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Isolation of Bacteria Associated with Palm Wine Sold in Otefe Metropolis

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ABSTRACT

Palm wine is the common name for the alcoholic beverage prepared from the fermentation process of various palm trees. The main objective of this study was to isolate, identify and characterize palm yeast-associated bacteria (and potential pathogens) in Otefe Metropolis, Western Ethiopia, Delta State, Nigeria. Ready-to-drink palm wine was purchased from various locations in Otefe-Oghara town, samples were collected using a sterile bottle and plated using dilution agar medium. Sample A had the highest colony count of 4.2 x 102 and 2.0 x 104 cfu/ml, while Sample B had the lowest colony count of 1.6 x 102 and 1.0 x 104 cfu/ml. A total of six bacteria were found, including the genera Staphylococcus, Lactobacillus, Micrococcus, Serratia, Bacillus and Streptococcus. Staphylococcus aureus had the highest detection frequency with 22.2%, followed by Micrococcus, Serratia, Bacillus, and Streptococcus with a frequency of 16.6%. The lowest incidence was seen in Lactobacillus species with 11.1%. The presence of these organisms in wine is a sign of poor tap hygiene, the equipment used, and the tap method used. These events are very important in public life because wine is used for its benefit.

Keywords: Palm wine, beverage, counts, plates microbial

INTRODUCTION

Palm wine is the common name for the alcoholic beverage prepared from fermented yeasts obtained from various palm species (Okafor, 2002). It is commonly obtained from Raphia rinfera, R. hookeri and Elaeis guineensis using the method described by Bassir (2002). The Raphia palm typically produces more fruit than the oil palm, but the Raphia palm can only be used once in its lifetime because the flower is destroyed during flowering (Okafor, 2008). During fermentation, the sugar in the palm turns into alcohol and organic acids, causing the resulting fruit juice to lose its flavor (Okafor, 2005). The type of bacteria present depends on the stage of fermentation and the composition of the juice (Bassir, 2002; Okafor, 2007). Although alcohol production is common in yeasts, it is rare in bacteria (Ingraham and Ingraham, 2004). The presence of S. cerevisea was reported while S. cereviseae was isolated from Ampicultа wine palm in Nigeria (Owuana and Saunder, 2000) (Ezeronye and Okertuba, 2000). The mesentery of Lactobacillus plantariun and Leuconostoc were recently identified as the dominant lactic acid bacteria responsible for the flavor of palm wine extracted from palm trees growing in Ghana (Uzochukwu et al., 2004). Ferments used in the production of alcoholic beverages. Wine is used in parts of Africa, Asia and South America. In Nigeria, the two main sources of sap for palm wine fermentation are oil palm (Elaeis guineensis) and Raphia palms (Raphia spp).

The quality of the wine is stable and depends on other factors depending on the type of palm from which the sap is extracted. When palm wine is examined under a microscope, abundant yeast and bacteria are seen. One of the factors that contribute to the formation of palm wine is the nature of the yeast and other microorganisms it contains. Unfermented Raphia palm sap is pure, sweet, colorless syrup containing up to 10-12% sucrose (Bassir, 2002). Sugar and other products are used when microbial fermentation is common (Obire, 2005), the soup turns milky white due to the proliferation of microbes resulting from the high proliferation of yeast organisms (Okafor, 2005). Palm wine is characterized by gaseous emissions resulting from the fermentation of sucrose (Bassir, 2002) and yeast organisms. Studies focusing on the microbiology of E. guineensis and R. hookeri identified a diverse bacterial flora and yeasts involved in the fermentation process. In addition, SPP types are used in press equipment, equipment and environment etc. It has also been reported that it originates from many sources such as (Faparunsi and Bassir, 2002).

Fermentation of Raphia palm wine is recognized as a cheap and effective way of producing food in Nigeria; Fresh palm sap tends to contaminate the sap during harvest and there are changes in the biological composition of palm wine (Faparunsi and Bassir, 2002). Palm wine loses flavor as fermentation continues and the original colorless juice becomes milky. If not used or bottled within 24 hours of production, it will turn sour due to malolactic fermentation and prolonged fermentation with bacterial microflora (Ezeronye, 2003). To make palm wine, a number of microorganisms mainly target bad bacteria. Wine contains approximately 3% alcohol and is a source of some protein and various vitamins, since the bacteria and yeasts are edible. Palm wine has a short shelf life, so it is best used for about 48 hours. Various methods have been developed to preserve palm wine, such as pasteurization, the use of chemicals such as Sorbate and Sulphite, and other preservatives such as Alstonia boonei (Egbu) and Saccoglottis gabonensis (Nche). Palm wine is the favorite drink of the belt people (Southern Nigeria and Southern Belt of Benue State). Palm wine is sold in various parts of Oghara city. When

this palm wine is offered for sale to consumers, the wine becomes contaminated with various microorganisms through improper handling and cleaning. Lack of cleanliness cannot be left behind. Wine poured into an unknown container (glass), not opened and exposed to the air where flies, various insects and vectors dwell, can contaminate the wine and become undrinkable. Microorganisms are everywhere. They can easily contaminate beverages sold to consumers (Ogbulie, 2004).

The primary objective of this study was to isolate, identify and characterize palm yeast-associated bacteria (and their potential pathogens) in Otefe Metropolis, Western Ethiopia, Delta State, Nigeria. The aims of the research are as follows: To determine the number of bacteria loaded by palm wine; Provide strategies and treatments to improve wine production.

METHODOLOGY

Study area

Oghara is a tropical metropolis in Ethiope West local government area of Delta State. It is found within the tropical rainforest belt in Nigeria. Oghara located on a geographical coordinate of longitude $5^{0}59'0.83''$ N and latitude $5^{0}45'$ $47.24''$ E is made up of three sub clans, Ogharefe, Ovade and Oghareki governed by a chieftaincy system with the Ovie of Oghara as the king. The area has a relatively high temperature ranging from 25^{0}C to 27^{0}C in the wet season but rises a little to between 27°C to 32°C during the dry season. The inhabitants of this metropolis are a mixture of people from various ethnic groups in Nigeria, although the majorities are the Urhobo speaking tribe of Delta State. Specifically, this study was carried out in Delta State Polytechnic in Otefe-Oghara with over 15000 students who rely mainly buying from various vendors with respect to what they are buying.

Inhabitants of Oghara metropolis are mainly civil servants, farmers, artisan workers, transport work and fisherman and sand dredgers who rely mainly on water from Jameson River and personal borehole water which runs occasionally. The heavy flooding during the rainy season occasionally increases sanitation problem. This can be attributed to the inadequate sewage and refuse disposal facilities in the community.

Collection of samples

Retailed ready to drink Palm wine were purchased from various locations in Otefe-Oghara town, samples were collected using sterilized bottle, and transported in a cool container to the Microbiology laboratory, Faculty of Applied Science and Technology, Delta State Polytechnic in Otefe-Oghara, for Microbiological analysis. Non-probability sampling method was employed as described by (Barnette, et al; 2000). This means not all vendors in Oghara had the same chance to be selected but only 2 Palm wine vendors were selected based on willingness and support of the owners.

Preparation of nutrient agar

The nutrient agar was prepared according to the manufacturer's instruction. 7g of nutrient agar was weighed on a weighing balance and aluminum foil. The nutrient agar was poured into a conical flask, then 250ml of water was measured with a measuring cylinder and the water was added to the nutrient agar in the conical flask. The solution was properly mixed together and was sterilized by autoclaving for 45minutes. After the end of the 45minutes, it was allowed to cool to palm temperature.

Preparation of the dilution

The serial dilution was prepared according to (Risiquat, 2013). A micropipette with sterile tip was held vertically and introduced not more than 3cm below the surface of the sample (Palm wine) and then 1ml was taken to the first tube of the dilution (which contain 9ml sterile water) series without touching the diluting fluid, the tip was discarded and the tube was labeled as the first dilution tube 10^{-1} or 1/10. A fresh sterile tip was used to mix the content of the first dilution and 1ml of the first tube was transferred to the second tube of dilution series (which contain 9ml sterile water) also without touching the diluting fluid. Then the tip was discarded and the tube was labeled as the second dilution tube 10^{-2} or 1/100. Further dilutions of 10^{-3} or 1/1000, 10^{-4} or 1/10000 and 10^{-5} or 1/100000 were prepared similarly.

Heterotrophic plate count

Serial dilution of Palm wine samples was prepared with sterile distilled water. The 10^{-2} or 1/100 and 10^{-4} or 1/10000 dilution was used.

The 1ml of the Palm wine sample was aseptically transferred into the sterile petri dish containing the suitable media. The Palm wine sample was spread uniformly using sterilized glass rod and the media was incubated at 37˚C for 24 hours. After the period of incubation, the colonies on the plates were

counted and recorded as colony forming unit per milliliter (cfu/ml) as described by Saxena, R. and Patil, (2012). Each of the bacterial colonies on the agar plates was sub-cultured and the pure culture obtained.

Colony count

Colonies were counted according to surface colony count method. An average colony count from at least 5 drops of each dilution was obtained, the conversion factor was 50 to obtain figure for the bacteria /ml in the original sample. The formula used for counting was: the total number of bacteria = the average of colonies count x dilution factor x 50

Identification of isolates

Isolates were identified using Gram staining, and biochemical tests which include; catalase, coagulase, oxidase, citrate utilization, indole, methyl red, and urease as described by Cheesbrough, (2004).

Gram Staining: The method used was that described by Ercolini, (2000) smear of the isolates was prepared and heat fixed on clean grease free slides. The smears were stained for one minute with crystal violet. This was washed out with a gentle running tap water. The slides were flooded with dilute gram iodine solution. This was washed off with water and smear were decolorized with 95% alcohol till the blue colour no more dripped out (about 30 seconds) the smear were then counter stained with saffranin solution for about 10 seconds finally the slides were washed with gentle running tap water, air dried and observed under oil immersion objective (Cruikshank, et al; 2002).

Catalase Test: This test was used to demonstrate which of isolated could produce the enzymes that release from hydrogen peroxide. It is also used as an aid to different staphylococci from streptococci and to differentiate other catalase positive organism from catalase negative. A loopful of pure colony was transferred into a plane; clean glass slide the sample was then mixed a drop of 3% v/v hydrogen peroxide. The reaction was observed immediately. The gas production indicated by the production of gas bubbles confirmed the presence of catalase (Cruikshank, et al; 2002).

Oxidase Test: It is used in the identification of organisms such as *pseudomonas, Neisseria, Vibrobrucecla* and *pasteurella species, Bacillus, mycobacterium* and other gram-negative cocci. All organisms that are oxidase positive produce the enzyme cytochrome oxidase negative organisms such as *staphylococcus, Escherichia coli and klebsiella species* etc. A freshly prepared reagent 0.2g of tetra-methyl-phenylene-diamine hydrochloride in 20ml of distilled water was poured over inoculated agar plate 24hrs incubation and the excess immediately poured off positive colonies turn bluish purple.

Indole Test: This test was used to determine which of the isolated has the ability to split indole from tryptophone present in buffered peptone water. This test is used for identification of Enterobacteria such as *Escherichia coli, Bacilli species* and gram negative (Babalola, 2000). The tube of peptone water were inoculated with young culture of the isolated, the tube were incubated at 37°C for 48hours about 4drop of koval reagent autoclaving at 121°C for 15minute one percent of solution of the sugar was prepared and sterilized separately at 115°C for minutes. This was aseptically dispensed in 5ml aliguot volume into the tube containing the peptone water and indicator. The tubes were inoculated with young culture of the isolated and incubated at 37°C. Acid and gas production were observed after 24hours of incubation. Add 0.5ml of kovals reagents shake gently and examine for colour the surface layer within 10 minutes. Red surface layer show positive indole test. Absence of red surface layer show Negative indole tests.

Coagulase Test: The coagulase test identifies whether an organism produces the exoenzyme coagulase which cause the fibrin of blood plasma to clot. Organism that produce catalase can form protective barriers of fibrin around themselves, making themselves highly resistants to phagocytosis, other immune responses and some other antimicrobial agent. The coagulase slide test is used to identify the presence of bound coagulase or clumping factor, which is attached to the cell walls of the bacteria. The coagulase test is useful for differentiating potentially form a pure culture is transferred aseptically to the serum indicative of a positive test. Positive coagulase test indicate the presence of *staphylococcus areus* because of the clumping within 10seconds. Negative coagulase test indicate *Eschericha coli*.

RESULTS

Table 1 showed the total heterotrophic counts and the isolated organisms. Sample A had the highest number of colony count of $4.2x10^2$ and 2.0×10^4 cfu/ml while Sample B had the lowest colony count of 1.6×10^2 and $1.0x10^4$ cfu/ml respectively.

Table 1: Total heterotrophic count and the isolated organisms from Palm wine drink

S. No	Samples	Total heterotrophic count (cfu/ml)	Isolates
1	A	4.2×10^2	*Salmonella spp. E. coli, Proteus spp. S. aureus, Klebsiella spp.* and *Pseudomonas spp*
		2.0×10^4	*Salmonella spp. E. coli, Proteus spp. S. aureus, Klebsiella spp.* and *Pseudomonas spp*
2	B	1.6×10^2	*Salmonella spp. E. coli, Proteus spp. S. aureus, Klebsiella spp.*
		1.0×10^4	*E. coli, Proteus spp.* and *Klebsiella spp.*

The morphological and biochemical characteristics of the bacterial isolates are shown in Table 2. A total of six bacteria isolates were identified which included strains of the genera *Staphylococcus Species, Lactobacillus, Micrococcus, Serratia, Bacillus,* and *Streptococcus species*.

Table 2: Morphological and Biochemical Characteristics of the Isolated organisms from Palm wine drink

Morphological Characteristics	Gram Staining	Biochemical Characteristics					Suspected Organism
		Catalase	Indole	Methyl red	Oxidase	Urease	
Smooth entire margin round	+ve cocci in cluster	-	-	-	-	-	*Staphylococcus Species*
Large smooth and glistering colony	+v rod	-	+	+	-	-	*Lactobacillus Species*
Circular convex colony	+ve cocci	-	-	-	-	+	*Micrococcus Species*
Round Mucoid	+ve	+	-	-	-	-	*Serratia species*
Raised, irregular wits colonies	+ve rod	-	-	-	-	-	*Bacillus species*
Small colourless colony	+ve cocci in chains	+	-	-	-	+	*Streptococus Spp*

Table 3 showed the distribution of the different pathogenic bacteria isolated from each sample. *Staphylococcus aureus* had the highest frequency of occurrence of 22.2%, followed by *Micrococcus, Serratia, Bacillus,* and *Streptococcus species* which had the frequency of 16.6%. *Lactobacillus Species* has the least frequency of occurrence of 11.1%.

Table 3: Frequency of bacterial occurrence in Palm wine drink

S. No	Isolated Organism	Frequency of occurrence (%)
1	*Micrococcus spp*	15 (16.6)
2	*Staphylococcus aureus*	20 (22.2)
3	*Serratia spp*	15 (16.6)
4	*Bacillus,*	15 (16.6)
5	*Streptococcus spp*	15 (16.6)
6	*Lactobacillus Species*	10 (11.1)
	Total	90 (100)

DISCUSSION

All over the world, alcoholic beverages are made from the sap of local mature plants such as coconut palm, palmira and wild palm. The terms toddy and palm wine are both used to describe alcoholic beverages. The term is used in many countries (Chandrasekhar et al., 2012). In Nigeria, the most common wine sources are Raphia rinfera, Raphia hookeri and Elaeis guineensis. The sap is usually collected from growing palm trees. This is done by striking the palms and making small incisions in the skin about 6 inches from the top of the head. A clear soup is tied to a tree to catch the bark penetrating it. In general, both brands of palm wine have many nutritional, medicinal, religious and social effects mentioned elsewhere (Iheonu, 2000) to increase the demand for natural products. sweet palm tree (Ogbulie et al, 2007; Amoa-Awua et al., 2007; Naknean et al., 2010; Santiago-Urbina et al., 2013) serves as a rich substrate for the growth of various types of microorganisms soon after the sap is collected and within an hour (or) two becomes reasonably high in alcoholic content (up to 4%). If allowed to continue to ferment for more than a day, it starts turning into vinegar. The sap undergoes spontaneous fermentation, which promotes the proliferation of yeasts and bacteria for the conversion of the sweet substrate into several metabolites mainly ethanol, lactic acid and acetic acid (Amoa-Awua et al., 2007; Stringini et al., 2009; Ouoba et al., 2012; Santiago-Urbina et al., 2013).

In this study, the microbial composition of palm wine obtained from different locations was investigated; The results showed that craft wine samples contained more microbial species; this can be attributed to good hygiene practices involved in the acquisition, collection and distribution of wine. However, Obi et al., (2015) also reported the presence of similar organisms in a palm wine sample collected from Ikwuano district of Abia State. Karamoko et al., (2012) also reported that yeasts and other bacteria were isolated from palm wine. However, according to Ogbulie (2007), the method of processing palm wine and harvesting palm trees affects the sap microorganism. The presence of these organisms in wine is a sign of poor tap hygiene, the equipment used, and the tap method used. These events are of great public health importance because wine is used almost everywhere in the country for its nutrition, health and nutrition benefits. Public awareness campaigns about the consumption of these wines are needed to help improve the quality of the product and prevent potential health risks associated with drinking contaminated wine (Kurtzman, 2002).

CONCLUSION

The presence of these organisms in the wine is an indication of the poor hygienic state of the tappers, materials used and the method of tapping involved. This occurrence is of public health importance as the wine is consumed due to its nutritional significance. There is therefore the need for public awareness in the consumption of these wines to help promote the quality of these products as well avoiding the health risk that may be associated with the consumption of contaminated palm wine.

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