Chapter One

Introduction

1.1 Background to the Study

Antimicrobial resistance is one of the major challenges facing global public health[1]. To ensure that the effectiveness of antibiotics is preserved to successfully treat infections caused by resistant bacteria, alternative approaches are required that can be used instead of antibiotics or after they have failed. One possible alternative currently being investigated for some applications is honey. The discovery and development of alternative therapeutic strategies that present novel avenues against resistant bacteria infections are increasingly demanded and gaining more attention.

According to epidemiological research, about 700,000 individuals die each year as a result of antibiotic-resistant bacterial illnesses. The overall resistance of *P. aeruginosa* isolated from European populations was 12.9%[2]. The World Health Organization (WHO) has recently listed carbapenem-resistant *Pseudomonas aeruginosa* as one of three bacterial species in which there is a critical need for the development of new antibiotics to treat infections. There is an urgent call for the development of new antibiotics or alternative therapeutic strategies for treatment of *P. aeruginosa* infections for the patients whose infections are resistant to conventional antibiotics. *Pseudomonas aeruginosa* is a gram negative motile ubiquitous bacterium most frequently isolated in wounds, infected burn injuries, community acquired and ventilator-associated pneumonia, and is an important opportunistic pathogen in the healthcare system known to cause nosocomial infections notorious for antimicrobial resistance[3].

Pseudomonas aeruginosa contains *gshA* and *gshB* genes, which encode enzymes involved in glutathione biosynthesis which is important in biofilm formation, bacteria virulence and stress protection[4]. The expressions of these genes have been shown to increase in the presence of oxidative stress due to hydrogen peroxide and superoxide as well other hydroperoxides[2]. Glutathione plays a primary protective role in the detoxification of these oxidative stresses.

Honey has been in use for ages in the treatment of infections ranging from its traditional use in the treatment of eye diseases, bronchial asthma, throat infections, tuberculosis, thirst, hiccups, fatigue, dizziness, hepatitis, constipation, worm infestation, piles, eczema, healing of ulcers, and wounds and used as a nutritious supplement. It has been widely researched to be effective in the control and treatment of wounds, diabetes mellitus, cancer, asthma, and also cardiovascular, neurological, and gastrointestinal diseases[5]. Honey has been found to have antibacterial properties against a wide range of bacteria species[6]. Hydrogen peroxide is the major contributor to the antimicrobial activity of most honey types. Concentrations of hydrogen peroxide in different honey types result in their varying antimicrobial effects[5].

Much has been known about the inhibitory and bactericidal effects of honey and antimicrobial effects of honey from different parts of Nigeria have been tested against some common bacteria isolated from clinical and environmental samples[7,8]. However, little is known about the resistance and virulent genes associated with honey therapy in Nigeria. *Pseudomonas aeruginosa* is one of the most common pathogens in wounds, infected burn injuries, community acquired and ventilator-associated pneumonia, and is an important opportunistic pathogen in the healthcare system known to cause nosocomial infections. It is well known that an ancient honey known has Manuka honey has an excellent effect on various strains of commonly isolated pathogenic bacteria including

multi drug resistant *Pseudomonas aeruginosa* and it has potentials to inhibit and eliminate biofilms produced by it[9], but weak antibacterial activity and resistance of *Pseudomonas aeruginosa* to honey from different parts of Nigeria have been reported in different studies[10,11,12].

There are two honey types that can be found in Nigeria, one is the sweet honey commonly known around the world. Another type recently becoming popular among the natives because of its medicinal effects is known as the bitter honey, so called because of its bitter taste. These honeys have been used not only as food additives but for its medicinal effects in the treatment of ailment.

1.2 Statement of the Problem

The constant increase in the resistance of bacteria to antibiotic drugs has given rise to the shift to natural products with less side effects for the treatment of infections and illnesses caused by pathogenic agents. Since antibiotic resistance has greatly increased over the past few decades, investigation into *P. aeruginosa's* resistance mechanism has become vital. Despite the great interest, the variability and complexity of drug resistance as well as the paucity of a thorough understanding of *P. aeruginosa's* pathogenic mechanisms cause the development of new antibiotics and other therapeutic strategies for *P. aeruginosa* infections to proceed at a painfully slow pace. Designing efficient treatment strategies to combat *P. aeruginosa*'s invasion has become more urgent and requires coordinated efforts.

One of the natural products used to combat antimicrobial resistance especially in wound infection in recent times is honey. Some honeys are effective in the complete treatment of infections; however, studies have shown that some bacteria are resistant to certain types of honey found in Nigeria. *Pseudomonas aeruginosa* is one of the commonly isolated bacteria responsible for causing hospital acquired infections and are

commonly isolated from surgical wounds and burns. This bacterium is usually found with multidrug resistant properties hence pose a big problem to clinicians, clinical laboratory scientists as well as patients and their relatives. Honey that is commonly recommended by clinicians as an alternative and effective means of treatment for wound infection has also been shown to be not totally effective in eradicating *Pseudomonas aeruginosa*. It is therefore necessary to find a means to modify and improve our locally produced honey to a therapeutic state with natural or synthetic additives that will have the potency for a bactericidal action against *Pseudomonas aeruginosa.* One of the ways to achieve this is to study this organism at the molecular level and understand its genes and mechanism for antibiotic resistance and virulence especially against honey. Since the glutathione enzyme through the expression of its encoding genes *gshA* and *gshB* is actively involved in the elimination of oxidative stresses caused by hydrogen peroxide, it is necessary to confirm the presence of these genes and ascertain that their presence will significantly inhibit the action of honey especially the ones whose main antimicrobial agent is due to the presence of hydrogen peroxide such that we have in Nigeria.

1.3 Justification of the Study

Several studies have addressed different aspects of Nigerian honey varieties, including their physicochemical properties, their chemical composition[13], their antibacterial and antibiofilm activities[14] , and their therapeutic usefulness[15]. However, it is not yet known whether these anti-biofilm and antimicrobial activities, as well as any possible anti-quorum sensing and anti-virulence activities possessed by this honey could be attributed to alteration of bacterial gene expression. The *gsh*A and *gsh*B genes have been shown to be important for the detoxification of H_2O_2 which is valuable for the effectiveness of honey as an antimicrobial agent. The information about the prevalence and expression of these genes in the presence of honey is valuable for other studies in the

future seeking to study the resistant mechanisms of *Pseudomonas aeruginosa* to honey. Study of the effects of these genes in relation to honey susceptibility will also help researchers discover novel targeted therapeutic strategies to combat *P. aeruginosa* infections using honey.

Knowledge about resistant and virulence genes makes it more likely for scientists to find and hopefully tackle new forms of multi-resistant bacteria. The more that is known about how bacteria can defend themselves against antibiotics or honey in this case, the better are the odds for developing new effective drugs and drug alternatives. Variation in the genome of *Pseudomonas aeruginosa* can have a great impact on the bacterium's ability to cause disease. The knowledge about these genes will make it easier to hand pick natural or synthetic substances that have properties to overcome such resistance and effectively kill the pathogen, hopefully for a long period of time. Furthermore, the knowledge will help us explore synergistic options and modify honey for its therapeutic use in healing wounds and other systemic infections.

1.4 Aim and Objectives of the Study

The aim of this study therefore is to determine the antimicrobial effect of two Nigerian bee honey types and detect the presence and expression of the virulence factor genes *gshA* and *gshB* in the presence of honey in clinical isolates of *Pseudomonas aeruginosa.*

The specific objectives of this research are to;

i. Isolate and identify *Pseudomonas aeruginosa* from clinical specimen

ii. Carry out the antibiotic susceptibility tests on the *Pseudomonas aeruginosa* isolates to determine its antimicrobial resistance pattern.

iii. Determine the antimicrobial effect of the two Nigerian bee honey types on the *Pseudomonas aeruginosa* isolates.

iv. Detect the presence of *gshA* and *gshB* genes on the selected clinical isolates of *Pseudomonas aeruginosa.*

v. Determine the expression of the genes (*gsh*A and *gsh*B) in the presence of the honey types.

The long-term aim of this study is to find a solution to the problem of antibiotic resistance to *Pseudomonas aeruginosa* by exploring natural alternatives such as honey in the treatment of illnesses associated with the bacteria, this involves genetically exploring the bacteria's genome for hindrances to this goal.

1.5 Research Questions

More specifically, the following research questions need to be addressed;

1. What are the antimicrobial effects of the studied honey types on clinical strains of *Pseudomonas aeruginosa*?
2. Could the poor antimicrobial effects of honey on some infections by *Pseudomonas aeruginosa* strains in Nigeria be attributed to the presence of the glutathione producing genes (*gshA* and *gshB*) in their genome or not?
3. Are these genes responsible for anti-honey resistant properties in *Pseudomonas aeruginosa*?
4. Can these virulent genes be targeted for manipulation to eradicate anti-honey resistant *Pseudomonas aeruginosa*?

1.6 Significance of the Study

It is important to answer the questions that this research poses so that it will not be a waste of time and resources using honey to combat a glutathione producing *Pseudomonas aeruginosa* strain. This research adds more to the knowledge and insight of the antimicrobial activity of our locally produced honey in its use to combat resistance in bacteria. Knowledge gathered from this research will add to the information base of the

research community about this notorious nosocomial multidrug resistant organism and could aid the fight of the World Health Organization (WHO) against antimicrobial resistance.

Furthermore, the knowledge from this study will help us explore synergistic options to modify our locally produced honey for its therapeutic use in healing wounds and other systemic infections. Also, *gshA* and *gshB* gene detection in bacteria could be used in a screening test to identify infections that can be successfully treated using honey.

1.7 Scope of the Study

This study covers the detection of the presence and expression of the glutathione biosynthesis genes *gshA* and *gshB* in clinical isolates of *Pseudomonas aeruginosa* using conventional and real time qPCR method. The isolates were gotten from clinical samples collected from patients in a tertiary health institution in South West Nigeria.

1.8 Limitation of the Study

This study could not analyze the concentrations of H_2O_2 in the honey samples used. The analysis is being proposed as a follow up study to back up the findings of this work.

1.9 Operational Definition of Terms

Gene- The gene is considered the basic unit of inheritance. Genes are passed from parents to offspring and contain the information needed to specify physical and biological traits. Most genes code for specific proteins, or segments of proteins, which have differing functions within the body. Humans have approximately 20,000 protein-coding genes.

Gene Amplification- Gene amplification refers to an increase in the number of copies of a gene in a genome.

Gene Expression- The process by which a gene's information is used to produce either RNA molecules that code for proteins or non-coding RNA molecules with additional functions is known as gene expression. When and where RNA molecules and proteins are produced, as well as their quantity, are controlled by gene expression, which also serves as a "on/off switch" and a "volume control" for those processes.

Genome- The genome is a cell's entire set of DNA instructions.

Glutathione- Glutathione (GSH) is a thiol containing compound that plays an important role in antioxidative and detoxification mechanisms in bacteria cells and plant tissues

Polymerase Chain Reaction- Polymerase chain reaction (PCR) is a laboratory technique for rapidly amplifying millions to billions of copies of a specific segment of DNA, which can then be studied in greater detail. PCR involves using short synthetic DNA fragments called primers or oligonucleotides to select a segment of the genome to be amplified, followed by multiple rounds of DNA synthesis to amplify that segment.

Copy DNA (cDNA)- cDNA (also known as complementary DNA) is synthetic DNA that has been transcribed from a specific mRNA using the reverse transcriptase enzyme. While DNA contains both coding and non-coding sequences, cDNA only contains coding sequences. cDNA is frequently synthesized and used by scientists in gene cloning and other research experiments.

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Chapter Two

Literature Review

2.1 Conceptual Review

2.1.1 Honey and its Uses

Stone Age artwork show that humans used honey for health around 8000 years ago. Aside from its vital function in traditional medicine, natural honey has been exposed to laboratory and clinical examinations by numerous research organizations over the last few decades, and it has found a position in contemporary medicine. Honey has been used as food and also for therapeutic purposes since the ancient time. Honey has been one of the most cherished and prized natural goods presented to humanity. Honey is utilized not only as a nutritious food, but also in traditional medicine to treat a variety of clinical ailments ranging from wound healing to cancer therapy.

Honey has traditionally been used to treat eye disorders, bronchial asthma, tuberculosis, throat infections, thirst, hiccups, weariness, dizziness, hepatitis, constipation, worm infestation, piles, dermatitis, wound healing, and as a nutritional supplement. Honey's constituents have been shown to have antioxidant, antibacterial, anti-inflammatory, antitumor, anticancer, and antimetastatic properties. The use of honey as therapeutic substance has been rediscovered recently and has been accepted as an antibacterial agent for treatment of ulcer, bed sore, surface wounds and surface infection resulting from wounds. Also, honey has been found to contain antioxidant properties and it is effective in the treatment of bacterial gastroenteritis in infants. Honey has been reported to have an inhibitory effect on around sixty species of bacteria, some species of fungi and viruses[1]. Honey has also been found to have anti-cancer properties.

According to the texts of Ayurveda, honey is useful for those with weak digestion as it aids digestive system[5]. It is also used in the treatment of irritating cough. Honey is

regarded by Ayurvedic experts, as valuable in keeping the teeth and gums healthy and strong[5]. It has been in use for ages for the treatment of insomnia because it has hypnotic action[5]. Honey reduces cardiac pain, palpitation and all imbalances of the lungs and anaemia. Honey also has a long history of Ayurvedic use for various eye ailments and improves the eye-sight.

2.1.2 Composition of Honey

Honey is formed from floral plant nectar gathered by honeybees and is mostly constituted of glucose and fructose. It does, however, include vitamins, minerals, amino acids, enzymes, organic acids, and other substances such as beeswax, pollen, pigment and phenol. Its composition is influenced by seasonal fluctuations as well as the geographic region where the bees collected the nectar. The moisture level of the deposited nectar mixture decreases and dries out, causing the honey to become more concentrated and viscous. Natural honey contains around 82% water, carbs, proteins, phytochemicals, antioxidants, and minerals. The concentration of the carbohydrate in honey is around 80% and the water content is from 15% to 20% depending on the botanical origin and the level of the honey.

Few of the components that affect the biological and medicinal potential of this substance have been shown to differ between different varieties of honey. In decreasing order, the sugars in honey are: "fructose (38.2%), glucose (31.2%), disaccharides and certain other tri-saccharides and higher saccharides (9%), and sucrose (0.7–1%). Honey rich in active components such as flavonoids, organic acids, phenolic acid, vitamins, and enzymes may aid in wound healing[7].

The intrinsic characteristics and the complex composition of honey, in which different substances with antimicrobial properties are included, make it an antimicrobial agent with multiple and different target sites in the fight against bacteria[5].Honey consists

mainly of carbohydrates, water and other minor components. Those minor ingredients include: proteins, minerals, phytochemicals and antioxidants. Findings have shown that those minor ingredients are the ones that are responsible for medical and biological activities of honey in the treatment of infections, burns, wounds and ulcers. Hydrogen peroxide is the major contributor to the antimicrobial activity of honey[8]. Concentrations of hydrogen peroxide in different honeys result in their varying antimicrobial effects. It has further been reported that physical property, geographical distribution and different floral sources may play an important role in the antimicrobial activity of honey.

The high concentration of sugars combined with the low moisture content are responsible for the low water activity values of honey which causes osmotic stress in microorganisms. The sugar content, phenolic chemicals, hydrogen peroxide, 1,2-dicarbonyl compounds, and bee defensin-1 all contribute to honey's antibacterial activity. All of these components are present in varying amounts depending on the nectar source, bee type, and conditions of storage. These components interact together to make honey effective against a wide range of pathogens, including multidrug resistant bacteria, and to modify their resistance to antimicrobial drugs. Honey's efficiency and potency against infections are determined by the type of honey produced, which is determined by its botanical origin, bee health, provenance, and processing technique[7]. Fortunately, in Nigeria the use of naturally occurring substance such as honey as alternative medicine has gained ground in the fight against antimicrobial resistance.

2.1.3 Honey and Wound Dressings

Honey is a biological wound dressing with a large number of bioactive components that can accelerate the wound healing process. It has been in use from ancient times for the healing and treatment of wounds. The high concentration of amino acids in honey may potentially increase fibroblast deposition and collagen production.

Honey is frequently used in the treatment of chronic wounds, especially diabetic foot injuries, decubitus ulcers, venous and arterial ulcers. Honey is very effective and has yielded positive results in the treatment of chronic wounds,. One study determined that the results of silver-coated dressing, silver with honey, and honey-coated dressings were not significantly different in terms of bacterial colonization and wound size contractions in malignant wounds.

Manuka honey is a popular honey with proven effective antibacterial properties against a wide range of bacteria including resistant strains. Manuka honey impregnated dressing was reported to provide better diabetic foot wound healing with a stronger antibacterial effect than other kinds of honey[,,]. Studies have also shown the superior effects of honey in burn wounds compared to other materials in the treatment of wounds in humans[,] except for an herbal burn ointment in one research where honey was observed to be less superior.The studies showed that inflammation was less in honey-treated groups and epithelization developed on the 6th-9th days[16,17]. Honey was also demonstrated to be superior and more cost-effective than mafenide in first-degree burns in terms of hastening wound healing and providing more aesthetic results[17]. Honey impregnated dressings have also been found to reduce surgical wound complications and give better aesthetic results in the healing of caesarean incision wounds[,].

Honey has been demonstrated to be effective most especially against *Escherichia coli*, *Pseudomonas aeruginosa*, methicillin resistant *Staphylococcus aureus* MRSA, *Acinetobacter* and much more[7]. The viscosity and hyperosmolarity of honey make the bacteria dehydrated and prevent their proliferation though antibacterial potential among different honey is known to vary up to 100 times.

Quite a number of substances in honey have been suggested as the key component to its antimicrobial potential; these are polyphenolic compounds, hydrogen peroxide,

methylglyoxal, and bee-defensin 1[22]. These components vary significantly across honey samples due to botanical origin, geographical location and secretions from the bee.

Honey is applied topically for treatment of infected wounds[5]. Honey and other plant-based products such as aloe vera that are capable of displaying antimicrobial activities are currently gaining considerable attention in indigenous medicine. Over the years honey have been studied for its antibacterial effects and its role in wound healing. The studies includes both in vitro and in vivo using humans and mice models. Honey whether used in isolation or in combination with other therapeutic agents have in the past showed excellent effects against many bacteria colonising and infecting wounds. Honey have been combined with antibiotics, natural agents (such as aloe vera, ginger extracts, royal jelly, Cinnamaldehyde and carvacrol), herbal extracts, ethanol, Vitamins C and E etc.. They have also been incorporated into wound dressings.

Worthy of mention amongst all other honeys is the Manuka honey, it is cited as the best-known non-peroxide-based honey. This popular honey that got its name from the product of the New Zealand scrub plant is quite potent against a number of bacteria including those that produce biofilms. When bees pollinate from the manuka plant, their honey is more potent than standard bee honey. This is because it has a higher concentration of methylglyoxal (MGO) which is its active antimicrobial agent. Manuka honey is known to be effective against antibacterial resistant strains such as Methicillin-resistant *S. aureus* and Vancomycin-resistant *Enterococcus* spp.. However, it has also been reported from a comparative study that *Melipona beecheii* honey from Cuba stood out among three other honeys that included the manuka honey because of it being particularly acidic and producing the highest antibacterial and antibiofilm action against *Staphylococcus aureus* and *Pseudomonas aeruginosa* cells isolated from wound infection,

and was connected to induced structural alterations seen by transmission electron microscopy.

Medical grade honey is defined as organic honey that has been sterilised by gamma irradiation, is processed in accordance with safety regulations and standards, and is safe for medical application. It has been shown to have broad spectrum antimicrobial activity against common wound infecting microorganisms such as *Staphylococcus aureus,* *Pseudomonas aeruginosa,* and *Escherichia coli..*

2.1.4 Antimicrobial Mechanism of Action of Honey

Honey exhibits antibacterial activity against numerous bacteria in different environments this is due to the natural components of honey which have various activities against different microorganisms. Honey's antimicrobial capability is influenced by a number of factors, including its low water content (low water activity), high viscosity, acidity, and H_2O_2 concentration as previously stated. Furthermore, honey is connected with numerous substances that offer its antibacterial potential, such as phytochemicals, peptides, non-peroxidase glycopeptides, and proteins, which are prominent properties of honey and are related with its antimicrobial activities.

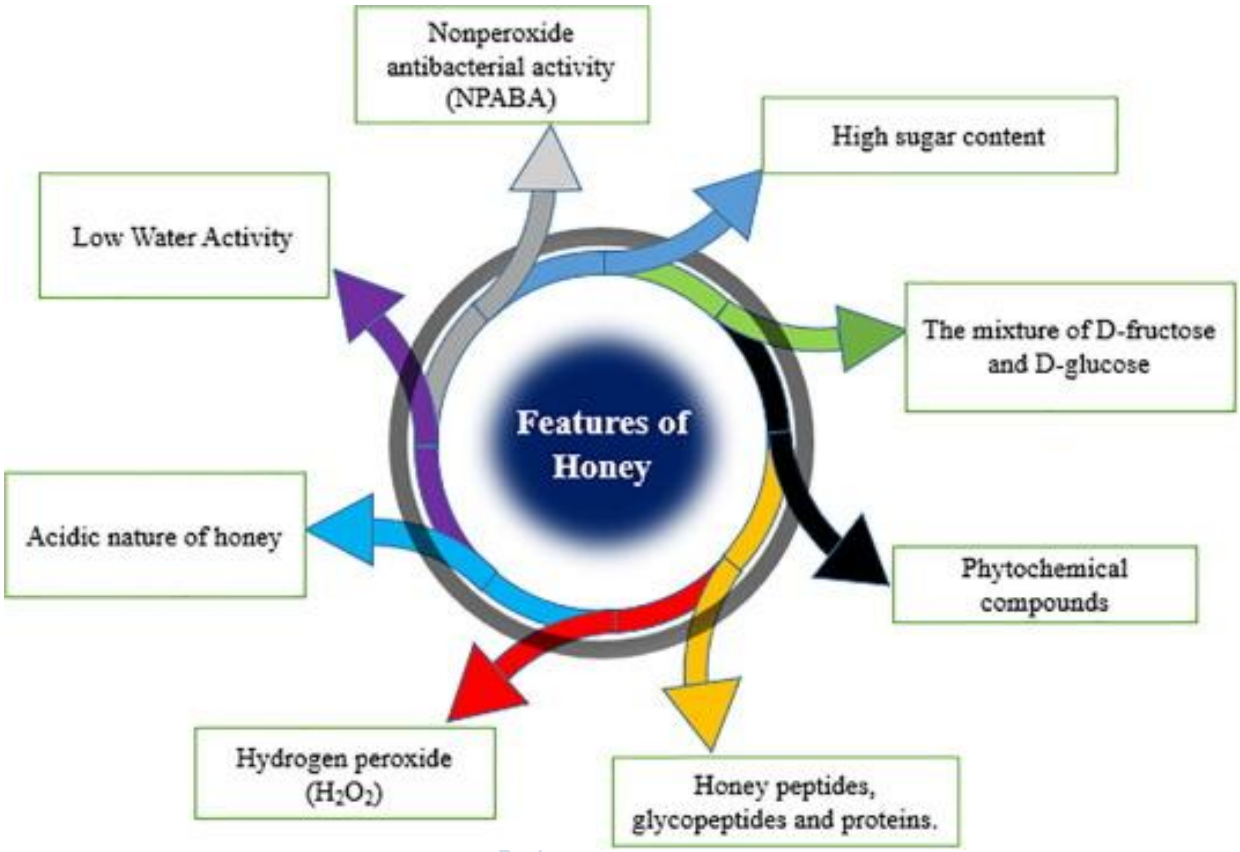

Fig. 1. Schematic diagram showing the parameters/agents that contribute to the antimicrobial potential of honey. Source

In undiluted pure honey, there are many factors that could contribute to its antibacterial potency. One of it is the low pH, however, pH alone is not sufficient to inhibit the growth of many types of bacteria when diluted in food or in other bodily liquids. Also, because of its sugar content, pure, undiluted honey inhibits bacterial development by exerting osmotic pressure on bacterial cells, forcing water to flow out of the bacterial cells via osmosis. As a result of the dehydration, the cells shrivel and become unable to live in the hypertonic sugar solution. When honey is combined with body fluids at infection sites, its antibacterial potential decreases. The acidity of honey which is between pH 3.2 and pH 4.5, is a very marked characteristic of its antibacterial efficacy inhibiting most bacteria whose optimal growth is at 6.5- 7.5.

In addition, glycogenic acid is generated from glucose oxidation in bacteria by an endogenous glucose oxidase enzyme and is an extremely potent antibacterial agent. Numerous published research has demonstrated that the main royal jelly proteins have antibacterial, anticancer, and anti-inflammatory properties. Except for Manuka honey, the antibacterial peptide defensin-1 (Def-1) derived from bees is one of the major components responsible for honey's antibacterial properties. Bee Def-1 is effective against Gram-positive bacteria. Recombinant Def-1 studies indicated its efficacy against Gram-negative bacteria such as *P. aeruginosa* and *Salmonella choleraesuis*. Phenolic compounds are also found at high levels in honey and may contribute to its antibacterial activity[33].

2.1.5 Hydrogen Peroxide

H_2O_2 is known to be a disinfectant and a powerful oxidizing agent that gives honey antibacterial properties and it is created enzymatically (Fig. 2). Due to the low pH circumstances, the enzyme glucose oxidase is naturally present in honey in an inactive state. When honey is diluted, the enzyme glucose oxidase is triggered and reacts with

natural glucose to form H_2O_2[34]. In reality, the highest amount of hydrogen peroxide may be obtained by diluting honey by 30%-50%, resulting in a concentration of 5 to 100 g H_2O_2/g honey (equal to 0.146-2.93 mM)[32]. There is a linear relationship between honey's H_2O_2 concentration and its antibacterial properties because hydrogen peroxide generation in some honey samples might grow continuously over time to a point dependent on the dilution utilized. It was reported that H_2O_2 levels in honey can reach 2.5 mmol in 30 minutes and can reach a twofold increase with continued incubation. The study showed that a 30-40 v/v dilution yielded up to 2.5 mM of hydrogen peroxide when incubated for 30 mins[32]. 1 mM has been shown to be sufficient to kill E. coli in 15 minutes

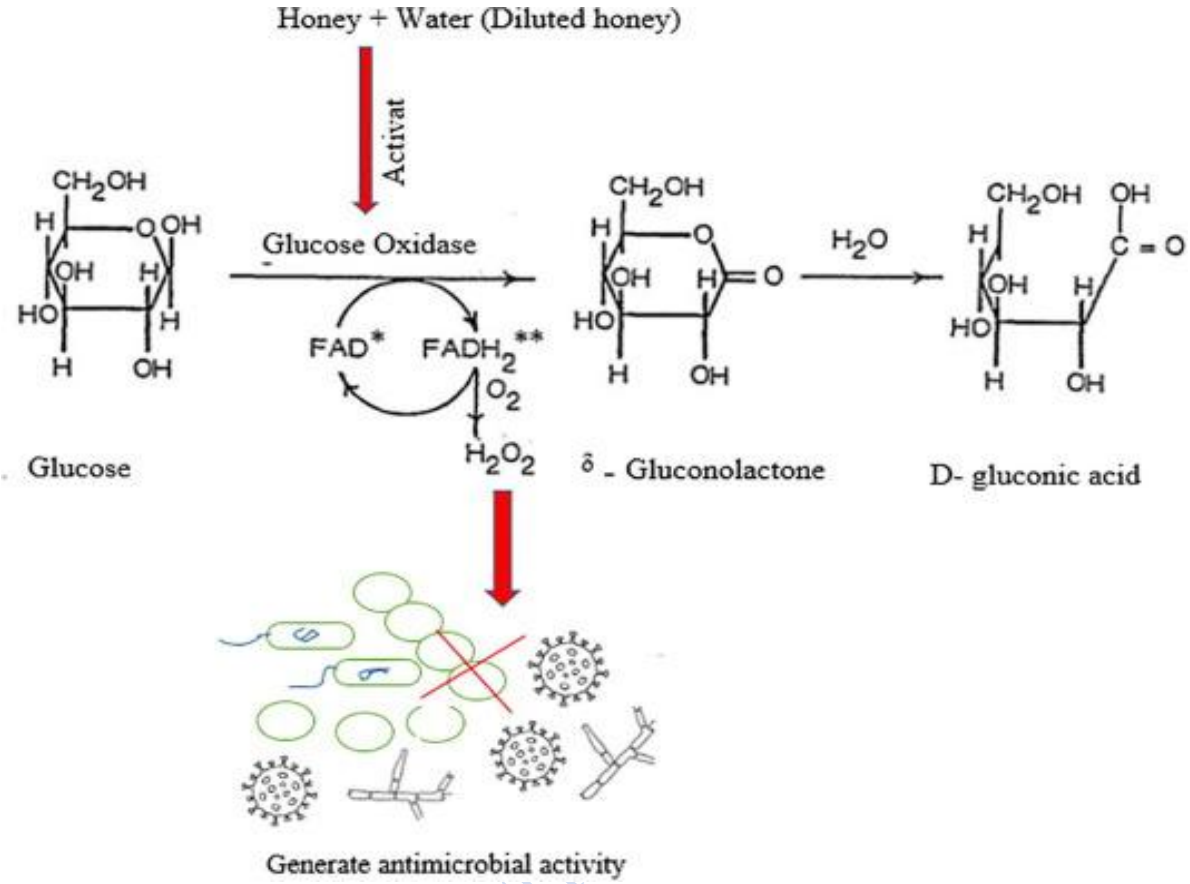

Fig. 2. Glucose oxidase catalyzes the generation of hydrogen peroxide (H_2O_2). Source[34]

2.1.6. Antibacterial Effect of Honey

The antibacterial efficacy of honey was initially recognized in 1892; however, in modern medicine, it is used only to a limited extent due to the absence of scientific support until recently with increased research on the natural product[7].

Around 60 types of bacteria, including gram-positive and gram-negative, aerobes and anaerobes, have been shown to be inhibited by honey. Numerous pathogens have been identified to be susceptible to honey's anti-infective effects. Its effectiveness against a number of bacteria has been demonstrated, including *Pseudomonas aeruginosa ,Staphylococcus aureus, Bacillus anthracis, Corynebacterium diptheriae, Haemophilus influenzae, Klebsiella pneumoniae, Listeria monocytogenes, Mycobacterium tuberculosis, Pasteurella multicoda, Yersinia enterocolitica, Proteus species,* and *Acinetobacter species*[36].

It has been shown in a study that diluted honey treated urinary tract infections because certain bacteria causing urinary tract infections such as *E. coli*, *Proteus* species and *Strep. faecalis*, were found to be sensitive to the antibacterial activity of honey. *Likewise, H. pylori* isolates which cause gastritis have been shown to be inhibited by a 20% solution of honey invitro. Compared to most conventional antibiotics, it has been reported that honey does not lead to development of antibiotic-resistant bacteria, and it may be used unceasingly[1]. A study from Nigeria showed that honey has antibacterial activity against some selected bacteria associated with diarrhea that were resistant to conventional antibiotics and therefore recommended to be exploited as an alternative to conventional antibiotics to treat infections.

The antibacterial activity of honey is likely to depend on the pasture on which the bees were raised, climatic conditions, as well as the natural composition of the flower nectar. Honey's antibacterial activity can also be attributed to its osmotic impact, high

sugar content, low water content, and presence of gluconic acid, which creates the antiseptic H_2O_2[35]. An invitro study linked honey's antibacterial effects to H_2O_2, methylglyoxal (MGO), and the antimicrobial bee protein defensin-1. Each of these components has a unique mode of action. As shown in Fig 2, glucose oxidase is an enzyme found in honey that transforms glucose to gluconolactone and finally to H_2O_2. To create a persistent antibacterial action, this H_2O_2 is released slowly and continuously.

Genomic sequencing and experiments with single-gene knockouts showed a key mechanism by which bacteria increased their honey resistance was by mutating genes involved in detoxifying methylglyoxal, which contributes to the antibacterial activity of *Leptospermum* honeys

2.1.7 Types of Honey Found in Nigeria

There are different types of honey that can be found in Nigeria. One is the blended honey which is a combination of honey from different floral sources Alfalfa, wildflowers, and clover are a few examples of the floral sources of blended honey. To establish a flavour profile with the lowest common denominator for mass-merchandising, blending is done with the more widely available and less distinctively flavoured honeys. In the end, the flavour is typically "sweet" and “honey” with no additional flavour characteristics. Varietal honey contrasts with blended honey. Unprocessed honey is raw honey, it is honey in the beehive or as extracted, settled, or strained without the use of heat (pasteurization). A little amount of pollen and possibly some waxes are present in raw honey. One of the purest foods on the table, raw honey comes from the comb and goes into the bottle.

Strained honey or filtered honey has been run through a mesh filter to remove particles (e.g., wax fragments) but not pollen. Because of the pollen, it has a hazy look and crystallizes faster than ultrafiltered honey[42]. Ultrafiltered honey is treated through

very fine filtration under high pressure to remove all extraneous substances and pollen grains. Because it crystallizes more slowly, ultrafiltered honey is highly clear and has a longer shelf life. The supermarket trade prefers it and it is popular with health food buyers. Unpasteurized honey is honey found in supermarkets and it is frequently pasteurized to help to avoid crystallization on the shelf. The crystals will dissolve in the microwave when heated for 30seconds or in a pan of boiling water if they crystallize (10 to 15 minutes).

Varietal or monofloral honey is honey made from a single flower, such as orange blossom, lavender, or sage[42]. In addition to the honey flavour, it will express secondary flavour characteristics such as lavender, sage, raspberry, and so on.

Honeys found in Nigeria are generally known and classified as the unprocessed raw honey or the processed ones. Raw honey is of the finest organic quality and is considered to be completely pure

It is mostly known that honey has a sweet taste but few are knowledgeable about the existence of a bitter honey. Bitter honey is so called because it has a bitter taste. Bitter honey is commonly produced by bees found in Sardinia. Sardinia is a large Italian Island in the Mediterranean Sea. It has nearly 2,000km of coastline, sandy beaches and a mountainous interior crossed with hiking trails. Many scholarly and eminent individuals have expressed their interest in and curiosity in the bitter honey produced in Sardinia. They have expressed varied, frequently conflicting opinions on the reasons behind its bitterness as well as on its quality and therapeutic capabilities. Sardinian bitter honey is derived from the fall flowering of the strawberry tree (Arbutus unedo L.), has a long history of popular use, particularly as a medication. Its expertise spans over 2000 years, from the Greeks and Romans to the present. Many literary references from prominent persons of the past, such as Cicero, Horace, Virgil, and Dioscorides, have been associated

with the distinctive peculiarity of its taste, which lends itself to literary and lyrical metaphors, up until modern times. The curiosity of its bitter taste is also what prompted the earliest research, which began in the late 1800s and tried to discover its origin[45].

Up until the most recent examinations, other studies on the botanical origin and characteristics of the Sardinian bitter honey had been conducted. These studies proved its potential for application in the medical area because of its antioxidant, antiradical, and cancer-prevention qualities. These advantages have been linked to its phenolic component, and more specifically, the predominant phenolic acid (homogentisic acid)[45]. Later, additional Mediterranean strawberry tree honey also demonstrated the same qualities. The geographical and historical identity of Sardinian bitter honey is still present, and other Mediterranean civilizations are aware of it. Other Italian regions of production of this honey are Liguria, Tuscany, and Campania. In the Mediterranean basin, it is produced also in Corsica, Portugal, Spain, Albania, Croatia, and Turkey. According to recent research on the subject, it appears that manuka honey and other studied honeys, have antibacterial activity on average that is on par with or higher than that of sardinian bitter honey. However, the data are not always directly comparable since they pertain to various bacterial strains and different techniques of measuring antibacterial activity, making a comparison amongst honeys while very useful, extremely challenging[46].

Interestingly, bitter honey has been reported as being harvested in Nigeria. Only a small number of research have described bitter honey in writing. Their respective botanical sources were predominately made up of plants like *Peronema canescens*, types of *Rhododendron, Robinia pseudoacacia*, and *Arbutus unedo*. However, the botanical origin of most Nigerian bitter honey remains mostly unknown. A study that aimed at characterizing the actual botanical source of a Nigerian bitter honey harvested in Osun State, Nigeria to establish a relationship between the plant precursor of its bioactive

components and potential health benefit using Melissopalynology analysis (via acetolysis and microscopic assessment) found pollen samples of medicinal plants such as *Elaeis guineensis*, *Irvingia gabonensis*,*Chromolaena odorata*, *Blighia sapida*, *Canavalia ensiformis* in the honey[48]. The most predominant phytochemical was found to be alkaloid, and it was stated that it could determine the plant's major therapeutic value. The bitter honey contained (15.53 ± 0.22) moisture, (0.86 ± 0.02) ash, (5.95 ± 0.02) protein, (77.66 ± 0.23), carbohydrate, (334.44 ± 0.80) energy, (1.43 ±0.0007) specific gravity and pH (3.38 ± 0.0033). The mineral calcium was the most abundant, followed by iron, zinc, phosphorus, sodium, potassium, and potassium. The exact same honey is the one used in this study.

A study that compared the antinutritional composition, qualitative and quantitative phytochemicals of Nigerian sweet and bitter honey revealed the presence of alkaloids, tannins, flavonoids, saponins glycoside with absence of anthraquinones and phlobatannins, while steroids was detected only in sweet honey. Subsequent quantification revealed no significant difference ($p>0.05$) in the Saponins and flavonoids contents of the two honey varieties. However, the tannins content of bitter honey (0.02+0.10) was significantly higher ($p<0.05$) was observed in the phytate, oxalate and cyanide content of the two honey varieties[49]. The study suggested that irrespective of the taste of both honey types, they are rich in nutrients and phytochemicals of medicinal significance.

2.2 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a gram negative non lactose fermenting bacteria that is usually associated with community and hospital acquired infections especially in persons with compromised immunity. It is notorious for being multi drug resistant to common antibiotics hence pose a big therapeutic challenge to clinicians, laboratorians and patients. Resistance in *Pseudomonas aeruginosa* can either be intrinsic or acquired leading to the occurrence of resistant strains that cannot be eradicated by common antibiotics such as aminoglycosides, beta lactams, quinolones, carbapenems and more recently colistin.

P. Aeruginosa is mainly considered an opportunistic, nosocomial gram-negative pathogen (responsible for 13–19% of hospital-acquired infections, mostly found in intensive care units and surgical theatres, where the prolonged use of antimicrobials has allowed for the acquired resistance of microorganisms. Practically all healthcare institutions have reported *P. Aeruginosa* outbreaks and intrahospital infections, as these bacteria have the ability to persist on a wide range of inanimate surfaces spreading via aerosols. Under normal circumstances, *P. Aeruginosa* can colonize the intestinal tract for a short while especially in immunocompromised patients in which case can be prolonged, nevertheless, 8–20% of nosocomial infections and outbreaks are associated with colonized individuals. To avoid nosocomial outbreaks, the strict adherence to infection control protocols, environmental cleaning plans, and hand hygiene practices are of critical importance, in addition to the identification and elimination of possible reservoirs of infection[51].

2.2.1 The Genome of *Pseudomonas aeruginosa*

Recent work has been done to herald the birth of the comparative genomics era for analysis of the important pathogen *Pseudomonas aeruginosa*. Comprehensive systematization of microorganisms requires extensive genome-wide comparisons of conserved and variable regions located in the genomes, determination of expression profiles, gene complements and correlation of phenotypic characteristics. An important stimulus to such systematic studies is expected from decoding and comparison of genome sequences for interesting strains within species of a bacterium. Genome comparison to determine relatedness between isolates of the same species can support or refute the probability of pathogen transmission and outbreaks in healthcare settings and the community. Microbial genome sequence information is critical for detecting low frequency mutations, finding key deletions and insertions, and discovering other genetic changes among microbial strains.

The first *P. aeruginosa* genome to be sequenced belonged to strain PAO1 which was first isolated from a chronic wound in the 1950s and is now a popular laboratory strain. The 6.3 Mbp length of the PAO1 sequence (5570 projected open reading frames) makes it one of the biggest bacterial genomes to be sequenced in 2000. It is significant to highlight that the PAO1 strains kept in various laboratories across the world exhibit phenotypic and genetic heterogeneity, which is a factor in the repeatability of tests between various research organizations. Another often used laboratory strain is PA14, which was first found in the environment as a very virulent strain that severely damaged plants. It was later found in burn wounds on people.

The genome of *P. aeruginosa* resembles a classical "secretor" genome, which includes a large proportion of regulatory genes (i.e., efflux pumps and other transport proteins, motility and chemotaxis), genes controlling metabolic pathways and genes

encoding a surplus of virulence factors and determinants of antibiotic resistance,. The genome of *P. aeruginosa* also contains a large number of transcriptional regulators and genes involved in the catabolism, transport, and efflux of organic compounds. *P. aeruginosa's* capacity to colonize and flourish in a range of environments likely depends on its metabolic and genetic flexibility[57].

A study that compared the genomes of clinical strains of *P. aeruginosa* identified and defined a set of 321 core essential genes, representing 6.6% of the genome. They came to the conclusion that analysis of four strains of P. aeruginosa was typically sufficient to converge on a set of core essential genes that are likely to be essential across the species under a wide range of conditions relevant to in vivo infection and, as a result, to represent appealing targets for novel drug discovery.

2.2.2 Treatment of *Pseudomonas aeruginosa* Infections with Antibiotics

The standard antibiotics used for the treatment of *Pseudomonas aeruginosa* infections are of eight categories which includes aminoglycosides (gentamicin, tobramycin, amikacin, netilmicin), carbapenems (imipenem, meropenem), cephalosporins (ceftazidime, cefepime), fluoroquinolones (ciprofloxacin, levofloxacin), penicillin with β-lactamase inhibitors (BLI) (ticarcillin and piperacillin in combination with clavulanic acid or tazobactam), monobactams (aztreonam), fosfomycin and polymyxins (colistin, polymyxin B)[59]. The strains of *P. aeruginosa* are categorized thus: (1) MDR when resistance is observed in more than 1 agent in more than 3 categories; (2) extensively drug-resistant (XDR) when a resistance is observed in greater agents in all but lesser categories; and (3) pandrug-resistant (PDR) when the strain is non-susceptible to all antimicrobial agents[59]. MDR, XDR, and PDR strains evolve in a timely manner due to changes in regulatory mechanisms that govern the expression of resistance determinants, mutations, changes in membrane permeability, and horizontal acquisition of antibiotic-

inactivating enzymes or enzymes that induce target alterations. For all severe infections that are known or suspected to be brought on by P. aeruginosa, empirical antibiotic therapy usually comprise two medicines from distinct classes with in vitro action against P. aeruginosa. The idea behind the so-called "double coverage effect" is to make it more likely that *P. aeruginosa* will respond to antibiotic therapy, particularly in situations where there is a significant risk of antimicrobial resistance. The definitive therapy is usually adapted in accordance with the susceptibility results once they are available, utilizing a single in vitro active agent with the highest antibacterial activity and the lowest tendency to select resistance.

2.2.3 Antimicrobial Resistance Pattern of *Pseudomonas aeruginosa*

Significant numbers of the conserved genes of *Pseudomonas aeruginosa* encode regulatory proteins. This suggests that *P. aeruginosa* have the capacity to respond to various environmental stresses. *P. aeruginosa* also exhibits inherent antibiotic resistance due to the existence of resistance-nodulation-division efflux pumps, which physically sequester entering antibiotics. Furthermore, biofilms serve as a barrier to antibiotic penetration. *P. aeruginosa* can also develop antibiotic resistance genetically through mutations or horizontal transfer of relevant genes.

The fast emergence of antibiotic resistance is a result of *Pseudomonas aeruginosa's* huge and complex genome, which contains high amounts (up to 20%) of transferable genetic components. Genomic divergence and phylogenetic connections, as well as genes for antibiotic resistance and virulence markers, in 22 strains recovered from eye and cystic fibrosis patients in Australia and India between 1992 and 2007 has been compared and the study showed the size of the accessory genome varied significantly between the 22 stains[62]. According to analysis of the pangenome, variation was connected with the number of genomic islands, insertion sequences, and prophages. The isolates

differed from P. aeruginosa clones from the worldwide outbreak in terms of sequence type.

Pseudomonas aeruginosa has inherent resistance to several drugs and is known to actively acquire genetic alterations for further resistance. A study that sought to understand the genomic and transcriptome landscapes of *P. aeruginosa* clinical isolates that were extremely resistant to several medications, the route of antibiotic resistance and the transcriptional responses of genes that confer antibiotic resistance sequenced the whole genomes and evaluated genome-wide RNA transcripts from three separate phylogenetically distant multi-drug resistant (MDR) clinical isolates. Multi-layered genome comparisons with antibiotic-susceptible *P. aeruginosa* strains and 70 additional antibiotic-resistance strains revealed both well-characterized conserved gene mutations and different antibiotic-resistant gene (ARG) distributions among strains.

In MDR strains, transcription of genes involved in quorum sensing and type VI secretion systems was always downregulated. Virulence-associated characteristics were investigated further, and the results show that the MDR strains are obviously avirulent. Transcription of 64 genes, which were rationally chosen to be associated with antibiotic resistance in MDR bacteria, was active under normal growth conditions and remained unaltered under antibiotic treatment. Their findings suggest that antibiotic resistance is obtained by a "constitutive" response scheme, in which ARGs are actively produced even when antibiotic stress is not present, rather than a "reactive" response[63].

2.2.4 *Pseudomonas aeruginosa* Virulence Factors and Mechanism of Antimicrobial Resistance

Pseudomonas aeruginosa is among the critical pathogen group of bacteria that made the top ten WHO list of priority pathogens for research and development of new antibiotics. *Pseudomonas aeruginosa* has both intrinsic and acquired resistance to

antibiotics. Its intrinsic properties include its restricted outer membrane permeability to drugs, multiple efflux pumps that pump out antibiotics that manage to get in through its cell walls and the presence of antibiotics inactivating enzymes such as beta lactamases (encoded by the *ampC* gene) and aminoglycoside modifying enzymes (aminoglycoside phosphotransferase, aminoglycoside acetyltransferase and aminoglycoside nucleotidyltransferase)

Antibiotic penetration is specifically blocked by the asymmetric bilayer of phospholipid and lipopolysaccharides that makes up P. aeruginosa's outer membrane. Porins embedded in this bilayer create protein channels with barrel shapes. Quinolones and β-lactams manage to penetrate cell membranes through porin channels to act against P. aeruginosa and aminoglycosides and polymyxins promote their own uptake by interacting with *P. aeruginosa* lipopolysaccharides on the outer membrane. The outer membrane permeability of P. aeruginosa is significantly lower than that of other bacteria, which may be due to the presence of mainly closed *Opr*F channels. Furthermore, the lack of the P. aeruginosa OprF causes an increase in biofilm formation by upregulating the crucial messenger for regulating biofilm formation, bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP).

Bacterial efflux pumps play an important role in expelling toxic compounds out of the *P. aeruginosa* cell, and can be classified into five families: resistance-nodulation-division (RND) family, major facilitator superfamily (MFS), ATP-binding cassette (ABC) superfamily, small multidrug resistance (SMR) family, and multidrug and toxic compound extrusion (MATE) family. More importantly, the proteins belonging to the resistance-nodulation-division family of efflux pumps play a key role in antibiotic resistance in *P. aeruginosa*. They consist of cytoplasmic membrane transporters, periplasmic linker proteins and outer membrane porin channel proteins. Multidrug efflux

(Mex) and a letter are used to identify the cytoplasmic and periplasmic parts of P. aeruginosa RND pumps, while Opr and a letter are used to identify the outer membrane porin. *Pseudomonas aeruginosa* expresses twelve resistance-nodulation-division family efflux pumps, four of them (MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM) contribute to antibiotic resistance. MexAB-OprM is responsible for efflux of β-lactams and quinolones. MexCD-OprJ is able to pump out β-lactams. MexEF-OprN is capable of extruding quinolones, while MexXY-OprM expels aminoglycosides.

Persistent and recalcitrant infections in cystic fibrosis patients are caused by the formation of *P. aeruginosa* biofilms and persister cells[51].

2.2.5 Antimicrobial Resistant Genes of *Pseudomonas aeruginosa*

In recent years there has been progress in the creation of new antibiotics with novel mechanisms of action, resistance to modification by bacterial enzymes, and drug delivery efficiency increases. Traditionally *Pseudomonas aeruginosa* can be treated with carbapenems, quinolones and β-lactams. However, *P. aeruginosa* has a remarkable ability to evolve or acquire novel resistance mechanisms to these new antibiotics, raising severe concerns about antibiotic overuse and abuse. The virulence genes found in recent research studying *Pseudomonas aeruginosa* strains isolated from wounds and responsible for the worsening of wounds included the LasB, plcH, toxA, and exoU which were detected among all multidrug resistant, extensively drug resistant, and pan-drug resistant pulsotypes found[41]. Carbapenemase activity was phenotypically detected in 45% pulsotypes and the responsible genes were blaGES (100%), blaVIM (58%), blaIMP (4%), and blaNDM (4%)[41]. Real-time polymerase chain reaction showed the concomitant use of multiple mechanisms such as oprD under-expression, enhanced efflux pump activity, and ampC overexpression in the resistant isolates. Polymyxin was found as the only class left to eradicate most of the strains[41]. Other studies have revealed the the ESβL-encoding

gene *bla*$_{\text{OXA-2}}$, *bla*$_{\text{IMP-7}}$ and *bla*$_{\text{OXA-50}}$ and metallo β-lactamase production genes (blaVIM, blaCTX-M and blaTEM) in *Pseudomonas aeruginosa* .

A recent study reports a high prevalence of colistin resistance and *mcr-1* gene in *P. aeruginosa* strains isolated from Egypt. The co-existence of OXA-48 and NDM-1 genes in colistin-resistant *P. aeruginosa* ST235 high-risk clone has also been reported. The strains were said to have caused infections with 30-day mortality rate of 87.5% (7/8). The ST235 clone harbours nearly 39 types of beta-lactamases especially IMP, NDM and VIM type Metallo-β-lactamases (MBLs). The ST235 high-risk clone of *P. aeruginosa* has high capacity to acquire antibiotic resistance and is fast disseminating worldwide[70].

Table 2.1: Summary of Resistance Mechanisms of *Pseudomonas aeruginosa*

Location	Resistance mechanisms	Targeted antibiotics	Type of resistance
Intrinsic (chromosomal)	AmpC–type cephalosporinase	β-lactams	Antibiotic inactivation
	Class D oxacillinase OXA-50	β-lactams	Antibiotic inactivation
	Aminoglycosides inactivating enzymes	Aminoglycosides	Antibiotic inactivation
	Efflux systems (overexpression)	Multiple antibiotic classes	Efflux systems
	Decreased membrane permeability	Multiple antibiotic classes	Membrane impermeability and purines
	DNA gyrase and topoisomerase IV	Fluoroquinolones	Target modification
	LPS modification	Colistin	Target modification
Imported (Mobile genetic elements)	Class A serine β-lactamases (PSE, CARB, TEM)	β-lactams	Antibiotic inactivation
	Class A serine ESBL (TEM, SHV, CTX-M, PER, VEB, GES, IBC)	β-lactams	Antibiotic inactivation
	Class D ESBL (OXA-types)	β-lactams	Antibiotic inactivation
	Class B Metallo-β-lactamase (IMP, VIM, SPM, GIM)	Carbapenems	Antibiotic inactivation
	Class A serine carbapenemase (KPC)	Carbapenems	Antibiotic inactivation
	Class D carbapenemase (OXA-types: OXA-40)	Carbapenems	Antibiotic inactivation
	Aminoglycosides inactivating enzymes	Aminoglycosides	Antibiotic inactivation
	Ribosomal methyltransferase enzymes	Aminoglycosides	Target modification

Source[59]

2.2.6 Treatment of *Pseudomonas aeruginosa* Infections with Honey

Honey has been reported to have an excellent activity against *Pseudomonas aeruginosa*. An invitro study has revealed that honey is potent to eradicate biofilms produced by the bacteria. The study assessed four well-characterised New Zealand manuka honeys, quantified for their key antibacterial components, methylglyoxal, hydrogen peroxide and sugar, for their capacity to prevent and eradicate biofilms produced by the common wound pathogen *Pseudomonas aeruginosa*. They demonstrated that honey used at substantially lower concentrations compared to those found in honey-based wound dressings inhibited *P. aeruginosa* biofilm formation and significantly reduced established biofilms. They also established that the anti-biofilm effect of honey was largely driven by the sugar component in the honey. However, bacteria cells recovered from biofilms treated with sub-inhibitory honey concentrations had slightly increased tolerance to honey. Also, honey used at clinically obtainable concentrations completely eliminated established *P. aeruginosa* biofilms. Their findings show that manuka honey-based wound dressings are a promising treatment for infected chronic wounds, particularly those with *P. aeruginosa* biofilms.

Manuka honey have been found to be effective as much as antibiotic ear drops for the treatment of ear infection. The study found that the healing of the mastoid cavity was almost similar in both test groups and bacteria isolates tested included *Pseudomonas aeruginosa*.

Other honeys such as chestnut honey, pine honey, the Malaysian tualang honey,, and honey in combination with natural and synthetic agents have shown positive antimicrobial activity against *P. aeruginosa*.

Honey from Nigeria has also proven to be quite effective especially against wound and respiratory tract bacteria including *Pseudomonas aeruginosa.* However, there have

been more literatures reporting the resistance of *P.aeruginosa* against honey from different parts of Nigeria.

2.2.7 Current Treatment Strategies of Resistant *Pseudomonas aeruginosa* Infections

Non-antibiotic therapeutic approaches, particularly quorum sensing inhibition, phage therapy, and the use of nanoparticles, have demonstrated significant antimicrobial effects in vitro or in animal models against antibiotic-resistant strains of *P. aeruginosa*, and they are being considered as alternatives or adjuncts to conventional antibiotics.

2.2.8 Glutathione and its Encoding Genes

Glutathione

Glutathione is a substance made from the amino acids; glycine, cysteine, and glutamic acid. It is produced by the liver in humans and is involved in many body processes. Glutathione is involved in tissue building and repair, making chemicals and proteins needed in the body, and in immune system function. This low-molecular-weight tripeptide thiol glutathione is also a component of eukaryotes and several bacteria, including *P. aeruginosa*. Glutathione is the predominant low molecular weight thiol in Gram-negative bacteria. Glutathione is produced by just a few Gram-positive bacteria, including *Listeria monocytogenes* and *Streptococcus agalactiae*[82]. The enzyme is produced when *gsh*A-encoded -glutamylcysteine synthetase ligates the amino group of cysteine to the -carboxyl group of glutamate, the resultant -glutamylcysteine is then condensed with glycine by the *gsh*B-encoded glutathione synthase to produce glutathione.

In bacteria, glutathione not only plays an important role in maintaining the normal oxidation state of protein thiols, but it also protects the cell against the effects of low pH, chlorine compounds, and oxidative and osmotic stressors. Furthermore, by directly modifying proteins via glutathionylation, glutathione has emerged as a posttranslational regulator of protein function under oxidative stress[82]. Aside from their usual roles as

redox regulators in bacteria, glutathione has been shown to influence virulence and bacterial disease. GSH plays a variety of roles in virulence, including stimulation of virulence gene expression and contribution to optimum biofilm development. GSH may also be transformed to hydrogen sulfide (H_2S), which is required for some bacteria's pathogenicity. Aside from GSH, several bacteria create additional low molecular weight thiols that impact bacterial virulence, such as mycothiol and bacillithiol[60].

***gsh*A and *gsh*B genes**

The oxidant expression profiles of the *gsh*A and *gsh*B genes suggest that these genes play a role in protecting cells from oxidants that highly induce their expression[84]. The genes *gsh*A (PA5203), which codes for the glutamate-cysteine ligase, and *gsh*B (PA0407), which codes for -glutamylcysteine synthetase and glutathione synthase, are both found in the P. aeruginosa PAO1 genome. *gsh*A and *gsh*B also operate in the production of glutathione and another antioxidant, ergothioneine, as well as of the glutathione analogues ophthalmate and norophthalmate.

In research to study the genes responsible for virulence in *P. aeruginosa* PA14 in Caenorhabditis elegans, both the *gsh*A and *gsh*B transposon mutants showed decreased infectivity. It was also discovered that a *gsh*A transposon mutant could spread in a neutropenic mouse model but did not vary in its capacity to colonize the gastrointestinal tract, whereas another study discovered an elevated abundance of a *gsh*A transposon mutant in both acute and chronic wounds.

It has been demonstrated that a substantial level of -glutamylcysteine is present in *gsh*B mutants of other bacteria, including E. coli, and this serve as a partial GSH substitute. Transposon mutants disrupted in the *gsh*A glutamate-cysteine ligase gene were studied in order to determine the function of GSH without interference from -glutamylcysteine and it was found that these mutants absolutely lacked GSH and -

glutamylcysteine, according to thiol analysis. Additionally, introducing the natural *gsh*A gene into one of the mutants caused GSH levels to return to those of the wild type. The study showed that there are several effects of *P. aeruginosa* lacking GSH. Although the mutant strain and wild-type strain grew equally well on enriched media (TSB), the mutant showed a little growth deficit in minimum medium (M9). Also, the *P. aeruginosa gsh*A mutant is less susceptible to H_2O_2 and CHP exposure, but it is more vulnerable to oxidative stress brought on by the redox cycler methyl viologen. This contrasts with a Salmonella enterica *gsh*A mutant, which was responsive to H_2O_2 but not to methyl viologen[56].

2.3 Review of Empirical Studies

Studies have showed that honey possess the ability to inhibit or eradicate *Pseudomonas aeruginosa* including its biofilm. However, some bacteria especially *Pseudomonas and Proteus* have shown to be resistant to some honey found in Nigeria. A study reported that honey showed an inhibitory action against *Pseudomonas aeruginosa* isolated from wound sites however, it was less effective compared to *Staphylococcus aureus*, this they explained could be due to emerging resistivity of *Pseudomonas aeruginosa.* In contrast, a study conducted in Abuja, the capital territory of Nigeria showed that *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* were resistant to five market samples of honey at all concentrations tested. Resistance to honey bought from the eastern, southern, northern and western part of Nigeria has also been reported.

In a recent study in Nigeria, it was found that none of the honey tested had bactericidal activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus* but had only weak antibacterial activity with inhibitory zone diameter of 6-12mm. Another study showed that honey from most parts of Nigeria had no antimicrobial effect against *Pseudomonas aeruginosa*. A study that assessed the antibacterial activities of three honey

samples collected from Nsukka in Enugu State showed that the different honey samples had antibacterial activity on all the strains of bacteria tested except for *Pseudomonas aeruginosa* with minimum inhibitory concentration ranging from 10mg/ml to 27 mg/ml[78]. Since it is known that some strains of *Pseudomonas aeruginosa* are resistant to honey from Nigeria, one should seek to know factors responsible for this resistance and the genes associated with it in order to accurately propose a suitable additive for synergistic action against the pathogen.

2.3.1 Molecular Studies

Molecular studies that have examined the impact of honey on *Pseudomonas aeruginosa* have used transcriptomic analysis to study its response to honey. A study investigated the antibacterial effects of pine honey against *P. Aeruginosa* PA14 at the molecular level using a global transcriptome approach via RNA-sequencing. Pine honey treatment was applied at sub-inhibitory concentration and short exposure time (0.5× of minimum inhibitory concentration –MIC- for 45 min). The mechanism of action of manuka honey and its key antibacterial components using transcriptomic approach in *Pseudomonas aeruginosa* has also been investigated. In a current study, a transcriptome investigation was performed to explore the mechanism underlying the biofilm dispersal of *P. Aeruginosa* after the exposure to *the Malaysian stingless bee* honey.

A study that investigated the effects of Trigona honey on the gene expression of two virulence genes of the *Pseudomonas aeruginosa* ATCC 10145 strains showed a decrease of the *algD and oprF* genes after exposure to 20% concentration of the honey. Another study that used kelulut honey assessed the gene expression profile of some biofilm formation genes of *P. aeruginosa*, *fli*A, *fli*C, *opr*B and *opr*H were down regulated while the *opr*C, *las*R, and *alg*U were upregulated.

Real-time RT-PCR was used to examine the *gsh*A and *gsh*B gene expression patterns in response to stress. Superoxide generators (0.5 mM plumbagin, 0.5 mM menadione, and 0.5 mM paraquat), organic hydroperoxides (1 mM cumene hydroperoxide and 1 mM t-butyl hydroperoxide [tBH]), 1 mM H2O2, and a thiol-depleting agent were used to challenge PAO1 cultures (0.5 mM N-Ethylmaleimide). In contrast to uninduced levels, *gsh*A expression was significantly elevated by peroxides such as H_2O_2 (2.6 0.3-fold), cumene hydroperoxide (6.3 0.2-fold), and t-butyl hydroperoxide (2.7 0.2-fold). Superoxide generators and N-Ethylmaleimide, however, did not significantly increase the expression of *gsh*A[85].

A Salmonella mutant defective in the *gsh*A gene was attenuated in the acute form of salmonellosis, demonstrating a function for glutathione in pathogenesis. Additionally, mice's susceptibility to infection by *Listeria monocytogenes* mutants with deficient glutathione production was 150 times lower. These GSH-induced virulence traits in *L. monocytogenes* could be connected to its allosteric binding.

The effects of Nigerian honey on gene expression in *P. Aeruginosa* have not been empirically studied in the past. The presence or prevalence of the glutathione producing genes have not been researched either.

2.4 Summary of Gaps in Literatures Reviewed

The studies above have shown the effects of honey on different virulence genes of *P. aeruginosa* and hydrogen peroxide-the active antimicrobial agent in honey, however, no study from my research and literature review have shown the gene expressions related to honey found in Nigeria. Few molecular studies exist that targets the presence and prevalence of virulence factor genes in Nigeria. Most of the work done focus on the prevalence of antibiotic resistant genes in Pseudomonas. Molecular studies on honey from Nigeria are scarce and little is known about the mechanisms of action of honey at the

molecular level on pathogenic bacteria. Roles of the glutathione producing genes, *gsh*A and *gsh*B have not been researched in relation to honey but only the expressions of the genes in response to H_20_2 have been recorded.

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Chapter Three

Methodology

3.1 Research Design

This study is a descriptive study with an experimental research design that was conducted between July to October, 2022.

3.2 Population of the Study

The study population is the *Pseudomonas aeruginosa* strains isolated from clinical samples submitted to the state government-owned teaching hospital in Ogbomoso, Oyo State. Test isolates of *Pseudomonas aeruginosa* were obtained from cultures of clinical samples from the Medical Microbiology Laboratory of the Ladoke Akintola University of Technology Teaching Hospital (LTH) in South West, Nigeria. Only isolates that have been confirmed to be *P. aeruginosa* through biochemical test and the polymerase chain reaction were considered for this study. Only confirmed isolates during the period of the research were included in this study.

The strains used and the samples site of collection are shown in the table 2.

Table 3.1- *Pseudomonas aeruginosa* strains used, samples and their sites of isolation

Isolates	Sample site of collection	Type of sample
P1	Wound	Wound Swab
P2	Wound	Wound Biopsy
P3	Ear	Ear Swab
P4	Ear	Ear Swab

P5	Throat	Sputum

Source[13]

3.3 Honey Samples and Bacteria Strains

3.3.1 Bacteria Strains

Eight selected *Pseudomonas aeruginosa* isolates from clinical samples obtained during the duration of study was used for the research. The strains of *Pseudomonas aeruginosa* were isolated from samples (such as wound swabs, urine, blood culture, ear swab etc.) from patients visiting with various infections at the medical microbiology laboratory of the teaching hospital. The samples are usually collected aseptically by attending physicians/nurses in various clinics and wards in sterile universal containers, swabs and blood culture bottles.

3.3.2 Honey Samples

The honey samples (bitter and sweet) were collected at apiary farms in Southwestern Nigeria. The Bitter honey was harvested at a branch of Community Lifestyle Improvement Project Farm (RC:2930642) located at Modakeke (7° 27' 19.6704"N and 4° 32' 39.8112" E), Osun State, Nigeria and the sweet honey was harvested at the Federal University of Technology Akure (FUTA) (7 15'0 2.7756" N and 5 12' 36.9576" E), Ondo State Nigeria as was used in a previous study.

The honey was extracted aseptically from the comb, stored in an air-tight sterile universal sample bottle, and kept in a dark, cool, dry place at room temperature before use in the laboratory.

3.4 Culture and Isolation of Bacterial Strains

The samples were inoculated onto the surface of sterile Blood and MacConkey agar plates and incubated for 24 hours at 37 °C. The suspected colonies were isolated in a

pure form by subculturing for further studies on a Mueller Hinton (Oxoid) agar plate. Distinctive morphological properties of each pure culture such as colony form, elevation, pigmentation and cyanin production were observed. Further standard microbiological and biochemical identification using gram staining and biochemical characterization including catalase, coagulase and oxidase tests were done based on the methods previously described. Identified isolates were stored in mueller hinton broth in a -20 freezer before molecular analysis.

3.5 Susceptibility Testing

3.5.1 Antibiotic Susceptibility Testing

Antibiotic susceptibility testing was done for 8 *P. aeruginosa* isolates by disk diffusion method on a Mueller Hinton agar plate. A random selection of one out of 3 colonies grown on the plate were picked from a pure culture as previously described. A suspension of each isolate was made with normal saline and was standardized using the Mac Farland standard (0.5 barium sulphate solution) and antibiotic susceptibility testing to eight antibiotics in four classes was done using the Kirby Bauer disc diffusion method as described.

The antibiotics disks used for this study were Amikacin, cefepime, Cefuroxime, Levofloxacin, Azithromycin, Ceftazidime, Augmentin, Ceftriaxone, Ofloxacin and Gentamycin. The test organisms were evenly seeded over the Mueller-Hinton agar surface with the aid of a swab stick. The antibiotic discs were carefully placed at equidistance using a sterile forceps on the Mueller Hinton agar plate, and then incubated for 16–18 hours at 37°C. Using a ruler and a standard table provided by the CLSI, the diameters of the zone of inhibition surrounding the discs were measured to the closest millimeter and classified as sensitive, intermediate, and resistant.

3.5.2 Honey Susceptibility Testing

Susceptibility testing of the honey was performed using the agar well diffusion technique[3] according to criteria set by CLSI, 2018[4]. The inoculums were made by selecting portions from the tested isolates using a sterile wire loop and suspending them in sterile normal saline. A comparison of the suspension's density with an opacity standard on a McFarland 0.5 barium sulphate solution was made. A sterile swab was used to disperse the isolate over the agar plate after being dipped into the suspension of the isolate and squeezed to remove excess fluid against the tube's wall. The Mueller-Hinton agar surface was evenly seeded with the test organism, and the plates were left on the bench to allow the extra fluid to be absorbed. Wells in the agar media were drilled using a sterile borer (6 mm in diameter, 4 mm deep, and spaced roughly 2 cm apart). Using a sterile syringe, 0.1 ml(100µl) of raw undiluted honey was introduced to the wells in the plate. The plates were incubated for 24 hours at 37°C.

The average widths of inhibitory zones were measured in millimeters and reported. Imipenem antibiotic disc was used as a positive control while the negative control well was filled with sterile distilled water as previously described.

The zones of inhibition around the antibiotic disc used and the wells containing honey were measured and the results of the test determined to be sensitive, intermediate or resistant when compared with the CLSI M100 2020 guidelines. The honey's sensitivity result was interpreted in comparison with a conventional broad-spectrum antibiotic (gentamycin).

3.5.3 Bacteria Preparation for Extraction

The bacterial isolates were exposed to honey following a previously used broth tube dilution method. Each bacterial isolate was suspended in 1mL of nutrient broth to a turbidity equivalent to 0.5 Mac Farland standard and were tested in the presence of different concentrations of honey appropriate dilutions of the honey samples, each isolate

was incubated with 1mL of 100%, 50%, and 25% concentrations (v/v) of the broth diluted honey samples at 37°C. In a stand, six sterile test tubes were arranged. Nutrient broth was made according to the manufacturer's directions and used to make the dilutions. Two milliliters of nutrient broth without bacterial suspension served as the negative control. For the next five test tubes, honey samples were serially diluted in 1 mL final volumes of nutritional broth to achieve concentrations. Except for the negative control, each tube was infected with 1ml of bacterial suspension (108 cfu/mL) and incubated at 37 °C for about 3 hours[8].

3.6 Molecular Analysis

3.6.1 DNA Extraction

DNA extraction was done for all isolates presumed to be *Pseudomonas aeruginosa* by biochemical tests for molecular confirmation**.** Genomic DNA was extracted from isolates cultured on blood agar, followed by individual inoculation into nutrient broth and incubation for 24 h at 37 °C. The isolates were extracted using the boiling method as previously described.

About 3-4 colonies of the *P. aeruginosa* were picked from the culture plates with flamed wire loop and emulsified in 500µl of distilled water in Eppendorf tubes. The cells were washed thrice with distilled water by centrifugation at 1000rpm for one minute. The sediments were eluted with 500µl of distilled water and heated at 100°C for 7mins in a digital dry bath. The tubes were then transferred into ice to be cold shocked for 2 mins. The extracts were frozen in a -20°C freezer for storage before use.

3.6.2 Quantification of Extract

The quantity and quality of the purified extract was checked using the DeNovix DS-11 FX spectrofluorometer/Fluorometer and A260/A280 ratios of approximately 2

were considered adequate for inclusion in the study. The DNA were stored at -20 °C before their further use for the purpose of qPCR.

3.6.3 Primers

The primers used for this study are shown in Table 2.

Table 3.2

DNA Sequences Used in PCR and Real-time RT-PCR

Gene	Sequence (5'-3')	Reference
GshA	F-CGCTACGGCAAGACCATG R-GCGCTCCAACTGGCTCGG	Wongsaroj et. al.10
GshB	F-CGCATGCGCCCGCTGAAGG R-GCGCGCCAGGCAGTAGGG	Wongsaroj et. al.10
16S rRNA	F-GCCCGCACAAGCGGTGGAG R-ACGTCATCCCCACCTTCCT	Wongsaroj et. al.10

The *16S rRNA* gene was used for amplification and was also used as an internal control and reference gene to normalize the cDNA samples. The primers were synthesized in the NIMR-MTN oligosynthesis laboratory, Yaba, Lagos, Nigeria.

3.7 PCR Analysis

3.7.1 PCR Amplification

The prevalence of the two virulence factor genes was determined in a separate reaction for each gene. For a preliminary confirmation study, conventional PCR for amplification of the glutathione producing genes was performed and the procedure was carried out with methods previously described.

The amplification PCR was performed with the Eppendorf Thermocycler at the Center for Remerging Infectious Diseases (CERID) LAUTECH, Ogbomoso. The 16SrRNA, *gsh*A and *gsh*B primers were first used to amplify and confirm the *Pseudomonas aeruginosa* specie and also to check the presence of the *gsh*A and *gsh*B genes in the bacterial genome.

Each reaction mixture contained 2µl of buffer, 10µl PCR water,0.4µl of forward and reverse primers, 1.6µl of $MgCl_2$ and 0.2µl each of dNTPs and Taq polymerase aseptically dispensed and vortexed in PCR tubes before being placed in the PCR machine. The thermocycling conditions used for the amplification were 95°C for 2 mins and 94°C for 20 seconds for denaturation, 56°C for 30 seconds for annealing, 72°C for 1 minute for extension and a 10°C hold between cycles for the 16SrRNA amplification. The cycling conditions were also adapted to the melting temperatures of the primers used for amplification of the two studied genes. Thirty cycles were used for the amplification.

3.7.2. Agarose Gel Electrophoresis

To prepare 1% agarose gel, 1g of the agarose powder was dissolved in 100ml of Tris/ Borate/ EDTA (TBE) buffer using a magnetic stirrer and hot plate. 1µl of ethidium bromide was added to the mixture before pouring into the electrophoretic gel tray.

The amplicon from the PCR were mixed with the loading buffer and SYBR green dye and electrophoresed one hour in TBE buffer. For each target, two reactions were carried out, and the amplicons, a positive, a negative control and DNA ladder were subjected to gel electrophoresis for one hour and the bands were viewed with the azure biosystems 200 transilluminator. The expected base pair for the 16SrRNA gene is about 1400 base pairs(bp) while that of *gsh*A and *gsh*B were about 1500bp and 1000bp respectively

3.7.2 qPCR Analysis of Isolates' Gene Expression

After the 16S rRNA gene's presence was established, the genes' expression was also examined. 3 isolates with glutathione producing genes *gsh*A and *gsh*B found by PCR in their genomic DNA were subjected to RT-qPCR for the gene expression analysis. Each isolate's relative quantification of glutathione gene expression was compared to that of the strains that received no honey. For relative quantification, the C_T value was obtained. Melting curves were constructed for each gene studied and each curve had only one peak where the variation in temperature was not greater than 0.5 °C per sample in each of the genes analyzed. Real-time PCR was performed as previously described using cDNA to determine gene expression levels. The Line Gene Bioer 9600 was used for the amplification. This was done at the molecular laboratory of the Babcock University Teaching Hospital, Ilishan Remo, Ogun State, Nigeria.

3.7.3 qPCR Sample Preparation

RNA Extraction

Total RNA samples were extracted from the exponential phase (OD_{600nm} of approximately 0.5 after 3 h of growth) cultures of the 3 selected isolates treated (at 100%, 50%, 25%, and untreated with the sweet and bitter honeys at 37° C. After 3 hours, the bacteria were pelleted by centrifuging at 14,000 *x g* (~14,000 RPM) for 1 minute. The supernatant was decanted and the remaining media or honey suspension was carefully removed by aspiration. The bacteria were resuspended in 100µL of the TE buffer by vortexing. It was incubated at room temperature for 5 minutes.300 µL of the Buffer RL provided by the manufacturer which contains guanidinium salt was added and the solution was vortexed vigorously for 15 seconds. 200 µL of 96 – 100% ethanol was then added to the lysate and mixed by vortexing for 10 seconds.

Total RNA was extracted using Norgen's Total RNA Purification Kit (Biotek Incorporation) prior to performing cDNA synthesis. The protocol for extraction was done strictly following the manufacturer's instruction.

Nucleic Acid Measurement (RNA)

The concentration and purity of the samples were determined using Thermo Scientific NanoDrop Lite spectrophotometer, at A260 nm.

cDNA Synthesis

This was achieved using FIREScript® RT cDNA synthesis kit by Solis BioDyne DS-06-15 v3. A total reaction volume of 50 µL with final concentration of Oligo (dT) primer (100 µM) at 12.5 µM, random primers (100 µM) at 12.5 µM, dNTP MIX (20 mM of each) 1250 µM, 10× RT reaction buffer with DTT at 2.5×, FIREScript® RT at 25

U/µL, RiboGrip™ RNase inhibitor (40 U/µL) at 2.5 U/µL were used, Nuclease-free H_2O was added to make up the reaction volume and the template was added.

The RT PCR program for the cDNA synthesis include: primer annealing at 25°C for 5-10 minutes, Reverse transcription at 50°C for 15-30 minutes and enzyme inactivation at 85°C for 5 minutes.

Nucleic Acid Measurement (cDNA)

The concentration and purity of the cDNA samples were again determined using Thermo Scientific NanoDrop Lite spectrophotometer, at A260 nm. A ratio not more than 2.0 was considered suitable for the analysis.

3.8 RT-qPCR Procedure

1microgram of RNA was added to a total volume of 20 µL with final volume of Luna Universal One-step Reaction mix (2x), Luna warmstart RT enzyme Mix (20x), Forward primer (0.4 µM), Reverse primer (0.4 µM) and Nuclease free Water. The RT PCR program and cycling processes are; Reverse Transcription (55°C for 10 minutes), Initial, denaturation (95°C for 1 min), Denaturation (95°C for 10 seconds) and Extension (60°C for 30 seconds) with Melt curve set at 60-95 °C with varying time limits. The internal reference (IC) and gene of interest were captured at FAM/SYBR.

3.9 Data Analysis

Relative expression analysis was calculated and is expressed as fold-expression relative to the level of the bacteria isolate grown under uninduced condition. The result analysis was done using the Livak equation, that is; the $2^{-\Delta\Delta}C_T$ was used to analyze the relative changes in gene expression. The normalization against a reference gene method was used where Cycle Threshold (C_T) of the target gene first normalized to that of the reference genes, then the change in C_T (ΔC_T) of the test sample was normalized to the ΔC_T of the calibrator (control) sample. Normalization is calculated thus;

ΔC_T (Calibrator)= C_T (target gene, calibrator) – C_T (reference gene, calibrator)

$\Delta\Delta$ C_T= ΔC_T (test)- ΔC_T (calibrator)

The fold difference in the expression was then calculated using the expression ratio $2^{-\Delta\Delta}C_T$. If the delta-delta Ct has a negative value, the gene of interest is downregulated, because the fold change will be smaller than 1. On the other hand, if the delta-delta Ct has a positive value, the gene is upregulated and the fold change is >1.

The descriptive statistics and graph showing fold change expression was plotted using the Microsoft word Excel application.

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Chapter Four

Results and Discussion of Findings

4.1 Presentation of Data

4.1.1 Antibiotics and Honey Susceptibility

The isolates showed varying degrees of susceptibility to the antibiotic discs used. The strains however showed lesser sensitivity to the honey samples compared with the positive control antibiotic disc used. All the isolates were interpreted to be intermediate to the imipenem disc used as positive control showing clearance zones of diameters of about 16mm.

The result of the honey sensitivity of the *Pseudomonas aeruginosa* strains showed that all except one of the isolates were resistant to the honey samples using the CLSI standard of ≥ 15mm sensitive zone of inhibition for gentamycin against *Pseudomonas*. Only one of the isolates (P1) was sensitive to the sweet honey.

The results of the tests are shown in the tables 4, 5 and 6.

Table 4.1- Antimicrobial Susceptibility of the five selected *P. aeruginosa* isolates against selected antibiotics by disk diffusion test method

Isolate	Sensitive	Intermediate	Resistant
P1	Meropenem Imipenem Azithromycin		Cefepime, Ofloxacin, Ceftazidime, Ceftriaxone, Levofloxacin, Cefuroxime
P2	Augmentin Amikacin Ceftazidime Levofloxacin	Cefepime	Cefuroxime Amoxicillin Azithromycin
P3	Ceftazidime, Gentamycin	Levofloxacin	Cefepime, Augmentin, Cefuroxime
P4	Ofloxacin Ceftriaxone	Ceftazidime	Augmentin Cefepime Amikacin
P5	Amikacin Ceftazidime	Cefepime	Cefuroxime Levofloxacin Azithromycin

Source[22]

Table 4.2- Sweet and Bitter Honey susceptibility of the five selected *P. aeruginosa* isolates by agar well diffusion method

Isolate	Sweet Honey Susceptibility Diameter zone of Inhibition	Bitter Honey Susceptibility Diameter zone of Inhibition (mm)
P1	16mm (Sensitive)	7mm (Resistant)
P2	10mm (Resistant)	4mm (Resistant)
P3	8mm (Resistant)	8mm (Resistant)
P4	9mm (Resistant)	7mm (Resistant)
P5	0mm (Resistant)	3mm (Resistant)

Source[22]

4.1.2 Genes expression of *P.aeruginosa* to Sweet Honey

The two genes (*gsh*A and *gsh*B) involved in glutathione synthesis were identified from extensive searches of the current literature as possible targets limiting the efficacy of honey therapy, and differential regulation in response to sweet and bitter Nigerian honey treatment was determined by qPCR. An 8.04 and a 9.0-fold reduction in the expression of *gsh*A was observed in the 25% and 50% respectively of the sweet honey treatment samples in the strain isolated from a wound site(P1). P2 strain that was also isolated from a wound source showed an 8.6 and an 9.2-fold reduction for 25% and 50% sweet honey treatment respectively for same gene. Lastly, the strain isolated from an ear infection showed 8.5 and 8.9-fold reduction in the *gsh*A gene for the 25% and 50% sweet honey treatment. Sweet honey was first considered for the expression analysis because it has more antimicrobial effect on the isolates than the bitter honey.

There was a significant reduction in the expression of genes *gshA* and *gshB* of *P.aeruginosa* after being treated with 25% and 50% of sweet honey. As shown in Table 7 and Figure 3, results showed all genes were downregulated and different degrees of downregulation were observed. All the isolates treated with 100% of the honey (undiluted honey samples) did not show any expression of the genes of interest.

Table 4.3- Effect of Sweet honey on the expression of *gsh*A gene in *P.aeruginosa* detected by RT-qPCR

Test Isolate in Honey Dilutions	Expression Fold Change $\Delta\Delta C_T$	Expression Fold Change $2^{-\Delta\Delta C_T}$
$P1_{25\%}$	31.16	-9.4
$P1_{50\%}$	29.76	-9.0
$P2_{25\%}$	28.69	-8.6
$P2_{50\%}$	30.56	-9.2
$P3_{25\%}$	28.25	-8.5
$P3_{50\%}$	25.36	-8.9

Source[22]

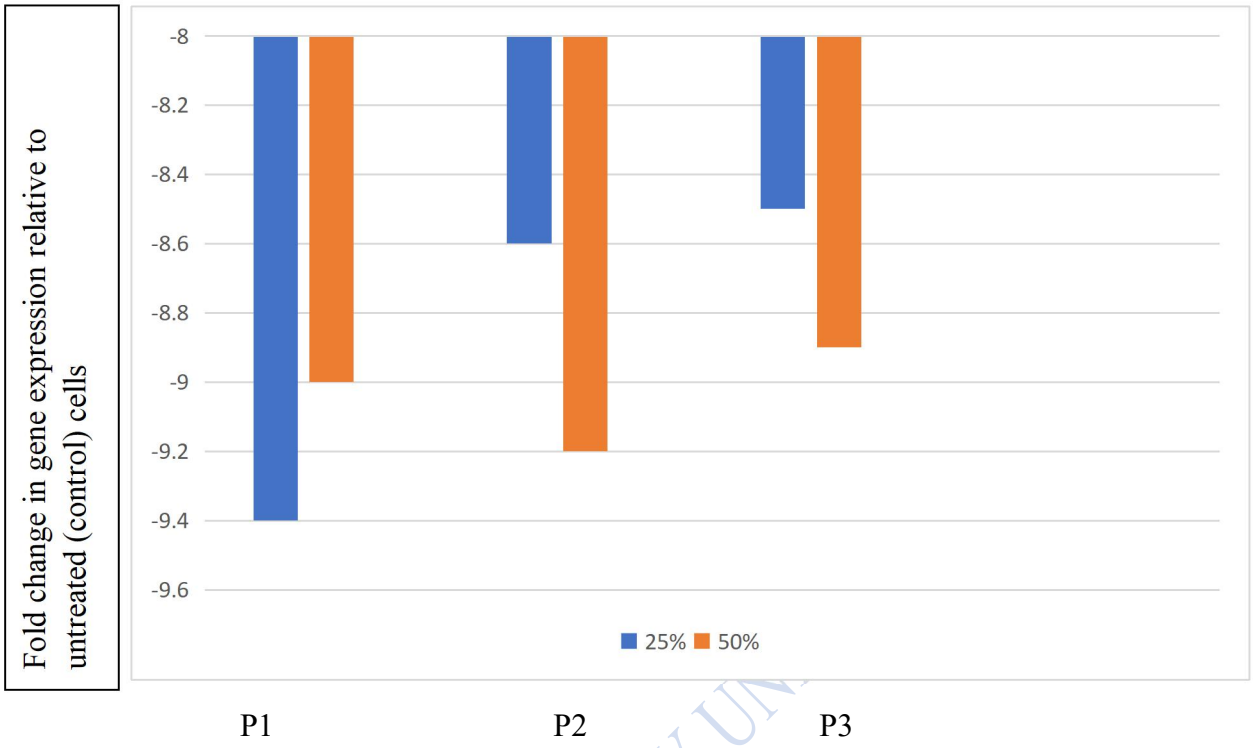

Figure 3: Graph showing the alterations in gene expression of the *gsh*A gene associated with exposure of *P.aeruginosa* in the three selected isolates (P1, P2 and P3) to sweet honey as determined by RT-qPCR. Values of fold changes are shown in relation to untreated (control) cells.

Table 4.4- Effect of Sweet honey on the expression of *gsh*B gene in *P.aeruginosa* detected by RT-qPCR

Test Isolate in Honey Dilutions	Expression Fold Change $\Delta\Delta C_T$	Expression Fold Change $2^{-\Delta\Delta C_T}$
$P1_{25\%}$	21.13	-6.4
$P1_{50\%}$	22.22	-6.7
$P2_{25\%}$	26.5	-7.98
$P2_{50\%}$	26.76	-8.1
$P3_{25\%}$	20.62	-10.6
$P3_{50\%}$	24.37	-6.21

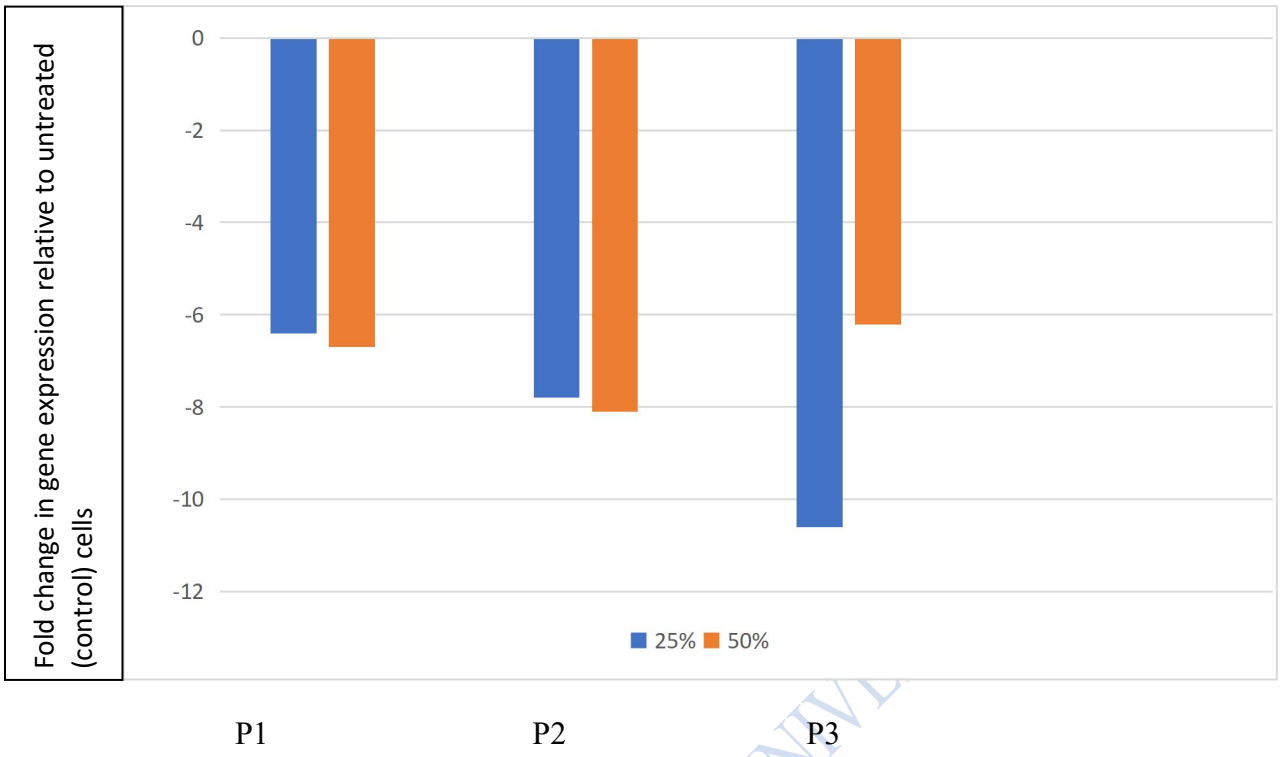

Figure 4- Graph showing the alterations in gene expression of the *gsh*B gene associated with exposure of *P.aeruginosa* in the three selected isolates (P1, P2 and P3) to sweet honey as determined by RT-qPCR. Values of fold changes are shown in relation to untreated (control) cells.

4.1.3 Genes expression of *P.aeruginosa* to Bitter Honey

For the purpose of comparison, expression analysis of the two genes was also done for one strain isolated from wound with bitter honey. It also showed 8.04 and 8.42 reduction in expression of the *gsh*A gene and 7.16 and 7.31 reduction in expression of the *gsh*B gene in 25% and 50% honey treatment respectively. It was found that both studied genes were expressed and down regulated in the test strain. Table 9 and figure 5 shows a summary of the results.

Table 4.5- Effect of Bitter honey on the expression of *gsh*A and *gsh*B genes in *P.aeruginosa* isolate (P1)detected by qPCR.

Test Isolate in Honey Dilutions	Expression Fold Change $\Delta\Delta C_T$	Expression Fold Change $2^{-\Delta\Delta C_T}$
***Gsh*A**		
$P1_{25\%}$	26.71	-8.04
$P1_{50\%}$	26.35	-8.42
***Gsh*B**		
$P1_{25\%}$	24.28	-7.16
$P2_{50\%}$	22.54	-7.31

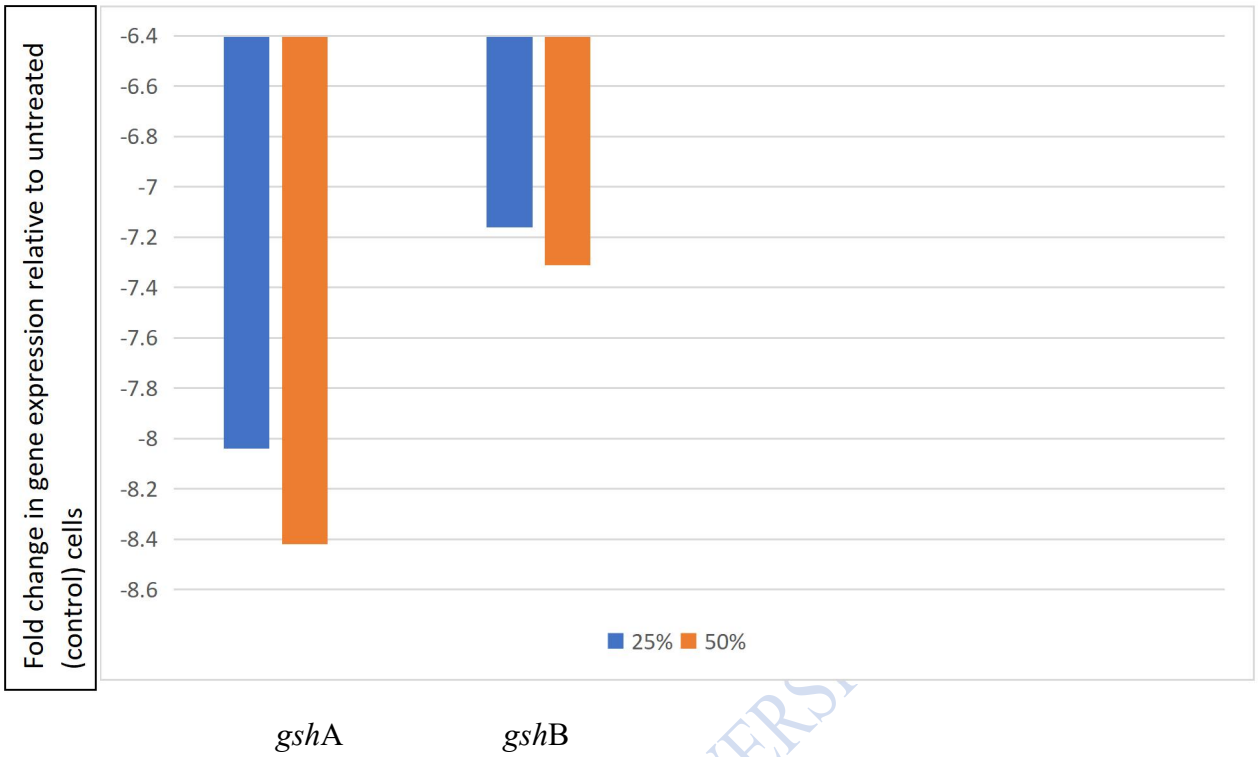

Figure 5- Graph showing the alterations in gene expression of the *gsh*A and *gsh*B genes associated with exposure of *P.aeruginosa* isolate (P1) to bitter honey at 25% and 50% concentrations as determined by RT-qPCR. Values of fold changes are shown in relation to untreated (control) cells.

4.2 Research Question

From this study, we can answer the following research questions of the study.

5. What are the antimicrobial effects of the studied honey types on clinical strains of *Pseudomonas aeruginosa*?
6. Could the poor antimicrobial effects of honey on some infections by *Pseudomonas aeruginosa* strains in Nigeria be attributed to the presence of the glutathione producing genes (*gshA* and *gshB*) in their genome or not?
7. Are these genes responsible for anti-honey resistant properties in *Pseudomonas aeruginosa*?
8. Can these virulent genes be targeted for manipulation to eradicate anti-honey resistant *Pseudomonas aeruginosa*?

4.3 Discussion of Findings

Pseudomonas aeruginosa is well known to be a notorious multidrug resistant bacteria pathogen[16]. The profiles of the susceptibility of the selected five distinct clinical isolates of *Pseudomonas aeruginosa* to antimicrobial agents using disk diffusion agar method is shown in tables 4. The result of the test showed that all of the strains exhibited intermediate susceptibility to imipenem (positive control), while the highest resistance rate (60%) was reported against cefuroxime. A recent study in Nigeria showed 100% resistance of clinical isolates of *Pseudomonas aeruginosa* to cefuroxime[17]. *Pseudomonas aeruginosa* strains that are resistant to colistin- the last line of antibiotics used to combat the bacteria have been isolated in different parts of the world[18]. This is due to the bacteria's constant mutation against antibiotic agents[19]. There is high incidence of metallo beta lactamase (MBL) encoding genes and integrons in different clinical *P. aeruginosa* from southwestern Nigeria[20]. The recent study that showed resistance in

general with a gradient manifested based on genotypic variation suggests that effective surveillance programs and antibiotic stewardship are urgently needed.

The isolates used in this study were mostly resistant to the tested honey, this confirms a recent study in Nigeria that found that none of the honey samples tested had bactericidal activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus* but had only weak antibacterial activity with inhibitory zone diameter of 6-12mm[21]. Another study also showed that honey from most parts of Nigeria had no antimicrobial effect against *Pseudomonas aeruginosa*[22]. A study that assessed the antibacterial activities of three honey samples collected from Nsukka in Enugu State showed that the different honey samples had antibacterial activity on all the strains of bacteria tested except for *Pseudomonas aeruginosa*[23].

This study describes the first systematic analysis of the effect of Nigerian honey on the level of gene expression of *P.aeruginosa* . *gsh*A and *gsh*B has been shown to be genes important for the virulence of *Pseudomonas aeruginosa*[24]. The presence and the expression of the genes in the presence of honey in this study suggests that the genes were activated for glutathione production and this could be responsible for the reduction in the antimicrobial quality of the honey tested. The downregulation of the genes is also similar to studies that have studied genes responsible for stress response and biofilm formation in bacteria. Recent research found that about 13.5% of the down-regulated genes in *P. aeruginosa* biofilm treated with Trigona honey were genes related to biofilm[25]. Additionally, it was found that the D-GMP signaling pathway and genes for diguanylate cyclases, which are involved in the formation of c-di-GMP (in the pathways involved in biofilm formation), eventually exhibit lower expression levels when exposed to trigona honey. Honey has been proven to induce alterations in the expression of different genes responsible for virulence activity in bacteria.

Numerous studies have shown that exposure to honey affects the expression of various genes related to the bacterial stress response. The universal stress protein *usp*A is downregulated in Methicillin Resistant *Staphylococcus aureus* (MRSA) after exposure to manuka honey, which decreases the bacteria's capacity to survive under cellular and metabolic stress[26]. Another more recent study demonstrated that manuka honey induced differential expression in a wide range of genes involved in the emergency and the oxidative stress response of *P. aeruginosa* cells[27]. They showed that honey affects the expression of genes in the SOS response, oxidative damage, and quorum sensing.

It has also been demonstrated that manuka honey changes the expression of genes in the *evg*AS regulon that are involved in bacterial adaptive responses to acid, osmotic, and drug resistance[28]. Later studies that used different types of honey (clover, citrus, and marjoram) showed that the gene expression profile varied depending on the honey variety, partially corroborating the findings of the initial study. Clover honey treatment resulted in an increase in *evg*A expression; however, citrus or marjoram honey exposure resulted in a decrease[8]. This was explained stating that since the main antimicrobial activity of manuka honey is not related to H_2O_2, while the other honey varieties were shown to be primarily peroxide-dependent, the differences in the expression patterns may reflect compositional differences among honey varieties as well as differences in their action mechanisms. Methylglyoxal is the main antibacterial compound in manuka honey[29]. Also, the variations in the antimicrobial mechanisms of the tested honey varieties and the variable effects they can induce on specific genes may be used to explain variations in expression pattern observed in them.

The result of this study generally showed a greater reduction of expression of these genes in the 50% honey dilutions (though not statistically different at $p > 0.05$) of both honey types which may indicate that H_2O_2 has a higher concentration in an equal

dilution of the honey which inhibited the expression of the glutathione producing genes. A study stated that H_2O_2 is highest in concentration at 30-50% dilution of honey resulting in a concentration of 5 to 100 g H_2O_2/g honey (equal to 0.146-2.93 mM)[30]. A study that also assessed the antibacterial activity of honey attributed to hydrogen peroxide, compared honey treated with bovine catalase with untreated honey[31]. They found that there was an increased minimum inhibitory concentration of treated honey compared to the untreated honey at 50% v/v honey in Mueller Hinton broth showing that the antibacterial activity of the tested honey was attributable to hydrogen peroxide[16].

The *gsh*B gene generally seem to have a lesser expression fold than the *gsh*A gene expression values in this study, this is because the *gsh*A gene is more involved in glutathione biosynthesis than *gsh*B gene[32]. It has been demonstrated that a substantial level of glutamylcysteine is present in *gsh*B mutants of bacteria, including *P. aeruginosa*, and this serves as a partial glutathione substitute while the *gsh*A mutants had no substitutes according to the study[17]. A study showed that the *gsh*A mutant strain of *Pseudomonas aeruginosa* was more susceptible to hydrogen peroxide than the *gsh*B mutant strain, while its parent strain was resistant establishing the level of importance of the genes in H_2O_2 detoxification[33].

Also, a study that manipulated the *gsh*A gene of *Pseudomonas aeruginosa gshA* showed that a *gsh*A mutant strain is defective in biofilm formation, swarming, and pyocyanin production[9]. The knock out technique was done to mutate the study strain and it was observed that a *gshA* transposon mutant has no detected glutathione production which is responsible for the protection of the bacteria from oxidative stress.

In our study we saw that these genes were expressed in the control strain (isolate without honey) and the isolates in diluted honey samples. They were however not expressed in the 100% honey sample. This could mean that other properties of the honey may be

responsible for inhibiting the *Pseudomonas aeruginosa* strains and because there was a short time of incubation, the bacteria could not resist the effects of the honey.

Undiluted pure honey has many other factors that could contribute to its antibacterial potency. One of it is the low pH, however, pH alone is not sufficient to inhibit the growth of many types of bacteria when diluted in food or in other bodily liquids. Also, because of its sugar content, pure, undiluted honey inhibits bacterial development by exerting osmotic pressure on bacterial cells, forcing water to flow out of the bacterial cells via osmosis. As a result of the dehydration, the cells shrivel and become unable to live in the hypertonic sugar solution. The acidity of honey which is between pH 3.2 and pH 4.5, is also a very marked characteristic of its antibacterial efficacy inhibiting most bacteria whose optimal growth is at 6.5-7.5. In addition, glycogenic acid is generated from glucose oxidation in bacteria by an endogenous glucose oxidase enzyme and is an extremely potent antibacterial agent[34].

Furthermore, results from a study have shown that there are multiple mechanisms of antimicrobial activity in honey[14]. The study investigated the antibacterial effects of pine honey against *P. aeruginosa* PA14 at the molecular level using a global transcriptome approach via RNA-sequencing. They observed the differential expression of 463 genes, 274 of which were down-regulated and 189 of which were up-regulated (greater than two-fold change). The Pine honey had a significant impact on a variety of biological processes, according to gene ontology analysis employed. Oxidation-reduction process, transmembrane transport, proteolysis, signal transduction, biosynthetic process, phenazine biosynthetic process, bacterial chemotaxis, and antibiotic biosynthetic process (of which glutathione also plays a role) were the most impacted down-regulated biological processes[14]. The study also concluded that multiple mechanisms of action were implicated in antibacterial activity exerted by pine honey against *P. aeruginosa*.

The expression of the studied genes in the nutrient broth suspension without honey(control) is normal and characteristics of the activation of the synthesis of glutathione in response to stress, that may have been caused by the constituents of the broth (beef extract, yeast extract, peptone and sodium chloride). Glutathione is a thiol molecule found in the majority of Gram-negative bacteria and all eukaryotic cells. It is an important chemical in cells because it is involved in cellular homeostasis, sulfur transport regulation, metabolite conjugation, xenobiotic detoxification, antibiotic resistance, enzymatic regulation, and stress response gene expression[35].A research that studied gene expression at different bacterial growth phases (lag, exponential and stationary) in the *Bifidobacterium longum* strain observed upregulation and downregulation of genes in the bacterium suspended in broth culture (Lactobacillus MRS Broth culture medium)[36] showing the activity of bacteria at the molecular level even in the absence of stress caused by antibacterial agents or chemical substances.

This study shows that the studied genes can be targeted for manipulation to eradicate anti-honey resistant *Pseudomonas aeruginosa.* Research that employed gene knock-out techniques has showed that the *gsh*A mutant strain of *Pseudomonas aeruginosa* was more susceptible to hydrogen peroxide than the *gsh*B mutant strain, while its parent strain was resistant[7]. This establishes the level of importance of these genes in H_2O_2 detoxification and marking them out as virulence genes to be targeted when honey is considered for the effective eradication of *Pseudomonas aeruginosa* infections especially as a topical agent for wound therapy or as honey drops for ear infection.

Even though this study is the first gene expression study using the Nigerian honey and glutathione producing genes, the data found supports previous findings that has studied the effects of H_2O_2 and honey on the expression of the genes responsible for stress protection and biofilm formation in *Pseudomonas aeruginosa* and it describes the effects

of the Nigerian sweet and bitter honey on *P. aeruginosa* at a molecular level. Whether these are the only targets that remains to be determined in relation to bacteria anti-honey resistance will be the subject of future research.

Endnotes

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Chapter Five

Conclusion

5.1 Summary of Findings

This study was able to establish that honey harvested in Nigeria has some antimicrobial effect against clinical isolates of *Pseudomonas aeruginosa*. Sweet honey tends to have more bactericidal properties than the bitter honey and can be explored for its antibacterial potentials.

The study has been able to establish the presence of the glutathione producing genes *gsh*A and *gsh*B in clinical isolates of *Pseudomonas aeruginosa*. Their existence means that hydrogen peroxide present in honey is exposed to the risk of deactivation by the production of glutathione. Honey with a low content of H_2O_2 may exhibit a weak antibacterial potency because of the presence of glutathione.

The study also showed that the genes were expressed in some of the isolates at different degrees of exposure to honey indicating that the genes are actively involved in the protection of *Pseudomonas aeruginosa* against the action of honey and its hydrogen peroxide content. The genes were not expressed when the isolates were exposed to 100% of the honey which could suggest that hydrogen peroxide concentration is more in diluted than raw honey samples and also depicts that raw honey have other components that have inhibitory effects on the growth of the Pseudomonas specie.

The genes showed down regulation in its expression in all the diluted honey treated samples studied indicating the ability of our locally harvested honey to suppress the expression of the glutathione producing genes.

5.2 Conclusion

Honey is a gift to humankind from God and its benefits are enormous, apart from it being used as a sweetener, it is beneficial for the treatment of infections that are caused

by *Pseudomonas aeruginosa*. This is the first study of the level of gene expression of *P. aeruginosa* after exposure to the Nigerian sweet and bitter honey. In this study, we presented the patterns of gene expression in *P. aeruginosa* strains treated with and without the Nigerian sweet and bitter honey. The study has also shown that raw undiluted Nigerian honey was able to inhibit the growth of *P. aeruginosa* isolated from wounds and ear infection. Differential gene expression in response to honey exposure exhibited downregulation of the glutathione producing genes (*gsh*A and *gsh*B) of *P. aeruginosa*. The obtained results indicate that while Nigerian honey may represent hopeful treatment for infections involving *P.aeruginosa*, there is still need for modifying our local honey to effectively eradicate infections caused by the organism. This study shows that our local honey has a lot of potentials and could be better if modified for its use in the treatment of bacterial infections.

This study has exposed and shed light to one of the hinderances to the effectiveness of honey when used as an antibacterial agent especially against a high load of bacterial infection. The virulence genes studied in this research could be targeted when manufacturing a medical grade honey used for the treatment of bacterial infections.

5.3 Recommendations

Based on the findings of this study, it is recommended that

1. Honey should be a first sought option for the treatment of infections involving *Pseudomonas aeruginosa* especially wound infection because resistance against them have been scarcely reported. Clinicians ought to routinely utilize honey for its curative function by prescribing them for patients.
2. Honeys harvested in Nigeria should be improved on to a medical grade standard to disarm the detoxifying effects of glutathione produced by most bacteria.
3. The antimicrobial potentials of honey could increase if used with H_20_2.

4. The glutathione producing genes could be targeted when producing a medical grade honey used for the treatment of infections.
5. Our locally produced honey can be used as a combination therapy with antibiotics and other antibacterial plant extract in the fight against antibiotic resistance.
6. Medical scientists should also work on including our locally produced honeys that have been certified and approved by NAFDAC as medical grade into their routine antibiotic susceptibility testing for its efficacy against infections.
7. Honey is best used diluted for its antibacterial potentials as it has been proven in previous researches and suggested in this study that a higher concentration of hydrogen peroxide could have caused a down regulation of the studied genes[1], however, further research in this area is needed.

5.4.1 Contribution to Knowledge

This research has been able to stress the importance of honey in the fight against bacterial infection especially that caused by *Pseudomonas aeruginosa*. Honey has potentials to be a good alternative for antibiotics in curing infections especially for the topical treatment of wounds and ear infections. *Pseudomonas aeruginosa* has been found to be susceptible to the sweet honey type used in this study despite being a catalase producing organism. The organism however may be effectively eradicated in the presence of higher volumes of hydrogen peroxide.

5.5 Suggested Areas for Further Research

The hydrogen peroxide concentration of all our locally produced honey ought to be analyzed to determine their antibacterial potential. Further research on this is suggested. Studies that involve detection of the presence of the glutathione producing genes is also recommended for other bacteria species especially the ones that can be easily treated using honey. The prevalence of these genes in clinical pathogenic bacteria ought also to

be known to establish their importance to be targeted for the success of honey therapy in Nigeria.

Studies should also be developed to investigate drugs capable of mutating or deactivating genes studied in this research to enable the use of our natural antibacterial substances such as honey to be more potent for the cure of infections.

Endnotes

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Appendix I

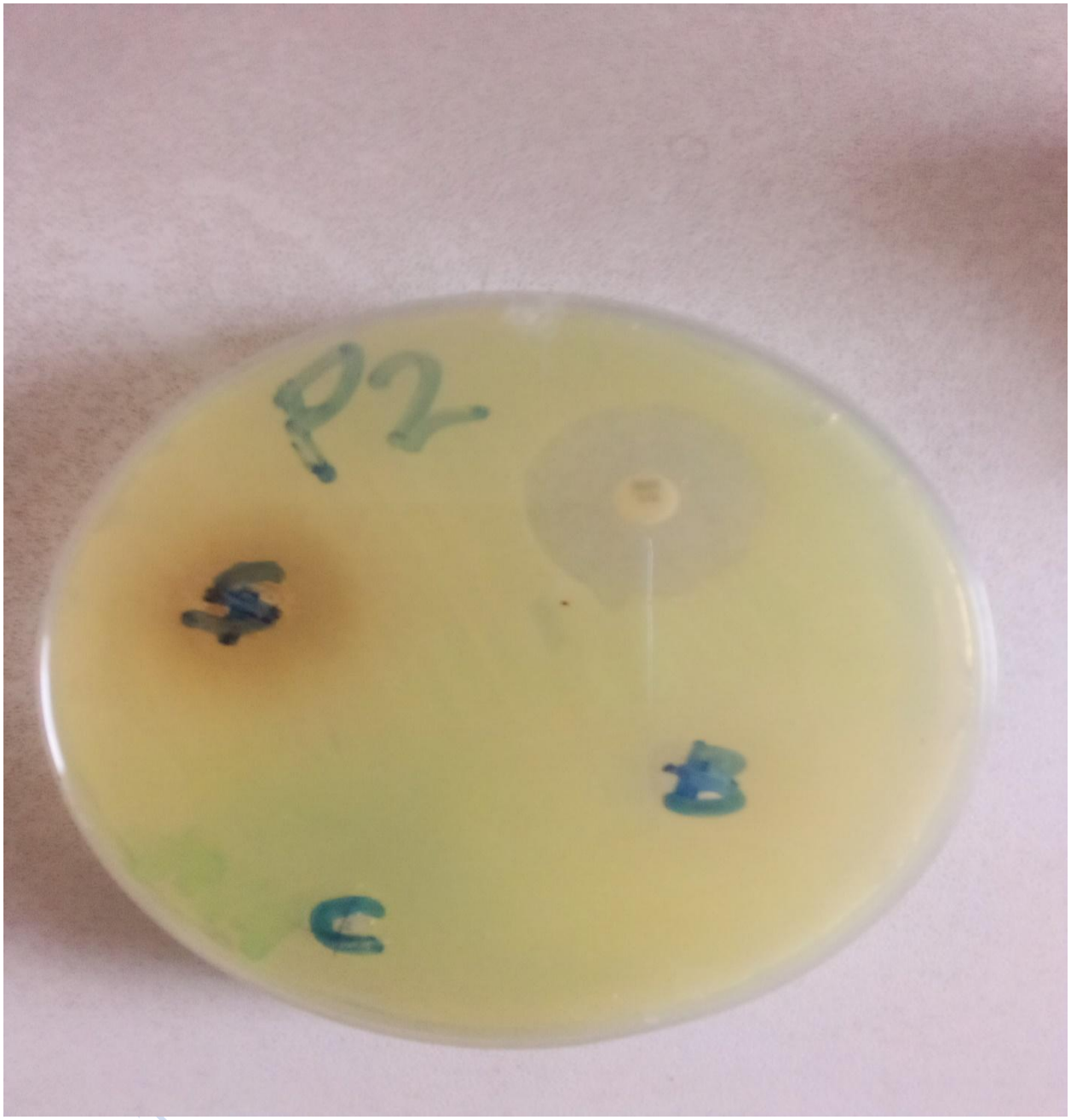

Plate 1- Shows the sensitive *Pseudomonas aeruginosa* strain to sweet honey by a brown clearance around the well S and its resistance to bitter honey at well B, C is the negative control well while the positive control (imipenem disc) showed a wide and clearer zone of inhibition. Source: Author's Field work, 2022.

Appendix II

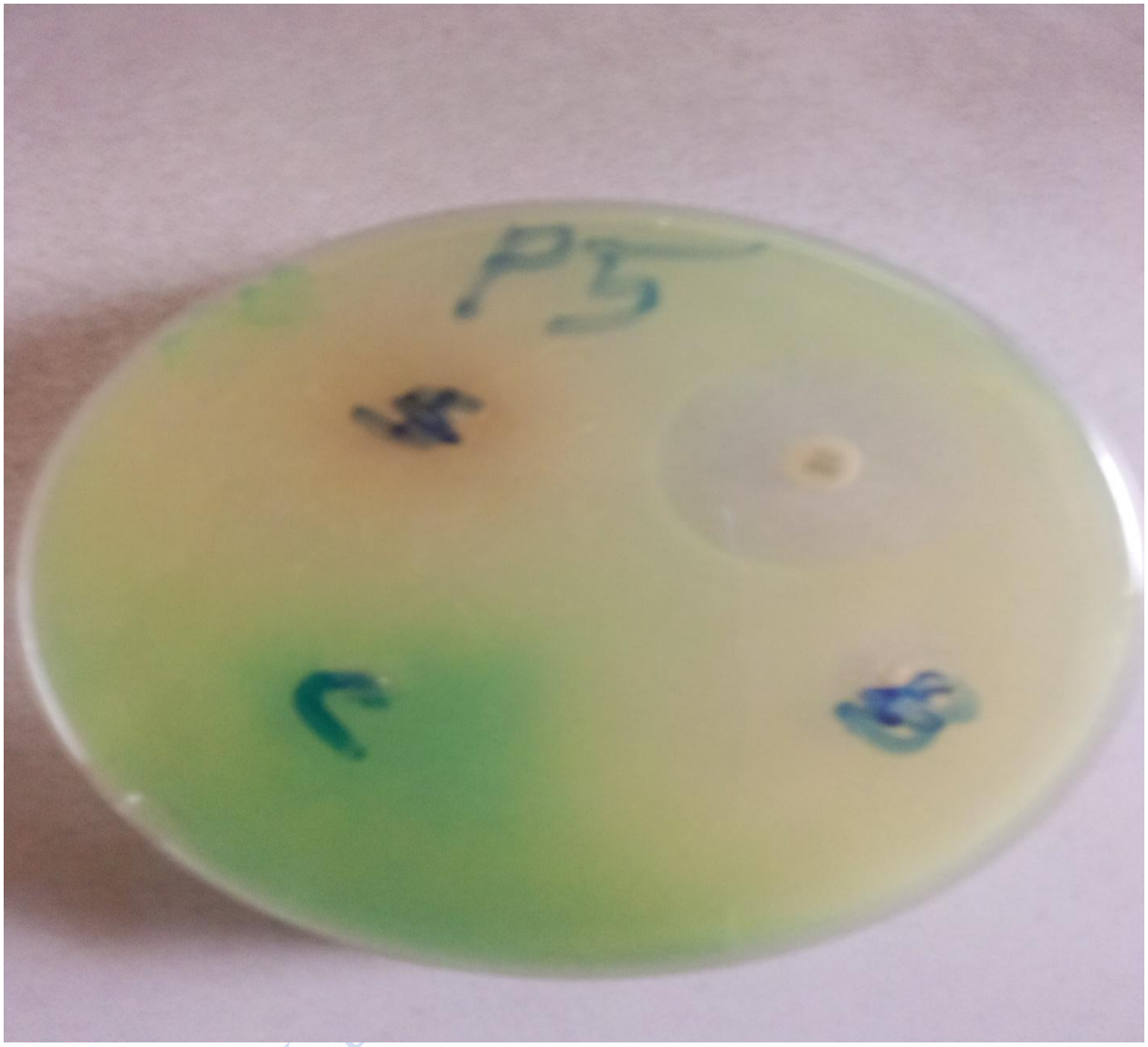

Plate 2- Shows a resistant plate of *Pseudomonas aeruginosa* against the bitter and sweet honey. Source: Author's Field work, 2022.

Appendix III

FULL GENE SEQUENCE FOR PA5203 |*gsh*A from NCBI Genbank

TTGAGCGATCTTCTCTCCCGCCGCCTGGCTCTGCTCGGCGCCGCAGCCAACCT

GCCCCTGCTGACCGAGTGCCTGCATGGCATCGAGCGCGAATGCCTGC

GAGTCGATAGCGACGGCAAGCTGGCGCTCACCCCCCACCCCCGGGCCCTGGG

TTCGACCCTGACCCATCCGCAGATCACTACCGACTACTCCGAGGCCTT

GCTGGAGTTCATCACGCCCACGGAAACCGACGTCGCCGACACTCTGGGCGAT

CTCGAGCGCATCCACCGCTTCGCCAGTTCGAAACTGGACGGCGAATAC

CTGTGGAGCCCATCGATGCCCTGCGAGCTGCCGGACGAGGAGAGCATCCCGA

TCGCCCGCTACGGCAGCTCGATGATCGGCCGCCTGAAATACGTCTACC

GCAAGGGCCTGGCGCTGCGCTACGGCAAGACCATGCAGTGCATCGCCGGCAT

CCACTACAACTTCTCCCTGCCGGAGAGGCTCTGGCCGCTGCTGCGCCA

GGCCGAAGGCAGCGAGCTGTCGGAACGCGACTACCAGTCCGCCGCCTACATC

GCCCTGATTCGCAATTTCCGCCGCTACAGCTGGCTGCTGATGTACCTG

TTCGGCGCCTCGCCGGCCCTCGACGCCGGCTTCCTGCGTGGCCGCCCGAGCCA

GTTGGAGCGCCTCGACGAACACACGCTTTACCTGCCCTACGCCACCA

GCCTGCGGATGAGCGACCTGGGCTACCAGAACAACGCCCAGGCCGGCCTGAC

GCCCTGCTACAACGACCTGCAGAGCTACATCGACAGCCTGCGCCAGGC

GGTCAGCACGCCCTACCCGCCCTACGAGAAGGTCGGCACCAAGCAGGATGGC

GAATGGGTGCAGCTGAACACCAACATCCTGCAGATCGAGAACGAGTAC

TACTCGAGCATCCGGCCAAAGCGCGTCACCTACACCGGCGAGCGTCCGGTGC

AGGCCCTGGCCGCTCGCGGCGTGCAGTACGTGGAAGTGCGCTGCCTGG

ACATCAACCCGTTCCTGCCGCTGGGCATCGACCTGGACGAGGCGCGCTTCCTC

GACGCCTTCCTGCTGTTCTGCGCGTTCAGCGACAGCCCGCTGCTGAA

CGGCGAGTGCAGCGACGCCACCGACAACTTCCTCGCCGTGGTCAAGGAAGGC

AGGCGGCCGGGCCTGCAATTGCAACGTCGCGGCCAGCCGGTGGAATTG

CAGGTCTGGGCGAACGAGCTGCTCGAACGAATCGCCGACACCGCGGCGCTGC

TCGACCGCGCGCGCGGCGGCGAAGCCCACGCGGCGGCGCTGGCAGCGC

AGCGGGCGAAGGTGGCCGATGCGGAACTGACGCCTTCGGCGCAGGTGCTGAA

GGTCATGCGCGAGCGCGGCGAGAGCTTCGAAGCGTTCTCCCTGCGCCA

GAGCCGCGAACACGCGGAGTATTTCCGCCAGCATCCGCTGGCCGCCGAAGAG

CAGGCGCGCTTCGAGAAGATGGCCAGCGACTCCCTGGCCGAACAGACG

GAGCTGGAACGCGACCAGGATGGCGACTTCGATACCTTCGTCGCCGCCTACC

AGGCGAGCATCCTCGGCCTGATCAGCAACTGA

Appendix IV

FULL GENE SEQUENCE FOR PA0407 |*gsh*B from NCBI Genbank

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AGAAGGACAGCTCGCTCGCCATGCTGCTGGCTGCCCAGGCCCGCGGCT

GGTCGCTGTTCTACATGGAACAGCAGGACCTCTACCAGAAGGCCGGCGTCGC

GCGTGGACGCATGCGCCCGCTGAAGGTCTTCAACGATGCCTCCCGCTG

GTTCGAACTGGAAGCGGAAAGCGACCAACCGCTGCACGAACTGGACGTGATC

CTGATGCGCAAGGACCCGCCCTTCGATAACGAATTCGTCTATTCGACC

TACCTGCTGGAACAGGCCGAGCGTGCCGGCGCCCTGGTGGTGAACCGGCCGC

AGAGCCTGCGCGACTGCAACGAGAAGTTCTTCGCCACGCAGTTCACCC

AGTGCACTCCGCCGACGATGGTCAGCCGGCGATCCGATATTCTGCGCGAGTTT

GCCGCCGAGCATCGTGACATCATTCTCAAACCCCTGGACGGAATGGG

CGGTTCGTCGATATTTCGTCACCGCGAAGGCGACCCGAACCTCTCGGTGATCC

TCGAGACCCTCACCCAGCATGGCAGCCAGCAGATCATGGCACAGCGC

TATCTGCCGGAAATCAAGGACGGCGACAAGCGCATCCTGATGATCGACGGCG

AACCGGTGCCCTACTGCCTGGCGCGCATCCCCGCCCAGGGCGAGACCC

GCGGCAACCTCGCCGCCGGCGGCCGCGGCGTGGCCCAGCCGCTGAGCGAGCG

CGACCGCTGGATCGCCGCCGAGGTCGGCCCGCACCTGCGCGAGCGCGG

CCTGCTGTTCGTCGGCCTCGACGTGATCGGCGATTATCTGACGGAAATCAACG

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GA

Appendix V

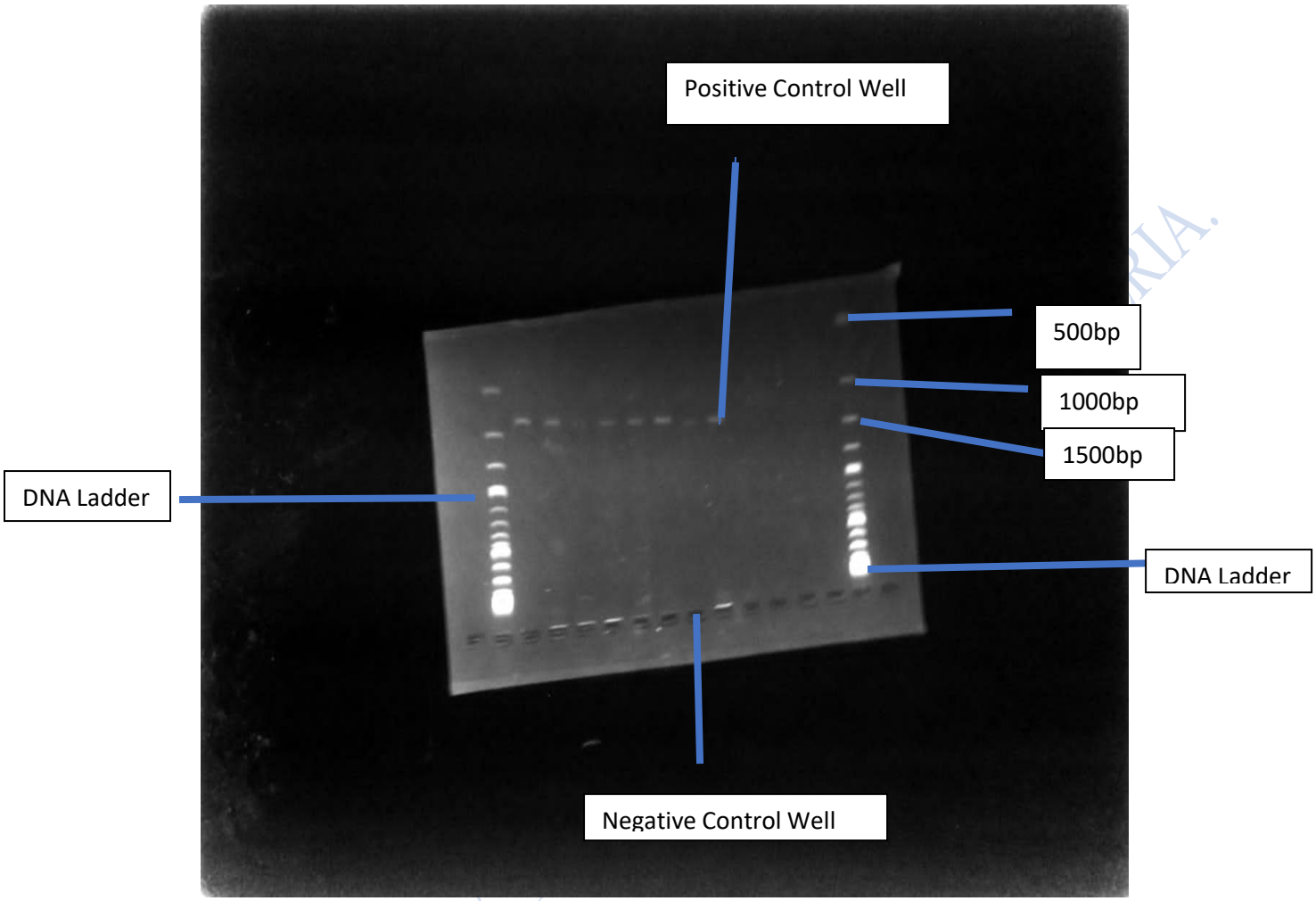

Figure 6-A picture of the gel electrophoresis of the 16SrRNA with an expected size of about 1400bp after amplification with the isolates extracts with conventional PCR. The 1kb DNA ladder was used for comparison.

Source: Author's Field work, 2022.

Appendix VI

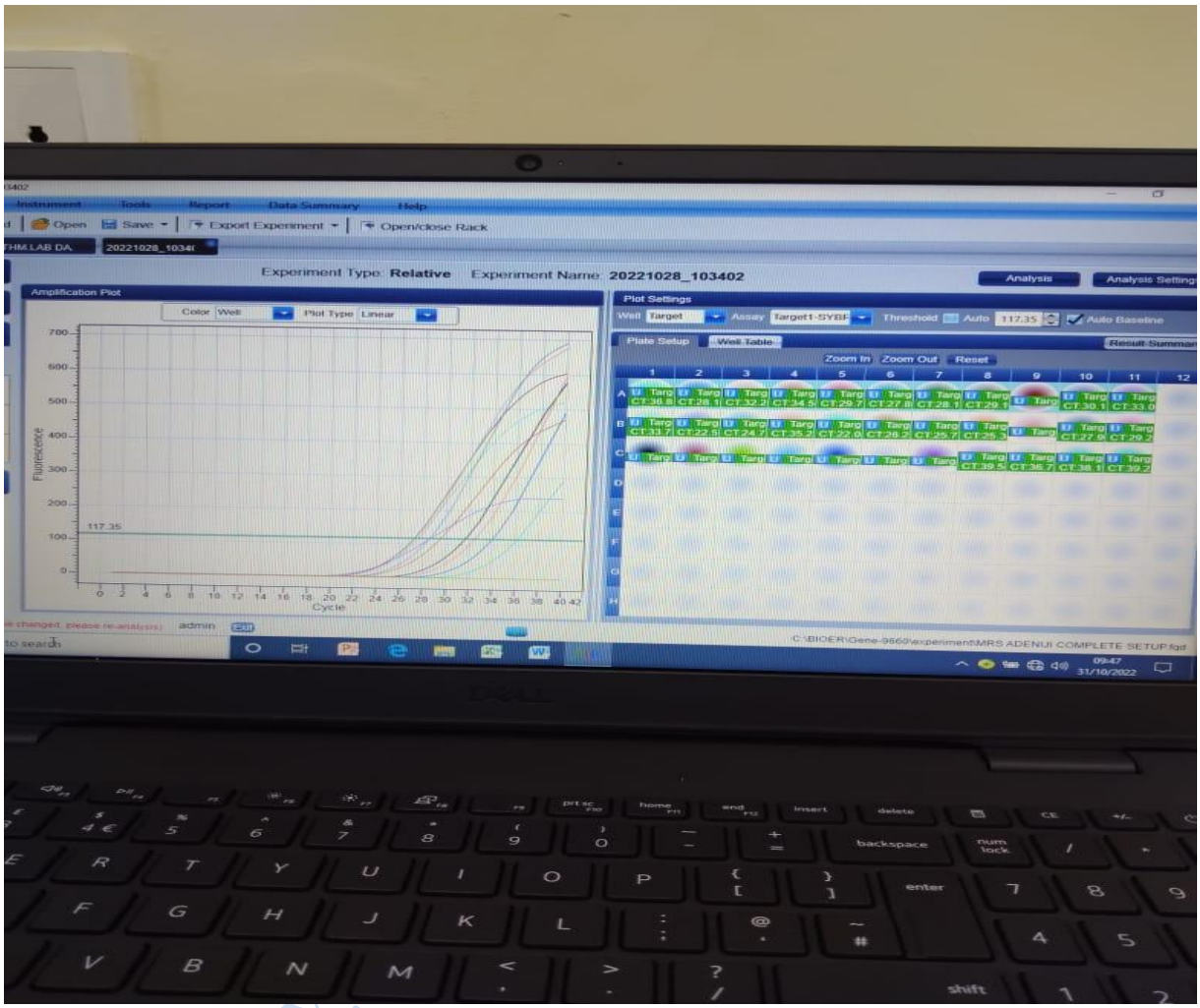

Figure 7: *gsh*A Amplification qPCR Full Spectrum Image

Source: Author's Field work, 2022.

Appendix VII

Figure 8- *gsh*B qPCR Amplification qPCR Full Spectrum Image

Source: Author's Field work, 2022.

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Bio-data

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- Member, Association of Medical Laboratory Scientists of Nigeria (AMLSN).
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- Member, Blood Drive Initiative, Nigeria

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(i)The microbial profile of the hands of food traders in Ekpoma main market, Edo State, Nigeria, In Partial Fulfilment of the Requirements for the Award of Bachelor of Medical Laboratory Science (BMLS) Degree in Medical Laboratory Science, 2012

(ii)A. J Oke, A. A Oke, A. Adegbenro, O. A Adeniji (2019) Chemical inhibition of pilus in Uropathogenic Escherichia coli: a strategy to combact infection. IOSR Journal of Dental and Medical Sciences Vol. 18, Issue 5, Series. 15, May 2019.

(iii)H.A. K Obiazi, O. A Adeniji, F.E Oviasogie, M.I Ebadan. (2018) Bacteriological and Fungi Characterization of Hand washing of Food Traders in Ekpoma Market, Edo State. Nig. Journal of Applied Science Vol. 36, pg. 179

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- Effects of Nigerian Honey on Expression of Glutathione-Encoding Genes in Clinical isolates of *Pseudomonas aeruginosa.* Presented at the Faculty of Natural and Applied Sciences, (FASCON), 3rd International Conference, 2nd- 4th November, 2022.
- AMLSCN Oyo State Annual CPD Workshop 2021
  The Science of COVID 19 Testing in Nigeria: Counting the Cost and Costing the Count. (Feb 2021)
- Bioethics in Nigeria Conference 2020
  Theme: Enhancing awareness of research ethics oversight among health researchers, clinical trialists and the public (September, 2020)
- AMLSCN UCH Ibadan 2019 Annual CPD Workshop
  Theme: Medical Laboratory Practice in P6 Medicine (July, 2019)
- AMLSCN Oyo State 2018 Annual Scientific Conference.
  Theme: Strengthening Medical Laboratories and Medical Laboratory Scientists towards Public Health Emergencies and Epidemics Management (August, 2018)
- AMLSCN Oyo State 2017 Annual Scientific Conference/Workshop
  Theme: Strengthening Medical Laboratory Systems to achieve sustainable development goals (SDGs): People, Process and Policy (August, 2017)
- AMLSCN-GSK Antibiotic discourse and lecture on overcoming challenges to Good Laboratory practice in resource limited settings (May, 2016)

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SIGNATURE **DATE**

University Compliance Certification

This is to certify that this thesis written by Oluwatobi Ajoke ADENIJI with matric no. LCU/PG/001588 in the department of Biological Sciences, Faculty of Natural and Applied Sciences, Lead City University, Ibadan is in full compliance with the approved University format and style.

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Signature **Date**