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THE BACTERIAL PROPERTIES OF THE RED PALM WEEVIL RHYNCHOPHORUS PHOENICIS (COLEOPTERA: CURCULIONIDAE) CONSUMED IN BAYELSA STATE, NIGERIA

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ABSTRACT

Studies on the bacterial properties of nymphs and adults of the red palm weevil *Rhynchophorus phoenicis* tested as pristine (treatment A) and in palm wine soaked forms (treatment B) was carried out in Biological Sciences laboratory of Niger Delta University in Bayelsa State. Using pour plate method on nutrient agar medium, the enumeration of the heterotrophic bacteria showed marked differences in the Bacterial population with weevils of treatment A having higher bacterial counts than those of B. Enumeration of the Coliform bacteria counts were higher in the test samples of treatment A, with tcfu range of 9.4×10^4 to 1.16×10^4 while that of treatment B was 4.9×10^4 to 6.6×10^4. Bacteria culture of the samples indicated a number of bacteria morphologies with treatment A showing higher diversity of bacteria (6-10) as against treatment B with 4-5. Biochemical tests and characterization of Bacteria isolates in treatment A was positive for *Bacillus subtilis*, *Proteus vulgaris*, Citrobacter, Escherichia, Serratia, Micrococcus and Staphylococcus species while treatment B showed positive for *Escherichia coli*, *Staphylococcus epidermidis*, *Citrobacter fruendii*, *Pseudomonas aeruginosa*, Salmonella, Proteus and Bacillus species respectively. The percentage occurrence of the Bacterial isolates revealed highest percentage (19%) for Citrobacter specie and *Escherichia coli* while Pseudomonas and Micrococcus species had the least percentage occurrence of 6%. Hence, we hereby recommend that caution be raised on the consumption of raw palm weevils, as the insects harbor soil and water bacteria in their bodies that could pose as health risk factor to consumers.

Index Terms - *Rhynchophorus phoenicis,* Coliform bacteria, Treatment, Occurrence, Nutrient agar.

I. INTRODUCTION

*Rhynchophorus sp*ecies also known as the red palm weevil (RPW) is widely described as the most devastating insect pest of palms in the world (Ju *et al*., 2011; Hou *et al*., 2011; Wang *et al*., 2015).

According to Durst *et al.*, (2010) insects are the most successful group of organism in the animal kingdom proving high biodiversity in terms of species richness, animal biomass and critical ecological functions. Insects are

valuable to humans as plant pollinators and as biological control agents in pests control (Choate and Drummond, 2011, 2012). Some insects provide commercial value products such as honey (FAO, 2015), tonnes of silk (Yong-woo, 1999), Carmine, (red dye) used to colour foods, textiles and pharmaceuticals, resilin used in medicine to repair arteries (Elvin *et al.*, 2005), silk proteins of arthropods as biomaterials thus inspiring technology and engineering methods Lewis, (1992). According to Pemberton, (1999) arthropods are generally regarded as a source for the development of drugs with immunological, analgesic, antibacterial, diuretic, anesthetic, and anti-rheumatic properties. Besides their commercial and medicinal value, insects also constitute an important component of the diet of many cultures around the world and their consumption makes efficient use of available natural resources (Ana Marı´aAcun˜ *et al*., 2011). Recently, it has also been established that insects are important natural source of food for many vertebrae animals, including birds, lizards, snakes, amphibians, insectivores and other mammals (Banjo *et al.*, 2010). The traditional use of insects as food continues to be widespread in the tropical and subtropical countries as it provides significant nutritional, economic and ecological benefits for rural communities. In Africa and Asia, people generally eat insects as part of their diet (Manary and Sandige, 2008; Nonaka, 2009; Ramos-Elorduy, 2009; Gahukar, 2011).

However, Insect intestinal tracts harbour rich communities of nonpathogenic microorganisms with a single gut housing about 105–109 prokaryotic cells (Butera, *et al*., 2012). It is increasingly evident that the microbiota of animals (humans included) plays a remarkable role in the host life. The insect gut microbiota affects many aspects of host phenotype. It can increase the digestive efficiency of soluble plant polysaccharides and can mediate interactions between the host and potential pathogens. The complexity, dynamics and types of interactions between the insect hosts and their gut microbiota are far from being well understood though the gut microbiota provide nutrients and is also involved in the development and maintenance of the host immune system. Understanding the relationship dynamics between insects and their microbiota can improve the biocontrol of these insect pests, hence the reason for this studies. Despite the economic and environmental damages caused by the RPW in all the areas where it is endemic, little is known about its microbiota. Radwan, *et al.*, (2012), disclosed that the bacterial community that is embedded in the frass produced inside the tunnels of palms by the RPW larvae is dominated by Enterobacteriaceae with a facultative fermentative metabolism.

Insects are long known for their economic importance to man. However, the presence of contaminating microorganisms, especially pathogenic ones pose challenge to insect farmers, health risks to the consumers and

influences the quality of the organisms. Insects have long been reported to have different forms of associations with microorganisms and there have been cases of disease outbreak resulting from the consumption of edible insects. The presence of high loads of Bacteria especially pathogenic types in the red palm weevil, pose great health issues to the consumers. Hence, there is need to assess the microbiological quality of the palm weevil, so as to determine the bacteria community associated with it.

II. MATERIALS AND METHODS

2.1 Sample collection

Sixty samples were collected from the palm wine camp along Amassoma road in Bayelsa State (30 adults and 30 nymphs) using sterile bottles in April 2019 and sent to the laboratory of Microbiology department of Niger Delta University, Wilberforce Island for microbial analysis.

2.2 Bacteriological analysis

The test samples were analyzed, while adhering to standard operation procedures and good laboratory practices in order to obtain accurate results as stipulated by Cheesbrough, (2006). The standardization of the amount of media, reagents and incubation time was ensured.

2.3 Sterilization and disinfection of materials

Using sterility principles prescribed by Cheesbrough, (2006), aseptic techniques were practiced so as to discriminate contaminating microorganisms. The materials used for this study were sterilized and/or disinfected before and after use. The glass wares including petri dishes, test tubes, nutrient media, normal saline were sterilized by being autoclaved (moist heat sterilization). The glass wares were wrapped with aluminum foil and autoclaved at 121^{o}C for 15 minutes to ensure the destruction of bacterial endospores as well as vegetative cells. All steps in the use of autoclaves following the standard operation procedures strictly as instructed by the manufacturer were adhered to. After autoclaving, the materials were transferred to the hot air oven for drying. Plastic materials not suitable for sterilization via autoclave were disinfected with absolute ethanol including the bench top which was disinfected before and after work.

2.4 Preparation of nutrient media

The nutrient media used for this study were sterilized by autoclaving. Nutrient agar, Macconkey agar, and Mannitol salt agar were used for the cultivation and enumeration of the bacterial population of the samples. Nutrient agar was for the cultivation of less fastidious microorganisms, Macconkey agar for the selective isolation and differentiation of lactose fermenting and lactose non-fermenting enteric bacteria and Mannitol salt agar for selective isolation of pathogenic *Staphylococci species.* Other nutrient media were used for biochemical test of the isolates. Kliger iron agar was used for the detection of lactose and glucose fermentation, gas and hydrogen sulfide production for differential identification of gram negative enteric Bacilli. Simmon citrate agar was used for the detection of citrate utilization as a sole carbon source for the differentiation of gram negative bacteria. Tryptone water was used for the detection of Indole production. The powder media were weighed and dissolved in distilled water according to the manufacturer's instructions. The dissolved media were autoclaved at 121^0 C for 15 minutes, following standard operation procedures.

2.4 Treatment groups

Treatment A- Pristine condition (not soaked in palm wine)

Treatment B- Soaked in palm wine

Thirty of the test weevils were put into the separate groups A and B. A total of six (60) palm weevils were used. During the bacteriological analysis, the different treatment groups were subjected to uniform analytical microbiology procedures.

2.5 Experimental design

The study was conducted in a complete randomized experimental design (CRD). The test insects were randomly separated into two treatment groups.

2.5.1. Experimentation

2.5.1.1 Serial dilution and inoculation

The test organisms were put into separate beakers containing 10ml of 0.85% normal saline to form stock bacteria culture. The beakers were vigorously shaken to discard the bacteria associated with the samples into the saline solution Thereafter, ten-fold dilution was done to reduce the bacterial load of the samples, in order to obtain an acceptable colony number (30-300).1ml from the stock culture was transferred to the first dilution tube (1:10) containing 9ml of normal saline. From the first dilution tube, 1ml was collected aseptically and transferred to the second dilution tube. The samples were diluted up to the fifth dilution tube (1:100000). The tubes were covered swiftly with cotton wool to prevent the contamination of the samples.

Plating was done in triplicates with the third dilution tube (10^{-3}) using pour plate method. 1ml of the inoculum was aseptically collected with a syringe and was poured into the petri dishes. 20ml of the molten agar was poured into the petri dishes and were swirled gently to spread the inoculum evenly in the medium. The plates were allowed to set (solidify) and were inverted and thereafter incubated at 37^{o} C for 24 hours. After the incubation time, the plates were observed for the number of colonies and colony morphology. The number of colonies were recorded and expressed in tcfu/ml.

2.5.2. Isolation and characterization of bacterial isolates

After the incubation of the agar pour plates, the colonies were randomly selected and were picked off with sterile wire loop. The colonies were sub-cultured on fresh nutrient agar plates by streaking colonies on the agar surface. The sub cultured plates were inverted and incubated at 37^{o}c under aerobic condition to obtain pure isolates which were identified by carrying out morphological and biochemical tests using Gram staining, catalase test, oxidase test, indole test, citrate utilization test, Kliger Iron agar slant test, indole test and oxidase test (Chessbrough, 2006).

2.5.3. Biochemical characterization of bacterial isolates

2.5.3.1 Gram Staining Technique

Adopting the procedure of Cheesbrough, (2010), colonies from different pure culture plates were emulsified into a drop of distilled water on a slide and a thin preparation was made. The smear was allowed to air dry. The smear was covered with crystal violet stain for 60 seconds and was rapidly washed off with clean water. The Lugol's iodine was added for 60 seconds and was washed off. The smear was decolorized with alcohol and washed off rapidly. The smear was counter stained with safranin for 60sec and washed off. The smear was examined microscopically under the x100 objective lens.

2.5.3.2 Catalase Test

This test was performed in test tubes, 3ml of hydrogen peroxide was discarded into sterile test tubes using a sterile glass rod, colony of the pure culture was picked and dipped into the test tube and observed for production of gas bubbles Cheesbrough, (2010).

2.5.3.3 Citrate Utilization Test

10ml of Simmon citrate slants were prepared in test tubes as slants. Using a wire loop, the test isolate was picked off and streaked on the slope of the medium, while the test tubes were inoculated at 37^{o}c for 24 hours (Cheesbrough, 2010).

2.5.3.4 Kliger Iron Agar Slant Test

10ml of Kliger Iron Agar was prepared in test tubes as slants. Using a stab, the butt of the test tubes was first inoculated. Thereafter, the slope was streaked with the test organism with a wire loop. Tubes were closed with cotton wool and incubated at 37^{o}c for 24 hours. At the end of the incubation period, the color changes, blackening and cracking of the medium were observed in the tubes and results were interpreted appropriately.

2.5.3.5 Indole Test

10ml of thee tryptophan broth was prepared in tubes. Using a wire loop, the medium was inoculated with the test organism and incubated for 48 hours. Thereafter, five drops of Kovac reagent was added to the medium (Cheesbrough, 2010).

2.5.3.6 Oxidase test

A piece of filter paper was placed in a sterile petri dish and 3 drops of freshly prepared oxidase reagent was added. Using a plastic wire loop, a colony of the test organism was smeared on the filter paper **(**Cheesbrough, 2010).

III. RESULTS AND DISCUSSION

Table 1 below shows the result for the total heterotrophic count of the samples in the treatments A and B. The enumeration of the heterotrophic Bacteria was done on nutrient agar medium, using pour plate method. The table shows marked differences in the Bacterial population associated with the samples and treatment groups. Treatment A (palm weevils not soaked in palm wine) showed higher Bacterial counts than treatment B (Palm weevils soaked in palm wine). However, the table indicates there are differences in the Bacteriological quality of the red palm weevils within the treatment groups.

Table 1: Total heterotrophic Bacteria count of Treatment Groups

TREATMENT A	MEAN	TCFU	TREATMENT B	MEAN	TCFU
T. A1	86	8.6×10^4	**T. B1**	52	5.2×10^4
T. A2	101	1.01×10^5	**T. B2**	67	6.7×10^4
T. A3	96	9.6×10^4	**T. B3**	58	5.8×10^4

KEY: T. A1 (T)- Treatment, (A)- Treatment A, (1)- Treatment A, rep 1, (B)- Treatment B

The number of Bacteria morphologies obtained from the Bacteria culture of the samples in the different treatment groups are represented on Fig 1. The chart showed that the samples in treatment A had higher diversity of Bacteria (ranging from 6 -10 different morphologies), while treatment B recorded a lower Bacteria diversity (ranging from 4-5 different morphologies).

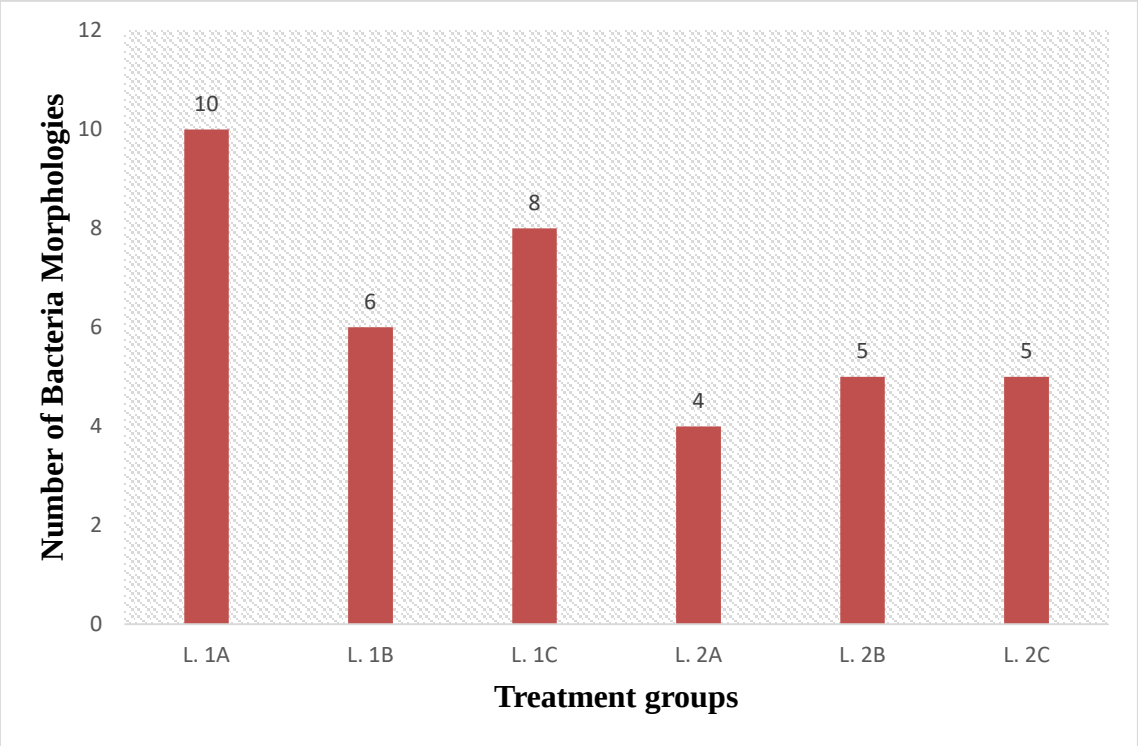

Fig. 1: Number of different bacteria morphologies per treatment Groups

Table 2 shows the results for the enumeration of the coliform Bacteria associated with the samples. The table indicated that the coliform Bacteria counts were higher in samples in

treatment A, with tcfu ranging from 9.4×10^4 to 1.16×10^4 while tcfu of the coliform Bacteria in treatment B ranged from 4.9×10^4 to 6.6×10^4.

Table 2: Population of Coliform bacteria in treatment groups

TREATMENT A	MEAN	TCFU	TREATMENT B	MEAN	TCFU
T. A1	95	9.5×10^4	**T. B1**	58	5.8×10^4
T. A2	116	1.16×10^5	**T. B2**	66	6.6×10^4
T. A3	94	9.4×10^4	**T. B3**	49	4.9×10^4

Table 3: Results for the biochemical tests and characterization of the Bacteria isolates in treatment A

	Bacillus subtilis	*Proteus vulgaris*	*Citrobacter sp*	Escherichia coli	*Serratia Sp.*	*Micrococcus Sp.*	*Staphylococcus Sp.*	*Citrobacter sp*
GRAM STAIN	+VE ROD	-VE ROD	-VE ROD	-VE ROD	-VE ROD	+VE COCCI	+VE COCCI	-VE ROD
CATALASE	+	+	+	+	+	+	+	+
GLUCOSE	+	+	+	+	+	+	+	+
LACTOSE	-	-	+	+	+	-	+	+
GAS	_	+	+	+	-	-	+	+
H_2S		+	+	-	-	-	-	+
CITRATE	+	-	+	-	+	+	+	+
INDOLE	_	+	-	-	-	-	-	-
OXIDASE	_	-	-	-	-	+	-	-

The identification of the Bacterial isolates was done based on the results from the Gram stain reaction, catalase test, glucose and lactose fermentation test, gas and hydrogen sulfide production, citrate utilization test, indole production test and oxidase test. The advanced bacteria identification software and Bergey's manual of determinative bacteriology were used as identification guide.

Table 4: Biochemical tests and characterization of Bacterial isolates in treatment B

	Salmonella Sp.	*Escherichia coli*	Proteus sp.	*Staphylococcus epidermidis*	*Citrobacter fruendii*	*Pseudomonas aeruginosa*	*Bacillus sp.*	*Escherichia coli*
GRAM STAIN	-VE ROD	-VE ROD	-VE ROD	+VE COCCI	-VE ROD	-VE ROD	+VE ROD	-VE ROD
CATALASE	+	+	+	+	+	+	+	+
GLUCOSE	+	+	+	+	+	-	+	+
LACTOSE	-	+	-	+	+	-	-	+
GAS	-	+	+	+	+	-	_	+
H_2S	+	-	+	-	+	-	-	-
CITRATE	-	-	-	+	+	+	+	-
INDOLE	-	-	+	-	+	_	_	-
OXIDASE	-	-	-	-	-	+	_	-

Graphical representation of the percentage occurrence of the Bacterial isolates is presented on figure 2. Indications from the graph showed that Citrobacter specie and *Escherichia coli* recorded the highest percentage (19%). The least occurrences were recorded by Pseudomonas and Micrococcus species (6%).

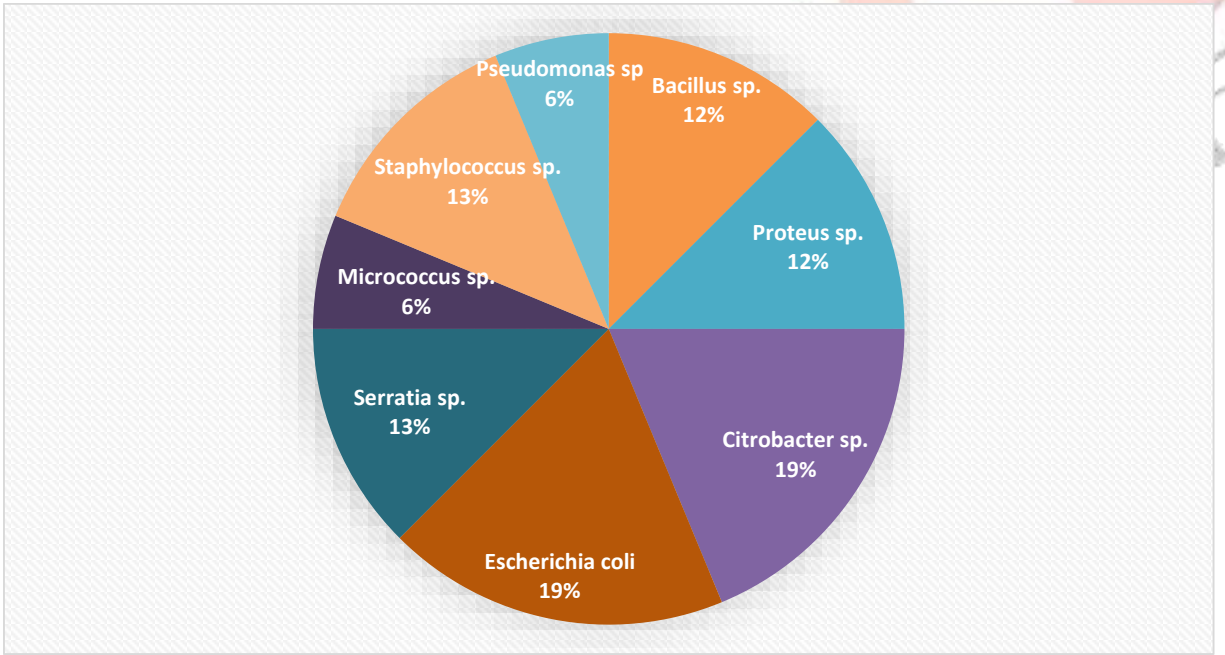

Fig. 2: Percentage occurrence of Bacterial isolates.

The African red palm weevil, *Rhynchophorus phoenicis,* have proven often to exhibit a negative interaction with the ecosystem, as they feed on plants, thus reducing crop yield and quality, notably the oil and coconut palms. Red

palm weevils serve both as food and rich source of vital nutrients. However, these weevils just like other edible insects have its associated Bacteria. The presence of Bacteria in the Red palm weevil has lot of impacts, especially on the safety of consumption and shelf-life, hence this study was undertaken to investigate the Bacteria associated with fresh palm weevils. The study also investigated the difference in the microbial load of red palm weevil soaked in palm wine and those not soaked. The two treatment groups were analyzed using conventional microbiology methods to assess the associated bacterial load.

The results obtained from the study showed that there is positive and significant difference in the bacterial population of the two different treatment groups. Table 1, shows the results for the total heterotrophic Bacteria count of the samples. The tcfu of the samples in treatment A ranged from 8.6×10^{4} to 1.01×10^{5} indicating that red palm weevil in its pristine state is associated with Bacteria. The Bacteria associated with the red palm weevil may as well come from the environment such as soil, water and palm it infects, which may influence the bacterial population associated with it.

In contrast to the results obtained from the pristine samples in treatment Group A, the bacterial load was shown to be smaller. Table 1 showed the bacterial population to range from 4.9×10^{4} to 6.6×10^{4}. This is lesser than the results obtained from the first treatment groups. The reduction in the bacterial load of the samples in treatment group B (soaked in Palm wine) may be as a result of the presence of alcohol in the palm wine. Alcohol has been reported to have antibacterial activity against bacteria, especially at high concentrations. The reduction in the bacterial population is an indication that the shelf-life of the Red palm weevils could be improved by preserving them in palm wine, especially fermented ones.

Figure one provided insight about the effect of the palm wine on the Bacteria population associated with red palm weevils. The chart shows the percentage of different bacteria morphologies, which is a reflection on the diversity of the Bacterial species associated with the samples. It showed reduction in the diversity of the associated bacteria. The highest diversity recorded was 10, in the pristine palm weevils, while lowest diversity was 4, recorded in the palm wine treated samples. The number indicated that the effect of palm wine reduced the diversity of the bacteria associated with the palm weevils by half, thus implying that microorganisms, including those capable of causing spoilage can be inhibited when the weevils are soaked in palm wine.

Figure two shows graphical representation of the percentage occurrence of the Bacterial isolates. Indications from the graph showed that Citrobacter specie and *Escherichia coli* recorded the highest percentage (19%). The least occurrences were recorded by Pseudomonas and Micrococcus species (6%). Enteric bacteria such as *E. coli* which forms part of the normal gut flora has been reported to cause several disorders including bladder infection (cystitis), urinary tract infection and gastrointestinal infection. Some strains of *E. coli* produce toxins that result in severe illness that can potentially lead to death, if untreated (Allocati *et al*., 2013). Enteric bacteria can compete for essential nutrients leading to adverse effects in the host. Also, enteric bacteria-derived metabolites and enzymes, colonization of pathogenic bacteria in the intestinal tract, compromised local immunity, and poor diet are some drivers of disease condition in the host (Batt *et al*., 1996).

This study also investigated the presence of Coliform bacteria in the samples. The Coliform bacteria were enumerated on Macconkey agar plates. The counts were similar with that of the total heterotrophic bacteria count on Nutrient agar plates. The Coliform counts for the samples in treatment A ranged from 9.4×10^4 to 1.6×10^4 in the pristine samples while the Treatment B samples showed lower Coliform counts ranging from 4.9×10^4 to 6.6×10^4 thus reflecting on the safety of consuming fresh red palm weevils. Coliform Bacteria are a group of gram negative rod, non-spore forming bacteria associated with the colon of man and animals. They have been implicated in several cases of disease outbreaks. The reduction in the number of coliform bacteria in the samples is indicative of the potential of using palm wine to control the growth of Coliform bacteria associated with red palm weevils.

In this study, Bacteria from 8 different genera were isolated. *Citrobacter sp. and Escherichia coli* recorded the highest frequency of occurrence (18.75%). In contrast, Pseudomonas specie, Micrococcus specie and Serratia specie recorded the least occurrence (6.25%). The bacteria isolates from this study are in conformity with those obtained in the previous study of Wang *et al.*, (2007) who reported that during 16S rDNA sequencing of the larvae of the red palm weevil, the bacteria isolated were mostly Gram positive and belonged to the genera *Bacillus*, *Brevibacillus* and *Paenibacillus*. The result obtained in this study is also in agreement with that of Ebenebe and Okpokpo, (2015) who reported the presence of three species of bacteria such as *Staphylococcus aureus, Escherichia coli* and Salmonella species in live oil palm weevil. Furthermore, Nrior *et al*., (2018) documented the isolation of higher loads of Bacillus

species, Staphylococcus species, Enterobacter species and Serratia species from the head and body fluids of the adults and nymphs of *R. phoenicis*.

IV. CONCLUSION

The data results generated from the study indicated that red palm weevils are contaminated with Bacteria obtained from the soil, water and tree they inhabited. The presence of Coliform bacteria in the weevils is an indication that the consumption of raw palm weevils could pose health risks to the consumers. However, this investigation positively showed a significant difference in the bacterial load of palm weevils soaked in alcohol and the pristine ones. The soaked palm weevils were found to have lower bacteria counts. This suggests that palm wine may be used as a mild preservative of red palm weevils which in return will have beneficial effects on consumers by way of reducing the microbial load of red palm weevils.

V. RECOMMENDATIONS

From the results obtained, we hereby recommend that caution be raised on the consumption of raw palm weevils, as the insects are proven to harbor soil and water bacteria in their bodies which could pose health risks to the consumers. Also, further studies to ascertain the extent at which palm wine can preserve red palm weevil and prolong its shelf life should be researched upon.

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