Research Article

Transcriptomic Response in \textit{Pseudomonas aeruginosa} towards Treatment with a Kaempferol Isolated from \textit{Melastoma malabathricum} Linn Leaves

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\textit{Pseudomonas aeruginosa} is one of the main causes of nosocomial infections and is frequently associated with opportunistic infections among hospitalized patients. Kaempferol-3-O-(2′,6′-di-O-trans-p-coumaroyl)-\beta-D glucopyranoside (\(K_F\)) is an anti-pseudomonal compound isolated from the leaves of the native medicinal plant \textit{Melastoma malabathricum}. Herein, an RNA-seq transcriptomic approach was employed to study the effect of \(K_F\) treatment on \textit{P. aeruginosa} and to elucidate the molecular mechanisms underlying the response to \(K_F\) at two time points (6h and 24h incubation). Quantitative real-time PCR (qRT-PCR) was performed for four genes (\(uvrD\), sodM, \(fumC1\), and \(rpsL\)) to assess the reliability of the RNA-seq results. The RNA-seq transcriptomic analysis revealed that \(K_F\) increases the expression of genes involved in the electron transport chain (NADH-I), resulting in the induction of ATP synthesis. Furthermore, \(K_F\) also increased the expression of genes associated with ATP-binding cassette transporters, flagella, type III secretion system proteins, and DNA replication and repair, which may further influence nutrient uptake, motility, and growth. The results also revealed that \(K_F\) decreased the expression of a broad range of virulence factors associated with LPS biosynthesis, iron homeostasis, cytotoxic pigment pyocyanin production, and motility and adhesion that are representative of an acute \textit{P. aeruginosa} infection profile. In addition, \textit{P. aeruginosa} pathways for amino acid synthesis and membrane lipid composition were modified to adapt to \(K_F\) treatment. Overall, the present research provides a detailed view of \textit{P. aeruginosa} adaptation and behaviour in response to \(K_F\) and highlights the possible therapeutic approach of using plants to combat \textit{P. aeruginosa} infections.

1. Introduction

\textit{Pseudomonas aeruginosa} sp. is deemed one of the major etiological agents of both acute and chronic human infections ranging from minor skin infections to persistent and often life-threatening diseases in hospitalized or immunocompromised patients [1, 2]. Infections caused by this organism are difficult to treat due to the ability of this bacterium to resist multiple classes of antibiotics [3]. Strains of \textit{P. aeruginosa} are well known to employ their high levels of intrinsic and acquired resistance mechanisms to combat most antibiotics [4]. In addition, pathogenesis of \textit{P. aeruginosa} is multifactorial, and many virulence factors are produced that include secreted factors such as cytotoxic pigment pyocyanin, siderophores, alkaline protease, elastase, exotoxin A, rhamnolipid structural component lipopolysaccharide, pili, flagella, and biofilm formation [5]. Therefore, alternative drugs and new therapeutic strategies that present novel avenues against \textit{P.
aeruginosa infections are increasingly required and gaining more and more attention [4]. Previous studies by our research group demonstrated that K\textsubscript{F} can induce P. aeruginosa cell wall damage [6, 7]. Thus, we decided to investigate the gene expression profile of P. aeruginosa growing in kaempferol-3-O-(2',6'-di-O-trans-p-coumaroyl)-\(\beta\)-D-glucopyranoside isolated from Melastoma malabathricum known to locals in Malaysia as “senduduk.” Next-generation sequencing (NGS) technology may provide a detailed view of P. aeruginosa adaptation and behaviour in response to K\textsubscript{F} and could help researchers further understand the transcriptomic response of P. aeruginosa to K\textsubscript{F} exposure [8]. We compared the transcriptional responses of P. aeruginosa upon exposure to K\textsubscript{F} at an early time point (6 h incubation) and at a late time point (24 h incubation) to provide information about the K\textsubscript{F} mechanism of action. Transcriptomic data highlighted a marked modulation of gene expression characterized by the induction of the expression of several genes involved in pathogenesis, iron acquisition, DNA replication and repair, and metabolic adaptation to K\textsubscript{F} growth conditions. The results presented in this study provide a detailed view of gene expression changes in P. aeruginosa in response to K\textsubscript{F} exposure, facilitating the understanding of the cellular strategies that are utilized under K\textsubscript{F} exposure conditions and identifying a potential mechanism for the inhibition of P. aeruginosa after K\textsubscript{F} exposure.

2. Materials and Methods

2.1. Bacteria and Growth Conditions. Pseudomonas aeruginosa strain ATCC 10145 was cultivated in Nutrient Broth (Oxoid, UK) with a shaking incubator at 151 rpm for 3 to 6 h at 37°C to achieve log phase growth. At the log phase (\(\sim 6\) h incubation), K\textsubscript{F} was added to the P. aeruginosa culture in Mueller Hinton Broth (Oxoid, UK) at a density of \(4 \times 10^5\) CFU/mL to achieve a final concentration of 0.5 mg/mL dissolved in 5% dimethyl sulfoxide (DMSO). DMSO (5%) was used as a negative control for untreated cells. The cultures were incubated at 37°C with a shaking incubator at 200 rpm.

2.2. RNA Extraction, cDNA Library Construction, and Illumina Sequencing. Total RNA was extracted from P. aeruginosa (treated or untreated) and harvested after 6 h and 24 h of incubation. RNA was extracted using an innuPREP RNA Mini Kit (Analytik Jena Biometra, Germany). The quantity and integrity were first determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The total RNA was depleted of rRNA using a ScriptSeq™ Complete Kit (Bacteria; Epicenter, San Diego, CA, USA). Total RNA samples were used for cDNA synthesis. Magnetic beads with attached poly T oligos were used to purify mRNA from the total RNA. The mRNA was then cleaved into small fragments by the addition of RNA fragmentation solution. First strand cDNA was synthesized using random hexamer adaptors and StarScript Reverse Transcriptase, followed by the synthesis of second strand cDNA using ScriptSeq v2 Terminal Tagging Premix and DNA polymerase. Exonuclease and polymerase were used to blunt and adenylate the 3’ ends of the DNA fragments, and Illumina PE adapter oligonucleotides were ligated to prepare for hybridization. The cDNA fragments (280 bp) were purified using the Pure AMXP system (Beckman Coulter, Beverly, CA, USA). The cDNA fragments with ligated adaptor molecules were enriched using Illumina PCR Primer Cocktail in a 15-cycle PCR. Finally, the cDNA library was sequenced on the Illumina MiSeq platform (San Diego, CA, USA) using single-end technology in a single run at the Institute of Biosciences, Universiti Putra Malaysia. The Illumina MiSeq software was used to perform the original image processing for sequencing, base calling, and quality value calculations, where 50 bp single-end reads were obtained.

2.3. Analysis of the Differentially Expressed Genes (DEGs). The raw reads were filtered to obtain the high-quality clean data by removing adaptor sequences and low-quality reads with the Phred quality score \(\leq 30\). The clean reads were then mapped to the P. aeruginosa PA01 genome (NCBI reference sequence, NC_002516.2; GenBank accession number AE004091.2). FASTQ read values were calculated and normalized to transform into expression values by using CLC Genomics Workbench version 6.5. Differential expression analysis (fold changes) for RNAseq data was performed to compare two different samples (untreated versus treated samples) using Kal’s Z-test. Genes with average fold changes >2 and adjusted \(p\) values less than 0.05 (i.e., false discovery rate less than 5%) were identified as significant DEGs. To better understand the biological functions and the metabolic pathways of the identified genes, the DEGs were functionally classified due to Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases. The significant DEGs at both 6 h and 24 h were compiled and used to generate a Venn diagram through an online interactive tool [9]. The gene lists of unique and shared genes in each group identified in the Venn diagram were analysed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov/home.jsp). The DAVID database provides a comprehensive set of functional annotation tools to understand the biological meaning behind the DEGs, including visualizing genes on KEGG pathway maps. In addition, the obtained data were then compiled with public datasets downloaded from the Pseudomonas Genome Database (http://www.pseudomonas.com) for further analysis. The raw RNA-seq data have been submitted to the NCBI Sequence Read Archive (NCBI SRA) under GenBank accession no. SRP060687 (NCBI SRA, http://www.ncbi.nlm.nih.gov/sra?term=SRP060687).

2.4. Validation of DEGs by Quantitative Real-Time PCR (qRT-PCR). In order to validate the RNA-seq data and to have a concise view of P. aeruginosa gene expression profiles over time, qRT-PCR was employed and gene expression levels were analysed on a subset of genes whose functions were documented to contribute to P. aeruginosa virulence. Four genes with different expression patterns at two time points
were chosen for the validation of the RNA-seq results. The template cDNAs were synthesized from 1 μg of total RNA using oligo (dT)\textsubscript{18}, random hexamer primers, and reverse transcriptase enzyme mix (Maxima First Strand cDNA Synthesis Kit; Thermo Scientific, USA). A Luminaris Color HiGreen Fluorescent qPCR Master Mix (Thermo Scientific, USA) was used as a labelling agent, and L-aspartate oxidase (nadB) served as an internal reference gene. The reaction mixture (20 μL) contained 2 × Master Mix (10 μL), 10 μM forward and reverse primers (1.2 μL and 0.6 μL of each), template cDNA (2 μL), and RNase-free water (6.8 μL). The PCR program was as follows: 2 minutes (min) at 50°C, 10 min at 95°C, followed by 40 cycles for 15 seconds (sec) at 95°C, 40 cycles of 30 sec at 53°C and 40 cycles of 30 sec at 72°C. The reaction was performed on an iCycler iQ5 instrument (Bio-Rad Laboratories, Inc., Hercules, Canada). Two independent biological replicates were included for each sample. The relative expression of a target gene in comparison to a reference gene expression level was calculated using Relative Expression Software Tool Multiple Condition Solver REST-MCS®-version 2 (http://rest.genequantification.info).

3. Results

3.1. Transcriptomic Analysis. Genome-wide transcriptomic analysis was conducted to elucidate the mechanism through which K\textsubscript{F} exerts its killing effect on P. aeruginosa using NGS technology. After statistical analysis (Kal’s Z-test), 2405 of the 5681 genes that comprise the P. aeruginosa genome were found to be significantly differentially regulated (p ≤ 0.05). A total of 2405 differentially expressed genes were classified based on their expression change direction (Figure 3).

Further analysis revealed that 1031 genes showed statistically significant upregulation (>2.0-fold) or down-regulation (<2.0-fold) of expression at 6h and 24h of exposure to K\textsubscript{F}. Figure 1 illustrates that more downregulated genes in the functional classes were generally observed at 24h compared to 6h of incubation. The most noticeable number of downregulated genes among all functional classes was hypothetical, unclassified, and unknown (HUU) with unknown function.

Note that 803 of 1031 genes were excluded as hypothetical proteins (HUUs). The Venn diagram for the remaining 228 genes at the two time points shows more uniquely over-represented genes at 24h (115) than at 6h (53), suggesting a difference between the early and late responses of P. aeruginosa to K\textsubscript{F} (Figure 2). A total of 228 genes were placed in six groups based on their expression change direction (Figure 3).

3.1.1. Group I: Genes with Upregulated Expression at 6h and 24h. Group I consisted of genes with upregulated expression at both 6h and 24h of exposure to K\textsubscript{F} (Table 1). Growth under K\textsubscript{F} exposure conditions induced changes in the expression of genes associated with ATP-binding cassette (ABC) transporters (agtABCD operon, PA4500), carbohydrate transporters (PA3190), and inorganic ion transporters (PA3514).

Group I also contained genes coding for the type III secretion system (T3SS). These genes with upregulated expression include those involved in the secretion and translocation machinery into the host cell plasma (popBD and pcrV); transcription and initiation (exsCED); chaperones that bind secreted proteins to facilitate the secretion process (specS, pcrH, pscG, and exsC); and effector proteins that are injected into host cells (exoSTY; Table 1).

3.1.2. Group II: Genes with Upregulated Expression at 6h. Group II is composed of genes with expression levels that increased only at 6h of exposure to K\textsubscript{F} (Table 2). The expression of genes involved in the biosynthesis of several amino acids, including histidine (hisCl and hisE), arginine (argF and argJ), isoleucine (ileA1), leucine (leuA), and phenylalanine (pheA), was increased after K\textsubscript{F} exposure. In addition, we observed the overexpression of genes related to translation class, including genes encoding 30S and 50S ribosomal proteins (the two most upregulated genes, 30S and 50S, are listed in Table 2); aminoacyl-tRNA synthetases associated with tryptophan (trpS), tyrosine (tyrZ), glycine (glyQ), glutamine (glnE), valine (valS), proline (proS), cysteine (cysS), and isoleucine (ileS); translation initiation factor (infC); elongation factor G (fusA2); and peptide chain release factor (prfC) in response to K\textsubscript{F} treatment.

As shown in Table 2, the upregulation of the expression of genes involved in the first step of long-chain fatty acid biosynthesis was also observed. The genes with upregulated expression include those encoding biotin carboxyl carrier protein (accB) and acetyl CoA carboxylase beta subunit (accD). In prokaryotes, this step involves the ATP-dependent carboxylation of acetyl coenzyme A (CoA) to form malonyl CoA by the enzyme acetyl CoA carboxylase. In addition, the expression of fabA and fabB genes, which are involved in the biosynthesis of unsaturated fatty acids (UFAs), was increased in K\textsubscript{F} samples. Under anaerobic conditions, P. aeruginosa can utilize nitrate, nitrite, or nitrous oxide instead of oxygen as a terminal electron acceptor in the denitrification process. The expression of the nitric oxide reductase gene (norB) required for denitrification was upregulated. Furthermore, the most obvious upregulation of gene expression was found in the oxidative phosphorylation pathway. NADH created by the Krebs cycle can be fed into the oxidative phosphorylation pathway. The expression of NADH dehydrogenase I chain (nuoBDFGHIJLMN) in the oxidative phosphorylation pathway was increased. The ann gene encodes the transcriptional regulator Anr, which is involved in controlling P. aeruginosa gene expression under anaerobic conditions was significantly increased in K\textsubscript{F}-treated samples, with log2-fold changes of 3.84. Table 2 also shows that the expression of genes related to the flagella assembly pathway (fliBCDEFGHJK and fliEFG) was also increased after K\textsubscript{F} exposure.

3.1.3. Group III: Genes with Downregulated Expression at 6h. The expression of genes associated with adaptation, protection, and secreted factor functional class was
The genes with downregulated expression include those associated with pyocin S2 (pys2) and pyocin S2 immunity protein (imm2). The expression of cobODUJ genes, which are involved in the aerobic cobalamin biosynthesis process (a cofactor for numerous enzymes mediating methylation, reduction, and intramolecular rearrangements), was reduced.

3.1.4. Group IV: Genes with Upregulated Expression at 24 h.

Exposure to KF increased changes in the expression of genes associated with tripartite ATP-independent periplasmic transporters, including dctP (a C4 dicarboxylate-binding protein) and dctQ and dctM (C4 dicarboxylate transporters) (Table 4). Pseudomonas aeruginosa preferentially uses C4 dicarboxylates, such as malate, fumarate, and succinate, as carbon and energy sources under anaerobic conditions.

3.1.5. Group V: Genes with Downregulated Expression at 24 h.

Group V is composed of genes with downregulated expression at 24h of exposure to KF (Table 5). The expression of the pchR gene, which encodes elements involved in iron Fe3+ acquisition, was reduced. In addition, growth under KF exposure conditions reduced the changes in the expression of genes including members of the extracytoplasmic factor (ECF) subfamily (PA0471-PA0472, PA1300-1301, PA3895-PA3896, PA0149, PA1912, and PA2896). The expression of the tonB gene (TonB-dependent siderophore receptor) required for chelating Fe3+ was reduced. Furthermore, the expression of genes encoding fumarate hydratase (fumC1), superoxide dismutase (sodM), haemooxygenase (hemO), and oxidoreductase (PA0853 and PA3768) was downregulated.

Table 5 also shows that the expression of virulence-associated genes that are involved in phenazine-1-carboxylic acid (PCA) biosynthesis (phzA1B1C1A2B2) and the conversion of PCA to pyocyanin (phzMS) were decreased. The expression of several genes associated with Sec system proteins was significantly altered. Exposure to KF reduced the changes in the expression of genes such as the inner membrane translocase subunit proteins (secD), a cytoplasmic membrane-associated ATPase (secA), and a chaperone (secB) that binds to presecretory target proteins. The results also showed a downregulation of the expression of the mexGHI-opmD efflux pump system in the KF-treated samples (Table 5). In addition, the expression of several genes involved in the LPS biosynthesis process, including lpxA, lpxB, waaF, waaG, waaP, PA4998, PA5007, PA5008, and rmlA, was decreased. Transcription data of P. aeruginosa showed a downregulation in the expression of type VI pili composed of pilDFMNOPQUVWXYZ. The expression levels of vfr (virulence factor regulator) and pilGHIJ-chpAB (Chp chemosensory system) genes were significantly decreased.
### Table 1: List of the group I genes with upregulated expression at 6h and 24h.

| Genes   | 6h fold change | 24h fold change | Description                                                                 | Functional class |
|---------|----------------|-----------------|------------------------------------------------------------------------------|------------------|
| agtA    | 20.45          | 9.21            | ABC-type spermidine/putrescine transport systems, ATPase components         | TSMs             |
| agtB    | 32.34          | 8.09            | ABC-spermidine/putrescine-binding periplasmic protein                       | TSMs             |
| agtC    | 25.25          | 4.57            | ABC-type spermidine/putresine transport system, permease component I       | TSMs; MPs        |
| agtD    | 15.20          | 3.00            | ABC-type spermidine/putresine transport system, permease component II      | TSMs; MPs        |
| PA4500  | 8.02           | 3.11            | ABC-type dipeptide transport system, periplasmic component                  | TSMs             |
| PA3190  | 7.60           | 2.09            | ABC-type sugar transport system, periplasmic component                      | TSMs             |
| PA3514  | 6.95           | 5.08            | ABC-type nitrate/sulfonate/bicarbonate transport system, ATPase component  | TSMs             |
| spcS    | 35.38          | 3.94            | Specific Pseudomonas chaperone for ExoS, SpcS                              | SFs; PSEA        |
| pcrV    | 14.54          | 5.89            | Type III protein secretion system complex                                   | PSEA             |
| pcrH    | 7.52           | 4.94            | Regulatory protein PcrH                                                     | SFs; PSEA        |
| popB    | 11.31          | 4.79            | Translocator protein PopB                                                   | PSEA             |
| popD    | 9.67           | 3.48            | Translocator outer membrane protein PopD precursor                         | PSEA             |
| exsC    | 10.98          | 4.09            | ExsC, exoenzyme S synthesis protein C precursor                            | PSEA             |
| exsE    | 8.61           | 4.62            | ExsE                                                                        | PSEA             |
| exsD    | 11.895         | 4.572           | ExsD                                                                        | PSEA             |
| pscG    | 7.60           | 2.31            | Exoenzyme S                                                                | SFs              |
| exoS    | 9.36           | 6.71            | Exoenzyme T                                                                | SFs              |
| exoY    | 31.08          | 5.82            | Adenylate cyclase ExoY                                                      | SFs              |

**Figure 3:** Classification of differentially upregulated and downregulated (total of 228) genes into six groups based on their functional classes at 6h and 24h exposure to KF (0.5 mg/mL). Group I consisted of genes with upregulated expression at 6h and 24h. Group II consisted of genes with upregulated expression at 6h without significant changes at 24h. Group III consisted of genes with downregulated expression at 6h without significant changes at 24h. Group IV consisted of genes with upregulated expression at 24h without significant changes at 6h. Group V consisted of genes with downregulated expression at 24h without significant changes at 6h. Group VI consisted of genes with downregulated expression at 6h and 24h.

**Table 1:** List of the group I genes with upregulated expression at 6h and 24h.
Table 2: List of the group II genes with upregulated expression at 6h.

| Genes            | 6h (fold change) | p value | Description                                      | Functional class |
|------------------|------------------|---------|--------------------------------------------------|------------------|
| hisCI            | 4.814            | 0.013   | Histidinol-phosphate aminotransferase            | AABM             |
| HisE             | 3.111            | 4.06E-08| Phosphoribosyl-ATP pyrophosphohydrolase          | AABM             |
| ArgF             | 2.723            | 2.16E-03| Ornithine carbamoyltransferase, anabolic         | AABM             |
| ArgG             | 2.601            | 9.75E-03| Glutamate N-acetyltransferase                    | AABM             |
| ilvA1            | 2.117            | 0.021   | Threonine dehydratase, biosynthetic             | AABM             |
| LeuA             | 5.244            | 0       | 2-Isopropylmalate synthase                      | AABM             |
| LeuC             | 2.387            | 7.87E-12| 3-Isopropylmalate dehydratase large subunit     | AABM             |
| PheA             | 2.1              | 0.018   | Chorismate mutase                               | AABM             |
| RpsL             | 2.56             | 0       | 30S ribosomal protein S12                       | TPTMD            |
| RplA             | 3.679            | 0       | 50S ribosomal protein L1                        | TPTMD            |
| TrpS             | 3.501            | 1.62E-05| Tryptophanyl-tRNA synthetase                    | TPTMD; AABM      |
| TyrZ             | 3.239            | 0       | Tyrosyl-tRNA synthetase 2                       | TPTMD; AABM      |
| GlyQ             | 2.431            | 0       | Glycyl-tRNA synthetase alpha                     | TPTMD; AABM      |
| GlnE             | 2.912            | 3.52E-07| Glutamine synthetase adenyllytransferase        | TPTMD            |
| ValS             | 2.356            | 3.57E-10| Valyl-tRNA synthetase                           | TPTMD; AABM      |
| ProS             | 2.274            | 2.36E-09| Prolyl-tRNA synthetase                          | TPTMD; AABM      |
| CysS             | 2.152            | 2.30E-05| Cysteinyl-tRNA synthetase                       | TPTMD; AABM      |
| ltc              | 2.02             | 1.72E-08| Isoleucyl-tRNA synthetase                       | TPTMD; AABM      |
| InfC             | 7.182            | 0       | Translation initiation factor IF-3              | TPTMD            |
| fusA2            | 2.313            | 5.73E-07| Peptide chain release factor 3                  | TPTMD            |
| PA5195           | 3.589            | 0.028   | Probable heat shock protein                     | CHSPs            |
| HscB             | 3.187            | 2.35E-05| Heat shock protein HscB                         | CHSPs            |
| AccB             | 6.303            | 0       | Biotin carboxyl carrier protein (BCCP)          | FAPM             |
| AccD             | 3.579            | 6.23E-12| Acetyl-CoA carboxylase beta subunit             | FAPM             |
| FabA             | 2.258            | 2.67E-06| Beta-hydroxydecanoyl-ACP dehydrase              | FAPM             |
| FabB             | 2.96             | 0       | Beta-ketoacyl-ACP synthase I                    | FAPM             |
| NorB             | 8.614            | 1.58E-03| Nitric oxide reductase subunit B                | EM               |
| Anr              | 3.84             | 0       | Transcriptional regulator Anr                   | EM               |
| NuoB             | 4.162            | 0       | NADH dehydrogenase I chain B                    | EM               |
| NuoC             | 2.982            | 0       | NADH dehydrogenase I chain C,D                 | EM               |
| NuoF             | 2.657            | 5.14E-09| NADH dehydrogenase I chain F                    | EM               |
| NuoG             | 2.761            | 0       | NADH dehydrogenase I chain G                    | EM               |
| NuoH             | 3.026            | 9.62E-06| NADH dehydrogenase I chain H                    | EM               |
| NuoI             | 7.185            | 3.21E-14| NADH dehydrogenase I chain I                    | EM               |
| NuoJ             | 4.172            | 3.27E-03| NADH dehydrogenase I chain J                    | EM               |
| NuoL             | 3.459            | 0       | NADH dehydrogenase I chain L                    | EM               |
| NuoM             | 2.884            | 5.40E-04| NADH dehydrogenase I chain M                    | EM               |
| NuoN             | 5.008            | 2.12E-07| NADH dehydrogenase I chain N                    | EM               |
| FlgB             | 5.197            | 0       | Flagellar basal body rod protein FlgB           | CWLC; MA         |
| FlgC             | 2.907            | 8.96E-10| Flagellar basal body rod protein FlgC           | CWLC; MA         |
| FlgD             | 4.125            | 4.38E-14| Flagellar basal body rod modification protein FlgD | CWLC; MA       |
| FlgE             | 4.984            | 0       | Flagellar hook protein FlgE                    | CWLC; MA         |
| FlgF             | 4.623            | 2.03E-14| Flagellar basal body rod protein FlgF           | CWLC; MA         |
| FlgG             | 3.297            | 0       | Flagellar basal body rod protein FlgG           | CWLC; MA         |
| Flgl             | 2.4              | 0.01    | Flagellar P-ring protein precursor Flgl         | CWLC; MA         |
| Flgl             | 4.057            | 3.23E-13| Flagellar protein Flgl                         | CWLC; MA         |
| FlgK             | 3.85             | 2.24E-10| Flagellar hook-associated protein 1 FlgK       | CWLC; MA         |
| FliE             | 4.534            | 1.18E-11| Flagellar hook-basal body complex protein FliE | CWLC; MA         |
| FliF             | 3.433            | 0       | Flagella M-ring outer membrane protein precursor | CWLC; MA      |
| FliG             | 2.635            | 6.34E-10| Flagellar motor switch protein FliG            | CWLC; MA         |

According to the Log2-fold changes. The virulence-associated flIC gene, which encodes flagellin type B, was downregulated under KE exposure conditions.

Growth under KE conditions reduced the expression of genes associated with translation class, including genes encoding the 30S and 50S ribosomal proteins (the two most downregulated 30S and 50S genes are listed in Table 5) and aminoacyl-tRNA synthetase associated with glutamine (glnS), glycine (glyS), leucine (leuS), lysine (lysS), proline (proS), valine (valS), and aspartate (aspS). In addition, the expression of genes involved in the biosynthesis of several amino acids, including histidine (hisF1 and hisG), arginine (argB, argG, and argH), cysteine (cysM), and tryptophan (trpA and trpB), was also decreased after exposure to KE.

RNA-seq data showed a downregulation in the expression of genes associated with DNA replication (dnaA,
### Table 3: List of the group III genes with downregulated expression at 6 h.

| Genes   | 6 h (fold change) | 24 h (fold change) | Description                                      | Functional class |
|---------|-------------------|---------------------|--------------------------------------------------|------------------|
| Pys2    | −4.084            | 0                   | Pyocin S2                                          | AP; SFs          |
| imm2    | −2.56             | 0                   | Pyocin S2 immunity protein                        | AP               |
| cobO    | −2.684            | 8.27E − 04          | Cob (I) alamin adenosyltransferase                 | BCPCGs           |
| cobD    | −4.229            | 0.012               | Cobalamin biosynthetic protein CobD               | BCPCGs           |
| cobU    | −4.306            | 7.36E − 04          | Nicotinate-nucleotide-dimethylbenzimidazole        | BCPCGs           |
| cobJ    | −4.9              | 0                   | Precorrin-3 methylase CobJ                        | BCPCGs           |

### Table 4: List of the group IV genes with upregulated expression at 24 h.

| Genes   | 24 h (fold change) | p value      | Description                                      | Functional class |
|---------|--------------------|--------------|--------------------------------------------------|------------------|
| dctP    | 2.879              | 0            | DctP                                             | MPs; TSMs        |
| dctQ    | 2.419              | 1.04E − 06   | DctQ                                             | MPs; TSMs        |
| dctM    | 2.206              | 4.35E − 05   | DctM                                             | MPs; TSMs        |

### Table 5: List of the group V genes with downregulated expression at 24 h.

| Genes   | 24 h (fold change) | p value      | Description                                      | Functional class |
|---------|--------------------|--------------|--------------------------------------------------|------------------|
| pchR    | −3.116             | 2.23E − 15   | Transcriptional regulator PchR                   | TRs              |
| PA0471  | −2.863             | 1.61E − 05   | Fe^{2+}-dicitrate sensor, membrane component     | TCRSs; MPs; TRs  |
| fui     | −2.171             | 1.62E − 03   | Fe^{2+}-dicitrate sensor, membrane component     | TRs              |
| PA1300  | −2.201             | 5.72E − 09   | Sigma-70 factor, ECF subfamily                  | TRs              |
| PA1301  | 2.386              | 0.014        | Probable transmembrane sensor                    | MPs; TRs         |
| PA3899  | −3.481             | 0            | Probable sigma-70 factor, ECF subfamily          | TRs              |
| PA3900  | −2.505             | 0.019        | Fe^{2+}-dicitrate sensor, membrane component     | MPs; TRs         |
| PA4895  | −5.965             | 1.26E − 09   | Fe^{2+}-dicitrate sensor, membrane component     | MPs; TRs         |
| PA4896  | −3.644             | 8.33E − 11   | Sigma-70 factor, ECF subfamily                  | TRs              |
| PA0149  | −3.859             | 1.15E − 08   | Probable sigma-70 factor, ECF subfamily          | TRs              |
| feml    | −3.628             | 0            | ECF sigma factor, Feml                           | TRs              |
| PA2896  | −2.495             | 0            | Probable sigma-70 factor, ECF subfamily          | TRs              |
| tonB1   | −2.067             | 0            | Periplasmic protein TonB, links inner and outer membranes | TSMs         |
| PA4156  | −11.36             | 0            | Probable TonB-dependent receptor                 | TSMs             |
| fumC1   | −4.599             | 0            | Fumarate hydratase                               | EM               |
| sodM    | −3.955             | 0            | Superoxide dismutase                             | AP               |
| hemO    | −3.581             | 0            | Heme oxygenase                                   | BCPCGs           |
| PA0853  | −3.242             | 2.66E − 13   | Oxidoreductase                                    | PEs              |
| PA3768  | −2.622             | 0            | Probable metallo-oxidoreductase                  | PEs              |
| phzA1   | −4.295             | 1.53E − 07   | Probable phenazine biosynthesis protein          | SFs              |
| phzB1   | −4.708             | −4.708       | Probable phenazine biosynthesis protein          | SFs              |
| phzC1   | −2.577             | −2.577       | Phenazine biosynthesis protein PhzC              | SFs              |
| phzA2   | −2.625             | −2.625       | Probable phenazine biosynthesis protein          | SFs              |
| phzB2   | −3.848             | −3.848       | Probable phenazine biosynthesis protein          | SFs              |
| phzM    | −2.125             | 1.14E − 10   | Probable phenazine-specific methyltransferase    | PEs              |
| phzS    | −2.241             | 0            | Flavin-containing monoxygenase                   | PEs              |
| secA    | −2.166             | 0            | Secretion protein SecA                           | PSEA             |
| secB    | −2.579             | 0            | Secretion protein SecB                           | PSEA             |
| secD    | −3.234             | 0            | Secretion protein SecD                           | PSEA; MPs        |
| mexG    | −2.119             | 7.29E − 03   | Membrane protein                                 | MPs              |
| mexH    | −9.088             | 0            | Probable resistance-nodulation-cell division (RND) efflux membrane fusion | TSMs             |
| mexI    | −5.758             | 0            | Probable resistance-nodulation-cell division (RND) efflux transporter | TSMs; MPs        |
| opmD    | −3.241             | 0            | Outer membrane protein precursor                 | TSMs; MPs        |
| lpxB    | −2.148             | 7.24E − 03   | Lipid A-disaccharide synthase                    | CWLC             |
| lpxA    | −2.274             | 0            | UDP-N-acetylglucosamine acyltransferase          | CWLC             |
| waaP    | −3.174             | 2.75E − 14   | Lipopolysaccharide kinase WaaP                    | CWLC             |
| waaG    | −2.437             | 0            | UDP-glucose-(heptosyl) LPS alpha 1.3-glucosyltransferase WaaG | CWLC             |
| waaF    | −2.283             | 1.48E − 08   | Heptosyltransferase II                           | CWLC             |
| PA4998  | −2.482             | 6.80E − 12   | Aminoglycoside 3′-phosphotransferase (APH) and choline kinase family | CWLC             |
| Genes  | 24h (fold change) | p value | Description                                                                 | Functional class |
|--------|-------------------|---------|------------------------------------------------------------------------------|------------------|
| PA5007 | –3.124            | 4.80E–08| Mn\(^{2+}\) dependent serine/threonine protein kinase                        | PEs              |
| PA5008 | –3.187            | 7.22E–15| RIO-like serine/threonine protein kinase fused to N-terminal HTH domain      | PEs              |
| rmlA   | –2.554            | 0       | Glucose-1-phosphate thymidyltransferase                                      | CWLC             |
| pilD   | –2.377            | 0       | Type 4 preprotein peptidase PilD                                             | SFs; PSEA; MA    |
| pilF   | –2.053            | 0       | Type 4 fimbrial biogenesis protein PilF                                       | PSEA; MA         |
| pilM   | –3.074            | 0       | Type 4 fimbrial biogenesis protein PilM                                       | MA               |
| pilN   | –3.871            | 0       | Type 4 fimbrial biogenesis protein PilN                                       | MA               |
| pilO   | –4.476            | 0       | Type 4 fimbrial biogenesis protein PilO                                       | MA               |
| pilP   | –3.956            | 0       | Type 4 fimbrial biogenesis protein PilP                                       | MA               |
| pilQ   | –3.219            | 0       | Type 4 fimbrial biogenesis outer membrane protein PilQ precursor              | MA               |
| pilU   | –2.226            | 0       | Twitching motility protein PilU                                              | MA               |
| pilV   | –2.382            | 0       | Type 4 fimbrial biogenesis protein PilV                                       | MA               |
| pilW   | –2.516            | 0       | Type 4 fimbrial biogenesis protein PilW                                       | MA               |
| pilX   | –2.366            | 0       | Type 4 fimbrial biogenesis protein PilX                                       | MA               |
| pilF   | –2.053            | 0       | Type 4 fimbrial biogenesis protein PilM                                       | MA               |
| pilG   | –2.713            | 0       | Twitching motility protein PilG                                              | TCRSs; MA; CT    |
| pilH   | –3.112            | 0       | Twitching motility protein PilH                                              | TCRSs; MA; CT    |
| pilI   | –2.731            | 2.20E–09| Twitching motility protein PilI                                              | MA; CT           |
| pilJ   | –5.282            | 0       | Twitching motility protein PilJ                                              | MA; CT           |
| Vfr    | –2.047            | 0       | Transcriptional regulator vfr                                                | TrRs             |
| chpA   | –2.124            | 0       | Component of chemotactic signal transduction system                          | TCRSs; MA; CT    |
| cheB   | –2.315            | 1.15E–05| Probable methylesterase                                                     | CT               |
| flIC   | –2.145            | 0       | Flagellin type B                                                            | MA               |
| rpsK   | –2.592            | 0       | 30S ribosomal protein S11                                                    | TPTMD            |
| rplA   | –2.375            | 0       | 50S ribosomal protein L1                                                     | TPTMD            |
| glnS   | –2.102            | 0       | Glutamyl-tRNA synthetase                                                     | TPTMD; AABM      |
| glyS   | –2.162            | 2.11E–13| Glycyl-tRNA synthetase beta chain                                            | TPTMD; AABM      |
| leuS   | –2.131            | 0       | Leucyl-tRNA synthetase                                                       | TPTMD; AABM      |
| lysS   | –2.38             | 0       | Lysyl-tRNA synthetase                                                        | TPTMD; AABM      |
| proS   | –2.764            | 0       | Prolyl-tRNA synthetase                                                       | TPTMD; AABM      |
| valS   | –2.13             | 0       | Valyl-tRNA synthetase                                                        | TPTMD; AABM      |
| aspS   | –2.129            | 0       | Aspartyl-tRNA synthetase                                                     | T-RNA-PD; TPTMD  |
| hisF1  | –3.599            | 1.02E–11| Imidazole glycerol-phosphate synthase, cyclase subunit                        | AABM             |
| hisG   | –2.863            | 2.04E–10| ATP-phosphoribosyltransferase                                                | AABM             |
| argB   | –2.436            | 0       | Acetylglutamate kinase                                                       | AABM             |
| argG   | –2.428            | 0       | Argininosuccinate synthase                                                   | AABM             |
| argH   | –2.127            | 0       | Argininosuccinate lyase                                                      | AABM             |
| cysM   | –3.436            | 0       | Cysteine synthase B                                                          | AABM             |
| trpA   | –4.405            | 0       | Tryptophan synthase alpha chain                                              | AABM             |
| trpB   | –6.527            | 0       | Tryptophan synthase beta chain                                               | AABM             |
| hslU   | –4.255            | 0       | Heat shock protein HslU                                                      | CHSPs            |
| hslV   | –3.742            | 0       | Heat shock protein HslV                                                      | CHSPs            |
| htrG   | –2.835            | 0       | Heat shock protein HtpG                                                      | CHSPs            |
| htrX   | –2.557            | 0       | Heat shock protein HtpX                                                      | AP               |
| dnaA   | –2.631            | 0       | Chromosomal replication initiation protein                                   | DNA-RRMR         |
| dnaJ   | –2.528            | 0       | DnaJ protein                                                                 | DNA-RRMR; CHSPs; AP |
| dnaK   | –3.087            | 0       | DnaK protein                                                                 | DNA-RRMR         |
| holC   | –2.701            | 5.25E–09| DNA polymerase III, chi subunit                                              | DNA-RRMR         |
| mutL   | –2.563            | 0       | DNA mismatch repair protein MutL                                             | DNA-RRMR         |
| Phr    | –3.012            | 2.14E–12| Deoxyribodipirimidine polynucleotase                                         | DNA-RRMR         |
| sbcD   | –2.056            | 2.40E–13| Exonuclease SbcD                                                            | DNA-RRMR         |
| recG   | –2.105            | 7.52E–16| ATP-dependent DNA helicase RecG                                              | DNA-RRMR; TRs    |
| uvrC   | –2.622            | 0       | Excinuclease ABC subunit                                                     | DNA-RRMR         |
| uvrD   | –3.412            | 0       | DNA helicase II                                                              | DNA-RRMR         |
| ccmE   | –2.297            | 7.41E–10| Cytochrome C-type biogenesis protein CcmE                                    | EM               |
| ccmG   | –2.001            | 3.24E–06| Cytochrome C biogenesis protein CcmG                                          | TPTMD; CHSPs; EM |
| PA1600 | –2.819            | 2.31E–06| Probable cytochrome c                                                        | EM               |
The expression of NADH dehydrogenase I chain subunits (nuoD and nuoE) in the oxidative phosphorylation pathway was significantly decreased in KF-treated samples, with log2-fold changes of −2.216 and −2.162, respectively. Table 5 also shows that the expression of genes involved in energy production in the absence of oxygen through denitrification was decreased after KF treatment. These genes with downregulated expression include those encoding elements in cytochrome c (ccmEG, PA1600, and PA4571) and cbb3-1 cytochrome c terminal oxidases (ccoQ1 and ccoO1).

The expression of NADH dehydrogenase I chain subunits (nuoD and nuoE) and cbb3-1 cytochrome c terminal oxidases (ccoQ1 and ccoO1) and cbb3-1 cytochrome c are highly expressed under microaerobic conditions. These genes with downregulated expression include those encoding elements in cytochrome c (ccmEG, PA1600, and PA4571) and cbb3-1 cytochrome c terminal oxidases (ccoQ1 and ccoO1).

3.1.6. Group VI: Genes with Downregulated Expression at 6 h and 24 h. This group consisted of genes with downregulated expression at both 6 h and 24 h (Table 6). Growth under KF exposure conditions induced the downregulation of the expression of genes encoding heat shock proteins (hslUV, grpE, and recG) (Table 5).

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### Table 5: Continued.

| Genes      | 24 h (fold change) | p value | Description                              | Functional class |
|------------|--------------------|---------|-----------------------------------------|------------------|
| PA4571     | −2.708             | 0       | Probable cytochrome c                  | EM               |
| PA4133     | −5.344             | 0       | Cytochrome c oxidase subunit (cbb3-type)| EM               |
| ccoQ1      | −2.475             | 0       | Cytochrome c oxidase, cbb3-type, CcoO subunit| EM               |
| ccoQ1      | −2.235             | 1.16E−04| Cytochrome c oxidase, cbb3-type, CcoQ subunit| EM               |
| nuoD       | −2.216             | 0       | NADH dehydrogenase I chain C,D          | EM               |
| nuoE       | −2.162             | 9.15E−13| NADH dehydrogenase I chain E            | EM               |
| narK1      | −2.541             | 2.23E−15| Nitrite extrusion protein 1             | MPs; TSMs        |
| narK2      | −5.637             | 0       | Nitrite extrusion protein 2             | MPs; TSMs        |
| narG       | −4.157             | 0       | Respiratory nitrate reductase alpha chain| EM               |
| narJ       | −2.386             | 0.014   | Respiratory nitrate reductase delta chain| EM               |
| narL       | −2.09              | 0       | Two-component response regulator NarL   | EM; TCRSs        |
| Dnr        | −2.065             | 8.03E−14| Transcriptional regulator Dnr           | Trs              |

This group also contained several genes related to cytochrome c, which is highly expressed under anaerobic conditions. These genes with downregulated expression include those encoding elements in cytochrome c (ccmEG, PA1600, and PA4571) and cbb3-1 cytochrome c terminal oxidases (ccoQ1 and ccoO1) and cbb3-1 cytochrome c.

The expression of NADH dehydrogenase I chain subunits (nuoD and nuoE) and cbb3-1 cytochrome c terminal oxidases (ccoQ1 and ccoO1) and cbb3-1 cytochrome c are highly expressed under microaerobic conditions. These genes with downregulated expression include those encoding elements in cytochrome c (ccmEG, PA1600, and PA4571) and cbb3-1 cytochrome c terminal oxidases (ccoQ1 and ccoO1) and cbb3-1 cytochrome c.

The type III secretion system (T3SS) regulates the virulence of many pathogenic bacteria [10]. The T3SS system is essential for the export of effector proteins through a needle-like structure directly inside target host cells [10]. Transcriptome data showed the continuous upregulation of all T3SS apparatus, regulators, and effector proteins in *P. aeruginosa* at 6 h and 24 h of KF treatment (Table 1). Interestingly, the expression of *P. aeruginosa* genes involved in the flagella assembly pathway, which mediates swimming motility and functions in biofilm development, was increased [11]. These findings indicate that the T3SS system and flagella assembly pathway are tuned by different environmental stresses, which might be an essential survival strategy for this bacterium [12].

As shown in Table 2, gene expression analysis of *P. aeruginosa* grown in KF for 6 h displayed an upregulation of the operon fabAB (Table 2), which is involved in the biosynthesis of unsaturated fatty acids (UFAs). UFAs are required to maintain the fluidity of bacterial membranes [13]. Thus, we assume that the membrane lipid composition might be altered to allow growth under KF exposure conditions.

### 3.2. Validation of NGS Results Using Quantitative Real-Time PCR (qRT-PCR).

Four genes identified from RNA-seq data (uvrD, sodM, fumC1, and rpsL) were selected for qRT-PCR analysis. *nadB* (PA0761) was chosen as the reference control gene that exhibited no change in our transcriptomic data at two treatment times. qRT-PCR data showed the same trend of either upregulation or downregulation of the genes as that in NGS, thereby validating our NGS results (Table 7). The variations were due to the difference in the sensitivity of the two assays.

### 4. Discussion

Previous studies have elucidated that KF can inhibit *P. aeruginosa* growth [6, 7]. In regard to this inhibitory effect, the approach of transcriptomic analysis is useful to identify the differentially expressed genes in this bacterium. The transcriptome profiles of *P. aeruginosa* treated with KF were examined to demonstrate the changes in gene expression at two time points (6 h and 24 h incubation). Functional analyses were performed to clarify the possible mechanisms underlying the changes in gene expression from a global perspective. In addition, qRT-PCR was used to confirm the RNA-seq results of select genes.

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Pseudomonas aeruginosa has a highly complex respiratory chain with multiple terminal oxidases and can respire both oxygen and nitrogen oxides [14, 15]. Under anaerobic conditions, *P. aeruginosa* can respire through denitrification [16]. In this process, four reductases (nitrate-, nitrite-, NO-, and nitrous oxide reductases) allow bacterial growth [17]. In this process, four reductases (nitrate-, nitrite-, NO-, and nitrous oxide reductases) allow bacterial growth [17].

Table 6: List of the group VI genes with downregulated expression at 6 h and 24 h.

| Genes | 6 h (fold change) | p value | 24 h (fold change) | p value | Description | Functional class |
|-------|------------------|---------|------------------|---------|-------------|-----------------|
| htpG  | -4.483           | 0       | -2.835           | 0       | Heat shock protein HtpG | CHSPs |
| HslV  | -5.567           | 0       | -3.742           | 0       | Heat shock protein HslV | CHSPs |
| HslU  | -3.379           | 0       | -4.255           | 0       | Heat shock protein HslU | CHSPs |
| grpE  | -2.265           | 0       | -3.718           | 0       | Heat shock protein GrpE | DNA-RMR; CHSPs |
| ClpB  | -5.018           | 0       | -2.312           | 0       | ATP-binding subunits of clp protease and DnaK/DnaJ chaperones | TPTMD |
| BfrR  | -2.707           | 0.033   | -2.584           | 0       | Response regulator | TRs; TCRSs |
| BfrS  | -4.934           | 6.31E-15| -2.714           | 0       | Signal transduction histidine kinase regulating C4-dicarboxylate transport system | TCRSs |

Table 7: Transcript level comparison of *P. aeruginosa* genes between qRT-PCR and NGS. qRT-PCR is the mean of two biological replicates with three technical replicates for each gene. Reference gene (nadB): L-aspartate oxidase, *uwrD*, and *sodM* were downregulated at 24 h with no change at 6 h; *fumC1* was upregulated at 6 h and downregulated at 24 h; *rpsL* was upregulated at 6 h with NC at 24 h exposure.

| Genes ID | Gene symbol | NGS Fold change | qRT-PCR Fold change | Primers | Length (bp) | Description |
|----------|-------------|-----------------|---------------------|---------|-------------|-------------|
| PA5443   | uwrD        | NC              | NC                  | GTGCAGCTGTCAATAC | 17       | DNA helicase II |
|          |             | -3.412 ± 0      | -2.54 ± 0.01        | GCCCTGAATGGTGGTAGA | 20       |                |
| PA4468   | sodM        | NC              | NC                  | GAGCAGCCGTTGAAGCTCT | 20       | Superoxide dismutase |
|          |             | -3.955 ± 0      | -2.44 ± 0.01        | GGCACATACGGTCCACAGAC | 20       |                |
| PA4470   | fumC1       | 3.618 ± 0       | -3.59 ± 0.01        | TCGGGCAACCTCGAAGCTGGA | 20       | Fumarate hydratase |
|          |             | -4.599 ± 0      | -5.39 ± 0.01        | GAGCTTGCCCTGGTGGACCT | 20       |                |
| PA4268   | rpsL        | 2.56 ± 0        | NC                  | CGGCACTGCTGAAGCTGGA | 20       | 305 ribosomal protein S12 |
|          |             |                 | 3.53 ± 0.53         | CCCGGAAAGTTCTTTACCG | 20       |                |
| PA0761*  | nadB        | Reference gene  | 22                  | ATCTCTTATAACCAGCAATCCC | 22       | L-aspartate oxidase |
|          |             |                 |                     | CGGTGATGAGGAAATCCTTTG | 20       |                |

Subunits (nuo-operon) in the oxidative phosphorylation pathway were increased in *K*<sub>F</sub>-treated samples at 6 h. The NADH-I chain is coupled to the denitrification pathway [22, 23]. The upregulation of genes encoding NADH-I chain was paralleled by the increased expression of *anr* gene involved in controlling *P. aeruginosa* gene expression under anaerobic conditions, suggesting that *K*<sub>F</sub>-treated cells underwent a switch to anaerobic respiration in response to oxidative stress. Zimmermann et al. [24] noted that the *anr* deletion mutant of *P. aeruginosa* does not grow anaerobically. In addition, the expression of genes encoding several elements of the ATP-binding cassette transporters (ABCs), which exist in all bacterial species and provide a pathway for substrates to cross the cell membrane [25], was upregulated (Table 1). Interestingly, the growth of *P. aeruginosa* under *K*<sub>F</sub> exposure conditions at two time points led to the increased expression of genes encoding ABC transporters of amino acids, carbohydrates, and inorganic ions (Table 1). As amino acids are key intermediates in bacterial metabolism, the increase in the ABC transporter proteins led to increased amino acid or peptide uptake. In conclusion, to maintain energy consumption, the cell increases the oxidative phosphorylation...
pathway and the expression of ATP synthase to produce ATP.

During host infection, *P. aeruginosa* utilizes several systems to acquire iron from the surrounding environment [26]. The iron uptake mechanisms include the production of siderophores (pyoverdine and pyochelin) and heme uptake [27]. Transcriptomic analysis showed a downregulation of the expression of genes involved in iron acquisition in *K*<sub>F</sub>-treated samples at 24 h of *P. aeruginosa* growth. As shown in Table 5, TonB-dependent siderophore receptor (*tonB*) and hae-moxygenase (*hemO*) showed a reduction in the expression level at 24 h. The downregulation of the expression of *pchR* encoding elements involved in iron Fe<sup>3+</sup> acquisition was also observed. In addition, the expression of genes highly regulated by iron starvation was repressed by *K*<sub>F</sub> treatment. These genes encode members of the ECF subfamily, which is mainly associated with extracellular functions that include the regulation of periplasmic stress, iron transport, metal ion efflux systems, alginate secretion, and synthesis of membrane-localized carotenoids [28]. Consequently, the results suggested that *K*<sub>F</sub>-treated cells underwent conditions of excess intracellular iron, which led to the downregulation of the expression of genes regulated by the ferric uptake regulator (Fur) required for iron acquisition. Ochsner et al. [29] reported that the Fur protein uses Fe<sup>2+</sup> as a cofactor and binds to Fur-Fe<sup>2+</sup>, resulting in the repression of the genes encoding pyochelin and pyoverdin proteins in iron-replete environments. Furthermore, transcriptomic analysis also showed the downregulation of the expression of genes coding for the components of the DNA replication and repair machinery in *P. aeruginosa* at 24 h of *K*<sub>F</sub> treatment. A superoxide (O<sub>2</sub>−) byproduct is formed by the autoxidation of a variety of reduced electron carriers and redox enzymes [30]. O<sub>2</sub>− is implicated in the production of oxidative DNA damage by the steady release of iron from storage proteins into the cytosol, and thus, the free iron binds DNA and catalyses electron transfer from the reductant to H<sub>2</sub>O<sub>2</sub> [31, 32]. The resultant ferryl or hydroxyl radical attacks the adjacent DNA [33]. The repression of genes encoding DNA repair proteins was coupled with the repression of genes involved in iron regulation at 24 h, suggesting that *K*<sub>F</sub>-treated cells were exposed to an excess concentration of intracellular free iron, leading to either hydroxyl or ferryl radical production, which promotes oxidative DNA damage by increasing the amount of DNA-bound iron. Oxidative DNA damage was also evident by the downregulation of the expression of genes involved in defence (*sodM*) against reactive oxygen species.

*P. aeruginosa* pathogenicity depends on the production and secretion of a large variety of virulence factors, including pyocin S2, in response to host environments. Pyocin S2 is a protease-sensitive bacteriocin produced by *P. aeruginosa* that kills sensitive cells by damaging chromosomal DNA through its DNase activity and the inhibition of lipid synthesis [34]. RNA-seq analysis showed reduced expression levels of pyocin S2 protease at 6 h of exposure to *K*<sub>F</sub> (Table 3). As shown in Table 5, the growth of *P. aeruginosa* under *K* treatment conditions at 24 h led to the decreased expression of genes involved in the LPS biosynthesis process (*lpxA, lpxB, waaF, waaG, waaP, PA4998, PA5007, PA5008, and rmlA*). LPS is the major component defining the outer membrane of Gram-negative bacteria. The outer membrane is essential for viability and mediates virulence and resistance to toxic and antibacterial agents [35]. Interestingly, a previous study revealed that the *waaP* gene in *P. aeruginosa* is required to produce full-length LPS, which is recognized by the outer membrane transport assembly machinery in this bacterium [36]. Therefore, *waaP* may constitute a good target for the development of novel antipseudomonal agents. Our previous observation is consistent with this finding. Transmission electron microscopy studies have revealed that cells treated with *K*<sub>F</sub> exhibit severe membrane damage concurrent with the disruption of membrane integrity, leading to the loss of intracellular material at 24 h of incubation [7]. These results suggest that LPS biosynthesis may be inhibited at 24 h *K*<sub>F</sub> exposure. In addition, the MexGHI-OpmD efflux pump system has been implicated in the efflux of xenobiotics, including the antibiotic norfloxacin and the heterocyclic dye acriflavine [37], and the transport of phenazine molecules [38]. Interestingly, the downregulation of the MexGHI-OpmD system observed in the RNA-seq data was coupled with a reduction in the phenazine biosynthesis process *K*<sub>F</sub> at 24 h of *P. aeruginosa* treated with *K*<sub>F</sub> (Table 5). The opportunistic pathogen *P. aeruginosa* is well known for its production of bright blue phenazine pyocyanin, which contributes to the colouration of sputum and pus associated with infections and interferes with multiple host cellular functions [39]. In response to *K*<sub>F</sub> treatment, *P. aeruginosa* repressed the expression of the secretory machinery (Sec system), responsible for the secretion of virulence factors, extracellular degradative enzymes, and other toxins, enabling adaptation to a wide range of ecological niches [40]. Therefore, taken together, these data reveal the marked remodelling of gene transcription characterized by an early and late reduction in the expression of several genes associated with virulence factors of *P. aeruginosa* in response to *K*<sub>F</sub> treatment.

Bacteria can form biofilms on living or nonliving surfaces and can be prevalent in natural, industrial, and hospital settings. Bacterial motility and adhesion are critical for biofilm development [41]. The type IV pili in *P. aeruginosa* play an important role in the adherence to epithelial cells and microbial intra and interspecies competition, while flagella filament-mediated motility enables bacteria to reach a surface and then divide and spread along the surface [42]. In response to *K*<sub>F</sub> treatment at 24 h, the downregulation of type IV and flagellin type B (*fliC*) genes observed in RNA-seq (Table 5) was paralleled by the decreased expression of genes involved in biofilm formation in *P. aeruginosa* (Table 6). These findings may indicate that *K*<sub>F</sub> treatment affects genes involved in biofilm formation and motility. As a consequence of these combined factors, we thus hypothesise that the swimming and biofilm formation ability of *P. aeruginosa* would be inhibited under *K*<sub>F</sub> treatment conditions.

The treatment of *P. aeruginosa* with *K*<sub>F</sub> for 24 h led to the decreased expression of genes encoding the aminoacyl-tRNA synthetases glutamine, glycine, leucine, lysine, proline, valine, and aspartate. Furthermore, genes associated
with the biosynthesis of several amino acids, including histidine, arginine, cysteine, and tryptophan, were also expressed at reduced levels in 5F-treated samples at 24 h (Table 5). RNA-seq data showed that the downregulation of the expression of genes hslVU, htpG, and gppE involved in the degradation of unfolded or misfolded proteins that accumulate in the periplasm [43], following heat shock or other stress conditions was coupled with the decreased expression of the clpB gene encoding an ATP-dependent protease, which functions as part of the chaperone network essential for the recovery of stress-induced protein aggregates [44] (Table 6). The altered expression of these genes at two 5F exposure time points may be indicative of their essential function in cellular responses to environmental stress. As a consequence of these combined factors, we thus assume that protein synthesis in P. aeruginosa might be affected by 5F treatment.

5. Conclusion
The crisis of the antibiotic resistance demands to be met with concerted efforts across many disciplines and areas of expertise. Natural products are mainstays of drugs and still play an essential role in providing chemical diversity, despite a reduced interest shown by pharmaceutical companies. Herein, we could prove efficacy of 5F against one of the most notorious pathogen P. aeruginosa. The 5F compound is more likely to have multitargets inside the test P. aeruginosa. To the best of our knowledge, the current study is the first report describing the antibacterial effect of 5F on P. aeruginosa at the gene expression level through transcriptomic analysis, revealing the regulation of various genes involved in cellular processes that lead to the destabilization of this bacterium. The transcriptomic analysis showed that 5F increases the expression of genes involved in the electron transport chain (NADH-I), resulting in the induction of ATP synthesis. 5F also increased the expression of genes associated with ATP-binding cassette transporters, flagella, type III secretion system proteins, and DNA replication and repair, which may further affect nutrient uptake, motility, and growth. The major mechanisms through which 5F seems to exert its antibacterial effect on P. aeruginosa are by the repression of a broad range of virulence factors associated with LPS biosynthesis, iron homeostasis, cytotoxic pigment pyocyanin production, and motility and adhesion that are representative of an acute P. aeruginosa infection profile. Taken together, the present study is a good demonstration of the therapeutic usefulness of the natural product from plant in validating the traditional medicine, i.e., M. malabathricum, very common in Malaysia. Specifically, attenuations of bacterial virulence factors are likely to be effective solutions in this therapeutic area. Although the current study offers a possible regulatory network of P. aeruginosa induced by 5F treatment, further studies will focus on the protein level expression of the target genes. In general, this study has generated scientific evidence that natural product research is perfectly positioned to address and solve the present bacterial resistance crisis and the closely linked antibiotic discovery gap.

Abbreviations

AP: Adaptation, protection
SFs: Secreted factors (toxins, enzymes, and alginate)
AABM: Amino acid biosynthesis and metabolism
T-RNA: Transcription, RNA processing, and degradation
BCPGCs: Biosynthesis of cofactors, prosthetic groups, and carriers
TRs: Transcriptional regulators
CWLC: Cell wall/LPS/capsule
TPTMD: Translation, post-translational modification, degradation
CHSPs: Chaperones and heat shock proteins
TSMs: Transport of small molecules
CT: Chemotaxis
TCRSs: Two-component regulatory systems
DNA: DNA replication, recombination, modification, and repair
HUU: Hypothetical, unclassified, and unknown
EM: Energy metabolism
PSEA: Protein secretion/export apparatus
FAPM: Fatty acid and phospholipid metabolism
PEs: Putative enzymes
MA: Motility and attachment
MPs: Membrane proteins.

Data Availability

The data used to support the findings of this study are included within the supplementary information files.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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