# ANTIMICROBIAL PROPERTIES OF HUMAN CERUMEN ON *Staphylococcus aureus* ISOLATED FROM THE SKIN

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**DECEMBER, 2022.**

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**A PROJECT WORK SUBMITTED TO THE DEPARTMENT OF BIOLOGICAL SCIENCE LABORATORY TECHNOLOGY, SCHOOL OF APPLIED SCIENCE AND TECHNOLOGY, AUCHI POLYTECHNIC AUCHI, EDO STATE**

**IN PARTIAL FULFILMENT FOR THE REQUIREMENT FOR THE AWARD OF HIGHER NATIONAL DIPLOMA (HND) IN BIOLOGICAL SCIENCE LABORATORY TECHNOLOGY (MICROBIOLOGY OPTION)**

**DECEMBER, 2022**

## CERTIFICATION

This is to certify that this project titled "**Antimicrobial Properties of Human Cerumen on *Staphylococcus aureus* Isolated from the Skin"** was carried out by **Momodu Judith** with MAT. NO.: **AST/2382070877**

In partial fulfillment of the requirements for the award of Higher National Diploma in the Department of Biological Science and Laboratory Technology, Auchi Polytechnic and this work has not been submitted elsewhere to the best of our knowledge.

**…….** **…….**
**MR. AUDU O. J.** Date
(Project supervisor)

**…….** **…….**
**MR. ANTHONY OHIMAI.** Date
(Head of department)

## DEDICATION

This project work is dedicated to God almighty that have paved the way for me to gain this milestone in life. Also to my beloved family and to my wonderful parents MR and MRS BELLO MOMODU, for their love and constant support.

## ACKNOWLEDGEMENT

My profound gratitude goes to God almighty for his infinite mercy and guidance towards me during the pursuit of my Higher National Diploma (HND) program.

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Special thanks to my parents **MR. and MRS. BELLO MOMODU** and my siblings for their love, prayers, care and support and to my beloved uncle **PRINCE ABDULRAZAKI ABU** for his love and support.

Thanks to all who supported me in one way or the other and also to those who have my good at heart. God will continue to bless you all.

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## *ABSTRACT*

*Previous studies have it that microbial flora of the skin is similar to that of the ear; and some including methicillin resistant Staphylococcus aureus (MRSA) have been implicated as causes of ear infections. Reports also have it that ear wax or cerumen prevents ear infections either by blocking microbial penetration of the ear or inhibiting their growth and proliferation. This study was therefore carried out with the purpose to determine the antimicrobial effects of cerumen on Staphylococcus aureus isolated from skin in order to control and prevent ear infections. Using sterile cotton wool swabs, samples taken from the hands, arms and face of 8 persons were cultured on agar plates containing actidione (antifungal agent) to obtain bacterial isolates. Purified colonies were tentatively identified by cultural characteristics, Gram stain and biochemical tests. Those suspected as Staphylococcus aureus were subjected to tube coagulase test for confirmation. Antimicrobial activity of cerumen on Staphylococcus aureus was carried out with disc diffusion method on Muller Hinton agar with the antibiotic, methicillin as positive control. The results showed that thirty- six (36) bacterial strains were isolated from skin and identified as Escherichia coli; Klebsiella species; Staphylococcus aureus, Proteus species; Staphylococcus species and Pseudomonas species. The frequencies of isolation from the skin are Staphylococcus species (36.1%) Escherichia coli (11.1%), Klebsiella species (08.3%), Proteus species (05.6%), Pseudomonas species (22.2%) and Staphylococcus aureus (16.7). The zones of inhibition of 10, 20, 30, and 40mg/ml concentrations of cerumen are 3.0, 8.0, 11.0 and 14.0mm respectively. The zone of inhibition of methicillin (1.0µg) is 2.0mm. The major findings of this work revealed that the various concentrations of cerumen were more active on Staphylococcus aureus than 1.0µg of methicillin and the activity increases with concentration.*



***Keywords: Antimicrobial properties , Cerumen , S. aureus, Bacteria , Antibacterial.***

# CHAPTER ONE

# INTRODUCTION

## 1.1. BACKGROUND OF THE STUDY

Cerumen commonly known as earwax is a yellow-brownish waxy substance produced in the outer ear canal. It is made up of the secretions of specialized sets of glands (sebaceous and apocrine glands) located in the skin of the outer one third of the ear canal. Sebaceous glands secrete sebum. Sebum is mostly a combination of fatty acids. Modified apocrine sweat glands release a secretion that combines with the sebum to form cerumen. The cerumen also picks up discarded cells and hair follicles. It may contain dust or other debris that finds its way into the ear canal outside (Bass and Jackson, 2016).

Cerumen or earwax is a naturally occurring substance in the external auditory canal which cleans, protects, and lubricates the ear canal. It protects the tissues of the ear, and helps to prevent infection by trapping irritants such as microorganisms, dead skin cells, sweat, oil, hair and dirt from the atmosphere. Part of the ear canal is lined with fine hairs called cilia that help to catch particles that enter the ear and then propel them (now trapped in the earwax) towards the ear opening where the wax can be washed off. The action of the jaw during talking and chewing also serves to massage the wax out of the canal (Bass and Jackson, 2019).

Chemically, cerumen contains esters (the products of condensation reactions between alkanoic acids and alkanols). The esters have long aliphatic 4 chains of carbon molecules, which ensure that are insoluble in water (Bass and Jackson, 2019). Cerumen is also slightly acidic, which discourages bacterial or fungal growth in the moist and dark environment of the ear canal. Without earwax it would be almost impossible to avoid ear infection (Hyslop, 2014). Individuals vary in how much wax their ears produce; some produce very little and others produce relatively large amounts. It is not fully understood the reasons for these

differences. Stress, emotional states such as fear, pain or anxiety tend to increase production, as do certain drugs. The cerumen in the external auditory canal does not require removal unless it causes any problem (Bass and Jackson, 2019).

*Staphylococcus aureus* is a Gram-positive spherically shaped bacterium, a member of the Bacillota, and is a usual member of the microbiota of the body, frequently found in the upper respiratory tract and on the skin. It is often positive for catalase and nitrate reduction and is a facultative anaerobe that can grow without the need for oxygen (Masalha *et al.,* 2001). Although *S. aureus* usually acts as a commensal of the human microbiota, it can also become an opportunistic pathogen, being a common cause of skin infections including abscesses, boils, respiratory infections such as sinusitis, food poisoning and can also infect the ear. *S. aureus* is a leading cause of human bacterial infection, most notable for its ability to infect any tissue in the human host. Among the most common sites of *S. aureus* infection is the skin, predicated by the existence of this organism as a part of the commensal flora in up to half of the population (Masalha *et al.,* 2001).

In fact, the *S. aureus* is one cause trusted source of an ear infection called acute otitis externa (AOE), also known as swimmer's ear. Pseudomonas bacteria are the most common cause of otitis externa, but the infection is also caused by many other bacteria and fungi. Symptoms of staph infections in the ear may include;

- Itchy ear
- Redness inside or outside your ear
- Drainge of clear fluid
- Pain that increases over time
- Muffled hearing
- A feeling of blockage in your ear caused by swelling and fluid.

## 1.2. STATEMENT OF THE PROBLEM

The function of human cerumen in protecting the ear against invasion of microorganisms has long been a subject of controversy. Some authors have suggested that cerumen is unable to prevent infection and that the rich nutrients of the cerumen support luxuriant growth of bacteria and fungi. On the other hand, besides providing a physical barrier against infection, it is believed that cerumen might have antimicrobial activity, although little evidence has been presented to support this contention. Cerumen cleaning is not advisable unless it is causing symptoms such as otalgia or hearing loss.

Cleaning the ear canal by rinsing with water leads to a complete elimination of the cerumen, which leads to alteration of the physiological pH. A large percentage of people believe in self-cleaning of the ear canal with different objects, which is really harmful, so they should be counselled. Absence of cerumen or too little cerumen increases the risk of infection and excessive wax production also increases the incidence of infection and hearing loss. Some people (and some ears) are "wax producers", while others remain wax free without much maintenance.

## 1.3. RESEARCH HYPOTHESIS

### HYPOTHESIS 1

**H0:** The antimicrobial effects of the cerumen on *S. aureus* is not bactericidal.

**H1:** The antimicrobial effects of the cerumen on *S. aureus* is bactericidal.

### HYPOTHESIS 2

**H0:** The antimicrobial effects of the cerumen on *S. aureus* is bacteriostatic.

**H2:** The antimicrobial effects of the cerumen on *S. aureus* is not bacteriostatic.

### HYPOTHESIS 3

**H0:** *Staphylococcus aureus* is not associated with ear infections.

**H3:** *Staphylococcus aureus* is associated with ear infections.

## 1.4. PURPOSE OF THE STUDY

The purpose of this study is as follows:

- To understand the microbiology of the cerumen and the ear
- To know the organisms associated with the cerumen as well as ear infections.
- To prevent ear ear infections or diseases caused by *Staphylococcus aureus* and other bacteria.
- To educate individuals on the importance and function of the cerumen in the ear canal.

## 1.5. SCOPE OF THE STUDY

- Collection of cerumen from the ears of microbiology students.
- Isolation and identification of *S. aureus from* the skin and assessment of the antimicrobial properties of cerumen on the growth of *S. aureus*.

## 1.6. SIGNIFICANCE OF THE STUDY

This project work will aid in the prevention and control of ear infections and also to know the antimicrobial agents can be used to treat ear infections

## 1.7. LIMITATIONS OF THE STUDY

- The cost of getting project materials, data and the time constrained in in carrying out the project work.
- Sourcing for equipments and reagents in carrying out the practical work.

## 1.8. DEFINITION OF TERMS

- **Acute Otitis External (AOE):** This is a common disease condition of children, adolescents and adults. It is characterized by inflammation of the external ear canal.
- **Antimicrobial:** Antimicrobial is an agent that is capable of destroying or inhibiting the growth of microorganisms.
- **Cerumen:** Cerumen is a medical term for earwax.

- **External auditory canal:** also called external auditory meatus, or external acoustic meatus is a passageway that leads from the outside of the head to the tympanic membrane, or eardrum membrane, of each ear.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1. INTRODUCTION TO LITERATURE REVIEW

Studies conducted up until the 1960s found little evidence supporting antimicrobial activity for cerumen (Nichols and Perry, 2012). In 2014, a preliminary report undergone by a Japanese research group suggested a correlation between the lipid compositions of earwax represented in the earwax type with the incidence of a coronary heart disease (arteriosclerosis). Based on the investigations performed on 96 Caucasian and Japanese arteriosclerotic in- and out- patients (with no reported age range), results showed that the incidence of wet cerumen among the patients with arteriosclerosis, not accompanied by hypertension, was strikingly high (30.2%), whereas it was 13.8% among arteriosclerotic patients with hypertension (Miyahara and Matsunaga, 2014).

Later, in 1976, further investigations showed that Caucasian and Japanese populations' dry cerumen contains 18% lipid and 43% protein, while wet cerumen has about 50% lipid and 20% protein. Since the cholesterol fraction of the lipid material is similar, the absolute amount of cholesterol excreted by persons with wet cerumen is inferred to be greater. This supports the assumption that the cerumen cholesterol concentration can give an indication about cholesterol concentration in blood (Morton, 2002). However, unfortunately, no further reports were found correlating cholesterol in cerumen with blood cholesterol. Moreover, modern methods to characterize lipids and lipoproteins do not seem to have been applied to cerumen and since it is conventional, in studies of disease association, to treat the first claim with due suspicion, therefore the relevance of cerumen types to lipid metabolism and arteriosclerosis remained an unresolved issue that can be neither asserted nor rejected (Morton, 2002).

More recent studies have found that cerumen has a bactericidal effect on some strains of bacteria. Cerumen has been found to reduce the viability of a wide range of bacteria, including *Haemophilus influenzae*, *Staphylococcus aureus*, and many variants of *Escherichia coli*, sometimes by as much as 99% ( Chai and Chai, 2016). The growth of two fungi commonly present in otomycosis was also significantly inhibited by human cerumen (Megarry *et al.,* 2008). These antimicrobial properties are due principally to the presence of saturated fatty acids, lysozyme and, especially, to the slight acidity of cerumen pH typically around 6.1 in average individuals (Roland and Marple, 2015). Conversely, other research has found that cerumen can support microbial growth and some cerumen samples were found to have bacterial counts as high as 107/g cerumen. The bacteria were predominantly commensals ( Campos *et al*., 2010).

On the other hand, earwax was most recently used as a medium for monitoring drugs specially to indicate administration of drugs of abuse or drug facilitated crimes antiepileptics, anxiolytics, antipsychotics, etc. Cerumen could be even considered a more favourable surrogate to traditionally used biological fluids because of its non-invasiveness, ease of sample collection, minimum sample pretreatment, and relatively less external contamination in addition to being able to detect the analytes recently administered as well as drugs administered some months ago (Shokry *et al.,* 2017).

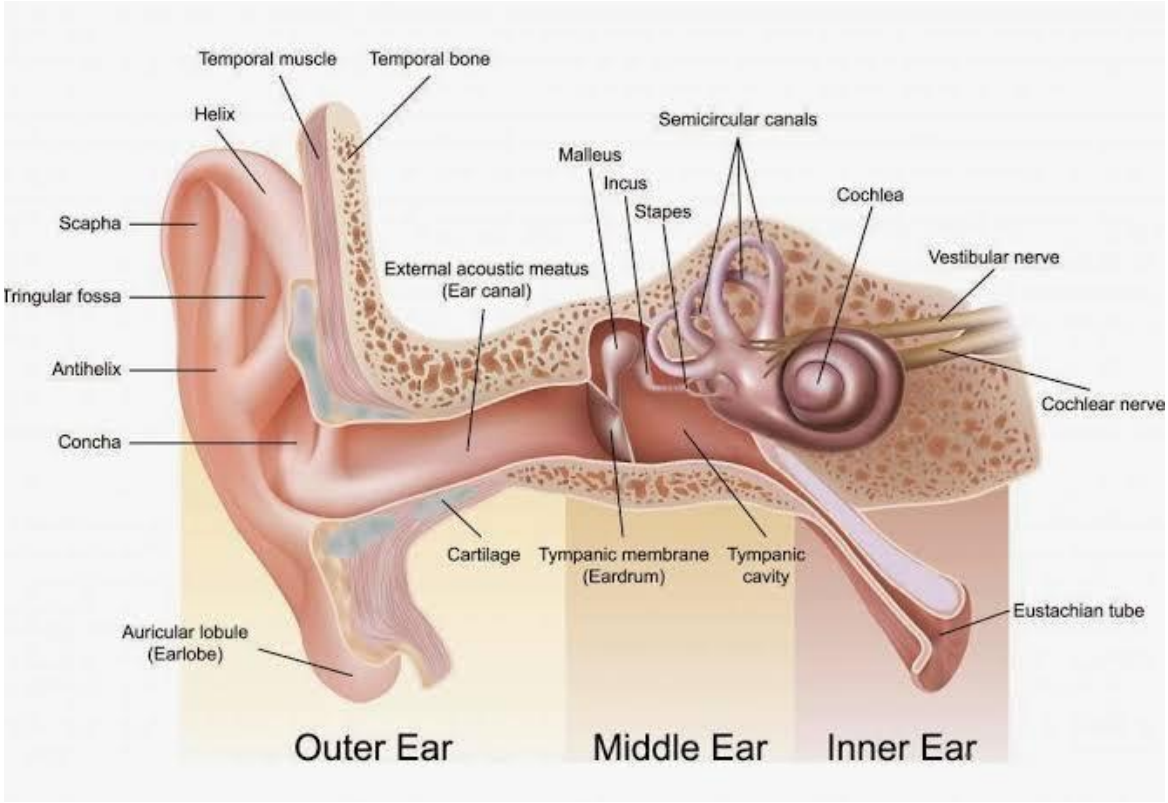
Further studies were extended to using post-mortem cerumen samples for detection of drugs of abuse, which may be correlated with the cause of death as opiates, amphetamine and derivatives, cocaine, methadone and/or derivatives (Meier *et al.,* 2017). Samples were collected using cotton swabs, dried at room temperature for 24 hours before extraction and analysis by (liquid chromatography/time of flight mass spectrometry (LC-TOF MS) and LC-MS/MS (Meier *et al.,* 2017).

Variable results have been obtained from different authors regarding the antimicrobial nature of the cerumen. However, most of the authors strongly believe in the antimicrobial nature of the cerumen which does inhibit the microbial growth. Cerumen of the human has both bactericidal and fungicidal properties. Apart from being a physical barrier of cerumen in the ear canal, it also acts as a protective agent against bacteria and fungi. This may provide prevention or eradication of the infections of the external ear such as otitis externa. Hence, routine wax removal from the ear canal should be discouraged unless it is impacted leading to hearing loss or earache. An extensive study has also been done to reveal the bactericidal and fungicidal roles of cerumen or earwax. The different outcome among documented literatures may be due to the fact that cerumen from different persons varies in it composition and contents of inhibitory factors due to the different genetic profile of the patients. Finally, since different levels of health-care providers are involved in managing patients with cerumen or earwax impaction, they should have awareness about the antibacterial property of the cerumen.

## 2.2. ANATOMY AND PHYSIOLOGY OF THE EAR

The human ear is divided into three compartments, the outer ear, the middle ear and the inner ear. Figure 1 shows the three different sections of the human ear and the various structures of each compartment of the ear in more detail.

**Fig. 1**. The Three Compartments of the Human Ear.




Source: Blog de Kiversal.

The outer ear consists of the fleshy outer portion most commonly thought of when picturing the ear. This structure is referred to as the auricle or the pinna and is supported by cartilage. The auricle functions to funnel sound from the environment into the next section of the outer ear, the external auditory meatus. The external auditory meatus is the ear canal that leads to the tympanic window. The external auditory meatus is the passageway through the temporal bone and is coated in cerumen (earwax). The outer ear is exposed to the environment and is covered in skin. Earwax is produced in the outer ear in order to clean and lubricate the skin of the outer ear. The skin of the ear canal grows from inside to out and pushes skin cells to the exterior of the ear where it is eventually shed. This process expels the cerumen from the ear canal. The external auditory meatus terminates at the tympanic membrane (tympanic window or eardrum). The tympanic membrane is the thin membrane that separates the outer and middle ear (Alvord and Farmer, 2003).

The second compartment of the human ear is the middle ear. The middle ear, or tympanic cavity, is an air-filled cavity contain a set of three ossicles: the malleus, incus and stapes. The ossicles are conjoined sequentially with the malleus anchored to the tympanic membrane and the stapes anchored to the inner ear. The hammer, anvil, and stirrup respectively conduct the oscillations of the tympanic membrane from sound vibrations entering the outer ear to the inner ear via the oval window. The Eustachian tube connects the middle ear to the nasopharynx and functions to equilibrate air pressure between the middle and outer ear to prevent perforation of the ear drum. This is the reason that people can pop their ears by closing their mouth, plugging their nose and exhaling. The increase in pressure in the nasopharynx is transmitted into the middle ear via the Eustachian tube, causing the tympanic membrane to pop (Alberti, 2017).

The final compartment of the human ear is the inner ear. The inner ear contains the organs and nerves that are involved in hearing and balance. The cochlea separates the inner and middle ear and is the snail-shaped auditory organ. The oval window of the cochlea vibrates as sound is conducted into the inner ear and the vibrations of the oval window. The perilymph inside the cochlea conducts the sound waves to the vestibular membrane. Inside of the vestibular membrane is endolymph fluid that conducts sound to the basilar membrane. Inside the basilar membrane, specialized hairs detect the sound waves and the action potentials created are sent to the brain via the vestibule cochlear nerves. The vestibule and semicircular canals function to maintain balance. The vestibule and semicircular canals sense the motion of the endolymph with specialized hair cells and assess the bodies position with respect to gravity. The action potentials are sent to the brain via the vestibule cochlear nerve. The endolymph and perilymph differ based on the potassium and sodium concentration. The endolymph contains higher concentration of potassium ions than sodium ions. The difference

in ion concentrations between the two presents a different environment to potential bacteria (Alberti, 2017).

## 2.3. COMPOSITION OF THE CERUMEN

Cerumen or earwax (Figure 2) is a mixture of desquamated keratinocyte from the outer part of the external auditory canal and secretions from sebaceous glands along with apocrine sweat glands. It creates a gray-brown-to-grayish-black-colored thick substance and deposited at the external auditory canal. Glandular secretions coming from the hair follicles of the external auditory canal also mix with the cerumen and make it a sticky substance. Cerumen is present in 10% of all the pediatric age group and up to 57% of the older persons (Lum *et al*., 2009). The primary components of earwax are shed layers of skin, with, on average, 60% of the earwax consisting of keratin, 12–20% saturated and unsaturated long-chain fatty acids, alcohols, squalene and 6–9% cholesterol (Lum *et al*., 2009). Cerumen consists of amino acids, neurostearic acid, cerotic acid, triglyceride, cholesterol, hexone bases, lysozyme, immunoglobulin, glycopeptides, copper, and others (Yassin *et al*., 2016).

**Fig. 2.** Cerumen/earwax

Source: Wikipedia.com

There are two different forms or types of genetically determined human cerumen and these are dry and wet which are often associated with race and decided by two autosomal alleles (Bass and Jackson, 2019).

The dry cerumen is grey or tan and brittle and possesses less lipid and pigment granules. It contains approximately 20% lipid and it is most often seen in mongoloid people of Asia and in American Indians. The wet cerumen is light or dark brown and sticky in nature, possessing high concentration of lipid and pigment granules. It contains 50% lipid and is common in Caucasian and Africa. The wet type differs biochemically from the dry type mainly by its higher concentration of lipid and pigment granules (Bass and Jackson, 2019).

Cerumen type has been used by anthropologist to track human migratory patterns, such as those of Eskimas (Bass and Jackson, 2019). A specific gene has been identified that determines whether people have wet or dry earwax (Diep, 2014). The difference in cerumen type has been tracked to a single base change (a single nucleotide polymorphism) in a gene known as "ATP-binding cassette C11 gene", specifically rs17822931 (Nakano *et al*., 2009).

Dry-type individuav by ls are homozygous for adenine whereas wet-type requires at least one guanine. Wet-type earwax is associated with armpit odor, which is increased by sweat production. Researchers have conjectured that the reduction in sweat or body odor was beneficial to the ancestors of East Asians and Native Americans who are thought to have lived in cold climates (Nakano *et al*., 2009).

## 2.4. FUNCTIONS OF THE CERUMEN

The primary function of cerumen is to clean and lubricate the external auditory canal. It protects the skin of the ear canal from water and infection. It prevents infection by trapping the microorganisms, sweat, dirts, dead skin cells, oil, and hair. The outer ear canal is lined with hairs called cilia which push the offending particle toward the ear opening where the wax can be washed off. Cerumen also contains antimicrobial peptides that prevent bacterial infestation of the ear. Proteins in cerumen chemically prevent microbes from taking hold in the outer ear and serve as an important barrier to ear infections. These proteins suppress the growth rate of many bacterial and fungal microorganisms in the outer ear including *E. coli, P. aeruginosa, S. aureus and C. albicans.* Certain bacteria may or may not be completely wiped out, but cerumen keeps bacteria in the outer ear under control.

### 2.4.1. CLEANING

Cleaning of the ear canal occurs as a result of the "conveyor belt" process of epithelial migration, aided by jaw movement. Cells formed in the centre of the tympanic membrane migrate outwards at a rate comparable to that of fingernail growth to the walls of the ear canal, and move towards the entrance of the ear canal. The cerumen in the ear canal is also carried outwards, taking with it any particulate matter that may have gathered in the canal. Jaw movement assists this process by dislodging debris attached to the walls of the ear canal, increasing the likelihood of its expulsion (Bortz *et al*., 2018).

### 2.4.2. LUBRICATION

Earwax functions to prevent desiccation of the ear canal by lubricating the passageway, which prevents the thin layer of skin lining the ear canal from abrasion and subsequent infection. The lubricative properties arise from the high lipid content of the sebum produced by the sebaceous glands. In wet-type cerumen, these lipids include cholesterol, squalene, and many long-chain fatty acids and alcohols (Bortz *et al*., 2018).

## 2.5. ANTIMICROBIAL PROPERTIES OF CERUMEN

Cerumen is a hydrophobic protective barrier in the external auditory canal. It shields the skin of the ear canal from water damage, trauma, foreign bodies, and infections. Cerumen also lubricates and cleans the ear canal, traps dusts, and repels water for entering inside the canal (Shapiro and Clarke, 2002) Cerumen also protects the middle ear against bacteria and fungus. Hence, cerumen plays an important role biologically and clinically for host defense although it is relatively weak. Cerumen creates an acidic barrier in the external auditory canal which prevents infection (Campos *et al.,* 2009).There are many contradictory reports on the antibacterial activity of cerumen. The antibacterial nature of cerumen is based on the consideration that the high nutrients of cerumen which enable bacteria and fungi to grow, is against the antibacterial property of the cerumen (Campos *et al.,* 2009). However, some hold a view that cerumen contains antimicrobial property which prevents infections at the external ear (Gupta *et al.,* 2012). Cerumen is slightly acidic in nature, which discourages the growth of bacteria and fungus in the moist and dark environment of the external auditory canal. It is almost impossible to avoid infection at the ear canal without the presence of cerumen (Hyslop, 2014). It is expected that if cerumen provides immunity, its composition should alter in response to infection and exposure to bacteria and should induce antibacterial components of the cerumen at the ear canal. However, in otitis externa, cerumen does not provide antibacterial polyunsaturated fatty acids than without any infection at the external

auditory canal (Osborne and Baty, 2016). Cerumen inhibits the growth of bacteria and fungi at various concentrations. Few studies demonstrated the antibacterial and anti-fungal properties which show its protective role toward the external auditory canal. The human earwax shows more antibacterial property in comparison to the anti-fungal property and this may be due to some protective mechanism of the fungus, leading to lower inhibition of fungal growth as compared to bacteria. In one study, authors demonstrated the bactericidal properties of cerumen on *Pseudomonas aeruginosa* (Lum *et al*., 2009). Some other studies reported that cerumen has no bactericidal effect on *P. aeruginosa*. Cerumen inhibits the growth of *Escherichia coli*, which is consistent with other study, whereas one study shown insignificant bactericidal effect and stated that *E. coli* is not a normal commensal bacterium in the external auditory canal and so may not be considered by the immune system of the ear canal. Human cerumen has antibacterial and anti-fungal properties against common bacteria and fungi as done in a study, which was in the following order: *E. coli* > *P. aeruginosa* > *Staphylococcus aureus* > *Candida albicans* (Campos *et al.*, 2000). As cerumen is secreted in the external auditory canal, it often does not come in contact with bacteria in middle-ear infection. Immunohistochemical studies of cerumen show that antibody-mediated immune reactions rather than cerumen protect the ear canal from infective microorganisms. The epidermis and dermis of the skin lining the external auditory canal contain ceruminous and sebaceous glands as well as piliary follicles which are capable of activating local immunity by immunoglobulin (Ig) A and IgG (Sirigu *et al.*, 2007). However, it needs more studies to confirm the nature of host defense in this anatomical location. There are many controversial reports in medical literature explained on the basis of culture media, methodology, and virulence of microorganisms. Microorganisms such as *S. aureus*, *P. aeruginosa*, and *C. albicans* are common microorganisms that cause otitis externa, whereas their presence in cerumen in the external auditory canal reduces the chance of infection (Lum *et al*., 2009).

There is little known about the chemical composition of the human cerumen and its antimicrobial role. Several proteins are found in the cerumen such as antimicrobial peptides of human beta defensin (hBD) 1-3, lactoferrin, LL-37, bactericidal permeability-increasing (BPI), hSLPI, and HNP1-3. All have some role to prevent bacteria and fungi infections at the external auditory canal. If this local defense system gets disturbed, infections of ear canal occur (Schwaab *et al.,* 2011). These proteins are described below.

### 2.5.1. Antimicrobial peptides

Different antimicrobial peptides from cerumen are hBD1-3, lactoferrin, LL-37, BPI, hSLPI, and HNP1-3. The antibacterial peptides in cerumen prevent bacteria and fungi from causing infections in the ear canal.

### 2.5.2. Human beta defensins

The hBD is named after beta sheet structure which is stabilized by intramolecular disulfide bonds. It has strong antimicrobial property on Gram-negative bacteria (Schneider *et al.,* 2005). hBD2 has a strong antimicrobial effect on *E. coli, P. aeruginosa*, and *C. albicans,* whereas a weak effect on *S. aureus.* hBD3 induced by tumor necrosis factor-alpha attaches with P. aeruginosa or S. aureus, for example, keratinocytes. hBD3 has antimicrobial properties against *S. aureus* including methicillin-resistant *S. aureus*, *Streptococcus pyogenes, P. aeruginosa, E. coli, Haemophilus influenzae, C. albicans*, and vancomycin-resistant *Enterococcus faecium* (Schneider *et al.,* 2005).

### 2.5.3. Human LL-37

Human LL-37 (LL-37) is a 37-aminoacid long C terminus which has active antimicrobial component of the human cathelicidin antimicrobial peptide 18 (Hcap-18). This protein is expressed on leukocytes like neutrophils, monocytes, B-cells and T-cells), epithelial cells like skin, respiratory tract and gastrointestinal tract which is secreted into the wound and surface fluid ( Wang *et al.,* 2004). It has antibacterial activity against Gram-positive and Gram-

negative bacteria. LL-37 also plays a role in angiogenesis, cancer development, and neutralization of bacterial lipopolysaccharide and chemotactic for monocytes, neutrophils, and CD4 T-lymphocytes (Von *et al.,* 2008).

### 2.5.4. Human secretory leukoprotease inhibitor

Human secretory leukoprotease inhibitor (hSLPI) is a heavy protein particle expressed on macrophages, epithelial cells, and neutrophils. It has antimicrobial properties against Gram-positive and Gram-negative bacteria at the N-terminal domain of the protein (Wahl *et al.,* 2012).

### 2.5.5. Human lactoferrin

Human lactoferrin can be seen in saliva, tears, milk, nasal mucosa, neutrophils, and granulocytes. The antibacterial spectrum of human lactoferrin includes *Streptococcus mutans, Vibrio cholera, E. coli, Actinobacillus actinomycetemcomitans, Enterobacteriaceae, Legionella pneumophila, C. albicans,* and *P. aeruginosa* (Schwaab *et al.,* 2011).

### 2.5.6. Human bactericidal permeability increasing protein

This is a single-chain cationic protein which is divided by proteolysis into two segments with antibiotic- and endotoxin-neutralizing functions in the N-terminal segment (Reichel *et al.,* 2003). BPI is mainly seen in the granules of neutrophils, dermal fibroblasts, and excretory lacrimal gland and often selective to the Gram-negative bacteria. It has significant protective effect in meningococcal infection (Von *et al.,* 2014). Cerumen has a protective antimicrobial potency, and cleaning the ear canal by rinsing with water leads to a complete elimination of the cerumen, which leads to alteration of the physiological pH. This explains why otitis externa occur after cleaning the ear canal with water. Removal of the earwax or cerumen manually by a hook and leaving some wax in the ear canal would maintain the physiological antimicrobial potency of the ear canal.

## 2.6. MICROORGANISMS ASSOCIATED WITH THE EAR

According to reports of many studies, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Proteus mirabilis*, *Klebsiella pneumoniae* and *Escherichia coli* are the common organisms isolated from cases of ear infection (Abera and Kibret 2011; Muluye *et al.*, 2013; Seid *et al.*, 2013).

### 2.6.1. OUTER EAR

The outer ear is exposed to the external environment and much like skin on other parts of the human body is in contact with microbial life. Both the auricle and the external auditory meatus house a variety of microbes under healthy conditions. The outer ear is exposed to the outside oxygen-filled environment, the majority of the bacterial flora on the auricle and in the external auditory canal is made up of aerobic species. The outer ear is home to a diverse set of microbes including bacteria, viruses, and fungi (Belkaid and Segre, 2014). The skin of the external auditory canal and auricle is predominantly occupied by Gram-positive over Gram-negative bacteria. The main Gram-positive bacteria are *staphylococci*, *coryneforms*, *streptococci* and *enterococci*, *micrococci*, and *bacillus*. Of the Gram-positive bacteria, the predominant species are *Staphylococcus auricularis*, *S. capitis* (both *capitis* and *ureolyticus*), *S. epidermidis*, *S. warnen*, *Turicella otitidis*, *Alloiococcus otitis*, *Micrococcus luteus*, and *E. coli*. Gram-negative species inhabit the auricle and skin of the external auditory meatus to a much lesser extent with *Pseudomonas aeruginosa* and *Moraxella osloensis* in relative abundance. Some fungal microbes can be found in the skin of the outer ear, but are less abundant than either Gram-positive or Gram-negative bacteria. *Candida parapsilosis*, *C. albicans*, and *Penicillium species* form the majority of fungal isolates of the ear integument (Campos *et al.*, 2000).

The skin and the cerumen present different microbial populations in the external ear. Gram-positive bacteria still dominate the bacterial flora and the species distribution is relatively

similar to that in the skin. However, the Gram-negative bacteria species are less common in the cerumen than the canal and almost non-existent in the cerumen with *Pseudomonas* present. Interestingly, fungal microbes across the board are more common in cerumen than the canal with similar species distribution (Stroman *et al.*, 2001).

*P. aeruginosa* is a Gram-negative rod-shaped bacterium with a flagellum at one pole. *P. aeruginosa* is a facultative anaerobe, but prefers aerobic respiration. This makes it well suited for life on the skin and the outer ear which is exposed to the oxygen-filled atmosphere. *P. aeruginosa* is able to utilize a wide variety of metabolites, it is also an opportunistic pathogen that causes multiple different diseases such as pneumonia, UTIs, and other skin diseases including acute diffuse otitis externa (Stroman *et al.,* 2001).

*S. epidermidis* is a Gram-positive firmicute commonly found on the skin of humans. As the name suggests *S. epidermidis* is cocci shaped and unlike *S. aureus* is nonpathogenic for the most part. *S. epidermidis* has been known to infect immune compromised individuals. Naturally a part of the normal human skin flora it is no surprise that *S. epidermidis* is found in the outer ear due to the similarity in environments between the auricle and the rest of the human skin (Belkaid and Segre, 2014).

*S. auricularis* is a Gram-positive firmicute also found in the outer ear. *S. auricularis* is a cocci shaped microbe that is nonpathogenic and is a part of the normal ear flora (Stroman *et al.*, 2001).

*Penicillium chrysogenum* is a fungal microbe that grows in damp environments and produces beta-lactam antibiotics, which is the active ingredient in penicillin. This microbe may contribute to the antibiotic properties of cerumen and help the human body keep ear infections at bay (Ho *et al.,* 2006).

*Aspergillus niger & Candida albicans* are fungal microbes that causes symptoms of pain, hearing loss, aural fullness, and itching. In extreme cases otomycosis can permanently

damage the ear canal and the tympanic membrane leading to hearing loss. *C. albicans*, like *P. aeruginosa*, is present in normal outer ear microbiota. *C. albicans* is also an opportunistic pathogen, attacking immune compromised individuals. Most of the *C. ablicans* in the human body resides in the human gut, but *C. albicans* is also found in the exterior of the human ear. Otomycosis is treatable with the usage of antifungal drops into the ear (Ho *et al.*, 2006).

### 2.6.2. MIDDLE EAR

Although the middle ear is segregated from the external portion of the ear via the tympanic membrane, the middle ear is connected to the nasopharynx by the way of the Eustachian tube. In this way the middle ear is somewhat in contact with the external environment. However, bacteria would still need to travel through the nasal cavity and up the Eustachian tube which is no easy task. The mucous and cilia in the nasal cavity function to trap and expel foreign particles including bacteria that may travel up to the middle ear (Dahl and Mygind, 2015). That being said microbes are present in the middle ear. For instance alpha hemolytic *streptococci* are present in a healthy middle ear. Alpha hemolytic *streptococci* are known to inhabit the middle ear. There is a possibility that the alpha hemolytic *streptococci* crowd out other bacteria and prevent middle ear infections. Lower incidence of alpha hemolytic streptococci has been observed in children with recurrent middle ear infections. This is of course a promising path to effective treatment of ear infections. Middle ear infections are termed otitis media. Since the middle ear is not directly accessible by bacteria from the external environment, bacteria must either travel up the Eustachian tube or through a perforated tympanic membrane in order to access the middle ear. (Belkaid and Segre, 2014). Some causes of otitis media are caused by bacteria found in the outer ear. These bacteria are facultative anaerobes which makes them well-suited for the tympanic cavity environment. They access the middle ear via the Eustachian tube connecting to the nasopharynx.

*S. pneumoniae* are lancet-shaped, gram-positive, facultative anaerobic bacteria with more than 100 known serotypes. Most S. pneumoniae serotypes can cause disease, but only a minority of serotypes produce the majority of pneumococcal infections.

*H. influenzae* is a gram-negative, non-motile, coccobacillary, facultatively anaerobic, pathogenic bacterium of the family Pasteurellaceae. The bacteria are mesophilic and grow best at temperatures between 35-37$^0$C.

*Moraxella catarrhalis* is a fastidious, nonmotile, Gram-negative, aerobic, oxidase-positive diplococcus that can cause infections of the respiratory system, middle ear, eye, central nervous system, and joints of humans. It causes the infection of the host cell by sticking to the host cell using trimeric autotransporter adhesins.

*Alloiococcus otitis and Turicella otitidis* are commensal in the external auditory meatus yet possibly pathogenic in the middle ear. *A. otitis* is a specie of bacteria first isolated from human middle-ear fluid, the type species of its monotypic genus and *T. otitidis* is a non-fermenting Gram-positive bacillus isolated almost exclusively from ear exudates. Its significance in acute or chronic otitis media is controversial (Belkaid and Segre, 2014).

### 2.6.3. INNER EAR

Unlike the outer and middle ear, the inner ear is completely secluded from the outside environment by the bony labyrinth that encases the cochlea, semicircular canals, and vestibule. This makes it much more difficult for bacteria to translocate across the oval window. The immune system is more prevalent in the inner ear which also reduces the possibility of microbial entry into the inner ear (Alberti, 2017). There is no access to the external environment via the external auditory canal and Eustachian tube for the outer and middle ear respectively. In most cases the cause of inner ear infections are usually viral. The two main inner ear maladies are vestibular neuritis and Labyrinthitis. Both of these conditions affect the balance of an individual and cause vertigo and sometimes nausea. Both vestibular

neuritis and Labyrinthitis onset are preceded by an upper respiratory infection such as the common cold, herpes simplex virus, or flu (Marill, 2011).

## 2.7. *Staphylococcus aureus*

*Staphylococcus aureus* is a Gram-positive bacterium and causative agent of wide range of infectious diseases such as skin infections, bacteremia, endocarditis, pneumonia and food poisoning. The organism was originally a leading nosocomial pathogen and afterwards epidemiologically distinct clones emerged in community settings. *S. aureus* expresses number of virulence factors which help to establish infection by facilitating tissue attachment, tissue invasion and evading from host immune response. The ability to acquire resistance to multiple antibiotics classes makes *S. aureus*, a challenging pathogen to treat. Emergence and spread of *S. aureus* strains which are resistant to methicillin, referred to as methicillin-resistant *S. aureus* (MRSA) resulted in high morbidity, high mortality and increased treatment costs. Vancomycin remained gold standard drug to tackle these strains for years but the emergence of resistance restricted its clinical utility. Newer anti-MRSA antibiotics which were approved by U.S. FDA came as respite for clinicians. However, new antibiotic discovery efforts and non- antibiotic approaches to tackle MRSA should not be diminished considering the ability of the pathogen to acquire resistance to newer drugs quickly after their introduction in clinics (Licitra, 2013).

### 2.7.1. Microscopic morphology of *S. aureus*

S. aureus cells are Gram-positive and appear in spherical shape. They are often in clusters resembling bunch of grapes when observed under light microscope after Gram staining. The name ‘Staphylococcus’ was derived from Greek, meaning bunch of grapes (staphyle) and berry (kokkos) (Licitra, 2013). The scanning electron microscopic observation reveals roughly spherical shaped cells with smooth surface. The diameter of the cells ranges from 0.5

to 1.0 μM. The transmission electron microscopy of cells shows thick cells wall, distinctive cytoplasmic membrane and amorphous cytoplasm (Licitra, 2013).

### 2.7.2. General cultural and biochemical characteristics of *S. aureus*

S. aureus is an aerobic and facultative anaerobic organism that forms fairly large yellow or white colonies on nutrient rich agar media. The yellow colour of the colonies is imparted by carotenoids produced by the organism. The term 'aureus' is derived by Cy from Latin, which refers to the colour of gold. The organism is often haemolytic in blood agar due to production of four types of haemolysins (alpha, beta, gamma and delta). Nearly all isolates of S. aureus produce coagulase enzyme, a virulence factor that also helps in identification of the organism. The organism is salt tolerant, which is able to grow in mannitol-salt agar medium containing 7.5% sodium chloride. The organism is catalase positive and oxidase negative (Liu *et al.,* 2005).

### 2.7.3. Medical laboratory diagnosis of *S. aureus*

The primary objective in laboratory diagnosis is to identify whether the diagnosed *S. aureus* isolate is methicillin resistant. Since MRSA emerged as problematic pathogen, a systematic diagnostic approach is necessary for early diagnosis so that treatment with appropriate antibiotics can be initiated as early as possible. For the species identification, slide and tube coagulase tests, latex agglutination tests and PCR-based tests are used. For detection of MRSA, determination of minimum inhibitory concentration (MIC) of methicillin or oxacillin or cefoxitin using broth micro-dilution method, cefoxitin disk screen, oxacillin agar screen and latex agglutination test for PBP2a and molecular methods for detection of mecA are employed (Tong *et al.,* 2015).

### 2.7.4. General pathogenesis and clinical diseases of *S. aureus*

The process of S. aureus infections involves five stages. They are (1) colonization, (2) local infection, (3) systemic dissemination and/or sepsis, (4) metastatic infections and (5) toxinosis.

The organism is in carrier state in the anterior nares and can remain so without causing infections for weeks or months. The colonization proceeds to infection under certain predisposing factors such as prolonged hospitalization, immune suppression, surgeries, use of invasive medical devices and chronic metabolic diseases. Localized skin abscess develop when the organism is inoculated into the skin from a site of carriage. This can further spread and results in various clinical manifestations of localized infections such as carbuncle, cellulitis, and impetigo bullosa or wound infection. The organism can enter into blood and spread systemically to different organs causing sepsis. This haematogenous spread may result in endocarditis, osteomyelitis, renal carbuncle, septic arthritis and epidural abscess. Without a blood stream infection, specific syndromes can occur due to extra cellular toxins of *S. aureus*. These are toxic shock syndrome, scalded skin syndrome and foot borne gastroenteritis (Tong *et al.*, 2015).

*S. aureus* causes wide range of infections in human. The clinical infections of *S. aureus* are classified into community and nosocomial categories based on origin of infection. These two types are distinct in clinical manifestations of the infections, antibiotic susceptibility and the genetic background of the infecting *S. aureus* strains. For decades, *S. aureus* has been predominately a nosocomial pathogen and is a leading cause of mortality and morbidity in hospitals. However, the community S. aureus infections are in rise. The important clinical *S. aureus* infections are bacteraemia, infective endocarditis, skin and soft tissue infections, osteoarticular infections and pleuropulmonary infections. Other clinical infections are epidural abscess, meningitis, toxic shock syndrome and urinary tract infections (Tong *et al.*, 2015).

### 2.7.5. Virulence factors of *S. aureus*

*S. aureus* possess battery of virulence factors. These factors enable the organism to be successful as pathogen that causes wide range of human and animal infections. Virulence

factors help in attachment to host cells, breaking down the host immune shield, tissue invasion, causing sepsis and elicit toxin-mediated syndromes. This is the basis for persistent staphylococcal infections without strong host immune response (Tong *et al.*, 2015). The virulence factors of Staphylococcus aureus includes; Antigens (Capsule, Adhesins), Enzymes (Coagulase, Lipase, Hyaluronidase, Staphylokinase, Nuclease), Toxins (Alpha (α )–Toxin, Beta (β) –Toxin, Gamma (γ)-Toxin, Delta (δ)-Toxin, Leukocidin, Enterotoxin, Exfoliative Toxin,Toxic Shock Syndrome Toxin).

**2.7.5.1. Antigens**

**Capsule:** Most strains of *S. aureus* have capsules (slime layer- polysaccharide). *S. aureus* capsular antigens are surface-associated, limited in antigenic specificity, and highly conserved among clinical isolates. Capsule inhibits phagocytosis, promotes adherence of the organism to host cells and in prosthetic devices and serves as a diffusion barrier (O'Riordan and Lee, 2004).

**Adherence Factors (Adhesins):** The attachment of *S. aureus* to the host cell surface initiating the colonization process is mediated by several adhesins. One major class of *S. aureus* adhesins comprises proteins covalently anchored to cell peptidoglycan (via the threonine residue in the sorting signal motif at their C-terminus), which specifically attach to the plasma or extracellular matrix (ECM) components and collectively are termed the microbial surface component recognizing adhesive matrix molecules (MSCRAMMs). These molecules recognize the most prominent components of the ECM or blood plasma, including fibrinogen, fibronectin, and collagens. Typical members of the MSCRAMM family are staphylococcal protein A (SpA), fibronectin-binding proteins A and B (FnbpA and FnbpB), collagen-binding protein, and clumping factor (Clf) A and B proteins (Chambers and DeLeo, 2009).

**Teichoic acid:** Teichoic acids are major constituents of Staphylococcus aureus. There are two types of teichoic acid (TAs); Lipo-Teichoic Acid (LTA): anchored in the cytoplasmic membrane and cell wall Teichoic Acid (WTA): covalently linked to peptidoglycan in the bacterial cell wall. Teichoic acids contribute to staphylococcal adhesion and colonization, cell division, and biofilm formation. Over expression of teichoic acid increases the virulence of *S. aureus*. In addition, D-alanine (D-Ala) residues on teichoic acids contribute to resistance to cationic antimicrobial peptides such as defensins or cathelicidins, and to glycopeptide antibiotics such as vancomycin or teicoplanin (Mistretta *et al.,* 2019).

**2.7.5.2. Enzymes**

**Coagulase:** Activates a coagulase reacting factor (CRF) normally present in plasma, causing the plasma to clot by conversion of fibrinogen to fibrin may act to coat the bacterial cells with fibrin making them resistant to opsonization and phagocytosis can be detected by tube coagulase test.

**Lipase:** Degrades lipids.

**Proteases:** Cleave and Degrade host proteins.

**Staphylokinase:** Binds to plasminogen and activates it to the fibrinolytic enzyme plasmin. Antigenically and enzymatically distinct from that produced by Streptococcus**.** It breaks fibrin clot and allow the spread of infection to contiguous tissues.

**Hyaluronidase**: Aids in invasiveness by breakdown of Hyaluronate rich tissue barriers (accounts for persistence of *Staphylococcus* in tissues) thus facilitating the spread of the organism to adjacent areas (O'Riordan and Lee, 2004).

**2.7.5.3. Toxins**

**α-Toxin (α-Hemolysin):** Alpha toxin is the major cytotoxic agent released by *S. aureus,* and it was the first bacterial exotoxin to be identified as a pore former. Pore formation on susceptible host cell membranes triggers alterations in ion gradients, loss of membrane

integrity, activation of stress-signalling pathways, and cell death. *S. aureus* α-toxin is known to play an important role in the pathogenesis of staphylococcal diseases, as *S. aureus* mutants lacking hla display reduced virulence in invasive disease models. α-toxin possesses additional biological functions such as binding to a putative glycoprotein receptor on host cells, activation of intracellular signalling, and modulation of several processes (Wardenburg and Schneewind, 2008).

**β-Toxin (β-Hemolysin):** Among *S. aureus* toxins, the least is known about the function of beta toxin in pneumonia and lung injury. Based on literature data, *S. aureus* β-toxin is a Mg2+-dependent neutral sphingomyelinase that hydrolyzes sphingomyelin of the host cell plasma membrane to generate phosphocholine and the bioactive secondary messenger, ceramide. Depending on the chain length of their fatty acids or the mode of metabolism, these ceramides may have a number of effects in eukaryotic cells, including stimulation of second messenger systems, activation of mitogen-activated protein kinases (MAPKs), changes in cell shape, and even apoptosis (Wardenburg and Schneewind, 2008). Beta toxin does not lyse most types of host cells but leaves them susceptible to a number of other lytic agents, such as α-toxin and leukocidin. In fact, the cytotoxic effect of β-toxin is cell type-specific and species-specific, suggesting that its primary virulence activity is to modulate host processes that affect pathogenesis, rather than to directly kill host cells (Hayashida *et al.*, 2009).

**γ-Toxin (γ-Hemolysin):** Gamma toxin is a bicomponent pore-forming toxin composed of LukF and Hlg2. These proteins are expressed as water-soluble monomers and then assemble into the oligomeric pore form on the target cel

**δ-Toxin (δ-Hemolysin):** It is among other toxins produced by *S. aureus* and is part of the phenol-soluble modulin peptide family. It has a wide spectrum of cytolytic activity. Its alpha-helical, amphipathic structure gives it detergent-like properties, allowing it to disrupt and

attach to the cytoplasmic membrane of a cell non-specifically, without a receptor, and integrate into the membrane. Delta toxin degrades the membrane on contact and forms short-lived pores, causing cell lysis and subsequent cell death.

**Leukocidin:** Also called Panton-Valentine leukocidin (PVL) is a cytotoxin, one of the β-pore-forming toxins. It damages polymorphonuclear leucocytes and necrosis.

**Enterotoxin:** Pyrogenic toxin Super-antigen and heat stable. Enterotoxin is responsible for Staphylococcal food poisoning. It acts by increasing intestinal peristalsis by increased vagal stimulation. Once formed enterotoxin is not destroyed even if food is heated sufficiently to kill all viable Staphylococci (Mistretta *et al.,* 2019).

**Exfoliative Toxin:** Has Super-antigen activity. Two forms of epidermolytic toxins (ETA and ETB) of *S. aureus* split human skin at a site in the upper epidermis. ET A (thermostable), ET B (heat labile). It is a serine protease which causes splitting of desmosomes or intercellular bridges in stratum granulosum and intraepidermal blistering leading to SSSS (Staphylococcal Scalded Skin Syndrome) in which the outer layer of epidermis gets separated from the underlying tissue. Severe form of the SSSS is known as Ritter's disease in the new born while milder forms are pemphigus neonatorum and bullous impetigo. Clinical effects are most common in infants (Mistretta *et al.,* 2019).

**Toxic Shock Syndrome Toxin:** It is also a Super-antigen, leading to a systemic release of a variety of cytokines which is the cause of multi system involvement in TSS. Major roles of TSST-1 includes; Induce cytokine release from macrophage and T lymphocytes, capable of enhancing the toxic effects of endogenous endotoxin, produce leakage of endothelial cells and penetrate mucosal barrier. Staphylococcal superantigen is responsible for almost all menstrual toxic shock syndrome cases (Mistretta *et al.,* 2019).

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.0 MATERIALS

Autoclave, hot air oven, light microscope, spatulas, pippetes, sterile cotton wool swab, inoculating loop, incubator, forceps, petri dishes, slides and cover slip, test tubes, flasks, Whatman filter paper, single antibiotic (methicillin) disc, Nutrient agar, peptone water, Mueller-Hinton agar; reagents for Gram stain reaction, capsular, oxidase, catalase, motility, citrate, coagulase, indole, manitol glucose, sucrose and lactose fermentation tests;

## 3.1 STERILIZATION OF GLASS WARES AND APPARATUS

All glasswares were properly washed and rinsed with clean water. The methods of sterilization adopted were the use of the autoclave at 121$^{o}$C for 15 minutes and hot air oven at 160$^{0}$C for 2 hours. The inoculating loop was sterilized using the flame from a Bunsen burner. The flaming of the wire loop was repeatedly done at the end of every inoculation. The table top was disinfected using eth  l using an autoclave.

## 3.2 PREPARATION OF MEDIA

### 3.2.1 Preparation of Nutrient Agar

This was done by weighing 28.0g of dehydrated nutrient agar powder using a chemical balance which was poured into a conical flask containing 1000ml of distilled water. The mixture was stirred with sterile spatula and placed over a Bunsen burner flame on a tripod stand to fully dissolve the entire powdered agar. The dissolved mixture in the flask was covered with cotton wool and aluminium foil and placed in an autoclave and was sterilized at 121$^{o}$C for 15 minutes. Once the sterilization was complete the autoclave was allowed to cool and the molten agar was brought out and allowed to cool to about 45$^{o}$C.

### 3.2.2 Preparation of Mueller-Hinton agar

This was done by weighing 16.8g of Mueller-Hinton agar base powder with a chemical balance and transferred into a conical flask containing 500ml of distilled water. The mixture was stirred with sterile spatula and placed over a Bunsen burner flame on a tripod stand to fully dissolve the entire powdered agar. The dissolved mixture in the flask was covered with cotton wool and aluminium foil and placed in an autoclave and was sterilized at 121$^{o}$C for 15 minutes.

## 3.3 COLLECTION OF SAMPLES

Using sterile cotton wool swabs, samples were taken from the hands, arms and face of 8 persons, transferred into duly labeled sterile universal bottles containing peptone water, transported to the laboratory and incubated at 30$^{0}$C for 24 hours.

## 3.4 ISOLATION AND CHARACTERIZATION OF SKIN BACTERIA

Using sterile inoculating loop, samples were taken from the incubated peptone water and streaked on fresh agar plates containing actidione (antifungal agent). Colonies from the nutrient agar plates were purified by repeated subculturing on fresh nutrient agar plates. Using sterile loop, the purified isolates were transferred onto agar slants for storage in the refrigerator until required. Purified isolates from overnight broth culture were characterized by cultural characteristics, Gram's, capsule, motility test and biochemical tests. The isolates were identified by comparing their characteristics with those of known taxa, as described by (Benneth and Lancette, 2001 and Harley, 2011).

### 3.4.1 Gram Staining

Test organisms were heat-fixed on slides and flooded with crystal violet for about 60 seconds and rinsed with water for about 5 seconds. The slides were then flooded with iodine solution for about 60 seconds and rinsed with water. Ethanol was then added as a decolourizer and rinsed with water afterwards. Finally, the slides were flooded with saffranin for about 60

seconds, rinsed with water, blotted dry, and viewed under a microscope. Gram-positive organisms appeared blue/purple under the microscope while Gram-negative organisms appeared red/pink. The cell shapes were also viewed under this procedure.

### 3.4.2 Capsule Stain

Slide smears of the bacterial isolates were placed on a beaker of boiling water and the smear covered with 1.0% aqueous solution of crystal violet for one minute. After which they were washed with 20% copper sulphate solution, blot dry and viewed under oil immersion of microscope. Capsules appear as faint blue-violet zone surrounding purple bacterial cell.

### 3.4.3 Motility Test

A wire loop was used to inoculate motility medium with the isolates by making a stab to the bottom of the tube and afterwards incubated 37$^{o}$C for 24-48 hours. If the organism is motile, the tube will appear cloudy and the organisms will spread out of the stab line. Non-motile organisms will grow along the streak line only and the media will not be cloudy.

### 3.4.4 Oxidase Test

Fresh growth of isolates was removed from the agar plate using a sterile plastic inoculating loop or a sterile swab. The oxidase test strip is moistened slightly with oxidase reagent and the growths were rubbed into the moistened paper of the strip. If the microbe has cytochrome oxidase, it will add electrons to the reagent, changing it from its colourless appearance to a deep indigo blue in a matter of 10-20 seconds. If the colour does not turn blue within 20 seconds, the test is negative for oxidase.

### 3.4.5 Coagulase Test

A drop of sterile distilled water was placed on each end of a sterile slide. With the aid of sterile loop cultures of the test organisms were emulsified on each spot to make two thick suspensions. A loop-full of plasma was added to one of the suspensions and mixed gently.

The slide was examined for clumping or clotting of the organisms within 10 seconds. Plasma was not added to the second suspension, which served as control.

### 3.4.6 Citrate Utilization Test

Cultures of the test organisms were aseptically inoculated into sterile slants of Simmons's citrate agar in capped tubes, and incubated at $37^0$C for 3-4 days. A positive result is indicated by change in medium colour from green to blue.

### 3.4.7 Urease Test

Urea broth with pH indicator, phenol red was inoculated with test organisms and incubated at $37^0$C for 2-5 days. Intense pink/red colour indicates a positive test and yellow or no colour change indicates a negative result. Uninoculated urea broth tubes were kept as control.

### 3.4.8 Indole Test

Test organisms were inoculated into tryptophan broth and incubated $37^0$C for 24 hours. Drops of Kovacs reagent were then added to the broth. The formation of a red ring at the surface of the broth signified a positive result.

### 3.4.9 Catalase Test

Two drops of hydrogen peroxide were placed on the surfaces of clean grease free glass slides A and B (control). Using a clean glass rod the test organisms were transferred to A; and gas bubbling or effervescence indicates a positive reaction.

### 3.4.10 Sugar fermentation test (glucose, lactose sucrose and manitol)

The basal medium used was peptone water, and sterilized water. A drop of phenol red was added as pH indicator to 1.0% of sugar prepared in 99ml of peptone water and sterilized in the autoclave at $121^0$C for 15minutes. A pure culture of the test organism was inoculated into the sterile solution of sugar and incubated at $37^0$C for 48-72 hours. At the end of the incubation, the colour formed due to acid production was yellow and gas production in the Durham tube immersed in the medium indicates a positive result.

### 3.5 CONFIRMATION OF *Staphylococcus aureus*

Using sterile inoculating loop, overnight broth culture of suspected *Staphylococcus aureus* was streaked on fresh nutrient agar plates and incubated at $35^{O}C$ for 24 hours(Baird-Parker *et al*;2000). Colonies from the nutrient agar plates were subjected to tube coagulase test. Those colonies which show four (4) positive coagulase test reaction were confirmed as *Staphylococcus aureus* (Benneth and Lancette, 2001).The colonies with between 1-3 coagulase positive reactions require additional tests for confirmation (Scabo, 2000). These colonies were subjected to Gram stain reaction, catalase production test and anaerobic utilization of manitol test. The colonies which appeared as Gram positive cocci produce catalase and utilize manitol were confirmed as *Staphylococcus aureus.* All isolates were inoculated unto agar slants and stored in the refrigerator until required.

### 3.6 COLLECTION OF CERUMEN

Earwax or cerumen was collected from male and female healthy persons in and outside the polytechnic. This was collected using sterile ear buds, pooled, weighed to obtain 10g and stored in refrigerator until required.

### 3.7 DETERMINATION OF THE ANTIMICROBIAL ACTIVITY OF CERUMEN

To determine antimicrobial activity of cerumen on the test organism, *Staphylococcus aureus* the disc diffusion method of Ambika *et al* (2015) was used. Cerumen was weighed as 1g, 2g, 3g and 4g amounts and then dissolved in separate 100ml tubes containing sterile buffer (pH 8.2) made by mixing $NaHCO_3$ (5%) and glycerol (30%) to give 10, 20, 30, and 40mg/ml concentrations. Small discs made from Whatman filter papers with a punching machine were sterilized by autoclaving and soaked in the cerumen buffer (pH 8.2) of the different concentrations. Muller Hinton agar was prepared, autoclaved, poured in to sterile Petri plates. After solidification plates were labeled as corresponding to the cerumen concentrations. On the surface of the medium overnight broth culture of *Staphylococcus*

*aureus* was spread as a thick layer using sterile cotton bud.

The soaked discs were gently placed on the surface inoculated plates with a sterile forceps. As negative control, buffer soaked discs (without cerumen) and as positive control, commercially available single antibiotic disc of methicillin (1.0µg) was aseptically placed on the surface of the inoculated agar plates with sterile forceps'. After incubation at 37°C for 24hrs, clear zones were measured and zones of inhibition of the cerumen calculated and compared with that of the antibiotic.

# CHAPTER FOUR

# RESULTS AND DISCUSSION

## 4.1 RESULTS

Thirty- six (36) bacterial strains were isolated from skin and identified as *Escherichia coli*; *Klebsiella* species; *Staphylococcus aureus*, *Proteus* species; *Staphylococcus* species and *Pseudomonas* species (Table 1).The frequency of isolation from the skin are *Staphylococcus* species (36.1%) *Escherichia coli* (11.1%), *Klebsiella* species (08.3%), *Proteus* species (05.6%), *Pseudomonas* species (22.2%) and *Staphylococcus aureus* (16.7)(Table 2).

Based on the zones of inhibition, the various concentrations of cerumen were more active on *Staphylococcus aureus* than 1.0µg of methicillin and the activity increases with concentration. The zones of inhibition of 10, 20, 30, and 40mg/ml of cerumen are 3.0, 8.0, 11.0 and 14.0mm respectively. The zone of inhibition of methicillin (1.0µg) is 2.0mm (Table 3).

**Table 1: Characterization of bacteria isolated from human skin**

| **Tests** | **Response of isolates to test** | | | | | |
|---|---|---|---|---|---|---|
| | A | **B** | **C** | **D** | **E** | **F** |
| **Gram stain** | - rod | - rod | - rod | + cocci | -rod | + cocci |
| **Capsule stain** | - | + | - | - | - | - |
| **Oxidase** | - | - | + | - | - | - |
| **Catalase** | + | + | + | + | + | + |
| **Urease** | - | - | - | - | + | - |
| **Citrate** | - | + | + | - | + | - |
| **Motility** | + | - | + | - | + | - |
| **Indole** | + | - | - | + | - | + |
| **Glucose** | A &G | A &G | + | A | A &G | - |
| **Sucrose** | A &G | A &G | - | A | A &G | - |
| **Lactose** | A &G | A &G | - | A | - | - |
| **Tentative identification** | ***E.coli*** | ***Klebsiella*** sp | ***Pseudomonas*** sp | ***Staphylococcus aureus*** | ***Proteus sp*** | ***Staphylococcus sp***. |

A = acid G = gas + = Positive - = negative

**Table 2; Frequency of isolation of bacteria from the skin**

| **Bacteria** | **No. of isolates** | **Percentage (%)** |
|---|---|---|
| *Staphylococcus* species | 13 | 36.1 |
| *Escherichia coli* | 04 | 11.1 |
| *Klebsiella* species | 03 | 08.3 |
| *Pseudomonas* species | 08 | 22.2 |
| *Staphylococcus aureus* | 06 | 16,7 |
| *Proteus* species | 02 | 05.6 |
| **Total** | **36** | **100** |

**Table 3: Antimicrobial activity of cerumen on *Staphylococcus aureus***

| Cerumen (mg/ml) | Diameter of disc **D** (mm) | Diameter of zone **d (**mm) | Zone of inhibition **D – d (**mm) |
|---|---|---|---|
| B | 5.0 | no zone | 0 |
| 10 | 5.0 | 8 | 3.0 |
| 20 | 5.0 | 13 | 8.0 |
| 30 | 5.0 | 16 | 11.0 |
| 40 | 5.0 | 20 | 14.0 |
| Methcillin | 6.0 | 8 | 2.0 |

B = buffer without cerumen

## 4.2 DISCUSSION

This study revealed that the human skin is a dwelling place for microorganisms including bacteria. The bacteria isolated in this study were *E.coli*, *Klebsiella* sp; *Staphylococcus* sp; *Proteus* sp; *Staphylococcus aureus*, and *Pseudomonas* sp. They are similar to the ones reported by previous workers (Grice, 2011 and Kumar and Chordia, 2017). It is also a known fact that these organisms are a leading cause of skin and ear infections (Campos *et al*; 1998 and Naqi, 2016).

In this study, *Staphylococcus* species (36.1%) and *Staphylococcus aureus* (16.7%) were the most frequent bacteria isolated from skin. According to Kumar and Chordia, (2017); the primary colonizers of human skin are *Staphylococcus epidermidis* and other coagulase – negative Staphylococci.

*Staphylococcus aureus* is a ubiquitous organism occurring in the air, dust, in water and on the skin and mucous membranes of most warm-blooded animals including all food animals and humans. It is part of the bacterial flora indigenous to humans and it possesses a resistant cell wall with a cross-linked peptidoglycan layer that may protect the cell from the host's defenses (Schmitt *et al*; 1990). In humans, the main reservoirs of these organisms are the skin and nasal cavity (Le Loir and Gautier, 2003) and approximately 50% of humans are carriers of this organism. Methicillin resistant *Staphylococcus aureus* (MRSA) strains have been implicated in many human infections. . Though, there is no link between methicillin resistance and virulence in *Staphylococcus aureus*; and there seem to be no correlation between the antibiotic resistance and enterotoxin production (Klaske, 2007). However, MRSA infections and intoxications are more difficult to treat and dangerous because of their resistance to commonly used antibiotics.

The detection of coliform bacteria, *E.coli* and *Klebsiella* sp in is of public health concern because they are indicators of faecal contamination and their presence may indicate the likely presence of other pathogenic organisms.

*Pseudomonas aeruginosa*, which occur in soil, vegetation, and surface of plants, animals and humans (Field, 2002) is a versatile bacterium with minimum nutritional requirements; and it is resistant to many antibiotics and are the major cause of nosocomial infections in many hospitals (Yetkin *et al*; 2006).

In this study, cerumen was more active on *Staphylococcus aureus* than 1.0µg of methicillin and the activity increases with concentration. This is similar to the observation of Ambika *et al* (2015); that cerumen showed appreciable activity against methicillin resistant *Staphylococcus aureus* (MRSA) strains. Efficacy of antimicrobial property of human cerumen has been a subject of debate for many years. Some authors believe that absence of cerumen mediates an alkaline environment which promotes growth of different microbes (Baumann, 2015). Role of cerumen in creating a physical barrier between external and internal ear has also been given. When cerumen is removed, this barrier will be lost, and results in bacterial growth, leading to infections (Stone and Fulghum; 2018). However, there is no concrete evidence to support this view. The role of common resident flora in the region might also play a role which again questions the as antimicrobial. In other studies, cerumen has been shown to have significant antibacterial properties.

This study demonstrated that cerumen has antibacterial properties, which play a role in the protection of the ear canal because *Staphylococcus aureus*, *P. aeruginosa* and *E. coli* are common pathogens which cause otitis external, and the presence of cerumen in the ear canal may reduce the likelihood of infection by these microorganisms( Lum *et al*; 2009). Interestingly, Sokolov *et al. (*2017) proved that the cerumen of some mammals possess antistaphylococcal, antimicrococcal and antiherpes activities.

Burtenshaw (2014) reported inconsistent bactericidal activity of cerumen against *Staphylococcus aureus*. Stone and Fulghum (2018) and Ambika *et al* (2015) reported a significant bactericidal activity similar to this study.

# CHAPTER FIVE

# CONCLUSION AND RECOMMENDATIONS

## 5.1 Conclusion

The major findings of this present work showed that the skin do not only harbour normal flora but also pathogenic organisms like *Staphylococcus aureus* and the recalcitrant MRSA, which are difficult to control. The implication of this is that these organisms may find their way into the ear to cause chronic ear infections.

The findings of this study also showed that apart from being a physical barrier, cerumen acts as protective coating over the external auditory canal due to its antibacterial properties.

## 5.2 Recommendations

In the light of the findings of this project work, I thus recommend that;

1. Routine wax removal and ear cleaning should not be mandatory unless impacted wax is leading to earache or conductive hearing loss. When cerumen is removed, the protective barrier will be lost, and results in bacterial growth, leading to ear infections (Stone and Fulghum; 2018).
2. Contaminated cotton buds should not be used for the removal of impacted cerumen from the ear because it may transmit skin pathogenic organisms like *Staphylococcus aureus* into the ear. Previous research have showed that *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* are the common pathogens which cause ear infections Ambika *et al* (2015).
3. Future research should be carried out to determine the minimum inhibitory concentration (MIC) of cerumen against *Staphylococcus aureus* isolated from skin. This is necessary because the basic parameter for the determination of

antimicrobial agents with antimicrobial potential is the minimum inhibitory concentration (Ajiboye and Olawoyin, 2020).

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**APPENDIX**

**Composition of Media**

**Nutrient agar**

Peptone……...5.0g

Beef extract…….3.0

Agar…… 15.0g

Distilled water……1 litre

**Mueller-Hinton agar**

Beef infusion……30.0g

Casein hydrolysate……1.75g

Starch……0.15g

Agar…….1.70g

Distilled water…….1.0 litre

$P^H$…….Neutral