Anti-biofilm Activity of Indigenous Dentrifices *MassulariaAcuminata Linn*and*Distemonanthusbenthamianus Baill* on biofilm forming cariogenic bacteria

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Abstract: Biofilms are heterogeneous conglomerate of microorganisms which play a significant role in causing dental caries in human. This study investigated the characteristics and antibiotics susceptibility profile of biofilm forming bacteria associated with dental caries. Questionnaire was administered and dental caried swab samples were collected from patients in two dental centers, in Abeokuta, Ogun state, Nigeria. Biochemical and molecular techniques were used for further identification and characterization of bacterial isolates. Tube assay method was used to screen for biofilm forming bacteria. Sensitivity test was done using agar well-diffusion techniques on commonly used toothpaste, indigenous dentifrices (*Massularia acuminata* and *Distemonanthus benthamianus*) and antibiotics. Prevalence of caries was higher among females (59.8%) compared to males (40.2%). The age of patients mostly affected ranged between 20 and35 years. The molar teeth were majorly affected with caries (72.5%), with the left lower jaws mostly infected (46.1%). Out of 274 bacterial isolates, *Streptococcus mutans* was the most prevalent (22.6%), *Staphylococcus aureus* (19.7%), *Enterobacteraerogenes* (15.0%), *Klebsiellapneumonia* (12.8%), followed by *Lactobacillussalivarius* (11.3%), *Bacillus subtilis* (10.7%) and *Escherichia coli* (8.0%) respectively. Biofilm formation assay showed that out of 274 isolates 197 (71.9%) were biofilm formers. There was a significant difference ($p<0.05$) between the toothpastes used. There was no significant difference ($p>0.05$) in inhibitory effect between *M. acuminata* aqueous compared with *D. benthamianus*. Gram-positive bacteria were sensitive to erythromycin, while the Gram negative bacteria were sensitive to septrin. This study showed that dental caries in Abeokuta affects all ages and groups with pathogenic *Streptococcus mutans* been the most frequently occurring cariogens. Toothpastes are more effective than the use of indigenous dentifrices in preventing dental caries.

Keywords: biofilm forming microorganisms, indigenous dentrifices, *Masulariaacuminata*, *Distemonathusbenthaminus, Streptococcus mutans*

Introduction

Biofilms are defined as complex matrix-enclosed microbial population that are adherent to each other and to living or nonliving surfaces. These microorganisms can either be planktonic or free living. Biofilm is not a mere homogeneous monolayer of slime, but heterogeneous mass of microbes, which allow flow of essential nutrients and oxygen to the cells growing within the biofilm (Evans and Lewandowski, 2000).

Whenever a living or nonliving surface is contaminated withmicroorganisms, several factors determine whether abiofilm develops or not (Donlan, 2001). Microorganisms undergo different steps which lead to the formation and maturation of the biofilm (Parsek and Faqua, 2003). Firstly, microorganisms initially attach to available surfaces, after which the planktonic microorganisms undergo phenotypic change with significant attachment through a thin extracellular polymeric substance.

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Microorganisms in biofilm proliferate and optimally interact to ensure their sustainability in the imposed environment. This is followed by the production and deposition of thick extracellular matrix (i.e. matured biofilm) which procures both chemical and physical protection for the microorganisms. The matured biofilm then undergoes focal dispersion through specific signals leading to microbial release. Free microbes are then able to spread to other locations to form new biofilm (Soni and Nannapaneni, 2010).

. Cell-to-cell signaling systems have been found to play an important role in matured biofilm communities. The role played is that they regulate the morphological transformation of bacteria when they become a permanent part of the biofilm: flagellin synthesis is decreased and the production of exopolysaccharide is increased (Watnick and Kolter, 2000). Exopolysaccharide production is favoured since it reinforces the biofilm structure (Danese *et al.,* 2000). An increase in dietary carbohydrates (sucrose), results in additional acid production that may exceed both the capacity of the saliva to remove acid end-products and the neutralizing power of the salivary/plaque buffer system (Sofia *et al.,* 2010). The resistant characteristic of biofilms leads to persistent infections in the human

body, as well as to troublesome biofilms in industrial processes. National Institute of Health showed that over 80% of microbial infections in the body are related to biofilms (Cogan, 2006).Bacteria in a mature biofilm are far more resistant to antimicrobials (biocides and antibiotics) than freely swimming cells due to exchange of genetic materials (Whitehead et al 2001).

Dental caries also called tooth decay is one of the most accessible biofilm found in the human oral cavity (in the crown and root portions of both primary andpermanent teeth). Dental caries is a chronic disease of the tooth hard tissues or a disease that involves in the destruction of the calcified tissues of the teeth, characterized by alternating phases of demineralization and remineralization, which can lead to cavitations and eventually tooth loss (Shamsher *et al*., 2010). Humans are not born with cariogenic bacteria, but it can be transmitted from caregivers (usually mothers), by mouth-to-mouth transmission via kissing or by sharing a spoon during feeding. It has been reported that the first bacteria to adhere to the teeth, determine the eventual pathogenicity of the plaque biofilm and once the species of bacteria has established itself, other bacteria introduced at a later date will have more difficulty in colonizing (Michelle *et al*., 2010).

Dental caries is considered by the World Health Organization (2003) as one of the most important oral health burdens that affect people of various age groups all over the world.

Seventy to eighty (70-80%) of the solid material in oral plaque is made up of aerobic and anaerobic species of bacteria (Fourrier *et al.*, 1998), most of which are harmless, but under certain conditions some can cause oral infections like caries or periodontal disease (Sakamato *et al*., 2005). In addition to bacteria, other organisms such as fungi, diatoms and other algae, amoebae and ciliates can also be found in biofilms, but their quantity depends on their specific environment (Aparna and Yadav, 2008).

Fluoride is available in many forms, including varnish, mouthrinse, toothpaste and mouthgel. Although fluoride has an important role in preventing caries, its overuse in some cases can result in fluorosis (Eichmiller *et al*., 2005), while some oral wash do not contain fluoride. Chewing sticks commonly called "pako" in south west Nigeria, are parts of higher plants that are cut into small lengths that is used for oral hygiene due to their low costs and ready availability **(**Ojo *et al*., 2007). Some workers has confirmed that some bacteria are susceptible to some commonly used chewing sticks and that their use could be more effective as the use of toothbrush and dentrifices . The main mechanisms by which plaque can be controlled is by the use of chemotherapeutic agents which help to reduce the overall rate of accumulation of new plaque, to reduce or remove existing plaque, to inhibit only the growth of those species implicated in disease and to inhibit the production of extrapolymeric substances (EPSs) (Marsh and Bradshaw, 1993).

Although bacterial biofilms and their role in disease have been investigated over a number of years (Douglas, 2003), paucity of information exist on dentine biofilm in relation to caries in Abeokuta, Ogun state, Nigeria. Thus, this study screened for bacterial diversity in dentine biofilm on tooth decay and the susceptibility of the bacterial isolates to local, commercial dentrifices and antibiotics.

Materials and Methods

Study area and population: This study comprises of caries infected patients from State Dental Center (SDCA) and Errix Dental Center (EDCA) in Abeokuta, Ogun State, Southwest, Nigeria. Personal data of patients (sex, age, literacy, tribe, educational level and occupational status), oral hygiene status (the use of toothpaste, type of toothbrush or local dentifrices) and caries details (tooth affected with caries, jaw affected, use of antibiotics and other treatment) were recorded in the questionnaire used for the study. Patients aged five to eighty years with signs and symptoms of dental caries (especially tooth decay) whose consent has been given were enrolled in the study.

Collection of samples: The sample size number (n) used for this study was calculated according to Dimeji (2010)

$$n = \frac{NZ^2P(1-P)}{d^2(N-1) + Z^2P(1-P)}$$

Where, N is the total population of dental caries, Z is standard normal deviation (at 95% confidence level), P is the expected proportion in the population and d is margin of error (set at 5%)

The estimated sample size needed to be 95% confident that the estimate of the population prevalence of tooth decay was within 5% of the true per cent frequency is equal to 75. Thus, a minimum of 377 patients were recruited into the study and , a total number of 102 samples were collected from patients in the dental unit for analysis. Samples were collected over a period of four months between March and June, 2016.

Samples were collected by rolling sterile swab sticks containing normal saline on the decayed tooth. This was done after tooth examination by the dentist, before adminstering antibiotics.All the samples were later transported to the laboratory for processing (Mohapatra *et al*., 2012). Swab samples collected were streaked directly on Brain Heart Infusion Agar (BHIA), blood agar, MannRogosa and Sharpe agar (MRS) and Eosin Methylene Blue agar (EMB) maintained in anaerobic jar for 24 hrs all at 37^{o}C.The pure isolates obtained were maintained on nutrient agar slants stored at 4^{o}C inside the refrigerator (Mohapatra *et al*., 2012).The pure bacterial isolates were identified using morphological and biochemical tests .

Procurement and Processing of local dentrifices: *Massularia accuminata*and *Distemonanthus benthamianus*

The indigeneous chewing sticks used were bought from Kuto market in Abeokuta and identified as *Massularia accuminata Linn* (pako Ijebu) and *Distemonanthus benthamianus Baill* (orin ayan) by the Herbarium Unit of Forest Research Institute of Nigeria, Jericho Ibadan. Also two commonly used toothpastes (designated as A & B) were bought from a supermarket in Abeokuta. The active ingredients for A include basil oil, 5% herbal extract (obtained from bullet wood, *Acacia Arabica*, lotur bark, pellitory root, bark of blackberry), calcium carbonate, sodium lauryl sulphate, spearmint coriander, ginger, eucalyptus, peppermint and lemon oil. Active ingredients for dentifrice B include sodium fluoride (1450 ppm), eucalyptus, peppermint, sage and *Aloevera* leaf extract.

Tube Adhesion Assay for biofilm formation

Glycocalyx (biofilm) production was determined by the method of (Mushtak, 2011). Bacterial colonies were inoculated into 5 ml of Brain Heart Infusion broth supplemented with 2% glucose and without glucose in polystyrene tubes and were incubated at 37°C. After 24 hours, the content was aspirated and stained with 0.1% crystal violet for 10 mins. Excess stain was removed by washing the tubes with distilled water. Finally, biofilm formation was observed by the presence of visible stained or unstained film lining the wall of the tube.

Quantification of biofilm formed by bacterial isolates from carried teeth

Quantification of biofilm formed was determined using a spectrophotometric method described by Yassien and Khardori (2001) and Stepanovic *et al*. (2003). Columbia agar supplemented with 5% rabbit blood was prepared, inoculated with biofilm forming bacterial isolates and incubated aerobically at 35°C for 24 hrs. The 24 hrs old bacterial cultures were used to prepare standardized suspension in sterile distilled water adjusted to 0.5 McFarland turbidity standard and the suspensions obtained were inoculated on brain heart infusion broth with glucose. Then, 200 µL of standardized culture was added to each well of sterile 96 wells Microtiter plate and incubated at 37°C for 18 hrs. After 18 hrs, the content of each well was aspirated and washed thoroughly with sterile distilled water and the remaining attached bacteria were fixed with 200 µL of methanol per well. After 15 mins, the wells were emptied and left to air dry. Thereafter the wells were stained for 5 mins with 160 µL per well of crystal violet Excess stain was rinsed off by washing the Microtiter plate with distilled water. The Microtitre plate was air-dried and the dye which was bound to the adherent cells was re-solubilized with 160 µL of 33% glacial acetic acid per well. Optical density (OD) of each well was measured at 570 nm and 630 nm using spectrophotometer (Jenway, UK). Biofilm forming bacteria were classified according to the criteria of Christensen *et al*. (1985) as follows: strong producer is greater than 0.240 (> 0.240), weak producer ranged between 0.120-0.240 and non-biofilm producer is (< 0.120).

Antibiotic susceptibility patterns of bacterial isolates to extracts from indigenous dentifrices

Indigenous dentifrices samples were washed under running tap water to remove dirt and then air-dried for 7 days. The dried samples were cut into small pieces and pulverized into a fine powder with a grinder (Phillips, USA). Extraction was carried out by addition of sterile distilled water (150 ml) to 50 g of powdered materials for 3 days (Ojo *et al*., 2007). The supernatant was filtered through membrane filter, having a pore size of 0.45 µm . A sterile nutrient agar slant was inoculated with the filtered aqueous extract for checking the sterility of extract. The extract was distributed in screw capped bottles and stored in deep freezer at -20^{0}C until used (Sorna *et al*., 2011). Muller-Hinton agar was prepared and poured into Petri dishes to solidify, isolate suspension of 0.5 McFarland turbidity standard (equivalent to 1.5 x 10^8 CFU/ml) was spread on the agar plates. Thereafter, 6 mm diameter wells were made into the Petri dishes with cork borer and aliquots (0.1ml) of each extract was added to it. The plates were then incubated at 37^{0}C for 24 hrs and examined for zone of inhibition.

Antimicrobial effectiveness of commonly used toothpastes on the isolates

Muller-Hinton agar plates were prepared and allowed to solidify. The plates were allowed to dry for 1 hr under a Laminar flow hood. Thereafter, isolate suspension of 0.5 McFarland turbidity standard (equivalent to 1.5 x 10^8 CFU/ml) was spread on the agar plates (Aneja *et al*., 2010).. The selected dentifrice solutions were made by mixing 2g of toothpastes with 2 mL of distilled water to give 1:1 dilution.Further dilutions of 1:2 and 1:4 (wt/v) were prepared. A sterile 6 mm cork borer were used to make well and 0.1 ml of the dentifrice solutions were introduced into one of the wells while the same amount of sterile distilled water was introduced into the first well as control. The plates were then incubated at 37^{0}C for 24 hrs and examined for zone of inhibition (Manupati, 2011).

Sensitivity test of commercial antibiotics against biofilm form isolates.

Sensitivity of biofilm producing bacteria to commercial antibiotics were determined and the zone of inhibition was recorded in milimetre (mm) using a metre rule. This was carried out on Mueller-Hinton agar using disc diffusion techniques (Bauer *et al*., 1996). Cultures were incubated for 24 hrs at 35^{o}C. The following antibiotic discs were used: Amoxycilin, Ofloxacin, Streptomycin, Chloramphenicol,

Ceftriazone, Gentamycin, , Cotrimoxazole, Ciprofloxacin, Erythromycin, Septrin,Sparfloxacin Amoxicilin, Augmentin, Perfloxacin, Streptomycin) and Tarivid (Fondiscs Laboratory,).

Extraction of DNA and identification of biofilm forming isolates

Extraction of isolates DNA was carried out as described by van Tongree et al., (2011). Isolates were initially centrifuged to collect the cell pellets which were resuspended in Tris-EDTA (TE) buffer. Sodium dodecyl sulfate (SDS) and protease K were added to the sample prior to which was then incubation at 37°C for 1 hr. Sodium chloride (NaCl) and 10% CTAB in 0.7 M NaCl were added to the mixture which was incubated at 65°C for 20 mins. An equal volume of chloroform: isoamyl alcohol (24:1) was added after which the mixture was centrifuged to separate the phases. RNase was added to the aqueous phase and this mixture was allowed to stand. An equal volume of isopropanol was added and mixed until the stringy white DNA pellet precipitated out of the solution and condensed into a tight mass. This was centrifuged and the supernatant was once again discarded. The washed and air-dried DNA pellet was dissolved in DNA-grade water and stored at -20^0 C until used for PCR. The concentration and purity of the extracted genomic DNA were evaluated by agarose gel electrophoresis and by ultraviolet spectrophotometry (Hutter *et al*., 2003).

DNA Sequencing and identification of biofilm forming bacterial isolates

Amplification of 16SrRNA genes was performed under standard conditions using universal selective primers (5 pMol forward and backward primer) for detection of bacteria, depending on the amplification efficiency and success (Hutter *et al*., 2003). Polymerase chain reaction (PCR) amplification for 16SrRNA was performed in 10 µl volume of the following reagents: 2.9 µl of sterile water, 1.0 µl dNTP (10 µM), 1.0 µl 10x buffer, 0.5 µl forward primer (10 µM), 0.5 µl reverse primer (10 µM), 0.3 µl Taq and 1.0 µl of 10x fold dilutions of DNA template. The PCR protocol is as follows : 94°C for 5 mins followed by 36 cycles of 94 °C for 30 secs, annealing temperature ranging from 55°C for 30 secs each cycle with extension temperature at 72 °C for 45 secs with final extension period of 7 mins at 72°C.

Statistical analysis of results

All data were analyzed using the SPSS statistical program version 16.0, at $P \leq 0.05$ level of significance.. Statistical significance was taken with *p* value < 0.05. The significant differences were detected by using either chi-square test or independent sample-test.

Results

The socio-demographic characteristics of the patients with tooth decay showed that the females (59.8%) were more affected compared to males (40.2%). The age of patients mostly affected ranged between 20 and 35 years. Students (48.0%) of tertiary level (59.0%) were mostly affected with dental caries. This was followed by the civil servants (24.5%) that fall within the age range of 35-50yrs. Literates (83.3%) were more affected with caries than the illiterate (16.7%) (Table 1). The oral hygiene status of the caried patients showed that almost all the patients (99%) accepted that good oral hygiene is essential. Findings also revealed that 89.2% of caried patients uses toothbrush while only 10.8% used local dentifrices. Soft (54.9%) and hard (30.4%) toothbrushes were majorly used. Commonly used toothpaste was toothpaste A (38.2%), followed by toothpaste B (35.3%) while the commonly used local dentifrices were M. acuminata (pako Ijebu) (8.8%) and D. bnethamianus (orin ayan) (5.9%).

Table 3 showed the clinical findings of patients affected with caries. Sugar intake by patients was as high as 79.6% and as low as 20.6%. Molar (72.5%) teeth were mostly affected, followed by premolar (20.6%), canine (5.9%) and incisors (1.0%). Lower jaw (both right and left) were mostly affected with caries.

Results showed that out of 274 total bacteria isolates, 22.6% were *Streptococcus mutans* UA 159, 19.7% *Staphylococcus aureus* NCTC 8325, 15.0% *Enterobacteria aerogenes KCTC 2190*, 12.8% *Klebsiella pneumonia* HSII 286, 11.3% *Lactobacillus salivarius* UCC 118, 10.7% *Bacillus subtilis* STR 46 and 8.0% *Escherichia coli* NRG 856 respectively (Fig 1).

Out of the 274 isolates screened for tube assay biofilm formation, only 197 bacterial isolates formed biofilm (Figure 2). *Staphylococcus epidermidis* (ATCC 35984) showed high slime productivity. In spectrophotometric assay method of quantifying biofilm, all the 197 bacterial isolates also formed biofilm, with 115 bacterial isolates recorded as strong biofilm producer (optical density greater than 0.240) while the remaining 82 bacterial isolates were weak biofilm producers with (optical density equals 0.120-0.240) (Figure 3).

Table 1: Socio-demographic profile of patients with tooth decay

Parameters	Variables	Frequency (n=102)	Positive dental caries (%)
Sex	Male	41	40.2
	Female	61	59.8
Age (years)	5-20	22	21.6
	20-35	39	38.2
	35-50	27	26.5
	50-65	9	8.8
	65-80	5	4.9
Race	Igbo	10	9.8
	Yoruba	92	90.2
Literacy	Yes	85	83.3
	No	17	16.7
Occupation	None	3	2.9
	Self employed	22	21.6
	Civil servant	25	24.5
	Students	49	48.0
	Others	3	2.9
Education	None	15	14.7
	Primary	12	11.8
	Junior	3	2.9
	Senior	13	12.2
	Tertiary	59	57.8

Table 2: Personal hygiene of dental caries patients

Parameters	Variables	Frequency (n=102)	Percentage (%)
Oral hygiene	Yes	101	99.0
	No	1	1.0
Use of toothbrush	Yes	91	89.2
	No	11	10.8
Types of brush	None	11	10.8
	Ex-hard	4	3.9
	Hard	31	30.4
	Soft	56	54.9
Product of paste	None	9	8.8
	DB	39	38.2
	CLUP	36	35.3
	ORB	10	9.8
	Others	8	7.8
Use of chewing stick	Yes	18	17.6
	No	84	82.4
Type of chewing stick	None	87	85.3
	Pako ijebu	9	8.8
	Orin ayan	6	5.9

Table 3: Clinical findings of caried patients

Parameters	Variables	Frequency(n=102)	Percentage (%)
Purpose of visit	Complaint	31	31.4
	Filling	7	6.9
	Removal	64	62.7
Sugar intake	No	11	10.8
	High	78	70.6
	Low	21	20.6
Tooth affected	Molar	74	72.5
	Premolar	21	20.6
	Canine	6	5.9
	Incisors	1	1.0
Number ofcaried teeth	One	96	94.1
	More	6	5.9

Table 4: Biochemical characterization of bacterial isolates from dental caries

S/N	S	GR	SS	CS	CT	CO	MO	IN	OX	CI	UR	MR	VP	G	L	M	Suspected organisms
1	Cocci	+	-	-	-	-	-	-	-	-	-	-	-	A	A	A	*Streptococcus mutans*
2	Rod	+	-	-	-	-	-	-	-	-	-	-	-	A	A	-	*Lactobacillus salivarius*
3	Cocci	+	-	-	+	+	-	-	-	-	-	-	+	A	A	A	*Staphylococcus aureus*
4	Rod	+	+	+	-	+	-	-	-	-	-	+	-	A	-	-	*Bacillus subtilis*
5	Rod	-	-	+	-	-	-	-	-	-	-	+	-	A	-	-	*Klebsiella pneumonia*
6	Rod	-	-	-	+	-	+	+	-	-	-	+	-	A	A	-	*Escherichia coli*
7	Rod	-	-	-	+	-	+	-	-	+	-	+	-	A	A	-	*Enterobacter aerogenes*

Keys: AG- Anaerobic growth, S- Shape, GR- Gram stain, SS- Spore stain, CS- Capsule stain, CT- Catalase ,C0- Coagulase, MO- Motility, IN- Indole, OX- Oxidase, CI- Citrate, UR- Urease, MR-Methyl-red, VP- Voges Proskaeur, G- Glucose, L-Lactose, M-Mannitol, A-Acid production, (+)- positive, (-)- Negative.

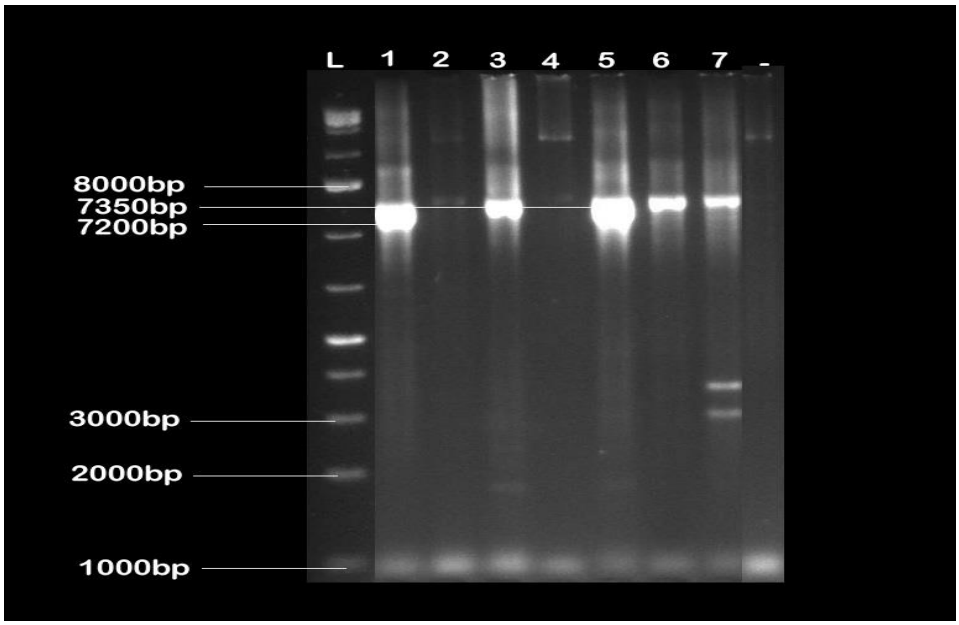

Plate 1: Gel electrophoresis of polymerase chain reaction (PCR) of bacterial isolates
KEY: L= ladder, bp= base pair, 1= *Streptococcus mutans*, 2= *Lactobacillus salivarius*, 3= *Staphylococcus aureus*, 4= *Bacillus subtilis*, 5= *Klebsiella pneumoniae*, 6= *Enterobacter aerogenes*, 7= *Escherichia coli*, (-) = Negative control

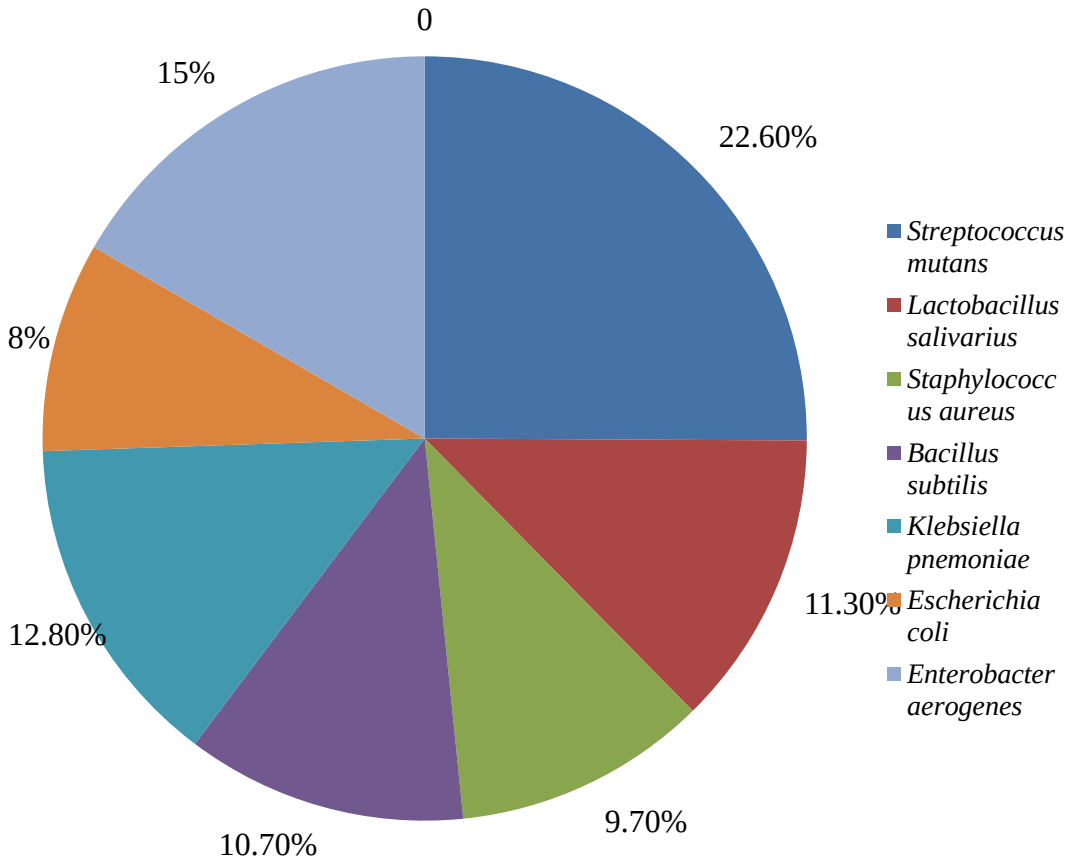

Fig 1: Occurrence rate of bacteria isolates in dental caries.

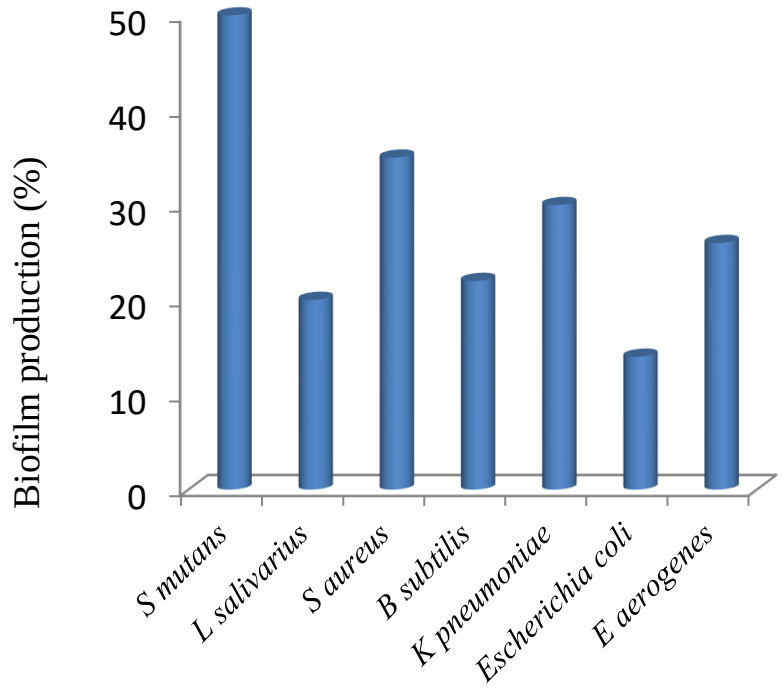

Fig 2: Comparison of biofilm production among bacterial isolates.

Toothpaste A 1:1, 1:2 and 1:4 (v/v) had the highest mean zones of inhibition values of 17.3 mm, 15.0 mm and 11.0 mm respectively while toothpaste B 1:1, 1:2 and 1:4 (v/v) had 18.0 mm, 15.3 mm and 11.3 mm respectively to *Strept. mutans*. The solutions 1:1 had the highest zone of inhibition followed by 1:2 solutions and then 1:4 solutions respectively and that *Strep mutans* are more susceptible to toothpaste B solution (18.0 mm) compare to toothpaste A solution (17.0 mm). There were significant differences between the groups ($p<0.05$). *Lactobacillus salivarius* had the mean values of 15.3 mm (toothpaste A 1:1) and 19.3 mm (toothpaste B 1:1). *Staph aureus* had the mean values of 15.0 mm and 19.0 mm while *Bacillussubtilis* had 17.3 mm and 19.0 mm to toothpaste A 1:1 and toothpaste B 1:1 solution respectively. *Klebsiella pneumoniae* had highest susceptibility value of 20.4 mm both in toothpaste A and toothpaste B (1:1). There was significance difference between the toothpastes used ($p<0.05$). *Escherichia coli* had values of 18.3 mm and 20.0 mm while *Enterobacter aerogenes* had 17.0 mm and 18.0 mm to toothpaste A 1:1 and toothpaste B 1:1 solution respectively.. There were significant differences within and between these groups ($p<0.05$). In susceptibility patterns of biofilm forming bacterial to indigenous dentifrices solution, *Massularia acuminata* (16.0 mm) had the highest inhibitory effect compared to 14.4 mm in *Distemonanthus benthamianus* (Table 6).. There were significant differences ($p\leq0.05$) between and within the use of the two local dentifrices against the biofilm forming bacteria,.

Table 5: Susceptibility patterns of biofilm forming bacteria to some toothpastes.

Pastes(g/ml)/isolates	*Strep. mutans*	*L.salivarius*	*Staph .aureus*	*B.subtilis*	*K.pneumoniae*	*E. coli*	*E.aerogene*
Paste A 1:1	17.3 ± 1.76^{b}	15.3 ± 2.91^{bc}	15.0 ± 2.52^{bc}	17.3 ± 0.67^{bc}	20.4 ± 2.60^{c}	18.3 ± 0.88^{c}	17.0 ± 2.65^{a}
Paste B 1:1	18.0 ± 1.16^{b}	19.3 ± 0.88^{c}	18.0 ± 1.00^{c}	19.0 ± 1.53^{c}	20.4 ± 0.88^{c}	20.0 ± 0.58^{c}	18.0 ± 1.16^{a}
Paste A 1:2	15.2 ± 1.53^{ab}	13.7 ± 2.85^{ab}	8.33 ± 1.00^{ab}	13.0 ± 0.57^{a}	12.0 ± 1.16^{abc}	8.67 ± 4.33^{a}	15.7 ± 2.33^{a}
Paste B 1:2	15.3 ± 0.88^{a}	15.0 ± 2.08^{b}	16.7 ± 1.16^{c}	14.3 ± 1.20^{ab}	14.3 ± 1.76^{bc}	15.3 ± 1.20^{bc}	14.0 ± 1.50^{a}
Paste A 1:4	11.0 ± 1.00^{a}	6.33 ± 3.18^{a}	5.33 ± 2.96^{a}	11.3 ± 0.67^{a}	6.67 ± 3.53^{a}	11.0 ± 0.58^{a}	11.0 ± 0.58^{ab}
Paste B 1:4	11.3 ± 1.33^{a}	11.3 ± 0.88^{a}	11.7 ± 1.20^{ab}	10.7 ± 1.76^{a}	7.67 ± 3.84^{ab}	10.7 ± 0.88^{ab}	12.0 ± 3.00^{a}

Values are Means of triplicate readings ± Standard Error
Values with the same subscript on each row are not significantly different ($p>0.05$).

Table 6: Susceptibility patterns of biofilm forming bacterial to indigenous dentrifices extracts

Isolates/sticks	*Strepyococcus. mutans*	*Lactobaccillus.salivarius*	*Staphylococcus.aureus*	*Baccillus.subtilis*	*Klebsiella pneumoniae*	*Escherichia. coli*	*Enterobacter. aerogenes*
Control	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]
Massularia accuminata(pako Ijebu)	7.33±1.76[b]	8.33±3.84[b]	13.3±3.53[b]	8.67±2.60[b]	16.0±2.31[b]	5.00±1.53[b]	8.67±3.53[b]
*Distemonanthusbenthamianus(*orinayan)	5.33±2.91[a]	1.33±1.33[a]	11.0±2.65[b]	8.33±1.76[b]	14.4±1.45[b]	9.33±2.03[c]	10.0±1.16[b]

Values are means of triplicate readings ± Standard Error values with the same subscript on each row are not significantly different (p>0.05).

The susceptibility patterns of the bacterial isolates to antibiotics is shown in table 7 and table 8. Erythromycin showed the highest zones of inhibition of 18.4 mm against *Bacillus subtilis* while Cotrimoxazole, Amoxycillin and Cefriazone showed no zone of inhibition against the isolates (Table 7).. Ciprofloxacin was the most active while augmentin was the least active antibiotic aginst the Gram negative isolates (Table 8).There were significant differences (p<0.05) between antibiotics reactions in *Strep mutans, L. salivarius* and *Staphaureus.* There were no significant differences (p>0.05) between antibiotics used against *Klebsiella pneumoniae.*

Ciprofloxacin showed highest zone of inhibition of 19.3 mm against *E. coli* while augmentin showed the lowest inhibition zone of 3.33 mm on *E. coli .E coli* and *Enterobacteria aerogenes* had zones of inhibition (19.4 mm and 18.4 mm respectively) against ciprofloxacin (Table 8). There were significant differences (p<0.05) between antibiotics reactions on Gram negative bacteria (Table 8).

Table 7: Susceptibility pattern of Gram positive bacteria to commercial antibiotics

Diameter of zone of inhibition (mm)

Values are means of triplicate readings ± Standard Error

Antibiotics/isolates	*S. mutans*	*L. salivarius*	*S. aureus*	*B. subtilis*
Cotrimoxazole	0.00±0.00[a]	0.00±0.00[a]	9.33±1.76[a]	9.67±2.03[a]
Ciprofloxacin	9.33±1.76[bc]	8.33±2.33[cd]	17.7±2.03[b]	16.3±3.48[a]
Ofloxacin	13.0±4.36[bc]	5.00±1.73[abc]	17.7±2.96[b]	13.3±2.91[b]
Gentamycin	6.67±1.76[ab]	3.00±1.73[ab]	10.7±1.20[a]	8.00±1.16[a]
Amoxycilin	0.00±0.00[a]	0.00±0.00[a]	0.33±1.20[a]	10.3±2.03[a]
Erythromycin	12.3±3.18[bc]	11.3±1.76[d]	8.00±3.22[a]	18.4±3.28[a]
Ceftriazone	0.00±0.00[a]	8.00±1.53[bcd]	11.7±2.03[a]	9.33±2.91[a]
Streptomycin	14.7±2.91[c]	13.0±1.16[d]	7.33±1.76[ab]	13.3±4.67[a]

Values with the same subscript on each row are not significantly different (p>0.05).

Table 8: Susceptibility pattern of Gram negative bacteria to commercial antibiotics

Antibiotics/isolates	Diameter of zone of inhibition (mm) *K. pneumonia*	*E. coli*	*E. aerogenes*
Gentamycin	17.3±2.91^{a}	13.3±1.76^{bcd}	10.0±3.06^{a}
Sparfloxacin)	15.0±3.21^{abcd}	14.0±2.31^{bcd}	15.3±2.03^{abc}
Tarivid	18.7±1.76^{cde}	8.00±2.31^{ab}	12.7±2.91^{a}
Chloramphenicol	12.3±1.45^{abc}	17.0±1.72^{cd}	17.3±2.91^{c}
Augmentin	16.0±3.46^{de}	3.33±1.76^{a}	11.3±2.40^{abc}
Amoxycilin	16.0±2.31^{abcde}	12.7±1.76^{a}	15.3±2.40^{a}
Pefloxacin	8.00±2.65^{a}	12.7±1.76^{a}	18.3±2.03^{bc}
Ciprofloxacin	18.0±3.46^{cde}	19.3±3.53^{d}	18.3±3.48^{bc}
Septrin	14.8±1.33^{e}	13.0±2.65^{bcd}	13.3±2.33^{abc}

Values are means of triplicate readings ± Standard Error
Values with the same subscript on each row are not significantly different (p>0.05).

Discussion

Findings from thestudy showed that prevalence of dental caries was high in females compared to male. This is in agreement with the study of Mohapatra *et al.* (2012) which stated that females showed a greater tendency of caries than males, and might be due to the fact that the dentition appears to erupt earlier in girls . Contrary to this is the study of Folayan *et al.* (2007) which stated that when caries experienced in temporary and permanent dentition were combined, there was a decrease in caries risk for females when compared to males which can be attributed to better oral hygiene practices of female.

Results also showed that there is correlation between caries and intake of sugar because majority of the caried infected patients preferred high intake of sugar containing diet. This was stated by Naseem (2004) that the role of refined carbohydrates, especially the disaccharide sucrose is the cause of dental caries. Paula (2005) stated that groups of people with high intake of sugars also have higher levels of caries. It has been suggested that the increase in the consumption of sweet drinks and food in Nigeria combined with poor oral hygiene might lead to a higher level of caries (May *et al*., 2006).

Patients within the age range of 20-35 years were mostly affected. This is in line with the study of Maatouk *et al*. (2001) which concluded that 70% of population aged between 18 and 22 years had caries. Mohapatra *et al*. (2012) stated that age group 21-50 years had highest incidence of caries, with age group 1-20 years having the lowest incidence. Patients that are students followed by the civil servants are mostly infected with caries. This two groups fall within the literacy group. This is in agreement with the study of Akpata (2004) which stated that the dental caries diseases appear to be on increase among certain segment of the urban communities and that dental caries in primary dentition in Nigerians are reported to be low.

Clinical findings showed that molar teeth were mostly infected; this is followed by the premolar, canine and incisors respectively. Caries prevalence varied from one tooth to another, this might be due to variability in their shapes, surface and the site in which they are located inside the oral cavity(Mohapatra). The canine and incisor had lower percentage of infection which might be due to their regular cleansing with saliva and that they are not majorly used for chewing compared to molar and premolar. Akpata (2004) stated that the caries prevalence in second permanent molar is higher than the first permanent molar, even though the first permanent molar erupted six years earlier than the second permanent molar. This is also in correlation with the study of Adekoya-Sofowora *et al*., (2009) which stated that caries was most prevalent in the molar. This study showed that the lower jaws (mandible) were highly infected than the upper jaws (maxillary). Also the lower left jaws were mostly affected with caries compared with the lower right jaw. This is in agreement with the study of Adekoya-Sofowora *et al.* (2009) which revealed that caries occurred more often in the mandibular molars (69.8%) than in the maxillary molars (23.2%).

Microorganisms isolated include both Gram-positive and Gram-negative bacteria namely; Dolan

(2001) stated that the most common biofilm-forming bacteria were *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus viridans*, *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter sp, Proteus mirabilis* and *Pseudomonas aeruginosa* which fall under Gram positive and Gram negative bacteria. Out of these isolates, higher percentages are biofilm formers. This is in line with the study of Shaymaa (2008) in his biofilm assay by tube method which showed that some isolates produced biofilm, while some do not produce biofilm. Dolan (2002) stated that cells may communicate through quorum sensing which may in turn affect biofilm processes like detachment. Mushtak *et al.* (2011) showed that there is significant difference observed between groups of isolates in their ability to produce biofilm.

Herbal medicine has made significant contribution to modern medical practice (Almas, 2001). Both herbal toothpastes had good antimicrobial effect with Paste B having higher inhibitory effect compared to paste A. Contrary to this study is the study of Pai *et al*. (2004) and Jabbarifar *et al*., (2004). The effectiveness of fluoride toothpaste is concentration dependent (Ferjeskov and Kidd, 2003).

The local dentifrices used in this study were commonly used in Abeokuta. Chewing sticks have inhibitory effect on some common microorganisms including those commonly found in the oral cavity (Sote and Wilson, 1995 Oyagade, 1999 and Ojo *et al*., 2007;). In susceptibility patterns of biofilm forming bacterial to local dentifrices solution, *Massularia acuminata* (pako ijebu) had the highest inhibitory effect than *Distemonanthus benthamianus* (orin ayan). This is in correlation with the study of Bankole *et al*. (2012) which stated that *Massularia accuminata* is better for orodental care and hygiene than *Distemonanthus benthamianus.* The high antibacterial effect observed in the chewing sticks was as a result of tannin present in them (Kareem *et al.*, 2012).

Sofia *et al*. (2010) stated that the main mechanisms by which dental plaque can be controlled is by the use of chemotherapeutic agents to reduce an existing plaque. The susceptibility patterns of the the gram positive biofilm forming bacteria isolates were all sensitive to erythromycin . while cotrimoxazole, amoxycilin and ceftriazone showed low activity against some of the isolates..Most of the biofilm forming gram negative organisms were sensitive to ciprofloxacin This is contrary to the study of Devi *et al*. (2012) which stated that some dental plague forming bacteria are resistant to t erythromycin and ciprofloxacin.

Conclusion

Analysis of caried patients in Abeokuta showed that all ages are affected. Even though literates are majorly affected, high occurrence was seen in females. Molar teeth of the left lower jaw (mandible) are the mostly affected. *Streptococcusmutans* and *Staphylococcus aureus* are the most occurring pathogenic cariogens. Although biofilm forming bacteria are susceptible to antimicrobials, non-biofilm forming bacteria are the most susceptible to antibiotics. Ciprofloxacin and erythromycin can be used for treatment of dental caries, but not for its prevention. Toothpastes used have good antimicrobial effects compared with the use of local dentifrices, thus they can be used regularly to prevent tooth decay.

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