AN EVALUATION OF THE MICROBIAL QUALITY OF FRESH SCENT LEAVES (*Ocimum gratissimum*) OBTAINED FROM THREE MARKETS IN BENIN-CITY NIGERIA.

BY

Hope Chizoba UBAWUIKE (Miss)

AGR1500129

TO STAFF AND STUDENTS

DEPARTMENT OF ANIMAL SCIENCE

FACULTY OF AGRICULTURE

UNIVERSITY OF BENIN

BENIN CITY

AUGUST, 2021

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A PROJECT SUBMITTED TO THE DEPARTMENT OF ANIMAL SCIENCE, FACULTY OF AGRICULTURE, UNIVERSITY OF BENIN , BENIN CITY, IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE BACHELOR OF AGRICULTURE(B. AGRIC)(DEGREE IN ANIMAL SCIENCE)

AUGUST, 2021

CERTIFICATION

This is to certify that **Hope Chizoba UBAWUIKE (Miss)** with matriculation number **AGR1500129** carried out this project which is adequate in scope and quality in partial fulfillment of the award of B. Agric Degree in Animal Science of the University of Benin, Benin City, Edo State, Nigeria.

_____ Date_____

Mr. E.S. ABEL
Project Supervisor

_____ Date_____

Prof. J. M. OMOYAKHI
(Head of Department)

DEDICATION

This project is dedicated to God Almighty for His mercies and grace all through my stay as a student in University of Benin; may all glory be unto His name. Also to my Parents Mr. and Mrs. Isaiah Ndidi Ubawuike and all my family members who have been my support all through.

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ABSTRACT

Scent leaf is a common vegetable and spice in the diet of most Nigerians, cherished as a result of its contribution to health and sensory qualities. However, its safety is usually compromised especially because it is usually consumed raw or slightly cooked. Hence, this study was conducted to determine its microbiological quality. Samples of scent leaf (9) were randomly purchased from New Benin, Oba and Uselu markets in Benin. Each sample was divided into 2- one group was washed with sterile distilled water and the other group was left unwashed and they were blended to aid homogenization. Samples were analyzed for bacteria and yeasts using conventional plate culture procedures. This was followed by the characterization of bacterial and fungal isolates using cultural and biochemical characteristics. The identity of isolates were confirmed using Polymerase Chain Reaction (PCR). The mean bacteria count (log_{10} cfu/g) across the three markets for the unwashed samples was 2.30 while that of the washed was 1.92. For fungi, the mean count in the unwashed scent leaf was 1.67 while that of the washed samples was 1.20. Statistically, there was a significant difference ($P<0.05$) in bacteria counts (log_{10} cfu/g) between the unwashed (2.27, 2.28 and 2.34) and washed scent leaves (1.96, 1.86 and 1.96) for New Benin, Oba and Uselu markets respectively. Significant differences ($P<0.05$) were also recorded in fungal counts (log_{10} cfu/g) between the raw scent leaves (1.71, 1.66 and 1.63) and washed samples (1.25, 1.18 and 1.16) obtained from New Benin, Oba and Uselu markets respectively. Also, the bacteria count across the markets was always higher than the fungal count; an indication of more bacterial contamination. The bacteria isolated from the scent leaf samples were identified and was found to be *Enterobacter aerogenes, Enterobacter cloacae, Escherichia coli, Bacillus cereus, Proteus mirabilis and Pseudomonas aeruginosa.* PCR tests was carried out and confirmed the identity of three of the isolates specifically as *Escherichia coli*, *Proteus mirabilis and Pseudomonas aeruginosa.* The fungi isolates were identified by cultural and morphological characteristics as *Trichoderma spp, Pennicillium spp and Curvularia*

spp. Thus it is recommended for raw spices and herbs to be washed with clean water to reduce the microbial load and make them safer for consumption. The effect of other methods of decontamination such as washing with water and salt or with vinegar should be tested in this study location since most vegetables are either consumed raw or partially cooked.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of study

Green leafy vegetables are important as food both from economic and nutritional standpoint (Bolaji *et al*., 2006). They occupy an important place among the food crops as they provide adequate amount of many vitamins and minerals for humans (Fasuyi, 2006). Leafy vegetables represent cheap but high quality nutritional source for the poor segment of the population especially where malnutrition is widespread (Nnamani *et al*., 2007).

Scent leaf *(Ocimum gratissimum)* is both a leafy vegetable and spice that belongs to the plant family *Labiatae* and is widely used in cooking and medicine (Ighodaro *et al.,* 2010). The plant is cultivated in abundance in different part of Nigeria and it contains some bioactive substances such as tannins, saponins, alkaloids, glycosides, phenols and flavonoids, also referred to as phytochemicals (Kin *et al*., 2018). Phytochemicals and essential oils inherent in *O. gratissimum* have been shown to have a wide range of properties including antibacterial (Prabhu *et al*., 2009), antifungal (Matasyoh *et al*., 2007), antiviral (Schnitzler *et al*., 2011) insecticidal (Essam, 2001), antioxidant properties (Ganiyu, 2006).

Increased consumption of vegetables has been associated with low incidence of chronic diseases such as cancer, cardiovascular diseases, chronic obstructive pulmonary diseases, osteoporosis, etc. (Pem and Jeewon, 2015). International organizations including the World Health Organization (WHO), the Food and Agricultural Organization (FAO), and Centers for Disease Control and Prevention (CDC) have encouraged people to eat more fresh fruits and vegetables (Mahmoud, 2019). Nigeria is endowed with favorable climate and seasons which enables the growth of varieties of fruits and vegetables (Rasaq *et al,* 2015). They are also affordable, accessible and readily available to consumers

(Nwachukwu and Chukwu, 2013). Besides the health and economic benefits of fruits and vegetables, there is much concern about their contamination by human pathogens, after they have been consumed fresh, or moderately cooked (Iyoha and Agoreyo, 2015). Fruits and vegetables can be easily contaminated with microbiological, chemical, and physical hazards, because they are often grown in open environment (Mahmoud, 2008). Microorganisms, bacteria, fungi and viruses are ubiquitous and could be found resident on fresh fruits and vegetables as sources of nutrients for their survival (Orji *et al.,* 2016). Outbreak of food borne pathogens due to contamination of fruits and vegetables beyond acceptable limits over the past decades cannot be overemphasized (Ailes *et al.,* 2008). In both developed and developing countries, there is increase in the incidence of food poisoning and gastroenteritis due to consumption of raw foods and vegetables (Adesetan, 2013, Istifanus, 2018).

Public awareness of healthy eating habits has been intensified in recent years and prompted an increased demand for fresh fruits and vegetables (Olaimat and Holley, 2012). Despite the health benefits derived from consuming fresh produce, the risk of microbiological contamination in vegetables is of concern as the contamination can possibly occur through the food chain, from farm to fork. Over the past decade, numerous foodborne disease outbreaks caused by *Listeria monocytogenes, Escherichia coli, and Salmonella spp*. were related to the consumption of contaminated fresh vegetables (Beuchat, 2002; Centers for Disease Control and Prevention, 2011, 2012; Maffei *et al.,* 2013).

Microbiology is the study of the microorganisms that inhibit, create, or contaminate food. Fruits and vegetables are widely exposed to contamination by micro-organisms through contact with soil, water and dust or by handling at harvest and post-harvest processing. This contamination could be from human or plant pathogens (Carmo *et al*.,2004). Safe fruits and vegetables are essential to maximize the nutritional and health benefits associated with the intake of these produce.Food safety problems especially of fresh

vegetables still persist across the globe and remain a challenge to the general public and government. Scent leaves is a popular vegetable consumed in Nigeria hence the need to determine the microbiological quality and safety of the vegetable.

Bacteriologically safe vegetables are essential to maximize the health benefits promised by adequate consumption of these produce. Proper washing of fruits and vegetables is essential for decontamination. Water supplemented with varying concentrations of organic acids, such as acetic, citric and sorbic acids, has been shown to reduce microbial populations on fruits and vegetables (Beuchat, 1998).

1.2 Aims and Objectives of the study

Scent leaves are known to be highly nutritious with many health benefits and so is taken by many Nigerians to alleviate gastro-intestinal microbial load, diabetes, hypertension, diarrhea etc. either as a tonic or as a condiment in foods hence the aim of this study is to study and evaluate the microbial quality of fresh harvested scent leaves and its safety for use.

Specific objectives of the study were to:

1) determine the microbial status of fresh scent leaves
2) isolate, characterize and quantify microorganisms found in fresh Scent leaves
3) evaluate the effect of washing with clean water on the microbiological quality of fresh scent leaves.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Morphology and Taxonomy of *Ocimum gratissimum*

Ocimum gratissimum is a perennial plant that is widely distributed in the tropics of Africa and Asia. It is a member of the *Labitae* family and is the most popular of the *ocimum* genus (Abdullahi *et al.,* 2003, Idris *et al*., 2011). It is a scented shrub with lime green leaves and has a woody base with an average height of 1-3 metres (USDA., 2008). The leaves measure up to 10 x 5cm and are ovate to ovate-lanceolate, sub-acuminate to acuminate at apex, cuneate and decurrent at base with a coarsely crenate, serrate margin, pubescent and dotted at both the edges. Stomata are few or absent on the upper surface while they are present on the lower surface. Petioles are up to 6cm long and racemes up to 18cm long. The peduncles are densely pubescent, calyx is up to 5mm long, Nutlets are mucilaginous when they are wet (Bhat, 2003).

It is commonly called Basil fever plant or Tea bush and vernacular names include *nchanwu* (Igbo), *Daidoya tagida* (Hausa), *Tanmotswangiwawagi* (Nupe) and *Efinrin* (Yoruba) (Abdullahi *et al*., 2003; Idris *et al*., 2011).

Scent leaves are known to have many uses. In culinary, it is used in salads, soups, pasta, vinegars and jellies in many part of the world. The Thai people are also known to use it in food flavoring. In traditional medicine, applied as a lotion for skin infections, and taken internally for bronchitis the leaves have been used as a general tonic and anti-diarrheal

agent; the leaf oil when mixed with alcohol is applied as a lotion for skin infections (Iwu, 1993).

Table 1: Scientific classification of *Ocimum gratissimum*

Kingdom:	Plantae
Clade:	Tracheophytes
Clade:	Angiosperms
Clade:	Eudicots
Clade:	Asterids
Order:	Lamiales
Family:	Lamiaceae
Genus:	*Ocimum*
Species:	*O. grattisimum*

Source: USDA (2009)

Fig 1: Scent leaf Plant Source: University of Benin (2021)

Fig. 2: Leaves of *Ocimum gratissimum* Source: New Benin Market, Benin-city (2021).

2.2 Traditional Uses of Scent leaf

O. gratissimum leaf extract is used in Nigeria in the treatment of diarrhea while the cold infusions are used for the relief of haemorrhoids and stomach upset (Kabir *et al*., 2005). It is used by Ibos in the Southern-eastern Nigeria to disinfect and keep the wound surface of a baby's cord sterile. It is also used in the treatment of fungal infections, cold and catarrh (Ijeh *et al*., 2005). The flowers and leaves of this plant are rich in essential oils and so can be used in making teas and infusions (Rabelo *et al*., 2003).

Fig 3: Scent Leave Juice Source: University of Benin (2021).

Some communities in Kenya and sub-saharan Africa the leaves of this plant are rubbed between the palms and sniffed as a treatment for blocked nostrils, treatment of abdominal pain, sore eyes, ear infections coughs, barenness, fever, convulsions, tooth gargle, regulation of menstruation and a cure for the prolapse of the rectum (Matasyoh *et al*., 2007).

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In India, *O. gratissimum* is used in the treatment of sunstroke, headache, influenza as a diaphoretic, antipyretic and for its anti-inflammatory activity. (Tania *et al*.,2006).

In the North-east of Brazil, it is used as condiment, culinary, medicinal purposes (Rabelo *et al.,* 2003). *O. gratissimum* is also known to have mosquito-repellent and mosquitocidal potential (Oparaocha *et al.,* 2010). It is also used in the treatment of miscarriage (Ogbe *et al.,* 2009).

2.3 Published Pharmacological Properties of *Ocimum gratissimum* (Scent Leaf)

2.3.1 Ovicidal Activity

The ovicidal activity of the essential oil of *O. gratissimum* and its main component eugenol was evaluated against Haemonchus contortus, a gastrointestinal parasite of small ruminants, the oil and eugenol were diluted in Tween20(0.5%) at five different concentrations. In the egg hatch test, H. Contortus eggs were obtained from the feaces of goats experimentally infected. At 0.50% concentration, the essential oil and eugenol showed a maximum eclodibilty inhibition. These results indicate a possible utilization of the essential oil of *O. gratissimum* as an aid to the control of gastrointestinal helminthosis of small ruminants (Pessoa *et al,* 2002).

2.3.2 Antidiarrheal Effect

The aqueous extract of the leaves of *O. gratissimum* was screened for antidiarrheal effects. The extract inhibited castor oil induced diarrhea in rats as judged by a decrease in the number of wet faeces in the extract treated rats. In addition, the extract inhibited the propulsive movement of the intestinal contents. Findings suggested that the aqueous extract of the leaves of *O. gratissimum* might elicit an antidiarrheal effect by inhibiting intestinal motility, partly via muscarinic receptor inhibition (Offiah and Chikwendu, 1996). *O. gratissimum* leaf extracts have been extensively demonstrated to be effective against the various aetiologic agents of diarrhoea, including Shigellae.

2.3.3 Wound Healing

Persistent microvascular hyperpermeability to plasma proteins is a characteristic feature of normal wound healing. Evan's blue dye (200mg/kg body weight) in normal saline was administered intravenously through marginal ear vein of experimental rabbits (n=5). Each animal served as its own control. One hour after evan's blue administration, 0.1 mL each of *O. gratissimum* oil, histamine dihyrochloride (30µg/ mL) and normal saline were randomly administered by intra- dermal injection at the prepared sites on each of the animals. Increase in vascular permeability was assesses by dye effusion test. Analysis of the differences in vascular permeability between treatment groups showed that *O. gratissimum* oil in intensity and duration was significantly ($p<0.05$) more effective in increasing cutaneous capillary permeability over a 24h period after treatment. The ability of *O. gratissimum* oil in increasing vascular permeability may be one of the factors that contribute to its wound healing property (Orafidiya *et al*., 2006).

2.3.4 Anti-Inflammatory

The following study report the inhibitory effect produced by chemical constituents of essential oils of three plants used in traditional medicine as anti-inflammatory and analgesic drugs, in vitro, on soybean lipoxygenase L-1 and cyclooxygenase function of prostaglandin H synthase (PGHS), the two enzymes involved in the production of mediators of inflammation. The essential oils were extracted from plants *O. gratissimum* along with two other oils*, O. gratissimum* inhibited the two enzymes, cyclooxygenase

function of PGHS and lipoxygenase L1, with an IC50= 125g/ mL and 144g/ mL (Sahouo *et al.,* 2003).

2.3.5 Antimicrobial and Antifungal Activity

The antibacterial activity of dispersions of Ocimum oil (2%) in methanol, honey, a macrogolblend, nonionic and ionic emulsifiers were assessed by cup plate method using type bacterial and wound isolates. Honey enhanced the antibacterial activity of ocimum oil to a greater extent than the macrogol blend (Orafidiya *et al*., 2005).

An investigation of antifungal activity of the essential oil obtained by steam-distillation (1.1%w/w) of the aerial parts of *O. gratissimum* and of an ethanolic extract from the steam distillation residue has been carried out using the agar diffusion method. The results revealed that the essential oil inhibited the growth of all fungi tested, including the phytopathogens, *Botryoshaeriarhodina, Rhizoctonia sp.* and two strains of *Alternaria sp*., while the extract from the residue was inactive. *O. gratissimum* has been reported earlier with in vitro activity against some bacteria and dermatophytes.

The essential oil of *O. gratissimum* inhibited S. aureus at a concentration of 0.75 mg/ mL. The essential oil was also active against members of the family Enterobacteriaceae.

The antibacterial effect of *O. gratissimum* extracted from the aromatic plant was investigated against Listeria monocytogenessero type 4a. Agar well diffusion and tube dilution method were used and the data recorded demonstrated antibacterial activity of the essential oil against the test bacteria. The bacterium was grown at 37 ℃ in a chemically

defined or a complex medium, containing essential oil obtained from *O. gratissimum*. At concentrations from 20 to 250g/ mL, the essential oil progressively inhibited the bacteria growth. The bacteria cultivated on chemically defined medium were more sensitive to essential oil at concentrations of 50, 62.5 and 100g/ mL in relation to those cultivated in complex medium at 37°C. The agar well diffusion was also evaluated. The results yielded a zone of inhibition of 25mm. These established good support to the use of this plant in herbal medicine and a base for the development of new drugs and phytomedicine (Mbata *et al*, 2007). The antibacterial activity of different extracts from the leaves of *O. gratissimum* was tested against *S. aureus, E. coli, S. typhi and S. typhimurium*, pathogenic bacteria that cause diarrhea. The extracts evaluated included cold water extract, hot water extract and stream distillation extract. Only the stream distillation extract had inhibitory effects on the selected bacteria. *O. gratissimum* leaves from Cameroon are a potential source of essential oil. Bioactivities were tested on the insect pest *sitophilus zeamais*, which is the major pest of stored maize. Insecticidal activity was tested by putting 20 adult representatives of *S. zeamais* with 20g of maize grains powdered with various mixtures of essential oil and Kaolin (5 and 10%). The tested essential oils of *O. gratissimum* protected 74% of the test-material against the *S. zeamais* population after 4 days. A direct application of the *O. gratissimum* on the test insects was found to be 85.7% by knock down effect (Okigbo and Ogbonnaya, 2006). Mbata *et al.* (2007) showed that *O. gratissimum* oils have properties that can inhibit growth of psychrophils and heat resistant

organisms and suggested that the plant and its derivatives can be used for the primary purpose of flavoring foods and for antimicrobial activities (Mbata and Saiki, 2007).

2.3.6 Antihypertensive Effect

Intravenous treatment of conscious deoxycorticosterone acetate DOCA-salt hypertensive rats with the essential oil of *O. gratissimum* (EOOG) induced a hypotensive effect that seems related to an active vascular relaxation. Research show that intravenous treatment with EOOG or Eugenol dose- dependently decreased blood pressure in conscious DOCA-salt hypertensive rats, and this action is enhanced when compared with uninephrectomized controls. This enhancement appears related mainly to an increase in EOOG induced vascular smooth relaxation rather than to enhance sympathetic nervous system activity in this hypertensive model (Interaminense *et al.*, 2005).

2.3.7 Antidiabetic Effect

The hypoglycemic effects of the aqueous leaves extract of *O. gratissimum* has been investigated in streptozotocin-induced diabetic rats. (Ehigesie *et al*., 2006). The extract was administered once at the dose of 250,500 and 10000mg/kg body weight. The aqueous extract of *O. gratissimum* at the dose of 500mg/kg, significantly lowered blood glucose level ($p<0.05$) of the diabetic rats by 81.3%, after 24h of extract administration. Preliminary phytochemical screening revealed the presence of reducing sugars, cardiac glycosides, resin, tannis, saponins, glycosides, flavonoids, glycerin and steroids. The median lethal dose (LD50) in rats was calculated to be 1264.9mg/kg body weight. The

leaves extract of *O. gratissimum* was reported to possess anti-diabetic activity in streptozocin-induced in diabetic rats (Mohammed *et al*., 2007).

2.3.8 Hepatoprotective Effect

Aqueous extract of the leaves of *O. gratissimum* has also been used to evaluate the hepatoprotective and diuretic effects. (Effraim *et al*., 2003). Extracts were administered orally by means of polythene cannula to male rabbits. The drug given at dose of 0.4g/kg body weight showed increase in luminal diameter of the collecting duct. At 0.8 g/kg, body weight further increase in luminal diameter was observed. Marked increase in the luminal diameter of the renal tubules was observed when the extract dose was increased to 1.6g/kg body weight, showing a dose response effect of the extract on the structure of the kidney, thus indicating the use of *O. gratissimum* as antidiuretic. The structure of the liver also showed dose-dependent changes when exposed to various doses of the extract. At a dose of 0.4/kg body weight of the extract, there was a generalized edema/hypertrophy of the hepatocytes resulting in a marked widespread, sinusoidal congestion. About 80% of the hepatocytes showed cytoplasmic compaction and disintegration, with some apoptic bodies as well as nuclear piknosis. Kupfer cells were many and were trapped within the sinusoids indicating a degenerative/necrotic process. Increasing the dose of extract to 0.8g/kg body weight produced similar results. There was a reduction in all the parameter observed. There was less hepatocytic edema/hypertrophy resulting in slightly widened sinusoidal spaces. Hepatocytes showed reduced cytoplasmic compaction and disintegration with less prominent apoptotic bodies. In addition, there was mild leukocyte infilteration and

compaction was observed in the hepatocytes with mild tissue lesion or damage as compared with the o.4g/kg treated group. The group of animals treated with 1.6g/kg of the extract depicted an establishment of the normal structure of the liver. Hepatocytes showed no sign of oedema hypertrophy resulting in sinusoids with larger (normal) diameter thereby indicating the usefulness of *O. gratissimum* as a hepatoprotective agent (Effraim *et al*., 2003).

2.3.9 Antioxidant Capacity

The antioxidant capacity of essential oils obtained by steam hydro distillation from five species of the genus *Ocimum*, were evaluated using a high performance liquid chromatography-based hypoxanthine/xanthine oxidase and DPPH assays. The yield of oils from the leaves of the five species was variable with the greater amount obtained from *O. gratissimum* (3.5%). In the hypoxanthine/xanthine oxidase assay, strong antioxidant capacity was evident in all the oils. Anti-oxidant capacity was positively correlated ($r = 0.92$, $p<0.05$) with a high proportion of compounds possessing aphenolic ring such as eugenol, while a strong negative correlation ($r = 0.77$, $p>0.1$) with other major volatiles was observed. These correlations were confirmed to a large extent in the DPPH assay. The data generated with *ocimum* species indicates that essential oils obtained from various herbs and species may have an important role to play in cancer chemoprevention, functional foods and in the preservation of pharmacologic products (Trevisan *et al*., 2006). Extracts from the leaves of *O. gratissimum* were investigated for their phytochemical constituents and for antioxidant activity. Tests for tannins, steroids, terpenoids, flavonoids

and cardiac glycosides were positive in both methanolic and acqueos extracts. These findings suggest the rich phytochemical content of *O. gratissimum* and its good anti-oxidant activity (Akinmoladun *et al*. 2007).

2.4 Microbial Contamination of Vegetables

Imafidor *et al*. assessed the microbial content of lettuce sold in Benin metropolis, Edo State. Whole and soft rot samples of the vegetables part were bought, processed and analyzed. Nutrient agar plated lettuce samples had bacterial counts in the range of 2.0×103 to 4.7×107 cfu/ mL. Bacteria isolated were Pseudomonas spp. and Bacillus spp., where Pseudomonas spp. dominated the most. For Mac Conkey agar, Enterobacter spp., Escherichia coli, and Klebsiella spp. were isolated, with bacterial counts ranging from 2.3×103 -5.7×107 cfu/ mL.

Kemajou *et al*. evaluated 300 vegetable leaves sold in Elele markets, River State for microbial content. Vegetables studied included *Telfairi occidentallis, Genetum gnecanum, Talilum triangulare, Celosta argentea and Vernonia amygdalina*, 60 samples each. From the study, *Talilum triangulare* recorded the highest frequency of infected samples (16.0%), followed by pumpkin leaves (15.0%), while bitter leaves had the lowest (13.3%). Species of bacteria isolated included *Escherichia coli* (29.3%), *Staphylococcs aureus* (22.9%), *Enterobacter aerogenes* (18.3%), *Pseudomonas aeruginosa* (8.2%), *Shigella spp.* (5.5%), *Alcaligenes feacalis* (4.6%), *Micrococcus spp*. (3.7%) and *Salmonella spp*. (2.1%). The

authors recommended that vendors and consumers should be educated to imbibe good sanitary practices, as the result is an indication of poor handling at point-of-sale.

De Oliveira *et al*., 2011 also examined the microbial contamination of 162 samples of minimally processed ready-to-eat vegetables in Brazil were Salmonella spp., *Escherichia coli*, Psychrophilic aerobic bacteria, total and thermotolerant coliforms and Listeria spp. were recorded at levels above WHO recommended limits. The various microbial contaminations have caused serious health implications when ingested by consumers. Annually, about a million cases of foodborne salmonella illness is reported in U.S, and about 19,000 hospitalizations and 380 death cases are reported every year (CDC, 2014).

According to the Centre for Food Safety, Food and Environmental Hygiene (2014) the microbiological limits for ready-to-eat food in general consist of three components:

1) Aerobic colony count (ACC)-
2) Hygiene indicator organisms – E. coli and Enterobacteriaceae;
3) Specific foodborne pathogens – ten specific bacterial pathogens.

The microbiological assessment of ready-to-eat food on these three components lead to the classification of microbiological quality into one of the following three classes:

(a) Satisfactory: test results indicating good microbiological quality.

(b) Borderline: test results that are not unsatisfactory but are also not satisfactory, are on the upper limit of acceptability and which indicate the potential for development of public health problems and of unacceptable risk.

(c) Unsatisfactory: For ACC, test results which indicate investigating reasons for high count may be considered. For hygiene indicator organisms, test results that require remedial action. For pathogens, test results at levels which indicate a product that is potentially injurious to health and/or unfit for human consumption and require immediate remedial action.

2.5 Sources of Microbial Contamination of vegetables

Fruits and vegetable possess normal or natural microbial flora, as also found in other living organisms. They could also be contaminants from the soil, environment, and inoculation through irrigation water. But, this may be altered in the course of harvesting, transportation and processing for consumption (Ofor *et al*., 2009). According to WHO, level of microbial contamination in production systems can change due to several factors such as post-harvest practices, water, local environment, fertilizer, workers' health and hygiene, and consumption patterns and practices (WHO, 2008). Some pathogenic microorganisms could have access to fruits and vegetables via damaged surfaces, and such organisms adapt, survive and reproduce in them and subsequently pose health hazard to consumers (Brooks, 2004). Although, microbial contamination of vended vegetables can occur at every stage of the food chain, from cultivation to processing and point of

consumption, poor hygienic conditions and environmental pollution during cultivation could also increase the risk of contamination (Wadamori *et al*., 2017). The use of animal manure for fertilization, and poor hygiene of workers are not exceptions (Ofor *et al*., 2009). A study by Moses and co-workers on the prevalence of *Escherichia coli* in fruits, vegetables and animal faecal waste used as manure in farms of some communities of Akwa Ibom State, Nigeria identified cow and goat dung as potential sources of *E. coli* contamination of farm productions, when used as manure (Moses *et al*., 2016). On this basis, contaminated irrigation or water channel, poor sanitation and improper maintenance of equipment, the use of animal manure for fertilization, and poor hygiene of workers are contributing factors (Ofor *et al*., 2009). Various pathogenic microbes can contaminate fresh fruits and vegetables at any point in the chain. *Salmonella spp., Escherichia coli* O157:H7, *Staphylococcus aureus, Campylobacter spp. and Listeria monocytogenes* are the most common pathogens that contaminate fresh fruits and vegetables (Wadamori *et al*., 2017). During harvest, asymptomatic human carriers might contaminate the products, and at the postharvest level, products become contaminated by contact with polluted water, other asymptomatic human carriers, or the production process environment. Over the last 30 years there has been at least a 24% of increase in the average amount of fresh vegetable consumed per person in the USA (Pem and Jeewon, 2015).

2.6 Effect of Microbial Contamination in Vegetables

Despite the nutritional and health benefits of vegetables, outbreaks of human infections associated with the consumption of fresh or minimally processed vegetables have

increased in recent years (Beuchat, 2002). Enteric pathogens such as *Escherichia coli* and *Salmonella spp* are among the greatest concerns during food-related outbreaks (Buck *et al*., 2003). Several cases of typhoid fever outbreak have been associated with eating contaminated vegetables grown in or fertilized with contaminated soil or sewage (Beuchat, 1998). Recent evaluation revealed that vegetables are carriers of protozoan cysts and oocysts (Istifanus and Panda, 2018). Consumption of improperly washed fruits and vegetables among children have led to worm infestation, impaired cognitive functions and growth retardation (Adeleke *et al*., 2012). Consumption of raw or partly cooked vegetables such as salad and other fruits is a common practice among the populace, which does not allow complete removal of microbial pathogens following exposure to the digestive system (Kibitok and Nduko, 2016, Imafidor *et al*, 2018). Over the past decade, numerous foodborne disease outbreaks caused by Listeria monocytogenes, Escherichia coli, and Salmonella spp. were related to the consumption of contaminated fresh vegetables (Maffei *et al*., 2013). It is reported that listeria can cause listeriosis a serious and deadly foodborne illness that can be dangerous when ingested especially by pregnant women, fetuses and embryos including individuals with a weakened immune system (Centre for Disease Control and Prevention, 2008).

2.7 Methods of Reducing Contamination of Vegetables

Various approaches of curtailing microbial contamination of vegetables have been recommended by authors. They include adoption of good sanitary condition, while handling, soaking fruits and vegetables in appropriate amount of vinegar for at least 10

minutes, so as to minimize the level of microbial contamination (Nwachukwu and Chukwu, 2013). Mahapatra *et al*. recommended that appropriate and feasible disinfection system should be developed by the government (Mahapatra, 2015). Kibitok and Nduko recommended that the government should establish safety control measure as well as a hazard analysis and critical control point control principles (Kibitok and Nduko, 2016). It was also reported that most venders of fruits and vegetables are neither educated on proper hygiene practice (Orji *et al*., 2016). This calls for adequate public sensitization by the constituted authorities. Oluwatoyin *et al*., 2015 recommended the use of high concentration of salt or chlorinated water in washing sliced fruits.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Experimental Site

This research was conducted in The University of Benin, Benin-City, Edo State, Nigeria. The microbial analysis was carried out in the laboratory of the Department of Microbiology while the Polymerase Chain Reaction (PCR) was conducted in International Institute of Tropical Agriculture (IITA), Ibadan.

3.2 Sources of Experimental Materials

A total of 9 different samples of *Ocimum gratissimum* were randomly purchased from Uselu, New Benin market and Oba markets all in Benin-City, Edo State Nigeria in May, 2021.Three samples were collected from three different traders in each of the markets. The samples were collected in sterile polythene nylons each and was transported to the University of Benin where it was stored in a refrigerator for about 24h before the microbial analysis.

3.3 Materials and Equipment used

Some of the materials used in this experiment were Petri dishes, Sterile distilled water, Pipettes, Micropipettes, Beakers, Electric blender, Nutrient media, Sample bottles, Autoclave, Incubator, Polythene bags, Masking tape, Gas flame, Marker, Muslin cloth, Conical flasks, Bijou bottle, Test tubes.

3.4 Culture Media

According to the conventional plate culture technique, the culture media used were prepared according to standard. Plate count agar (PCA) was used for the culture of bacteria while Potato Dextrose agar (PDA) was used for the culture and isolation of fungi.

3.4.1 Plate count Agar (PCA)

This was used to culture bacteria organisms. This medium was prepared from commercially available dehydrated powder sold by suppliers of culture media. In the preparation, 23.5g was dissolved in 1 litre of distilled water in a conical flask covered with cotton wool and aluminium foil paper. It was mixed thoroughly and sterilized by autoclaving at 121 ℃ for 15 min. The medium was cooled to 45-50 ℃ and then dispensed carefully into the petri dishes.

3.4.2 Potato Dextrose Agar (PDA)

This was used to culture fungi. The medium was prepared from commercially available dehydrated powder sold by suppliers of culture media. In the preparation, 39g of the Potato Dextrose Agar was dissolved in 1 litre of distilled water in a conical flask and was sterilized in an autoclave at 121℃ for 15 min. The medium was cooled to 45-50℃ and then dispensed carefully into the petri dishes.

3.5 Preparation of Samples

Fresh scent leaves were collected from three different markets in Benin. Each sample from the market was divided into two. One group was unwashed designated A and the second group was washed with sterile distilled water and designated B. The leaves from each location were shredded and then blended separately using an electric blender with 40 mL of sterile distilled water each to aid homogenization and then labelled accordingly. Each sample was filtered using a white muslin cloth to filter the solid leaving the liquid which was used for culturing the microorganisms.

3.6 Microbial Analysis and Isolation of Bacteria and Fungi

From the filtered liquid, 0.5 mL aliquot was taken aseptically with the aid of a micropipette in the lamina flowhood and inoculated in an already prepared well labelled petri-dishes containing already solidified Plate Count Agar (PCA) for isolation of bacteria and Potato Dextrose Agar (PDA) for the isolation of Fungi while avoiding splashes and over-filling. The samples were lightly rocked for even spreading and was then incubated at 37 ℃ for 24h for the bacteria and 28 ℃ for 72h for the fungal isolates.

3.7 Enumeration of Microorganisms

The method used is as described by Holt *et al*. (2000) for estimating the total viable counts of the isolates. The discrete colonies on the Nutrient agar and Potato Dextrose agar were selected and counted. The mean colony count on the nutrient agar and potato

dextrose agar plates was used to estimate the total viable count for the samples in colony forming units per gram (cfu/g).

$$\text{Count (cfu/g)} = \frac{mean\ count}{volume\ of\ sample\ used} \times dilution\ factor$$

3.8 Subculturing of bacterial and fungal isolates

From the plates, single colonies were picked up with the aid of a sterilized wire loop and was cultured in, Potato Dextrose agar (PDA), Eosin methylene blue (EMB) agar, Plate Count Agar (PCA), Bacillus cereus Agar (BCA) and Salmonella Shigella Agar (SSA) cultured individually in petri dishes to properly identify the bacteria and fungi isolates. The fungal colonies were then identified using morphological features (Barnett and Hunter, 1972). The fungal isolates were purified by subcultured transplanting to new set using PDA. /The pure strains of isolated fungi were identified using fungal identification keys (Domssch *et al.,* 1993; Klich, 2002; Samsin and Varga, 2007).

3.9 Characterization and Identification of Bacterial isolates

3.9.1 Cultural characteristics

The bacterial isolates were cultured using Nutrient agar. The cultural characteristics which include size, shape, colour, margin and elevation was observed and determined by visual observation.

3.9.2 Gram staining test

The Gram staining technique was used for differentiation between gram positive and gram negative bacterial strains. A drop of sterile distilled water was placed on a clean glass slide and a single colony of 24h old culture was mixed in it. The smear was made by spreading the culture and was then air-dried and fixed by rapidly passing the slide three times over the flame. It was then flooded with crystal violet for 1minute and then washed off using sterile distilled water. Gram's iodine solution was then added to the smear, the glass slide was left for one minute and rinsed with distilled water followed by the application of decolourizing agent (ethanol) for 30seconds. It was then washed with distilled water and the smear was counter-stained with safaranine for one minute. The slide was washed with distilled water, air dried and observed under a microscope.

3.9.3 Catalase production test

1 mL of hydrogen peroxide solution was discharged into a clean glass slide and a sterile inoculating loop was used to collect the colonies of the test organism which was subsequently immersed in the hydrogen peroxide solution. A positive result was indicated by the production of gas bubbles while the absence of gas bubbles is regarded as a negative result.

3.9.4 Citrate utilization Test

5 mL of Simmon citrate broth was inoculated with the test organisms from the cultures. The broth was incubated at 37 ℃ for 48h. A positive reaction is indicated by a change in

colour of the medium from green to blue colour. Negative tubes were observed daily for 4 days to detect any delayed reaction.

3.9.5 Indole Test

This test was carried out according to the method described by Cheesbrough (2000). The test was inoculated into Bijou bottle containing 3 mL of peptone water incubated at 35-37 ℃ for 48 h. This was followed by the addition of 0.5 mL Kovac's reagent. Red colour on the surface layer within 10mins indicated positive test for indole (Cheesebrough, 2000).

3.9.6 Oxidase test

Filter paper (Whatman) was soaked with 2 drops of freshly prepared oxidase reagent. Colony of the test organisms was smeared on the filter paper using sterile wire loop. Positive oxidase was indicated by the production of a deep purple/blue colour within 10 seconds (Cheesebrough, 2000).

3.9.7 Sugar Fermentation Test

Many bacteria species can be identified and differentiated based on the sugars they ferment and utilize. The fermentation medium was prepared by the addition of 0.1g of peptone, 0.1g of sodium chloride and 0.1g of fermentable sugar (glucose, sucrose, lactose and mannitol) in 10 mL of distilled water. About 4 mL of the medium was pipetted into Bijou bottles containing Durham tubes. About 1 mL of phenol red indicator was added to the tubes also. The Bijou bottles containing the sugar solutions were inoculated with the

test bacterial isolates and incubated at 37 ℃ for 34-48 h. After incubation, a change of colour from red to yellow indicates acid production and the presence of gas in the inverted Durham tubes was indicates gas production.

3.10 Identification of Fungal Isolates

A drop of lactophenol blue stain was dropped on a clean grease free sterilized glass slide and after this a sterile inoculating wire loop was used to pick the mycelium unto the glass slide from the mold culture. The mycelium was spread evenly on the slide and then teased to separate the mycelium in order to get a homogenous mixture and the mixture was then covered with the cover slips gently and allowed to stay for some seconds and was then observed using a $\times 40$ microscope. The microscopic examination of actively growing molds was on the basis of structures bearing spores, presence or absence of septate, nature of hyphae and colour.

3.11 Molecular Characterization and Phylogenetic Analysis

DNA was extracted using boiling method described by Igbinosa *et al*. (2018). Briefly, 2 mL of 24 h broth culture was transferred to Eppendorf tube and centrifuged at high speed (10,000 xg) for 5 min. The supernatant was discarded after which 200 µL of sterile distilled water (SDW) was added to the pellets and vortexed using a vortex mixer for 1 min before it was heated at 100 ^{o}C for 15 min. Lastly, it was thereafter centrifuge at high speed for 2 minutes. The supernatant from the second spin was now regarded as the pure DNA and from which, 10 µL of DNA was used for amplification of the gene by PCR.

Successful amplification of the genomic bacterial DNA was made possible using the 27F primer with the sequence 5'-AGAGTTTGATCMTGGCTCAG and the 1540R primer with the sequence 5'-TACGGYTACCTTGTTACGACT.

3.12 Polymerase chain reaction

PCR sequencing preparation cocktail consisted of 10 µL of 5x GoTaq colourless reaction, 3 µL of 25mM MgCl2, 1 µL of 10 mM of dNTPs mix, 1 µL of 10 pmol each 27F 5'-AGAGTTTGATCMTGGCTCAG-3' and - 1540R, 5′-TACGGYTACCTTGTTACGACT-3′ primers and 0.3 units of Taq DNA polymerase (Promega, USA) made up to 42 µL with sterile distilled water 8 µL DNA template. PCR was carried out in a GeneAmp 9700 PCR System Thermal cycler (Applied Biosystem Inc., USA) with a PCR profile consisting of an initial denaturation at 94 °C for 5 min; followed by a 30 cycles consisting of 94 °C for 30 secs, 50 °C for 60 secs and 72 °C for 1 min 30 secs; and a final termination at 72 °C for 10 min. After the aforementioned processes, the product was kept in the refrigerator at 4 ºC until ready for use.

3.13 Integrity

The integrity of the amplified about 1.5Mb gene fragment was checked on a 1% Agarose gel ran to confirm amplification. The buffer (1XTAE buffer) was prepared and subsequently used to prepare 1.5% agarose gel. The suspension was boiled in a microwave for 5 minutes. The molten agarose was allowed to cool to 60 °C and stained with 3 µL of 0.5 g/ mL ethidium bromide (which absorbs invisible UV light and transmits

the energy as visible orange light). A comb was inserted into the slots of the casting tray and the molten agarose was poured into the tray. The gel was allowed to solidify for 20 min to form the wells. The 1XTAE buffer was poured into the gel tank to barely submerge the gel. Two microliter (2 l) of 10X blue gel loading dye (which gives colour and density to the samples to make it easy to load into the wells and monitor the progress of the gel) was added to 4µL of each PCR product and loaded into the wells after the 100 bp DNA ladder was loaded into well 1. The gel was electrophoresed at 120V for 45 min visualized by ultraviolet trans-illumination and photographed. The sizes of the PCR products were estimated by comparison with the mobility of a 100bp molecular weight ladder that was ran alongside experimental samples in the gel.

3.14 Purification of Amplified Product

After gel integrity, the amplified fragments were ethanol purified in order to remove the PCR reagents. Briefly, 7.6 µL of Na acetate 3M and 240 µL of 95% ethanol were added to each about 40µL PCR amplified product in a new sterile 1.5 µL tube eppendorf, mix thoroughly by vortexing and keep at -20 °C for at least 30 min. Centrifugation for 10 min at 13000 g and 4 °C followed by removal of supernatant (invert tube on trash once) after which the pellet were washed by adding 150 µL of 70% ethanol and mix then centrifuge for 15 min at 7500 g and 4 °C. Again remove all supernatant (invert tube on trash) and invert tube on paper tissue and let it dry in the fume hood at room temperature for 10-15 min. then resuspend with 20 µL of sterile distilled water and kept in -20oC prior to sequencing. The purified fragment was checked on a 1.5% Agarose gel ran on a voltage of

110V for about 1 h as previous, to confirm the presence of the purified product and quantified using a nano-drop of model 2000 from thermo scientific.

3.15 Sequencing

The amplified fragments were sequenced using a Genetic Analyzer 3130xl sequencer from Applied Biosystems using manufacturers' manual while the sequencing kit used was that of BigDye terminator.

3.16 Blasting of Amplified PCR Sequences and Deposition of Sequences in Gene bank

Following sequencing of the amplified bacteria DNA, blasting was carried out on the sequences using the national centre for biotechnology information (NCBI) blast website (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.17 Statistical Analysis

The data obtained from the microbial count of the scent leave vegetables were converted to $\log_{10}$ cfu/g and subjected to statistical analysis of variance (ANOVA) using SPSS (16.0) at 5% probability level while means were separated using Duncan Multiple Range Test (DMRT) of the same statistical software to evaluate if there was any significant difference between the bacteria and fungi load of the washed and unwashed samples.

CHAPTER FOUR

4.0 RESULTS

4.1 Microbial Population

This study shows variations in bacteria and fungi presence and their levels in scent leaves samples collected from three markets in Benin city, Nigeria. This may be attributed to the different hygienic practices by food vendors as well as the typess of cultivation from which these vegetables were obtained. Bacteria dominant in all analysed samples was *Enterobacter spp*, which was detected in 50% of them. 16.7% samples also tested positive for *Escherichia coli*, 8.33 % for *Bacillus cereus*, 16.7% for *Proteus mirabilis and* 8.33% for *Pseudomonas aeruginosa*. The fungi detected in the samples were identified morphologically and culturally as *Trichoderma spp, Penicillium spp and Curvularia spp* (Table 11).

4.2 Mean Bacterial Count of the Unwashed Scent Leaves

The mean bacterial count ($\log_{10}$ cfu/g) of the unwashed scent leaves from the three markets evaluated (New Benin market, Oba market, and Uselu market) are presented in Table 2 below. In the fresh scent leaves, New Benin, Oba and Uselu markets recorded mean values of 2.27, 2.28 and 2.34 respectively for the unwashed. The results from the statistical analysis indicated that there was no significant difference ($p > 0.05$) in the means of the unwashed samples from the three markets (Table 3).

Table 2: Mean Bacterial Counts (log_{10} cfu/g) of Unwashed Scent Leaves across the three markets

Market	Total Heterotrophic Bacterial Count	Standard deviation (+/-)
New Benin	2.27 [a]	0.09
Oba Market	2.28 [a]	0.12
Uselu	2.34 [a]	0.06

4.3 Effect of Washing on the Mean Bacterial Counts of Scent Leaves

Results demonstrating the effect of washing on fresh scent leaves are as shown in Table 3 below. The Total Heterotrophic Bacteria Count (log_{10} cfu/g) recorded in washed samples were 1.96, 1.86 and 1.96 for New Benin, Oba market and Uselu markets respectively. This implies that there was a significant difference ($p< 0.05$) between the unwashed and washed scent leaves. There was however no significant difference ($p> 0.05$) in the means of the washed samples from the three markets.

Table 3: Effect of Washing with Clean Water on the Mean Bacterial Counts of the Fresh Scent Leaves in Selected Benin Markets.

TREATMENT	NEWBENIN MARKET	OBA MARKET	USELU MARKET
Unwashed Scent leaves	2.27 [b]	2.28 [b]	2.34 [b]
Washed Scent Leaves	1.96 [a]	1.86 [a]	1.96 [a]
SEM	0.08	0.11	0.09

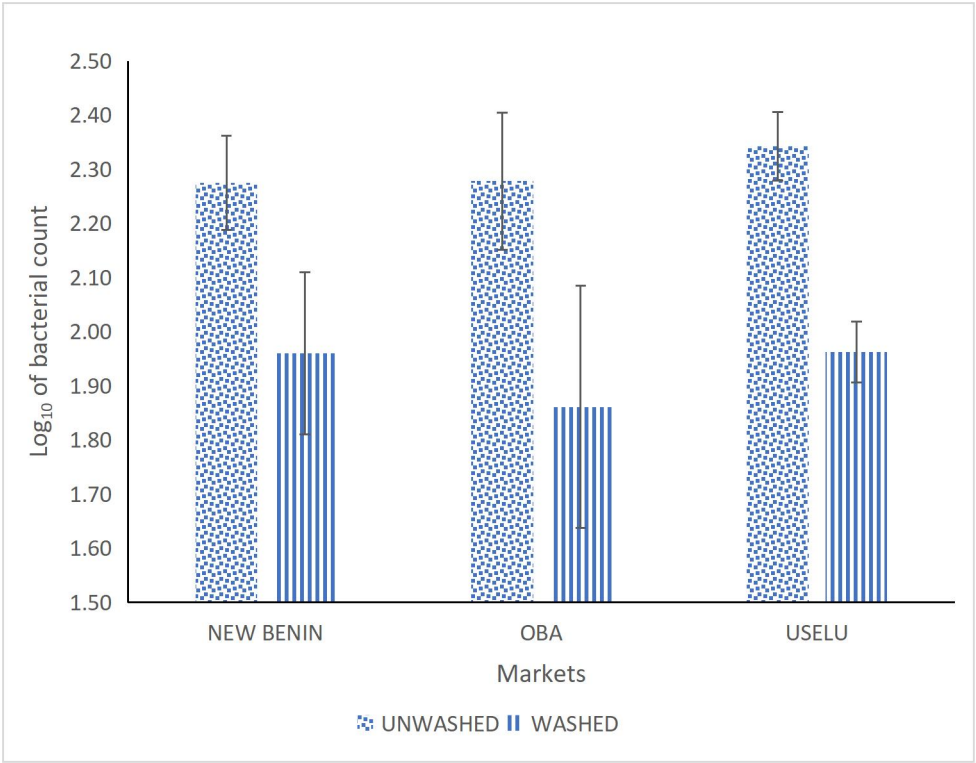

Fig 4: Bacterial count of the washed and unwashed samples (log10 Cfu/g)

4.4 Mean Fungi Count of Unwashed Scent Leaves

The mean fungi count (log10 Cfu/g) of the scent leaves from the three markets (New Benin market, Oba market, and Uselu market) are presented in Table 4 below. From the results, New Benin market recorded a mean value of 1.71, while 1.66 and 1.63 were recorded for Oba and Uselu markets respectively in the unwashed samples. The results from the statistical analysis indicated that there was no significant difference ($p > 0.05$) in the means of the unwashed samples from the three markets as shown in Table 4.

Table 4: Mean fungi count (log10Cfu/g) of Scent Leaves across the three markets

MARKET	Total Heterotrophic Fungi Count	STANDARDDEVIATION (+/-)
New Benin	1.71^{b}	0.03
Oba	1.66^{a}	0.01
Uselu	1.63^{a}	0.05

4.5 Effect of Washing on the Mean Fungi Count of Scent Leaves

Results demonstrating the effect of washing on the fungi count of fresh scent leaves are as shown in Table 5 below. The Total Heterotrophic Fungi Count (log_{10} cfu/g) recorded in washed samples were 1.25, 1.18 and 1.16 for New Benin, Oba market and Uselu markets respectively. This implies that there was a significant difference ($p < 0.05$) between the unwashed and washed scent leaves indicating that washing with clean water significantly reduces the fungi load of scent leaves. There was however no significant difference ($p > 0.05$) in the means of the washed samples from the three markets.

Table 5: Mean fungi counts of washed and unwashed samples across the markets

TREATMENT	NEWBENIN MARKET	OBA MARKET	USELU MARKET
Unwashed	1.71 [b]	1.66 [b]	1.63 [b]
Washed	1.25 [a]	1.18 [a]	1.16 [a]
SEM	0.10	0.11	0.11

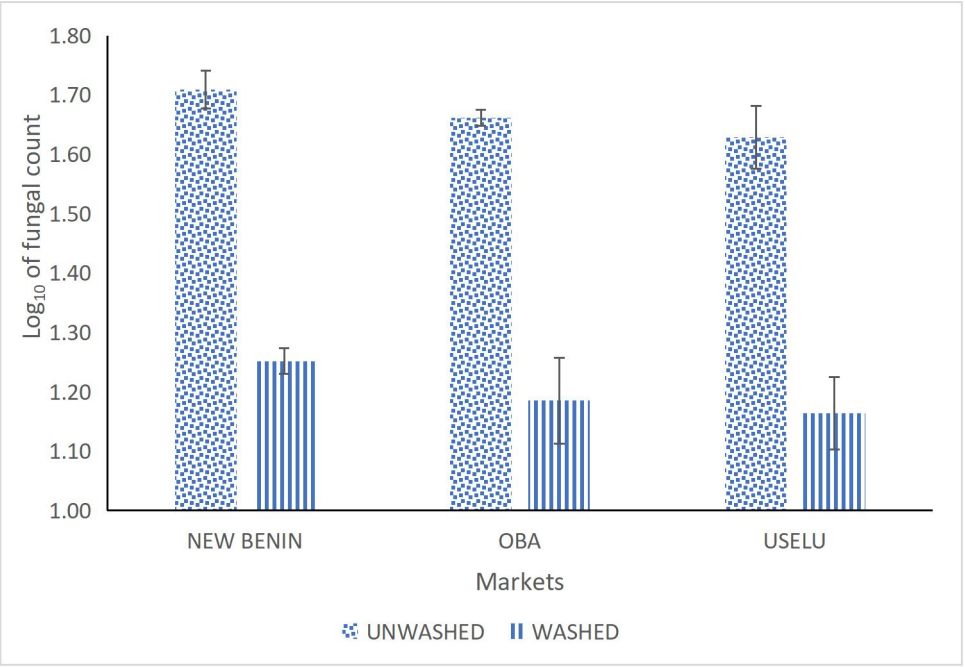

Fig 5: Fungi count of the washed and unwashed samples (log10 Cfu/g)

4.6 Comparison of the Bacteria and Fungi Count in Scent Leaves

The mean bacteria and fungi count from the three markets were compared as shown in Table 6,7 and 8 below. The bacteria and fungi count (log10 Cfu/g) of the unwashed samples from all three markets were all found to be significantly different and lower than the bacteria count as presented in Table 6 below.

Table 6: Mean bacteria and fungi counts of the unwashed samples across the three markets

TREATMENT	NEWBENIN MARKET	OBA MARKET	USELU MARKET
Bacteria	2.27 [b]	2.28 [b]	2.34 [b]
Fungi	1.71 [a]	1.66 [a]	1.63 [a]
SEM	0.13	0.14	0.16

From the results in Table 6, Uselu market recorded the highest bacteria count (log10 Cfu/g) of 2.34 while New Benin and Oba markets had 2.27 and 2.28 respectively whereas New Benin had the highest fungi count (1.71) while Uselu market had the lowest count (1.63).

A different trend was recorded in the washed samples, New Benin and Uselu market had a bacteria count of 1.96 while Oba market had a count of 1.86.The fungi count of the washed samples from all three markets were all found to be significantly different ($p > 0.05$) and lower than the bacteria count as presented in Table 7 below in log10 Cfu/g. with

New Benin Market having a mean Fungi count of 1.25 and bacteria count of 1.96, Oba market with fungi count of 1.18 and bacteria count of 1.86 while Uselu market had a fungi count of 1.16 and bacteria count of 1.96.

Table 7: Mean Bacteria and Fungi Counts of the Washed Samples across the three Markets

TREATMENT	NEWBENIN MARKET	OBA MARKET	USELU MARKET
Bacteria	**1.96 [b]**	**1.86 [b]**	**1.96 [b]**
Fungi	**1.25 [a]**	**1.18 [a]**	**1.16 [a]**
SEM	**0.16**	**0.16**	**0.18**

The mean of the unwashed and washed samples for the bacteria and fungi count was found to be significantly different ($p< 0.05$) across the three markets as presented in Table 8. The bacteria and fungi count for the unwashed are 2.30 and 1.67 respectively while that of the washed samples was 1.92 and 1.20 respectively. This implies more bacteria contamination in the leaves than fungi contamination.

Table 8: Mean of Bacteria and Fungi count of the samples

MARKET	Total Heterotrophic Bacteria Count	Total Heterotrophic Fungi Count
Unwashed	2.30[b]	1.67[b]
Washed	1.92[a]	1.20 [a]

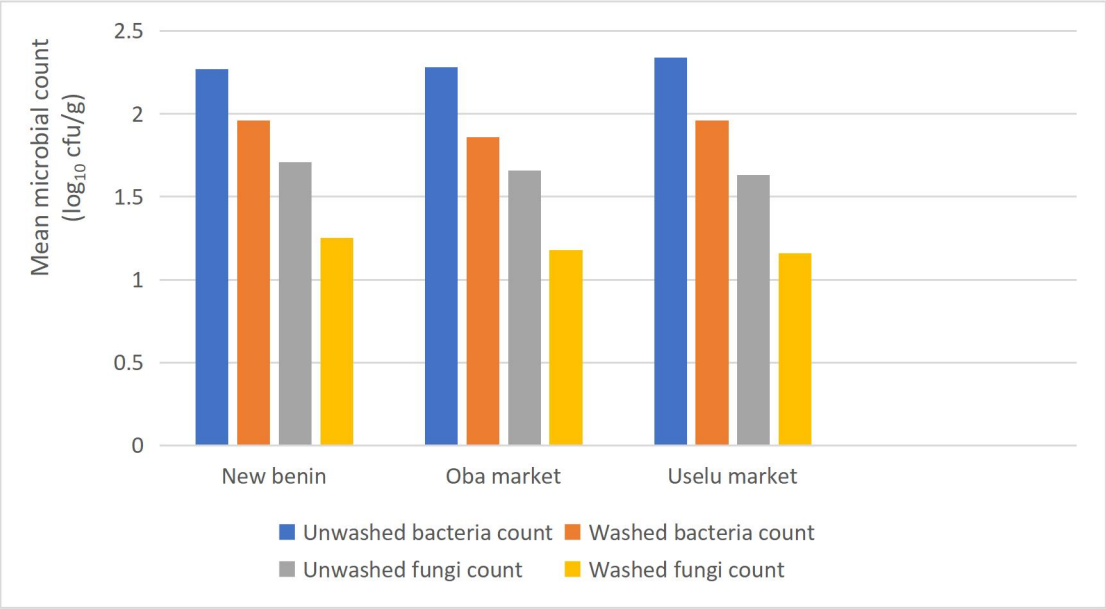

Fig 6: Microbial count of the washed and unwashed samples (log10 Cfu/g)

4.7 Characterization of the Bacteria Isolates

The cultural and morphological as well as the biochemical analysis of the bacteria isolates from Scent leaves samples as tested are presented in table 9 below.

Table 9: Characteristics of the bacterial isolates

Appearance	2	4	2	1	2	1
Colony						
Elevation	Flat	Flat	Flat	Flat	Flat	Raised
Margin	Undulate	Undulate	Undulate	Undulate	Entire	Entire
Color	Cream	Cream	Cream	Cream	Cream	Lemon
Shape	Irregular	Irregular	Irregular	Irregular	Circular	Circular
Size	Large	Large	Large	Large	Medium	Medium
Gr. diff. agar	EMB	EMB	EMB	BCA	SSA	PCA
Colour	Pink	Purple	Green	Straw	Black	Green
Morphological						
Gram stain	-	-	-	+	-	-
cell type	Rod	Rod	Rod	Rod	Rod	Rod
Arrangement	Disperse	Disperse	Disperse	Disperse	Disperse	Disperse
Color	Pink	Pink	Pink	purple	Pink	Pink
Biochemical						
KOH test	+	+	+	-	+	+
Indole	-	-	+	-	-	-
Citrate	+	+	-	+	+	-
Oxidase	-	-	-	-	-	+
Urease	-	-	-	-	+	+
Glucose	+	+	+	+	+	-
Sucrose	+	-	+	+	-	-
Lactose	+	-	+	+	-	-
Mannitol	-	-	-	-	-	-
Gas formation	-	+	+	-	+	-
H_2S formation	-	-	-	-	+	-
Identity	*Enterobacter aerogenes*	*Enterobacter cloacae*	*Escherichia coli*	*Bacillus cereus*	*Proteus mirabilis*	*Pseudomonas aeruginosa*

Key:

+…….. Positive to test

-…… Negative to test

Gr. Diff. Agar = Growth on differential agar.

Findings from the experiment showed that bacteria contaminants in Fresh scent leaves sold in Benin City were *Enterobacter aerogenes, Enterobacter cloacae, Escherichia coli, Bacillus cereus, Proteus mirabilis and Pseudomonas aeruginosa*.

The PCR (molecular) tests in Table 4.4 confirms the identity of the prominent most bacteria in the sample cultures as *Escherichia coli, Pseudomonas aeruginosa, Proteus mirabilis.*

Table 10: Molecular identity (PCR) of bacterial isolates from the unwashed scent leaf samples

Source	Closest Similarity in Gene Bank	Homology (%)	Genebank Accession number
New Benin	*Escherichia coli*	100.00	MK371829.1
Oba	*Pseudomonas aeruginosa*	100.00	MN326502.1
Uselu	*Proteus mirabilis*	100.00	CP053718.1

4.8 Characterization of Fungi isolates

The cultural and microscopic characteristics of the fungi isolates are presented in Table 11. Three different colonies of fungi from the cultures were subcultured. Observation of the cultural characteristics and microscopic examination revealed their identity as *Trichoderma spp, Pennicillium spp and Curvularia spp.*

Table 11: Characterization of the Fungal isolates

Parameter			
Cultural Morphology	A	B	C
Colour of mycelium on agar plate	Green mycelium	Green mycelium	Dark brown to black mycelium
Plate culture reverse	Pale yellow	Pale White	Brown to black
Microscopic characteristics			
Nature of hyphae		Septate	Septate brown hyphae
Type of Spore	Loosely branched conidiophore	Conidiospore	Conidiophores produce conidia
Appearance of special structure	Green conidia	Phialids	None
Class of fungi	Ascomycetes	Ascomycetes	Ascomycetes
Possible Isolates	*Trichoderma* sp.	*Penicillium* sp,	*Curvularia lunata*

CHAPTER FIVE

5.0 DISCUSSION

5.1 Bacteria Load of Scent Leaf

The total bacterial count for the scent leave samples tested ranged between 1.86 to 2.34 $\log_{10}$ cfu/g as shown in Table 3. These values were lower than that reported by Brackett and Splittstoesser (1992) found that bacteria counts in vegetables can be as high as 7 $\log_{10}$ cfu/g but is in agreement with Allydice-Francis and Brown (2012) who found bacteria contamination levels to be up to 10^2 though it may also be as high as $10^{6.}$ It is important to note however that bacteria on the produce may multiply over time depending on the storage conditions especially those that are psychrotrophic (Montville and Matthews, 2008; Abadias *et al*., 2008). Kayomo and Mayo (2018) found contamination levels in vegetables to be between 10^4 to 10^8 in local markets. It was also stated that contamination in markets were higher than that gotten directly from farms. Contamination levels of vegetables from the markets may be due to various factors, including the dirty surrounding environment with various small pools filled with dirty water and trickling of dirty water on vegetables to keep vegetables fresh. It was observed that the vegetables displayed for sale are exposed and often touched by unwashed hands of customers and vendors. It was also suggested by Chaturvedi *et al*. (2013) that frequent handling by unhygienic hands is a factor contributing to the high microbial loads of vegetables gotten from the market.

5.2 Bacteria Contaminants of Scent Leaf

Different bacteria species were isolated from the scent leaves samples. They include *Enterobacter cloacae, Enterobacter aerogenes, Escherichia coli, Bacillus cereus, Proteus mirabilis and Pseudomonas spp*. Abakari *et al*. isolated *Escherichia coli, Bacillus cereus, Shigella spp. and Salmonella spp* from ready-to-eat salad vegetables. (Abakari *et al.,* 2012). All the bacteria isolated from the scent leaves were Gram-negative except *Bacillus cereus* which was Gram-positive. Samapundo *et al.*, stated that *Bacillus cereus* was recorded in almost every friendly environment (Samapundo *et al.*, 2011). Akusu *et al*., stated that Salad vegetables contamination with *Bacillus cereus* might have resulted also from the source of production of the vegetables based on the nature of water used in irrigating them, contamination of salads vegetables from coming into contact with soils and also using contaminated utensils during the preparation of salads (Akusu *et al*., 2016).

Enterobacter cloacae had the highest occurrence and occurred in about 33% of the scent leaves. This is in agreement with the findings of Ma *et al*. (2018) in which *Enterobacter cloacae* also had the highest occurrence of 36% in fresh lettuce samples. Enterobacter cloacae which is usually considered part of the normal flora, is an opportunistic pathogen causing infection in immunocompromised patients if suitable opportunity arises. These bacteria spp are harmful to human health and may lead to cases of food poisoning. *Enterobacter spp* and *E. coli* are examples of coliform bacteria. High coliform count on vegetables from super market might be due to poor handling from harvesting, packing and transportation. Bakobie *et al*., 2017 stated that the presence of *E. coli* in food samples

was an indication of faecal contamination and improper hygienic practices by food vendors (Bakobie *et al*., 2017). Some strains of *E. coli* when in food could cause gastroenteritis and diarrhea in humans when ingested (Akter, 2016). Adams and Moss, (2008) established that *E. coli* do not usually lead to foodborne illness in humans but can however cause diarrhea in children in less developed countries. *E. coli* O157:H7 produces a powerful toxin that damages the lining of the small intestine, which can cause bloody diarrhea. One can develop an Escherichia coli infection when this strain of bacteria is ingested (Lynch *et al*., 2009). It is known to be very resistant unless is destroyed by thorough cooking or pasteurization. It can survive for extended periods of time in water and soil, under frozen and refrigerated temperatures, and in dry conditions as well. It also can adapt to acidic conditions (Chang and Fang, 2007) and they are widely distributed in air, dusts and soils (Oladele and Olakunle, 2011).

Pseudomonas aeruginosa was found in about 8.33% of the scent leaves. *Pseudomonas aeruginosa* is an oxidase-positive, non-fermentative, motile, and gram-negative bacterium that is ubiquitous and very versatile. While *P. aeruginosa* is considered an opportunistic pathogen, several reports indicate that the organism can also cause infections in healthy hosts. *P. aeruginosa* contamination was more likely from non-manure sources, particularly soil, flies, cockroaches, or rodents. In other words, the organism's presence in the environment and increased temperature and humidity, such as those normally experienced in tropical countries, may be predisposing factors for growth or colonization of vegetables (Allydice-Francis and Brown, 2012).

Proteus mirabilis was detected in about 16.7% of the scent leave samples. *Proteus mirabilis* is the dominant *Proteus* spp. causing human infections and has been suggested as a possible causative agent of outbreaks of gastroenteritis, resulting from the consumption of contaminated food. Its role as a causative agent of acute gastroenteritis is very difficult to assess because of the high carrier rate in healthy individuals. *Proteus spp.* also causes food spoilage and decomposition of organic matter especially of vegetables. *Proteus* is found in the human and animal gastrointestinal tract, skin, and oral mucosa, as well as in feces, soil, water, and plant The detection of *Proteus mirabilis* in the scent leaves indicates that it was not prepared in hygienic surroundings. (Yong and Xiaoling, 2014).

5.3 Fungi Load of Fresh Scent Leaves

In the current research, the mean counts for the fungi in both the unwashed and washed samples were lower than the bacteria counts as shown in Table 5. These findings were in tandem with the data obtained from previous studies by Oliveira *et al*., 2010 and Maffei *et al*., 2013 in which fungi counts were lower than the bacteria counts. The mean of the fungi counts range from 1.16 to 1.71 as presented in Table 6 and 7. This is lower than the findings of Tsado *et al*., 2013 who reported a fungal of 5.0 x104 cfu/g in fluted pumpkin and 2.8x104 cfu/g in Amaranth. In contrast to the present study, Badosa *et al*. (2008) reported fungi counts in most of the vegetable samples, ranging from 4 to 7 log CFU/g. Acevedo *et al*. (2001) also detected molds in the levels of 4.5×10^4 CFU/g in salad samples. Yeasts and molds, depending on genus and species, are the main culprit of most fresh

produce spoilage and can also be pathogenic. According to some authors, the high density of mycotoxin-producing molds generally correspond to poor cleaning practices and/or use of unhygienic techniques and contaminated equipment (Lynch *et al*., 2009).

5.4 Fungi Contaminants of Scent Leaf

The current work showed that scent leaves were contaminated with fungal species of *Epidermophyton floccosum, Aspergillus fumigatus and Penicillium spp*. (Table 11). This agrees with Yaffeto *et al*. (2019) who also isolated *Aspergillus* and *Penicillium* spp from cabbage and lettuce. Acevedo *et al*. (2001) also detected *Penicillium*, *Aspergillus,* and *Fusarium* spp in salad samples. These microbial groups can invade fresh produce in the field prior to harvest and during storage. All *Aspergillus* and *Penicillium* species either are commensals, growing in crops without obvious signs of pathogenicity, or invade crops after harvest and produce toxins during drying and storage. The presence of yeasts and molds not only link to food spoilage problems in vegetable, they can also pose health risks due to mycotoxins production (Tournas, 2005; Tournas and Katsoudas, 2005) such as aflatoxins, ochratoxins, and fumonisins The ingestion of these mycotoxins causes several health issues leading in the worst case to cancer in humans and animals. However, Trichoderma species are effective biofungicides, enzymatically degrading other fungi, producing anti-microbial compounds that kill pathogenic fungi, and outcompeting pathogenic fungi for space and nutrients (Siddiquee, 2014). This could be responsible for the low fungi count in the vegetables.

Although these organisms were found at low levels in the scent leaves samples, these can grow fast and cause spoilage in short time. In addition to the differences in local sanitary conditions from farm to the market, various methodologies used in these studies could yield different results.

5.5 Effect of Washing on the Microbial Load of Vegetables

Regardless of the cultivation methods, fresh produce can be contaminated starting from the pre-harvest stage, for example, through the use of fresh or non-composted animal manure, irrigation water, wild animals, pests, and insects (Beuchat and Ryu, 1997; Mandrell, 2009; Talley *et al*., 2009; Mishra *et al*., 2017). Post-harvest handling activities, such as selection, trimming, precooling, washing, grading, packaging, storage, and transportation can exacerbate the situation (Mandrell, 2009; Buchholz *et al*., 2012; Maffei *et al*., 2013). Hence it is important that fresh vegetables be properly decontaminated before consumption. In this study, the comparison of microbiological quality of washed and unwashed vegetables showed significant difference. Washing with sterile distilled water significantly lowered the bacteria and fungi count of the samples. Beuchat, 1998 stated that proper washing of fruits and vegetables is essential for decontamination. Water supplemented with varying concentrations of organic acids, such as acetic, citric and sorbic acids, has been shown to reduce microbial populations on fruits and vegetables (Beuchat, 1998). Previous studies revealed that a vinegar dip resulted in a 3 to 6 log10 decrease in the number of aerobic bacteria on parsley leaves, depending on vinegar concentration used and incubation time (Beuchat, 1998). In this study, washing with

sterile distilled water only reduced the bacterial load by 0.38 – 0.48 log10 cfu/g from the highest mean of unwashed samples of 2.34 and lowest of 2.27 to 1.96 and 1.86 in washed samples as shown in Table 3.

The fungi load was also reduced by 0.46-0.47 log10 cfu/g from the highest mean of 1.71 in New Benin market and lowest mean of 1.63 in Uselu markets in the unwashed samples to 1.18 and 1.16 respectively for the washed samples (Table 5).

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATION

6.1 Conclusion

This study shows that both bacteria and fungi are present in fresh Scent Leaves and can act as a potential vehicle for transmission of these organisms both pathogenic and non-pathogenic, and thus, constitutes a likely health risk to consumers in terms of microbial quality. The contamination could be attributed to poor post-harvest handling technique by farmers and vendors, improper food handling, source contamination of the produce from production sites and generally along the value chain and environmental conditions. Despite the microbial counts obtained for some of the samples in this study, it is important to note that these samples did not show any visible signs of spoilage. Thus outward appearance may not be a good criterion for judging the microbial quality of fresh produce and so fresh vegetables, spices and herbs may potentially cause illnesses since they were all found contaminated with disease causing organisms such as *Escherichia coli, Proteus ssp., Pseudomonas spp, Enterobacter spp, Bacillus spp, Trichoderma sp., Penicillium sp., and Curvularia lunata*. Sterile distilled water significantly reduced the microbial load of the scent leaf vegetables thus, all vegetables should therefore be adequately washed before consumption either by the consumer or the processor and consumers where possible. The current research has shown that washing can effectively reduce the microbial load of

vegetables which is necessary as most leaves and vegetables are consumed raw or just partially cooked.

6.2 Recommendation

From the findings of this research, the following recommendations have been proposed:

i. Proper washing and decontamination of scent leaves in order to reduce the microbial load of the vegetables to prevent or reduce the incidence of food-borne diseases.

ii. Adequate sterilization of organic manure, fertilizer or compost prior to use for cultivation of vegetables to reduce contamination, improvement of the sanitary condition of vegetables facilities.

iii. Public awareness programmes on the handling of vegetables and health risk associated with the consumption of improperly vegetables and the need for proper decontamination of fruits and vegetables prior to consumption should be encouraged.

iv. Microbiological quality of fresh fruits and vegetables can also be affected by the postharvest handling e.g. packaging and storage condition. It is therefore, necessary to ensure good quality of water especially in areas with inadequate or no cold-chain for proper washing of vegetables and ensure that the vegetable vendors get their water from appropriate source as vegetables are mostly consumed raw or just partially cooked.

v. The government should establish safety control measure as well as a hazard analysis and critical control point control principles. Vegetable processors should be educated on the adverse effect of using untreated or polluted water for processing as these could serve as sources of contamination. Vendors and marketers in Nigeria should be compelled to sell, store and transport sliced ready to eat fruits and vegetables in cool temperature controlled environments to prevent microbial proliferation in the vegetables and also to also to observe strict hygienic measures to ensure that they do not serve as source of chance inoculation of microorganisms and contamination.

vi. Even though Sterile distilled water reduced the microbial load of the vegetables they were still contaminated with these microorganisms. Therefore, further studies should be done on other methods of reducing the microbial load of scent leaves and other vegetables without reducing their nutritional value.

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APPENDIX

Mean values of the bacteria counts of the samples (log10 Cfu/g)

MARKET	Treatment	Replications			Mean	S.D
New Benin	Unwashed	2.19	2.36	2.27	2.27	0.09
	Washed	2.11	1.95	1.81	1.96	0.15
Oba Market	Unwashed	2.16	2.26	2.41	2.28	0.13
	Washed	1.61	2.04	1.93	1.86	0.22
Uselu	Unwashed	2.28	2.37	2.38	2.34	0.06
	Washed	1.97	1.90	2.01	1.96	0.05

Total bacteria and fungi count of New Benin Market (cfu/g)

SAMPLE	Total bacteria count	Total fungi count
NSL1A	2.19	1.75
NSL2A	2.36	1.69
NSL3A	2.27	1.69
NSL1B	2.11	1.25
NSL2B	1.95	1.23
NSL3B	1.81	1.27

Key: NSL= New benin market Scent Leave; A=Unwashed, B= Washed

Total bacteria and fungi count of Oba Market (cfu/g)

SAMPLE	Total bacteria count	Total fungi count
OSL1A	2.16	1.66
OSL2A	2.26	1.68
OSL3A	2.41	1.65
OSL1B	1.61	1.23
OSL2B	2.04	1.10
OSL3B	1.93	1.22

Key: NSL= Oba market Scent Leave; A=Unwashed, B= Washed

Total bacteria and fungi count of Uselu Market (cfu/g)

SAMPLE	Total bacteria count	Total fungi count
USL1A	2.38	1.69
USL2A	2.27	1.61
USL3A	2.38	1.59
USL1B	1.97	1.10
USL2B	1.90	1.17

Key: NSL= New benin market Scent Leave; A=Unwashed, B= Washed

USL3B	2.01	1.22

Mean values of the fungi counts of the samples (log10 Cfu/g)

MARKET	Treatment	Replications			Mean	S.D
New Benin	Unwashed	1.75	1.69	1.69	1.71	0.03
	washed	1.25	1.23	1.27	1.25	0.02
Oba Market	Unwashed	1.66	1.68	1.65	1.66	0.01
	Washed	1.23	1.10	1.22	1.19	0.07

ANOVA(Fungi)

		Sum of Squares	Df	Mean Square	F	Sig.
NB	Between Groups	4.698	2	2.349	4.405E3	.000
	Within Groups	.003	6	.001		
	Total	4.701	8			
OB	Between Groups	4.397	2	2.199	1.207E3	.000
	Within Groups	.011	6	.002		
	Total	4.408	8			
US	Between Groups	4.228	2	2.114	985.808	.000
	Within Groups	.013	6	.002		
	Total	4.241	8			

Uselu	Unwashed	1.69	1.61	1.59	1.63	0.05
	Washed	1.10	1.17	1.22	1.16	0.06

Multiple Comparisons(Fungi)

Dependent Variable	(I) group	(J) group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
NB	Tukey HSD unwashed	Washed	.46000*	.01886	.000	.4021	.5179
		Mean	1.71000*	.01886	.000	1.6521	1.7679

		washed	unwashed	-.46000*	.01886	.000	-.5179	-.4021
			Mean	1.25000*	.01886	.000	1.1921	1.3079
		mean	unwashed	-1.71000*	.01886	.000	-1.7679	-1.6521
			Washed	-1.25000*	.01886	.000	-1.3079	-1.1921
	LSD	unwashed	Washed	.46000*	.01886	.000	.4139	.5061
			Mean	1.71000*	.01886	.000	1.6639	1.7561
		washed	unwashed	-.46000*	.01886	.000	-.5061	-.4139
			Mean	1.25000*	.01886	.000	1.2039	1.2961
		mean	unwashed	-1.71000*	.01886	.000	-1.7561	-1.6639
			Washed	-1.25000*	.01886	.000	-1.2961	-1.2039
OB	Tukey HSD	unwashed	Washed	.48000*	.03485	.000	.3731	.5869
			Mean	1.66333*	.03485	.000	1.5564	1.7703
		washed	unwashed	-.48000*	.03485	.000	-.5869	-.3731
			Mean	1.18333*	.03485	.000	1.0764	1.2903
		mean	unwashed	-1.66333*	.03485	.000	-1.7703	-1.5564
			Washed	-1.18333*	.03485	.000	-1.2903	-1.0764
	LSD	unwashed	Washed	.48000*	.03485	.000	.3947	.5653
			Mean	1.66333*	.03485	.000	1.5780	1.7486
		washed	unwashed	-.48000*	.03485	.000	-.5653	-.3947
			Mean	1.18333*	.03485	.000	1.0980	1.2686
		mean	unwashed	-1.66333*	.03485	.000	-1.7486	-1.5780
			Washed	-1.18333*	.03485	.000	-1.2686	-1.0980
US	Tukey HSD	unwashed	Washed	.46667*	.03781	.000	.3507	.5827
			Mean	1.63000*	.03781	.000	1.5140	1.7460
		washed	unwashed	-.46667*	.03781	.000	-.5827	-.3507
			Mean	1.16333*	.03781	.000	1.0473	1.2793

	mean	unwashed	-1.63000*	.03781	.000	-1.7460	-1.5140
		Washed	-1.16333*	.03781	.000	-1.2793	-1.0473
LSD	unwashed	Washed	.46667*	.03781	.000	.3741	.5592
		Mean	1.63000*	.03781	.000	1.5375	1.7225
	washed	unwashed	-.46667*	.03781	.000	-.5592	-.3741
		Mean	1.16333*	.03781	.000	1.0708	1.2559
	mean	unwashed	-1.63000*	.03781	.000	-1.7225	-1.5375
		Washed	-1.16333*	.03781	.000	-1.2559	-1.0708

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

NB

	Group	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a]	Mean	3	.0000		
	Washed	3		1.2500	
	unwashed	3			1.7100
	Sig.		1.000	1.000	1.000
Duncan[a]	Mean	3	.0000		
	Washed	3		1.2500	
	unwashed	3			1.7100
	Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

OB

	Group	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a]	Mean	3	.0000		
	Washed	3		1.1833	
	unwashed	3			1.6633
	Sig.		1.000	1.000	1.000
Duncan[a]	Mean	3	.0000		
	Washed	3		1.1833	
	unwashed	3			1.6633
	Sig.		1.000	1.000	1.000

US

	group	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a]	mean	3	.0000		
	washed	3		1.1633	
	unwashed	3			1.6300
	Sig.		1.000	1.000	1.000
Duncan[a]	mean	3	.0000		
	washed	3		1.1633	
	unwashed	3			1.6300
	Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

BACTERIA Report

Group		NB	OB	US
UNwashed	Mean	2.2733	2.2773	2.3433
	N	3	3	3
	Std. Deviation	.08505	.12491	.06351
	Std. Error of Mean	.04910	.07211	.03667
Washed	Mean	1.9567	1.8600	1.9600
	N	3	3	3
	Std. Deviation	.15011	.22338	.05568
	Std. Error of Mean	.08667	.12897	.03215
Total	Mean	2.1150	2.0687	2.1517
	N	6	6	6
	Std. Deviation	.20491	.28009	.21665
	Std. Error of Mean	.08366	.11435	.08845

Notes

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
NB	2.603	2	6	.154
OB	5.171	2	6	.049
US	5.316	2	6	.047

ANOVA

		Sum of Squares	Df	Mean Square	F	Sig.
NB	Between Groups	9.097	2	4.548	458.409	.000
	Within Groups	.060	6	.010		
	Total	9.156	8			

OB	Between Groups	8.820	2	4.410	201.981	.000
	Within Groups	.131	6	.022		
	Total	8.951	8			
US	Between Groups	9.480	2	4.740	1.993E3	.000
	Within Groups	.014	6	.002		
	Total	9.494	8			

Post Hoc Tests

Multiple Comparisons

Dependent Variable		(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
NB	Tukey HSD	BACTERIA	FUNGI	.56333*	.04329	.000	.4305	.6962
			MEAN	2.27333*	.04329	.000	2.1405	2.4062
		FUNGI	BACTERIA	-.56333*	.04329	.000	-.6962	-.4305
			MEAN	1.71000*	.04329	.000	1.5772	1.8428
		MEAN	BACTERIA	-2.27333*	.04329	.000	-2.4062	-2.1405
			FUNGI	-1.71000*	.04329	.000	-1.8428	-1.5772
	LSD	BACTERIA	FUNGI	.56333*	.04329	.000	.4574	.6693
			MEAN	2.27333*	.04329	.000	2.1674	2.3793
		FUNGI	BACTERIA	-.56333*	.04329	.000	-.6693	-.4574
			MEAN	1.71000*	.04329	.000	1.6041	1.8159
		MEAN	BACTERIA	-2.27333*	.04329	.000	-2.3793	-2.1674
			FUNGI	-1.71000*	.04329	.000	-1.8159	-1.6041
OB	Tukey HSD	BACTERIA	FUNGI	.61333*	.05975	.000	.4300	.7967
			MEAN	2.27667*	.05975	.000	2.0933	2.4600
		FUNGI	BACTERIA	-.61333*	.05975	.000	-.7967	-.4300

			MEAN	1.66333*	.05975	.000	1.4800	1.8467
		MEAN	BACTERIA	-2.27667*	.05975	.000	-2.4600	-2.0933
			FUNGI	-1.66333*	.05975	.000	-1.8467	-1.4800
	LSD	BACTERIA	FUNGI	.61333*	.05975	.000	.4671	.7595
			MEAN	2.27667*	.05975	.000	2.1305	2.4229
		FUNGI	BACTERIA	-.61333*	.05975	.000	-.7595	-.4671
			MEAN	1.66333*	.05975	.000	1.5171	1.8095
		MEAN	BACTERIA	-2.27667*	.05975	.000	-2.4229	-2.1305
			FUNGI	-1.66333*	.05975	.000	-1.8095	-1.5171
US	Tukey HSD	BACTERIA	FUNGI	.71333*	.03897	.000	.5938	.8329
			MEAN	2.34333*	.03897	.000	2.2238	2.4629
		FUNGI	BACTERIA	-.71333*	.03897	.000	-.8329	-.5938
			MEAN	1.63000*	.03897	.000	1.5104	1.7496
		MEAN	BACTERIA	-2.34333*	.03897	.000	-2.4629	-2.2238
			FUNGI	-1.63000*	.03897	.000	-1.7496	-1.5104
	LSD	BACTERIA	FUNGI	.71333*	.03897	.000	.6180	.8087
			MEAN	2.34333*	.03897	.000	2.2480	2.4387
		FUNGI	BACTERIA	-.71333*	.03897	.000	-.8087	-.6180
			MEAN	1.63000*	.03897	.000	1.5346	1.7254
		MEAN	BACTERIA	-2.34333*	.03897	.000	-2.4387	-2.2480
			FUNGI	-1.63000*	.03897	.000	-1.7254	-1.5346

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

NB

	GROUP	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a]	MEAN	3	.0000		
	FUNGI	3		1.7100	
	BACTERIA	3			2.2733
	Sig.		1.000	1.000	1.000
Duncan[a]	MEAN	3	.0000		
	FUNGI	3		1.7100	
	BACTERIA	3			2.2733
	Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Molecular identity of bacterial isolates from scent leaf

Sample code	Closest Similarity in Gene Bank	Homology (%)	Genebank Accession number
NSL1A	*Escherichia coli*	100.00	MK371829.1
OSL1A	*Pseudomonas aeruginosa*	100.00	MN326502.1
USL1A	*Proteus mirabilis*	100.00	CP053718.1

> Escherichia coli

CATTACCACCTACTTCTTTTGCAACCCACTCCCATGGTGTGACGGGCGGTGTGT
ACAAGGCCCGGGAACGTATTCACCGTGGCATTCTGATCCACGATTACTAGCGA
TTCCGACTTCATGGAGTCGAGTTGCAGACTCCAATCCGGACTACGACGCACTT
TATGAGGTCCGCTTGCTCTCGCGAGGTCGCTTCTCTTTGTATGCGCCATTGTAG
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CGAGCTGACGACAGCCATGCAGCACCTGTCTCACGGTTCCCGAAGGCACATTC
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CATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATT
TGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCGACTTAACGCGTTAGC
TCCGGAAGCCACGCCTCAAGGGCACAACCTCCAAGTCGACATCGTTTACGGCG
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TCAGTCTTCGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTAC
GCATTTCACCGCTACACCTGGAATTCTACCCCCCTCTACGAGACTCAAGCTTG
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CTACCTTTCCAGTAGTTATCCCCCTCCATCAGGCAGTTTCCCAGACATTACTCA
CCCGTCCGCCACTCGTCAGCGAAGCAGCAAGCTGCTTCCTGTTACCGTTCGA
>seq Pseudomonas

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GATGAGCCTAGGCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGA
CGATCCGTAACTGGTCTGAGAGGATGATCAGTCAACTGGAACTGAGACACGG
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GCTAGTCTAACCGCAAGGGGGACGGTTACCACGGAGTGATTCATGACTGGGG
TGAAGTCGTAACAAGA

>Proteus

ATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGTAACAGGAGA
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TCTACGGACCAAAGCAGGGGCTCTTCGGACCTTGCACTATCGGATGAACCCAT
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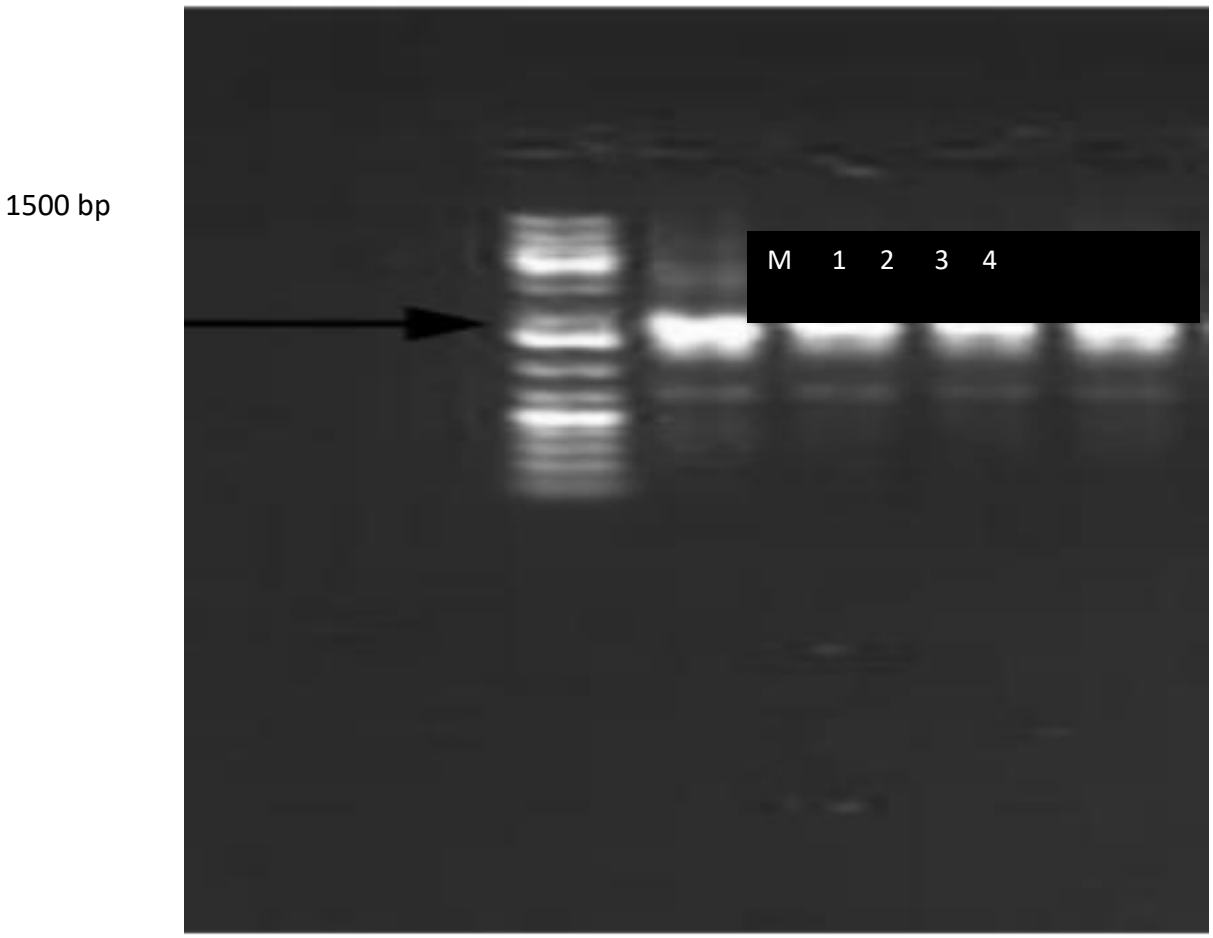

Plate 1. Gel pictures of the four bacterial isolates from crude oil, produced water and refinery wastewater.

Lane M - DNA ladder

Lane 1 sample bacterial DNA

Lane 2 sample bacterial DNA

Lane 3 Control bacterial DNA

Mean values of the bacteria counts of the samples (log10 Cfu/g)

MARKET	Treatment	Replications			Mean	S.D
New Benin	Unwashed	2.19	2.36	2.27	2.27	0.09
	Washed	2.11	1.95	1.81	1.96	0.15
Oba Market	Unwashed	2.16	2.26	2.41	2.28	0.13
	Washed	1.61	2.04	1.93	1.86	0.22
Uselu	Unwashed	2.28	2.37	2.38	2.34	0.06
	Washed	1.97	1.90	2.01	1.96	0.05

USELU MARKET, BENIN CITY, EDO STATE

NEW BENIN MARKET, BENIN CITY, EDO STATE

OBA MARKET, BENIN CITY, EDO STATE