Adeniji et al. *Afr. J. Clin. Exper. Microbiol.* 2025; 26 (1): 48 - 60 https://www.afrjcem.org

African Journal of Clinical and Experimental Microbiology. ISSN 1595-689X Jan 2025; Vol.26 No.1
AJCEM/2358. https://www.ajol.info/index.php/ajcem

Original Article **Open Access**

Honey-induced expression of glutathione-encoding genes (*gsh*A and *gsh*B) in clinical *Pseudomonas aeruginosa* isolates may reduce the antibacterial potency of honey

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Abstract:

Background: It has been reported in Nigeria that honey has low antibacterial properties against *Pseudomonas aeruginosa.* Hydrogen peroxide (H_2O_2) is the major contributor to the antimicrobial activity of honey. This research sought to determine whether stress protective glutathione biosynthesis genes (*gshA* and *gshB*) present in pathogenic *Pseudomonas aeruginosa* are associated with its anti-honey resistant nature
Methodology: The susceptibility of 5 *P. aeruginosa* clinical strains obtained from Ladoke Akintola University of Technology (LAUTECH) Teaching Hospital Ogbomoso, Nigeria to antibiotics and honey were assessed using disc and agar well diffusion techniques. Detection and expression of *P. aeruginosa gsh*A and *gsh*B genes in the presence of varying dilutions of sweet and bitter Nigerian honey (100%-undiluted, 50%, 25%) and untreated controls, were done using conventional and real-time qPCR, with 16S rRNA gene used as internal control and reference gene to normalize the cDNA samples.
Results: The *gsh*A and *gsh*B genes were detected and expressed in 3 of the 5 selected isolates of *P aeruginosa* in the controls (untreated), and in 50% and 25% honey dilutions where they showed down regulation, but in the 100%, the genes were not expressed.
Conclusion: The presence and expression of the glutathione producing genes (*gsh*A and *gsh*B) in *P. aeruginosa* may reduce the potency of honey as an antibacterial agent by interfering with antibacterial action of H_2O_2 component of honey. Further studies are needed to confirm these genes as hinderances against the successful treatment of bacterial infections caused by *P. aeruginosa* using honey.

Keywords: *Pseudomonas aeruginosa;* honey; gene expression; hydrogen peroxide; honey-resistance

Received Sept 17, 2024; Revised Oct 20, 2024; Accepted Oct 21, 2024

L'expression induite par le miel des gènes codant pour le glutathion (*gsh*A et *gsh*B) dans les isolats cliniques de *Pseudomonas aeruginosa* peut réduire la puissance antibactérienne du miel

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Résumé:

Contexte: Il a été signalé au Nigéria que le miel a de faibles propriétés antibactériennes contre *Pseudomonas aeruginosa*. Le peroxyde d'hydrogène (H_2O_2) est le principal contributeur à l'activité antimicrobienne du miel. Cette recherche visait à déterminer si les gènes de biosynthèse du glutathion protecteurs contre le stress (*gsh*A et *gsh*B) présents dans *Pseudomonas aeruginosa* pathogène sont associés à sa nature résistante au miel
Méthodologie: La sensibilité de 5 souches cliniques de *P. aeruginosa* obtenues auprès de l'hôpital universitaire de technologie de Ladoke Akintola (LAUTECH) d'Ogbomoso, au Nigéria, aux antibiotiques et au miel a été évaluée à l'aide de techniques de diffusion sur disque et sur puits d'agar. La détection et l'expression des gènes gshA et *gsh*B de *P. aeruginosa* en présence de diverses dilutions de miel Nigérian doux et amer (100% non dilué, 50%, 25%) et de témoins non traités, ont été réalisées en utilisant la qPCR conventionnelle et en temps réel, avec le gène 16S rRNA utilisé comme contrôle interne et gène de référence pour normaliser les échantillons d'ADNc.
Résultats: Les gènes *gsh*A et *gsh*B ont été détectés et exprimés dans 3 des 5 isolats sélectionnés de *P. aeruginosa* dans les témoins (non traités), et dans des dilutions de miel à 50% et 25% où ils ont montré une régulation négative, mais dans les 100%, les gènes n'étaient pas exprimés.
Conclusion: La présence et l'expression des gènes producteurs de glutathion (*gsh*A et *gsh*B) dans *P. aeruginosa* peuvent réduire la puissance du miel en tant qu'agent antibactérien en interférant avec l'action antibactérienne du composant H_2O_2 du miel. Des études complémentaires sont nécessaires pour confirmer que ces gènes constituent un obstacle au traitement efficace des infections bactériennes causées par *P. aeruginosa* à l'aide du miel.

Mots clés: *Pseudomonas aeruginosa*; miel; expression génique; peroxyde d'hydrogène; résistance au miel

Introduction:

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 (1). According to epidemiological research, about 700,000 individuals die each year as a result of antibiotic-resistant bacterial illnesses (2). The overall resistance of *P. aeruginosa* isolated from European populations was 12.9% (3). In Africa, there have been reports of a higher prevalence of *P. aeruginosa* in hospital environments in Nigeria 86.4% (4) and Uganda 33.0% (5). The World Health Organization (WHO) has recently listed carbapenem-resistant *P. aeruginosa* as one of three bacterial species in which there is a critical need for development of new antibiotics to treat infections caused by this pathogen (6).

Pseudomonas aeruginosa contains *gshA* and *gshB* genes, which encode enzymes involved in glutathione biosynthesis that are important in biofilm formation, bacteria virulence and stress protection (7). 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 (8). Glutathione plays a primary protective role in the detoxification of these products of oxidative stresses.

Antimicrobial resistance is one of the major challenges facing global public health (9). 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. 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 (10).

Honey has been found to have antibacterial properties against a wide range of bacteria species (11). Hydrogen peroxide is the major contributor to the antimicrobial activity of most honey types. The concentrations of hydrogen peroxide in different honey types result in their varying antimicrobial effects (11). One of the natural products used to combat antimicrobial resistance especially in wound infections in the recent times is honey. Some honey types 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 hospital-acquired infections and are frequently isolated from surgical wounds and burns (12, 13). This bacterium is usually found to have multidrug resistant properties hence tend to pose therapeutic challenge to clinicians, 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 ineffective in eradicating *P. aeruginosa*. It is therefore necessary to find a means to modify and improve locally produced honey to a therapeutic state with natural or synthetic additives that will have the

potency for a successful bactericidal action against *P. aeruginosa.* One way to achieve this is to study this organism at the molecular level to understand its genomes, and the mechanisms of resistance and virulence especially against honey.

The *gsh*A and *gsh*B have been shown to be genes important for the virulence of *P. aeruginosa* (14). 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 was necessary to confirm the presence of these genes in strains in Nigeria 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 is common in Nigeria (15).

Studies have shown the effects of honey (16) and hydrogen peroxide, the active antimicrobial component in honey (11), on different virulence genes of *P. aeruginosa*, however, no study or reviews have shown the gene expressions related to honey found in Nigeria. Few molecular studies exist that targets the presence and prevalence of virulence genes against honey therapy in Nigeria. Most of the work done focus on the prevalence of antibiotic-resistant genes in *P. aeruginosa*.

Molecular studies on honey from Nigeria are scarce in the literature and little is known about the mechanisms of action of honey at the molecular level on pathogenic bacteria. The roles of the glutathione encoding genes (*gsh*A and *gsh*B) have not been well researched in relation to honey but only the expressions of the genes in response to H_20_2 have been reported (17). This current study describes the first analysis of the effects of Nigerian honey on the level of gene expression in clinical *P. aeruginosa* isolates.

Materials and method:

Study setting and clinical samples for bacteria isolation:

The study involved the selection of 8 phenotypically-confirmed *P. aeruginosa* strains isolated from clinical samples analyzed for diagnostic purposes at the Medical Microbiology Laboratory of the Ladoke Akintola University of Technology Teaching Hospital (LTH) in Ogbomoso, southwest Nigeria.

The strains were isolated from samples of patients that are usually collected aseptically by attending physicians/nurses in the various clinics and wards of the hospital into sterile universal containers and swabs.

Honey samples:

The honey samples (bitter and sweet) used for the study were collected at the Apiary farms in southwest Nigeria. The bitter honey was harvested at a branch of the Community Lifestyle Improvement Project Farm (RC:293 0642) 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 previously described (18). 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.

Culture and isolation of *Pseudomonas* strains:

The clinical samples collected from patients 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 pure cultures by subculturing on Mueller Hinton agar (Oxoid, UK). Distinctive morphological properties of each pure culture such as colony form, elevation, pigmentation and cyanin production were observed.

Further standard microbiological identification with Gram stain and biochemical tests including oxidase and catalase, were done as previously described (19). Identified isolates were stored in Mueller-Hinton broth in a -20°C freezer before molecular analysis.

Susceptibility testing of *Pseudomonas* isolates to antibiotics and honey:

Antibiotic susceptibility testing was done for 5 *P. aeruginosa* isolates by the Kirby Bauer disk diffusion method on Mueller Hinton agar plate. A random selection of one out of 3 colonies grown on the plate (a total of about 5 colonies) were picked from a pure culture as previously described (20). A suspension of each isolate was made with normal saline and standardized using 0.5 MacFarland standard (0.5 barium sulphate solution) and 10 antibiotics in 4 classes were used as previously described (21). The antibiotics disks used were aminoglycosides (gentamicin, amikacin), beta-lactams (augmentin, cefuroxime, ceftriaxone, ceftazidime, cefepime), macrolides (azithromycin), and fluoroquinolones (ofloxacin and levofloxacin).

The test organisms were evenly seeded over the Mueller-Hinton agar surface with sterile swab stick. The antibiotic discs were carefully placed at equidistance using a sterile forcep on the Mueller Hinton agar plate, and then incubated for 16–18 hours at 37°C. Using a ruler, the diameters of the zone of inhibition surrounding the discs were measured to the closest millimeter and interpreted as sensitive, intermediate, and resistant using the CLSI guideline (22).

Susceptibility testing of the isolates to honey was performed using the agar well diffusion technique in line the criteria set by CLSI

(22). The inocula were prepared by selecting portions from the test isolates with a sterile wire loop and suspending them in a sterile normal saline. The inoculum was standardized by comparing with 0.5 McFarland standard. A sterile swab was used to dispense the inoculum over the agar plate after being dipped into the suspension of the isolate and squeezed to remove excess fluid against the tube 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 2cm apart). Using a sterile syringe, 0.1 m l(100µl) of raw undiluted honey was introduced to the wells in the plate. The plates were incubated for 24 hours at 37°C.

The mean diameter of inhibitory zones in millimeters were 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 in line with the CLSI M100 2020 guidelines.

Bacteria preparation for nucleic acid extraction:

The bacterial isolates were exposed to honey following a previously used broth tube dilution method (23). Each bacterial isolate was suspended in 1ml of nutrient broth to achieve a turbidity equivalent to 0.5 MacFarland 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% dilutions of the original honey samples (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 instruction 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 1ml final volumes of nutritional broth to achieve the required concentrations. Except for the negative control, each tube was inoculated with 1ml of bacterial suspension (10^8 CFU/ml) and incubated at 37°C for about 3 hours.

Molecular analysis:

DNA extraction:

Genomic DNA extraction was done for all isolates confirmed to be *P. aeruginosa* using the boiling method as previously described (24). About 3-4 colonies of *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 1000 rpm 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 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.

RNA extraction

Total RNA was extracted using the Norgen's Total RNA Purification Kit (Biotek Inc) prior to performing cDNA synthesis. The protocol for extraction was done strictly following the manufacturer's instruction. The RNA samples were extracted from the exponential phase (OD_{600nm} of approximately 0.5 after 3 h of growth) cultures of the 3 selected *P. aeruginosa* isolates treated at 100%, 50%, and 25% of the sweet and bitter honeys, and untreated control at 37°C. After 3 hours, the bacteria were pelleted by centrifuging at 14,000 *x g* (~14,000RPM) for 1 minute. The supernatant was decanted, and the remaining suspension was carefully removed by aspiration. The bacteria were resuspended in 100µl of TE buffer by vortexing, incubated at room temperature for 5 minutes, and 300µl of the Buffer RL provided by the manufacturer which contains guanidinium salt, was added and the solution was vortexed vigorously for 15 seconds. Approximately 200µl of 96-100% ethanol was then added to the lysate and mixed by vortexing for 10 seconds. The concentration and purity of the extracted samples were determined using ThermoScientific NanoDrop Lite spectrophotometer at A260nm.

$_c$DNA synthesis:

This was achieved using FIREScript® RT cDNA synthesis kit (Solis BioDyne DS-06-15 v3). A total reaction volume of 50µL with final concentration of Oligo (dT) primer (100 µM) at 12.5µM, random primers (100µM) at 12.5µM, dNTP mix (20mM of each) 1250µM, 10×RT reaction buffer with DTT at 2.5×, FIREScript® RT at 25U/µL, RiboGrip™ RNase inhibitor (40U/µL) at 2.5U/µl, were used. Nuclease-free H_2O was added to make up the reaction volume and the RNA template was then 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. The concentration and pu-

rity of the cDNA samples were again determined using ThermoScientific NanoDrop Lite spectrophotometer at A260nm. A ratio of not more than 2.0 was considered suitable for the analysis.

PCR amplification and analysis of 16S rRNA, gshA and gshB genes:

The prevalence of the two virulence 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 (25). The *gsh*A and *gsh*B primers (from previous study as shown in Table 1) were amplified using the extracted DNA templates to detect *gsh*A and *gsh*B genes in the bacterial genomes. The two genes (*gsh*A and *gsh*B) involved in glutathione synthesis were identified from extensive searches of current literature as possible targets, limiting the efficacy of honey therapy. The 16S rRNA gene was used as an internal control and reference gene to normalize the cDNA samples (17). The primers were synthesized in the NIMR-MTN oligosynthesis laboratory in Yaba, Lagos, Nigeria.

Table 1: Primers used in conventional and real-time qRT-PCR assay

Gene	Sequence (5'-3')	Accession number	Reference
*gsh*A	F-CGCTACGGCAAGACCATG R-GCGCTCCAACTGGCTCGG	PA5203	(17)
*gsh*B	F-CGCATGCGCCCGCTGAAGG R-GCGCGCCAGGCAGTAGGG	PA0407	(17)
16S rRNA	F-GCCCGCACAAGCGGTGGAG R-ACGTCATCCCCACCTTCCT		(17)

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 genes. Thirty cycles were used for the amplification.

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

Gene expression analysis by RT-qPCR:

After confirming the *gsh*A and *gsh*B genes on conventional PCR, expression of the genes was also examined. Three isolates with glutathione producing genes (*gsh*A and *gsh*B) were subjected to RT-qPCR for gene expression analysis. The relative quantification of glutathione gene expression for each isolate 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 descri bed (26) using cDNA to determine the gene expression levels. The Line Gene Bioer 9600 was used for the amplification.

For RT-qPCR procedure, 1µg of cDNA was added to a total volume of 20µL with final volume of Luna Universal One-step Reaction mix (2x), Luna warm start RT enzyme mix (20x), forward primer (0.4µM), reverse primer (0.4µM) 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.

Data analysis:

Relative expression analysis was calculated and 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 ($2^{-\Delta\Delta C_T}$) to analyze the relative changes in gene expression (27). 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 gene, then the change in C_T (ΔC_T) of the test sample was normalized to the ΔC_T of the calibrator (control) sample. Normalization was calculated as ΔC_T (Calibrator) = C_T (target gene, calibrator) – C_T (reference gene, calibrator). $\Delta\Delta\ C_T = \Delta C_T$ (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 ($\Delta\Delta\ C_T$) has a negative value, the gene of interest is down-regulated, because the fold change will be smaller than 1. On the other hand, if the delta-delta C_T has a positive value, the gene is up-regulated, and the fold change is >1. Descrip-

tive statistics and graph showing fold change expression were plotted using the Microsoft Word Excel application.

Results:

Susceptibility of *P. aeruginosa* to antibiotics and honey:

The 8 *P. aeruginosa* isolates were recovered from wound, ear and throat infections as shown in Table 2. The isolates showed varying degrees of susceptibility to the anti-biotics tested. The strains however showed lower sensitivity to the honey samples compared with the positive control antibiotic. All the isolates were interpreted to be intermediate to imipenem used as positive control, showing inhibition zone diameters of 16 mm.

The susceptibility test result of the *P. aeruginosa* strains to honey showed that all except one of the isolates were resistant to the honey samples when compared to the CLSI standard of ≥ 15mm zone of inhibition for sensitivity to gentamicin against *Pseudomonas.*

Table 2: *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
P6	Wound	Wound swab
P7	Throat	Sputum
P8	Ear	Ear swab

Only one of the isolates (P1) was sensitive to the sweet honey using this standard. The results of the tests are shown in the Tables 3 and 4.

Table 3: Antimicrobial susceptibility of five selected *Pseudomonas 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 Gentamicin	Levofloxacin	Cefepime, Augmentin, Cefuroxime
P4	Ofloxacin Ceftriaxone	Ceftazidime	Augmentin Cefepime Amikacin
P5	Amikacin Ceftazidime	Cefepime	Cefuroxime Levofloxacin Azithromycin

Table 4: Susceptibility of five selected *Pseudomonas aeruginosa* isolates to sweet and bitter honey by agar well diffusion method

Isolate	Inhibition zone diameter (mm) to sweet honey	Inhibition zone diameter (mm) to bitter honey
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)

Genes expression of *P. aeruginosa* to sweet honey:

An 8.04 and a 9.0-fold reduction in expression of *gsh*A was observed in *P. aeruginosa* strain (P1) isolated from a wound site treated with 25% and 50% sweet honey respectively. P2 strain, also isolated from a wound source, showed an 8.6 and 9.2-fold reduction in *gsh*A for 25% and 50% sweet honey treatment respectively.

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. There was a significant reduction in the expression of the *gshA* and *gshB* of *P. aeruginosa* after being treated with 25% and 50% sweet honey.

As shown in Tables 5 & 6 and Figs 1 & 2, results showed all genes were downregulated and different degrees of downregulation were observed. All the isolates treated with 100% of both honey types (undiluted honey samples) did not show any expression of the genes of interest.

Table 5: Effect of sweet honey on expression of *gsh*A gene in *Pseudomonas 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

Table 6: Effect of sweet honey on the expression of *gsh*B gene in *Pseudomonas 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

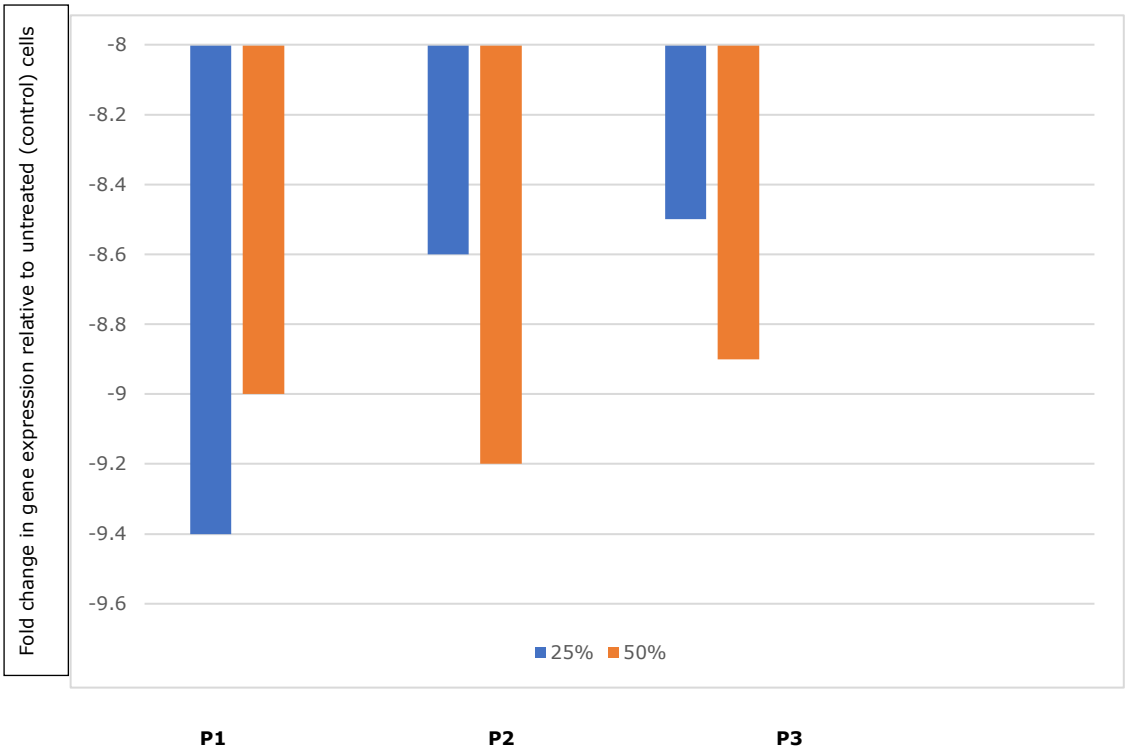

Fig 1: Graph showing the alterations in gene expression of the *gsh*A gene associated with exposure of *Pseudomonas aeruginosa* in the three selected isolates (P1, P2 and P3) to sweet honey as determined by RT-qPCR. Relative expression was normalized to the 16SrRNA gene, values of fold changes are shown in relation to the level of uninduced condition.

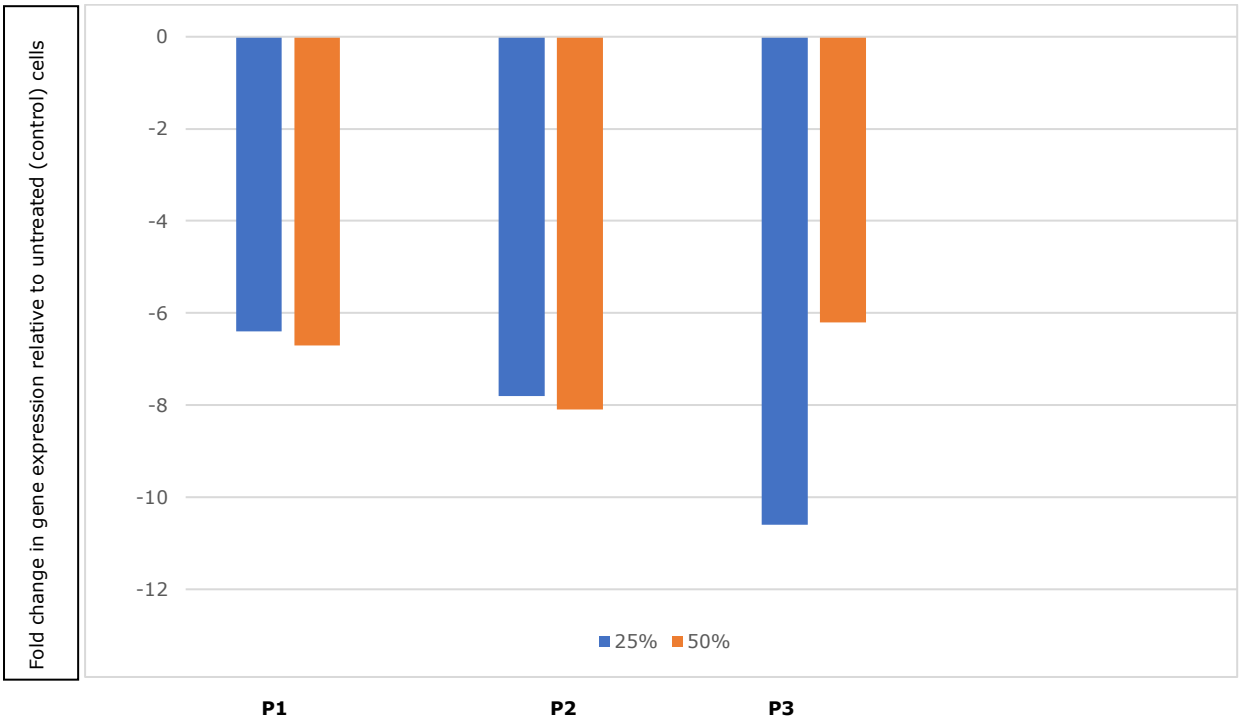

Fig 2: Graph shows the alterations in gene expression of the *gsh*B gene associated with exposure of *Pseudomonas aeruginosa* in the three selected isolates (P1, P2 and P3) to sweet honey as determined by RT-qPCR. Relative expression was normalized to the 16SrRNA gene, values of fold changes are shown in relation to the level of uninduced condition.

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. Both genes were expressed but down-regulated in the test strain (Table 7 and Fig 3).

There was a greater reduction of expression of the genes in the 50% honey dilution, although this was not significantly different (p=0.564) from that of the 25% dilution (Table 8).

Table 7: Effect of bitter honey on expression of *gsh*A and *gsh*B genes in *Pseudomonas aeruginosa* (P1) detected by RT-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
$P1_{50\%}$	22.54	-7.31

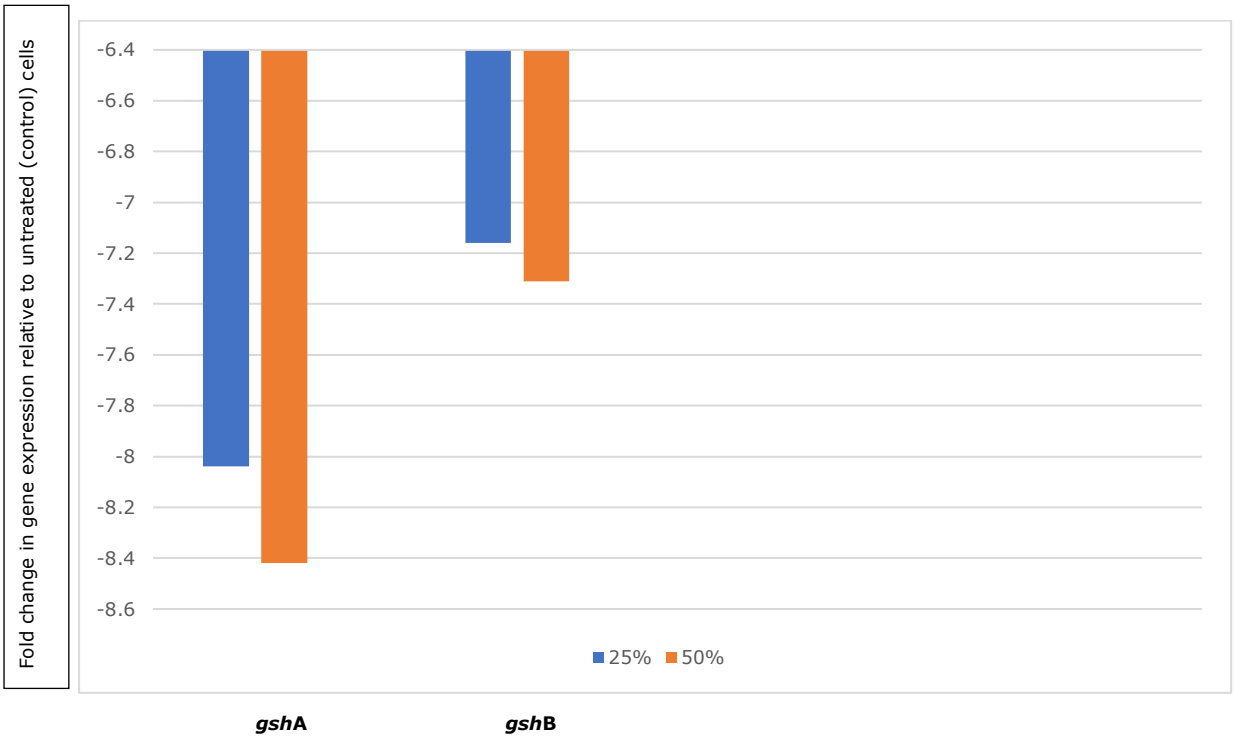

Fig 3: Graph showing the alterations in gene expression of the *gsh*A and *gsh*B genes associated with exposure of *Pseudomonas aeruginosa* isolate (P1) to bitter honey at 25% and 50% concentrations as determined by RT-qPCR. Relative expression was normalized to the 16SrRNA gene, values of fold changes are shown in relation to the level of uninduced condition.

Table 8: T-test showing the difference between values of downregulation of 25% and 50% concentrations of honeys tested

Paired sample T-test									
		Paired differences					**T**	**df**	**Sig (2-tailed)**
		Mean	**Std deviation**	**Std error of mean**	**95% confidence interval difference**				
					Lower	**Upper**			
Pair 1	Honey 25% - Honey 50%	-.35500	1.65681	.58577	-1.74013	1.03013	-.606	7	.564

Discussion:

This study describes the first systematic analysis of the effect of Nigerian honey on the level of gene expression in *P. aeruginosa*, well known to be a notorious multidrug resistant bacteria pathogen (28). The susceptibility profiles of the five selected distinct clinical isolates of *P. aeruginosa* used in this study to antimicrobial agents showed that all of the strains exhibited intermediate susceptibility to imipenem, while the highest resistance rate (60.0%) was reported against cefuroxime. A recent study in Nigeria showed 100% resistance of clinical isolates of *P. aeruginosa* to cefuroxime (29). *P. aeruginosa* strains that are resistant to colistin, the last line of antibiotic used to combat the bacteria, have been isolated in different parts of the world (30). This is due to the bacteria constant mutation against antimicrobial agents (31). There is high incidence of metallo-beta lactamase (MBL) encoding genes and integrons in different clinical *P. aeruginosa* from southwestern Nigeria (32). This 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 (32).

The isolates used in this study were mostly resistant to the tested honey types. This confirms a recent study in Nigeria which reported that none of the honey samples tested had bactericidal activity against *P. aeruginosa* but had only weak antibacterial activity with inhibitory zone diameter of 6-12mm (33). Another study also showed that honey from most parts of Nigeria had no antimicrobial effect against *P. aeruginosa* (34). A study that assessed the antibacterial activities of three honey samples collected from Nsukka, Enugu State showed that the different honey samples had antibacterial activity on all the strains of bacteria tested except for *P. aeruginosa* (35). This clearly reveals the lack of efficacy of most honey types in Nigeria against *P. aeruginosa*.

The expression of the genes (*gsh*A and *gsh*B) in the presence of honey in this study suggests that the genes were activated for glutathione production whose presence may affect the antimicrobial quality of the honey when used in the treatment of wound, however, these genes were downregulated. Downregulation of the genes suggests that the genes could not find full expression for the transcription of the glutathione enzyme. This varies from some other studies that showed an increased expression of the genes in the presence of only hydrogen peroxide (17). The downregulation of the genes in our study is similar to reports of research that have studied genes responsible for stress response and biofilm formation in bacteria in the presence of honey.

Honey has been proven to induce alterations in the expression of different genes responsible for virulence activity in bacteria (36). Numerous studies have shown that exposure to honey affects the expression of various genes related to the bacterial stress response. It has 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 (36). Later, studies that used different types of honey (clover, citrus, and marjoram) showed that the gene expression profile varied depending on the honey type, partially corroborating the findings of the former study. Clover honey treatment resulted in an increase in *evg*A expression; however, citrus or marjoram honey exposure resulted in a decrease (36). This was due to the fact that the main antimicrobial activity of manuka honey is not related to H_2O_2, while the other honey types 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 mechanisms of action. Methylglyoxal is the main antibacterial compound in manuka honey (37). Also, the variations in the antimicrobial mechanisms of the tested honey types and the variable effects they can induce on specific genes may explain the variations in expression pattern observed in them.

The result of our study generally showed a greater reduction of expression of the genes in the 50% honey dilutions (though not statistically different at $p>0.05$ as shown in Table 8) of both honey types. This may indicate that H_2O_2 has a higher concentration in an equal dilution of the honey which inhibited the expression of the glutathione genes. This is in line with a study which reported that H_2O_2 is highest in concentration at 30-50% dilutions of honey resulting in a concentration of 5 to 100g H_2O_2/g honey (equal to 0.146-2.93mM) (38). A study that also assessed the antibacterial activity of honey attributed to hydrogen peroxide, compared honey treated with bovine catalase with untreated honey (16). They found that there was an increased in 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.

The *gsh*A and *gsh*B have been shown to be genes important for the virulence of *P. aeruginosa* (14). The *gsh*B gene seems to generally have a lesser expression fold than the *gsh*A gene expression values in this study, which may be because *gsh*A gene is more involved in glutathione biosynthesis than *gsh*B gene (39). It has been demonstrated that a

substantial level of glutamyl cysteine is present in *gsh*B mutants of bacteria, including *P. aeruginosa*, and this serves as a partial glutathione substitute while the *gsh*A mutants has no glutathione substitutes (39).

Furthermore, a study has shown that *gsh*A mutant strain of *P. 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 *gsh*A gene in H_2O_2 detoxification (8). Likewise, a study that manipulated the *gsh*A gene of *P. aeruginosa* showed that a *gsh*A mutant strain is defective in biofilm formation, swarming, and pyocyanin production (14). 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 noticed that the genes were expressed in the control strain (isolate without honey) and the isolates in diluted honey samples, however they were not expressed in the 100% (undiluted) honey sample. This suggests that other properties of the honey may be responsible for inhibiting the *P. aeruginosa* isolates. Undiluted pure honey has many other factors that could contribute to its antibacterial potency. One of this 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 body fluids. Also, because of its sugar content, pure undiluted honey inhibits bacteria 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 unable to survive in the hypertonic sugar solution. In our previous study (18), we reported the pH of sweet and bitter honey used for this study to be 3.30 and 3.38 respectively. The acidity of honey which is between pH 3.2 and 4.5, is also a very marked characteristic of its antibacterial efficacy inhibiting most bacteria whose optimal growth is at pH 6.5-7.5. In addition, glycogenic acid is generated from glucose oxidation in bacteria by an endogenous glucose oxidase enzyme and this is an extremely potent antibacterial agent (40).

Furthermore, results from a study have shown that there are multiple mechanisms of antimicrobial activity in honey (16). A study that investigated the antibacterial effects of pine honey against *P. aeruginosa* PA14 at the molecular level using a global transcriptome approach by RNA-sequencing, observed the differential expression of 463 genes, 274 of which were down-regulated while 189 were up-regulated. 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 (16). The study conclusion was that multiple mechanisms of action were implicated in the antibacterial activity exerted by pine honey against *P. aeruginosa*.

Our study showed that *gsh*A and *gsh*B genes are important for neutralizing the effects of H_2O_2 and may be targeted for manipulation to eradicate anti-honey resistant *P. aeruginosa.* The presence of the genes could become a problem when a high load of bacterial infection is encountered and honey with low or diluted H_2O_2 content is employed for treatment. There is a possibility that the genes become fully expressed to detoxify the H_2O_2 present in such circumstances. While other factors are responsible for the antibacterial properties of honey, H_2O_2 plays the most important role (41).

Although our study appears to be the first gene expression study on Nigerian honey and glutathione producing genes, our data support previous research that have studied the effects of H_2O_2 and honey from other parts of the world on the expression of the genes responsible for stress protection in *P. aeruginosa*. Our study also confirmed the presence of *gsh*A and *gsh*B genes in clinical *P. aeruginosa* strains isolated in Nigeria. These genes were expressed implying that they are important for the detoxification of H_2O_2 of the honey used, however, they were downregulated, indicating that they are not totally responsible for the resistant nature of the *P. aeruginosa* strains studied against the honey used. Also, other components of the honey could have been responsible for the downregulation of the genes.

Nevertheless, our study describes the antimicrobial effects of a sample of Nigerian sweet and bitter honey on *P. aeruginosa* at the molecular level. We believe that these genes are important but are not the only targets that exists in relation to bacteria anti-honey resistance, and others should be identified in future research. Researches that study the expression of these genes when treated with honey in the presence of a higher load of bacteria is needed to confirm its threat to the efficacy of the use of honey as an antimicrobial agent.

Conclusion:

Our study emphasizes the importance of honey in the fight against bacterial infection especially that caused by *P. aeruginosa*. Honey has potential to be a good alternative antimicrobial for treatment of bacterial infections especially for the topical treatment of wounds and ear infections. *Pseudomonas aeruginosa*

was found to be susceptible (though weakly) to sweet honey type used in this study despite being a catalase producing organism. Our study also established the presence and expression of glutathione encoding genes, *gsh*A and *gsh*B, in clinical isolates of *P. aeruginosa* in southwest Nigeria in the presence of honey.

In this study, we presented the patterns of gene expression in *P. aeruginosa* strains treated with Nigerian sweet and bitter honey. The study showed that raw undiluted Nigerian honey is able to inhibit to some extent the growth of *P. aeruginosa* isolated from wounds and ear infections and also prevent the expression of the glutathione producing genes invitro. Differential gene expression in response to honey exposure exhibited down-regulation of the glutathione encoding genes in clinical isolates of *P. aeruginosa*. The results indicate that while Nigerian honey may represent a hopeful treatment for infections caused by *P. aeruginosa*, there is still need for modification of the honey types to more effectively eradicate infections caused by the organism. H_2O_2 should be considered as the most preferred antiseptic agent for the cleaning of infected wounds prior to honey treatment and as diluents for honey ear drops especially used for chronic ear infections.

This study showed that Nigerian sweet honey type has antimicrobial potentials and could be better if modified for its use in the treatment of bacterial infections. It also shed light on one of the hinderances to the effectiveness of honey when used as an antibacterial agent especially against *P. aeruginosa*. The virulence genes investigated in this study are good targets to consider when producing medical grade honey used for the treatment of bacterial infections.

Acknowledgements:

The authors acknowledge with thanks the staff of CERID, Mrs M. V. Adigun, Dr A. J. Oke and Mr Olalekan Kemiki of the Molecular Laboratory, Babcock University Teaching Hospital for their technical support, mentorship, and assistance rendered in the analysis of the molecular findings respectively.

Contribution of authors:

OA, FA and OO contributed to the study design and implementation of the study; TA isolated the bacteria from samples and performed antimicrobial susceptibility testing; AO carried out part of the molecular analysis; BA analyzed the honey samples and provided expert contributions in the discussion. All the authors read and approved the final manuscript submitted for publication.

Source of funding:

The research was personally funded by the authors and supported with reagents, consumables, and equipment by the Centre for Emerging and Re-emerging Infectious Diseases (CERID), LAUTECH, Ogbomoso, Nigeria.

Conflict of interests:

The authors declare that there are no conflicts of interest.

Data availability:

The data sets used and analyzed during the current study are available from the corresponding author upon reasonable request.

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