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Halitosis: The Antimicrobial Susceptibility Pattern of some Commercially Sold Toothpaste on Selected Isolates

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Abstract- This study aims to investigate the antimicrobial susceptibility pattern of some commercially sold toothpaste on some selected isolates. The isolates used were Escherichia coli, Klebsiella spp, and Streptococcus spp. The commercially sold toothpaste used in this study were CU, PD, and OB. This study was conducted to assess the efficacy of these toothpaste in controlling halitosis. The results of the study showed that all the toothpaste exhibited varying levels of antimicrobial susceptibility against the various isolates tested. CU exhibited the highest level of susceptibility against E. coli and Klebsiella spp while PD showed the highest level of susceptibility against Streptococcus spp. OB had the highest susceptibility against all the isolates. The results of this study suggest that all the toothpaste tested can be used for the control of halitosis.

Keywords- Halitosis, Toothpaste, Susceptibility, Antimicrobial Screening, CU, Klebsiella spp.

I. INTRODUCTION

Halitosis, commonly known as bad breath, is a pervasive problem affecting a significant percentage of the world's population. Halitosis is mostly brought on by an accumulation of oral bacteria that produce offensive gases such dimethyl sulfide, hydrogen sulfide, and methyl mercaptan. These volatile sulfur compounds (VSCs) are responsible for the characteristic foul odor associated with bad breath. While there are numerous factors that contribute to halitosis, including poor oral hygiene, smoking, alcohol consumption, and various medical conditions, the presence of VSC-producing bacteria in the mouth is a significant contributor to the problem (Yaegaki et al., 2000).

Personal image and interpersonal interactions are valued in today's society. As a result, halitosis may be a significant aspect in social communication and, as such, may be the source of concern not only for a probable health issue but also for frequent psychological changes that lead to social and personal isolation. Although oral malodor or foul breath is an unpleasant ailment that most people encounter, it is usually only temporary. According to Van Den Broek et al., (2017), in approximately 80% of all cases, halitosis is caused by microbial degradation of oral organic substrates and the major degradation products are volatile sulphur-containing compounds.

The mouth cavity of a human being is a sophisticated ecosystem that supports a wide variety of bacteria, fungi, and viruses. As a result of their interactions with one another and the host, these bacteria either play significant roles in preserving oral health or contribute to conditions including periodontitis, caries, and halitosis. The tongue, particularly the dorsum (top) of the tongue, is one of the primary locations in the mouth where VSC-producing bacteria gather. The tongue's surface provides an ideal habitat for microorganisms to grow and colonize due to its rough texture and numerous fissures that can harbor bacteria (Gunsolley, 2006).

Bosy (2014) investigated and stated that at least 50% of the population suffer from chronic oral malodour and approximately half of these individuals experience a severe problem that creates personal discomfort and social embarrassment. The mouth air of chronic malodour sufferers is tainted with compounds such as hydrogen sulphide, methyl mercaptan and organic acids, which produce a stream of foul air that is gravely offensive to the people in their vicinity. Sufferers often make desperate attempts to mask their oral malodour with mints and chewing gum, compulsive brushing, and repeatedly rinsing with mouthwashes.

Mouth diseases can be considerably reduced by reducing the microbial burden in the mouth cavity, which can be accomplished by regular oral hygiene (Okafor et al., 2016). Toothpaste is a gel dentifrice used with a toothbrush as an accessory to clean and maintain the aesthetics and health of teeth. It is a widely used oral care product that contains various active ingredients, including fluoride, abrasives, and antimicrobial agents such as triclosan, zinc chloride and chlorhexidine. These ingredients work together to remove plaque, prevent caries, and reduce the bacterial load in the oral cavity. According to the American Dental Association, triclosan and zinc chloride helps to reduce tartar and bad breath.

This research study gives insight of halitosis-causing bacteria, their susceptibility and resistance to various antibiotics and the degree of effectiveness of common commercially sold in the market. This would help create awareness and help for further researches for future inventions for treatment of halitosis.

II. MATERIALS AND METHODS

1. Study Area:
The study was conducted using toothpastes purchased from a store in Ifite, Awka, Anambra State. The study was conducted at the General Microbiology laboratory at Nnamdi Azikiwe University Awka, Anambra State.

2. Sample Collection:
6 labeled sterile swab sticks were given to different individuals to collect biological samples. Samples were collected in the early hours of the morning, before brushing or even talking. The samples were then stored at cold temperature for about 4 hours and were taken to the Microbiology Laboratory for laboratory analysis.

3. Materials and Instruments Used:
Nutrient agar, Blood agar, Mannitol salt agar, Eosin methylene blue agar, Mueller Hinton agar, three brands of toothpastes, sterile petri dishes, alcohol(70%), cotton wool, Aluminium foil, swab sticks, test tubes, spatula, graduated syringe, Micropipette, pasteur pipette, normal saline, beakers, conical flask, measuring cylinder, weighing balance, cotton wool, lighter, gas cylinder, paper tape, inoculating loop, glass slide, crystal violet, Certified Safranin O, 95% ethyl alcohol, potassium iodide, iodine, 100% alcohol, hydrogen peroxide, distilled water, bijou bottles, autoclave, light microscope, test tube racks, cork borer, immersion oil, gram negative and gram positive paper discs, 5ml syringe, tourniquet, Bunsen burner, wash bottles, basic ruler (cm and mm).

4. Isolation of Microorganisms:
The swab stick containing sample was then inoculated by streaking unto the already prepared and set blood agar to check for the growth and hemolysis. Each sample was inoculated unto two different plates (the idea was to culture each sample aerobically and anaerobically). After inoculation, the culture plates were inverted to prevent condensation of moisture into the media. Aerobic condition was allowed by leaving to sit at room temperature for 24 hours to allow for growth. Meanwhile, anaerobic condition was achieved using the candle jar method. The inoculated plates are placed in a jar in which a candle is introduced before sealing the container's airtight lid. The candle's flame burns until extinguished by oxygen deprivation, which creates a carbon dioxide-rich, oxygen-poor atmosphere in the jar. After 24hours, the plates were checked for growth and hemolysis. Colonies were also sub-cultured to obtain pure culture of isolate (Cheesbrough, 2006).

5. Pure Culture Maintenance:
The isolated organisms were purified through repeated subculture method. Streak plate methods were used for this purpose. Nutrient agar and was used as media. When a plate yielded only one type of colony, the organisms were considered to be pure.

6. Identification and Characterization of the Isolates:
The isolates were characterized by morphological, biochemical characteristics and were identified by using Bergey's Manual of Systematic Bacteriology (9th edition, 1994). The microbiological identification procedures included: Gram staining method, colour / pigmentation on various culture plates, smell. The biochemical tests such as catalase test, oxidase test, and hemolysis test.

7. Gram staining:
7.1 Preparation of Safranin:
1.25g of Certified Safranin O was measured (using a weighing balance) into 50ml 95% ethyl alcohol (42.5ml ethyl alcohol + 7.5ml distilled water) in a 1litre conical flask. The 50ml (w/v) mixture was added to 450ml distilled water to make a 500ml solution. It is then stored in a covered container at room temperature (Cheesbrough, 2006).

7.2 Preparation of Lugol's iodine:
In a 1litre conical flask, 15ml potassium iodide is dissolved in 500ml distilled water. 2.5g iodine crystal is also added. The mixture is heated gently with constant mixing until iodine crystal dissolves. It was then diluted to 50ml distilled water and stored in a covered container at room temperature (Cheesbrough, 2006).

7.3 Gram staining procedure:
A drop of normal saline was placed on a disinfected grease free slide. Using a sterile wire loop, a smear of the culture was made on the slide and heat fixed. The fixed smear was covered with crystal violet stain (using pasteur pipette) for 60 seconds. The stain was rapidly washed off with clean running water (from a wash bottle) and drained quickly. The smear was then covered with Lugol's iodine for 60 seconds and washed off with clean water. The slide was flooded with 95% ethanol (decolorizer) for 5 seconds, after which the slide was washed using distilled water. The smear was again flooded with safranin for 60seconds and then washed off. The back of the slide was then cleaned and placed in a draining rack for the stained smear to dry. The standard smear was then allowed to air dry and then viewed under the microscope using ×100 objectives lens with a drop of immersion oil (Cheesbrough, 2006). This procedure was repeated for each isolate.

8. Biochemical Tests:
Biochemical tests are used for microbial identification based on difference in their biochemical activities exhibited by different types of bacteria. Different

biochemical tests are listed below that are used for identification of gram positive and negative organism.

9. Catalase Test:
Catalase test is performed to check the ability of bacterial isolates to produce the enzymes catalase that breaks Hydrogen Peroxide (H2O2) into water (H2O) and Oxygen (O2). A suspension of the organism was made on a clean grease free slide by emulsifying the organism with a loop full of normal saline. 3 drops of 3% hydrogen peroxide was added to the suspension and checked for the production of active bubbles which is a positive result (Cheesbrough, 2006).

10. Oxidase Test:
The oxidase test detects the presence of a cytochrome oxidase system that will catalyze the transport of electrons between electron donors in bacteria and a redox dye-tetramethyl-p-phenylene-diamine. Cytochrome containing organisms produce an intracellular oxidase enzyme which catalyzes the oxidation of cytochrome. Oxidase positive bacteria possess cytochrome C oxidase. A piece of filter paper is placed in a clean petri dish and 2-3 drops of freshly prepared oxidase reagent is added. A small portion of the culture is places on the filter paper (with the help of a sterile glass rod) and a smear is made. It is then examined for immediate color change to blue to purple within 10 seconds (Cheesbrough, 2006).

11. Haemolysis Test:
It is a blood agar test. This test provides information on what hemolytic enzymes a bacterium possesses by providing a culture medium enriched with red blood cells, it is possible to determine whether a bacterium can destroy the cells and whether it can digest the hemoglobin inside. Hemolysis is determined by streaking for isolation on a blood agar plate, this might also include several stabs of the inoculum into the agar to encourage any anaerobic versions of the enzymes to digest blood cells. If the medium is discolored or darkened after growth, the organism has demonstrated alpha-hemolysis. If the medium has been cleared under growth, the organism is beta-hemolytic. No discernible change in the color of the medium constitutes gamma-hemolysis (Cheesbrough, 2006).

12. Antibiotics Susceptibility Testing of the Isolates:
For the antibiotics susceptibility testing, the modified Kirby-Bauer disk diffusion method as described by (Agu et al., 2013; Adindu et al., 2016; Awah et al., 2016; Awah et al., 2017; Umeoduagu et al., 2023) was adopted. 0.5 McFarland turbidity standard was used to get the desired of concentration of isolates in 2ml broth each. 0.5 McFarland turbidity standard was prepared by adding 0.5ml of 1.175% (w/v) barium chloride dihydrate (BaCl2.H2O) solution to 9.95ml of 1% (v/v) sulfuric acid (H2SO4). After getting desired turbidity, using the spread plating method, 0.1ml of the isolates was inoculated onto each of the Mueller Hinton agar plates using a micropipette and spread well using the bottom of a test tube. A forceps is used to place the suitable antibiotics discs into each plate. The plates are then incubated at 37°C for 24 hours and checked for zones of inhibition. It is important to note that this procedure was carried out in an aseptic condition (Cheesbrough, 2006).

13. Antimicrobial Assay of the Toothpastes on the Isolates:
The Kirby-Bauer agar well diffusion method was used. 0.5 McFarland turbidity standard was used to get the desired of concentration of isolates in 2ml broth each. The nutrient agar plates were divided each into 4 equal sections; for 3 toothpastes and control. Using the spread plating method, the isolates were inoculated onto each of the plates. Cork borer was used aseptically to bore 4 holes in the 4 marked sections. 20g of each of the toothpastes was diluted in test tubes of 2ml diluted water. Aseptically, 0.1ml of the solution was poured in the holes of its allocated labeled sections. For the control, water was used. The plates were incubated for 24 hours at 37°C and checked for reactivity and zones of inhibition (Cheesbrough, 2006).

III. RESULTS

Table 1. Morphology characterization of aerobic and anaerobic bacterial isolates colonies on blood agar.

Samples	Texture	Size	Color	Elevation	Odor	Hemolysis
A1	Smooth	Small	Gray white	Slightly elevated	Odorless	No hemolysis
A2	Smooth	Larger	White	Raised	Odorless	No hemolysis
A3	Smooth	Small	Golden yellow	Raised	Musty	β-hemolysis
A4	Mucoid	Small	Grayish white	Flat	Odorless	No hemolysis
A5	Smooth	Small	Grayish white	Convex	Odorless	No hemolysis
A6	Mucoid	Small	Grayish white	Flat	Odorless	No hemolysis
AN1	Smooth	Small	Greyish white	Flat	Odorless	No hemolysis
AN2	Mucoid	Small	Grayish white	Flat	Odorless	No hemolysis
AN3	Smooth	Small	White	Slightly convex	Odorless	No hemolysis
AN4	Smooth	Small	Grayish white	Flat	Foul	No hemolysis
AN5	Mucoid	Small	Grayish white	Flat	Odorless	No hemolysis
AN6	Mucoid	Small	Grayish white	Flat	Odorless	No hemolysis

A.Aerobe AN- Anaerobe

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Table 2. Characteristics of the isolates as seen under the microscope after Gram staining.

Isolates	Shape	Arrangement	Stain
A2	Rod	Singly	+
A3	Cocci	Cluster	+
A4	Cocci	Chain	+
A5	Rod	Chain	-
A6	Cocci	Chain	+
AN1	Cocci	Chain	-
AN2	Cocci	Chain	+
AN3	Rod	Short chains	-
AN4	Rod	Chain	-
AN5	Cocci	Chain	+
AN6	Cocci	Chain	+

A.Aerobic, AN- Anaerobic, +- Gram positive, - Gram N-.

Table 3. Observations from the culture of Gram negative bacteria on Eosin methylene blue agar.

Gram negative isolates	Observation	Suspected organism
A1	Growth; light purple	
A5	Pink mucoid growth	Klebsiella spp.
AN1	Almost insignificant growth	
AN3	Purple colonies with green metallic sheen	Escherichia coli
AN4	Growth	

A.Aerobic, AN- Anaerobic

Table 4. Observations from the culture of Gram positive bacteria on Mannitol salt agar.

Gram positive isolates	Observations	Suspected organism
A2	Small, convex, grayish white colonies	Bacillus spp.
A3	Yellow colonies	Streptococcus spp
A4	No growth	
A6	No growth	
AN2	No growth	
AN5	No growth	
AN6	No growth	

A.Aerobic, AN- Anaerobic

Table 5. Result of catalase and oxidase tests of the isolates.

Isolates	Catalase test	Oxidase test
A2	+	-
A3	+	+
A4	-	-
A5	+	-
A6	-	-
AN1	-	-
AN2	-	-
AN3	+	-
AN4	-	-
AN5	-	-
AN6	-	-

A.Aerobic, AN- Anaerobic

Table 6. An overview of the tests carried out.

Isolates	Shape	GS	Hemolysis	EMB agar	MSA agar	C	O	Probable organisms
A2	Rod	+	B		Small, convex, grayish white colonies	+	-	Bacillus spp.
A4	Cocci	+	Γ		No growth	-	-	Streptococcus spp.
A5	Rod	-	Γ	Pink mucoid growth		+	-	Klebsiella spp.
A6	Cocci	+	Γ		No growth	-	-	Streptococcus spp.
AN1	Cocci	-	Γ	Growth		-	-	Veillonella spp.
AN2	Cocci	+	Γ			-	-	Streptococcus spp.
AN3	Rod	-	Γ	Purple growth with green metallic sheen	No growth	+	-	Escherichia coli
AN4	Rod	-	Γ	Growth		-	-	Fusobacterium nucleatum
AN5	Cocci	+	Γ		No growth	-	-	Streptococcus spp.
AN6	Cocci	+	Γ		No growth	-	-	Streptococcus spp.

GS- Gram stain, C- Catalase, O- Oxidase, A- Aerobe, AN- Anaerobe, γ (gamma hemolysis)- no hemolysis, β (beta hemolysis)- partial hemolysis

Table 7. Result of the antibiotics susceptibility testing of Gram negative bacterial isolates on Mueller Hinton agar, showing the measurements of the zones of inhibition measured in millimeters.

GM solates	CPX (10 mcg)	CXM (30 ncg)	CIP (30 mcg)	PN (30 mcg)	SXT (30 mcg)	OFX (10 mcg)	S (30m cg)	CFX (5mcg)	CN (10m cg)	AU (10m cg)
A1	30	15	12	20	15	21	22	13	22	19
A5	25	17	19	19	R	16	20	18	16	18
AN1	20	12	16	18	R	R	15	R	11	10
AN3	13	R	R	R	R	R	10	R	18	20
AN4	R	R	17	R	R	R	R	R	20	R

GN- Gram negative, A- Aerobic, AN- Anaerobic, AU- Augmentin, CPX- Ciprofloxacin, CXM- Cefuroxime Axetil, CIP- Ceftrazon, PN- Penicillin, SXT- Septrin,

OFX- Tarivid, S- Streptomycin, CFX- Cefixime, CN- Gentamicin, mcg- microgram

Table 8. Result of the antibiotics susceptibility testing of Gram positive bacterial isolates on Mueller Hinton agar, showing the measurements of the zones of inhibition measured in millimeters.

GP isolates	CPX (10 mcg)	E (30 mcg)	LEV (20 mcg)	CN (10 mcg)	CH (30 mcg)	S (30 mcg)	RD (20 mcg)	NB (10 mcg)	APX (20 mcg)	AML (20 mcg)
A2	12	R	R	17	R	R	R	R	R	R
A3	20	15	20	17	R	20	R	R	R	R
A4	23	R	20	20	13	25	R	R	R	R
A6	26	R	15	7	R	17	R	R	R	R
AN2	12	R	R	15	R	R	12	R	R	R
AN5	25	17	20	16	15	25	R	R	R	R
AN6	20	R	20	17	R	20	R	R	R	R

A.Aerobic, AN- Anaerobic, GP- Gram positive, CPX- Ciproflox, E- Erythromycin, LEV- Levofloxacin, CN- Gentamycin, CH- Chloramphenicol, S- Streptomycin, AML- Amoxil, NB- Norfloxacin, APX- Ampiclox, RD- Rifampicin, mcg- microgram

Table 9. The reactivity of the toothpastes on the isolates.

Isolates	Control (distilled water)	CU(130g, SP-1450ppm)	PD (130g, SMFP-1450ppm)	OB (130g, SF-1100ppm)
A5	NR	R	R	R
AN1	NR	R	R	NR
AN4	NR	R	R	R
AN3	NR	R	R	R
A2	NR	R	R	R
A3	NR	R	R	NR
A4	NR	R	R	NR
A6	NR	R	R	R
AN2	NR	R	R	R
AN5	NR	R	R	R
AN6	NR	R	R	R

A.Aerobic, AN- Anaerobic, SF- Sodium fluoride, SMFP- Sodium monoflourophosphate, NR- Non-reactive, R- Reactive, ppm- parts per million.

Table 10 The measurements of the resultant zones of inhibition (as a result of the antimicrobial effect of the toothpastes on the isolates) in millimeters.

Isolates	CU	PD	OB
A5	16	13	10
AN1	10	15	NR
AN4	18	15	10
AN3	11	20	7
A2	12	12	6
A3	10	15	NR
A4	5	14	NR
A6	10	12	5
AN2	10	15	12
AN5	12	12	10
AN6	8	8	5

A.Aerobic, AN- Anaerobic, NR- Non reactive

IV. DISCUSSION

Halitosis is a common oral health issue that affects many people. Bacteria that can cause volatile sulfur compounds (VSCs) are usually responsible for causing oral health. The aim of this study was to isolate and identify the bacteria responsible for causing halitosis, and to test the antibiotics susceptibility and antimicrobial effects of some common toothpaste on these bacteria. Samples were collected from tongue swabs gotten from very early hours of the morning because major halitosis-causing bacteria are putrefactive in nature.

This putrefaction process is enhanced or encouraged by diets and low salivary flow rate during the night while resting or asleep. Samples were cultured properly on blood agar to detect hemolytic bacteria (Wybo et al., 2014). The result of this culture is shown in Table 4.6. Besides, morphological characteristics were observed, recorded and shown in Table 4.1. Nutrient agar is a basal medium that was used for sub-culturing and preservation of pure culture of the isolates in bijou bottles. Asides hemolysis test, other bacteriological tests were carried out the isolates for their identification. Gram staining was carried out to classify the isolates into Gram positive and Gram negative bacteria (Cheesbrough, 2006).

The result of the Gram staining is shown in Table 4.2. Five isolates were tested to be Gram negative while seven of them were Gram positive. Selective and differential media (Eosin methylene blue agar and Mannitol salt agar) were further used to culture the isolates for further identification. Gram negative bacteria were cultured on Eosin methylene blue agar and Klebsiella spp. and E. coli were suspected as they showed obvious results on the medium after 24 hours of the culture. This is shown in Table 4.3. Staphylococcus aureus and Bacillus spp. were suspected after 24 hours of culture of Gram positive bacteria on Mannitol salt agar. The result of this culture is found in Table 4.4.

Catalase and oxidase tests are biochemical tests used for further bacterial identification (Cheesebrough 2006). All the isolates were passed through this test and results are given in Table 4.5. A combination of these tests and matching with the Bergey's Manual of Systematic Bacteriology (9th Edition, 1994) gave a list of probable organisms (as shown in Table 4.6) and they are Neissera spp., Bacillus spp., Staphylococcus aureus, Streptococcus spp., Klebsiella spp., Veillonella spp., Escherichia coli and Fusobacterium nucleatum. This result is quite agreeable when compared to result of a similar study by Zaura et al., (2009).

For the antibiotics susceptibility testing, Mueller Hinton agar, which is known as a loose non-selective, non-differential agar, was used (Cheesbrough, 2006). From the results shown in Tables 4.7 and 4.8, it can be deduced that gentamicin was most effective on the Gram negative bacteria while septrin was the least effective. Table 4.7 shows that 100% of the Gram negative bacteria were susceptible to gentamicin while 80% of them were resistant to septrin. For the Gram positive bacteria, it is shown in Table 4.8 that 100% proved susceptible to gentamicin and ciproflox while 100% proved resistant to norfloxacin, ampiclox and amoxil. This result confirms the report by the World Health Organization that includes gentamicin on the list of broad-spectrum antibiotics and is approved as a strong antibiotic (Tacconeli and Magrini, 2017).

The antimicrobial effect of CU, PD and OB (140g) was assessed using the Kirby-Bauer agar well diffusion method. The images of the toothpastes are shown in Figure 1. Distilled water was used as the control. The control was non-reactive on all the isolates. CU and PD were reactive on all the isolates while OB was reactive on nine of them. The result is clearly shown in Table 4.9. Table 4.10 shows the measurement of the zones of inhibition as a result of their antimicrobial effects on the isolate.

The results of this research as seen in chapter four provides important insight to the oral microbiota and even, opportunistic bacteria found in the oral environment. It shows the effectiveness of antibiotics and the toothpastes in treating this condition. This information could be useful in the development of new oral care products and treatment strategies for halitosis. Also, it can be deduced that although OB is the most expensive and wanted of the toothpastes, its efficacy is the least. Comparing tables 4.7 and 4.8 to 4.10, it is obvious that the effects of these toothpastes are less than antibiotics which the bacteria are readily susceptible to, owing to the measured of zones of inhibition. This stresses the need for use of toothpastes regularly to prevent the buildup of these bacteria and their effects. This is because regular use of antibiotics instead gives rise to the problem of antibiotics resistance.

V. CONCLUSION

Mechanical plaque control, mouthwash, and antibiotics are already available as therapy alternatives. However, conventional treatments are frequently ineffectual, and there is a need to create new therapeutic methods that target the condition's underlying pathogenic pathways. The development of preventive therapies is another critical field of halitosis research. According to recent study, adopting oral hygiene techniques such as brushing and flossing on a regular basis, as well as using mouthwash, can considerably lower the occurrence of halitosis. Probiotics and prebiotics, which can encourage the growth of beneficial bacteria in the oral cavity and reduce the prevalence of halitosis, are two more potential preventive methods.

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