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HEAVY METAL TOLERANT BACTERIA ISOLATED AND DETECTED FROM THE EFFLUENT OF HAZARIBAGH TANNERY INDUSTRY IN DHAKA CITY

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ABSTRACT

The tanning industry is held to be an activity with the high budding for environmental pollution all over the world. Many Bacterial strains isolated from natural resources have been found to possess unique properties which make them useful for environmental cleans ups. So it is very important to find out an alternative ecofriendly way for the treatment of contaminated effluent. The objective of this study was to isolate, explore and pick out natural occurring bacteria capable of reducing heavy metals from tannery effluent collected from the Hazaribagh tannery industry of Dhaka. The pH value of all the effluents samples were ranged from 7.12 to 7.91. Five bacterial strains were confirmed as *Bacillus bataviensis*, *Bacillus aryabhattai*, *Micrococcus antarcticus*, *Bacillus proteolyticus* and *Bacillus paranthracis* on the basis of their morphological, cultural, biochemical, and 16S rRNA gene sequencing. Among these five strains, *Bacillus bataviensis*D1 exhibited higher resistance to cadmium (Cd), chromium (Cr), copper (Cu), lead (Pb) and arsenic (As) up to the amount of 550 µg/mL, 500 µg/mL, 500 µg/mL, 1050 µg/mL and 1100µg/mL respectively. *Bacillus aryabhattai* D2 and *Micrococcus antarcticus* D3 showed similar result to chromium (Cr) and Lead (Pb), but *Bacillus proteolyticus* B1 showed higher resistance to nickel (Ni) that is up to 250 µg/mL. From these results, it can be suggested that the identified heavy metal-adapted bacteria could be useful for the biosorption of heavy metal contaminated effluent.

Keywords: Bacterial strains, Tannery effluent, 16S rRNA sequencing, Heavy metals, *Bacillus sp*, *Micrococcus sp*.

INTRODUCTION

In Bangladesh government has identified the leather industry as one of the promising sectors with considerable growth and investment potential which secured fifth position in getting export currency. There are many factors existing in Dhaka city such as unavailability of proper facilities to manage tannery wastes, having inadequate knowledge regarding green industrialization threaten the quality of environment. Most of the tannery industries are discharging their wastes in the rivers and also in the surrounding soils that results in water pollution. Our country belongs to more than 113 tanneries in with an annual processing capacity of 180 million square feet of hides and skins. Most of the tanneries do not have proper effluent plants and generate nearly 20000 m^3 tannery effluents and 232 tones solid waste per year (**Paul *et al.*, 2013)**. From old times, tanning activities were organized to meet the local demands of leather shoes, belts, bags, upholstery, gloves, drums and musical instruments. The increasing demand of leather and its product lead to the increasing number of commercial tanneries globally. Tannery effluent is highly polluted because it contains imbalance suspended solids particles, electrical conductivity (EC), nitrogen, sulfide, sulfate and copper (Cu), chromium (Cr), cadmium (Cd) and manganese (Mn), chemical oxygen demand (COD) and biological oxygen demand (BOD) (**Mondal *et al.*, 2005; Zahid *et al.*, 2006**). Although some heavy metals are essential for life's physiological processes, their lavish accumulation in living organisms is always inimical. Various microbial growth are greatly hampered resulting in the abnormality in biomass and biodiversity **(Ayangbenro and Babalola, 2017**; **Roane and Pepper, 2000**). Lower number of microbes reside in the habitats with higher level of metal contamination than the uncontaminated habitats (**Tscherko *et al.*, 2000**). Heavy metals which are commonly used for the production of color pigments of dyes are chromium (Cr), copper (Cu), cadmium (Cd) and lead (Pb) (**Halimoon and Yin, 2010**). The impact of Zinc, Cadmium, and Mercury on microbial metabolism is dependent on the growth form, while it is consortia from mining sites; the resistance thresholds are lower in pure culture than in consortium (**Sprocati *et al.*, 2006**). Chlorinated organic compounds and heavy metals are major contaminants found in the environment, are extremely lethal to microbes, plants animals and human(s), which can destroy the DNA structure by damaging cell membrane. This toxicity is created by the rearrangement of elemental metals from their native binding sites or ligand interactions (**Olaniran *et al.*, 2013**).
There are some microbiological parameters such as the weight, number, and activity of microorganisms can be a good indicator of soil contamination with heavy metals (**Brookes, 1995**). Heavy metals have unusual attributes that they do not decay with time; they can be crucial or useful to plants at a certain level but can be poisonous when transcending lower limit; they strongly interact with the soil matrix consequently; heavy input in soils being related to weathering of parent rocks and pedogenesis; and they frequently present as cations which metals in soils can become portable as a results of changing habitat conditions. This situation is regarded to as "Chemical timing bomb" (**Facchinelli *et al.*, 2001)**. Tannery wastewaters are generally featured by high organic loading, high salinity, and certain pollutants such as Chromium (**Tunay *et al.*, 1994; Song *et al.*, 2000**). In tanning industries, many chemicals (salt, soda ash, lime, ammonium sulfate, ammonium chloride, fat liquors, sodium bicarbonate, sodium carbonate, chrome sulfate, sodium sulfate, magnesium oxide, vegetables oils and dyes) are commonly used to transform hide and skin of animal into commercial product. Alongside these, chemicals such as mercuric chloride, zinc chloride, and formaldehyde are used as disinfectants; sodium chloride in curing and as bleaching powder and sodium fluoride to prevent putrefaction; sodium sulfate, ammonium chloride, borax and hydrochloric acid in deliming agent; lime in liming; sodium for decreasing and various acidic or basic dyes are also used to process leather as finished product **(Saritha and Chockalingam, 2018**). A rigorous and substantial ruin of the environment is another point of concern from such a focal industry to sustain a billion dollar business due to the lack of a better and efficient waste water treatment. An excess of such chemicals in the water and soils is detrimental to the health of the people guzzle into the area (**Sundar *et al.*, 2010**). Several microorganisms have developed the process of removing poisons from a substance and respiration mechanism using heavy metals and thus become resistant to it (**Ezaka and Anyanwu, 2011**). Because of its metal accumulation ability and its resistance properties, the isolation and detection of heavy metal tolerant bacteria are crucial for removing and detoxifying of toxic metals. Therefore, the study focuses on isolation, biochemical and molecular characterization of naturally residing tannery effluent's bacteria, their characterization of heavy metal tolerance, in order to use them for detoxification in an incorporated biosorption strategy.

MATERIALS AND METHODS

The study was conducted in Soil and Environment Laboratory, Biological Research Division, Bangladesh Council of Scientific and Industrial Research, Dhaka-1205, Bangladesh.

Sampling sites

Hazaribagh tannery located at Dhaka city was selected for the collection of Tannery effluent samples (Fig. 1). Pre-sterilized bottles, used to keep the

collected samples, were well sealed with plastic bags and transferred very quickly to the BCSIR laboratory in ice-box; collected samples were preserved at 4ºC before analysis and during experiments.

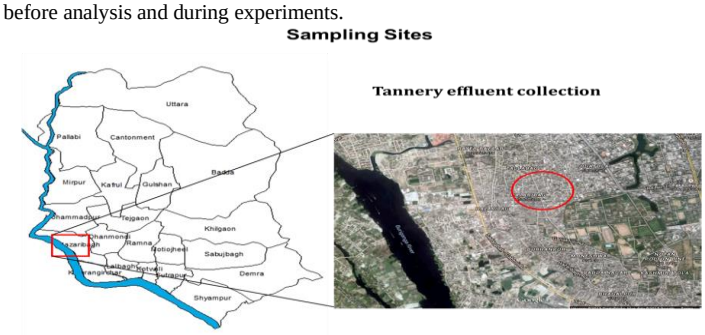

Figure 1 Image of the location of Hazaribagh Tannery industrial areas

Measurement of pH of the Samples

The pH of the effluent was measured immediately in the laboratory with pH meter (Sension Refillabe pH electrode, HACH) after transportation of the samples to the laboratory.

Isolation of Bacteria

Serial dilution plate techniques were carried out and subsequent serial dilutions were made up five times for the isolation of bacteria. Nutrient agar plates were seeded with each diluted samples in duplicate and incubated at 37ºC for at least 24h in an incubator (**Zahoor and Rahman, 2009; Sujatha *et al.*, 2012; Ezaka *et al.*, 2011**). A total of twenty-five isolates were isolated from colonies differing in morphological characteristics; among them five isolates were selected and used for further analysis. The purification of selected isolates was done by streak plate method through repeated plating (**Greenberg *et al.*, 1980**) and the identification of that isolates were performed based on gram staining methods, colony characteristics and by various biochemical tests as described by Bergey's (1974) Manual of Determinative Bacteriology.

Maintenance of Isolates

The fumigated isolates were then transplanted on nutrient agar slant. The slants were maintained as a stock culture in a refrigerator at 4ºC for further study. Periodical transfer of isolates on agar slants was done for maintaining the viability of the organisms after each week (**Cappuccino and Sherman, 2005**).

Physiological and Biochemical studies of Isolates

Selected bacterial strains were grown in nutrient broth culture medium containing 2.5% peptone, 1% yeast extract and 0.5% beef extract. Cultures (50 ml in 250 ml conical flasks) were inoculated with 5% (v/v) inoculums and incubated at 37ºC for 24h with uninterrupted vigorous orbital shaking at 150-180 rpm. After gram staining, we used a microscope to examine the color and shape of vegetative cells of selected strains. Whether the arrangement of cells exists in singles or in chains or clusters was carefully recorded. Isolates were physiologically and biochemically analyzed to identify unknown isolates for the activity of Oxidase, Catalase, Acid gas production from D-glucose, Hydrolysis of starch, Liquefaction of gelatin, Indole formation, MR test, VP test, Deamination of Phenylalanine, and Citrate utilization (Aneja, 2003). These tests were performed using Bergey's Manual of Determinative Bacteriology along with other manuals such as Manuals of Microbiological Methods (**SAB, 1957**), Microbiological Methods (**Collins and Lyne, 2004**).

Bacterial Genomic DNA isolation

Bacterial genomic DNA was primarily isolated as per the standard protocol (**Sohail, 1998**). It was then visualised on gel electrophoresis at 0.8% agarose gel with ethidium bromide (0.5 µg/mL) as per the standard protocols and compared with marker DNA to assess genomic DNA.

Automated sequencing and bioinformatics analysis

For molecular characterization, sequence analyses of 16S rRNA gene were performed by PCR, using the bacterial universal primers (Forward primer 5′-CCAGACTCCTACGGGAGGCAGC-3′ and reverse primer 5′-CTTGTGCGGGCCCCCGTCAATTC-3′) for the amplification of the 16S rRNA gene fragment. PCR amplified DNA of the 5 isolates was purified using PCR Purification Kit by silica- gel membrane absorption method and sent for automated sequencing (Applied Bio-system 3130) to the Centre for Advanced Research in Sciences at University of Dhaka. 16S rRNA gene sequences were conveyed into "Basic Local Alignment Search Tool (BLAST)" available from the website of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to distinguish matches with existing identified reference sequences. The result of BLAST searches was sorted based on highest matches with other genus or species names in Genbank records. The phylogenetic trees were constructed based on the 16S rRNA sequences of each bacterium with reference sequence in Genbank using CLC drug discovery workbench software version 1.0.2 to define the identities and conserved region (**CLC Inc A, Denmark**).

Screening of multiple heavy metal tolerant bacteria

For the selective screening of heavy metal tolerant bacteria, various concentrations (10 µg/mL -100 µg/mL) of each heavy metals i.e. Cadmium Nitrate (Cd(NO3)2), Chromium (III) Nitrate(Cr(NO3)3), Nickel Nitrate (Ni(NO3)2), Cupric Sulphate ($CuSO_4 \cdot 5H_2O$), Lead (II) Nitrate (Pb(NO3)2) and Sodium Meta Arsenate (NaAsO2) were incorporated on to LB (Luria Bertani) agar plates and screened by the standard spread plate method (**APHA, 1992**) observed at 37ºC/24h for any kind of development on the culture medium. After the primary screening of effluent samples containing heavy metal resistant isolates, a serial dilution was done as (**Greenberg *et al.*, 1980**) to isolate desired bacteria. Streak plate technique was followed during isolation. Controls plates also prepared with LB media without including any heavy metal to make a comparison. Colonies differing in morphological attribute were selected, picked, purified and then maintained on different plates for further studies.

Assessment procedures of minimum inhibitory concentration (MIC)

For the determination of MIC, selected heavy metal tolerant isolates were grown on heavy metal associated media against respective heavy metals (Cd, Cu, Cr, Ni and Pb) with varying concentrations ranged from 50 to 1200 µg/mL with gradual increase of the heavy metal concentration by 50 µg/mL each time on nutrient-agar plate. The starting concentration of heavy metals was 50 µg/mL and culture growing on last concentration was transferred to higher concentration each time while streaking on the agar plate. This method was based on the procedure described by Yamina et al (2012) with slight modification. The cultures were incubated for 24-72h and measured for optical density (OD) at 620nm in UV spectrophotometer. A culture, having an OD greater than 0.1 at 620nm, was considered resistant. MIC was determined according to standard protocol of European Food Safety Authority (**EFSA**), Parma, Italy, 2012 when the isolates failed to grow on petri dish.

RESULTS

pH measurement of effluent samples

Physical and chemical properties of effluents have partial effect on the resistance of the microorganisms (**Kermanshahi *et al.*, 2007**).

Table 1 pH value of the effluents samples

Sample No.	Location	pH
D1	Tannery Area, Hazaribagh	7.46
D2	Tannery Area, Hazaribagh	7.30
D3	Tannery Area, Hazaribagh	7.12
DY	Tannery Area, Hazaribagh	7.91
B1	Tannery Area, Hazaribagh	7.86

Since the resistance of the microorganisms would be affected by the variation of pH and the pH of the effluents samples were near about neutral, there would be

little effect on bacterial resistance. As given in table 1, the pH values of five respective samples varied from 7.12 to 7.91.

Isolation of heavy metal resistant bacteria

Being an industrial city, Dhaka is facing pollution problems. For example, Tanning and other industries discharge various types of heavy metals those are very toxic and accumulate in food chain, inhibits metabolic reactions causing the collapsion of marine biodiversity in nature. Prefatory screening of the collected samples for heavy metal tolerance ability showed that all samples were positively grown utilizing heavy metals (Cu^{2+}, Cd^{2+}, Ni^{2+}, Cr^{6+}, Pb^{2+}, and As^{2+}) in culture media. Preliminary screening of the characterization of bacteria were found belong to five bacterial species as *Bacillus bataviensis*, *Bacillus aryabhattai*, *Micrococcus antarcticus*, *Bacillus proteolyticus* and *Bacillus paranthracis* based on morphological characteristics (gram reaction, colony color, shape and arrangement of cells, motility) and biochemical characteristics (Oxidase test, Catalase Test, Oxygen Requirement, Gelatin Liquefaction, Starch Hydrolysis, Indole Formation VP Test, MR Test, Deamination of Phenylalanine, Utilization of Citrate, Acid Production from D-Glucose) which are mentioned in Table 2.

Table 2 Morphological and Biochemical Characteristic of bacterial isolates

Morphological Characteristics	**Bacterial isolates**				
	D1	**D2**	**D3**	**B1**	**DY**
Gram reaction	+	+	+	+	+
Colony color	Cream	Initially brown, later peach	Yellowish	Pink	Off White
Shape and arrangement of cells	Rod, rounded end, occur in chain	Rod shaped rounded	Spherical, occur in pairs and packets	Rod shaped	Rod, occur in chain
Motility	+	+	-	+	-
Biochemical test result					
Oxidase test	-	+	+	+	-
Catalase Test	+	+	+	+	-
Oxygen Requirement	Facultative anaerobes	Facultative anaerobes	Strictly aerobes	Facultative anaerobes	Facultative anaerobes
Gelatin Liquefaction	+	+	+	+	+
Starch Hydrolysis	-	+	+	+	+
Indole Formation	-	-	+	+	+
VP Test	-	+	+	-	+
MR Test	+	-	+	-	-
Deamination of Phenylalanine	-	-	-	+	-
Utilization of Citrate	-	-	+	+	+
Acid Production from D-Glucose	+	+	-	+	+
Name of the Isolates	*Bacillus bataviensis*	*Bacillus aryabhattai*	*Micrococcus antarcticus*	*Bacillus proteolyticus*	*Bacillus paranthracis*

Assessment of MIC against each heavy metal

The effluents sampled from tannery area had higher concentration of Cu^{2+}, Cd^{2+}, Ni^{2+}, Cr^{6+}, Pb^{2+}, and As^{2+}. It is mentioned that the isolates those showed high resistance to Cr, Cd, Cu, Pb and As were isolated from contaminated sites. The multi-metal resistance capacity approached two bacterial isolates *Bacillus bataviensis* and *Micrococcus antarcticus* are highly resistant to Cd, Cu, Pb, and As compared to *Bacillus paranthrac* which is reported in Table 3. Minimum inhibitory concentration (MIC) is the lowest concentration of the metal at which the isolate is completely suppressed (**Roane and Kellogg, 1993**). In this study, the metal resistant tests showed that some of the selected isolates had MIC above 600 µg/mL against cadmium (Cd); above 550 µg/mL against chromium (Cr); above 300 µg/mL against Nickel (Ni); above 450 µg/mL against copper (Cu); above 1000 µg/mL against lead (Pb), and above 1100 µg/mL against arsenic (As).

The MIC of identified strains for Cr^{6+} ranged from 400 to 600 µg/mL, for Cd^{2+} ranged from 300 to 650 µg/mL, for Ni^{2+} ranged from 250 to 350 µg/mL, for Cu^{2+} ranged from 400 to 500 µg/mL, for Pb^{2+} ranged from 600 to 1050 µg/mL, for As^{5+} ranged from 900 to 1150 µg/mL in nutrient broth**.**

In this study order of bacteria tolerance to Cadmium and Arsenic was found to be *Bacillus bataviensis*>*Micrococcus antarcticus*>*Bacillus proteolyticus*>*Bacillus aryabhattai*>*Bacillus paranthracis* and *Bacillus aryabatti*> *Bacillus bataviensis*> *Bacillusparanthracis*> *Bacillus proteolyticus*>*Micrococcus antarcticus.* Upon above experiment, the resistance level *Bacillus bataviensis*> *Bacillus proteolyticus*>*Bacillus aryabhattai*= *Bacillus paranthracis*> *Micrococcus antarcticus* showed for Pb and *Bacillus bataviensis*>*Bacillus aryabatti*= *Micrococcus antarcticus*> *Bacillus paranthracis*> *Bacillus proteolyticus* showed for Cr. Our study found multiple heavy metal tolerance capacity for *Micrococcus antarcticus* having MIC 950 µg/mL, 500 µg/mL, 400 µg/mL and 900 µg/mL against As, Cr, Cu and Pb respectively (Table 3). Among five isolates bacteria, *Bacillus aryabatti* and *Micrococcus antarcticus* were identified here as the lowest resistance to Nickel.

Table 3 Heavy metal resistance capacity and MIC of bacterial isolates

Resistance capacity	Strain No.				
	D1	**D2**	**D3**	**B1**	**DY**
Cd	++	+	++	+	+
Cr	++	++	++	+	+
Ni	+	+	+	+	+
Cu	++	+	+	++	+
Pb	+++	++	++	+++	++
As	+++	+++	++	+++	++
MIC (µg/mL**)**					
Cd	650	400	500	450	300
Cr	600	500	500	400	450
Ni	300	250	250	300	350
Cu	500	450	400	500	450
Pb	1050	950	900	1000	950
As	1100	1150	950	1000	900

+++ Indicates-High resistant, ++ indicates- Moderate and + indicates- Low

Analysis of 16S rRNA gene sequencing results

Comparative analysis of the sequences with already available database showed that the strains were closed to the members of genus *Bacillus* and *Micrococcus* (Fig. 2). To detect the possible similarity of organisms via the alignment of homologous sequences, NCBI BLAST (**http://blast.ncbi.nlm.nih.gov/**) program was subjected to analyze the sequence resulted from the automated sequencing PCR amplified DNA (**Marchler-bauer *et al*., 2002; Pruitt *et al*., 2005**)

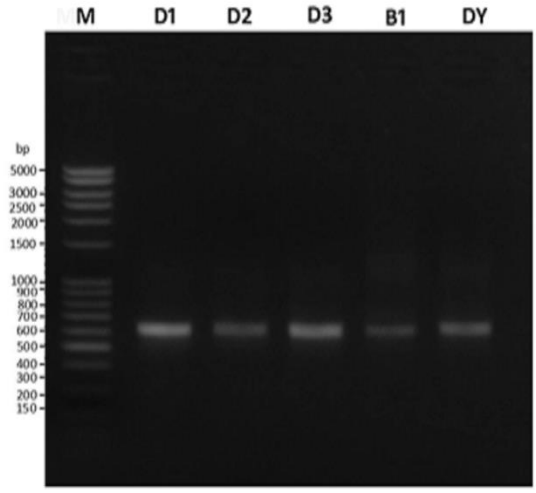

Figure 2 Gel image of 16S rDNA Amplicon, Line 1: 100 bp of DNA Marker [Lane 2, 3, 4, 5, 6: 16S rDNA Amplicon band of D1, D2, D3, B1, and DY isolates].

The highest sequence similarities of effluent bacteria are as follows: D1, *Bacillus bataviensis* strain NBRC102449 (Query coverage 94% and 99% similarity, accession number NR_114093.1); D2, *Bacillus aryabhattai* strain B8W22 (Query coverage 96% and 99% similarity, accession number NR_118442.1); D3, *Micrococcus antarcticus* strain T2 (Query coverage 93% and 99% similarity, accession number NR_025285.1); B1, *Bacillus proteolyticus* strain MCCC 1A00365 (Query coverage 96% and 99% similarity, accession number NR_157735.1); DY, *Bacillus paranthracis*strain MCCC 1A00395 (Query coverage 93% and 98% similarity, accession number NR_157728.1). All the closely related homologs of identified bacteria were used for the construction of the phylogenic dendrogram to know their evolutionary origin. The dendrogram showing the relation among *Bacillus bataviensis, Bacillus aryabhattai, Micrococcus antarcticus, Bacillus proteolyticus, Bacillus paranthracis,* and the close homolog's of them (fig. 3.(a-e))

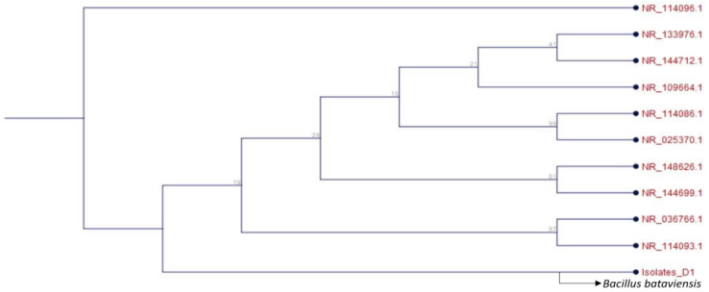

Figure 3 (a) Phylogenetic tree representing close homolog's of D1, *Bacillus bataviensis* strain NBRC 102449

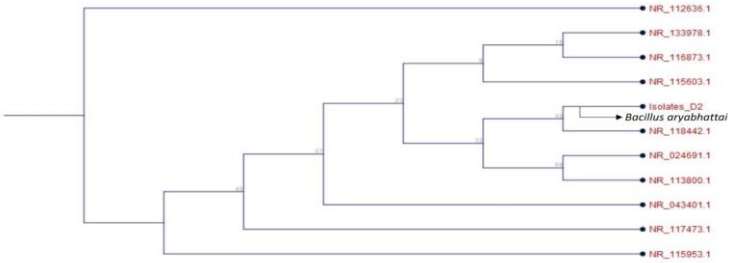

Figure 3 (b) Phylogenetic tree representing close homolog's of D2, *Bacillus aryabhattai strain B8W22*

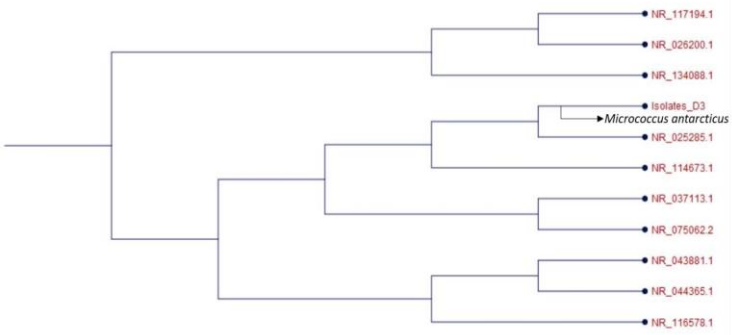

Figure 3 (c) Phylogenetic tree representing close homolog's of D3, *Micrococcus antarcticus* strain T2

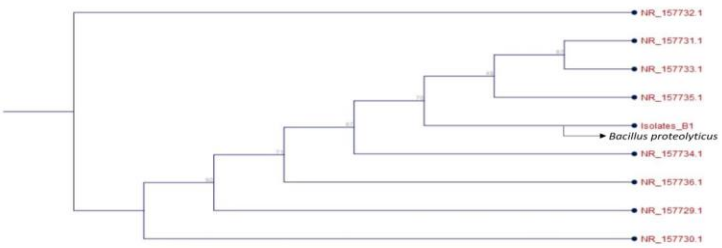

Figure 3 (d) Phylogenetic tree representing close homolog's of B1, *Bacillus proteolyticus* strain MCCC 1A00365

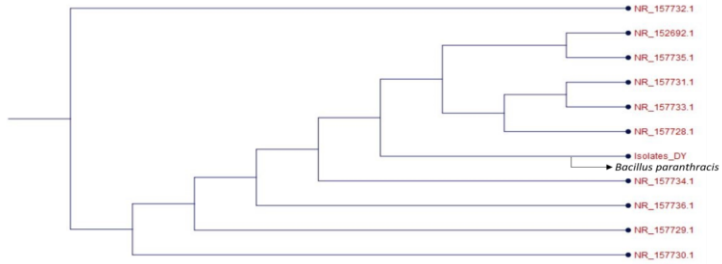

Figure 3 (e) Phylogenetic tree representing close homolog's of DY, *Bacillus paranthracis* strain MCCC 1A00395

DISCUSSION

In the present study, bacterial strains were isolated from the most common heavy metal contaminated sites of tannery areas in Hazaribagh, specially focused on the identification of Cupper (Cu), Cadmium (Cd), Chromium (Cr), Nickel (Ni), Arsenic (As) and Lead (Pb) tolerant bacteria in search of their metal resistance and minimum inhibitory concentration (MIC). pH value was observed in sample D1,D2, D3, B1, DY collected from tannery area, Hazaribagh and the pH value were found 7.46, 7.30, 7.12, 7.91, and 7.86 respectively. Therefore, high pH is one of the key characterization factors of the tannery waste (**Kumar and Mani, 2007**). The pH is one of the parameters of effluents by which the microbial activity is greatly affected. pH influences all the enzymatic reaction of microbial metabolism and so resistance of microbes may be influenced. The observed pH values were almost nearer to neutrality. So, microbial activities are not much more affected (**Kermanshahi, *et al*., 2007**).

The gram staining test showed that all isolates were gram-positive (Table 2). *Micrococcus sp*. is strictly aerobes and oxidase-positive, which can be used to differentiate them from other gram-positive bacteria like most *Staphylococcus sp*. (**Thelwell *et al.*, 1998**), which are normally oxidase negative. In our experiment, D3 isolates was identified as oxidase positive as well as it may be *Micrococcus antarcticus* (**Liu *et al*., 2018**). In growth medium, it generated yellow to brown colonies, on the contrary, *Micrococcus roseus* produced red colony (Table 2). *Bacillus proteolyticus* being a protease producing bacteria has the possible activity for use in aquaculture as a bioremediation (**Bhaskar and Sudeepa, 2007**). *Bacillus anthracis* is a member of the bacillus cereus group species that are non-punctilious facultative anaerobes and gram-positive spore forming bacteria (**Koehler, 2009**).

Sanjay *et al*., (2018) isolated two bacterial species *Klebsiella pneumoniea* strain MS 1.5 and *Mangrovibacter vixingensis* Strain MS 2.4 that showed high reducing ability for Cr (VI) upto 80 mg/l and 100 mg/l respectively. **Rajbanshi (2008)** also reported that the results of minimum inhibitory concentration of different bacteria 150 to 500 µg/mL for chromium and 200 to 300 µg/mL for copper. **Brocklehurst and Morby (2000)** reported that in response to toxic concentrations of heavy metal ions, Escherichia coli strain exhibited varying degrees of tolerance (3 to 14-fold) both to the adaptive metal and its congeners. **Singh *et al*., (2010)** isolated a bacterium *Bacillus cereus*, which exhibited a high degree of tolerance to heavy metal like lead, arsenic, and cesium.

In our experiments, the resistant level As>Pb>Cd>Cr>Cu>Ni showed for the maximum isolates. In this study order of MICs for the isolates D1, D2 and D3 was found to beAs>Pb>Cd>Cr>Cu>Ni and; As>Pb>Cr>Cu >Cd>Ni, and As>Pb>Cd=Cr>Cu>Ni. The order of other isolates B1 and DY was found to be As>Pb>Cu>Cd >Cr>Ni and Pb>As>Cr=Cu>Ni. It was also demonstrated that the resistant levels of heavy metal for sewage bacteria *Proteus vulgaris*, *Acinetobacter redioresistens* and *Pseudomonas aeruginosa* were shown to be As>Pb=Ni>Cd>Cr>Hg on the LB agar plates (**Raja *et al*., 2009**). Besides *Micrococcus antarcticus* shows sensitivity to Cr and Pb as well as their resistance capacity against Ni and Cu are also lower compared to other bacteria.

CONCLUSION

Genotypic characteristic for bacterial strain identification is accurate, simple and effective than identification by the conventional phenotypic method. We performed various biochemical characterization and molecular techniques; and identified *Micrococcus sp.* and *Bacillus sp.* This identification of bacterial species can be used to its multiple metal tolerant capacity and MIC which can be further used for the biosorption of heavy metal pollutants in the environment. In future, these bacteria can be employed for remediation of heavy metal released into the environment, used for extraction of novel metal and potential use to safeguard the ecosystem.

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PHYTOCHEMICAL AND ANTIMICROBIAL PROPERTIES OF LEAF EXTRACTS OF *Calliandra calothyrsus, Leucaena diversifolia* AND *Sesbania sesban*

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ABSTRACT

Phytochemical compounds are secondary metabolites of plants useful as antimicrobial agents. Botanicals are being explored for bioactive compounds with antimicrobial properties against phytopathogens. Little information is available on the phytochemical and antimicrobial activity of *Calliandra calothyrsus, Leucaena diversifolia* and *Sesbania sesban* against *Cercospora zeae-maydis* and *Xanthomonas campestris* pv. *musacearum*. The aim of the study was to determine the phytochemical and antimicrobial properties of leaf extracts of *C. calothyrsus, L. diversifolia* and *S. sesban* against *C. zeae-maydis* and *Xc.* pv. *musacearum*. Dried leaves were extracted in methanol and aqueous solvents and screened for phytochemical and antimicrobial activity using Kirby-Bauer's disk diffusion and poisoned food technique methods. *Sesbania sesban* extracts contained all the phytochemical tested; tannins, terpenoids, steroids, saponins, flavonoids, and alkaloids, *Leucaena diversifolia* lacked alkaloids while *Calliandra calothyrsus* lacked steroids and alkaloids. The extracts were active against *Cercospora zeae-maydis* and *Xc.*pv. *musacearum* with *Sesbania sesban* having greater radial inhibition activity. There was no significant difference in the antimicrobial activity between the lowest concentrations (25% and 25mg/ml) and highest concentrations (75% and 75mg/ml) in all the three plant extracts. Growth inhibition observed could be as a result of the different chemical compound observed in the extracts. Presence of alkaloids in *Sesbania sesban* could explain the greater growth inhibition of the pathogens under study. The results form the basis for further research that could lead to isolation and development of antimicrobial agents. Therefore, these plants can be used as an alternative to synthetic chemicals to control *Cercospora zeae-maydis* and *Xanthomonas campestris* pv. *Musacearum.*

Keywords: Phytochemical, antimicrobial**,** *Cercospora zeae-maydis* and *Xanthomonas campestris*pv. *musacearum*

INTRODUCTION

Plants have been a source of novel metabolites useful in therapeutics and antimicrobial since invent of traditional medicine (**Araya-Contreras and Bittner, 2019; Alemu *et al.*, 2017**). They synthesize array of mixtures of secondary metabolites called phytochemicals that are used in treatment of some diseases and management of microbial related diseases (**Ugweko *et al.*, 2017; Banu and Cathrine, 2015**). The type, quality and concentration of phytochemical in a plant is a function of both agronomic and environmental factors of ecological zone in which the plant is growing **(Borges *et al.*, 2018; Kumar *et al.*, 2017; Liu *et al.*, 2016; Liu *et al.*, 2015**). Besides environmental factors, age of the plant, relative humidity of harvested materials and method of extraction have great influence on the variation of phytochemical concentration and toxicological activity of the plant extracts (**Borges *et al.*, 2018; Izah 2018**). It is therefore important to screen different plant species from various regions to reveal their chemical compounds distributions. Plants with useful secondary metabolites may be cultivated to be used as food supplements (**Borges *et al.*, 2018; Hossain *et al.*, 2013**) or may be harvested for medicinal purposes (**Ugweko *et al.*, 2017**). However, most of the plants used in folk medicine are understudied in relation to their phytochemical composition which are pillars of traditional medicines. Phytochemical compounds which have been studied in different plants include flavonoids, phenols and phenolic glycosides, saponins and cyanogenic glycosides, stilbenes, tannins, nitrogen compounds (alkaloids, amines, betalains), terpenoids and steroids (**Borges *et al.*, 2018; Igbal *et al.*, 2015; Vaghasiya *et al.*, 2011**).

Most of the studies on the antimicrobial activity of plant crude extracts have focused majorly on human and animal pathogens (**Ugweko *et al.*, 2017; Zayed *et al.*, 2011**) while neglecting phytopathogens which have ravaged food crops hence compromising food security. Although little has been done on antimicrobial activity of plant extracts against phytopathogens, some plants have shown promising results in management of plant pathogens. For instance, some plant extracts have been reported to inhibit the growth of *Fusarium guttiforme* responsible for fusariosis in pineapples (**Sales *et al.*, 2016**). Extracts from *Bucida buceras*, *Breonadia salicina*, *Harpephyllum caffrum*, *Olinia ventosa*, *Vangueria infausta* and *Xylotheca kraussiana* are active against *Aspergillus niger* and *Aspergillus parasiticus* pathogens of fruits (**Mahlo *et al.*, 2016**). Similarly extracts from *Bidens pilosa* and *Euphorbia hirta* demonstrated antimicrobial activity against *Xanthomonas campestris* pv. *vescatoria* (**Emitaro *et al.*, 2018**). Considering the fact that most plants if not all have biologically active compounds against pathogens (**Salhi *et al.*, 2017; Mahlo *et al.*, 2016**), it is therefore necessary to screen *Calliandra calothyrsus*, *Leucaena diversifolia* and *Sesbania sesban* for phytochemical and antimicrobial properties.

Calliandra calothyrsus Meisn. is a small leguminous shrub that is predominantly cultivated as fodder for ruminant livestock (**Setyawati *et al.*, 2019**) but other uses are also found within different farming systems and include the provision of green manure, fuel wood, shade for coffee and tea, land rehabilitation, erosion control, and honey production (**Setyawati *et al.*, 2019; Abia *et al.*, 2006**). *Sesbania sesban* (L.) Merrill is a multipurpose tree that is widely distributed in tropics and subtropics of Africa and Asia and usually planted by smallholder farmers mostly for its fodder and soil improvement values **(Nigussie and Alemayehu, 2014; Mythili and Ravindhran, 2012**). It is used also as a source of green manure, anti-inflammatory activities, reproduction and milk production enhancement, nitrogen fixation, bioenergy source, antibacterial and anti-parasitic effect, antioxidant and mosquito repellant effects (**Nigussie and Alemayehu, 2013; Degefu *et al.*, 2011**). *Leucaena diversifolia* is an erect tree shrub that grows well in cool and seasonally wet locations and provides crude protein for livestock, control soil erosion and fix nitrogen in soils (**Walker, 2012; Orwa *et al.*, 2009**).

Even though there are no reports on the antimicrobial activity of *Calliandra calothyrsus* and *Leucaena diversifolia*, extracts from related species *Calliandra haematocephala* and *Leucaena leucocephala* have been reported to be active against Gram positive and Gram negative bacteria (**Josephine *et al.*, 2017; Chew *et al.*, 2011**). *Sesbania sesban* has been shown to possess phytochemical compounds with antimicrobial activity (**Gomase *et al.*, 2012; Kathiresh *et al.*, 2012**). While plants are being considered reliable sources of antimicrobial compounds, the antimicrobial activity of *C. calothyrsus*, *L. diversifolia* and *S. sesban* against *Cercospora zeae-maydis* and *Xanthomona scampestris* pv. *musacearum* has not been documented. Similarly, because of the effect of environment on phytochemical constituents, it is necessary to study the phytochemical composition and distribution among the three plant species. This will go along with identifying alternative measures of disease control using botanicals and understanding the variation in phytochemical components of plant in varying ecological zones. This study therefore aimed at identifying the phytochemical constituents and evaluating the antimicrobial properties of *C. calothyrsus*, *L. diversifolia* and *S. sesban* leaf extracts.

MATERIALS AND METHODS

Collection and processing of plant materials

Leaves of *C. calothyrsus*, *L. diversifolia* and *S. sesban* were collected in nonwoven bags from demonstration plots of Maseno University located 0° 10' 0" South, 34° 36' 0" East along Kisumu Busia road in western Kenya. They were transported to the botany laboratory for extraction. They were washed in tap water, air dried under shade for 14 days with periodic turning of two days then crushed into fine powder using electrical motor. Fine powder was used for extraction with both methanol and aqueous solvents according to Dent *et al.* (2013) where 50 grams of each powdered plant leaf materials were separately kept in 500 ml conical flask and 500 ml methanol and aqueous added and thoroughly mixed respectively. The mixtures were left to stand overnight on a shaker for complete extraction, filtered using muslin cloth followed by Whatman no 1 filter paper. Methanol was evaporated using rotary vacuum evaporator with the water bath temperature of 45ºC. The filtrate was used to test for the presence of phytochemicals and antimicrobial activity of the extracts.

Phytochemical screening of the extracts

The presence of steroids, alkaloids, flavonoids, saponins, tannis and terpenoids in leaves of *C. calothyrsus*, *L. diversifolia* and *S. sesban* were determined as indicated below.

Steroids: Fifty (50) mg of sample was dissolved in chloroform, filtered, then heated plus anhydrous acetic acid and cooled. Concentrated sulfuric acid was added through the walls of the tube drop wise and formation of a brown ring indicated the presence of steroids (**Setyawati *et al.*, 2019**).

Terpenoids: The crude extract was mixed with few drops of acetic anhydride, boiled and cooled. Concentrated sulfuric acid was added from the sides of the test tube and observed for the formation of a brown ring at the junction of two layers. Formation of deep red color in the lower layer indicated a positive test for terpenoids (**Bhandary *et al*., 2012**).

Saponins: Test solution was mixed with water, shaken and observed for the formation of froth, which is stable for 15 minutes for a positive result (**Gul *et al.*,2017**).

Alkaloids: The plant extract was warmed with 2% H_2SO_4 for two minutes, filtered and few drops Mayer's reagent added. Appearance of a creamy- white color precipitate indicated a positive result (**Sheel *et al.*,2014**).

Flavonoids: 2 ml of 2.0% NaOH mixture was mixed with aqueous plant crude extract. A concentrated yellow color was produced, which became colorless when 2 drops of dilute H_2SO_4 acid was added. Colorless appearance indicated presence of flavonoids (**Gul *et al.*, 2017**).

Tannins: A small quantity of the extract was boiled with 5 ml of 45% solution of ethanol for 5 minutes, cooled and filtered. 1ml of filtrate was diluted with distilled water and two drops of ferric chloride added. A transient greenish to black color indicated the presence of Tannins (**Sheel *et al.*, 2014**).

Isolation of fungal pathogen *Cercospora zeae-maydis.*

Maize leaves showing characteristic symptoms were collected from the fields, cut into pieces of approximately 5cm, placed on sterile moist blotter in a sterile petridish and incubated at 25ºC for 5 days for the pathogen to sporulate. Conidia were picked with an isolation needle under dissecting microscope and plated on PDA. Plates were incubated at 25ºC for 5-7 days and hyphal tips from the advancing colony margins with typical morphological characteristics were transferred onto PDA with isolating needle as pure culture and kept at 5ºC (**Nega *et al.*, 2016**).

Isolation of bacterial pathogen *Xanthomonas campestris* pv. *musacearum*

The bacterial pathogen was isolated from diseased banana leaves according the procedure of **Adriko *et al.* (2016)**. Approximately 1g sample of infected leaves was crushed in 1 ml of sterile distilled water in a petridish. The suspension was spread on semi-selective YPGA (Yeast extract-5 g l−1, Peptone-5 g /l, Glucose-4 g /1, Agar-12 g/1) medium containing antibiotics cephalexin (40 mg/1), 5-fluorouracil (10 mg/1) and cycloheximide (120 mg/1). The inoculated plates were incubated at 28ºC for 48–72 hr and mucoid yellow-pigmented colonies were picked and purified on nutrient agar (NA) medium.

Determination of antimicrobial activity

Methanol extracts were reconstituted by Dimethyl sulfoxide (DMSO) to make concentrations of 12.5mg/ml, 25mg/ml/ 50mg/ml and 75mg/ml while aqueous extracts were reconstituted into concentrations 12.5%, 25%, 50% and 75% and used for antimicrobial studies. Disc diffusion method was used to assess the sensitivity of the bacterial pathogen to plant extracts (Bauer *et al*., 1966). Colonies from pure culture were lawn spread on MHA plates and discs impregnated with 10µl of each test extract placed on the surface aseptically while discs impregnated with pure water and DMSO served as negative control. Every treatment was replicated thrice, plates arranged in completely randomized design and incubated at 30ºC for 48 hours and zone of inhibition measured in millimeters. Antifungal activity of the extracts was determined using poisoned food technique according to **Durgeshlal *et al.* (2019)**, by dispensing 4 ml of each extract in petri plates and adding 16 ml of PDA then mixing and allowing them to set. A 5mm mycelia plug from 7 day old mycelia was inoculated at the center of the plates and plates without extracts served as control in triplicates. Plates were incubated for 7 days at 28ºC where fungi mycelia radial growth was measured and inhibition percentage determined using the formula of **Durgeshlal *et al.* (2019)**;

Inhibition (%) = $[(D_c – D_T)/D_c] \times 100$

D_C and D_T are the colony diameters of the control and treated sets respectively

RESULTS

Phytochemical screening

Phytochemical screening revealed that terpenoids, flavonoids and saponins were present in the leaf extracts of the three plant species (Table 1). Tannins were present in *C. calothyrsus* and *S. sesban* but absent in *L. diversifolia*. Steroids were present in *L. diversifolia* and *S. sesban* but absent in *C. calothyrsus*. Alkaloids were present only in the leaf extracts of *S. sesban*. *Calliandra calothyrsus* had higher concentrations of tannins, terpenoids, saponins and flavonoids while *S. sesban* contained higher amounts of steroids and alkaloids.

Table 1 Leaf phytochemical compounds of *C. calothyrsus, L. diversifolia* and *S. sesban*

Plant species	Tannins	Terpenoids	Steroids	Saponins	Flavonoids	Alkaloids
C. calothyrsus	++	++	-	++	++	-
L. diversifolia	-	+	+	+	++	-
S. sesban	+	+	++	++	+	++

Key. - = Absent, += Present in low concentration, ++= Present in high concentration.

Antimicrobial activity of the extracts

Antimicrobial activity of leaf extract of *S. sesban, C. calothyrsus* and *L. diversifolia* against *Xc*.pv. *musacearum* and *Cercospora zeae-maydis*

The antimicrobial activity of leaf methanol and aqueous extracts of *S. sesban* , *C. calothyrsus* and *L. diversifolia* against *Xc*. pv *musacearum* and *Cercospora zeae-maydis* were significantly different with the P value of (P=0.0014 and P=0.0001) in methanol extract and (P=0.0016 and <.0001) in aqueous extract for *Xc*. pv *musacearum* and *Cercospora zeae-maydis* respectively. *Sesbania sesban* produced largest mean zone of inhibition of 13.9 mm for bacteria and inhibition percentage of 72.2% for fungi in methanol extracts and 13mm for bacteria and 78.3 % for fungi in aqueous extracts compared to *C. calothyrsus* and *L. diversifolia* (Tables 2 and 3). There was no significant ($p \leq 0.05$) difference in the mean zone of inhibition between different concentrations for both methanol and aqueous extracts against *Xc*. pv *musacearum* except for *L. diversifolia* whose concentrations exhibited significant difference against *Cercospora zeae-maydis* with concentration 75% having greatest mean inhibition percentage in both methanol and aqueous extracts (Tables 2 and 3).

Table 2 Antimicrobial activity of *S. sesban*, *C. calothyrsus* and *L. diversifolia* leaf methanol extract against *Xc*. pv*musacearum* and *Cercosporazeae-maydis*

Xc*. pv *musacearum				***Cercospora zeae-maydis***		
Plant species	Mean radial growth inhibition (mm)			% growth inhibition		
S. sesban	13.9[a]			72.2[a]		
C. calothyrsus	11.0[b]			68[a]		
L. diversifolia	10.0[b]			55.2[b]		
P value	0.0014			0.0001		
LSD	1.98			7.0789		
Xc*. pv *musacearum				***Cercospora zeae-maydis***		
Mean growth inhibition (mm)				**% growth inhibition**		
Treatments (mg/ml)	***S. sesban***	***C. calothyrsus***	***L. diversifolia***	***S. sesban***	***C. calothyrsus***	***L. diversifolia***
12.5	14.3[a]	10.3[a]	8.3[b]	74[a]	57[a]	64[a]
25.0	13.3[a]	11[a]	8.3[b]	75[a]	78.3[a]	61.6[a]
50.0	13.6[a]	10.3[a]	12.6[ab]	64.6[a]	62.6[a]	68.6[a]
75.0	14.3[a]	12.3[a]	11[ab]	75[a]	74[a]	23.3[b]
P value	0.95	0.65	0.09	0.2	0.17	<.0001
LSD	5.09	4.1	3.99	11.73	21.95	11.45

Means followed by the same letters down the column are not significantly different at P = 0.05.

Table 3 Antimicrobial activity of *S. sesban, C. calothyrsus* and *L. diversifolia* leaf aqueous extract against *Xc*. pv *musacearum* and *Cercospora zeae-maydis*

Xc*. pv *musacearum				***Cercospora zeae-maydis***		
Plant species	Mean radial growth inhibition (mm)			% growth inhibition		
S. sesban	13[a]			78.3[a]		
C. calothyrsus	9.4[b]			59.1[c]		
L. diversifolia	12[a]			71[b]		
P value	0.0016			<.0001		
LSD	1.87			2.7		
Xc*. pv *musacearum				***Cercospora zeae-maydis***		
Mean growth inhibition (mm)				% growth inhibition		
Treatments (%)	***S. sesban***	***C. calothyrsus***	***L. diversifolia***	***S. sesban***	***C. calothyrsus***	***L. diversifolia***
12.5	9.7[b]	8.7[ab]	11.6[a]	80[a]	55.3[b]	68.6[b]
25.0	15[a]	10.3[a]	11.3[a]	78[a]	53.3[b]	72a[b]
50.0	14.3[ab]	8.3[b]	12.3[a]	79[a]	55.6[b]	69.3[b]
75.0	13.3[ab]	10.3[a]	12.6[a]	80[a]	72[a]	74[a]
P value	0.16	0.08	0.91	0.71	0.0028	0.05
LSD	5.24	1.95	4.64	4.61	8.31	4.06

Means followed by the same letter down the column are not significantly different at P = 0.05.

DISCUSSION

Plant species used in traditional medicines continue to be reliable sources for discovery of useful compounds (**Musyimi *et al.*, 2008; Emitaro *et al.*, 2018**). Plants have become the subject of human curiosity in search for novel natural products relevant to pest and disease management in food crops (**Izah *et al.*, 2018; Sales *et al.*, 2016**). Medicinal plant extracts contain secondary metabolites such as alkaloids, quinones, flavonoids, glycosides, saponins, tannins and terpenoids with antimicrobial properties (**Izah *et al.*, 2018; Salhi *et al.*, 2017; Sales *et al.*, 2016; Musyimi *et al.*, 2008**). The variation in the concentration of the of phytochemicals in the leaf extracts of *C. calothyrsus* and *S. sesban* than *L. diversifolia* could be attributed to the type of solvent used and response of individual plant to biotic and abiotic factors as the plants were obtained in the same ecological zone. The concentration of bioactive compounds in each plant species depends on the environmental conditions, age of the plant, relative humidity of harvested materials and method of extraction (**Borges *et al.*, 2018;**

Izah 2018; Musyimi *et al*., 2008). The presence of all the phytochemical tested in *S. sesban* is in agreement with the results of **Nirosha *et al.* (2019)** and this explains its significance in antimicrobial activity (tables 2 and 3). *Callindra calothyrsus* extracts lacked steroids and alkaloids contrary to the results by **Setyawati *et al.* (2019).** This may be due to the fact that these plant occupy different ecological zones (**Kumar *et al*., 2017**). Phytochemicals differences can also occur depending on the type of solvents used in extraction. Phytochemical compounds take into account different parameters and factors such as species, ecological factors and environmental conditions (**Musyimi *et al*., 2008**).

The toxicological activity of plant extracts on the pathogens depends on the presence of bioactive compounds (**Pizzi, 2019; Nirosha *et al*., 2019**). Saponins and tannins have antibacterial and antifungal activity as well as anti-insect activity (**Hossein *et al*., 2013; Nirosha *et al*., 2019; Pizzi, 2019**). Flavonoids have been used as scavengers of superoxide anions, anti-inflammatory and antimicrobial agent (**Nirosha *et al*., 2019; Hossein *et al*., 2013**) while alkaloids act as anti-inflammatory, antimalarial, antimicrobial and cytotoxicity (**Iqbal *et al*., 2015; Matsuura and Fett-Neto, 2015; Hussain *et al*., 2018**). Similarly, steroids and terpenoids have been reported to possess cardiotonic effect, antibacterial, insecticidal properties (**Iqbal *et al*., 2015; Tholl, 2015; Bergman *et al*., 2019**).

There was a significant inhibition of radial growth of *Xc.* pv *musacearum* and *Cercospora zeae-maydis* by the leaf extracts from *S. sesban*, *C. calothyrsus* and *L. diversifolia*. *Sesbania sesban* extract was more effective against *Xc.* pv *musacearum* and *Cercospora zeae-maydis* pathogen as it produced large zones of inhibition compared to *C. calothyrsus* and *L. diversifolia.* The difference in performance could be attributed to high concentration of saponins, steroids and alkaloids in the leaf extract of *S. sesban* (Table 1). These compounds are known to have antibacterial and antifungal activity when they work synergistically with flavonoids. It may also be because active compounds were polar which dissolved in methanol and aqueous solvents readily than those in *C. calothyrsus* and *L. diversifolia.* The results are in agreement with the results reported by **Ahmed *et al.* (2013)** that *S. sesban* extracts are active against plant bacterial pathogen *Erwinia amylovora*. *Sesbania sesban* and plant pathogenic fungi *Curvularia lunata* and *Fusarium oxysporum* (**Mythili and Ravindhran 2012**).

The antimicrobial activity of the three plant leaf methanol and aqueous extracts have a wide range of activity because different concentrations of the extracts inhibited the growth of *Xc.* pv *musacearum* and *Cercospora zeae-maydis.* This could be probably due to the active ingredients in the extract which interfered with pathogen's cell functioning hence arresting growth. The ability of *S. sesban*, *C. calothyrsus* and *L. diversifolia* extracts to inhibit the growth of *Xc.* pv *musacearum* is attributed to the presence of secondary metabolites with antibacterial and antifungal properties. Leaves of all the three plants were found to have terpenoids, flavonoids and saponins which have antibacterial and antifungal properties (**Deivasigamani, 2018; Ahmed *et al*., 2013**). The antimicrobial activity of the plant secondary metabolites is due to their ability to denature protein, interfere with pathogen's cell signaling, DNA alkylation and altering the reproductive system of the pathogen (**Ramírez-Gómez *et al*., 2019**).

CONCLUSION

This study aimed at isolating, identifying and evaluating the antimicrobial properties of the compounds from the leaf extracts of *S. sesban*, *C. calothyrsus* and *L. diversifolia*. Phytochemical screening revealed that terpenoids, flavonoids and saponins were present in the leaf extracts of the three plant species. Tannins were only present in *C. calothyrsus* and *S. sesban*. Steroids were present in *L. diversifolia* and *S. sesban* while alkaloids were present only in the leaf extracts of *S. sesban*. The study found a variation in the concentration of phytochemical compounds in the leaf extracts of three plant species even though the plants were from the same ecological zone. Antimicrobial activity of plant extracts is depended on the secondary metabolites that the plant synthesise. *Sesbania sesban*, *Callindra calothyrsus* and *Leucaena diversifolia* extracts showed antimicrobial activities against *Xc.* pv *musacearum* and *Cercospora zeae-maydis* which could form a basis of developing botanical pesticides to avoid the adverse effects of synthetic chemicals. The results in this study supports the use of plant extracts in controlling plant pathogens as they are readily available, cheap and ecofriendly. Future studies should focus on identifying the active ingredients in the extracts of *S. sesban*, *C. calothyrsus* and *L. diversifolia* for development of chemicals to optimize their use by smallholder farmers in disease control to reduce dependence on synthetic pesticides.

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BACTERIOLOGICAL QUALITY AND SENSORY EVALUATION OF WINE PRODUCED FROM BLENDS OF DATE PALM FRUIT AND CUCUMBER JUICE USING *Saccharomyces cerevesiae*

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ABSTRACT

Bacteriological quality and sensory evaluation of wine produced from blends of date palm fruit and cucumber juice using *Saccharomyces cerevisiae* was investigated. The yeast was isolated and identified using standard techniques. The *S. cerevisiae* inoculum was produced using standard procedures and used to ferment the blends of date and cucumber to produce wine. Five (5) samples of wine were produced from date and cucumber at various concentration of substrates, anaerobically (Aerobic fermentation were terminated after 6 days and the fermented ''musts'' were sieved to remove the shaft and debris of the crushed fruits.During the anaerobic phase of fermentation, the filtrates obtained after sieving the ''musts'' were transferred into anaerobic fermentation round bottom flask (s) and incubated at room temperature. An air trap were fixed to the fermenting jars. Fermentation were terminated after four (4) weeks). Physicochemical, microbiological and sensory evaluation (using 7 point hedonic scale by 20 panelist) of the produced wine were studied. The yeast was identified as *Saccharomyces cerevisiae*. The physiochemical analysis of the produced wines showed that percentage of alcoholic content of wine B had the highest at 16.6 %, as Wine A had 15.8 %, Wine D had 13.17 %, Wine C had 11.17% and Wine E had 10.18% of alcohol content after fermentation. The suspected bacteria isolate were *Corynebacterium* sp. *Neisseria* sp, *Corynebacterium* sp., *Micrococcus* sp. *Enterobacteriaceae* sp. *Lactobacillu* sp.and *Micrococcus* sp. and some fungi sp. were isolated from the wines before pasteurization for sensory evaluation. But no microorganisms were detected after pasteurization of the produced wines. The sample C (Date and Cucumber) of ratio 3:1 was significantly preferred ($p<0.05$) from other wine A, B, D and E in respect to the aroma, test and appearance and over all acceptability. The *S. cerevisiae* used in the production of the wines gave excellent results. Harnesing the date and cucumber for wine production will reduce post-harvest loss of the fruit (wastage), improve, conribute to the economy and reduce unemployment.

Keywords: Physicochemical, microbiological, sensory evaluation, wine, yeast

INTRODUCTION

Wine was derived from the Greek word '*oines*' meaning wine while the science of wine is called 'oenology'(Okoro, 2007). It starts with the harvesting of grapes, separation of the juice before fermentation and concludes with the variety of storage and ageing steps. Apples, berries and black currants are sometime also fermented for wine production.Grape berries have a natural chemical balance which allows a completely fermentation without the addition of sugar, acid, enzymes or othernutrients (Okoro, 2007). It is a rich source of vitamins, many essential amino acids, minerals, fatty acid andothers; however other fruits with samecharacteristics have been discovered and used effectively for wine production. During fermentation, microscopic single-celled organism called 'yeast' such as *Saccharomyces cerevesiae* digest sugar found in fruit juice,producing alcohol and carbon dioxide gas in the process Wines are categorized using a number of different criteria, this include grape variety, regionof origin, by colour, by name of the wine makeror viticulturist, or by production technique. However, three basic groups of wines are most easily distinguishable for the consumer: table wines, sparkling wines, fortified wines, fruit wine, dry wine sweet wine (Okoro, 2007). They may also be classified on the basis of the Countries of origins or fruit. From which they were obtained for example red table wine are made from black grapes while whites wine are made from black or white grapes. It is now know that it can be produced from other fruit such as mango, oranges, date fruit, pineapple, cucumber, Lemons bananas (Robinson, 2006; Amerine *et al.*, 2012). Wine has been produced and enjoyed by many people, from peasants to kings, for thousands of years. The consumption of red wine is known to have a remarkable protective effect against oxidative stress in blood plasmas. Most wines consumed in Nigeria are completely fermented, aged, bottled and imported ones. Imported products are costly now due to high duties paid on them. This had made imported wines too expensive to local consumers and for these reasons, there arose the need for more wine from other plants species.

Date Palm fruit (*Phoenix dactylifera*L.) originated in Mesopotamia (currently Iraq) and its cultivation spread to the Arabian Peninsula, North Africa and the Middle Eastern countries in ancient times about 5000 years ago (Kader and Hussein, 2009). Dates are very cheap fruits but rich in nutrition. The mineral contents in the dates have the potential to provide a good source of zinc, potassium, calcium, and sodium in the diet. (Mowunmi , 2013).Egypt places top among the top date fruits exporting countries around the world by economizing 1,373 metric tonnes averagely each year (UN Food and Agricultural Organization,2016) . Fruits of the date palm are a main source of staple food in arid and semi-arid regions of North Africa, Middle East and South-Asian countries (Amina *et al.*, 2010). Dates have always played an important role in the economic and social lives of people of this area. These have abundant use as nutritional treat during Islamic holy month of Ramadan (Jamil *et al.*, 2012). Fresh dates compose of soft, easily consumable flesh and simple sugars like fructose and dextrose. When eaten, they replenish energy and revitalize the body instantly (Ragava *et al.*,2017). They are deseeded and stuffed or chopped and used in a great variety of ways. They are mixed with cereals, in pudding, bread, cakes, cookies, ice cream or candy bars. Dates are also made into cubes, paste, spread, powder (date sugar), jam, jelly, juice, syrup, vinegar or alcohol. Date brittle, date arrack, a wine like drink were also prepared in Egypt from dates (Ragava *et al.*,2017).

Dates are generally regarded as resistant to microbial spoilage. However some contaminants, especially some osmotolerant yeasts and moulds may survive longer times or even grow on the fruits (Siddig, 2012). Endophytic fungal species plays a major role in colonisation and survival of host plant. Date fruits also contains some anti-nutritional factors like tannins, phytates and oxalate contents. Consumption of tannin in large doses may cause bowel irritation, damage the liver, stomach and kidney irritation and gastrointestinal pain, chelate minerals and makes them unavailable to the body (Coe *et al.*, 2005). Phytate is a complex fibre content which cause mineral deficiency and pellagra (Coe *et al.*, 2005). Date extracts were contains stronger antibacterial activity against Gram positive bacteria than Gram negative bacteria and it has an observed notable antibacterial activity against *Staphylococcus saphrophyticus* (Salah and Otiabi, 2013). Cucumber is a creeping vine that bears cylindrical fruits. It isscientifically known as Cucumissativus it belongs to the gourd family cucubitacea (Abbey and other, 2017). Other vegetables which belong to this family include melon, squash, watermelon and pumpkins. It originated from theAsia continent. Cucumber plant can be cultivated in bothtemperate and tropical environment hence it is said to be a native ofmany regions of the world. There are several varieties of cucumberbut the edible cucumber is classified under two groups the slicing andpickling cucumber.(Abbey *et al.*, 2017). Cucumber fermentation relies on the process of naturally-occurring by LAB or in some cases inoculum from pure starter culture. The slicing cucumbers are longer and thinner when compared to thepickling cucumber. Cucumber contains nutrients vital for the bodydevelopment. It also has several health benefits such as: rehydratingthe body, health regulating the blood pressure, body weightmanagement, cholesterol reduction, cancer prevention, bone health, diabetes cure and antioxidant activity (Abbey *et al.*,2017). The microflora population of fresh cucumbers is dominated by Gram-negative aerobic bacteria, In general, LAB growth is primarily influenced by

various factors such as pH of the media, fermentation temperature, media composition (sugars and nutrients) and their mode of fermentation (Mussantto *et al*., 2008). Fermentation is a viable technique in the development of new products with modified physicochemical and sensory qualities, especially flavour and nutritional components. Alcohol and acetic and lactic acid fermentation are important for quality in production. of these, alcoholic fermentation is widely employed for the preparation of beverages in which alcohol is major constituent. Fermented beverages have been known to human kind from time immemorial. An alcoholic beverage is a drink that contains ethanol. These are divided into three general classes for taxation and regulation of production, namely, beers, wines, and spirits distilled beverages such as whisky, rum, gin, and vodka *(*Saranraj *et al*., 2017). On the other hand fermentation is biotechnology in which desirable microorganisms are used in the production of value-added products of commercial importance. Fermentation occurs in nature in any sugar-containing mash from fruit, berries, honey, or sap tapped from palms. If left exposed in a warm atmosphere, available yeasts act on the sugar to convert it into alcohol and carbon dioxide (Saranraj *et al*., 2017). The study was aimed at bacteriological quality and sensory evaluation of wine produced from blends of date palm fruit and cucumber juice using *Saccharomyces cerevisiae.*

MATERIALS AND METHODS

Study Area

The research was carried out at Applied Food Microbiology and Food Safety Laboratory of Ibrahim Badamasi Babangida University, Lapai, Niger State of Nigeria.

Collection of Samples

The Date fruit and cucumber used in this research work were purchased from local market at Lapai, Niger State and were deposited in sterile sampling bag. Both substrate (sample purchased) were transported down to Applied Microbiology and Food safety Laboratory, Ibrahim Badamasi Babangida University, Lapai and stored in the refrigerator at 4^0C .

Media Preparation

The media: Nutrient Agar (NA), Potato Dextrose Agar (SDA) were prepared according to the manufacturers instructions.

Inoculum Preparation of Starter Culture

Pure culture of *Saccharomyces cerevisiae* used to ferment the fruit blends/mixtures was obtained from Applied Food Microbiology and Food Safety Laboratory of Ibrahim Badamasi Babangida University, Lapai, Niger State. The inoculum of *Saccharomyces cerevisiae* was prepared from 24 hours old culture and was serially diluted and turbidity was compared with 0.5 Mcfarland standard to get 10^8 cell/ml.

Preparation of Samples

The dried date fruit and fresh cucumber were weighed, washed, sliced, rewashed, with the removal of the seeds for date and then reweighed. The date fruit were diluted with sterile water to obtained softness of the date. Date fruit and cucumber were then minced and homogenised using a sterile blender and then filtered. The overall water added during the blending was 3000 ml distilled water to avoid friction in the blender. Exactly 3000 ml of distilled water was added to extract the "must" by filtering the juice with muslin cloth. After filtration, juice were then pasteurized at 60^0C for 1hr and allowed to cool before 1000 ml was measured in to round bottom flasks of 2000 ml capacity in ratio of 1:1, 1:2, 1:3, 2:1, and 3:1 date and cucumber juice combination labelled as A, B, C, D and E respectively, all in duplicates.

Fermentation Process to Produce Wine

Standardized inoculum (10^8cells/ml) of *Saccharomyces cerevesia*e were added to the ''musts'' in 2000 ml round bottom flask containing the date and cucumber juice blends, this was achieved by sprinkling it over the surface of the juice then stirred;the inoculated musts were covered with sterile muslin cloth and incubated at room temperature (28±2°C); and was aerated daily by stirring twice to encourage yeast multiplication. The physicochemical parameters such as temperature, pH, total titrable acidity (TTA) and specific gravity during fermentation were determined using standard techniques. Aerobic fermentation were terminated after 6 days and the fermented musts were sieved to remove the shaft and debris of the crushed fruits.During the anaerobic phase of fermentation, the filtrates obtained after sieving the musts were transferred into anaerobic fermentation jar and incubated at room temperature. An air trap were fixed to the fermenting jars. Fermentation were terminated after four (4) weeks; the wines were then stored at temperature of 28±2°C to allow the yeast to flocculate for 21 days. The resulting wines were then aged for two months. The aged wines were then filtered using a sterile given set kit, decanted into sterile bottles and corked as described by Victoria *et al.* (2017).

Microbial Enumeration of the Produced Wines

Populations of bacteria in the wine were assessed by standard pour plate method on nutrient agar.The microbial load was counted and the bacterial and fungal growth were also identified under a high power microscope. Twenty five (25) ml of the wine sample was added to 225 ml of normal saline (1:9) for each sample respectively. Exactly one (1) ml was collected from each serially diluted sample solution. And were pour plated by pipetting 1ml of each sample into empty sterile petri dish and pouring 15ml of the Nutrient agar medium.The plates were rocked properly to achieve uniform distribution of both the media and sample after which the plates were allowed to solidify on a flat surface. The nutrient agar plates for enumeration of bacteria were incubated at 37 °C for 24 hours while Sabouraud dextrose agar were used for the enumeration of fungi after serial dilution procedures (Tenfold serial dilution of wine made were aseptically pipette into 90 ml amount of sterile distilled water. 1ml of inoculum was transferred to the next test tube of distilled water (9ml) 10^{-5} dilution. The sample were diluted up to $10^{-6)}$ of the wine, pour plated and were incubated at 28 ± 2^0C for 72 hours. At the end of the incubation, the plates were retrieved and the number of colonies formed were counted as described by Awe and Nnadoze (2015). Colonies observed on the plates after incubation period were counter representative for bacteria and fungi were selected after random morhological screening for identification. Colonies were repeatedly subculture to purify by employing streaked plate method.

Identification and Characterization of Bacteria Isolates From Produced Wine

The identification and characterization of isolated organisms was done based on colonial morphology, cellular morphology and biochemical characterization tests.

Morphology of Bacterial Isolates from Produced Wine

Parameters, such as colour, shape, elevation surface, consistency edge and capacity were observed and recorded.

Cellular Morphology of Bacteria Isolates from Produced Wine

Microscopy of isolates were carried out using gram and spore staining for bacteria and lacto phenol cotton blue stain for fungal isolates.

Biochemical Characteristic of Bacterial Isolates From Wine

Gram Staining

Using sterile technique, smears of the fresh culture of 24 hours of the isolates were made by placing a drop of water on a clean slide with sterile cooled loop. The smear were air dried and heat fixed by passing the slides over flames. The slides were flooded with crystal violet for 1-2 min each. They were rinsed with tap water and Lugol's iodine was added and left to stand for 1 min. (acts as a mordant). The smears were then decolourised with until crystal violet failed to washed with water and then counter stain for 30 sec. The slides were washed with water and blot dried and further dried in air. The slides were then examined under an oil immersion lens (Oyeleke and Manga, 2008).

Catalase Test

Two drops of 3% hydrogen peroxide (H_2O_2) was placed on grease free slides using a rubber pipette. The test organisms were aseptically transferred onto the

drops on the slide using a sterile wire loop and were mixed properly. The smear on the glass slides were observed for gas bubbles as described by (Cheesbrough, 2000).

Coagulase Test
A small drop of distilled water was placed on clean glass slides. A sterile wire loop was used to pick the test organisms and was emulsified on the slides. Three drops of plasma was added and mixed properly (Cheesbrough, 2006).

Mannitol Salt Test
Exactly 2.8g of agar-agar and 0.08g of mannitose salt were dissolved in 250 ml of distilled water, and dispensed into a conical flask and autoclaved at 121ºC for 15 minutes. The media was allowed to solidify on plates. The test organisms were streaked on the surface of the agar-agar and incubated at 37ºC for 48 hours (Onyeagba, 2004).

Citrate Test
Twenty four 24g Simon citrate agar 24g was dissolved in 100/ml of distilled water, and dispensed into a conical flask and autoclaved at 121ºC for 15 minutes. The media was allowed to solidify on plates. The test organisms were streaked on the surface of the citrate agar and inoculated at 37ºC for 48 hours (Onyeagba, 2004).

Sugar Fermentation Test
One (1) % (i.e. 1g in 100ml) of peptone water was prepared and 9ml was dispensed into each test tube. 3-4 drops of phenol red was added and autoclaved at 121^{0}C for 15 minutes. Double strand of a glucose sugar was prepared (2% of glucose). 1m of the sugar (glucose) was added to each test tube i.e after the prepared peptone water has cooled. A colony of organism was picked from the slant bottle and inoculated into the test tube containing the sugar, peptone water and an inverted Durham tube. Lactose, sucrose and fructose were also prepared (1%) following the same procedure. The test tubes were incubated 3 days after which each test tubes were observed for acid and gas production by comparing them to the control (Cheesbrough,2006).

Urease Test
Exactly 2.8g of Nutrient Agar was dissolve in 90 ml of distilled water, phenol red was introduced into the media, mixed and autoclaved at 121^{0}C for 15 minutes. The urea was then added aseptically to the mixture of material agar and phenol red which was mixed properly and dispensed into test tubes and allowed to solidify in a slant position. The test organism were streaked on the entire slat surface and incubated at 37^{0}C for 48 hours.

Sensory Evaluation of the Produced Date – Cucumber Wines

The sensory evaluation of the wines were assessed by twenty (20) panelists drawn from staff and students of The Department of Microbiology of IBB University. Wine samples were assessed based on parameters such as appearance, texture, aroma, taste (sweet, sour and bitter) and the overall acceptability. The wine samples were assessed using a nine (9) point hedonic scale. Prior to sensory evaluation, ISO standards for selection, training (≥2 days) and monitoring of assessors (ISO 8586:2012), design of testing room (ISO 8589:2010), and methodology of monitoring performance of sensory panel (ISO 11132:2009) was followed. Each assessor was served with approximately 20 mL of test sample (18 ± 2^{0}C) with 250 mL wine tasting glasses (ISO 3591:1977); results were ranked (ISO 8587:2006) and expressed in accordance with the sensory vocabulary (ISO 5492:2008) (Balogu and Towobola, 2017).

Statistical Analysis of Data

Data generated were subjected to analysis of variance (ANOVA). Mean separation and comparison was done using SPSS version 23.0. Significance was accepted at P < 0.05 and results were expressed as mean ± Standard deviation from the mean.

RESULTS

Assessment of the Date-Cucumber Wine samples showed that the temperature profile were relatively stable from week 0 to week 4 during fermentation. This was shown that Wine A (1:1) slightly varied from 29.8 °C - 30.8 °C, Wine B (1:2) 30.8 °C - 31.0 °C, Wine C (3:1) 31.5 °C – 32.7 °C, Wine D (1:3) 29.9 °C – 31.7 °C and Wine E (2:1) 29.8 °C – 31.0 °C. Wine A had the lowest temperature of 29.8 °C – 30.8 °C and Wine C had the highest temperature (Fig1). The pH kinetic of Date-Cucumber wine showed maximum pH of (6.67) on Wine A followed by B (6.61), E (6.55), C (6.53) D (6.46) respectively on week 3 and steadily increased to pH (6.93) on week 6 (sample A) (Fig.2). There was a noticeable difference as the wine gradually reduce in quantity as the fermentation days of wine increases.The specific gravity of each wine samples decreased after fermentation. The gravity sample A range from (1.40-067), B (1.47-0.70), C(1.45-0.71), D(1.64-0.73),and E (1.75-0.72) (Fig.3). Fermentation velocity is the rate of fermentation. It tends to measure the rate or percentage sugar conversion to alcohol. Sample B had the best rate (0.770) and other (A, D, C and E) range from 0.5419-0.4126 (Fig4). The residual sugar mean sweetness in wine, it is usually measured in percentage (%). Basically, residual sugar or "RS" is the sugar form date-cucumber wines left over after fermentation.The more residual sugar remaining in the wine, the sweeter the wine. The residual sugar was stable from the first week and it starting reducing and sample C, D and E had the highest reduction factor (-95.828) and sample B and A (-72.313) had the lowest reduction rate or range (Fig.5). Alcoholic content of date-cucumber wine showed that sample B had the highest alcoholic content of 16.06 % followed by A (15.8 %) D (13.17 %), C (11.17 %) and E (10.18) (Fig.6). A steady progressive microbial growth curves of 7.8-8.3 were observed within 60 days of fermentation with sequential intervals week 0, week 2, week 4, week 6 and week 8, respectively (Fig.7). Morphological and biochemical characterization of bacterial isolates from date-cucumber wines are presented in table 1. The bacteria isolated include *Corynobacterium* sp, *Neisseria veinella*, *Microbacterium* sp., *Microbacterium* sp. *Enterobacteriaceae* sp. *Lactobacillus* sp, *Micrococcus* sp (Table1). Overall quality of date-cucumber wine were bars with different (P<0.05) where sample C and D were significantly preferred with rate of 1.5 and A, B, and E were less with preferred by seven points hedonic scale (Fig.8). The mean and standard deviation of the date-cucumber wines produced showed that wine C (3.1) and D (1.3) were significantly preferred. Post Hoc Duncan multiple range test found that factor that contribute to the acceptability were aroma, taste and appearance (Table2).

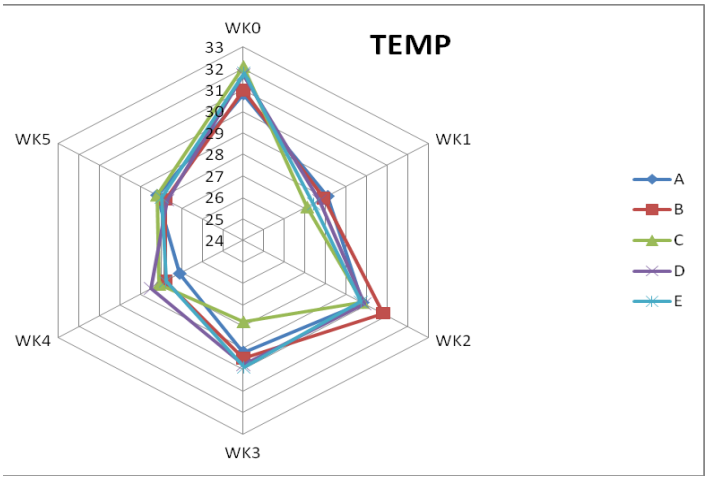

Figure 1 Temperature Profile of Wine Production during 8 weeks of fermentation
NB: Graph with different colour shows the different sample of Date-Cucumber Wine by ratio.
Wine A (1:1) =1000ml: 1000ml,Wine B (1:2) =500ml: 1000ml,Wine C (3:1) =500ml: 500ml
Wine D (1:3) =500ml: 1500ml,Wine E (2:1) =1000ml: 500ml

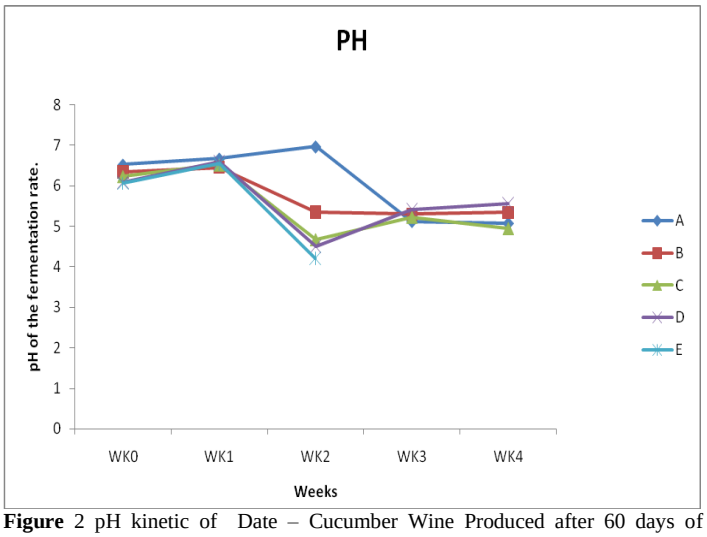

Figure 2 pH kinetic of Date – Cucumber Wine Produced after 60 days of fermentation
NB: Graph with different colour shows the different sample of Date-Cucumber Wine by ratio.
Wine A (1:1) =1000ml: 1000ml, Wine B (1:2) =500ml: 1000ml, Wine C (3:1) =500ml: 500ml
Wine D (1:3) =500ml: 1500ml, Wine E (2:1) =1000ml: 500ml

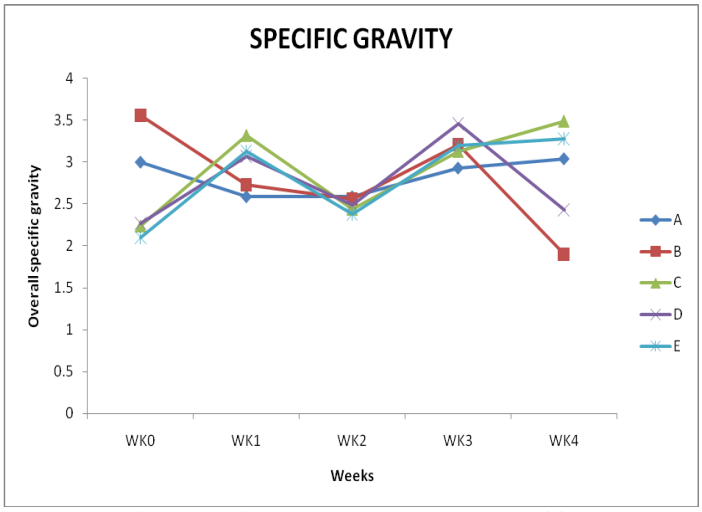

Figure 3 Specific gravity of Wine Production during 60 days of fermentation
NB: Graph with different colour shows the different sample of Date-Cucumber Wine by ratio Wine A (1:1) =1000ml: 1000ml, Wine B (1:2) =500ml: 1000ml,Wine C (3:1) =500ml: 500ml
Wine D (1:3) =500ml: 1500ml, Wine E (2:1) =1000ml: 500ml

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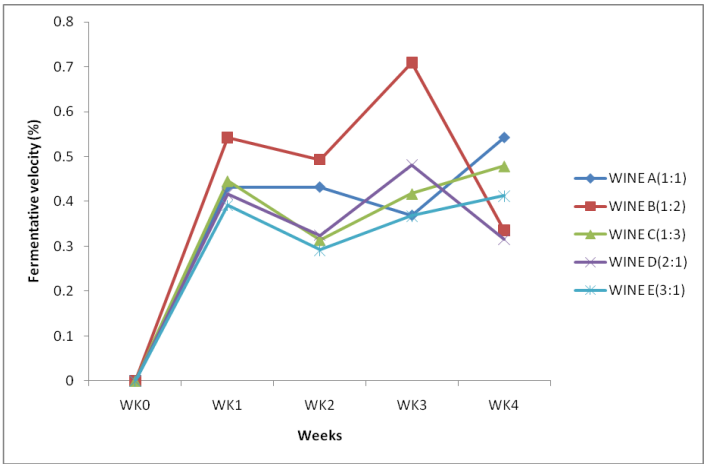

Figure 4 Fermentation Velocity of Date-Cucumber after 60 days Fermentation
NB: Graph with different colour shows the different sample of Date-Cucumber Wine by ratio.
Wine A (1:1) =1000ml: 1000ml, Wine B (1:2) =500ml: 1000ml, Wine C (3:1) =500ml: 500ml
Wine D (1:3) =500ml: 1500ml, Wine E (2:1) =1000ml: 500ml

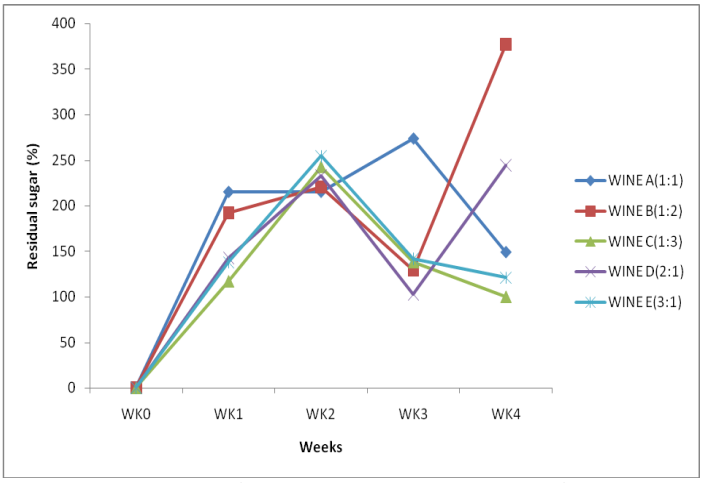

Figure 5 Residual Sugar of Date-Cucumber during the 60 days fermentation
NB: Graph with different colour shows the different sample of Date-Cucumber Wine by ratio.
Wine A (1:1) =1000ml: 1000ml, Wine B (1:2) =500ml: 1000ml, Wine C (3:1) =500ml: 500ml
Wine D (1:3) =500ml: 1500ml,Wine E (2:1) =1000ml: 500ml

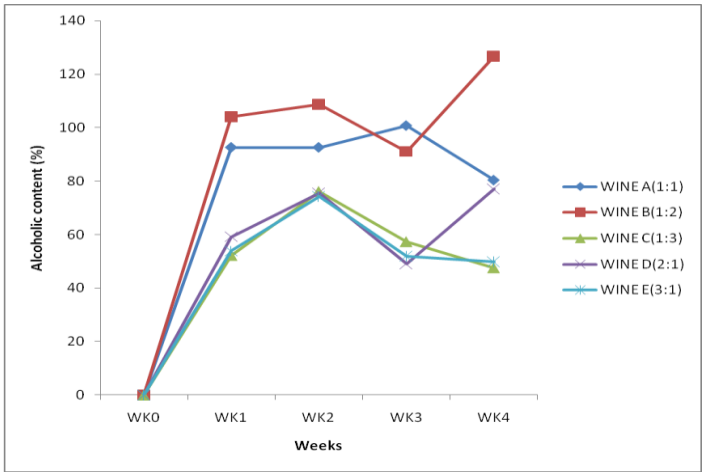

Figure 6 Alcoholic Content of Date-Cucumber Wine Produced after 60 days of Fermentation
Wine A (1:1) =1000ml: 1000ml, Wine B (1:2) =500ml: 1000ml, Wine C (3:1) =500ml: 500ml
Wine D (1:3) =500ml: 1500ml, Wine E (2:1) =1000ml: 500ml

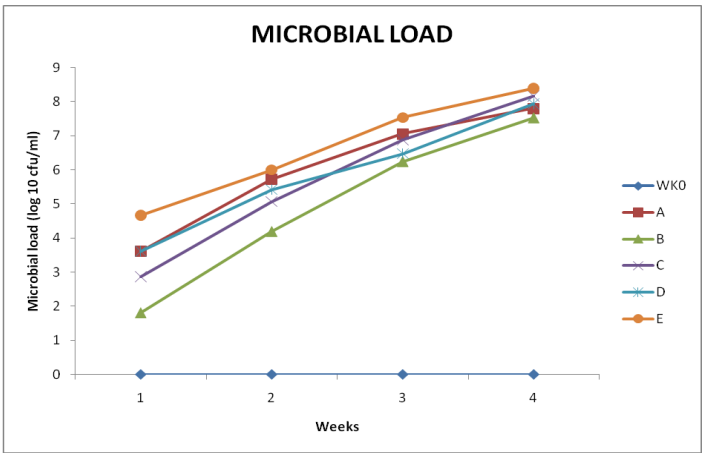

Figure 7 Microbial growth curve of Wine Production at 60 days Fermentation
NB: Graph with different colour shows the different sample of Date-Cucumber Wine by ratio
Wine A (1:1)=1000ml:1000ml, WineB (1:2)=500ml:1000ml, Wine C (3:1) =500ml: 500ml
Wine D (1:3=500ml:1500ml, Wine E (2:1) =1000ml: 500ml

Table 1 Morphological and Biochemical Characteristics of Babterial Isolates from Date-Cucumber Wines before Pastuerization

Sample Code	Morphology	Shapes and Arrangement	Gram	Catalase	Coagulase	Citrate	Oxidase	Galactose	Lactose	Fructose	Probable Bacteria
A1	Circular irregular Off white flat Colony	Single bacillus	+	+	-	+	+	G	AG	G	*Corynebacterium* sp
A2	Circular irregular Milk raise colony	Clusters Bacillus	+	+	-	+	-	G	AG	AA	*Corynebacterium* sp
B1	Smooth regular Milk raise colony	Single Cocci	-	-	+	-	-	AG	G	AG	*Neisseria, Veillonella*
B2	Smooth regular Milk dotted raise Colony	Single Cocci	+	+	-	+	-	G	G	A	*Micrococcus*sp
C1	Circular regular Milk slightly raise	Single Cocci	+	+	-	+	+	G	G	A	*Micrococcus* sp
C2	Round regular Mucoid slightly Raise	Single Bacillus	-	-	+	-	-	AG	G	A	*Enterobacteriaceace* sp.
D1	Smooth Regular milk flat colony	Clusters Bacillus	-	+	+	+	+	AG	AG	G	*Micrococcus varians*
D2	Smooth regular Dirty milk colony	Clusters Bacillus	+	+	-	-	-	A	G	AG	*Lactobacillus* sp
E1	Circular irregular Off whitecolony	Single Cocci	+	+	+	+	-	AG	AG	AG	*Micrococcus* sp.
E2	Circular irregular dirtyMilk colony	Single Bacillus	+	+	+	+	-	A	AG	AG	*Micrococcus* sp

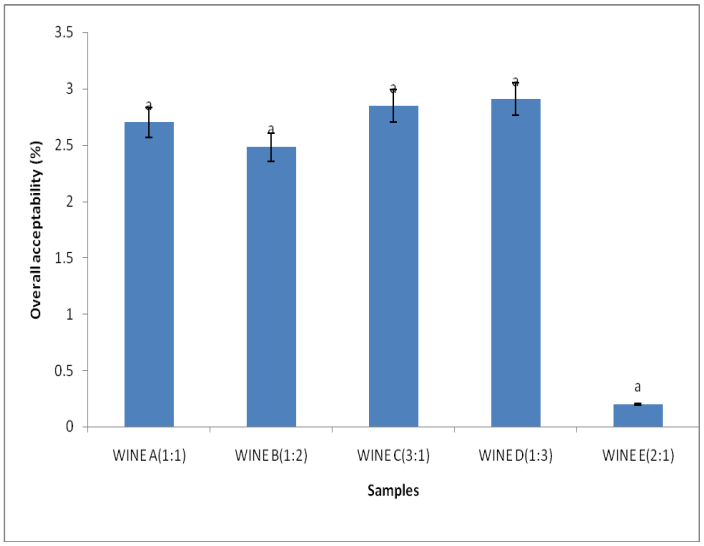

Figure 8 Overall acceptability of Date-Cucumber Wine Production after 60 days of fermentation
NB**:** Differentbar chart represent each sample by ratio.
Wine A (1:1) =1000ml: 1000ml,Wine B (1:2) =500ml: 1000ml,Wine C (3:1) =500ml: 500ml
Wine D (1:3) =500ml: 1500ml, Wine E (2:1) =1000ml: 500ml

Table 2 Sensory Evaluation of the Produced Date –Cucumber Wines

Wine	Colour	Aroma	Taste	General Acceptibility
Wine A (1:1)	B 3.20 ±0.36	B 3.80 ± 0.29	B 3.00 ± 0.22	B 3.87 ± 0.31
Wine B (1:2)	A 1.20 ± 0.13	A 2.70 ± 0.39	C 3.40 ± 0.45	A 2.43 ± 0.27
Wine C (3:1)	A 1.50 ± 0.22	A 2.40 ± 0.40	A 2.90 ± 0.34	A 2.26 ± 0.19
Wine D (1:3)	A 1.50 ± 0.22	A 3.40 ± 0.37	A 2.00 ± 0.29	A 2.30 ± 0.18
Wine E (2:1)	A 1.90 ± 0.31	A 2.50 ± 0.26	A 2.40 ± 0.16	A 2.26 ± 0.18

NB**:** mean of sensory analysis have different alphabet down to the group are significantly different (P < 0.05), Wine A (1:1) =1000ml: 1000ml,Wine B (1:2) =500ml: 1000ml, Wine C (3:1) =500ml: 500ml, Wine D (1:3) =500ml: 1500ml,Wine E (2:1) =1000ml: 500ml

DISCUSSION

The identified *S.cerevisiae* used in the production of the wines performed excellently well, this could be as a result of it stability during the fermentation processes to produce the date-cucumber wines. This is in agreement with Mohammed *et al.* (2018) who characterized *S.cerevisiae* isolated from palm wine and used it to produce wine from blends of cocnut milk and honey slurry and reported excellent results from the microbial and sensory evaluation of the produced wines. The physiochemical properties of Date-Cucumber wine revealed in this study include temperature, pH and specific gravity.In this study, the temperature of the wine produced varies and this could be as a result of microbial metabolism of viable nutrients to fabricate alcohol with resulting generation of heat. This result agrees with the report of Okafor (2007) and Robinson (2006). Based on this study, it is implicit that the pH of the wine throughout the period of fermentation ranged from 6.67-6.93, there was a significant differences among the samples.A comparable observation has been reported by Siddig (2013): in their study on Date wine, optimum pH value for quality wine production was 7.0 and should not exceed 7.0. Studies have shown that during fermentation: Low pH is the inhibitory to the growth of spoilage organisms but creates conducive environment for the growth of desirable organisms. Also, low pH and high acidity are known to give fermenting yeasts modest advantage in the natural environment. In the case of specific gravity of the wine produced in the study, there was an obvious difference as the wine gradually reduced as the fermentation days of the wine increased; this could be due to microbial progression: available nutrients, sugar and alcohol resulting in the production of acid. This result conforms to the report of Querol *et al.* (2003). The specific gravity of wine A ranges from (1.40-0.67), wine B (1.47-0.70), wine C (1.45-0.71), wine D (1.64-0.73) and wine E (1.75-0.72).These decreases were observed to be irrespective of the yeast strain used in the wine production. This result conforms to Nidhi *et al*.(2008). *Saccharomyces cerevisiae* has been reported to reduce specific gravity quality of fruit wines during fermentation (Okafor *et al*.,2007).
The fermentative capacity of different Date-Cucumber wine sample, on the week 4, wine B and A had the highest fermentative capacity of 76.9 and 73.2 respectively. While D, C and E had the lowest (44.1, 44.1 and 41.0), which is why sample B had the highest percent alcohol content. In increasing order, the alcoholic content of different Date-Cucumber wine where wine B (1:2) had the highest alcoholic concentration of 16.06, A (15.8), D (13.17) and wine C and E had the least alcohol content of 11.17 and 10.18 respectively. High alcohols are known to be important precursors for the formation of esters, which are associated with pleasant aromas (Clement-Jimenez *et al.*, 2005).
The microbial analysis of Date-Cucumber wine revealed that there was no any mould and coliform growth at the dilution factors used, while the total viable count ranged between 6.7-7.4x10^{-6}log CFU/ml. This implies that the wine was produced under hygienic conditions and safe for human consumption. This agree with the report of Frazier and Weshoff (1998).Therefore, the total viable count was within the acceptable limit according to Sri Lanka standard for all drinks/beverages and juice as reported by Ghana Standard Board (1995). Close observation on the predominant bacteria isolated from date-cocumber wines include: *Corynebacterium* sp, Neissaria sp.*Micrococcus* sp, *Enterobacteriaceace* sp, *Micrococcus* sp *and Lactobacillus* sp. The sensory scores of Date-Cucumber wine produced where wine C (1:3) ratio of Date-Cucumber was significantly preferred. The post Hoc Duncan multiple rang test found that factor contributed to the acceptability of wine C (1:3) were aroma, taste and appearance and colour.Colour is an important sensory characteristic on which consumer preferences are dependent, this result obey the rules with the result of Francis (1995) who reported that colour influences other sensory characteristics, which subsequently account for food acceptability, choice and preference. The result of sensory evaluation of the samples of wine produced from date-cucumber wine showed that wine C was rated highest in colour, taste, aroma and general acceptability followed by wine A, the least were wine B, C and D. There was a significant differences (P<0.05) among the wine samples with respect to flavor. The wine C was rated high generally thus can be prepared for commercial purposeto serve as a special wine with similar constituents as other already existing commercial wines as reported by Selli *et al*. (2013).

CONCLUSION

The present study has shown that acceptable fruit wines can be produced from Date fruit and Cucumber using *Saccharomyces cerevisiae* in a spontaneous fermentation. Therefore good wine can be obtained by controlled fermentation of a starter culture for 8 week using *Saccharomyce cerevisiae*.The statistical analysis showed that Sample C was significantly preferred at 5% confidence (P< 0.05).However, large-scale production of Date and Cucumber wines could be an alternative to extending the shelf-life of Date and Cucumber fruit and the level of post-harvest loss of the fruit.

Recommendations:

Recommendations were made at the enf of this study;

1. It was recommended that in the production of Date and Cucumber Wine, the fermentation temperature should be maintained at between 24°C to 27°C. The temperature should not exceed 29°C. Otherwise the growth of yeast cell will be stopped and encourage the bacteria growth.
2. The products (Date-Cucumber Wine) is recommended for commercial production in a large scale.

Conflict of interest: The authors of this research declare no conflict of interest.

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INVESTIGATION OF MICROBIOLOGICAL QUALITY OF SELECTED STREET VENDED FOODS SOLD IN UYO METROPOLIS, AKWA IBOM STATE, NIGERIA

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ABSTRACT

Street vended food play an important role in developing societies as they support livelihood of millions of people in the society. This study evaluated the microbial quality of selected street-vended foods in Uyo Metropolis, Akwa Ibom State, Nigeria. Popular food samples were collected from five different locations with intensive vending activities and analyzed for total bacterial count (TBC), total *Salmonella (*TSC), *E. coli* and *Vibrio cholera* counts (VCC). Results indicated that TBC ranged from 1.33 x 10^5 + 0.03 cfu/g to 6.00 x 10^5 + 0.03 cfu/g while TSC ranged from 1.00 x 10^3 + 0.02 cfu/g to 3.00 x 10^4 + 0.04 cfu/g. Total *E.coli* count ranged from 1.00 x 10^1 + 0.00 cfu/g to 2.09 x 10^3 + 0.01 cfu/g which was above WHO standard of 0 cfu/g. VCC was detected in 7 food samples with the highest count being 7.20 x 10^2 + 0.02 cfu/g. There were significant differences (P<0.05) in microbial counts among food samples and vending locations. Bacterial species isolated from the food samples include: *Aerococcus viridans, Cornynebacterium uberis, Escherichia coli, Klebsiella species, Lactobacillus salivarius, Micrococcus luteus, Micrococcus varians, Proteus mirabilis, Pseudomonas pyogenes, Pseudomonas pyogenia, Salmonella paratyphi, Salmonella typhi, Staphylococcus albus, Staphylococcus aureus, Staphylococcus epidermidis, Vibrio cholera.* Data obtained from the questionnaires showed poor handling and preparation of food by vendors, hence the need to train and educate vendors on food hygiene. Application of hazard analysis and critical control point (HACCP) is necessary to improve the safety of street foods and consequently safety of consumers.

Keywords: Street-vended-foods, microbiological-quality, Uyo, bacterial-count, *Salmonella*-count, *E. coli* and *Vibrio-cholera*-counts

INTRODUCTION

Food is any substance that people or animal eat or drink or that plant absorb to maintain life and growth. **Ezeronye (2007)** defined food as any substance consumed for support for the body; it is usually of plant or animal origin. In other words, foods are substances (either solid or liquid) that are consumed for adequate nutritional status. It is usually composed of carbohydrates, fats and oil, protein, vitamins, minerals, fibre and water that can be eaten or drunk by animals and humans for nutrition or pleasure **(Davidson, 2008).**

From microbiological perspective, food can be viewed as a fertile ecosystem in which organisms vie for nutrients **(Nester *et al*., 2004)**. They are complex organic substances which living organisms require for producing energy for metabolic processes and for building up of body tissues when broken down within the body of a living organism, they may be used for repairing worn out tissues and cells, replacement of dead old cells, fight against antigens and proper functioning of body system among others. The lack of food or its inadequacy will result in diverse disorderliness and malfunctioning **(Anibijuwon & Sunday, 2012)**. Excess food can be detrimental to living organism, so likewise do contaminated foods have effect in living organisms when ingested.

Street foods also known as 'Ready-to-eat' (RTE) foods could be defined as foods; raw or cooked, hot or chilled, that are ready for immediate consumption at the point of sales without any further treatment. The United States Department of Agriculture defined RTE food as food that is in a form that is edible without washing, cooking or heating by the consumer and is reasonably expected to be consumed in that form. Foods such as washed, cut fruits and vegetables are considered as RTE foods. Also, foods presented for consumption for which further washing or cooking is not required and from which rinds, peels, husks, shells are removed are also considered as RTE **(DBPR, 2002)**.

Processing of these value-added products require strict hygiene applications throughout the processing chain. This is because these foods are susceptible to microbiological spoilage and could harbor pathogens even under the best management conditions and practices.

On consumption of food, occasionally human beings consume undesirable biological agents and toxins. Food is not only of nutritional value to those who consumed them but also often are ideal culture media for the growth of microorganisms **(Gadaga *et al*., 2007)**. Foods are normally contaminated with bacteria and other microbes since the environment in which we live is colonized by them. Food prepared locally for human consumption is at greater risk of contamination and vice versa. Also, the health status of the individual preparing the food is a major determinant.

Safe food is a basic human right despite the fact many foods are frequently contaminated with naturally occurring pathogenic microorganisms which cannot be detected organoleptically but can cause diseases including death especially if the way they are conserved during exposition for sale provides condition for those microorganisms to grow and reach considerable levels of contamination **(WHO, 2000)**.

In Nigeria, there has been an increase in consumption of ready-to-eat foods over the last decade, because they are relatively cheap, easily accessible and convenient. Also, they are easily available and affordable. It provides variable food source, employment and with a potential of improving food security and national status and general social security. Most people are very much interested in satisfying their hunger and the convenient of RTE foods rather than its microbiological quality and hygiene. The fact that RTE food is an indispensable part of urban and rural diets, it has its own associated health problems. Since it does not require any further processing before consumption, street-food can be a good vehicle for the transmission of food-borne microorganisms **(Monday *et al*., 2014)**.

Street-vended foods provide major source of income for vast number of persons, particularly women, chance of self-employment and opportunity to develop business skills with low capital investment, least expensive and most accessible means of obtaining nutritionally balanced meal outside the home for many low income people **(WHO, 2000; Dipeolu *et al*., 2007)**. Street foods play an important role in developing societies as they support livelihood of millions of the urban poor. Despite the economic and nutritional benefits of street foods, the consumption of these foods is readily contaminated from different sources **(Tambekar *et al*., 2008).** There are number of factors that can result to food borne diseases; these may include failure to cook food thoroughly, holding food at ambient temperature optimal for bacterial growth, poor handling, storage and transportation of cooked foods, lack of hygienic practices among others **(Mensah *et al*., 2002)**. Food borne diseases are diseases resulting from the ingestion of bacteria, toxins and cells produced by microorganisms present in food. It is capable of reducing the productivity and economic output, and also imposes substantial stress on healthcare system.

Furthermore, studies on microbiological quality and safety of street vended foods in Uyo metropolis have received little or no attention from food agencies. Thus, this study forms a basis for justification of microbial examination owing to the general unhealthy and unhygienic practices used during food preparation by food vendors which has led to the growing concern for food safety. It is therefore pertinent to isolate, identify and characterize food borne pathogens associated with ready-to-eat foods and the relationship between their occurrence and the hygienic practices in order to create public awareness. The outcome of this study could provide useful information about potential food poisoning outbreak relating to street vended foods sold in Uyo metropolis. Again, food handlers in Uyo could be guided in their daily activities.

MATERIALS AND METHODS

Samples collection

Samples were collected in sterile packages after preparation in the same manner as how a consumer would buy them from different mobile and fixed vendors. It was transported without delay to the laboratory in aseptic conditions within one to two hours of collection where the product type, purchase date and place of purchase was recorded and kept in their original packages, not in the cooling box so as to replicate consumer behavior, until beginning of the analysis. Samples and locations were coded to aid identification as follows: A- Rice and stew, B- Porridge beans, C- Ekpangkukwo, D- Moi moi, E- Noodles, UTC- Uniuyo Town Campus, UMC- Uniuyo Main Campus, UTH- Uniuyo Teching Hospital, ITP- Itam Park, AAM- Akpan Andem Market.

Sterilization of media

All materials were adequately and appropriately sterilized before and after use. Glass wares such as test tubes, glass rod, pipette, measuring cylinder, beakers and conical flasks required for this research work were soaked and washed thoroughly with detergent and rinsed with distilled water properly and drained. They were wrapped with aluminum foil paper and dried in the oven in inverted position at 180 ^{o}C for 60 min. The working area was swabbed with ethanol. Contamination by microorganisms from the external environment was reduced by closing windows and putting off fans in the laboratory. Prepared media and distilled water was autoclaved at 121^{o}C for about 30 min at 15 psi (per square inch). Metal equipment like the inoculating loop was heated to redness in an open flame before and after use. Every isolation and inoculation was done near the flame to reduce contamination of the agar plates tube.

Preparation of culture media

The following culture media were used; nutrient agar for the determination of total bacterial count, potato-dextrose agar (fluka) for the determination of total fungal count, *Salmonella Shigella* agar for the determination of *Salmonella Shigella* count, eosin methylene blue agar for the determination of *Escheriche coli* count; thiosulphate citrate bile salt sucrose agar for the determination of total *Vibrio cholera* count and cysteine lactose electrolyte deficient (CLED) agar for differentiation of bacterial isolates. Each of the growth media were accurately weighed according to the manufacturer's specifications and mixed in a recommended quantity of water in a conical flask. The growth media were sterilized in an autoclave at a temperature of 121 ^{o}C for 15 psi. *Salmonella shigella* agar was melted at 100 ^{o}C in conical flask because it was already sterile.

Preparation of samples

About 10 g of each food sample was weighed and mixed with 90 ml of 0.1% sterile peptone water in a mortar and homogenized. The resultant homogenate was diluted serially down to 10. From the appropriate dilutions 1 ml was plated in duplicate onto the different media using pour plate technique on nutrient agar (in duplicate) for aerobic colony count (ACC). The plates were allowed to set for 15 min and were incubated in inverted position for 24 h. All plates were incubated at 37 ^{o}C for 24 – 48 h; EMBA-inoculated plates were incubated at 72 h. At the end of the incubation periods, plates containing 30 – 300 colonies were counted using illuminated colony counter to obtain viable bacterial colonies (Gallenkamp, England). The counts for each plate were expressed as colony forming unit per gram of sample (cfu/g). Morphological attributes of the colonies on the media were observed; discrete colonies on the different media were purified by repeated sub-culturing on nutrient agar. Pure cultures were stored on agar slants at 4 ^{0}C for further characterization.

Enumeration of total *Salmonella* count

About 1 ml of each sample was taken and added into test tube containing 9 ml of normal saline (0.85%W/V) and was mixed well using vortex. Then, the serial dilution was made up to 10^{-3} using test tube no.3. 1 ml suspension was transferred and poured on to a sterile *Salmonella-Shigella* agar (SSA) in duplicate for *Salmonella* count. The plates were incubated at 37 ^{o}C for 24 h for optimum growth of cells. After the incubation period, the result of the total *Salmonella Shigella* Species count were expressed in colony formation units per gram sample **(Feglo & Sakgi, 2012)**

Enumeration of *Escherichia coli* count

About 1 ml of dilution 10^{-3} levels was poured-plated in duplicate on eosin methylene blue agar (EMB). The plates were kept in an incubator at 44 + 0.5 ^{o}C for 72 h. After pour-plating and incubation on EMB agar, plates with colonies ranging from 30 – 300 with greenish metallic sheen which are indicative of *Escherichia coli* colonies were counted and recorded **(Nkere *et al.*, 2011).** The cell count was converted to colony forming units per gram (cfu/g).

Enumeration of *Vibrio cholerae* count

About 1 ml of sample diluent at 10^{-3} was transferred to a plate of thiosulfate-critrate-bile salts-sucrose (TCBS) agar. The colour of this media is deep green. The pour plate technique was done. The agar plate was incubated at 37 ^{o}C for 24 h. for samples with vibrio species present such as *V. cholera*, ferment sucrose, resulting in pH shift and production of yellow colonies. The result of the *Vibrio* species count was expressed in colony formation units per gram sample.

Characterization and identification of the isolates

As reported by **Edem *et al.* (2017),** standard inocula were prepared from the preserved stock culture by taking a loopful of the isolates and aseptically inoculating onto sterile nutrient agar plates. The plates were incubated at 28°C for 24 h. Morphological and biochemical characterization was carried out using standard methods **(Harrigan & McCane, 1976),** while the isolates were identified by reference to Bergey´s Manual of Systematic Bacteriology **(Holt *et al.*, 2000)**. Fungal isolates were identified using colonial appearance and microscopic characteristics **(Chessbrough, 1984; Barnett & Hunter, 1987).**

Statistical analysis

Data obtained were subjected to the analysis of variance (ANOVA), and the Duncan's test was used as a post-hoc mean separation technique for treatments. Differences among the data were determined at 5% level of significance. The data analysis was completed using SPSS version 20.

RESULTS

Total fungal count of street vended foods in Uyo metropolis

Table 1 presents the total fungal count of street vended foods in Uyo metropolis. Only porridge beans obtained from Uniuyo teaching hospital and Uniuyo Town Campus recorded fungal growth with a total fungal growth of $7.00 \times 10^{1} + 0.001$ cfu/g and $3.00 \times 10^{1} + 0.00$ cfu/g respectively

Table 1 Total fungal count in street vended food in the Uniuyo metropolis

	LOCATION				
Food sample	**UTC**	**UMC**	**UTH**	**ITP**	**AAM**
A	Nil	Nil	Nil	Nil	Nil
B	$3.0 \times 10^{1b} + 0.00$	Nil	$7.00 \times 10^{1a} + 0.001$	Nil	Nil
C	Nil	Nil	Nil	Nil	Nil
D	Nil	Nil	Nil	Nil	Nil
E	Nil	Nil	Nil	Nil	Nil

Values are means + SD of triplicate determinations. Means in the same row with different superscripts are significantly ($p<0.05$) different. UTC – Uniuyo Town Campus, UMC – Uniuyo Main Campus, UTH – Uniuyo Teaching Hospital, ITP – Itam Park, AAM – Akpan Andem Market, A – Rice and stew, B – Porridge beans, C – Ekpankukwo, D – Moi Moi, E – Noodles.

Total bacterial count of street vended foods sold in Uyo metropolis

Total bacterial count of street vended foods sold in Uyo metropolis is presented in Table 2. The results obtained show that total bacterial count was significantly (P<0.05) higher in rice and stew, moi moi and noodles obtained from Akpan Andem Market than in other food samples from other locations. However, total bacterial count was significantly (P<0.05) higher in porridge beans from Uniuyo Town Campus as well as Ekppangkukwo from Itam Park than in other locations. This indicated that location and type of food had significant (P<0.05) effect on total bacterial count.

Table 2 Total bacterial count in street vended foods in Uniuyo metropolis

	Location				
Food sample	**UTC**	**UMC (x10^5 cfu/g)**	**UTH**	**ITP**	**AAM**
A	2.33^{d}+0.02	2.15^{e} +0.01	4.00^{b} +0.05	3.60^{c} +0.03	4.30^{a} +0.02
B	4.10^{a}+0.02	3.90^{b} +0.04	1.66^{e} +0.03	2.50^{c} +0.05	2.30^{d} +0.02
C	2.04^{b}+0.04	1.96^{c} +0.02	1.33^{e} +0.03	2.50^{a} +0.05	1.80^{d} +0.02
D	2.80^{e}+0.04	4.50^{d}+ 0.03	5.00^{c} +0.01	5.10^{b} +0.05	5.20^{a} +0.03
E	5.70^{b}+0.05	4.10^{d} +0.02	3.90^{e} + 0.03	5.00^{c} +0.03	6.00^{a} +0.05

Values are means + SD of triplicate determinations. Means in the same row with different superscripts are significantly (p<0.05) different. UTC – Uniuyo Town Campus, UMC – Uniuyo Main Campus, UTH – Uniuyo Teaching Hospital, ITP – Itam Park, AAM – Akpan Andem Market, A-rice and stew, B – Porridge beans, C – Ekpankukwo, D – Moi Moi, E – Noodles.

Total *Salmonella shigella* count in street vended foods in Uyo metropolis

Total *Salmonella Shigella* count of street vended foods sold in Uyo metropolis is presented in Table 3. The result showed that total *Salmonella shigella* count was significantly (p< 0.05) higher in rice and stew and noodles from Uniuyo town Campus than in same food samples from the other locations. However, total *Salmonella Shigella* count was significantly (p< 0.05) higher in porridge beans from Uniuyo Teaching hospital than that from other locations. The result obtained indicated that total *Salmonella Shigella* count was significantly (p< 0.05) higher in ekpangkukwo from Uniuyo Main Campus than in other locations, while moi-moi obtained from Itam Park had a significantly (p<0.05) higher total *salmonella shigella* count than moi-moi from other local.

Table 3 Total *Salmonella Shigella* count in street vended foods in Uyo Metropolis

	Locations				
Food Sample	**UTC**	**UMC (in cfu/g)**	**UTH**	**ITP**	**AAM**
A	2.00 x 10^{4a} +0.05	1.50 x10^{4c}+0.01	1.80 x 10^{4b}+0.03	1.90 x 10^{3e}+ 0.02	8.70 x 10^{3d} +0.05
B	4.00 x 10^{3c}+0.01	3.00 x 10^{3e}+0.05	9.00 x10^{3a}+0.03	3.50 x10^{3d}+ 0.05	7.60 x10^{3d} +0.02
C	1.20 x 10^{3d} + 0.02	3.00 x10^{3a}+0.04	2.00 x10^{3b}+ 0.02	1.70 x10^{3c} +0.01	1.00 x10^{3e}+ 0.02
D	1.45 x10^{4b}+0.02	1.15 x10^{4c}+0.05	1.10 x10^{4c}+ 0.02	2.10 x 10^{4a}+ 0.02	2.10 x10^{4a} +0.03
E	3.00 x10^{4a}+0.04	5.00 x10^{3d}+0.05	4.00 x 10^{3e} +0.01	1.90 x 10^{4b}+ 0.04	1.10 x10^{4c}+ 0.05

Values are means SD of triplicate determinations. Means in the same row with different superscripts are significantly (p<0.05) different. UTC – Uniuyo Town Campus, UMC – Uniuyo Main Campus, UTH – Uniuyo Teaching Hospital, ITP – Itam Park, AAM – Akpan Andem Market, A – Rice and Stew, B – Porridsge beans, Ekpankukwo, D – Moi Moi, E – Noodles.

Total *Escherichia coli* count in street vended foods in Uyo metropolis

Total *Escherichia coli* count in Street vended Foods in Uyo metropolis is presented in Table 4. Result indicated that the type of food sample and the location had significant (p<0.05) effect on total *E. coli* count. Total *E. coli* count in Rice and Stew obtained from Uniuyo town Campus and Itam Park were significantly (p<0.05) higher than that obtained from other locations but not significantly (p<0.05) different from each other. Porridge beans obtained from Akpan Andem Market had significantly (p>0.05) higher total *E. coli* count than in food samples obtained from other locations. However, no *E. coli* was detected in porridge beans obtained from Uniuyo Town Campus. The result also showed that total *E. coli* count was significantly (p<0.05) higher in ekpankukwo and moi-moi from Uniuyo Teaching Hospitral than that obtained from other locations. However, noodles obtained from Itam Park had significantly (p>0.05) higher total *E. coli* count than other locations. No *E. coli* was detected in moi-moi, rice and stew and noodles obtained from Akpan Andem Market.

Table 4 Total *Escherichia coli* in street vended foods in Uyo Metropolis

	Location				
Food sample	**UTC**	**UMC (in cfu/g)**	**UTH**	**ITP**	**AAM**
A	1.00 x 10^{2a} + 0.02	6.00 x 10^{1b}+ 0.03	1.00 x 10^{1c}+ 0.00	1.00 x 10^{2a}+ 0.01	Nil
B	Nil	1.00 x 10^{1d}+ 0.00	1.00 x 10^{2c}+ 0.01	1.10 x 10^{2b}+ 0.03	4.10 x 10^{2a}+ 0.02
C	2.80 x 10^{2d} + 0.05	4.50 x 10^{2b}+ 0.05	5.30 x 10^{2a}+ 0.03	1.30 x 10^{2e}+ 0.03	3.50 x 10^{2c}+ 0.02
D	1.10 x 10^{2c} + 0.03	1.50 x 10^{2b}+ 0.02	2.10 x 10^{2a}+ 0.01	1.50 x 10^{2b}+ 0.03	Nil
E	4.10 x 10^{2d} + 0.05	8.10 x 10^{2b}+ 0.02	5.10 x 10^{2c}+ 0.02	2.09 x 10^{3a}+ 0.01	Nil

Values are means SD of triplicate determinations. Means in the same row with different superscripts are significantly (p<0.05) different. UTC – Uniuyo Town Campus, UMC – Uniuyo Main Campus, UTH – Uniuyo Teaching Hospital, ITP – Itam Park, AAM – Akpan Andem Market, A – Rice and stew, B – Porridge beans, C – Ekpankukwo D – Moi Moi, E – Noodles.

Total *Vibro cholerae* count in some street vended foods in Uyo Metropolis

Total *Vibro cholera* count in some street vended foods in Uyo Metropolis is presented in Table 5. The result showed that the total *Vibrio cholera* count was significantly ($p<0.05$) higher in moi-moi from Uniuyo Town Campus than in the same food samples gotten from other locations. However, no *Vibrio cholera* count was detected in moi-moi from Itam Park and Akpan Andem Market. Porridge beans from Uniuyo Town campus also showed total *Vibrio cholera* count significantly ($p<0.05$) higher than porridge beans from Itam Park while no Vibrio cholera count was detected in ekpangkukwo and noodles samples from all locations.

Table 5 Total vibrio cholerae count in some vended foods

	Locations				
Food samples	**UTC**	**UMC**	**UTH**	**ITP**	**AAM**
A	Nil	$1.00 \times 10^{1b} +0.00$	Nil	$1.00 \times 10^{2a} +0.01$	Nil
B	$5.80 \times 10^{2a} +0.05$	Nil	Nil	$1.00 \times 10^{2b} +0.02$	Nil
C	Nil	Nil	Nil	Nil	Nil
D	$7.20 \times 10^{2a} + 0.02$	$6.40 \times 10^{2b} +0.05$	$5.70 \times 10^{2c} +0.03$	Nil	Nil
E	Nil	Nil	Nil	Nil	Nil

Values are means SD of triplicate determinations. Means in the same rows with different superscripts are significantly ($p<0.05$) different. UTC – Uniuyo Town Campus, UMC – Uniuyo Main campus, UTH – Uniuyo Teaching Hospital, ITP – Itam Park, AAM – Akpan Andem Market, A – Rice and stew, B – porridge beans, C – Ekpankukwo, D – Moi Moi, E – Noodles.

Morphological characteristics and frequency of occurrence of fungal species in street vended foods in Uyo metropolis

Table 6 presents the morphological characteristics and frequency of occurrence of fungal species as identified in the street-ended foods in Uyo metropolis. The result showed that two fungal species were isolated. The fungal species: *Aspergillus fumigatus* and *Penicillum frequentans* were obtained from porridge beans from Uniuyo Town campus and Uniuyo Teaching Hospital respectively.

Table 6 Morphological characteristics and frequency of occurrence of fungal species in vended foods in Uyo metropolis

Colony Colour	**Somatic Structure**	**Nature of Hyphae**	**Special Vegetative structure**	**Asexual Spore**	**Special Reproductive Structure**	**Most Probable organism**	**Prevalence Frequency**	**Percentage Prevalence Frequency (%)**
Smoky or grey-green	Filamentous	Septate	Footcell	Globose conidia	Short conidiophores	Aspergillus fumigatus	1	50
Small blue colony	Filamentous	Septate	Broom-like shape	Sub globose Conidia	Branded conidiophores	Penicillum frequentans	1	50
TOTAL							2	100

Frequency of occurrence for bacterial species street vended foods in Uyo metropolis

Table 7 shows the frequency of prevalence of bacteria isolated from street-vended samples collected in different locations. Table 7 revealed that *Escherichia Coli* had the highest number of occurrence while *Micrococcus luteus* had the lowest number of occurrence.

Morphological and biochemical characteristics of bacterial species in street vended foods in Uyo metropolis

Results in Table 8 showed the biochemical and morphological characteristics, carbohydrate fermentations and names of bacterial species from isolated street – vended food samples in Uyo metropolis.

Table 7 Percentage frequency of prevalence of bacterial isolates in street ended foods in Uyo metropolis

S/N	**Bacterial Isolates**	**Prevalence Frequency**	**Percentage Prevalence Frequency (%)**
1	*Klebsiella species*	7	5.19
2	*Staphylococcus epidermidis*	8	5.92
3	*Pseudomonas pyogenes*	8	5.92
4	*Escherichia coli*	21	15.56
5	*Salmonella typhi*	18	13.33
6	*Corynebacterium uberis*	6	4.44
7	*Micrococcus varians*	5	3.70
8	*Aerococcus viridians*	6	4.44
9	*Staphylococcus albus*	7	5.19
10	*Proteus mirabilis*	7	5.19
11	*Salmonella paratyphi*	12	8.89
12	*Micrococcus luteus*	4	2.96
13	*Lactobacillus salivarius*	7	5.19
14	*Pseudomonas pyogenia*	7	5.19
15	*Staphylococcus aureus*	5	3.70
16	*Vibrio cholera*	7	5.19
TOTAL		**135**	**100**

Table 8 Morphological and biochemical characteristics of bacterial species in street vended foods in Uyo metropolis

Cultural Characteristics	Cell Shape	Gra	Motility	Met	Coa	Indo	Oxi	Cat	Citr	spor	Ure	Glucose	Lactose	Mannitol	Maltose	Xylose	Sucrose	Probable Bacterial Isolates Identified
Mucoid milky circular colonies	Rod	-	-	+	-	-	-	+	+	-	+	AO	OO	AO	AO	OO	AO	*Klebsiella species*
Tiny white circular colonies	Cocci	+	-	-	-	-	-	+	-	-	+	AO	OO	AO	AO	OO	OO	*Staphylococcus epidermidis*
Irregular milky colonies	Rod	-	+	+	-	-	+	+	+	-	+	AO	OO	OO	AO	AO	AO	*Pseudomonas pyogenes*
Greenish metallic colonies	Rod	-	+	+	-	+	-	+	-	-	-	AG	AG	AG	AG	AO	AO	*Escherichia coli*
Circular creamy colonies	Rod	-	+	+	-	-	-	+	-	-	-	AG	AO	AG	AG	AO	OO	*Salmonella typhi*
Milky raised colonies on N.A	Rod	+	-	-	-	-	-	-	+	-	+	AG	OO	OO	AO	OO	AG	*Cornynebacterium uberis*
Tiny white colonies on N.A	Cocci	+	-	-	-	-	-	+	+	-	+	AO	OO	AO	AO	OO	AO	*Micrococcus varians*
Tiny white colonies on N.A	Cocci	+	-	+	-	-	-	+	+	-	-	AO	OO	AO	AO	OO	AO	*Aerococcus viridiands*
White flat circular colonies	Cocci	+	-	-	-	-	-	+	+	-	+	AO	OO	AO	AO	OO	OO	*Staphylococcus albus*
Irregular greenish colonies	Rod	-	+	+	-	+	+	+	+	-	+	AG	AO	AO	AO	OO	AG	*Proteus mirabilis*
Circular creamy colonies	Rod	-	+	+	-	-	-	+	-	-	-	AO	OO	AO	AO	OO	OO	*Salmonella paratyphi*
Tiny white colonies on N.A	Cocci	+	-	-	-	-	-	+	+	-	+	OO	OO	OO	OO	OO	OO	*Micrococcus luteus*
Large circular colonies	Rod	+	-	-	-	-	-	-	-	-	-	AO	AO	AO	AO	OO	AO	*Lactobacillus salivarius*
Irregular milky colonies	Rod	-	+	+	-	-	+	+	+	-	+	AO	AO	AO	AO	AO	AO	*Pseudomonas pyogenia*
Golden yellow colonies	Cocci	+	-	-	-	-	-	+	-	-	+	AG	AG	AG	OO	OO	AG	*Staphylococcus aureus*
Irregular yellow colonies on TCBS	Rod	-	+	+	-	+	+	+	+	-	-	AO	AO	AO	AO	AO	AO	*Vibrio cholerae*

Profile of the street food vendors and personal hygiene in Uyo metropolis

Table 9, 10 and 11 summarized the food handling practices, characteristics of vending environment and food vendors who participated in the study. The result in Table 9 showed that 73.3% of the vendors interviewed were women. The age range of vendors was between 15 - ≥ 55 *years* with the highest percentage (40.00%) in the age group of 25 – 34 year's olds. Most of the vendors (60.00%) had primary school qualification, 26.7% had a secondary school qualification while 2% had no formal school.

Table 10 summarized the Preparation, storage and handling practices of the vendors of street foods in Uyo Metropolis. About 33.3% of the vendors stored their foods in a wheelbarrow while 20.0% displayed foods openly in the stalls and only 46.7% stored them in sealed containers. Only 6.7% (1/15) of the stalls covered their utensils and 86.7% of the vendors washed their utensils using cold and soapy water. About 53.3% of the vending sites were protected by the sun, wind and dust. Evidence of the presence of houseflies was observed at 66.7% of the stalls. It was observed that 60.0% of the stalls had garbage bins, while Table 11 presented the personal hygiene of the vendors and vending site which showed that 33.3% worked without aprons while 66.7% used their bare hands during handling, preparation and serving of foods. All of the vendors (100%) did not cover their hair during the handling, preparation and serving of foods. About 93.3% exchanged money during the handling.

Table 9 Profile of street food vendors in Uyo Metropolis (n=15)

Parameter (%)	Frequency
General Information	
Gender	
Female	11(73.3)
Male	4(26.7)
Age range	
15-24	3(20.0)
25-34	6(40.0)
35-44	3(20.0)
45-54	2(13.3)

n ≥ 55	1(6.7)
Educational Attainment	
Did not attend formal school	2(13.3)
Primary school completed	9(60.0)
Secondary school completed	4(26.7)
College completed	0
Vending Duration	
<1 years	1.(6.7)
1-2 years	2(13.3)
3-5 years	4(26.7)
6-9 years	6(40.0)
n≥ 10 years	2(13.3)
Types of vendor	
Stationary	10(66.7)
Mobile	5(33.3)
Undergone Food safety training	2(13.3)

Table 10 Preparation, storage and handling practices of vendors of street foods in Uyo Metropolis

Parameter **(%)** **Frequency**
Preparation time
Night before selling
6(40.0)
Morning of selling
4(26.7)
During day
5(33.3)
Place of preparation
At home
6(40.0)
At the site of sell
9(60.0)
Food storage
In wheelbarrows
5(33.3)
Openly in the stalls/uncovered
3(20.0)
In sealed (transparent/opaque) containers
8(46.7)
Leftovers
Consumed
7(46.7)
Stored for use next day
8(53.3)
Throw away
0
Care of Utensils by the vendors
Method of washing utensils before and after serving to another consumer
Hot water and soap
2(13.3)
Cold water and soap
13(86.7)
Frequency of washing of serving utensils (plates and cutlery)
Only at the end of the day
1(6.7)
Immediately after use
5(33.3)
When exhausted
9(60.0)
How many times water is used before replacement
Once
1(6.7)
Twice
3(20.0)
Several
11(73.3)
Covered utensils
1(6.7)

Table 11 Hygienic practices of vendors and vending environment of street foods in Uyo Metropolis

Parameter **Frequency** **(%)**
Use of apron
Half apron used
5(33.3)
Not used
10(66.7)
Hair
Covered
0
Uncovered
15(100)
Have long finger nails
6(40.0)
Handling money while serving food
14(93.3)
Handles food with bare hand
10(66.7)
Hand washing method
Using clean water
4(26.7)
Using clean water and soap
10(66.7)
Any water
1(6.7)
Hand washing
After blowing of nose and scratching
4(26.7)
After using toilet
15(100)
After touching money
0
Medical check up
Once a year
2(13.3)
When I feel sick
12(80.0)
No check-up
1(6.7)
Hygienic status of vending environment and waste disposal practices of food vendors
Vending stall protected from sun, dust and wind
8(53.3)
Far from rubbish, waste water, toilet facilities, open drains and animals
2(13.3)
There were adequate waste or food disposal facilities available
5(33.3)
Presence of houseflies in the stall

10(66.7)

Frequency of waste disposal
Daily
5(33.3)
Twice weekly
2(13.3)
Weekly
8(53.5)

Bins were available for garbage disposal
9(60.0)

DISCUSSION

In other to prevent the occurrence of food-borne diseases, it is necessary to ensure that foods sold to consumers are hygienic and safe for consumption. The microbial load and the presence of pathogenic microorganisms in food will reflect the food hygienic quality and the associated potential health hazards **(Hoque *et al.*, 2015).** Two fungal species were encountered; *Aspergillus fumigatus* and *Penicillum frequentans.* These fungal species are airborne and not part of the food analyzed, but in large numbers can produce poisonous carcinogens like aflatoxins **(Freese *et al.*, 1998; Acho-Chi, 2002; Rizzon and Miele, 2012; Oz *et al.*, 2014)**

All of the sampled foods contain total bacterial counts of 10^5 cfu/g. These foods are therefore considered fit for human consumption **(FAO, 2005).** It was also observed that *Salmonella Shigella* count were very high in all the food samples considering the standard for food which is 0 cfu/g for the *Salmonella Shigella* **(AOAC, 1999; FDA, 2001).** All the tested samples were infected or contaminated with *salmonella* or *shigella* species. The presence of *Salmonella Shigella* in food is an indication of poor handling, transportation, exposure of these food samples after cooking since these organisms are water borne *(Dilbaghi and Sharma, 2007; Cogan et al., 2013).*

Escherichia coli count in the food samples were considered high in relation with standard demands of 0 cfu/g *E. coli* count for quality **(WHO 2002; FDA, 2007; Chang *et al*., 2009).** *Escherichia coli* are the indicator bacteria that suggest either direct or indirect fecal contamination. The presence of *E. coli* might not possess health hazards but when reach an elevated numbers or the presence of certain enteropathogenic or toxigenic *E. coli* strains such as *E. coli* O;157:H7, food borne illness is more likely to occur **(Cabedo *et al*., 2008; Mhone *et al*., 2011).**

Vibrio cholera species is the ethologic agent of cholera disease (which is indicated with Rice and stew-water watery stools). It kills fast in a matter of hours from the inception of the symptoms **(Lando, 2006; FDA, 2007; Vigano *et al,* 2007***)***.** Cholera infection is a serious and dangerous disease in any community because of the speed of circulation even in air. The presence of *Vibrio cholera* species in food is an indication of very recent contamination of samples because the organism does not survive for a long time after separation from the host and is carried by water medium. It is also an indication of contamination after cooking since it responds fast to heating; it has no spores **(Seper *et al,* 2014).** Although the cell counts were low, but standard demands that there should be no *Vibrio cholera* cell in edible food or water because it is enough for one cell to initiate an epidermis infection **(Seper *et al,* 2014).**

Sixteen bacterial species were identified in the food samples. Bacterial species isolated and characterized was in line with taxonomic scheme of **Cowen and Steel (1996)**. The bacterial isolates included the following: *Aerococcus viridans, Cornynebacterium uberis, Escherichia coli, Klebsiella species, Lactobacillus salivarius, Micrococcus luteus, Micrococcus varians , Proteus mirabilis, Pseudomonas pyogenes, Pseudomonas pyogenia, Salmonella paratyphi, Salmonella typhi, Staphylococcus albus, Staphylococcus aureus, Staphylococcus epidermidis, Vibrio cholera* by comparing their morphological and biochemical characteristics with standard reference organisms **(Cheesbrough, 1984).** The findings of this work are in agreement with the work of **Oranusi and Braide (2012).**

Klebsiella species also obtained from this study produce exogenic slimy metabolic by-product which covers the cell and provides protection from chemical attacks. This organism is one of those that cause high drug resistant pneumonia once it gets to the blood stream and lower regions of the lungs.

Micrococcus varians which is a causative agent of tooth decay was found in Rice and stew, porridge beans and ekpangkukwo. The presence of *Micrococcus varians* is an indication of saliva contamination by food handlers and hawkers since it is not a normal flora of the environment **(Penrose *et al*., 2010).** The organism produces cementum at the root of the teeth which produces cementum at the root of the teeth which prevents the organism from chemical attack and heat. It produces an enzyme in the cementum which dissoles the calcium and phosphorus (teeth enamel) and tiny blood capillaries at the root of the teeth thus the teeth will shake with painful inflammation and subsequent removal. If the cementum is not removed in time, it will cause a plague and removal of all the teeth in the mouth **(Vigano *et al*., 2007; Makun *et al*., 2009).**

It was also observed that *Lactobacillus salivarius* was present in rice and stew, porridge beans, ekpankukwo and noodles. The presence of this organism in prepared food is a confirmation of poor food handling by the vendors. *Lactobacillus salivarius* is a normal flora of human gut (mouth), as well as a very recent oral contamination of the food sample.

Staphylococcus aureus was isolated from Rice and stew, porridge beans, moi moi and noodles. *Staphylococcus aureus* is a normal flora of the human body especially the nasal cavity and the ear. *Staphylococcus aureus,* a golden yellow colony on Nutrient Agar is a very serious pathogenic bacterium which is characterized by vomiting and diarrhea. It is gram positive cocci that produce exogenic by products one hour after ingestion of the organism with food **(Kateete *et al*., 2010; Di Ciccio *et al*., 2015).** The exogenic enzyme reacts with the antibodies in the cell wall of the stomach and causes serious contraction of the stomach to bring out its content. If the exogenic enzyme gets into the blood stream, the body will wall off the entire area to arrest the circulation of the bacteria by product which is capable of causing serious shock or stroke. Toxin production strain of *Staphylococcus* is the leading cause of gastro enteritis following handling of food by the person who carries the microorganism in their nose and skin **(Kadariya *et al*., 2014).** These bacteria present in about 60% of a given human population and can also survive on hand knives, chopping board and dish cloths.

Non-pathogenic bacteria isolated from the vended foods included; *Staphylococcus epidermidis, Cornyebacterium uberis, Aerococcus viridians, Staphylococcus albus, Micrococcus luteus.* They are environmental microorganisms that are present in the air or on human body surface. The act as protective organism to the skin thus preventing the skin from aggressive attack by other organisms. Their presence in food shows that the samples were not properly handled **(Christison *et al*., 2008),** *Pseudomonas pyogenes* and *Pseudomonas pyogenia* are always found in the soil. *Pseudomonas pyogenes* is the ethologic agent of gas gangrening and black spotted carbuncles of the skin; the infection has no cure. They were found in Rice and stew, porridge beans, ekpangkukwo and noodles. Other gram negative bacteria such as *Proteus mirabilis* were also isolated. This organism is always present in contaminated meat. It produces a greenish color on the meat 12 h after contamination because the enzyme that turns the meat green is endogenic and can only be released when the cell is dead. This is a character for all gram positive bacteria that produces endotoxins **(Donkor, 2009; Powell, 2010)**. These foods are often prepared by heating but gets cold by the time it is served because the sellers are not able to keep the food at a good holding temperature and therefore ambient temperatures provide a suitable protected from dust, sun and wind; condition for growth of the microorganisms **(Mensah *et al.,* 2002).** The most contaminated food samples were Rice and stew with 12 species and the least contaminated was moi moi samples. This could be attributed to the fact that the samples were put in plates and nylons before cooking, reducing contact of food samples with handlers.

The study revealed that street food trade was conducted by both males (26.7%) and females (73.3%). Generally, the higher proportion of female vendors can be explained by the fact that women are responsible for traditional cooking and child care and generally have lower education and skills levels, which result in their greater involvement in informal sector's such as street food vending. Higher proportions of women vendors may actually be advantageous as female vendors have been reported to provide street floods with higher nutritional quality than male counterparts **(Ohiokpehai, 2003).** This is in agreement with studies in other developing countries including Botswana, Ghana, Brazil, Kenya and Uganda **(Ohiokpehai, 2003; Hanashiro, 2005; Muinde & Kuria, 2005; Muyanja *et al*., 2011).**

There were two major types of vending units involved in this study: the stationary (66.7%) and the mobile. About 26.7% of the vendors cooked food on morning sale, 33.3% cooked food while selling, and 40.0% cooked food on advance consumption. Most of the vendors used the same water to rinse their utensils several times during the day while only 6.67% covered the utensils. The repeated use of the same water may lead to cross contamination from the water to cooked food via the rinsed utensils **(FAO, 2005; Mahale *et al.,* 2008)**. None of

the vendors covered their hai during preparing and serving of food; 40% of them kept long finger nails, 93.3% of them handled money while serving food and 66.7% handle food with bare hands. According to the FAO, the hands are a crucial faxtor in the contamination and spreading of faecal-oral transmitted bacteria; therefore, this risk greatly enhances when food is handled with bare hands **(FAO, 1997**); also money is dirty and may cause contamination of food **(FAO, 1997).** About 53.3% of the stalls were the stalls were protected from dust, sun and wind; only 13.35 were far from rubbish, waste water, toilet facilities, open drains and animals. Dust potentially carries pathogens and therefore may become a vector for their transmission to prepared foods. Similar observations were reported by **Muinde & Kuria (2005)** and **Mensah *et al*, (2002)** in studies conducted on street foods in Nairobi (Kenya) and Accra (Ghana), respectively.

About 60% of the stalls involved had bins for garbage disposal hence the remaining 40% disposed their garbage just near the stalls. According to the FAO, the place of food preparation should be kept clean at all times and should be far from any source of contamination such as rubbish, waste water, dust and animals **(Yassin & Almonuqatea, 2010).** In addition, the vendors disposed of their waste food and water next their stalls. Consequently, this dirty environment attracted flies, which are not only an indication of poor hygiene and sanitary conditions, but they are also vectors of fecal pathogens.

CONCLUSION

From the results obtained, it is observed that the selected vended foods analyzed contain different species of bacteria and fungi which poses health risks to consumers of these foods. However, these risks can be reduced if better sanitary, handling, processing and storage measures are employed as studies have shown that a greater percentage of contamination comes from unhealthy, prolonged exposure to the environment, unhygienic practices as well as handling. In summary, it can be generally concluded that street foods are vended in Uyo Metropolis under unsafe (unhygienic) conditions consisting of the dirty open air environments in which the foods compounded by poor food handling practices and often inadequate storage conditions.

RECOMMENDATIONS

From our research, it has been shown that some vended foods in Uyo metropolis are not microbiologically safe for human consumption as they are capable of causing different types of disease. Therefore, it is recommended that food vendors should be educated through seminars, workshops on the need to prepare foods only in hygienic environment, using sterile as well as adopted improved sanitary handling, processing and storage methods. It is also recommended that proper handling and packaging of foods by food vendors to avoid direct contact with hands etc. which could be sources of contamination. Also the microbiological quality of portable water used by street food vendors should be assessed because water is a potential source of contamination during the preparation, processing and vending of street foods.

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EFFECT OF GINGER EXTRACT ON THE VIABILITY OF LACTIC ACID BACTERIA AND SENSORY CHARACTERISTICS OF DAIRY YOGHURT AND SOY YOGHURT

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ABSTRACT

Yoghurt is usually produced from cow milk which is unsuitable for vegetarian diet. Chemical preservative used for its preservation may have adverse effect on the lactic acid bacteria. This research was designed to evaluate the effect of ginger extract on the viability of lactic acid bacteria, physico-chemical property and sensory attributes of bio preserved functional yoghurt produced from dairy milk and soy milk during storage at refrigerated temperature. The result obtained showed that yoghurt produced from soy milk (5.67 – 7.52 CFU/ml) had higher lactic acid bacteria count than yoghurt from dairy milk (4.71 – 7.31 CFU/ml) throughout the period of storage. Ginger extract was more active against fungi in dairy yoghurt than (0 - 5.09 CFU/ml) soy yoghurt (0 – 5.29 CFU/ml). The pH was generally lower in dairy yoghurt (4.29 - 4.67) than soy yoghurt (4.69 – 5.14) while the titratable acidity was higher in dairy yoghurt (1.30 – 2-01%) than soy yoghurt (0.7 – 1.54%). Addition of ginger extract improved the texture and flavour of soy yoghurt. It is therefore suggested that functional yoghurt with good sensory attributes can be produced from soy milk and dairy milk using ginger extract as bio-preservative.

Keywords: yoghurt, dairy, soy milk, ginger, lactic acid bacteria

INTRODUCTION

Yogurt is one of the most widely distributed dairy products. It has a smooth texture, sour taste and pleasant flavor. It is obtained by inoculating dairy milk with *Lactobacillus delbrueckii spp. bulgaricus* and *Streptococcus thermophilus* **(Qureshi *et al*., 2011)**. Its popularity and high consumption are due to its nutritional value and the therapeutic effects of starter bacteria during fermentation **(Gündoğdu *et al*., 2009)**. Lactic acid bacteria produce organic acids which exerts a positive effect on food through their activities during fermentation by imparting desirable flavors and inhibiting a variety of food spoilage and pathogenic organisms **(Fowoyo and Ogunbanwo, 2010; Okpara *et al*., 2014; Malomo *et al*., 2018)**. The desirable flavor of yogurt is associated with the presence of several compounds; nonvolatile acids such as lactic and pyruvic, volatile acids such as butyric and acetic, and other components like amino acids and carbonyl compounds such as acetaldehyde and **diacetyl (Yangilar and Yildiz, 2017)**. Yogurt produced from cow milk is consumed in both developing and developed countries. However, demands for alternatives to cow milk is growing day by day due to allergy, enriched nutrition and a desire for an alternative for vegetarian **(Ranjana *et al*., 2016)**.

Legumes are sources of low-cost dietary vegetable proteins and minerals when compared with animal products such as meat, milk, fish and egg **(Malomo *et al*., 2019)**. The usefulness of the legumes in developing high protein foods in meeting the needs of the vulnerable groups of the population is now well recognized, and several high protein energy foods have been developed industrially, in different parts of the world **(Ayo *et al*., 2012)**.

Soybean (*Glycine max*) has been reported to contribute significantly towards protein, mineral, fat and B complex vitamins needs of people in developing countries. Fermentation of soymilk could modify or improve its flavor and texture so that it becomes more acceptable and can also lead to production of new types of soy product similar to cultured dairy products **(Akabanda *et al*., 2010; Obadina *et al*., 2013)**. Different flavoring ingredients have been added during manufacturing of yoghurt to improve the flavor and artificial flavors are mostly used to improve yoghurt flavor during production.

The use of spices as flavor during yoghurt manufacturing could also be useful considering the fact that, they are plants rich in bioactive components with health benefits and constitute a potential source of minerals and vitamins in addition to their antimicrobial **activities (Aswal *et al*., 2012; Njoya *et al*., 2018)**. The increase in the demand for safe foods, with less chemical additives, increased the study of bio preservatives, which do not injure the host or the environment **(Olaniran *et al*., 2015).** Ginger **(*Zingiber officinale*)** contains a fusion of aroma oils such as volatile and nonvolatile oils, pungent compounds (gingerols, shogaols), which have local effect on digestive system **(Churbasik *et al*., 2005; Ozgoli et. al., 2009; Singh *et al*., 2017; Simeon *et al*., 2018).** Many authors reported that it is endowed with antibacterial and antifungal properties **(Al-Amin *et al*., 2006; Mendi, 2011; Njoya *et al*., 2018).** This study was carried out with a view to determining the effect of ginger extract on the viability of lactic acid bacteria, safety and consumer perception of dairy and soy yoghurt preserved with ginger extract and to advance the use of soy milk in the production of yoghurt.

MATERIALS AND METHODS

Procurement of materials

Cow milk was obtained from a farm at Alakowe, Ile – Ife, ginger and commercial yoghurt starter culture were obtained from Oja tuntun, Ile - Ife. Soybean was obtained from Institute of Agricultural Research and Training, Ibadan.

Production of dairy and soy yoghurt

Modified method of **Yangilar and Yildiz (2017)** was used for the production of dairy yoghurt. Cow's milk was heated up to 85ºC for 20 min and then cooled. Starter (*Streptococcus thermophilus, Lactobacillus delbrueckii spp. bulgaricus* and *Lactobacillus acidophilus*) was added in the milk (in the rate of 1.5%). Ginger extract was incorporated into inoculated milk in different concentrations (2 and 4%), incubated at 43ºC for 10 h in an incubator and stored in the refrigerator (4ºC ± 1) for further analysis.

Soy yoghurt was produced using modified method of **Ranjana *et al*. (2016)**. Soybean was cleaned, washed and soaked for 12 h in water at room temperature. The beans were boiled for 20 min and hulls were removed by rubbing between palm and the resulting cotyledons blended. The resultant slurry was filtered through 3 layers of cheese-cloth and the residue was discarded. The supernatant was boiled for 30 min at 100ºC and then cooled down to 35ºC. The soy milk obtained was divided into three portions. The first portion served as control, pasteurized aqueous ginger extract (2%) was added to second portion and (4%) to the third portion. Soy yoghurt was obtained by addition of commercial culture containing *Streptococcus thermophilus, Lactobacillus delbrueckii spp. bulgaricus* and *Lactobacillus acidophilus*. Starter culture (1.5 %) was added soy milk and incubated at 43ºC for 10 h and stored in the refrigerator (4 ºC ± 1) for further analysis.

Preparation of aqueous ginger extract

Ginger extracts was obtained from ginger paste by modified methods described by **Kaushik and Goyal (2011)** and **Abd El – Aziz *et al.* (2015)** described by **Njoya *et al.* (2018)**. Ginger root was washed, peeled and grated to form a paste. Decoction was done by boiling 50 g of ginger paste in 1.6 L of distilled water till one fourth (1/4) of the initial volume was attained. The solution was filtered twice using a muslin cloth, allowed to sediment and the supernatant was collected. Extracts collected was pasteurized at 75°C for 15 min, cooled at room temperature and kept in the refrigerator at 4 ± 1°C for further uses.

Microbial analysis

Yoghurt samples were analyzed on a weekly basis using the pour plate method. Samples (5 ml) were weighed into stomacher bag and homogenized with 45 ml of sterile maximum recovery diluent. The resulting mixture was serially diluted and 1.0 ml of appropriate dilution was dispensed into Petri dish. Nutrient agar was used for total viable count, de Man Rogosa and Sharpe (MRS) agar for Lactic acid bacteria count and potato dextrose agar (PDA) for fungi count **(Harrigan and McCance, 1976; Harrigan, 1998)**. Plates were incubated anaerobically for lactic acid bacteria at 35ºC for 72 h and incubated in inverted position for total viable microorganisms and fungi at 35ºC for 24 h and at 25ºC for 3 to 5 days respectively **(Harrigan, 1998)**. The colonies on each plate were counted using a Gallenkamp colony counter and pure isolates of the representative colonies were obtained by streaking on media of their primary isolation, incubated appropriately and kept in agar slant under refrigeration condition.

Bacteria isolates were identified using cultural and morphological characteristics, Gram's staining techniques and biochemical tests following the scheme of **Harrigan and McCance (1976)** and **Wood and Holzapfel (1995)**. Fungi isolates were identified using colony characteristics, cell shape, size, type of budding and cell aggregation was determined by microscopy (Leica DM500 Model 13613210), and the ability of isolates to assimilate various carbon sources and nitrate were assessed **(Beech *et al.*, 1968; Barnett *et al*., 2000)**.

pH of soy and dairy yoghurt

pH meter (Scholar 425) was standardized with buffer 4.0 and 7.0. Yoghurt sample (10 ml) was dispensed into a beaker and the electrode was inserted. The pH of the sample was recorded when the reading stabilized **(AOAC, 2005)**.

Titratable acidity of soy and dairy yoghurt

Sample (10 ml) was measured into conical flask, 10 ml of distilled water was added and stirred to homogenize the sample. Three drops of phenolphthalein indicator was added to 10 ml aliquots of filtrate and was titrated against 0.1 N NaOH **(AOAC, 2005)**.

Sensory evaluation

A group of 15 experienced panelists chosen from the students and staff of the Department of Food Science and Technology, Obafemi Awolowo University, Ile – Ife evaluated the dairy and soy yogurt samples preserved with ginger. Panelists were presented with a cup filled with 100 ml of each sample to score. Samples were evaluated for appearance, color, texture, flavor, and taste using 9 -point Hedonic scale where the lowest was 1 and highest was 7. All the samples were provided the same test conditions and the panelist were allowed to clean their palates with water after tasting each sample **(Yangilar and Yildiz, 2017)**.

Statistical analysis

Data obtained were subjected to Analysis of Variance using SPSS (version 17 incorporation, Chicago, Illinois, USA). Means of samples was separated using Duncan Multiple range Test.

RESULTS AND DISCUSSION

Effect of ginger extract on the viability of lactic acid bacteria count of dairy and soy yoghurt

The result of the lactic acid bacteria is shown in Table 1. The count was generally higher in soy yoghurt (5.67 – 7.52 CFU/ml) than dairy yoghurt throughout the period of storage (4.71 – 7.31 CFU/ml). Lactic acid bacteria count decreased in both dairy yoghurt and soy yoghurt with increase in ginger extract. Soy yoghurt support the growth of lactic acid bacteria more than dairy yoghurt during the period of storage. This may be due to the

Table 1 Effect of Ginger Extract on the Lactic Acid Bacteria Count of Yoghurt (CFU/ml)

Samples	Week 0	Week 1	Week 2	Week 3
SY0	7.52^{a}±0.03	7.22^{a}±0.20	7.31^{a}±0.02	6.45^{a}±0.05
SY2	7.46a±0.06	7.05^{b}±0.03	7.00^{b}±0.06	6.13^{b}±0.02
SY4	7.43^{ab}±0.03	6.45^{c}±0.04	6.53^{c}±0.01	5.67^{c}±0.05
DY0	7.31^{bc}±0.01	6.35^{d}±0.02	5.09^{e}±0.03	5.28^{d}±0.04
DY2	7.19^{cd}±0.08	6.90^{d}±0.02	5.68^{d}±0.05	4.94^{e}±0.04
DY4	7.11^{d}±0.04	6.40^{c}±0.05	5.59^{d}±0.02	4.71^{f}±0.01

SY0: Soy yoghurt; SY2- Soy yoghurt with 2% ginger extract; SY4- Soy yoghurt with 4% ginger extract; DY0: Dairy yoghurt; DY2: Dairy yoghurt with 2% ginger extract; DY4: Dairy yoghurt with 4% ginger extract. Values are means of three replicates ± standard error. Means followed by different superscript in the same column are significantly different at $p < 0.05$ lower pH of the dairy yoghurt.

The reduction in lactic acid bacteria count could be due to accumulation of organic acids and waste products produced by bacterial activity such as hydrogen peroxide **(Shah, 2000; Amal, 2013)**. The organic acid produced also gives the yoghurt its distinctive sour taste and pleasant aroma **(Adepoju *et al*., 2012)**. **Amal (2013)** reported that the presence of soybean in fresh cow or camel milk-yogurts significantly increased the *Lactobacillus* spp. compared to respective plain-yoghurt.

Effect of ginger extract on the total viable count of dairy and soy yoghurt

The total viable count of dairy and soy yoghurt is presented in Table 2. Count was significantly higher ($p < 0.05$) in soy yoghurt (6.05 - 7.76 CFU/ml) than dairy yoghurt (4.99 – 7.32 CFU/ml) throughout the period of fermentation. Ginger extract which was used as both flavoring agent and bio-preservatives reduced the growth of the microorganisms in both soy and dairy yoghurt (4.99 - 7.61 CFU/ml). The higher the concentration of ginger, the lower the growth of microorganisms. Though the effect was not significant in dairy yoghurt without preservative and dairy yoghurt preserved with ginger from the beginning of storage to the second week but it was significantly lower ($p > 0.05$) at the third week. According to **Adesokan *et al*. (2010)**, ginger could increase the shelflife of food due to its antimicrobial nature.

Table 2 Effect of Ginger Extract on the Total Viable Count Yoghurt (CFU/ml)

Samples	Week 0	Week 1	Week 2	Week 3
SY0	7.76^{a}±0.03	6.35^{c}±0.05	7.76^{a}±0.04	6.81^{a}±0.01
SY2	7.61^{ab}±0.09	6.09^{d}±0.04	7.37^{c}±0.02	6.53^{b}±0.03
SY4	7.54^{b}±0.04	6.05^{d}±9.05	7.61^{b}±0.07	6.13^{c}±0.02
DY0	7.32^{c}±0.02	6.91^{a}±0.01	5.70^{d}±0.03	5.19^{d}±0.06
DY2	7.28^{c}±0.04	6.88^{a}±0.03	5.63^{d}±0.03	5.03^{e}±0.03
DY4	7.21^{c}±0.03	6.70^{b}±0.05	5.13^{e}±0.05	4.99^{e}±0.05

SY0: Soy yoghurt; SY2- Soy yoghurt with 2% ginger extract; SY4- Soy yoghurt with 4% ginger extract; DY0: Dairy yoghurt; DY2: Dairy yoghurt with 2% ginger extract; DY4: Dairy yoghurt with 4% ginger extract. Values are means of three replicates ± standard error. Means followed by different superscript in the same column are significantly different at $p < 0.05$

Effect of ginger extract on the fungi count of dairy and soy yoghurt

The result of fungi count is shown in Table 3. There was no fungi growth in both dairy and soy yoghurt samples at week zero. Soy yoghurt had fungi count from week one to week three (3.64 - 5.05 CFU/ml) while dairy yoghurt had count at the third week (4.01 – 4.58 CFU/ml), Addition of ginger extract reduced the growth of fungi in the yoghurt samples. This showed that ginger is effective

against fungi. Increase in fungi count could be as a result of production of acid by lactic acid bacteria which reduced the pH and enhanced yeast multiplication. During fermentation, bacteria provide the rapid acidic environment for fungi and the fungi provide essential metabolites such as pyruvates, vitamins and amino acids for the bacteria **(Owuzu-Kwarteng and Akabanda, 2014; Malomo *et al*., 2019).**

Table 3 Effect of Ginger Extract on the Fungiount Yoghurt (CFU/ml)

Samples	Week 0	Week 1	Week 2	Week 3
SY0	Nil	4.48^{a}±0.04	4.58^{a}±0.03	5.29^{a}±0.01
SY2	Nil	3.70^{b}±0.01	4.46^{b}±0.03	5.05^{b}±0.03
SY4	Nil	3.64^{c}±0.02	4.46^{b}±0.05	4.89^{c}±0.06
DY0	Nil	Nil	Nil	4.09^{d}±0.04
DY2	Nil	Nil	Nil	4.01^{d}±0.03
DY4	Nil	Nil	Nil	4.58^{cd}±0.04

SY0: Soy yoghurt; SY2- Soy yoghurt with 2% ginger extract; SY4- Soy yoghurt with 4% ginger extract; DY0: Dairy yoghurt; DY2: Dairy yoghurt with 2% ginger extract; DY4: Dairy yoghurt with 4% ginger extract. Values are means of three replicates ± standard error. Means followed by different superscript in the same column are significantly different at $p < 0.05$

Effect of ginger extract on microorganisms during storage of dairy and soy yoghurt

The occurrence pattern of microorganisms in dairy and soy yoghurt (Table 4) showed the dominance of lactic acid bacteria during storage. *Lactobacillus delbrueckii spp. bulgaricus, Streptococcus thermophilus* and *Lactobacillus acidophilus* were viable till the third week of storage. *Bacillus subtilis* was only isolated from soy yoghurt without ginger extract. **Singh *et al*. (2017)** reported that ginger showed antimicrobial activity against *E coli, Salmonella typhi* and *Bacillus subtilis. Torulopsis versatilis* was isolated from soy yoghurt without ginger extract and with 2% ginger extract from week one to week three but were isolated in soy yoghurt containing 4% ginger extract in week two and week three. *Saccharomyces lactis* was also isolated from all dairy yoghurt samples at week three. Lactic acid bacteria (LAB) are prominent in fermentation of food. They produce organic acids such as lactic acid, butyric acid, acetic acid and other metabolites from sugar present in food and this has positive effect on aroma, taste, texture and shelf life of food. LAB also produces antimicrobial substances known as bacteriocin which inhibit the growth and activities of pathogenic microorganisms. Their common occurrence in food contributes to their acceptance **(Malomo *et al*., 2019)**. Bacillus spp. was also isolated from fermentation of legume-based food by different authors **(Ezeama and Ihezie, 2006; Farinde *et al*., 2014, Malomo *et al*., 2019)**.

Effect of ginger extract on the pH of dairy and soy yoghurt during storage

The pH generally decreased in dairy yoghurt throughout the period of storage (Fig. 2). pH was a lower in dairy yoghurt (4.29 - 4.67) than soy yoghurt (4.69 – 5.14) as seen in Figure 1. Increase was observed in all soy yoghurt samples from week one to week three. Adepoju *et al*. (2012) attributed decrease in pH to metabolism of sugar which led to acid production by the relevant microorganisms. Addition of ginger increased the pH of both dairy and soy yoghurt. **Ihemeje *et al*. (2015)** also reported an increase in pH with addition of ginger.

Table 4 Effect of ginger extract on the microorganisms during storage dairy and soy yoghurt.

Samples	Microorganism	Week 0	Week 1	Week 2	Week 3
SY0	*Lactobacillus acidophillus*	+	+	+	+
	Lactobacillus delbrueckii spp. bulgaricus	+	+	+	+
	Streptococcus thermophilus	+	+	+	+
	Lactobacillus plantarum	+	+	+	+
	Bacillus subtilis	-	+	+	+
	Torulopsis versatilis	-	+	+	+
SY2	*Lactobaccillus acidophillus*	+	+	+	+
	Lactobacillus delbrueckii spp. bulgaricus	+	+	+	+
	Streptococcus thermophilus	+	+	+	+
	Lactobacillus plantarum	+	+	+	+
	Torulopsis versatilis	-	+	+	+
SY4	*Lactobaccillus acidophillus*	+	+	+	+
	Lactobacillus delbrueckii spp. bulgaricus	+	+	+	+
	Streptococcus thermo nnnnnnnnilphilus	+	+	+	+
	Lactobacillus plantarum	+	+	+	+
	Torulopsis versatilis	-	-	+	+
DY0	*Lactobacillus acidophillus*	+	+	+	+
	Lactobacillus delbrueckii spp. bulgaricus	+	+	+	+
	Streptococcus thermophilus	+	+	+	+
	Lactobacillus plantarum	+	+	+	+
	Saccharomyces lactis	-	-	-	+
DY2	*Lactobacillus acidophillus*	+	+	+	+
	Lactobacillus delbrueckii spp. bulgaricus	+	+	+	+
	Streptococcus thermophilus	+	+	+	+
	Lactobacillus plantarum	+	+	+	+
	Bacillus subtilis	-	+	+	+
	Saccharomyces lactis	-	-	-	+
DY4`	*Lactobacillus acidophillus*	+	+	+	+
	Lactobacillus delbrueckii spp. bulgaricus	+	+	+	+
	Streptococcus thermophilus	+	+	+	+
	Lactobacillus plantarum	+	+	+	+
	Saccharomyces lactis	-	-	-	+

SY0: Soy yoghurt; SY2- Soy yoghurt with 2% ginger extract; SY4- Soy yoghurt with 4% ginger extract; DY0: Dairy yoghurt; DY2: Dairy yoghurt with 2% ginger extract; DY4: Dairy yoghurt with 4% ginger extract. +: present; -: absent

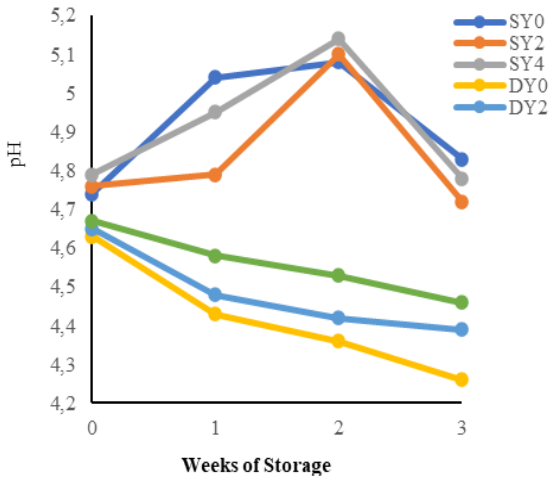

Figure 1 Effect of ginger extract on pH of dairy and soy yoghurt during storage
SY0: Soy yoghurt; SY2- Soy yoghurt with 2% ginger extract; SY4- Soy yoghurt with 4% ginger extract; DY0: Dairy yoghurt; DY2: Dairy yoghurt with 2% ginger extract; DY4: Dairy yoghurt with 4% ginger extract.
Effect of ginger extract on the titratable acidity of ginger spiced dairy and soy yoghurt sample during storage

The titratable acidity of ginger spiced yoghurt samples is compared in Fig. 2. The titratable acidity was generally lower in soy yoghurt (0.7 – 1.54%) than dairy yoghurt (1.30 – 2-01%) throughout the period of storage. The highest acidity in dairy yoghurt was observed in dairy yoghurt without ginger extract and it is the same with soy yoghurt. This is an indication that ginger can inhibit lactic acid bacteria from carrying out their normal activity. Acidity is accepted to take place among the significant factors influencing shelf life and consumer acceptability of yogurt **(Al–Otaibi and El–Demerdash, 2008; Yangilar and Yildiz, 2017)**. The titratable acidity decreased with increase in ginger extract in both soy and dairy yoghurt. The reduction of titratable acidity of yoghurt with the increase in ginger extract could be due to the dilution effect **(Njoya *et al*., 2018)**. **Obadina *et al.* (2013)** attributed decrease in pH and increase in titratable acidity to the accumulation of some organic acid and acetic acid resulting from the activities of some fermentative organisms such as lactic acid bacteria and yeasts in the fermenting foods.

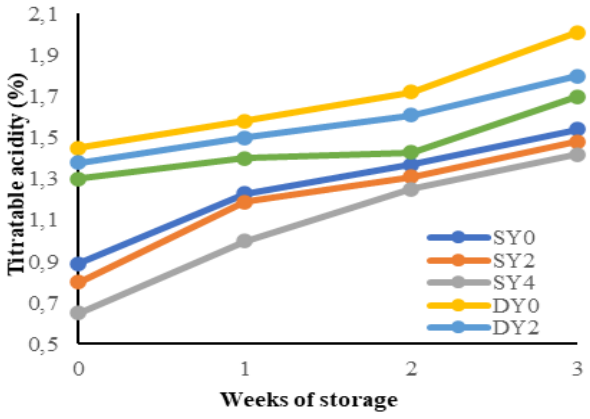

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Figure 2 Effect of ginger extract on the titratable acidity of dairy and soy yoghurt sample during storage.
SY0: Soy yoghurt; SY2- Soy yoghurt with 2% ginger extract; SY4- Soy yoghurt with 4% ginger extract; DY0: Dairy yoghurt; DY2: Dairy yoghurt with 2% ginger extract; DY4: Dairy yoghurt with 4% ginger extract.

Effect of ginger extract on sensory assessment of soy and dairy yoghurt

The result of the sensory assessment carried out on dairy yoghurt and soy yoghurt is seen in Table 5. Dairy yoghurt (5.8 – 6.7) had significantly higher ($p < 0.05$) score than soy yoghurt (4.4 – 4.7) and addition of extract ginger did not have significant effect ($p > 0.05$) on the color of both dairy and soy yoghurt samples. Soy yoghurt containing 4 % ginger had the highest score for texture and the score is not significantly different ($p > 0.05$) from other yoghurt samples. Ginger contains an important amount of starch (up to 40%, dry basis) with several potential applications which is considered as one of the most thickening agents used for yogurt manufacturing **(Ibrahim, 2015).** There was no significant difference ($p > 0.05$) in the flavor of spiced dairy and soy yoghurt. The flavor of dairy yoghurt decreased with increase in ginger extract while the flavor score of soy yoghurt increased. There was no significant difference ($p > 0.05$) in the taste of ginger spiced dairy and soy yoghurt. Ginger extract had no significant effect on both dairy and soy yoghurt samples but rather increased the overall acceptability of soy yoghurts. Score for overall acceptability shows similarities between spiced dairy and soy yoghurt. The refreshing pleasant aroma, biting taste and carminative property of ginger makes it as an indispensable ingredient of food processing throughout the world **(Singh *et al*., 2017).**

Table 5 Mean Sensory Score for Soy and Dairy Yoghurt Preserved with Ginger Extract

Samples	Colour	Texture	Flavour	Taste	Overall acceptability
DY0	$6.7^{a}\pm0.67$	$5.4^{a}\pm1.35$	$6.1^{a}\pm0.88$	$6.6^{a}\pm0.52$	$6.6^{a}\pm0.70$
DY2	$6.2^{a}\pm0.63$	$5.4^{a}\pm1.79$	$5.8^{ab}\pm0.63$	$5.7^{a}\pm0.67$	$5.9^{ab}\pm0.73$
DY4	$5.8^{a}\pm1.03$	$5.5^{a}\pm1.45$	$5.4^{ab}\pm0.84$	$5.6^{a}\pm0.84$	$5.7^{ab}\pm0.82$
SY0	$4.4^{b}\pm1.51$	$4.8^{a}\pm1.48$	$4.4^{bc}\pm1.65$	$4.4^{b}\pm1.65$	$4.2^{c}\pm1.75$
SY2	$4.7^{b}\pm1.57$	$5.4^{a}\pm0.70$	$5.4^{ab}\pm0.70$	$4.6^{b}\pm1.58$	$4.6^{bc}\pm1.42$
SY4	$4.4^{b}\pm1.65$	$5.6^{a}\pm1.35$	$5.3^{ab}\pm0.95$	$4.6^{b}\pm1.51$	$4.4^{bc}\pm1.51$

SY0: Soy yoghurt; SY2- Soy yoghurt with 2% ginger extract; SY4- Soy yoghurt with 4% ginger extract; DY0: Dairy yoghurt; DY2: Dairy yoghurt with 2% ginger extract; DY4: Dairy yoghurt with 4% ginger extract.

CONCLUSION

Functional yoghurt with appealing aroma and taste can be produced from both dairy milk and soy milk by using ginger extract as bio preservative. Ginger extract extended the shelflife of both dairy and soy yoghurt and had minimal effect on the viability of the lactic acid bacteria. It also inhibited the activities of fungi and pathogenic microorganisms during storage. Addition of ginger extract also improved the texture, taste and flavour of soy yoghurt. This research suggested that functional yoghurt similar to dairy yoghurt with acceptable microbiological and organoleptic quality can be also produced from soymilk for especially for vegetarian and low-income earners.

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SCREENING OF RHAMNOLIPIDS FROM *Pseudomonas* spp. AND EVALUATION OF ITS ANTIMICROBIAL AND ANTIOXIDANT POTENTIAL

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ABSTRACT

Biosurfactants are amphipathic compounds that are useful in industries and are also used in bioremediation. Rhamnolipids (RL) are biosurfactant mostly produced by the genus *Pseudomonas*. This study screened 6 strains of *Pseudomonas* isolated from underwater cave sediments to determine if they were capable of producing rhamnolipids. The biosurfactant production of potential strains was screened by Parafilm-M Test, Oil spreading technique, Microplate assay, Emulsification Index, CTAB Agar plate method, Anthrone test, and Thin Layered Chromatography. The study showed that only 3 strains had the potential to produce rhamnolipids, these were *Pseudomonas poae*, *Pseudomonas fluorescens* and *Pseudomonas libanensis*. The antimicrobial activity of the crude rhamnolipid extracts was tested against two Gram-negative bacteria (*Escherichia coli*, *Serratia marcescens*) and two Gram-positive bacteria (*Bacillus cereus*, *Staphylococcus aureus*) by the conventional minimum inhibitory concentration (MIC). The antioxidant activity of the crude rhamnolipid extracts was determined through DPPH free radical scavenging assay. All rhamnolipid extracts had a positive correlation between increasing concentrations and the zone of inhibition against test microorganisms. Rhamnolipids from *P. libanensis* had the lowest MIC among the other extracts, indicating its potency against the test bacteria. The rhamnolipid extracts were effective at inhibiting the test bacteria compared to the positive control, Nitrofurantoin. The antioxidant activity of the crude rhamnolipid extracts was determined through DPPH. Crude rhamnolipid extracts exhibited antioxidant activity but not as great as that of ascorbic acid.

Keywords: Rhamnolipids; Biosurfactant; *Pseudomonas*; Antimicrobial; Antioxidant

INTRODUCTION

Marine and freshwater ecosystems are home to a diverse array of organisms due to the fluctuating environmental parameters **(Molles, 2010)**. Biodiversity in these environments are high, the organisms have various adaptations in their physiology for them to survive. One adaptation is the production of metabolites **(Bhatnagar and Kim, 2010)**. These are substances that are not required for growth by the organism but may be used for protection. These can be extracted from organisms like plants, fungi, algae, and microorganisms. The substances, when processed, can have various uses such as antimicrobial medicine, biosurfactants, and more. *Pseudomonas* is a genus of Gram-negative, aerobic, and flagellated Proteobacteria **(Bauman, 2006)**. Species of this genus can be found in terrestrial, freshwater, and marine habitats. The terrestrial *Pseudomonas aeruginosa* is known to produce bioactive substances, other species of *Pseudomonas* are not well studied in terms of bioactive substances. The genus has been known to produce about 610 antibacterial substances **(Isnansetyo and Kamei, 2009)** and has the potential to produce Rhamnolipids, a type of biosurfactant **(Cortes-Sanchez *et al.*, 2013)**.

The emerging problems of multi-drug resistant bacteria have become a global problem **(Cardozo *et al.*, 2013)** and the discovery of novel antimicrobials coming from new sources like freshwater Pseudomonas might help lessen the problem. Oil pollution in water systems is also an arising issue that threatens the aquatic and marine ecosystems **(Wiedenhoft, 2017)**. Biosurfactants are amphipathic compounds found in the bodies of organisms. They are usually in the form of glycolipids. The extraction of biosurfactants from abundant and sustainable sources like bacteria is becoming an interest in the industry due to the wide array of functions it can offer. Biosurfactants can help disperse spilled oil more efficiently than commercial surfactants and has lesser toxicity **(Cortes-Sanchez *et al.*, 2013)** so this can help address the pollution of aquatic and marine ecosystems in a more sustainable way via bioremediation. *Pseudomonas aeruginosa* is known to produce biosurfactants in the form of Rhamnolipids. Aside from being used as oil dispersant, rhamnolipids have also been discovered to have antimicrobial properties against pathogens like *Serratia marcescens* **(Haba *et al.*, 2003)** contributing to the issue on the need for novel antimicrobial sources. Rhamnolipids can also inhibit the biofilm formation of other microorganisms so they can be spread onto substrates that need protection from microbial colonization **(Singh and Cameotra, 2004)**. A study on the produced biosurfactants and antimicrobial activity of other *Pseudomonas* species will be a timely and relevant solution to address the issues we face today. This study is important because it explored the other biosurfactant and antimicrobial activity that the metabolites of the genus *Pseudomonas* can produce. There have been studies showing the potential of biosurfactants to act as antioxidants so they can delay the spoiling of food while being less toxic than BHA (butylatedhydroxyanisole) and BHT (butylatedhydroxytoluene). However, none of the published studies investigated the antioxidant activity of rhamnolipids which is important because it helps the scientific community and industries in the innovation of more sustainable ways to preserve food that does not threat the health of the people.

This study aimed to screen for the bioactivity of rhamnolipid producing *Pseudomonas* spp. isolates from the underwater cave of Casili, Balamban, Cebu, Philippines sediments with the following specific objectives in mind to: qualitatively screen for biosurfactant production of the isolated *Pseudomonas* strains through (a) Parafilm M test, (b) oil spreading assay, (c) microplate assay, (d) emulsification capacity assay, (e) CTAB agar plates, (f) anthrone test and (g) thin layered chromatography; to measure the MIC of the crude rhamnolipid extract on two Gram-positive bacteria (*Bacillus cereus* and *Staphylococcus aureus*) and two Gram-negative bacteria (*Escherichia coli* and *Serratia marcescens*); to compare the zone of inhibitions of the crude extract to those of commercial antibiotics (Ciprofloxacin and Nitrofurantoin), and to determine if crude rhamnolipid extracts exhibit antioxidant activity through
DPPH free radical scavenging method.

MATERIAL AND METHODS

Bacterial strains

The *Pseudomonas* species that were acquired from the Microbiology Laboratory of the University of San Carlos which were isolated from aquatic sediment. The strains were stored and maintained in the laboratory using PYG agar prior to the screening of biosurfactant: (1) *Pseudomonas poae*, (2) *Pseudomonas reactans*, (3) *Pseudomonas parafulva*, (4) *Pseudomonas fluorescens*, (5) *Pseudomonas libanensis* and (6) *Pseudomonas* sp. These isolated *Pseudomonas* species were used and designated as Strains 1 to 6 throughout the experiment.

Rhamnolipid Production

Each *Pseudomonas* strain was cultured in a 250-mL shake flask for the production of rhamnolipid. The method of **Wang *et al.* (2007)** was followed where the strains were first grown in nutrient broth for 24 hours and shook at 200 rpm with a temperature of 30°C before being introduced to the rhamnolipid media and incubated at the same conditions for 7 days. Then 5.0 mL of nutrient broth was inoculated in 50 mL of rhamnolipid production media, following a 1:10 ratio, similar to that of **Wang *et al.* (2007)**. The rhamnolipid media utilized in this study follows that of **Thanomsub *et al.* (2007)**. After incubation, each

culture was filtered by using a 0.2 µm Whatman membrane filter to obtain cell free broths for biosurfactant screening.

Screening for biosurfactant

Parafilm M Test

Fifty µL was taken from each of the 6 cell-free broths and was dropped in the surface of hydrophobic side of the parafilm M. After 1 minute, the diameter of the drop was measured using a ruler. A negative control which was water was also set. A drop which had larger diameter than that of the distilled water was considered positive for biosurfactant.

Oil Spreading Assay

Five hundred µL of colored oil was added to the surface of 40 mL of water in a petri dish to form a thin oil layer. Then 250 µL of cell free broth for each strain was gently placed on the center of the oil layer and after 10 seconds, the diameter of any formed clearing zone was measured with a ruler. Three trials for each strain were observed.

Microplate Assay

A 100 µL cell-free broth of each strain was taken and put into separate microwells of a 96-microwell plate. The plate was viewed using a backing sheet of paper with grid lines. The presence of biosurfactant was indicated by optical distortion of the grid lines.

Emulsification Capacity Assay

To calculate the emulsification index, 2 mL of kerosene was added to 2 mL of the cell free broth in the test tube and was vortexed at high speed for 1 minute. After 24 hours, the height of the stable emulsion layer was measured. The percentage of the emulsification index was calculated as the ratio of the height of the emulsion layer and the total height of the liquid.

CTAB Agar Plates

The *Pseudomonas* strains were cultivated in a light blue mineral salts agar plates that contained the cationic surfactant cetyltrimethylammonium bromide (CTAB) and the basic dye methylene blue. After incubation, plates were placed at 4°C for 24 hrs. Positive reactions were caused to darken significantly and to make visible weak positive reactions that were not apparent upon initial inspection.

Rhamnolipid Recovery

Rhamnolipids in the cell free broths of the strains was made less soluble in the aqueous phase by acidifying with 5% HCl to pH 3.0 which neutralized the negative charges on the rhamnolipids and precipitate. The precipitated rhamnolipids were recovered by solvent extraction using ethyl acetate.

Anthrone Test

Fifty µL of each ethyl acetate layer from the recovered rhamnolipid was added. Then 1.0 mL of distilled water and 3.0 mL of the 0.2% anthrone in 75% sulfuric acid solution were also added in each. The test tubes were heated for 10 minutes in a water bath of boiling water. Extracts that were positive for glycolipids produced a dark green color in the anthrone solution.

Thin Layered Chromatography

Ethyl acetate layers of the strains which yielded a positive result in anthrone test were separated by spotting at a point of origin near the bottom of the pre-coated plate (silica gel 60, Sigma, USA). Then plates were developed in solvent system of chloroform: methanol: acetic acid (65:10:2, v/v/v).

Extraction and Partial Purification of Crude Rhamnolipid

Bacterial isolates that showed positive results in anthrone test and TLC were purified for its precipitated rhamnolipids by extracting three times with ethyl acetate at room temperature. The solvent in the organic layer was removed using rotary evaporator leaving behind relatively pure rhamnolipids having an oil-like appearance **(Zhang & Miller, 1992; Gunther *et al.*, 2005)**.

Determination of Minimum Inhibitory Concentration (MIC) of Crude Extract on Solid Medium

MIC was performed using Muller Hinton Agar. Five millimeter – diameter holes were bore on the 3mm-thick solid medium. A standard two-fold serial rhamnolipid dilution technique was applied to measure antibacterial activity **(Washington & Wood, 1995)**. Then 10 mg/ml stock solution of crude extract was diluted twofold to final concentrations: 100, 200, 400 and 800 µg/mL modified from **Lotfabad, Shahcheraghi, and Shooraj (2013)**. Following the standard AST, a standardized inoculum of the organism was swabbed onto the surface of a Muller-Hinton agar plate, then 30 µL of crude extract concentrations: 100, 200, 400 and 800 µg/mL were pipetted and added to the 5mm diameter holes. After plates were incubated for 24 hours at 37°C, the diameter of zone inhibition (ZI) of the different test bacteria around each hole was measured and MIC was determined.
Two antibiotics, 5.0 µg/mL of Ciprofloxacin and 300 µg/mL of Nitrofurantoin were used as positive controls and ethyl acetate as negative control respectively. The diameters of inhibition zone of the crude extract and the known drugs were compared.

Antioxidant Activity by DPPH Radical Scavenging

This was done by preparing 5, 10, 15, 20, 25 and 30 µg/mL concentrations of the rhamnolipid extracts in methanol. Similar concentrations of ascorbic acid were used as the standard antioxidant. 0.1 mM solution of DPPH in methanol was prepared and 1 mL of this solution was added to 3 mL of the extracts and to ascorbic acid in their corresponding concentrations, handling of DPPH was done in the dark. The solutions were shaken vigorously and allowed to stand for 30 minutes in complete darkness to complete the reaction.
Absorbance of the solutions were measured at 517 nm by using UV-Vis spectrophotometry. DPPH scavenging effect (%) or Percent inhibition was solved through the equation described by **Shekhar and Anju (2014)**: Percent inhibition $= A_0 - A_1 / A_0 \times 100$. Control reaction was the absorbance of the DPPH solution in methanol without any extract added. IC_{50}, the concentration at which 50% of the free radicals are oxidized, was determined through making inhibition curves in MS Excel.

Statistical Analysis of Data

For statistical analysis, Microsoft Excel 2013 and Minitab17 (Pennsylvania, USA) were used. Regression analysis was done in Microsoft Excel 2013 to determine the relationship of the zones of inhibition and concentrations of the extracts. The equations of the regression graphs for the extract against corresponding test bacteria were used to solve for the desired concentration that was needed for it to have a similar effect to that of the positive controls (Ciprofloxacin and Nitrofurantoin). The standard zone of inhibition for bacterial susceptibility to Ciprofloxacin is 21 mm **(Ali *et al.*, 2010)** while Nitrofurantoin is 17 mm **(RxList, 2016)**. Kruskall-Wallis one-way analysis of variance was done in Minitab17. This was used to determine which extract was the most effective at inhibiting the test bacteria and this was also made in comparison with the control Nitrofurantoin but not Ciprofloxacin. Extracts were not anymore compared with Ciprofloxacin because it was already evident that there really was a difference with this control and the extracts in inhibiting bacteria.

RESULTS AND DISCUSSION

Screening for Biosurfactant

Biosurfactant screening results were summarized in Table 1. The biosurfactant screening results showed that strains 1, 4 and 5 were positive (Table 1). The Parafilm M Test detects the presence of biosurfactants through the diameter of the drop when placed on parafilm. Biosurfactant would reduce the tension between polar water solution and nonpolar surface. Therefore more biosurfactants present in the drop, the larger the diameter of the drop. The diameter of the negative control was 5 mm and all of the strains exhibited a larger drop diameter than distilled water. Studies on biosurfactants showed that stability

of the test droplet would depend upon the amount of surfactant present in the drop **(Walter *et al.*, 2010)**. Droplets without or less biosurfactants would have hydrophilic molecules that would repel from the nonpolar surface. For the oil spread assay, positive biosurfactant production was observed of having a larger clear zone in comparison to the negative control. All strains except strain 2 were able to show a larger displacement of oil around its crude extract which signifies that these strains potentially had biosurfactants that can act as oil dispersants. Distortion of the grids in the microplate was observed in all strains which indicates for biosurfactant production. All strains were positive for this assay which indicated that these strains had enough biosurfactants to induce optical distortion.

The emulsification assay test of a biosurfactant is based on the formation of an emulsion layer that would decrease the surface tension between the water in the aqueous test solution and kerosene. None of the strains produced a biosurfactant that could form a stable emulsion layer after the standard 24 hours. The critical micelle concentration (CMC) of rhamnolipid is 0.08% **(Sekhon Randhawa & Rahman, 2014)**, it is quite low compared to biosurfactants. The CMC of a biosurfactant signifies the least amount of the biosurfactant needed in order for it to form micelles and emulsify oil efficiently. The lower the CMC, the more efficient it is as an emulsifier. The result implied that the extracts did not produce enough biosurfactant for it to reach the CMC. The CTAB agar plate detects the production of anionic biosurfactants **(Walter *et al.*, 2010)** secreted by the microorganism. All the strains yielded negative result for the CTAB agar plate wherein no blue halo was observed and this could be because of the minute amount of rhamnolipid present the *Pseudomonas* isolate extracts as explained that the size of the zone of precipitation is proportional to the amount of biosurfactant present in the sample **(Rahman *et al.*, 2010)**.

Table 1 Biosurfactant screening results of six *Pseudomonas* isolates

Strain	Parafilm M test (mm)	Oil spread (mm)	Microplate assay	Emulsification Index (%)	CTAB agar plates	Anthrone	TLC
1	6	52	+	0	-	+	+
2	6	6	+	0	-	-	-
3	6.5	10	+	0	-	-	-
4	7	34	+	0	-	+	+
5	7	10	+	0	-	+	+
6	7	9	+	0	-	-	-
Control	5	0	-	0	-	-	-

It was evident that *Pseudomonas reactans* (strain 2) was negative in all the assays for biosurfactant screening except for the Parafilm M test and Microplate assay. This indicated that *P. reactans* has the least potential to produce rhamnolipids. The biosurfactant screening tests are not yet enough to conclude that these strains have rhamnolipids. It only indicated that these strains have produced biosurfactants. The anthrone test determined if the biosurfactants detected in the previous assays were glycolipids. Thin-Layered Chromatography would then verify if the glycolipid is a rhamnolipid. The crude extracts of strains 1, 4, and 5 were able to form a distinct dark green colored complex that indicates the presence of carbohydrate (Figure 1). This was most evident in strain 5. The anthrone test is based on the reaction of sugar with the anthrone reagent under acidic conditions to yield a green colored complex. The carbohydrates in strains 1, 4, and 5 were hydrolyzed by acid treatment to form furfurals and hydroxymethyl furfural. These furfural are then condensed by anthrone reagent to form a blue green colored complex. Strain 1 had the faintest green colored complex followed by strain 4 while strain 5 had the darkest colored complex by which it could be generated that strain 5 had the highest amount of carbohydrates present followed by strain 4 and strain 1. These strains are determined to have reducing and non-reducing sugars because of the presence of the strongly oxidizing sulfuric acid.

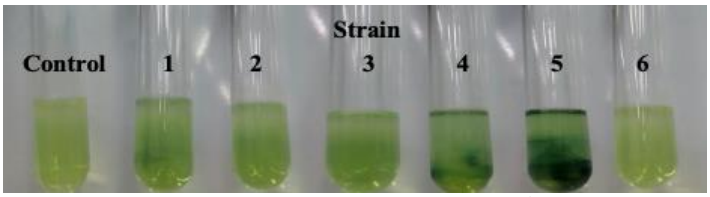

Figure 1 Anthrone test, negative control, and strains 1 - 6 (left to right)

Thin-layer chromatography (TLC) has been used extensively for determining the composition of culture broth extracts of rhamnolipids **(de Koster *et al.*, 1994; Rendell *et al.*, 1990; Syldatk *et al.*, 1985)**. The normal-phase chromatography on the silica 60 plates with the solvent mixture of chloroform-methanol-acetic acid (65:10:2) allowed for the division of the mono- and disaccharide polar head groups of the rhamnolipid. Though the staining and bands of the rhamnolipid standard were very dark and thick since it is highly concentrated, the crude extracts of strains 1, 4, and 5 were able to form light thin bands comparable to the standard (Figure 2). The organic solvent system was able to extract the lipid containing amphiphatic molecules present in strains 1, 4 and 5. This indicated that these strains were positive for rhamnolipids. It was noted that strain 1 had a very faint band.

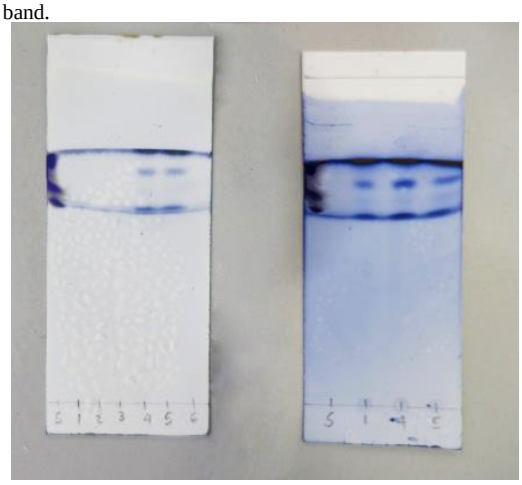

Figure 2 Only strains 1, 4, and 5 formed bands in the TLC test

The biosurfactant screening tests showed that only *Pseudomonas poae* (strain 1), *Pseudomonas fluorescens* (strain 4) and *Pseudomonas libanensis* (strain 5) were the only *Pseudomonas* species that could potentially produce rhamnolipids in the study. Studies that utilized terrestrial *P. fluorescens* and *P. libanensis* from oil polluted soils showed that these two species can really produce rhamnolipids. The environment where the *Pseudomonas* isolates used in the study was unlike those that are mostly experienced by *Pseudomonas* that are used in rhamnolipid studies. The underwater cave of Casili, Balamban was described by one of the Filipino Cave Divers to be free of oil pollution but was instead polluted by agricultural waste. Wastes coming from piggeries and farms near the area tended to seep into the waters of the cave. Other studies usually get their isolates from areas that encounter oil stress such as those that had an oil spill since exposure to hydrophobic pollutants in these environments become selective for biosurfactant producers. It is under this stressful condition that pseudomonads exhibit the greatest capacity to produce rhamnolipids **(Arino *et al.*, 1996)**. This explains why only 3 out of the 6 isolates were able to potentially produce rhamnolipids since the *Pseudomonas* isolated were not used to being in an oil polluted environment. The biosurfactant screening of the study imply that not all *Pseudomonas* species

have the capacity to produce biosurfactants and that *P. poae*, *P. fluorescens*, and *P. libanensis* can be induced to produce biosurfactants. Though the *Pseudomonas* used in the study were isolated from non-oil polluted areas, it was determined that as long as they are stressed under the proper conditions, they can produce biosurfactants.

Antibacterial Activity

Only the rhamnolipid extracts of *Pseudomonas poae* (strain 1), *Pseudomonas fluorescens* (strain 4), and *Pseudomonas libanensis* (strain 5) were tested for their antibacterial activity. This was because these were the strains that exhibited the greatest potential in producing rhamnolipids during the biosurfactant screening process as discussed earlier. Table 2 shows the comparison of mean zones of inhibition results of the crude rhamnolipid extract produced by Strains 1, 4 and 5 at different concentrations against two Gram-negative bacteria (*Escherichia coli*, *Serratia marcescens*) and two Gram-positive bacteria (*Bacillus cereus* and *Staphylococcus aureus*). All the strains exhibited their largest zone of inhibition at its highest concentration. Crude rhamnolipid extract produced by Strain 5 was observed to have its largest zone of inhibition, 10.89 mm, against *Escherichia coli*. Crude rhamnolipid extract of Strain 1 follows Strain 5 with the zone of inhibition of 9.67 mm against *Serratia marcescens* and *Escherichia coli.* The produced crude rhamnolipid extract of Strain 4 ranked third among the three strains' largest zone of inhibition with 8.11 mm against *Escherichia coli.* The crude rhamnolipid extracts produced by all of the strains affected more the growth of Gram-negative bacteria than Gram-positive bacteria. The negative control, ethyl acetate, was not able to affect the growth of any test organism while the positive controls, Ciprofloxacin and Nitrofurantoin inhibited the growth of all test organisms with Ciprofloxacin stronger than Nitrofurantoin and all other crude rhamnolipid extracts (Table 2).

Table 2 Mean zones of inhibition of crude rhamnolipid extracts of the 3 *Pseudomonas* strains at different concentrations versus commercial antibiotics (Nitrofurantoin and Ciprofloxacin).

Microorganisms	Strain 1				Strain 4				Strain 5				Nitrofurantoin	Ciprofloxacin	Ethyl acetate
	Extract Concentration (µg/mL)														
	100	200	400	800	100	200	400	800	100	200	400	800	300	5	100%
S. marcescens	5.11	6.11	8.44	9.67	6.22	5.67	6.00	8.00	5.50	6.00	7.22	9.44	5.11	36.67	5.00
E. coli	5.44	6.44	8.56	9.67	5.44	5.33	6.78	8.11	5.89	6.94	9.00	10.89	9.78	32.11	5.00
B. cereus	5.00	6.22	7.13	8.56	5.00	5.11	5.22	5.67	5.00	5.67	6.33	9.67	8.67	29.89	5.00
S. aureus	5.22	5.56	7.33	7.67	5.33	5.56	6.22	6.22	5.78	6.78	7.74	9.56	7.67	27.22	5.00

Note: Zones of inhibition were measured in millimetres (mm)

As observed, all of the rhamnolipid extracts of the 3 strains were able to inhibit the test bacteria to some degree with extracts coming from *Pseudomonas fluorescens* (strain 4) inhibiting the least. All of the extracts show a positive correlation between its concentration and the zone of inhibition against test organisms (Table 3) and the positive correlation between the concentration and the zone of inhibition has a statistical significance with the P value of 0.024214 which render the r^2 values reliable. This implies that if the extracts were found at higher concentrations, the greater zone of inhibitions would have been observed. Nonetheless, this also proves that the rhamnolipids in the crude extract did have antibacterial properties against the 4 test organisms.

Table 3 R^2 values for zone of inhibition and extract concentrations in regression analysis

Test Microorganism	Strain 1	Strain 4	Strain 5
S. marcescens	0.9025	0.7638	0.9995
E. coli	0.899	0.9557	0.958
B. cereus	0.9405	0.9858	0.9742
S. aureus	0.8131	0.7204	0.9805

p-value = 0.024214

The strain with least MIC was Strain 5 while Strain 2 consistently had 200 as MIC throughout (Table 4). A possible reason for the different responses of the test bacteria towards rhamnolipid extracts coming from the 3 strains could be that the rhamnolipids produced by each strain were different in chemistry. There are several types of rhamnolipids and the type produced is dependent upon the bacterial strain, carbon source used and process strategy **(Lotfabad, Shahcheraghi, and Shooraj, 2013)**. Characterization of the rhamnolipid extracts from each strain would be needed to determine the specific type of rhamnolipid produced.

Table 4 Minimum inhibitory concentration (MIC) of crude rhamnolipid extracts from the 3 *Pseudomonas* strains against test bacteria.

Test Organism	MIC (µg/mL)		
	Strain 1	Strain 4	Strain 5
S. marcescens	200	100	100
E. coli	200	400	100
B. cereus	200	800	200
S. aureus	200	200	100

Kruskall-Wallis test was done to determine which strain produced the most lethal extract against the test bacteria. It also elucidated whether the extracts had significantly different effects in inhibiting the test bacteria. A p-value of P= 0.000 (Table 5), indicating that there is a significant difference among the extracts when it came to inhibiting the test bacteria. Extracts coming from strain 1 were most effective against *Serratia marcescens* and *Staphylococcus aureus* while those from strain 5 were the most effective against *Escherichia coli* and *Bacillus cereus* (Table 5). The rhamnolipid extracts were better at inhibiting the test bacteria compared to the positive control, Nitrofurantoin (Table 5).

Table 5 Strain effectivity ranking through Kruskal-Wallis Test.

Treatment	Average Rank
6	110.2
10	99.8
1	96.4
9	93.1
12	90.4
4	90.1
14	89.6
3	82.4
7	80.6
5	67.1
15	66.4
2	55.5
16	55.3
11	37.1
8	27.9
13	18.2
P = 0.000	

LEGEND: Codes for treatment in Kruskal-Wallis Test

1 = S1 at 800 µg/mL against *Serratia marcescens*
2 = S4 at 800 µg/mL against *Serratia marcescens*
3 = S5 at 800 µg/mL against *Serratia marcescens*
4 = S1 at 800 µg/mL against *Escherichia coli*
5 = S4 at 800 µg/mL against *Escherichia coli*
6 = S5 at 800 µg/mL against *Escherichia coli*
7 = S1 at 800 µg/mL against *Bacillus cereus*
8 = S4 at 800 µg/mL against *Bacillus cereus*
9 = S5 at 800 µg/mL against *Bacillus cereus*
10 = S1 at 800 µg/mL against *Staphylococcus aureus*
11 = S4 at 800 µg/mL against *Staphylococcus aureus*
12 = S5 at 800 µg/mL against *Staphylococcus aureus*
13 = Nitrofurantoin against *Serratia marcescens*
14 = Nitrofurantoin against *Escherichia coli*
15 = Nitrofurantoin against *Bacillus cereus*
16 = Nitrofurantoin against *Staphylococcus aureus*

Rhamnolipids are reported to have strong antibacterial properties against gram-positive bacteria **(Lotfabad, Shahcheraghi, and Shooraj, 2013)** and a few gram-negative bacteria **(Kahlon, 2016)**. The crude rhamnolipid extracts produced by all of the strains affected more the growth of Gram-negative bacteria than Gram-positive bacteria and this is in contrast to the findings of **Lotfabad *et al*., (2013)** and **Sotirova *et al*., (2008)** which showed that biosurfactants were active against Gram-positive bacteria and its presence does not affect the growth of Gram-negative strains. The rhamnolipid in the experiment could have altered the permeability of the Gram negative cell membrane and this new information could be used for the formulation of antibiotic and reinforcement, highlighting rhamnolipid potential in biomedicine. *Pseudomonas libanensis* (strain 5) was observed to have the greatest antibacterial activity among the 3 strains extracted. Previous studies done on extract coming from other strains of *Pseudomonas libanensis* also reported similar results of its good antimicrobial activity. This antimicrobial activity of *Pseudomonas libanensis* could be attributed to its ability to produce a cyclic lipopeptide, viscosin **(Kahlon, 2016)** in addition to its ability to produce rhamnolipids.

Antioxidant Activity by DPPH Radical Scavenging

Antioxidant activity in rhamnolipids has not been fully investigated in published papers. One fast and relatively cheaper way to determine the antioxidant activity of a compound is through the DPPH radical scavenging method. This method utilizes the Absorbance of a DPPH and extracts solution at 517 nm after it undergoes reaction. The mean Absorbance of the reference standard, ascorbic acid, and the crude rhamnolipid extracts coming from *P. poae* (strain 1), *P. fluorescens* (strain 4), and *P. libanensis* (strain 5) at various concentrations are found in Table 6 below. It was seen that there a decreasing trend of absorbance as the concentration of the extracts and the ascorbic acid increases, this is most pronounced in ascorbic acid.

Table 6 Absorbance of ascorbic acid and crude rhamnolipid extracts at various concentrations measured at 517 nm.

Concentration (µg/mL)	Ascorbic Acid (Abs)	Strain 1 Crude RL (Abs)	Strain 4 Crude RL (Abs)	Strain 5 Crude RL (Abs)
5	0.191	0.196	0.221	0.166
10	0.145	0.183	0.228	0.156
15	0.125	0.180	0.160	0.156
20	0.092	0.165	0.145	0.151
25	0.042	0.154	0.157	0.146
30	0.033	0.084	0.122	0.144

Note: Abs of Control was 0.247

It was observed that ascorbic acid has the greatest % inhibition, followed by the crude rhamnolipid extracts of Strain 1 and Strain 4 while Strain 5 had the least % inhibition. The % inhibition of the extracts or standard when graphed against the concentrations through MS Excel, allows one to determine the equation of the line which allows the IC_{50} to be solved. The graphs are presented in Figure 3. The R^2, regression equation, and IC_{50} of the extracts are in Table 7.

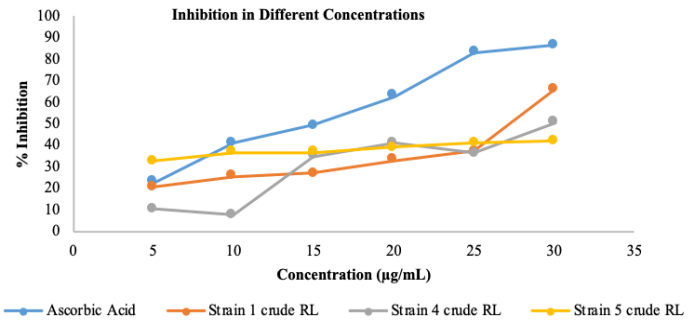

Figure 3 Percent Inhibition of ascorbic acid and crude RL extracts at various concentrations.

It was seen that ascorbic acid had the greatest antioxidant activity compared to the crude rhamnolipid extracts (Figure 3). It was also observed that at the lowest concentration, crude rhamnolipid extracts from strain 5 had the greatest % inhibition of the free radical but at the highest concentration, it was the least (Figure 3). Ascorbic acid had the least IC_{50} which further proves its great antioxidant activity since even at a low concentration, it can already reduce 50% of the free radicals. This is seen on Table 7 below. Among the crude rhamnolipid extracts, those coming from strain 1 (*P. poae*) exhibited the greatest antioxidant activity though its difference with strain 4 (*P. fluorescens*) is not that far off. Extracts from strain 5 (*P. libanensis*) relatively had the least antioxidant activity.

Table 7 R^2 value, regression equation, inhibitory concentration (IC_{50}) of ascorbic acid and crude rhamnolipid extracts.

Solution Tested	R^2	Linear equation	IC_{50}	P-value*
Ascorbic Acid	0.9776	y = 2.6115x+12.002	14.5502	0.000
S1 crude RL	0.7756	y = 1.5315x + 8.2411	27.2667	0.021
S4 crude RL	0.8191	y = 1.6757x + 0.9717	29.2584	0.013
S5 crude RL	0.9478	y = 0.3412x+32.074	52.5381	0.001

Note: calculated using Pearson correlation

The results clearly showed that ascorbic acid had the greatest antioxidant activity which was expected since ascorbic acid is one of the known standard antioxidants with an IC_{50} of 14.5502 µg/mL in the study. It was observed that all of the crude rhamnolipid extracts had antioxidant activity but it was not as great as that of ascorbic acid. The IC_{50} of crude rhamnolipid extracts from *P. poae* (Strain 1) was 27.2667 µg/mL, *P. fluorescens* (Strain 4) had 29.2584 while *P. libanensis* (Strain 5) had the greatest IC_{50} which was 52.5381. This indicated that the strains had antioxidant activity and that crude rhamnolipid extracts from *P. poae* had the greatest radical scavenging activity among the crude rhamnolipid extracts investigated in the study.

Ascorbic acid as an antioxidant deters oxidation of free radicals by donating an electron to the radical in order for the radical to be stable **(Nimse & Palb, 2015)**. The R^2 value of ascorbic acid and the crude rhamnolipid extracts had strong correlations since they ranged from 0.8191 – 0.9478 (Table 7). The average R^2 value of the crude rhamnolipid extracts was 0.88, a strong correlation. Strong positive correlation indicates that it is truly the extracts themselves that acted as the antioxidant **(Li, Wu, & Huang, 2009)**. This shows that the crude rhamnolipid extracts used in the study exhibited antioxidant activity and that increasing concentrations of the extracts would also result in greater free radical scavenging activity since the correlation is positive. No study describing the mechanism of how a biosurfactant can act as an antioxidant has been published so far. Though there are already studies that claim that some glycolipids such as sophorolipids and MELs do show antioxidant activity **(Kosaric & Sukan, 2015)**. This only shows that more research regarding the bioactivity of biosurfactants like rhamnolipids is needed to further determine and verify the possible application of these compounds in various industries.

CONCLUSION

The study has shown that not all of the isolated *Pseudomonas* strains from the freshwater sediment can produce rhamnolipids. The rhamnolipids produced from *Pseudomonas poae*, *Pseudomonas fluorescens*, and *Pseudomonas libanensis* exhibited antibacterial activity against Gram-positive and Gram-negative used in the study. Crude rhamnolipids at a higher concentration had larger zones of inhibition especially against *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus cereus*. The rhamnolipid extracts from *P. poae* and *P. libanensis* are comparable to commercial antibiotic Nitrofurantoin against all the test microorganisms (*Serratia marcescens*, *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus cereus*) but not to Ciprofloxacin. Through DPPH radical scavenging method, it was determined that all of the crude rhamnolipid extracts showed antioxidant activity but not comparable to that of the standard antioxidant, ascorbic acid.

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PRODUCTION OF LIPASE BY IMMOBILIZED *BACILLUS THURINGIENSIS* AND *LYSINIBACILLUS SPHAERICUS* AND THEIR BIODEGRADATION POTENTIAL ON DIESEL

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ABSTRACT

This study reported production of lipase by immobilized *Bacillus thuringiensis.* Bacteria isolates were screened on Bushnell-Hass Mineral Salt medium containing 1% v/v Diesel for oil degradation. The potent isolates were identified using 16S rRNA as *Bacillus thuringiensis*. The isolates were immobilized in gelatin matrix and cultured for lipase production in a submerged medium. The crude lipase extracted was used for degradation of Diesel. Optimum degradation of Diesel 41.4% was obtained by lipase from Immobilized *Bacillus thuringiensis* and 31.6% for *Lysinibacillus sphaericus* at pH 7 and 35 in 20 days. GC-MS analysis was carried out to show the compounds degraded after 20 days. This study therefore presented the use of immobilized bacterial lipase in degradation of Diesel as a simple and effective approach.

Keywords: *Bacillus thuringiensis*, *Lysinibacillus sphaericus*, Immobilization, Biodegradation, Lipase

INTRODUCTION

Lipases are serine hydrolases that catalyze both hydrolysis and synthesis of long-chain triacylglycerols. Lipases occupy a place of prominence among biocatalysts owning to their ability to catalyze a wide variety of reactions and are an important group of biotechnologically relevant enzymes and they find massive applications (**Mohammed, 2013**).

Lipases are carboxylic ester hydrolases currently attracting an enormous attention due to their versatile nature and widely used enzyme in biotechnological applications and owing to their unique properties (**Cristian, 2005**). Lipases are produced by microorganisms (bacteria and fungi), plants and animals, However, microbial lipase especially from bacteria are more useful than their plant and animal origin, since they have great variety of catalytic activities and microorganisms are easy to manipulate genetically and capable of rapid growth on inexpensive media (**Sirisha, 2010; Mongkolthanaruk and Boonmahome, 2013; Veerapagu *et al*., 2014; Jinyong, 2014**). Microbial lipases are commercially most important mainly are secreted into the culture medium by many of microbial species are belong to bacteria, fungi, yeasts and actinomyces (**Babu and Rap, 2007; Abada, 2008**). Lipases play a vital role in the manufacturing and services sectors for the mankind. Microbial lipases have gained special industrial attention due to their selectivity, stability and broad substrate specificity. Microbial enzymes are also more stable than their corresponding animal and plant enzymes and their production is more convenient and safer (**Veerapagu *et al*., 2013**). Generally, bacterial lipases are glycoproteins but some extracellular bacterial lipases are lipoproteins (**Abdul-Hammid *et al*., 2013; Sagar *et al*., 2013**). The production of extracellular lipases from bacteria is greatly influenced by medium composition besides physicochemical factors such as temperature, pH and dissolved oxygen. The major factor for the expression of lipase activity has always been reported as the carbon source, since lipase are inducible enzymes. These enzymes are generally produced in the presence of a lipid such as oil or any other inducer, such as triacylglycerols, fatty acids, hydrolysable esters, Tweens, glycerol and bile salts .However, nitrogen sources and essential micronutrients should also be carefully considered for growth and production optimization (**Veerapagu *et al*., 2013**). Considering the important of lipase enzyme, the present study was aimed to isolate, identification and immobilize lipase producing bacteria from different sources of oil contaminant soil and study the activity of these isolates. This study therefore aimed at production of bacterial lipase from immobilized bacteria organisms and subsequent use for degradation of Diesel.

MATERIALS AND METHODS

Sample Collection

Samples were collected from oil contaminated Atlantic Seawater and Sediment sites. The collected samples were packed in sterile bottle to the laboratory. The entire samples were stored at refrigeration temperature before the experimental work.

Isolation of bacteria:

Soil and water sample were serially diluted and plated on Nutrient Agar medium, (pH 7.0) by spread plate method. Plates were incubated at 37°C for 48 hours. Pure cultures of the isolates were maintained on nutrient agar slants and were sub-cultured every 15 days.

Screening of hydrocarbon degrading bacteria

The isolated bacteria were inoculated on an enrichment medium that contains mineral salt medium (MSM) supplemented with single hydrocarbon compound as sole carbon source (1% petrol and diesel). The MSM composition was made up of basal salt medium and trace element solution. The basal medium contain (g/L): K_2HPO_4, 1.8; KH_2PO_4, 1.2; NH_4Cl, 4.0; $MgSO_4.7H_2O$, 0.2; NaCl, 0.1; yeast extract, 0.1 and $FeCl_2.4H_2O$, 0.05 and trace elements solution contain: H_3BO_3, 0.1; $ZnSO_4.7H_20$, O.1; $CuSO_4.5H_2O$, 0.05 and $MnSO_4.H_2O$, 0.04 with the pH of 6.5 (**Balogun and Fagade, 2010**).

Biochemical tests of the isolates

Different types of biochemical tests were done such as Gram's staining, Indole test, Methyl red test, VP test, Citrate utilization test, Urease test, Catalase, Oxidase and Starch hydrolysis (**Cheesbrough, 2006**).

Molecular characterization of isolates

Dna extraction

Molecular characterization of isolates was carried out by extracting DNA (1 ml) of bacterial isolate using the method of (**Keramas *et al*., 2004**). Sterile distilled water was added into the eppendorf tubes. The bacterial isolates were added and mixed by vortexing. Centrifugation was carried out at 10,000 rpm for 5 min at 4 ^{0}C. The supernatant was discarded. 200 µl of sterile distilled water was added and vortex to homogenize the pellets. The tubes containing the homogenized pellets were boiled at 100 ^{0}C for 10 minutes. After boiling, the tubes were vortexed again and centrifuged at 10,000 rpm for 5 minutes. The supernatant were transferred into another pre-labeled eppendorf tube by gentle aspiration using a micropipette.

Polymerase chain reaction

Fragments of the gene of interest, the 16S ribosomal gene, were amplified using standard PCR protocol and the universal primer. The PCR reaction mixture (20 µl) consisting of 4 µl PCR master mix (Solis Biodyne)), 0.5 µl of each primer, 14.1 µl nuclease free water and 1.5 µl template DNA (**Keramas *et al*., 2004**).

Agarose gel electrophoresis

Agarose powder of (1.5 g) was added to 150 mls of 0.5x TAE buffer and dissolved by boiling using microwave oven. The mixture was allowed to cool to about 60 ºC. Ethodium bromide (10 ml) was added and mixed by swirling gently it was then poured into electrophoresis tank with the comb in place to obtain a gel thickness of about 4-5 mm. The tank was filled with 1x TAE buffer. Thereafter the comb was removed. 10 µl of sample were mixed with 1µl of the 10x loading dye. The samples were carefully loaded into the wells created by the combs. The electrodes were connected to the power pack in such a way that the negative terminal was at the end where the sample was loaded. The electrophoresis was allowed to run at 60- 100 V until the loading dye migrates about three- quarter of the electrodes. Electrodes were turned off and disconnected. The gel was observed on a UV- trans-illuminator (**Keramas *et al*., 2004**).

Screening of the isolates for lipase activity

Lipolytic microorganisms were screened by qualitative plate assay method of **Singh *et al*., 2006**. Bacterial strains were gown on nutrient medium substrate containing Tween-80 agar plates and incubated at 37°C for 24 to 48 hours and zone were observed.

Immobilization of *bacillus thurigiensis* and *lysinibacillus sphaericus* within a gelatin matrix

Immobilization of bacteria isolate was done within gelatin matrix using a modified method of **Osho *et al*. (2001)**. Aqueous solution of gelatin (7-10.0% w/v) was crossed linked by adding ethanolic formaldehyde to give a final formaldehyde concentration of 2% (v/v) and incubated for 20 min at 37 ºC. *Bacillus sp* were added to the crossed-linked gelatin under vigorous stirring at 35 ºC. The mixture was poured into a burette and added drop-wise into a beaker containing cold liquid paraffin to obtain droplets of gelatin (beads). The beads were hardened by suspending in the cross linking agent for 24 h. They were washed with sterile distilled water and stored in the refrigerator at 4 ºC.
For optimal bead size determination, an aqueous solution of gelatin (8%w/v) with 1ml of the cell culture was entrapped at 35ºC under vigorous stirring. Each preparation gel was poured through an improvised laboratory dropper (2.5 mm diameter) and a syringe (4.0 mm diameters) into a cold paraffin liquid respectively at a constant flow rate (2 ml/min). The gel bead were hardened by suspending in the cross linking agent for 24 h. They were washed with sterile distilled water and stored in the refrigerator at 4 ºC prepared.

Lipase production

Lipase production was carried out in a submerged medium containing peptone 0.2(%w/v); $NH_4H_2PO_4$ 0.1; NaCl 0.25; $MgSO_4.7H_2O$ 0.04; $CaCl_2.2H_2O$ 0.04; olive oil 2.0 (v/v); pH 7.0; 1-2 drops Tween 20 as emulsifier. Overnight cultures were suspended in 5ml of sterile deionised water and used as the inoculum. Submerged microbial cultures were incubated in 500 ml Erlenmeyer flasks containing 100 ml of liquid medium on a rotary shaker (150 rpm) at 36°C. After 24 hours of incubation, the culture was centrifuged at 10,000 rpm for 20 min at 4ºC and the cell free culture supernatant fluid was used as the sources of extracellular enzyme. The lipase activity in the supernatant was determined by the colorimetric method.

Determination of lipase biodegradation of diesel

The degrading activities of each enzyme were obtained using Mineral salt broth (MSB) in which 40 ml of each hydrocarbon (Diesel) was added and incubated at room temperature for 20 days. The enzyme activity was measured by taking the optical density (O.D) readings at 600 nm after 20 days against mineral salt medium as blank.

Optimization studies on degradation

Optimization studies of the enzymes on degradation of hydrocarbons was done, effect of temperature (20 ºC, 25 ºC, 30 ºC, 35 ºC, 40 ºC, 45 ºC, 50 ºC), effect of pH (5, 5.5, 6, 6.5, 7, 7.5, 8) and degradation time (5 days, 10 days, 15 days, 20 days). Degradation was carried out using the method described previously in section 2.7.

Gravimetric analysis

The amount of oil in culture was estimated using the Gravimetric method. Diethyl ether and acetone were taken in 1:1 ratio and was mixed with culture. The mixture was allowed to vaporize at room temperature. The oil residue obtained was weighed and taken as the gravimetric value for further calculation. (**Marquez-Rocha *et al*., 2001**)

$$\text{Percentage of Diesel oil degraded} = \frac{\text{Weight of Diesel oil degraded}}{\text{Weight of Diesel oil present originally}} \times 100$$

Where, the weight of Diesel oil degraded = (original weight of Diesel oil – weight of residual Diesel oil obtained after evaporating the extract).

Extraction and analysis of residual oil

Biodegradation of petroleum hydrocarbon in liquid culture was collected for analysis by gas chromatography (GC HP 680 series GC system, US90704303) (**Marquez-Rocha *et al*.,2001**).

RESULTS AND DISCUSSIONS

Table 1 Biochemical tests of the isolates

Code	Gram staining	Catalase	Oxidase	Indole	Motility	Methyl Red	Voges P	Citrate	Urease	Starch hydrolysis	Probable organisms
1	GPB	+	-	-	+	-	+	-	-	+	*Bacillus thuringiensis*
2	GNB	+	+	+	-	-	+	+	+	-	*Klebsiella 0xytoca*
6	GPC	+	+	-	-	-	-	+	+	NA	*Staphylococcus aureus*
7	GPB	+	-	-	+	-	-	-	+	NA	*Corynebacterium striatum*
11	GPB	+	-	-	+	-	+	-	-	+	*Lysinibaccillus sphaericus*

Key: GNB- Gram negative Bacilli, GPB- Gram positive Bacilli, NA- Not applicable

Table 2 Primary Screening and enzyme activity of the isolates

Isolates	Zone of hydrolysis	Enzyme activity(u/ml)
1	5.67 ± 1.45^{cd}	2.63 ± 0.04^{e}
3	4.68 ± 0.20^{cd}	1.53 ± 0.04^{d}
7	1.82 ± 0.11^{a}	1.65 ± 0.03^{c}
10	5.36 ± 0.52^{cd}	1.73 ± 0.04^{d}
11	6.40 ± 0.46^{d}	1.96 ± 0.07^{d}

Legend: - indicates negative reaction

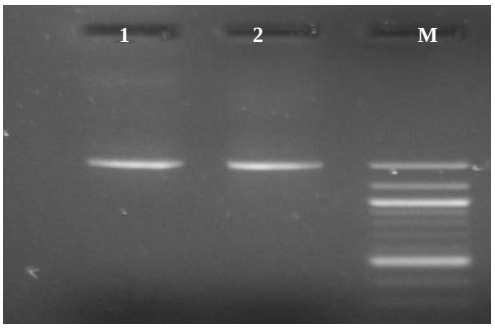

Plate 1: Gel electrophoresis of DNA extraction of bacteria isolates
M- 100bp marker
1- Sample 1- Sequencing result showed it to be *Bacillus thuringiensis*
2- Sample 2- Sequencing result showed it to be *Lysinibacillus sphaericus*

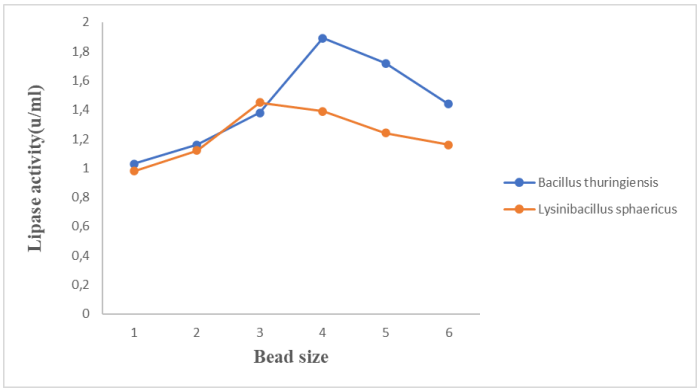

Figure 4 Effect of bead size on lipase activity by immobilized *Bacillus thuringiensis and Lysinibacillus sphericus*

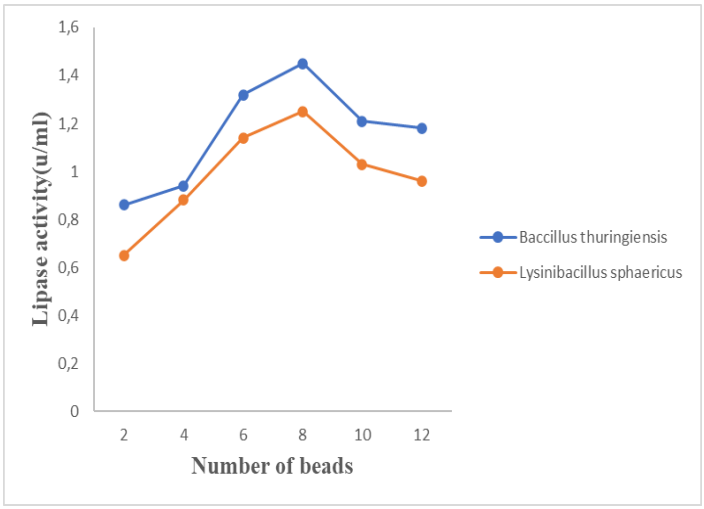

Figure 5 Effect of number of beads on lipase activity by immobilized *Bacillus thurigiensis* and *Lysinibacillus sphericus*

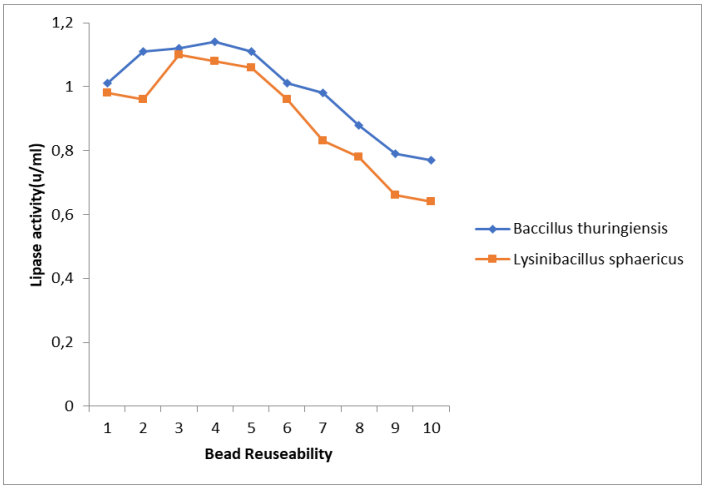

Figure 6 Bead reusability on lipase activity by immobilized *Bacillus thuringiensis* and *Lysinibacillus sphericus*

Table 3 Biodegradation of PMS by lipase from Immobilized *Bacillus thuringiensis*

	Bacillus thuiringiensis		*Lysinbacillus sphaericus*	
Test enzyme + Hydrocarbons	OD	Oil consumption (%)	OD	Oil consumption (%)
Free lipase + Diesel	0.52	38.2	0.32	26.8
Immobilized Lipase + Diesel	0.52	41.4	0.44	38.6
Diesel with no enzyme(Control)	1.82	0.0	1.82	0.0

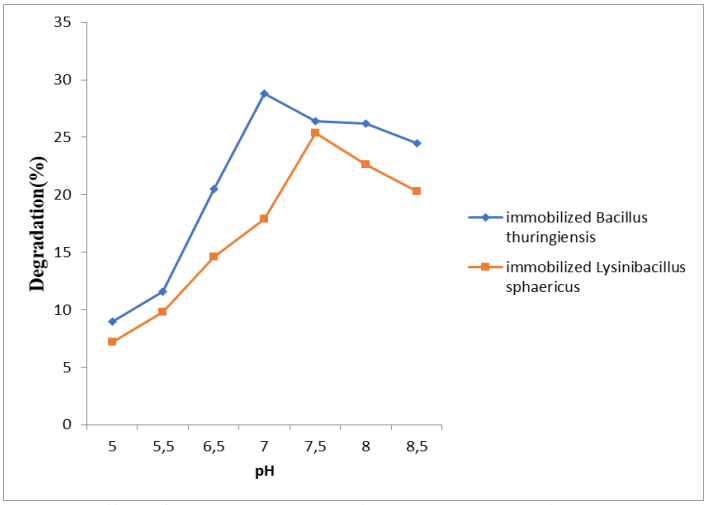

Figure 8 Effect of pH on degradation of Diesel using lipase from immobilized cells of *Bacillus thuringiensis* and *Lysinibacillus sphaericus*

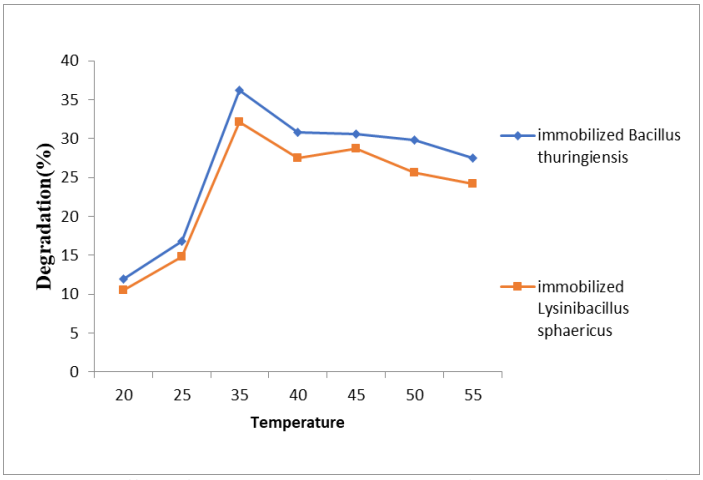

Figure 10: Effect of temperature on degradation of Diesel using lipase from immobilized cells of *Bacillus thuringiensis* and *Lysinibacillus sphaericus*

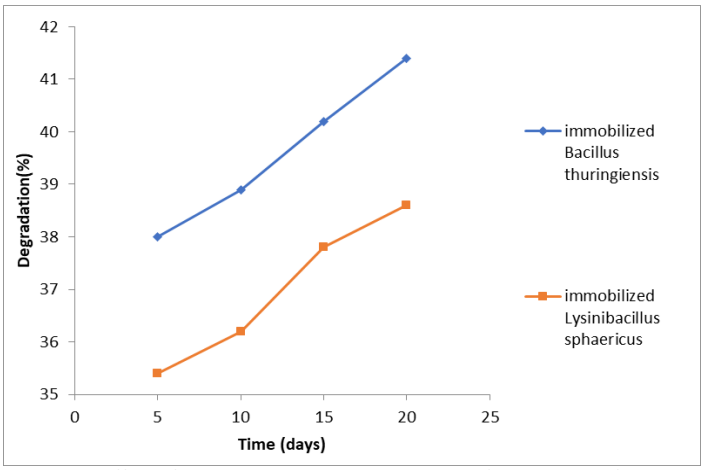

Figure 11 Effect of degradation time on degradation of Diesel using free lipase and lipase from immobilized cells of *Bacillus thuringiensis* and *Lysinibacillus sphaericus*

Table 4 GC-MS results on degradation of Diesel by lipase produced from immobilized cells of *Bacillus thuringiensis* and *Lysinibacillus sphaericus*

	Compound	R.T(min)	Area	PPM	Area (%)
Control	Hexadecane	14.828	136545	21.78	100
	Eicosane	19.171	1487708	151.23	100
	Tetracosane	22.977	7541703	685.45	100
	Hexacosane	24.487	63934	5.54	100
	Octacosane	26.152	2108528	172.87	100
Bacillus thuringiensis	Hexadecane	14.811	84	0.01	0.06
	Eicosane	19.194	222	0.02	0.01
	Tetracosane	22.862	232	0.02	0.003
	Hexacosane	24.499	208	0.02	0.33
	Octacosane	26.992	350	0.03	0.01
Lysinibacillus sphaericus	Hexadecane	14.788	609	0.10	0.45
	Eicosane	19.103	656421	66.73	44
	Tetracosane	22.862	3685954	335.01	49
	Hexacosane	24.395	28777	2.49	45
	Octacosane	26.055	1026102	84.13	48

Key: **R.T**- Retention time, **PPM**- Part per million

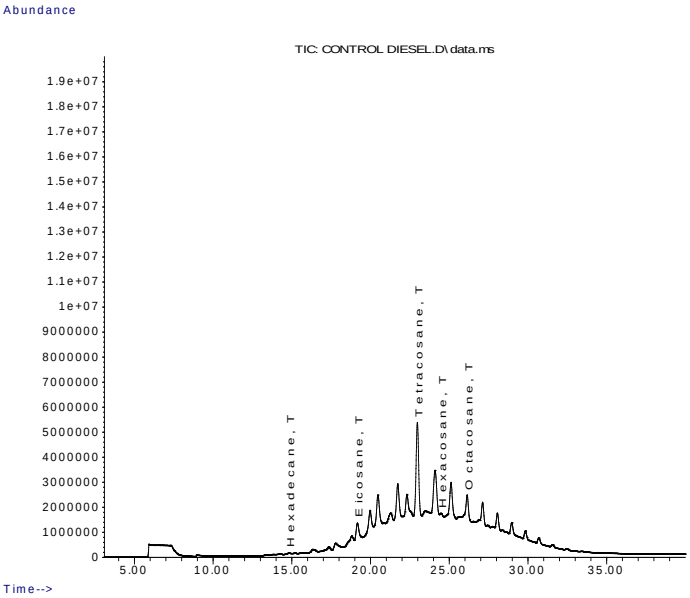

Figure 12 GC-MS of Diesel without enzyme as the control

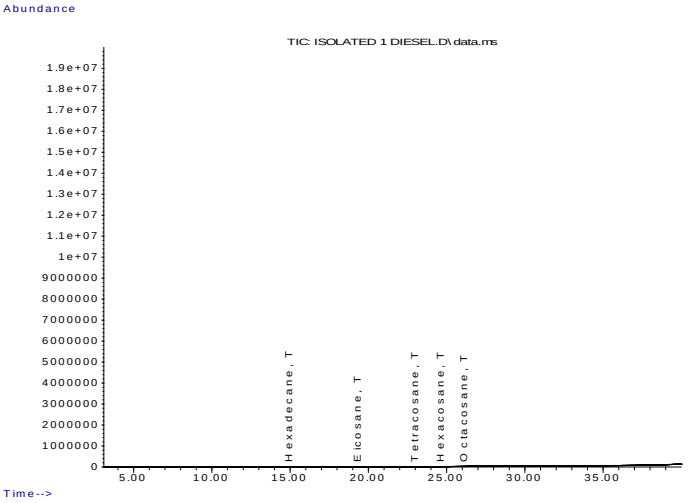

Figure 13 GC-MS result of Diesel degraded by lipase produced from immobilized *Bacillus thuringiensis*

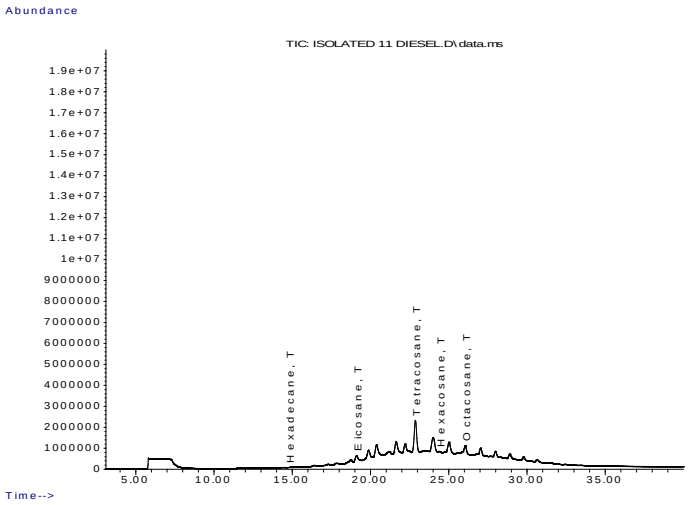

Figure 14 GC-MS result of diesel degraded by lipase from immobilized *Lysinibacillus sphaericus*

DISCUSSION

Many bacterial species are present in the soil normally and adapting to the soil conditions and almost the bacteria that degrade the oil present in the soil contaminated with oil, which help in cleaning the soil from oil products (**Cesarini *et al*., 2014**). Prominent among the bacteria found in the oil contaminated soil is *Bacillus sp*, this agrees with earlier reports by **Kumar *et al*, (2012)** stated that *Bacillus sp, Pseudomonas sp, Micrococcus, Aeromonas sp* are among bacteria found in oil contaminated areas. The biochemical of the various bacterial isolates was shown in Table 1. Dominant bacterial cultures were inoculated in mineral salts broth (MSB) medium with 1% Diesel as a carbon source to determine their biodegradative ability; all the isolates were able to utilize the crude oil as their carbon source. This corresponds to the findings of **Latha and Kalaivani (2012)** where the isolates were able to utilize hydrocarbons- as their carbon source. A total of 5 bacterial colonies were selected and isolated. Only two out of five 5 isolates showed good clear zone in the olive agar medium with phenol red as indicator. Plate 1 shows gel electrophoresis of DNA extraction of bacteria isolates using 100bp marker. The nucleotide sequence was 99% identical to *Bacillus thuringiensis* strain VITSJ-01 and *Lysinibacillus sphaericus* strain 2362. In the screening medium, *Bacillus thuringensis* shows maximum lipase production which produced 2.6 U/mL and *Lysinibacillus sphaericus* at 1.96U/ml was selected for further research while others showed less than 1.96 U/mL, (table 2). It was reported that maximum lipase production was at 72 hours for *Bacillus coagulans* (**PrasanthKumar *et al*., 2007**). *Bacillus thuringiensis* and *Lysinibacillus sphaericus* were immobilized on solid surfaces using gelatin matrix. Effect of varying bead size (1, 2, 3, 4, 5 and 6) on lipase production by immobilized bacteria cell is shown in Figure 1. Lipase activity increased gradually and further increased in bead size beyond 4 lead to decrease in lipase activity (Figure 4). Maximum lipase activity was achieved with immobilized *B. thuringiensis* (1.89U/ml**)** and *Lysinibacillus sphaericus* (1.45U/ml).

Effect of varying bead number (5, 10, 15, 20 and 30) on lipase production by immobilized -bacteria cell is shown in Figure 1. Lipase activity increased gradually and further increased in bead number beyond 8 lead to decrease in lipase activity (Figure 5). Maximum lipase activity was achieved with immobilized *B. thuringiensis* (1.45 U/ml**)** and *Lysinibacillus sphaericus* (1.25 U/ml).

Effect of varying bead reuseability on lipase production by immobilized bacteria cell is shown in Figure 2. Lipase activity increased gradually and further increased in bead reuseability beyond 4 lead to decrease in lipase activity (Figure 6). Maximum lipase activity was achieved with immobilized *B. thuringiensis* (1.14 U/ml**)** and *Lysinibacillus sphaericus* (1.11 U/ml). Contrary, Hung and coworkers, 2003; **Won and coworkers, 2005**) found that the immobilized lipase of *C. rugosa* entrapped in Ca-alginate gel beads retained 72% after three uses, also found that the repeated use for immobilized *C. rugos*a lipase on chitosan retained 74% after 10 reuses. Similarly **Yi and coworkers (2009**) showed that the activity of immobilized lipase of *C. rugosa* on alanine chitosan beads retained 77% of the initial activity after 10 times of reuse.

Bacterial strain isolated in this study was identified among hydrocarbon degrading microorganisms for crude oil. The results obtained clearly showed that lipase produced from this microorganism had biodegradable abilities and values of degraded Diesel varied after incubation at 20 days. Table 3 presented degradation of Diesel with immobilized lipase of *Bacillus thuringiensis* and *Lysinibacillus sphaericus.* Lipase from Immobilized *Bacillus thuringiensis* degraded 41.4% Diesel and *Lysinibacillus sphaericus* 38.6% Diesel. The result of pH on degradation of Diesel showed that degradation increased progressively with increase in pH from 5-8.5 reaching a maximum at 7 for Diesel degradation from lipase of immobilized *Bacillus thuringiensis* and *Lysinibacillus sphaericus* (figure 1). **Gupta *et al,* (2004**), states that maximum activity of lipases at pH values higher than 7 has been observed in many cases. Effect of temperature on lipase activity of the crude enzyme on degradation of Diesel showed that degradation of Diesel increased progressively with increase in temperature from 20 ºC reaching a maximum at 35 ºC for lipase of *Bacillus thuringiensis* and *Lysinibacillus sphaericus* (figure 2). An optimum temperature of 40 ºC for phenantheren degradation was reported by **Stringfellow and Aitken (1994**). Effect of degradation time on lipase activity of the crude enzyme on degradation of Diesel was presented in figure 3, an increase in degradation was observed along with the increase in time from 5 to 20 days. **Siddiqui *et al,* (2001**) reported the percentage degradation for individual *n*-alkanes increased with time and in the range of 43.5 - 53.9% on 7th day. The result of the GC-MS analysis of Diesel as presented in Table 4 showed 99.94% and 99.45% reduction in Hexadecane by lipases from Immobilized *Bacillus thuringiensis* and *Lysinibacillus sphaericus*. Maximum degradation of Diesel from lipase of Immobilized *Bacillus thuringiensis* and *Lysinibacillus sphaericus* was at 20 days. In contrary, **Verma *et al,* (2006**) tested the ability of *Bacillus* sp. SV9 to degrade *n*-alkanes fraction of oily sludge and reported that *Bacillus* sp. SV9 was able to degrade 88.9±1.24% of C_{12}-C_{30} *n*-alkanes in 5 days.

CONCLUSION

In this study, lipase from Immobilized *Bacillus thurigiensis* and *Lysinibacillus sphaericus* degraded diesel after 20 days. It can be concluded that enzymatic degradation of Diesel using bacterial lipase is an effective and eco-friendly biotechnological approach.

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A REVIEW OF STREET GRILLED MEAT (SUYA) IN BENIN CITY, NIGERIA: A POTENTIAL PUBLIC HEALTH RISK

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ABSTRACT

Suya originated in the northern parts of Nigeria is ready to eat grilled skewered meat products prepared in different forms spiced with locally sourced, commonly produced by the Hausas in Northern Nigeria. It has permeated Nigerian society, being affordable for all and available everywhere. It is produced from boneless meat hung on stick and spiced with peanut cake, salt, vegetable oil and other flavourings followed by roasting around a glowing charcoal fire. It's generally made with skewered beef, ram, or chicken. Innards such as kidney, liver, and tripe are also used. Suya are of three main forms namely Tsire, Kilishi, and Balangu, and Tsire is the most commonly preferred. In Benin City, which is located in the southern part of Nigeria, Suya has become a very common street food delicacy scattered most major areas and are sold mostly in the evening time, only very few vendors will sell Suya in the day time. Concerns have been raised in many research regarding the public health risk of consuming suya, part of which is food poisoning, as a result microbial contamination such as *Bacillus cereus*, *Staphylococcus aureus*, aflatoxigenic molds, *Staphylococcus epidermidis*, *Proteus vulgaris*, *Klebseilla pneumoniae*, *Escherichia coli*, *Candida* spp., *Salmonella enteritica* and *Enterobacter* spp. Trace metals such as zinc, lead, manganese, iron, and copper have been observed in suya in concentrations exceeding recommended tolerable upper intake levels which can consequently cause serious long term health issues. This study reviews the potential public health risk of consuming street grilled meat (Suya) in Benin City.

Keywords: Suya, public health, contamination, hygiene, food handling, food poisoning

INTRODUCTION

Meats are rich sources of essential and beneficial minerals needed for body morphological processes. Eating meat, a good source of proteins and fats, plays a major role in the intake of a number of nutritional and trace elements in human health (**Adebiyi *et al.*, 2008**). Ready to eat meat products in Nigeria are prepared in different forms, one of which is the grilled skewered meat, spiced with locally sourced, mixed dry ingredients that is prepared and sold on the street. Suya is commonly produced by the Hausas in Northern Nigeria. It is produced from boneless meat hung on stick and spiced with peanut cake, salt, vegetable oil and other flavorings followed by roasting around a glowing charcoal fire. It's generally made with skewered beef, ram, or chicken. Innards such as kidney, liver, and tripe are also used (**Eke *et al.*, 2012**). suya is classified into three main forms namely Tsire, Kilishi, and Balangu (**Igene &Mohanned, 1981; Ahmadu &Aduwa, 2015).** Among the three forms of suya product, Tsire is the most commonly preferred. Although Suya originated in the northern parts of Nigeria, it has permeated Nigerian society, being affordable for all and available everywhere. It has been called a unifying factor in Nigeria (**Ekanem, 1998**). Suya has become a Nigerian national dish with different regions claiming the superiority of their recipe and methods of preparation, but similar grilled meat recipes are common in many West African countries (**Egbebi & Seidu, 2011**). In Benin City, which is located in the southern part of Nigeria, Suya has become a very common street food delicacy scattered most major areas within Benin City (**Ahmadu and Aduwa, 2015**). Suya spots in Benin City can be located in almost any place there is a bar or any busy area and the vendors are usually from the Northern part of Nigeria. Suya in Benin City are sold mostly in the evening time, only very few vendors will sell Suya in the day time. Concerns have been raised about the hygienic standards of processing and safety of road side Suya (**Obadina *et al.*, 2014**). This review is aimed at contributing to knowledge on the potential public health risk of consuming street grilled meat (Suya) in Benin City, raising awareness to spark off public health monitoring on street grilled meat (Suya) in Benin City, Edo state, and consequently in all states in Nigeria where it is sold.

SOCIO-ECONOMIC CHARACTERISTICS OF SUYA

Suya, production in Nigeria remains largely in the hands of small-scale traditional producers and there is little or no information on the economics of its production, especially in Benin City of Edo State. The work of **Iliyasu *et al.*, (2013)** was on the economic aspect of suya production, the study was conducted in the northern part of Nigeria, Borno State precisely. It is important to know whether suya production is also profitable in the southern part of Nigeria especially Benin City. A study carried out by **Ahmadu and Aduwa, (2015)** in Benin City Metropolis which comprises mainly Oredo, Egor and Ikpoba-Okha Local Government Areas (LGAs), showed that all of the suya producers were males from northern Nigeria and mostly illitrates who produced suya majorly. The producers depended mainly on family labour for their production. Personal savings was the dominant source of finance available to the suya producers for their business. Consequently, majority of the producers had low initial capital investment in the suya production business. From this study suya and was found to be profitable in Benin City compared to Borno State as seen in the study of **Iliyasu *et al.*, (2013)** owing to the fact that there is low production of suya in Benin City which makes it expensive and hence higher returns compared with the high production of suya at Borno with higher population of Hausas that are involved in the business. According to economic theory, low production of a commodity which entails its shortage in the market will lead to increase in its price and consequently, higher returns, all things being equal (**Adegeye and Dittoh, 1985**). **Ahmadu et al., (2008)** in his study highlighted that the costs of meat, charcoal and labour significantly influenced the gross income from suya. The effect of frequent rainfall experience in Benin City during the wet season created unfavourable weather that often disrupted the suya production business. This is because majority of the producers had their production/sale stands in the open space (**Ahmadu and Aduwa, 2015**).

EFFECT PROCESSING AND PACKAGING METHODS OF SUYA

There is no standard recipe for the production of the complex mixture of spices and additives which make up the Suya and the spice mix served with it (**Akpamu et al., 2011**). Ingredients may vary according to personal and regional preferences (**Egbebi & Seidu, 2011**), and may include clove, ginger, red pepper, black pepper, table salt, and groundnut cake, as well as food additives such as monosodium glutamate and maggi cube (**Akpamu et al., 2011**). Packaging maintains the benefits of food processing after the process is completed, enabling food to travel safely for long distances from their point of origin and still be wholesome at the time of consumption (**Joshua *et al.*, 2016**). Most of the processors of this meat were found in strategic locations and were people who does not have much formal education and as a result still uses traditional methods of handling, processing and packaging the products, which are considered to be unhygienic, unsafe and can result in rapid deterioration of the processed meat if not consumed within a short period of time. The processors have been accustomed to collecting old newspapers from different homes and using same to package Suya meat for their customers, which are considered to be dirty and dusty, also in some homes where chemicals were being used to control insects like cockroaches and mosquitoes, there is tendency of the chemicals being sprayed on the newspapers, which the chemicals when in contact with the meat and being consumed can poised serious health issues. Besides the fact that the use of old newspapers in packaging of Suya meat product does not give a good professional image to the processor, the printed inks on the papers contain pigments, colorants, binders, additives and photo initiators which can be harmful to the health of the consumer (**Rokade *et al.*, 2012**). Packaging does not only ensure that foods contains and maintains the amount and forms of the required ingredient and nutrients but also improves the sensory quality and colour

stability. It has been demonstrated that food packaging can retard product deterioration, retain the beneficial effects of processing, extend shelf-life and maintain or increase the quality and safety of food (**Marsh & Bugusu, 2007**). Therefore it is important that food packaging materials should possess proper mechanical, thermal and optical properties for foods. In addition anti-microbial and barrier functions against gases, vapour and aroma are also important in food packaging materials (Chin *et al.*, 2015). Suya meats are to be stored between 50 to 600C to disfavour the growth of microbes (**Uzeh & Akinyemi, 2012**). The principal roles of packaging are to protect food products from outside influences and damages, to contain the food, and to provide consumers with ingredient and nutritional information (**Coles, 2003**). A study by **Joshua *et al.*, (2016)**, shows that processing methods and Packaging materials used in the preparation of Suya for consumption have effects on the quality attributes of the final products.

HYGIENE PRACTICES OF SUYA PRODUCERS

The contamination of street foods in Nigeria have been attributed to factors such as the unstructured nature of the street food industry, inadequate public health infrastructure, the poor state of environmental health and sanitation, and a lack of enforcement of sanitary regulations (**Hassan & Dimassi, 2014**) Understanding the factors implicated in the contamination of street foods as well as the health and socio-economic relevance of street foods in Nigeria is important in providing further clarity on the individual hygiene practice of suya producers (**Rahman*, et al.*, 2012**). Qualitative studies utilizing focus groups, surveys, and inspection reports however show that food handlers do not always adopt the safe practices that they report including proper hygiene, and the use of risk reduction tools such as thermometers and hand washing (**Clayton & Griffith, 2004; Chapman *et al.*, 2013; Green & Selman, 2005**). The choice of environment and clothing in which suya is prepared and sold is of concern. **Chukuezi, (2010)** highlighted that in Nigeria, 24% of street food vendors prepare food under unhygienic conditions, 48% handle food with their bare hands, 52% do not wear hair covering, and 62% handle money while serving food. It is further reported that 19% wore jewelry while serving food, 29% blow into polythene bags meant for serving food, and 43% did not wear appropriate clothing such as aprons. These facts are well observed in suya handlers in Benin City. The unsanitary conditions in which street food vendors operate, coupled with media reports on foodborne disease outbreaks seem to have created consumer mistrust of street foods (**Rheinlander *et al.*, 2008**). Improving food safety is therefore predicated on knowledge of personal hygiene, adequately cooked foods, avoidance of cross contamination, safe temperatures for stored foods, and avoidance of serving unsafe foods to consumers (**Food Safety and Zoonoses, 2006). Adebiyi *et al.*,(2008)** reported the accumulation of high levels of trace metals concentration like zinc, lead, manganese, iron and copper on suya owing possibly to the water removal from raw meat by fire during the roasting process thereby increasing its dry matter; this could have resulted in the increase in metals per mass of the Suya. Anthropogenic sources are additional reasons. He also pointed out the fact that Suya is commonly prepared and sold along roadsides that are usually very busy thus can be exposed to street dusts due to re-suspension mechanisms. Nigeria is known to be very poor in road maintenance; a situation that may explain the presence of dusts on the roads. Suya is roasted on an iron gauze platform using wood charcoal fires, causing exposure to wood smokes and iron rust. These also serve as sources of metal accumulation by Suya. Vehicular emissions comprise another source of contamination. It has been established that particulate matter from vehicle emissions contain some metals (wear metals) (**Trevor, 1981**). Like the vehicle emissions, particulates from the breakdown of vehicle brake parts can be another source of trace metals in Suya sold along the roadsides in the study locations (**Yeung, 2003; Todd, 2003**). Another study by **Enabulele and Uraih, (2009),** showed that in Benin City, improper handling of the suya before and after preparation and most especially from vectors like flies and hands of the sellers themselves, are responsible for contamination by organisms such as *E.coli* and *Salmonella* spp.

RISK SOURCES AND PUBLIC HEALTH IMPLICATIONS

The contamination of ready-to-eat meat products is a common phenomenon in Nigeria that has been reported by many researchers (**Chukwu & Imodiboh, 2009; Fonkem *et al.*, 2010; Salihu *et al.*, 2010; Iheagwara & Okonkwo, 2016**). Suya is normally sold wrapped in old newspaper, which has been criticized for serving as a possible source of contamination (**Apata *et al.*, 2013**). Tapeworm (*Taenia saginata*) from infested beef has been found to survive the temperatures used in preparing Suya and remain viable to infect humans (**Mosimabale & Belino, 1980**). Cases of haemolytic anaemia have been described after ingestion of Suya, possibly as a result of adulteration of food additives (**Williams, *et al.*, 1988**). **Edema *et al.*, (2008)** who evaluated the microbial hazards associated with processing of suya meat, reported that processing water, meat processing labs, utensils, spices, and raw meat revealed contamination with potential pathogens such as *Bacillus cereus, Staphylococcus aureus, Salmonellae* species, and aflatoxigenic molds with aerobic mesophilic counts in the order of 105 cfu/g, with the highest value (7.17) observed in the packaging material and the lowest value (1.47) observed in the processing water. **Adebiyi *et al.*, (2008)** reported the accumulation of trace metals such as zinc, lead, manganese, iron, and copper on suya and the concentrations of these metals exceeded recommended tolerable upper intake levels. This is as a result of exposure the suya to open air in busy areas with high human and vehicular movements. Ingestion metals at high doses can result to long term health conditions such as cancers, high noncarcinogenic risks, hormonal disorders, cardiac diseases, neurological disorders, renal failures, reproductive issues such as infertility, immunological disorders (**Ekhator *et al.*, 2017**).

E. coli, which are normal flora of the human and animal intestine, have been identified as a leading cause of food borne illness all over the world (**Agbeyegbe & Uraih, 1982; Hussein, 2007**). **Enabulele & Uraih, (2009)**, showed that in Benin City, high levels *E. coli* 0157: H7 strain was detected in some of the raw meat samples and suya. E. coli 0157:H7 prevalence rate where observed to be higher in suya than raw meat which will still be processed before consumption. The high occurrence of *E. coli* 0157: H7 in suya and raw meat could be as a result of improper handling of the suya before and after preparation and most especially from vectors like flies and hands of the sellers themselves and also poor sanitary environment in Benin City under which the animals are slaughtered and sold.

In similar study by **Inusa & Said, (2017)**, Suya samples from Kano metropolis of Kano State, Northern-Nigeria, were found to be contaminated by organisms that pose threat to public health. These include *Staphylococcus aureus, Staphylococcus epidermidis, Proteus vulgaris, Klebseilla pneumoniae, Escherichia coli, Candida* spp., *Salmonella enteritica* and *Enterobacter* spp. The microbial contamination that was detected in the locations for sample collection could be traced to unhygienic processing and low level of sanitation. Odey ***et al.*, (2013)** isolated *Staphylococcus aureus, Escherichia coli, Streptococcus* spp, *Salmonella* spp, *Bacillus* spp, *Pseudomonas* spp and *Proteus* spp. from selected suya samples on sale at Calabar, Cross River State, Nigeria. Okonko *et al*., (2013) isolated *Bacillus* spp, *Staphylococcus aureus* and *Escherichia coli* in suya samples from Port Harcourt, Rivers State. Study conducted on suya sold in Ado and Akure, South west Nigeria revealed bacteria, molds, yeast, and fungi (**Egbebi & Seidu, 2011**). **Osho, (2004)** also evaluated the bacteria contamination of suya processed in Abeokuta, south western Nigeria and found up to 103 cfu/g enterobacteriaceae in 40% of the 622 samples collected; more than 104 cfu /g aerobic mesophiles including Staphylococcus aureus in all collected samples. **Inyang *et al.* (2005)** also evaluated the bacterial quality of suya sold in Markurdi, northern Nigeria and concluded that fecal coliforms were the main bacterial contaminants, although they occurred within acceptable limit.

CONCLUSION AND RECOMMENDATION

Suya though a highly nutrient dense ready-to-eat meat product, it could be contaminated at the retail outlets. Consequent upon this, it is recommended that consumers cook their own meat properly before they are consumed and also avoid or indiscriminate eating of meat sold in the open. It is also recommended that the public must be aware of the consequences of selling and purchasing Suya in parks and must be discouraged. However, adequate covering of the Suya at the selling points may minimize its contamination from vehicle exhausts and dusts while, instead of direct heating of the raw meat with charcoal fire, an indirect heating such as using microwave oven should be encouraged to prevent direct contact of the meat with charcoal smokes and iron wire gauzes. Good manufacturing practices, proper packaging and storage are recommended to safeguard the health of consumers.

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ASSESSMENT OF SHELF LIFE AND BACTERIAL LOAD OF VIABLE EGGS OBTAINED AT THE POINT OF LAY FROM ROOM AND REFRIGERATOR STORAGE TEMPERATURES

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ABSTRACT

It is well established that storing hatching eggs over a longer period of time affects its quality. The current study evaluated the impact of egg storage duration in-relation to two different temperature conditions (room and refrigerator) to determine the bacterial load and shelf life of viable eggs. One hundred and twenty eggs were used for this study, 60 were boiled and 60 were raw. Thirty of the boiled eggs were stored at room temperature and the other 30 eggs were kept in the refrigerator. Similarly, 30 raw eggs were each stored at room and optimal refrigeration temperatures for eggs (< 7 ºC) respectively, while the egg weight, viability and sensory tests were performed daily on the eggs. However, the eggs kept in the refrigerator were viable for longer and relatively maintained higher physical appearance and sensory quality compared to eggs kept at room temperature. In the investigation of bacterial load, the total viable count ranged from 6.0×10^3 to 11.9×10^3 coliform forming unit per millilitre (cfu/ml) and 1.0×10^3 to 6.5×10^3 cfu/ml for the boiled eggs kept at room and refrigeration temperatures (BRT and BFT) respectively. More so, the bacterial counts in raw eggs obtained at room and refrigerator storage ranged from 4.8×10^3 to 6.5×10^3 cfu/ml. Subsequently, the characterization and identification of bacterial isolates indicated the presence of *Salmonella pullorum*, *Proteus mirabilis* and *Pseudomonas* sp. The *Salmonella pullorum* was isolated from all the egg samples (BRT, BFT, RRT and RFT). The *Proteus mirabilis* was isolated from boiled eggs kept in both room and refrigerator temperatures (BRT and BFT) while *Pseudomonas* sp. was obtained only from raw eggs stored in the refrigerator (RFT). In addition, the boiled eggs at room temperature started deterioration on Day 9, while its counterpart in the refrigerator began spoilage or decrease in quality from Day 16. The weight of the viable eggs in relation to the non-viable ones was statistically significant ($P < 0.05$). The refrigeration of eggs increases its longevity while proper hygiene and adequate boiling of eggs reduces the risk of acquiring infections through bacterial contamination.

Keywords: Egg shelf life, bacterial load, refrigerator storage temperatures, room temperatures

INTRODUCTION

The chicken egg is a perfect source of proteins, lipids, essential vitamins, and minerals that are nutritionally and medically beneficial to humans. It is therefore essential that the eggs are in perfect health conditions, devoid of contaminants at the point of lay to maintain their potential viability status. However, some factors have been reported to be responsible for egg spoilage after it had been hatched or laid (**Davies & Breslin, 2002**).

The outer layer of the egg known as the shell prevents microbial activity from penetrating the egg contents, moisture from escaping and to protect the egg during handling and transport. The bacteria at the surface are able to gain access through the pores of the shell to infect the inner part of the egg. Though the egg shell serves as physical barrier, the albumen (egg white) and yolk also contain anti-microbial properties such as protein components of Lysozyme and immunoglobulin Y (IgY) (a class of proteins formed by the immune system in reaction to certain foreign substances, and specifically able recognize them). These constituents are very effective during early stages of embryo development and to resist invasion and growth of microorganisms (**Barnhart *et al*., 1991**).

Despite the egg's innate properties to protect itself from bacterial attack, these barriers are transient and offer no permanent protection against the infiltration of bacteria through the shell and pores of the membrane. This results in formation of sliminess, jellying of albumen, offensive and pungent smell due to enzymatic, proteolytic and lypolytic substances released during bacterial growth (**Moore & Madden, 1993**). The egg shell can readily be infected when passing through the vent and contamination occurs through the cloaca area within a short duration of lay and from contact with dirty surfaces. Previous research has shown chicken eggs to be associated with the transmission of human pathogenic bacteria such as *Escherichia coli*, *Salmonella typhi*, *Listeria monocytogens* and *Yersinia enteriticus* in human populations (**Padron, 2005**). **Davies and Breslin (2002)** emphasized the need to remove any contaminant on the surfaces of eggs to reduce the risk of bacterial adhesion and entry into the egg contents. His proposed ways of achieving this were washing, boiling water, hydrogen peroxide application, storage in refrigeration temperature and pasteurization. The aim of this study therefore was to evaluate the bacterial counts of viable eggs obtained at the point of lay from room and refrigerator storage temperatures.

MATERIALS AND METHODS

Study Area

The study was carried out in Okada community, Ovia North-East Local Government Area, Edo State, Nigeria.

Sample Collections

The egg samples were collected from Fortune poultry farm located at Iguomo quarter in Okada town. The farm holds about two thousand (2,000) chicken layers, which were raised in cages, fed with Top Feed layer's match and supplemented with calcium bone meal (CBM). One hundred and eighty brown-coloured eggs were collected in sterile plastic crates and immediately transported to the microbiology laboratory, Igbinedion University, Okada for analysis.

Viability Test

A Candler made of carton with inserted bright light was used for the viability testing of the egg samples and carried out in the dark in order to obtain a clearer observation of the internal features of the egg. A score which ranges from 0 to 5 was allotted on quality or viable basis. The viable eggs with intact quality were scored 5 and decreased down to 0 for non-viable with irritating, pungent smell.

Measurement of Egg Weight

The egg weight was measured using an electronic weighing balance (Adventurer Ohaus Company, Model XP 1005. NJ. (USA) to get the initial weight of the samples. Sixty egg samples consisting of both 30 boiled eggs using a cooking pot and 30 raw eggs were weighed and recorded

Storage Procedure

The egg samples were divided into 4 portions; the 30 boiled eggs and 30 raw eggs were packaged on plastic crates and each was stored at room (28 ± 2 ºC) and refrigerator temperatures (4 ± 2 ºC) respectively. The samples were analysed for bacterial load at interval of 5 days for a period of 45 days.

Isolation and Enumeration of Bacteria

Isolation of bacteria from the egg samples was conducted by standard techniques of pour plating using nutrient agar and MacConkey agar following serial dilution of egg sample prepared from 1.0 up to 10^{-4} ml. Thereafter, 1.0 ml of the dilution was aseptically inoculated onto the corresponding labelled petri plates. The sterilized nutrient agar and MacConkey agar were separately poured slowly into the base of the petri plate with slight swirling for uniform distribution of the medium. The plates were allowed to solidify and incubated at 37 ± 2 ºC for 24 hr and distinct bacterial colonies in the plates were used to determine the total bacterial counts (TBC) and expressed as colony forming unit per millilitre of the test sample (cfu/ml) (**Barrow & Feltham, 2008 p. 60; Long *et al*., 2017**).

Identification and Characterization of Bacteria

Following the culturing and Gram staining processes, three bacterial colonies were picked based on their different colonial characteristics and each of them was phenotypically characterized by performing basic biochemical tests such as catalase, indole, urease, oxidase, citrate, methyl red and Voges-Proskauer as prescribed standard methods (**Adams & Moss, 2008 p. 141**; **Barrow & Feltham, 2008 p. 60**).

Statistical Analysis

The statistical significance was performed using students T – test to compare the means of bacterial counts and one-way analysis of variance (ANOVA) was applied to determine the means of bacterial loads of different storage temperatures (**Sokal & Rohif, 1994 p. 945**).

RESULTS

The mean viable sensory evaluation test carried out on both the boiled and raw eggs on daily basis from Day 1 to 46 revealed that; the boiled eggs at room temperature (BRT) started producing offensive odour from day 9 (4.7) while the bad odour of the boiled eggs at refrigerator temperature (BFT) started from day 16 (3.5). The raw eggs stored at room temperature (RRT) started developing unacceptable smell from day 26 (3.5) while the raw eggs stored in refrigerator temperature (RFT) began smelling from day 37 (3.0) (Tables 1a and b).

Table 1a Mean Sensory Evaluation of The Eggs Examined from Day 1 To Day 23

Code of eggs	**DAY 1 – 23**																						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
	Mean sensory evaluation (k) on each day of storage																						
BRT	5	5	5	5	5	5	5	5	4	4	3.5	3.5	3.3	3	3	2.3	-	-	-	-	-	-	-
BFT	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	3.5	3.3	3.3	3.3	3	2	-	-
RRT	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
RFT	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

Key: BRT – Boiled Room Temperature; BFT – Boiled Refrigerator Temperature; RRT – Raw Room Temperature; RFT – Raw Refrigerator Temperature

Table 1b Mean Sensory Evaluation of the Eggs Examined from Day 24 to Day 46

Code of eggs	**DAY 24 – 46**																						
	24	25	26	27	28	29	30	31	32	32	34	35	36	37	38	39	40	41	42	43	44	45	46
	Mean sensory evaluation (k) on each day of storage																						
BRT	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BFT	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RRT	5	5	3.5	3.7	3.7	3.7	3.7	3.3	3.3	3	3	2.3	1	-	-	-	-	-	-	-	-	-	-
RFT	5	5	5	5	5	5	5	5	5	5	5	5	5	4	4.7	4.7	4.7	4.3	4.2	4.2	3	2	1

Key: BRT – Boiled Room Temperature; BFT – Boiled Refrigerator Temperature; RRT – Raw Room Tempe ature; RFT – Raw Refrigerator Temperature

The bacterial load of the boiled egg stored at room temperature (BRT) determined at a 5- day interval showed that bacteria growth occurred in day 16 of storage with bacterial count ranging between 15 × 10^3 to 102×10^3 cfu/ml with higher bacterial count in the nutrient agar used. In the boiled eggs stored at the refrigerator temperature (BFT) the growth started at day 21 and the bacterial count ranged between 25 × 10^3 to 65× 10^3 cfu/ m (Table 2a). However, no bacterial growth was observed from raw eggs stored at both room and refrigerator temperatures from day 6 to day 31. Prolonged storage of the raw eggs at room and refrigeration temperatures resulted in growth at day 36 and 46 respectively and ranged between 17 × 10^3 to 65 x 10^3 (Table 2b).

Table 2a Bacterial Analysis of Eggs In-Relation to Number of Days

Code of eggs	**Average counts of bacteria on eggs (x 10^3)**																													
	DAY 6						DAY 11						DAY 16						DAY 21						DAY 26					
	NA			MA			NA			MA			NA			MA			NA			MA			NA			MA		
BRT	0	0	0	0	0	0	0	0	0	0	0	0	102	88	119	15	13	21.5	0	0	0	0	0	0	0	0	0	0	0	0
BFT	0	0	0	0	0	0	0	0	0	0	0	0	4	3	1	0	0	0	51	65	58	60	25	60	0	0	0	0	0	0
RRT	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RFT	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 2b Bacterial Analysis of Eggs In-Relation to Number of Days

Code of eggs	**Average counts of bacteria on eggs (x 10^3)**																							
	DAY 31						DAY 36						DAY 41						DAY 46					
	NA			MA			NA			MA			NA			MA			NA			MA		
BRT	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BFT	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RRT	0	0	0	0	0	0	48	65	55	10	18	15	0	0	0	0	0	0	0	0	0	0	0	0
RFT	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	42	40	40	40	65	50

Key: NA - Nutrient agar; MA- MacConkey agar; BRT – Boiled Room Temperature; BFT – Boiled Refrigerator Temperature; RRT – Raw Room Temperature; RFT – Raw Refrigerator Temperature

Three microorganisms were isolated from the eggs during the course of the study. The bacteria phenotypically isolated from the egg contents were *Salmonella pullorum, Proteus mirabilis* and *Pseudomonas sp.* (Table 3).

Table 3 Phenotypic Characterization of Microbial Isolates

Characteristics		**Isolate 1**	**Isolate 2**	**Isolate 3**
Cultural	Elevation	Low convex	Swarming	convex
	Margin	entire	Serrated	smooth
	Shape	circular	circular	circular
	Colour	grey	creamy	creamy
Morphological	Gram stain	-	-	-
	Cell type	rod	rod	rod
	Cell arrangement	single	single	single
	Gram stain	-	-	-
Biochemical	Catalase	+	+	+
	Indole	-	-	-
	Urease	-	+	-
	Oxidase	+	-	-
	Methyl red	+	+	+
	Voges Proskauer	-	-	-
	Citrate	+	+	+
	Acetate	-	-	-
	Lactose	-	-	-
	Mannitol	-	-	+
Probable Organism		*Pseudomonas sp.*	*Proteus mirabilis*	*Salmonella pullorum*

Salmonella pullorum, *Proteus mirabilis* and *Pseudomonas sp*. *Pseudomonas spp.* were isolated from the raw eggs stored in the refrigerator. *Proteus mirabilis* was isolated from boiled eggs stored in both room and refrigerator temperature. While Salmonella pullorum was isolated from all the eggs stored. The Pseudomonas sp. was only isolated from raw eggs stored in the refrigerator temperature (RFT) while *Proteus mirabilis* was isolated from boiled eggs stored in both room and refrigerator temperature (BRT and BFT). However, Salmonella *pullorum* was isolated from both raw and boiled eggs stored in both room and refrigeration temperatures (BRT, BFT, RRT and RFT) (Table 4).

Table 4 Occurrence of Isolated Bacterial in The Storage Temperatures

Organism	**BRT**	**BFT**	**RRT**	**RFT**
seudomonas spp	-	-	-	Present
Proteus* sp *mirabilis	Present	Present	-	-
Salmonella* sp *pullorum	Present	Present	Present	Present

BRT – Boiled Room Temperature; BFT – Boiled Refrigerator Temperature; RRT – Raw Room Temperature; RFT – Raw Refrigerator Temperature; - – Not Present

Table 5a Analysis of Variance to Test for Significant Difference in Weight Eggs Stored at Various Storage Temperatures

Source of variation	Degrees of freedom	Sum of squares	Mean square	F	F 0.05
Treatments	5	22.31	44.463	1.065	0.3853
Residuals	90	3758.2	41.758		

F-Calculated > F-critical, Ho is rejected (P < 0.05), Ho – No significant difference of the various weight of the boiled eggs stored at room temperature. On the other hand, the weight of boiled eggs stored in the refrigerator varied significantly at P < 0.05.

Table 5b Analysis of Variance to Test for Significant Difference in The Weight of The Boiled Eggs Stored in Refrigerator

Source of variation	**Degrees of freedom**	**Sum of squares**	**Mean square**	**F**	**F 0.05**
Treatments	5	1283.7	256.74	198.67	< 0.05
Residuals	120	155.08	1.292		

The P value is P < 0.05, considered significant. Variation among column means is significantly greater than expected by chance.

Table 5c Analysis of Variance to Test for Significant Difference in The Weight of The Raw Eggs Stored at Room Temperature

Source of variation	**Degrees of freedom**	**Sum of squares**	**Mean square**	**F**	**F 0.05**
Treatments	11	5388.5	489.86	12.220	<
Residuals	420	16836	40.086		0.05

The P value is P < 0.05, considered significant. Variation among column means is significantly greater than expected by chance.

Table 5d Analysis of Variance to Test for Significant Difference in The Weight of The Raw Eggs Stored in Refrigerator

Source of variation	**Degrees of freedom**	**Sum of squares**	**Mean square**	**F**	**F 0.05**
Treatments	11	6497.4	590.4	316834	<0.05
Residuals	264	0.4922	0.001864		

The P value is P < 0.05, considered significant. Variation among column means is significantly greater than expected by chance.

DISCUSSION

The eggs in the refrigerator maintained a relatively higher sensory quality than the eggs stored at room temperature. Storage of eggs at room temperature for few days was enough to substantially reduce the consumption quality of the eggs. The changes in the eggs standard may be as a result of differences in physicochemical composition of the eggs contents such as the albumen pH, yolk index and percentage weight loss. The sensory quality of the eggs also reduced as the storage duration decreased. The bacterial load of the boiled egg stored at room temperature (BRT) determined at a 5- day interval showed that the bacteria growth occurred in day 16 of storage with bacterial count ranging from 15 to 102 $\times 10^3$ cfu/ml with higher bacterial count in nutrient agar (NA). In the boiled eggs stored at the refrigerator temperature (BFT), the growth was observed at day 21 and the bacterial count ranged from 25 to 65 $\times 10^3$ cfu/ml. However, no bacterial growth was observed from the raw eggs stored at both room and refrigerator temperatures from day 6 to day 31. Prolonged storage of the raw eggs at room and refrigeration temperatures resulted in growth at day 36 and 46 respectively and ranged from 1.7 to 6.5 x 10^3 (Tables 2a and 2b). The above results demonstrated that, a higher bacterial count was recorded in raw eggs kept in room temperature than in refrigeration temperature. This goes to show that refrigeration temperature extends the shelf life of both raw and boiled eggs.

The bacteria isolates identified in this study are pathogenic to humans and possess the capacity to persist on and in the egg for a longer period under harsh environmental conditions. This supports the reports of **Dereu *et al.* (2005) and Jones *et al.* (1995),** that microorganisms can be found on the outside of egg shell as a result of the egg emerging from the hen's body through the same route the faeces-containing bacteria is excreted leading to faecal contamination. Microorganisms inside a non-cracked or whole egg may be due to the presence of pathogens within the hen's oviduct before the shell forms around yolk and albumen. Different researchers have indicated that the transovarian route is the most important route for *Salmonella* sp. contaminating egg. This is due to the ability of the *Salmonella* sp. to colonize the ovary and oviduct (vertical transmission) of laying hens for a long time. In the penetration through the egg shell (horizontal transmission), the eggs pass through the highly contaminated cloaca area at the point of lay leading to visible faecal contamination on the shell. Eventually, the shell acquires contamination and being wet, the egg cools down immediately with the egg content contracting and a negative pressure is established inside egg content, thereby moving the bacteria through the cell (**Radowski, 1995**). However, some factors enhancing bacterial infection of eggs

include physical chemical defence mechanisms that protect egg contact from invasion and multiplication. The egg shell and membrane physically hinder bacterial penetration into the egg albumen, while the vitellin membrane and chalazae reduce invasion into the nutritious component of the egg. The antimicrobial properties of the egg albumen (ovotransferin and avidin: chelating metal ions and biotin respectively) its viscosity and alkaline pH inhibit bacterial growth and egg yolk attack (**Long *et al*., 2017; Moore & Madden, 1993**).

Adams and Moss, (2008) notes that the most common egg spoilage bacterial genera are the *Salmonella*, *Escherichia*, *Pseudomonas*, *Aeromonas* and *Proteus* (p. 141). This indicates that the gram-negative bacteria are well equipped to suppress or overcome the antimicrobial defences of the egg. This has now been supported by the outcome of this study with the presence of *Salmonella* sp. in all the samples of both boiled and raw eggs stored in room and refrigeration temperatures. The genus *Proteus*; was recorded in boiled room temperature (BRT) and boiled refrigeration temperature (BRT) while the *Pseudomonas* sp. was present only in the raw refrigeration temperature (RFT) (Table 4). Consequently, the eggs stored under refrigeration temperature presented ($p < 0.05$) when compared with eggs kept at room temperature. It was apparently evidenced that the quality of eggs changes significantly ($p > 0.05$) according to the storage temperature and period of storage mainly due to weight loss and internal and external infections by the bacteria. However, it is worthy of note that once eggs have been refrigerated, they must be kept refrigerated to prevent condensation from forming on the shell. If they warm up, the moisture makes it easier for bacteria to penetrate the shell.

CONCLUSION

The mean total viable count for eggs stored at room temperature was higher than refrigeration storage temperature, as bacterial growth may partly be due to handling, storage equipment and immediate environmental conditions of the storage room. Therefore, consumers should be encouraged to store eggs in refrigerators and maintain good sanitary practices to reduce contamination. More so, the isolated bacteria being pathogenic organisms, would necessitate adequate cooking eggs before consumption and places where eggs are stored should be of satisfactory hygienic standard to reduce bacterial contacts. The practice of eggs undergoing a sterilizing process before they are sold like washing in hot, soapy water and sprayed with a disinfectant, which kills any bacteria on the shell should be encouraged.

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