000	-1.08	.75
		B	-.500	.316	.720	-1.42	.42
		D	-.062	.317	1.000	-.99	.86
		E	.155	.294	1.000	-.70	1.01
	D	A	-.105	.339	1.000	-1.09	.88
		B	-.438	.341	.898	-1.43	.56
		C	.062	.317	1.000	-.86	.99
		E	.217	.320	.999	-.72	1.15

		E	A	-.322	.317	.977	-1.24	.60
			B	-.655	.319	.368	-1.58	.27
			C	-.155	.294	1.000	-1.01	.70
			D	-.217	.320	.999	-1.15	.72
After taste	Tamhane	A	B	-.500	.320	.733	-1.43	.43
			C	-.167	.312	1.000	-1.08	.75
			D	-.402	.372	.965	-1.49	.68
			E	-.108	.341	1.000	-1.10	.89
		B	A	.500	.320	.733	-.43	1.43
			C	.333	.245	.862	-.38	1.05
			D	.098	.318	1.000	-.83	1.03
			E	.392	.281	.841	-.43	1.21

	C	A	.167	.312	1.000	-.75	1.08
		B	-.333	.245	.862	-1.05	.38
		D	-.236	.311	.998	-1.15	.68
		E	.059	.273	1.000	-.74	.85
	D	A	.402	.372	.965	-.68	1.49
		B	-.098	.318	1.000	-1.03	.83
		C	.236	.311	.998	-.68	1.15
		E	.295	.340	.993	-.70	1.29
	E	A	.108	.341	1.000	-.89	1.10
		B	-.392	.281	.841	-1.21	.43
		C	-.059	.273	1.000	-.85	.74
		D	-.295	.340	.993	-1.29	.70

Overall acceptability	Tamhane	A	B	-.5233	.2374	.274	-1.215	.168
			C	-.1433	.2216	.999	-.791	.504
			D	-.2444	.2668	.989	-1.022	.533
			E	.0092	.2410	1.000	-.692	.711
		B	A	.5233	.2374	.274	-.168	1.215
			C	.3800	.2032	.498	-.212	.972
			D	.2790	.2517	.959	-.456	1.014
			E	.5326	.2242	.190	-.120	1.185
		C	A	.1433	.2216	.999	-.504	.791
			B	-.3800	.2032	.498	-.972	.212
			D	-.1010	.2369	1.000	-.796	.593
			E	.1526	.2074	.998	-.452	.757

	D	A	.2444	.2668	.989	-.533	1.022
		B	-.2790	.2517	.959	-1.014	.456
		C	.1010	.2369	1.000	-.593	.796
		E	.2536	.2552	.980	-.491	.998
	E	A	-.0092	.2410	1.000	-.711	.692
		B	-.5326	.2242	.190	-1.185	.120
		C	-.1526	.2074	.998	-.757	.452
		D	-.2536	.2552	.980	-.998	.491

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Oxalate (%)	Between Groups	147.257	4	36.814	400.417	.000
	Within Groups	.919	10	.092		
	Total	148.177	14			
Saponins (%)	Between Groups	.003	4	.001	82.294	.000
	Within Groups	.000	10	.000		
	Total	.003	14			
Tannins (mg/100g)	Between Groups	112.301	4	28.075	355.742	.000
	Within Groups	.789	10	.079		
	Total	113.090	14			

Cyanide (mg/100g)	Between Groups	4.238	4	1.060	1382.030	.000
	Within Groups	.008	10	.001		
	Total	4.246	14			
Phytate (mg/100g)	Between Groups	457666.834	4	114416.709	8430.623	.000
	Within Groups	135.716	10	13.572		
	Total	457802.550	14			
Alkaloids	Between Groups	197.090	4	49.272	1409.932	.000
	Within Groups	.349	10	.035		
	Total	197.439	14			
Total Phenol GAE mg/g	Between Groups	166293.662	4	41573.416	2059.595	.000
	Within Groups	201.852	10	20.185		

	Total	166495.514	14			
Flavonoids (%)	Between Groups	163.999	4	41.000	598.711	.000
	Within Groups	.685	10	.068		
	Total	164.684	14			

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Protein Content (%)	Between Groups	10.554	4	2.639	17.456	.000
	Within Groups	1.512	10	.151		
	Total	12.066	14			
Fat Content (%)	Between Groups	34.057	4	8.514	390.925	.000
	Within Groups	.218	10	.022		
	Total	34.275	14			
Fibre Content (%)	Between Groups	19.925	4	4.981	23.193	.000
	Within Groups	2.148	10	.215		
	Total	22.072	14			

Carbohydrate/NFE Content (%)	Between Groups	24.259	4	6.065	19.093	.000
	Within Groups	3.176	10	.318		
	Total	27.436	14			
Moisture Content (%)	Between Groups	22.047	4	5.512	29.027	.000
	Within Groups	1.899	10	.190		
	Total	23.945	14			
Ash content (%)	Between Groups	.929	4	.232	8.260	.003
	Within Groups	.281	10	.028		
	Total	1.210	14			

Multivariate Tests[a]

Effect		Value	F	Hypothesis df	Error df	Sig.
Intercept	Pillai's Trace	.999	30621.902[b]	3.000	83.000	.000
	Wilks' Lambda	.001	30621.902[b]	3.000	83.000	.000
	Hotelling's Trace	1106.816	30621.902[b]	3.000	83.000	.000
	Roy's Largest Root	1106.816	30621.902[b]	3.000	83.000	.000
Microorganism	Pillai's Trace	1.258	20.469	9.000	255.000	.000
	Wilks' Lambda	.107	33.775	9.000	202.151	.000
	Hotelling's Trace	5.086	46.150	9.000	245.000	.000
	Roy's Largest Root	4.392	124.439[c]	3.000	85.000	.000
Days	Pillai's Trace	1.740	16.777	21.000	255.000	.000

	Wilks' Lambda	.023	31.019	21.000	238.881	.000
	Hotelling's Trace	14.649	56.968	21.000	245.000	.000
	Roy's Largest Root	12.919	156.868[c]	7.000	85.000	.000

a. Design: Intercept + Microorganism + Days

b. Exact statistic

c. The statistic is an upper bound on F that yields a lower bound on the significance level.

Tests of Between-Subjects Effects

Source	Dependent Variable	Type II Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	Temperature	419.357[a]	10	41.936	23.637	.000
	pH	115.955[b]	10	11.596	39.928	.000
	Total Titrable Acidity	8.713[c]	10	.871	105.794	.000
Intercept	Temperature	144347.693	1	144347.693	81361.333	.000
	pH	7129.430	1	7129.430	24549.695	.000
	Total Titrable Acidity	82.722	1	82.722	10044.476	.000
Microorganism	Temperature	67.160	3	22.387	12.618	.000
	pH	9.726	3	3.242	11.164	.000

	Total Titrable Acidity	2.663	3	.888	107.778	.000
Days	Temperature	352.196	7	50.314	28.359	.000
	pH	106.229	7	15.176	52.256	.000
	Total Titrable Acidity	6.050	7	.864	104.944	.000
Error	Temperature	150.803	85	1.774		
	pH	24.685	85	.290		
	Total Titrable Acidity	.700	85	.008		
Total	Temperature	144917.853	96			
	pH	7270.070	96			
	Total Titrable Acidity	92.135	96			
Corrected Total	Temperature	570.160	95			

	pH	140.640	95			
	Total Titrable Acidity	9.413	95			

a. R Squared = .736 (Adjusted R Squared = .704)

b. R Squared = .824 (Adjusted R Squared = .804)

c. R Squared = .926 (Adjusted R Squared = .917)