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Original Research Article

ISOLATION AND IDENTIFICATION OF BACTERIA OBTAINED FROM FEMALE STUDENTS USED LIPSTICK IN UNIVERSITY OF CROSS RIVER STATE.

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Abstract: The aim of the study was to isolate and identify bacteria from used lipsticks of female students. Samples were collected from different students, well labeled and transferred to the Microbiology laboratory for analysis. Serial dilution of the samples was carried out, and a dilution factor of 10^4 was plated on a sterilized nutrient agar using the spread plate method. The colonies developed were counted, recorded, and their morphological appearances were noted accordingly. The colonies developed were cultured repeatedly to obtain pure isolates, which were exposed to various biochemical identification tests. Data indicated that bacterial count was in the range of $1.8x10^5$ CFU/g to $6.5x10^5$ CFU/g. The identified bacterial species are *Pseudomonas*, *Rothia*, *Streptococcus*, *Escherichia*, *Corynebacterium*, *Klebsiella* and *Staphylococcus* species. *Staphylococcus* spp., were the most prevalent with a relative occurrence of 27.8%. *Streptococcus*, *Escherichia, Klebsiella. and Micrococcus* species. were least prevalent with a relative occurrence of 5.55% each. The identified bacterial species may be due to direct contact with skin, environmental exposure, co-sharing and absence of preservatives. Therefore, users should avoid sharing lipsticks and maintain good personal hygiene, and manufacturers should ensure that lipsticks contain an effective system and conduct regular preservative efficacy testing to inhabit microbial growth throughout the product shelf life.

Keywords: Lipstick, Identification, Isolation, Bacteria.

INTRODUCTION

Lipstick was created in France in 1869 as the first cosmetic product using beeswax and animal fat (Munawiroh *et al*., 2017). In 1915, lipstick was sold in metallic cylindrical

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tubes (Ragas and Kozlowski, 1998). Nowadays, lipstick is a necessary product for most people. Lipstick has a wide array and variety of textures and colors (Rasheed *et al*., 2020). Lipstick comes in a variety of colors due to the ever-growing demand, as cosmetic products contain nutrients that bring an environment for *microorganisms* to thrive and survive that include water, lipids, polysaccharides, alcohol, proteins, amino acids, glycosides, peptides, and vitamins (Kim *et al*., 2020). Lipstick is a mixture of wax, pigments, and oils that is used to lip color, and lip protect, and makeup (Joshi, 2013). Among other cosmetics, lipstick is the most common of all. Lipstick is therapeutic, social, and psychological (Lwin *et al*., 2020). Lipstick colors are frequently caused by a combination of several pigment compounds. Depending on the type of pigments, thin layer chromatography can be used to separate them (Verma, 2023). The presence of *microorganisms* in cosmetic items can cause them to deteriorate since these organisms change the chemical and physical characteristics of the products, which can be dangerous for consumers. Therefore, it should be crucial to ensure that the addition used can stop the growth of germs (Olson, 1967). Lipsticks contain preservatives, but they also contain bacteria that will develop and reproduce when they are moistened by breath after each use. (Parker, 1972).

2. METHODS AND MATERIALS

2.1 Sample Gathering

A sum of ten (10) used lipstick swap samples were collected from female students at the University of Cross River State. The samples were collected by using a sterile cotton swap stick to swap on the surface of the lipstick, and were carefully handled into the swap container, closed and labeled accordingly. The samples were immediately taken to the lab for examination.

2.2 Preparing and Analyzing Samples for Bacteria

Each lipstick swap sample was dipped into 9mls of distilled water within a test tube and permitted to stand for 2 minutes before shaking to make a stock. Serial dilution of the samples was carried out by transferring 1ml of the sample aseptically from the stock using a sterile syringe into another test tube containing 9mls of distilled water and was shaken to enhance even mixing of the sample, this was repeated till the ninth (9) test tube. Dilution factor 10^4 was plated on Nutrient agar for each of the sample. The plates were incubated at 37^0C for 24hrs. Bacterial colonies that grew were counted and noted as the samples' colony forming units (CFU) on the plates. In order to produce pure isolates, the colonies were also frequently subcultured on new media. The isolates of pure bacteria were preserved in slant bottles for further analysis. The pure bacterial isolates in slant bottles were subjected to different biochemical test and were identified using Bacterial Identification and Microscopic identification.

3. Identification of Isolates

Gram Staining

This method was used to distinguish between *microorganisms* that are Gram-positive and Gram-negative based on their ability to pick up the basic dyes. A

24-hour culture of each isolate were used. Each isolate's smear was created on clean slide with a loopful of distilled water, air dried then heat-fixed by moving the slide across burning flames several times. The slide was inundated with crystal violet, left for a minute, and then washed with water to clear surplus crystal violet. Lugols iodine was added to the stain and was left for 60 seconds, prior to being washed with water. After the smear was decolorized with acetone alcohol for 5 seconds, it was washed with water, and then counterstained with safranin for 60 seconds and rinsed. It was air dried, and oil immersion was applied and was viewed using x100 Len of the microscope.

Motility

A loopful of organisms from isolated colonies of 18-24hrs culture was inoculated in settings that promoted motility using a straight wire to insert into nutrient agar, making a single stab down the tube's center to roughly the medium's depth for about 24hrs at 37^{o}C. Motile bacteria swim to give a diffuse spreading out from the line that is easily recognized with the naked eyes, while non-motile organism remains in the line of streak.

4. Biochemical Test

The following biochemical tests were carried out to identify the isolates.

Catalase Test

Small inoculum was picked from the cultured plate and placed in a glass slide, and hydrogen peroxide (H_2O_2) was dipped on it. Immediately production of bubbles indicates positive result.

Oxidase Test

The oxidase reagent was prepared by dissolving 0.1g of pallav in 10ml of water and shake till dissolves and used immediately. A white filter paper was impregnated with oxidase reagent, using a sterile pasture pipette to place a drop on each of the several spot on the filter paper. A sterile wooden applicator stick was used to pick a discrete colony from a 24hr culture and smeared on the impregnated filter paper and the result was recorded within 5-10seconds. The development of a blue colour within 5-10seconds on the filter paper indicated a positive result.

Citrate Test

Simon citrate agar was made in accordance with the manufacturer's instructions and was introduced into the different test tubes and kept in slant position to become solid. A sterile wire loop was employed to choose the organism from a 24hrs culture and smeared on the agar's surface in the tubes. The tubes were incubated for 24hrs. Following incubation, the tubes were watched for a change in color and results were recorded. A shift in hue from green to blue signifies a positive result, while green slants indicate a negative result.

Indole Test

This was carried out in accordance with the procedure reported by Cheesebrough (2022). Peptone water was prepared and sterilized, allowed to cool and received an inoculation with a loopful test organism which was incubated at 37^{o}C for 48hrs. When the incubation period is over, 3 drops of kovacs was added. After adding the reagents, the mixture was shaken. A positive outcome was indicated by a red ring at the medium's interface, and the absence of red colour ring denoted a negative result.

Triple Sugar Iron (TSI)

The manufacturer's instructions were followed for preparing the agar. The TSI was allowed to cool before introducing 5mls into the different test tubes and kept in a slant position to solidify. A 24hr bacteria culture was inoculated into the different test tubes containing TSI and allowed for 24hrs. The tubes were observed for gas production, which was indicated by an air gap in tubes. The presence of black precipitate in the test tube indicated the generation of hydrogen sulfide, the slant and butt were observed for colour change and were recorded accordingly.

Test for Methyl Red

Peptone water was made in accordance with the manufacturer's instructions and 5mls of the prepared peptone broth was introduced into several test tubes and the isolates were inoculated and allowed for 72hrs. A 2-4 Methyl red reagent drops were applied to the broth culture. The presence of red colour indicates a favorable outcome, while the color yellow indicates an adverse outcome.

Voges Proskauer

Peptone water was made in compliance with the manufacturer's guidelines and 5mls of the prepared peptone water was introduced into different test tubes and the isolates were inoculated and allowed for 72hrs. 2 drops of Barit A solution 40% (i.e 40g of potassium hydroxide (KOH), and 2 drops of Barit B solution (alpha naptol) was added. Red/pink colour indicates positive result.

Sugar Fermentation

Peptone water was prepared according to manufacturer’s instructions. A gram of each sugar (glucose, sucrose, lactose, maltose and fructose) to be used was dissolved into the broth and 0.01g of phenol red indicator was introduced and shaked for even mixture, and was sterilized using the autoclave. A 5mls of the broth was distributed to sterile test tubes along with the Durham's tubes and the isolates were inoculated and incubated for 24hrs after which the results were recorded. A colour change from red to yellow indicates positive and the air space in the Durham tubes indicates the presence of gas.

Statistical Analysis

All data were analyzed using simple percentage and Microsoft word.

Ethical Consideration

Ethical approval was sought from the Department of Microbiology at the University of Cross River State. A letter of consent was issued, which was presented to the female students before commencement of the study.

5. RESULT

The bacterial loads on the ten (10) lipstick samples ranged from 1.8 x 10^5CFU/g to 6.5 x 10^5 CFU/g. Lipstick sample from AK showed the highest bacterial count with a value of 6.5 x 10^5 CFU/g. Sample from RA recorded the lowest count with a value of 1.8 x 10^5 CFU/g. Results are shown in the table below.

Table 1: Heterotrophic Bacterial Count

Sample Code	Bacteria count	($X10^4$CFU/g)
EA	52	$5.2x10^5$
RA	18	$1.8x10^5$
AO	35	$3.5x10^5$
OA	36	$3.6x10^5$
AK	29	$2.9x10^5$
AK	65	$6.5x10^5$
BA	45	$4.5x10^5$
UD	47	$4.7x10^5$
HT	49	$4.9x10^5$
J	44	$4.4x10^5$

Table 2: BACTERIA ISOLATES, CULTURAL, MORPHOLOGICAL AND BIOLOGICAL CHARACTERISTICS

		GRAM STAIN												TS.1				
S/N	Colour/Appearance	RXN	Shape	Arrangement	Catalase	Oxidase	Methyl Red	Indole	VogesProskauer	Citrate	Glucose	Sucrose	Maltose	Slant	Butt	H2S	Motility	Suspected Organism
1	Creamy Irregular Rough	+	Cocci	Short Chains	+	-	+	-	+	-	+	+	+	Y	Y	-	-	*Staphylococcus* spp
2	Creamy Irregular Smooth	-	Rod	Single	+	+	-	-	-	+	-	-	-	R	R	+	+	*Pseudomonas* spp
3	Creamy Irregular Rough Dry Opaque	+	Rod	Cluster	+	-	-	-	-	-	+	+	-	R	R	-	-	*Rothia* spp
4	Creamy Circular Smooth Opaque	+	Cocci	Long Chains	-	-	+	-	-	-	+	+	+	Y	Y	-	-	*Streptococcus* spp
5	Creamy Irregular Smooth, Opaque	-	Rod	Single	+	+	-	-	-	+	-	-	-	R	R	-	-	*Pseudomonas* spp
6	Creamy, Irregular Smooth Opaque	+	Cocci	Cluster	+	-	+	-	+	-	+	+	+	Y	Y	-	-	*Staphylococcus* spp
7	Creamy Irregular Rough Dry Opaque	+	Rod	Cluster	+	-	-	-	-	-	+	+	-	R	R	-	-	*Rothia* spp

8	Creamy Moist Circular Entire	-	Rod	Single	+	-	+	+	-	-	+	+	+	Y	Y	-	+	*Escherichia* spp
9	Creamy, Irregular Rough Dry Opaque	+	Rod	Single	+	-	-	-	-	-	+	+	-	R	R	-	-	*Corynebactenum* spp
10	Creamy Irregular Smooth Opaque	-	Rod	Single	+	+	-	-	-	+	-	-	-	R	R	-	-	*Pseudonomas* spp
11	Creamy, Irregular Rough	+	Cocci	Short Chains	+	-	+	-	+	-	+	+	+	Y	Y	-	-	*Staphylococcus* spp
12	Creamy Irregular Smooth Opaque	+	Cocci	Long Chains	-	-	+	-	-	-	+	+	+	Y	Y	-	-	*Staphylococcus* spp
13	Creamy Irregular Rough Dry Opaque	+	Rod	Cluster	+	-	-	-	-	-	+	+	-	R	R	-	-	*Rothia* spp
14	Creamy Irregular Rough Dry Opaque	+	Rod	Single	+	-	-	-	-	-	+	+	-	Y	Y	-	-	*Corynebacterium* spp
15	Creamy Irregular Smooth, Opaque	-	Rod	Single	+	+	-	-	-	+	-	-	-	R	R	-	+	*Pseudomonas* spp
16	Creamy, Convex Smooth	-	Rod	Single	+	-	-	-	+	+	+	+	+	Y	Y	-	-	*Klebsiella* spp
17	Creamy, Convex Dry	+	Cocci	Pairs	+	+	+	-	-	+	-	+	-	R	R	-	-	*Micrococcus* spp
18	Creamy, Irregular Rough	+	Cocci	Short Chains	+	-	+	-	+	-	+	+	+	Y	Y	-	-	*Staphylococcus* spp

The biochemical and physical traits of the bacteria that were separated from used lipstick swap were identified. Ten (10) samples were analyzed and a total of eighteen (18) bacteria were isolated from the samples, as represented in the table above. Their cultural morphology and biochemical characteristics were observed. The isolated organisms had different colour and appearance, some were creamy, irregular and rough, others were dry, opaque, convex and smooth. Some were entire in margin, and moist on surface. Some appeared to be in short chains, long chains, single and cluster in arrangement. The biochemical test carried out include catalase, oxidase, methyl red, indole, voges proaskauer, citrate, sugar fermentation (glucose, sucrose, maltose), triple sugar iron test and motility as represented above.

Table 3 shows the percentage occurrence of bacteria isolated from different used lipstick swap samples *Staphylococcous* spp having the frequency of (4) had the highest percentage occurrence of 27.8%, while *Streptococcus* spp, *Escherichia* spp, *Klebsiella* spp and *Micrococcus* spp had the lowest frequency of (1), with the percentage of 5.55% respectively.

Table 3: Percentage Occurrence of Bacteria Isolates

S/N	Bacteria	Frequency	Percentage (%) Occurrence
1	*Staphylococcus* spp	5	27.8
2	*Pseudomonas* spp	4	22.22
3	*Rothia* spp	3	16.66
4	*Streptococcus* spp	1	5.55
5	*Escherichia spp*	1	5.55
6	*Corynebacterium spp*	2	11.12
7	*Klebsiella spp*	1	5.55
8	*Micrococcus spp*	1	5.55
Total		**18**	**100**

6. Discussion

From this study ten (10) used lipstick swap samples were collected from female students. The sample were analyzed using standard microbiological methods. According to heterotrophic bacterial counts (Table 1), counts ranged from $1.8x10^5$ CFU/g to $6.5x10^5$ CFU/g. Environmental factors, and sharing can all be sources of contamination.

Bashir and Lambert (2020), also concluded that used cosmetic products were more contaminated by *microorganisms*, and this contamination increased with continued use of cosmetics. A total of eight (8) bacterial isolates were obtained from the used lipstick swab sample, of which three were identified as Staphylococcus spp and Pseudomonas spp, and Rothia spp. and the remaining five were Streptococcus spp, Escherichia spp, Corynebacterium spp, Klebsiella spp and Micrococcus spp. Bashir and Lambert (2020), reported the presence of Staphylococci in used cosmetic product and identified them as major contaminants associated with consumer use. Similar findings were reported by Razooki et al., 2017, who identified Pseudomonas spp., and Klebsiella spp among the common bacterial contaminants discovered from cosmetic product. Yazici and Eryilmaz (2023), identified the presence of Rothiia spp and Streptococcus spp in used lipstick due to contamination from saliva and repeated human contact. The used lipstick samples allowed for the identification of Corynebacterium spp, Escherichia spp, Staphylococcus spp and Pseudomonas spp, supporting the findings of Lee and Lee (2017). Using pyrosequencing, Lee and Lee identified 103 bacterial genera present in used lipsticks and reported the presence of Corynebacterium spp, Escherichia spp, Staphylococcus spp, Pseudomonas spp and Salmonella spp, as well as Mycobacterium spp and Neisseria spp, all of which are associated with the human skin and oral microbiota as well as environmental contamination. The detection of Micrococcus spp. among the used lipstick samples corroborates the findings of Saeed and Asif (2011), who isolated Micrococcus spp. from used lipsticks.

7. CONCLUSION

This study showed that many female students used lipsticks were contaminated with a variety of bacteria including the highest numbers of Staphylococcus spp and Pseudomonas spp. The high number of bacteria and the presence of bacteria like Escherichia spp., Klebsiella spp., Corynebacterium spp. Point out that used lipsticks may contain potentially harmful *microorganisms*. There is a concern that the repeated use and unhygienic storage and sharing of lipsticks may have caused the contamination, hence used lipsticks should be treated in a hygienic manner and not shared.

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