CHAPTER 4

RESULTS

4.1 Antimicrobial Properties of *L. rhamnosus* ATCC 7469 CFS

The antimicrobial properties of *L. rhamnosus* ATCC 7469 CFS against *P. gingivalis* ATCC 33277 were assessed based on two procedures which were disc diffusion assay and broth microdilution assay for susceptibility determination.

4.1.1 Disc Diffusion Assay of *L. rhamnosus* ATCC 7469 CFS

The CFS of *L. rhamnosus* ATCC 7469 showed high inhibition zone diameter (10.64 ± 0.44 mm) as shown in Table 4.1. The mean inhibition diameter of the positive control, 0.2% chlorhexidine was larger at 16.88 ± 2.16 mm. Meanwhile, as expected the negative control sterile MRS broth showed no inhibition at all. The CFS inhibition diameter were statistically significant in comparison to negative control at $p<0.001$ with the ANOVA test.

Table 4.1: The inhibition diameters of the tested samples against *P. gingivalis* in disc diffusion assay (significant results are marked by asterisk (*) with p-value<0.001 when being compared to negative control).

Test Samples	**Inhibition diameter, Mean±SD (mm)**	**Significance**
*L. rhamnosus*ATCC 7469 CFS	10.64 ± 0.44*	p-value <0.001
0.2% chlorhexidine	16.88 ± 2.16	
Sterile MRS broth	-	

4.1.2 Minimum Inhibitory and Bactericidal Concentration of *L. rhamnosus*ATCC 7469 CFS

The MIC and MBC were determined to identify the lowest concentration of *L. rhamnosus* ATCC 7469 CFS to inhibit *P. gingivalis* ATCC 33277. The MIC and MBC values of *L. rhamnosus* ATCC 7469 CFS were identified as 25% (v/v, CFS/sterile broth) (Table 4.2) compared to positive control, 0.2% chlorhexidine at 3.125%. The negative control, sterile MRS recorded no inhibitory and bactericidal effect on *P. gingivalis* ATCC 33277. The result indicated that the CFS was able to inhibit and simultaneously kill the pathogen at 25% v/v concentration. This was shown in Figure 4.1 where the clear well indicated that there was no growth of *P. gingivalis* which was marked by the MTT solution. The MTT solution stained the culture with specific metabolic activity related to growth. Meanwhile, the wells that showed colour changes indicated that there was growth of *P. gingivalis.* The determination of MBC was also demonstrated in Figure 4.1. The visualization of MBC values determination was as shown in Figure 4.2 where MBC was determined by the streak of culture that showed no culture at all or less than 10% colony on the plate. The results indicated that all *P. gingivalis* colony exposed to the particular concentration of CFS were killed. In this study, 25% v/v concentration of CFS was found to kill more than 90% of *P. gingivalis.*

Table 4.2: The minimum inhibitory and bactericidal concentration of *L. rhamnosus* ATCC 7469 CFS as determined by broth microdilution assay.

Test Samples	**Minimum Inhibitory Concentration, MIC (%, v/v)**	**Minimum Bactericidal Concentration, MBC (%, v/v)**
*L. rhamnosus*ATCC 7469	25	25
0.2% Chlorhexidine	<3.125	<3.125
Sterile MRS broth	>100	>100

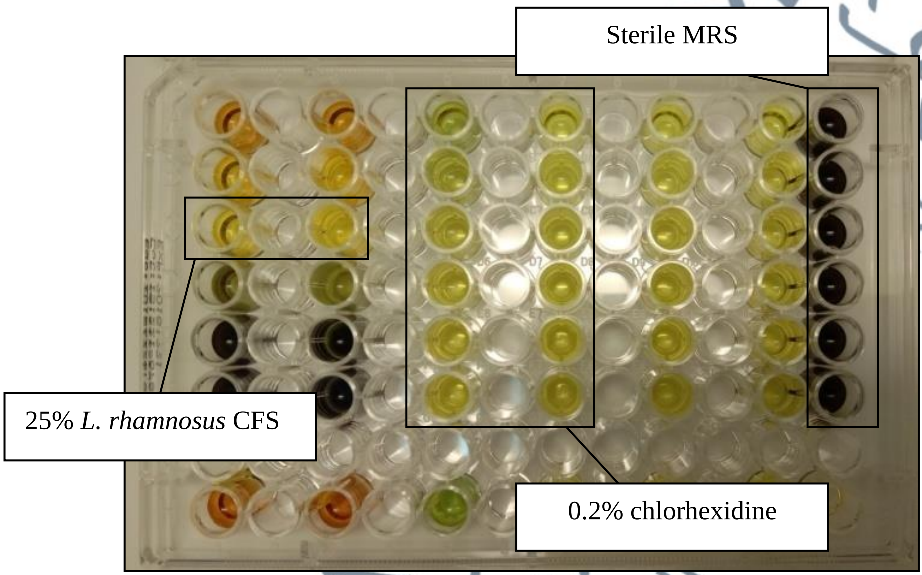


Figure 4.1: The representative plates for MIC after being stained with MTT solution.

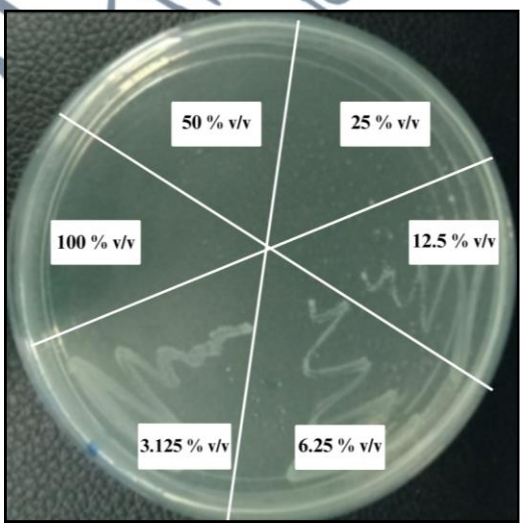


Figure 4.2: The representative streak plate for MBC determination.

4.2 Anti-biofilm Properties of *L. rhamnosus* ATCC 7469 CFS

The anti-biofilm properties were determined by measuring the optical density of biofilm that was stained by crystal violet solution in 96-well plate setups. The staining density was measured at 600nm by spectrophotometer to determine the intensity of stained biofilm in each well. The reduction of biofilm percentage varied according to the concentration of *L. rhamnosus* ATCC 7469 CFS. As shown in Figure 4.7, the highest concentration of *L. rhamnosus* ATCC 7469 CFS at 100% (v/v, CFS/sterile MRS) recorded the highest percentage of biofilm reduction at 84.94% while the lowest concentration of CFS recorded the percentage of biofilm reduction at 14.00%. Meanwhile, in positive control, the highest concentration of 0.2% chlorhexidine recorded 96.03% percentage of biofilm reduction. On the other hand, in the negative control, sterile MRS broth recorded no biofilm reduction at all. All results for test samples were statistically significant when compared to the untreated biofilm (p-value > 0.001).

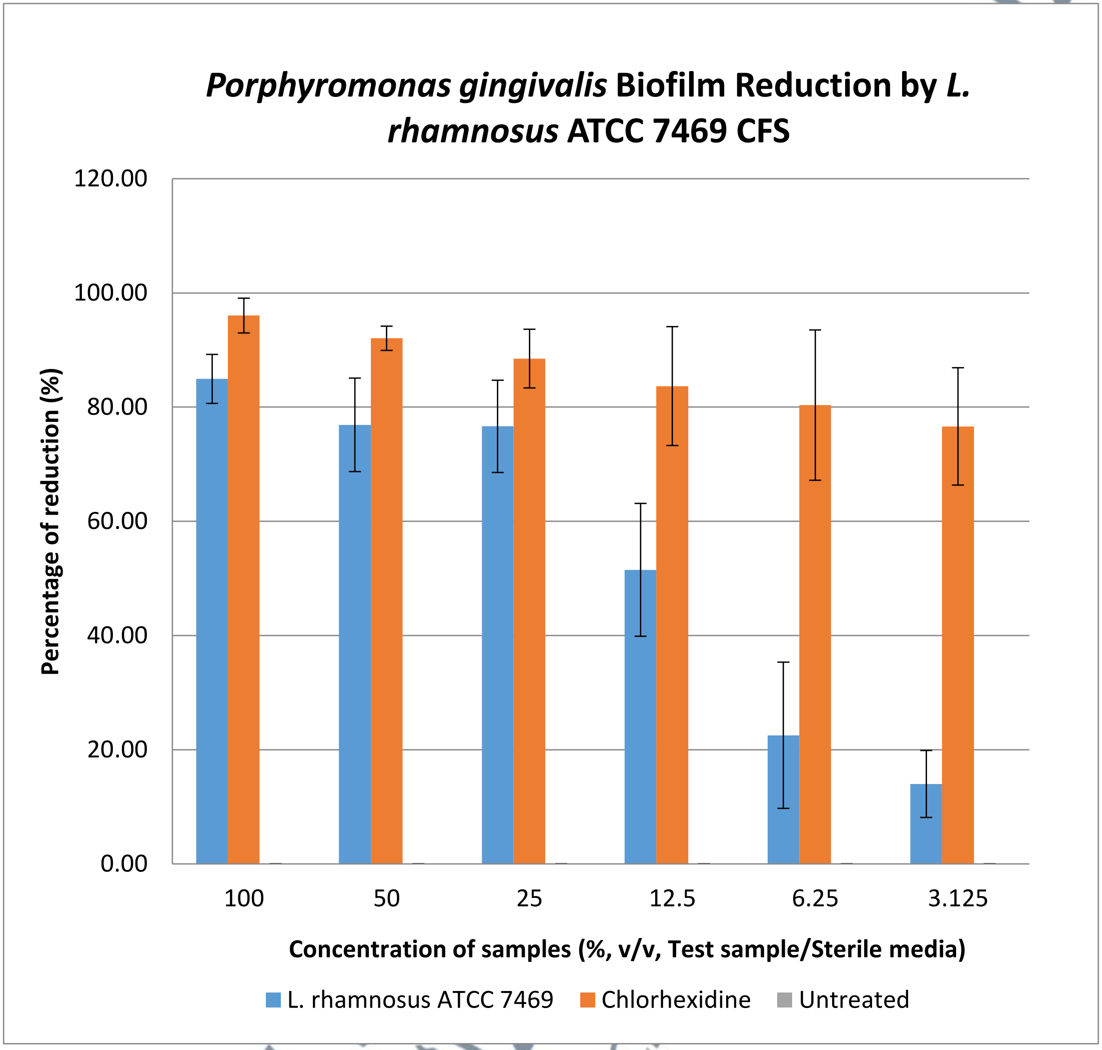


Figure 4.3: *P. gingivalis* biofilm inhibition by *L. rhamnosus* ATCC 7469 CFS.

4.3 Transcriptomic Analysis of *P. gingivalis*

4.3.1 Mapping Analysis of Treated and Untreated *P. gingivalis.*

The mapping analysis was carried with *P. gingivalis* ATCC 33277 as the reference genome. The total mapping rates (Table 4.3) were above 99% for both treated and untreated *P. gingivalis.* The preferred multiple mapping ratesare below than 10% and both samples reported less than 5% multiple mapping rate.

Table 4.3: The mapping results for treated and untreated *P. gingivalis.*

Sample name	**Treated *P. gingivalis***	**Untreated *P. gingivalis***
Total reads	17671550	18353180
Total mapped reads	17581281	18266026
Uniquely mapped reads	16771614	17743343
Multiple mapped reads	809667	522683
Total mapping rate	99.49%	99.53%
Uniquely mapping rate	94.91%	96.68%
Multiple mapping rate	4.58%	2.85%

4.3.2 Gene Expression Level

The gene expressions level of *P. gingivalis* was measured by transcript abundancy based on the gene length and sequencing depth. The analysis on *P. gingivalis* RNA showed that *L. rhamnosus* ATCC 7469 CFS had a siginifcant effect on the gene expression of *P. gingivalis* compared to the untreated *P. gingivalis.* As shown in Figure 4.4, out of 1848 genes, a total of 327 genes were expressed differentially. The differential gene expressions include 188 upregulated genes and 139 downregulated genes.

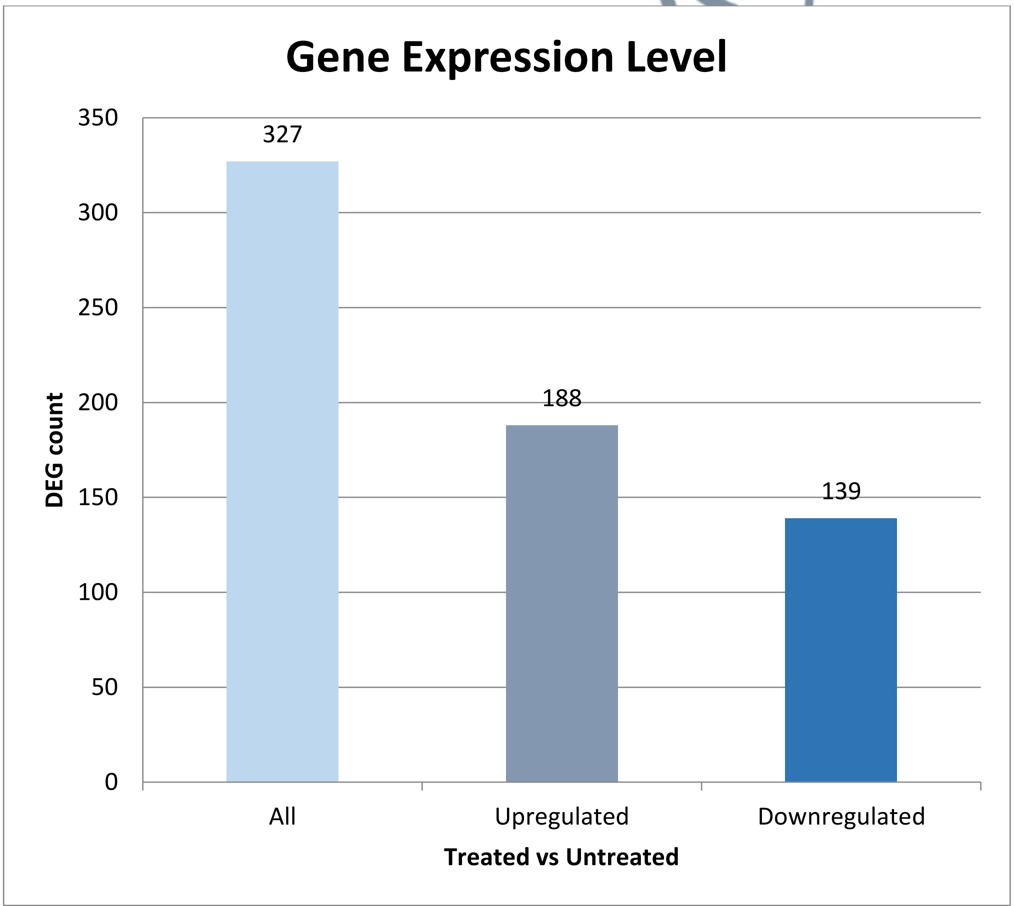


Figure 4.4: The comparison of upregulated and downregulated genes between the treated and untreated group of *P. gingivalis.*

The gene expression changes are as shown in the heatmap (Figure 4.5) where it showed genes with high fold change. The highly expressed genes are marked in red while the lowly marked genes are marked in blue or grey. The columns represented the two groups of samples, treated and untreated while each row the genes that were differentially expressed.

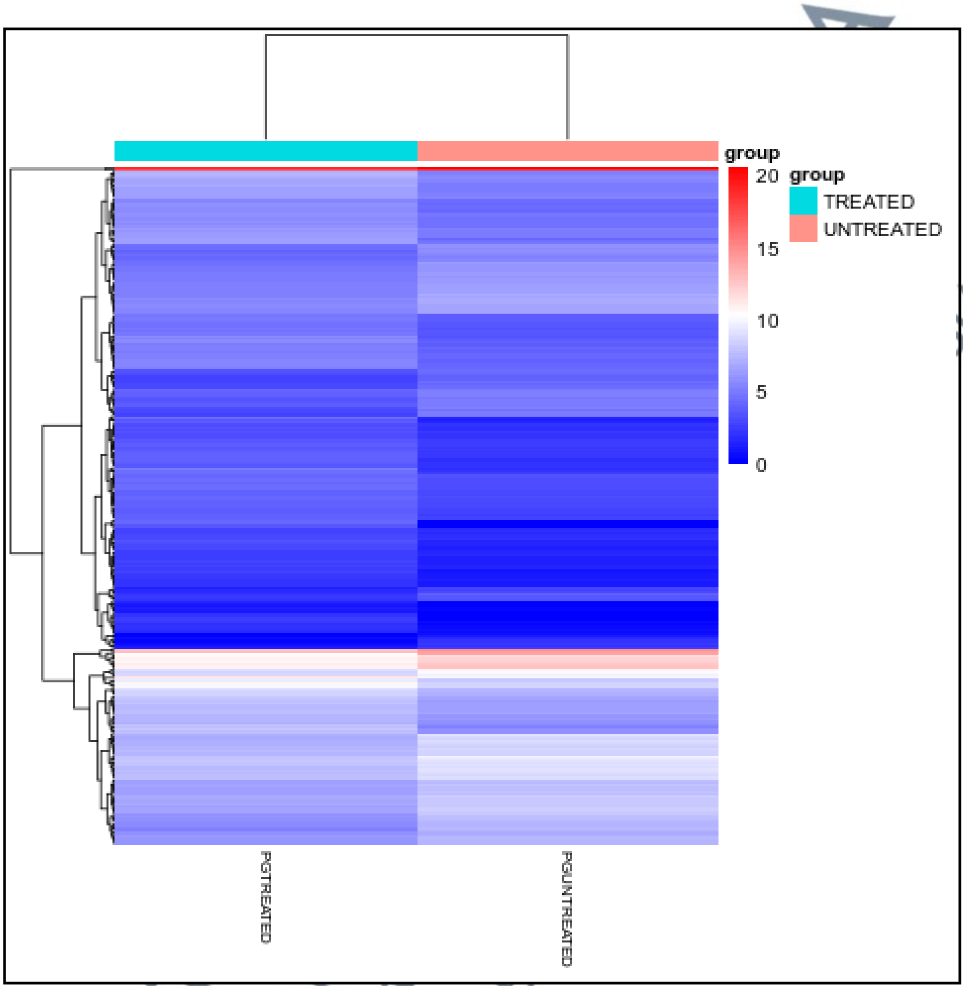


Figure 4.5: The heatmap of the differentially expressed genes.

Other than heatmap, the volcano plot is also important to infer the overall statistical distribution of the differentially expressed genes. As shown in Figure 4.6, the upregulated genes were marked in red while the downregulated genes were marked in green. The similarly expressed genes were marked in blue.The horizontal axis indicated the fold change of the genes while the vertical axis indicated the statistical significance of the gene expression. The smaller log p-value indicated higher statistical significance of the gene expressed.

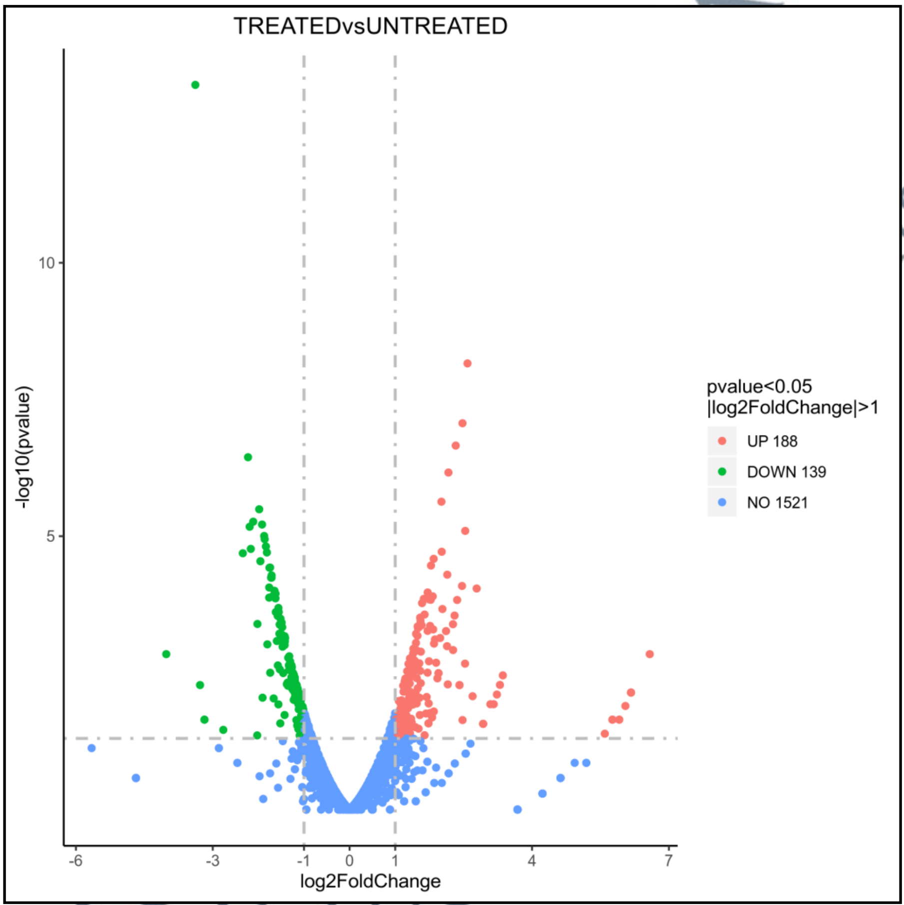


Figure 4.6: The volcano plot for all expressed genes.

4.3.3 Differentially Expressed Genes in Treated *P. gingivalis*

The treated group of *P. gingivalis* showed differential expression of some genes when being compared with the untreated group. Several genes related to DNA regulatory proteins, cellular metabolism, bacterial signalling, and virulent factors were significantly downregulated as shown in Table 4.4. Genes such as *dnaK, dnaJ*, and *polA* are regulatory and chaperone genes involved in the molecular activity of *P. gingivalis.* Meanwhile, genes like *pdxH, galE, ablA, kdsA, purH, hflX, folD, nifJ, mgtE, pdxB, purD*, and *rfbA* are involved in cellular metabolic activity especially in the glycolysis and electron transport chain. Additionally, genes like *mfa1*, *mfa3*, and *mfa4* are genes involved in fimbrial activity which is one of the virulent factors of *P. gingivalis.* The gene *ftsY* is the only geneinvolved in cellular signal recognition.

Table 4.4: The significantly downregulated genes in the treated group of *P. gingivalis.*

Gene name	Log_2 fold change	P-value	Gene description
dnaK	-1.8349	1.54E-05	Molecular chaperone DnaK
dnaJ	-1.7222	5.74E-05	Molecular chaperone DnaJ
galE	-1.7101	5.74E-05	NAD dependent epimerase/dehydratase family
ablA	-1.6468	9.98E-05	Lysine-2,3-aminomutase family
kdsA	-1.6250	0.000137	3-deoxy-8-phosphooctulonate synthase
purH	-1.5605	0.000205	Bifunctional phosphoribosylaminoimidazolecarboxamide formyltransferase
hflX	-1.5534	0.000243	50S ribosome-binding GTPase
pdxH	-2.0208	0.000403	Pyridoxamine 5'-phosphate oxidase
folD	-1.4239	0.000979	Tetrahydrofolate dehydrogenase/cyclohydrolase, NAD(P)-binding domain
nifJ	-1.3233	0.001570	Pyruvate:ferredoxin (flavodoxin) oxidoreductase
mfa1	-1.3333	0.001655	Minor fimbrial subunit Mfa1
mfa4	-1.3461	0.00167	Minor fimbrial subunit Mfa4
nrfA	-1.2660	0.002477	Ammonia-forming cytochrome C nitrite reductase
mgtE	-1.2717	0.002525	Cation (magnesium) transporter
pdxB	-1.2076	0.004307	D-isomer specific 2-hydroxyacid dehydrogenase, NAD binding domain
purD	-1.1343	0.007428	Phosphoribosylglycinamide synthetase, ATP-grasp (A) domain
mfa3	-1.1298	0.011486	Fimbrial tip subunit Mfa3
polA	-1.0701	0.011855	DNA polymerase I
ftsY	-1.0367	0.017097	Signal recognition particle-docking protein

On the other hand, genes related to ribosomal subunits, protein synthesis, protein folding, protein assembly, and some virulent genes were significantly upregulated in the treated *P. gingivalis*molecular expression. Table 4.5 shows the genes that are significantly upregulated. As shown in the table, a lot of ribosomal protein genes were upregulated which includes 50S and 30S ribosomal proteins. The genes are *rplQ*, *rpsD*,*rpmA*, *rplU*, *rpsN*, *rpsO*, *rpsM*, *rplC*, *rplS*, *rpsG*, and *rplX*. Other than that, there are a few metabolism related genes such as *gpmA*, *nadA*, *miaA*, *panC*, *coaE*, and *serC*. Additionally, a few assembly genes were also upregulated in the treated group of *P. gingivalis* including *rimP* and *bamD*. Other upregulated genes belong to virulent genes such as *rgpB* and *rgpA*, resolvase gene *ruvX* and heme chaperone gene *hemW*.

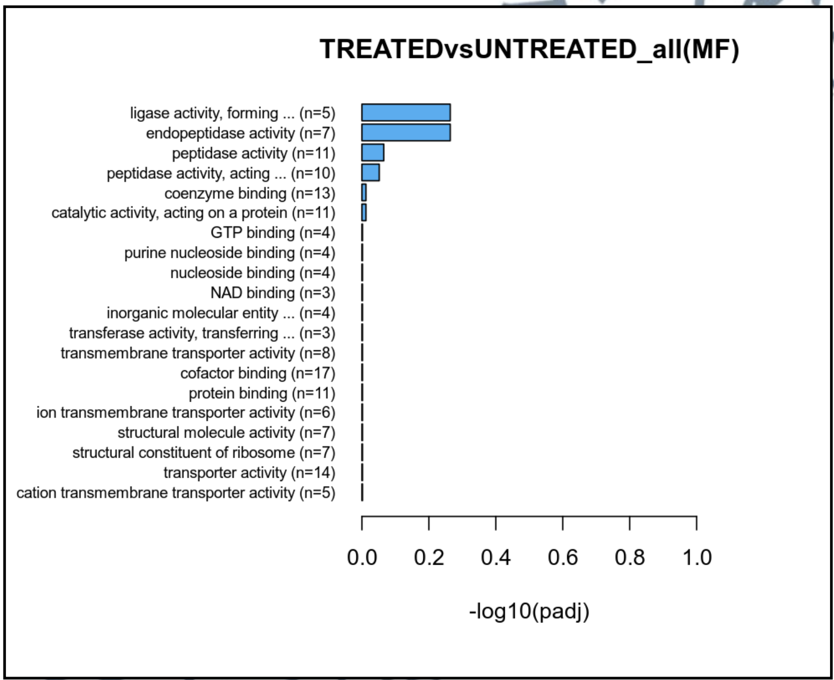
Table 4.5: The significantly upregulated genes in the treated group of *P. gingivalis*.

Gene name	**Log_2 fold change**	**P-value**	**Gene description**
ruvX	2.4745	8.59E-08	Holliday junction resolvase
gpmA	2.5345	8.00E-06	Putative 2,3-bisphosphoglycerate-dependent phosphoglycerate
rplQ	1.7843	3.44E-05	50S ribosomal protein L17
rpsD	1.7438	0.000144	30S ribosomal protein S4
nadA	2.3011	0.000283	Quinolinate synthase
rpmA	1.5461	0.000309	50S ribosomal protein L27
rplU	1.5543	0.000354	50S ribosomal protein L21
rpsN	1.4569	0.000611	30S ribosomal protein S14
rpsO	1.4881	0.000662	30S ribosomal protein S15
rgpB	1.3235	0.001720	Arg-gingipain RgpB

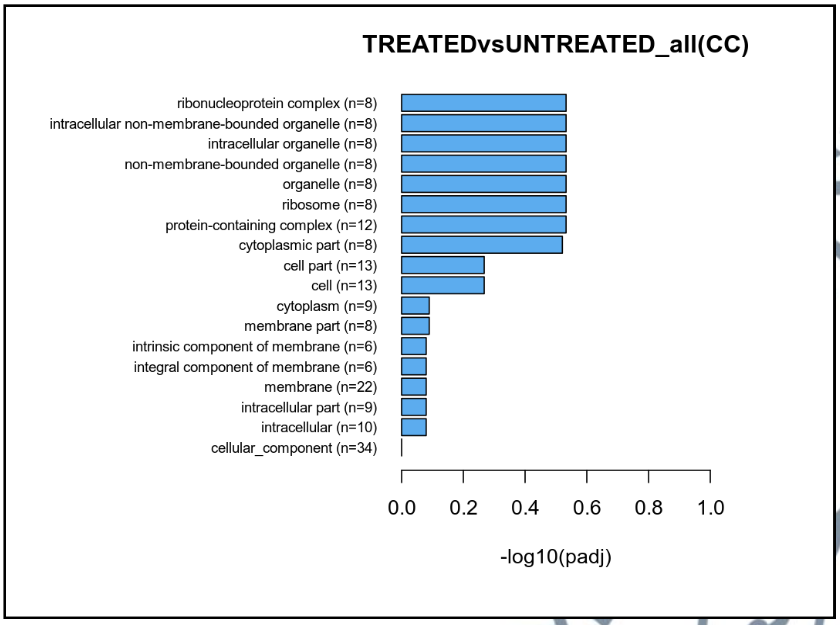
rgpA	1.2903	0.002164	Arg-gingipain RgpA
rpsM	1.3617	0.003352	30S ribosomal protein S13
rimP	1.3008	0.003534	Ribosome assembly cofactor RimP
miaA	1.4931	0.006048	tRNA (adenosine(37)-N6)-dimethylallyltransferase
rplC	1.2019	0.006903	50S ribosomal protein L3
rplS	1.1823	0.006995	50S ribosomal protein L19
bamD	1.4487	0.007262	Outer membrane protein assembly factor
panC	1.4722	0.011442	Pantoate-beta-alanine ligase
rpsJ	1.1781	0.011557	30S ribosomal protein S10
coaE	1.8469	0.016051	Dephospho-CoA kinase
serC	1.0163	0.024314	Putative phosphoserine transaminase
rpsG	1.1059	0.024467	30S ribosomal protein S7
hemW	2.9271	0.027042	Radical SAM family heme chaperone
rplX	1.1422	0.039811	50S ribosomal protein L24

4.3.4 Gene Ontology (GO) Enrichment Analysis

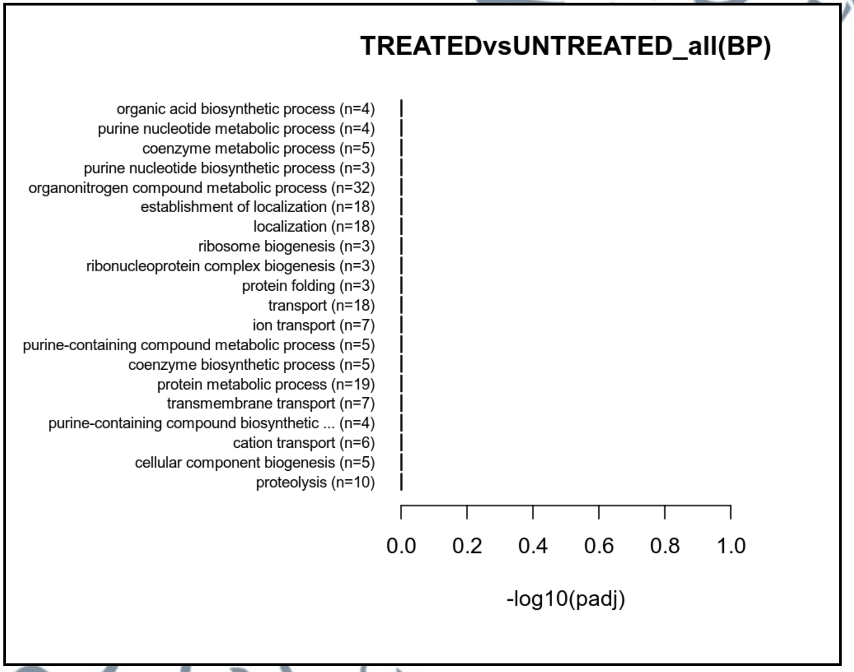
Investigation of the gene ontology (GO) of the treated *P. gingivalis* were also done to investigate the affected biological functions and metabolic pathway. The GO results for all differentially expressed genes are as shown in Figure 4.7. The GO of downregulated genes are as shown in Figure 4.8 while the GO of upregulated genes are as shown in Figure 4.9. The GO analysis is divided into three groups of biological functions which are molecular function (MF), cellular components (CC), and biological process (BP). The significant GO are marked with asterisk.



(a)

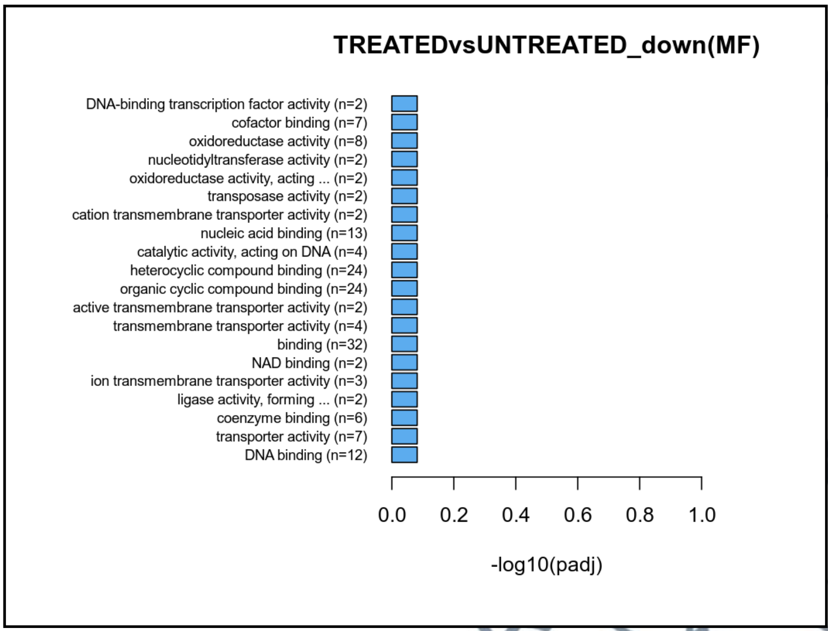


(b)

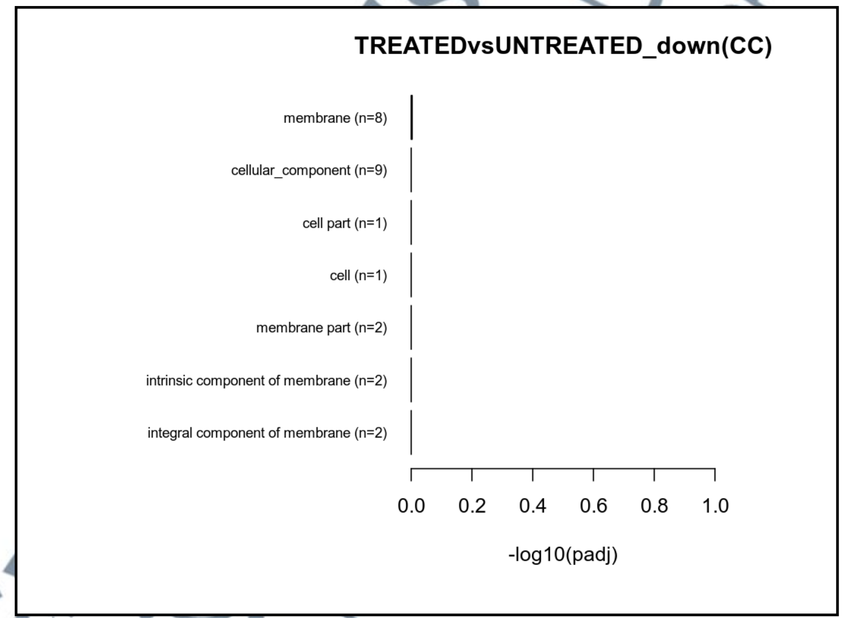


(c)

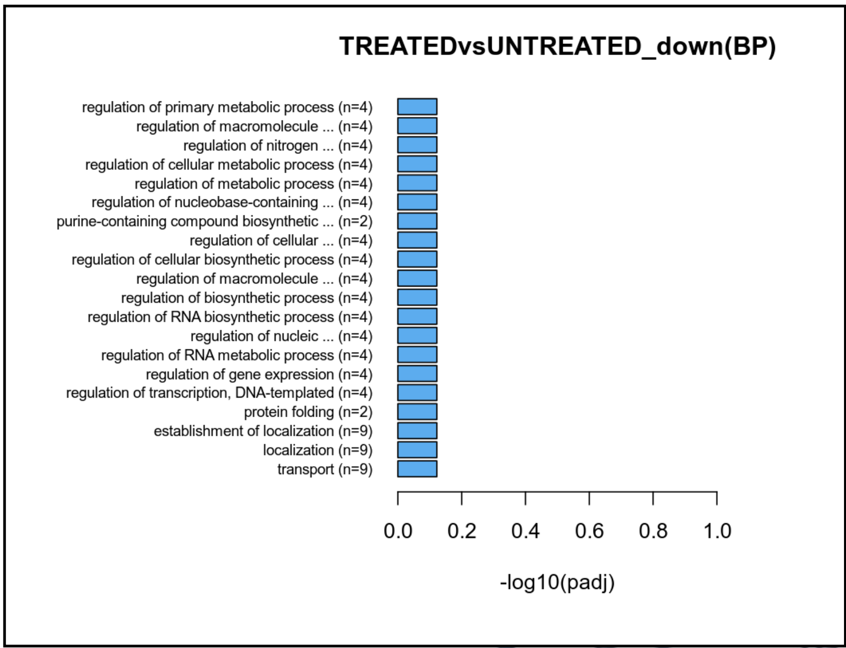
Figure 4.7: Overall gene ontology analysis (a) Gene ontology analysis of all differentially expressed genes in the MF category where the highest differentially expressed genes were in the ligase forming activity and endopeptidase activity group. (b) Gene ontology in the cellular components showed the differential expression of respective activity as shown in the figure. (c) Gene ontology in binding process category where all genes were expressed similarly in all categories.


TREATEDvsUNTREATED_down(MF)
DNA-binding transcription factor activity (n=2)
cofactor binding (n=7)
oxidoreductase activity (n=8)
nucleotidyltransferase activity (n=2)
oxidoreductase activity, acting ... (n=2)
transposase activity (n=2)
cation transmembrane transporter activity (n=2)
nucleic acid binding (n=13)
catalytic activity, acting on DNA (n=4)
heterocyclic compound binding (n=24)
organic cyclic compound binding (n=24)
active transmembrane transporter activity (n=2)
transmembrane transporter activity (n=4)
binding (n=32)
NAD binding (n=2)
ion transmembrane transporter activity (n=3)
ligase activity, forming ... (n=2)
coenzyme binding (n=6)
transporter activity (n=7)
DNA binding (n=12)
0.0
0.2
0.4
0.6
0.8
1.0
-log10(padj)

(a)

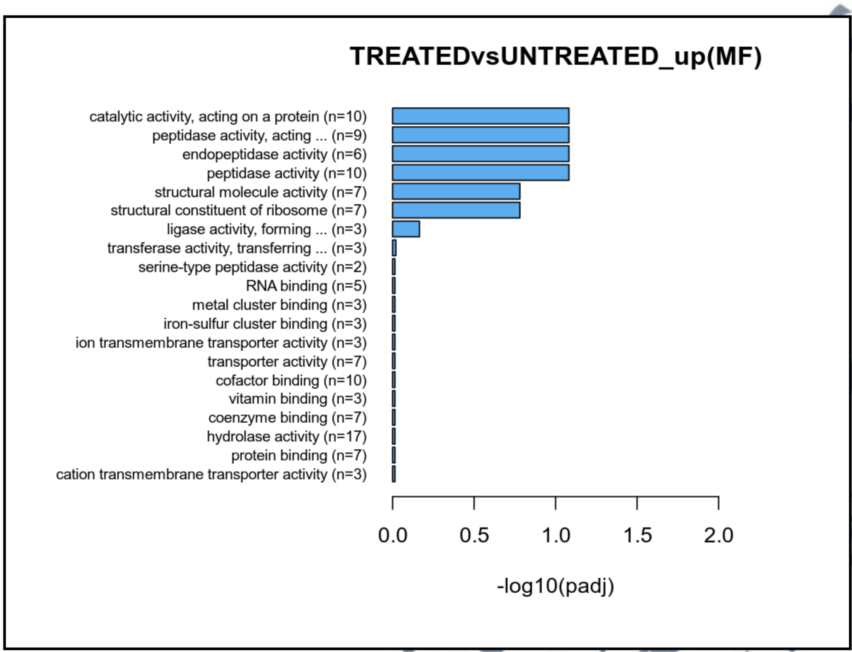

TREATEDvsUNTREATED_down(CC)
membrane (n=8)
cellular_component (n=9)
cell part (n=1)
cell (n=1)
membrane part (n=2)
intrinsic component of membrane (n=2)
integral component of membrane (n=2)
0.0
0.2
0.4
0.6
0.8
1.0
-log10(padj)

(b)

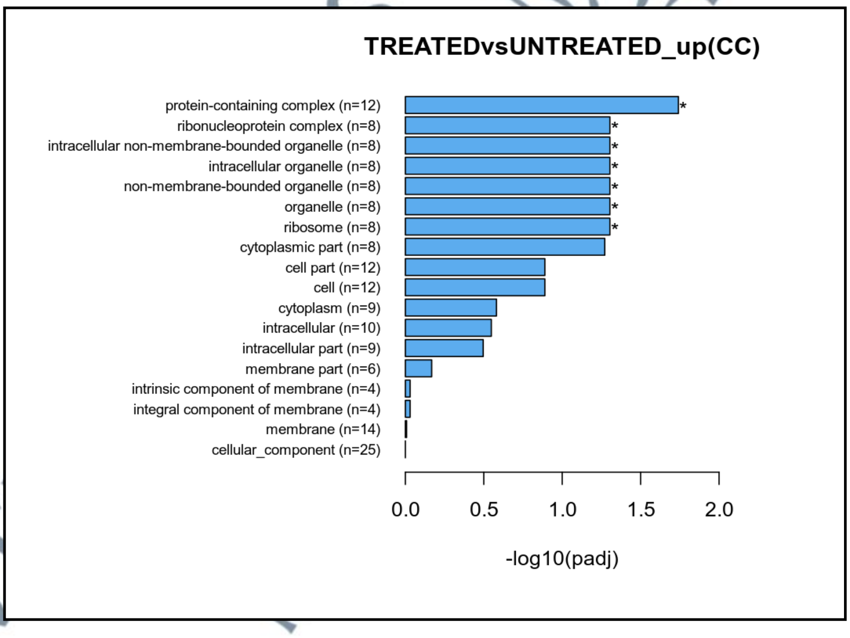


(c)

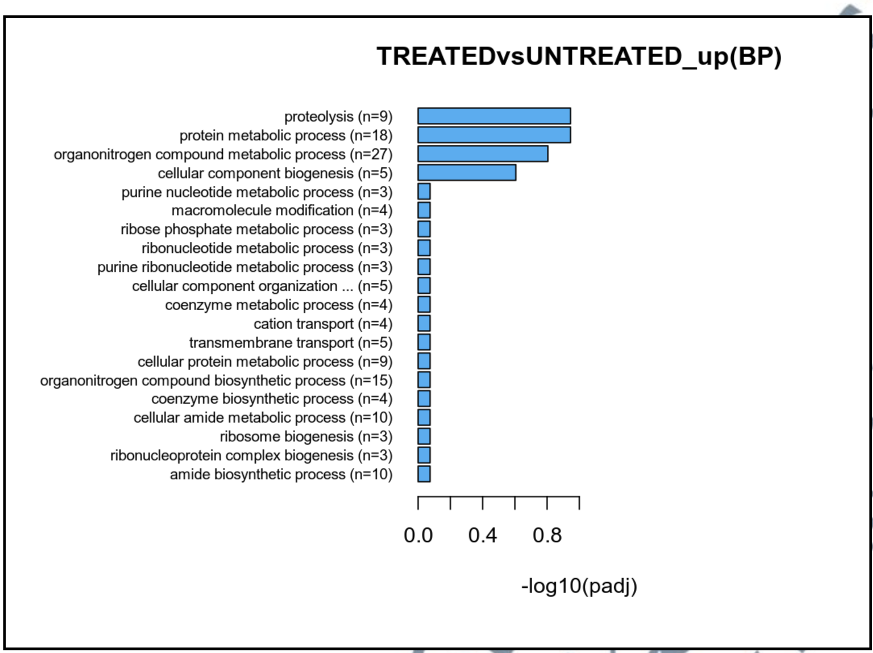
Figure 4.8: Downregulated gene oncology analysis (a) Gene ontology analysis of downregulated genes in the MF category showed the genes were downregulated in similar amounts for all MF activities. (b) Gene ontology analysis of downregulated genes in the CC category where genes under membrane components were slightly downregulated. (c) Gene ontology in BP category where all genes were similarly downregulated in all activities.


TREATEDvsUNTREATED_up(MF)
catalytic activity, acting on a protein (n=10)
peptidase activity, acting ... (n=9)
endopeptidase activity (n=6)
peptidase activity (n=10)
structural molecule activity (n=7)
structural constituent of ribosome (n=7)
ligase activity, forming ... (n=3)
transferase activity, transferring ... (n=3)
serine-type peptidase activity (n=2)
RNA binding (n=5)
metal cluster binding (n=3)
iron-sulfur cluster binding (n=3)
ion transmembrane transporter activity (n=3)
transporter activity (n=7)
cofactor binding (n=10)
vitamin binding (n=3)
coenzyme binding (n=7)
hydrolase activity (n=17)
protein binding (n=7)
cation transmembrane transporter activity (n=3)
0.0
0.5
1.0
1.5
2.0
-log10(padj)

(a)


TREATEDvsUNTREATED_up(CC)
protein-containing complex (n=12)
ribonucleoprotein complex (n=8)
intracellular non-membrane-bounded organelle (n=8)
intracellular organelle (n=8)
non-membrane-bounded organelle (n=8)
organelle (n=8)
ribosome (n=8)
cytoplasmic part (n=8)
cell part (n=12)
cell (n=12)
cytoplasm (n=9)
intracellular (n=10)
intracellular part (n=9)
membrane part (n=6)
intrinsic component of membrane (n=4)
integral component of membrane (n=4)
membrane (n=14)
cellular_component (n=25)
0.0
0.5
1.0
1.5
2.0
-log10(padj)

(b)



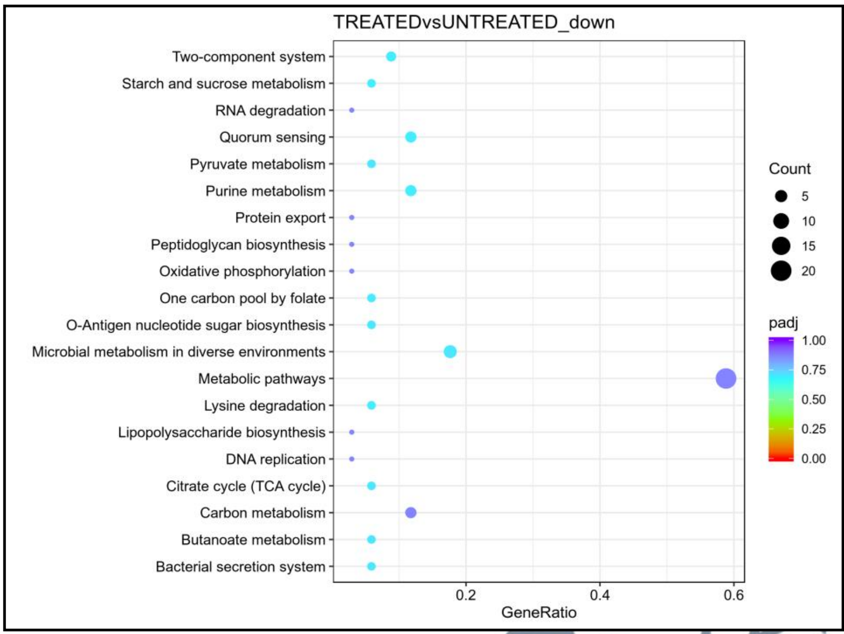
(c)

Figure 4.9: Upregulated gene oncology analysis (a) Gene ontology analysis of upregulated genes in the MF category showed that genes in catalytic activity and peptidase activity were the highest upregulated genes. (b) Gene ontology analysis of upregulated genes in the CC category where protein-containing complex were the highest upregulated activity. (c) Gene ontology in BP category showed that genes under proteolysis activity and preotein metabolic process were the highly upregulated genes in the BP category.

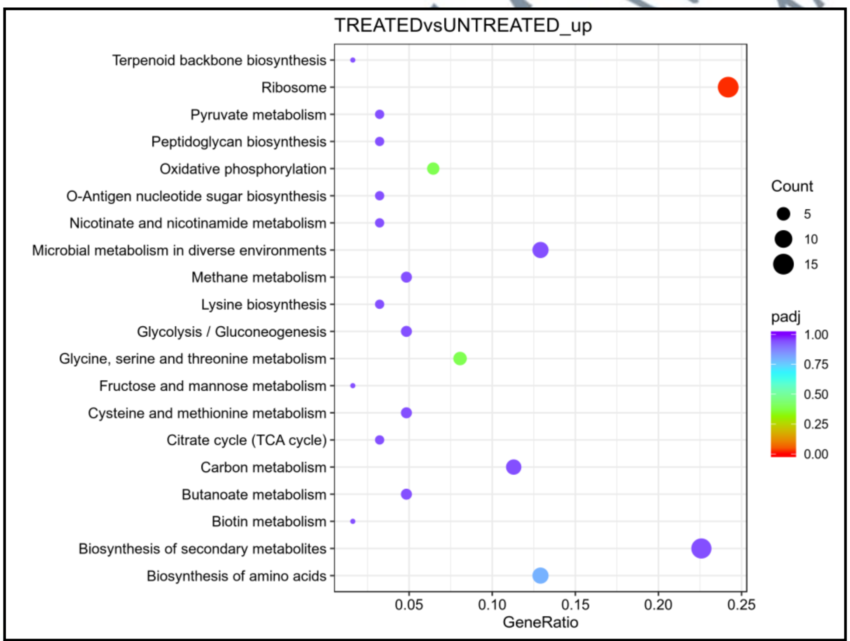
4.3.5 KEGG Enrichment Analysis

The connection of several genes might be integrated together in a biological function and it is studied in an analysis called KEGG enrichment analysis. KEGG is a database for genomes, biological pathways, disease pathways, drugs metabolism, and chemical reactions. KEGG is applied in bioinformatics to understand the genomics, transcriptomics, metabolomics, and a few other branches of biological study. This study utilized KEGG analysis to understand the significantly enriched pathways associated with the differentially expressed genes. The scatter plots for KEGG enrichment are as shown in Figure 4.10.

As shown in Figure 4.10 (a) the genes that are highly downregulated belongs to the two-component system, quorum sensing, purine metabolism, and microbial metabolism in diverse environment groups. Meanwhile, in the upregulated KEGG pathways shown in Figure 4.10 (b), the significantly upregulated genes belong to ribosome pathway, oxidative phosphorylation, glycine, serine, and threonine metabolism and biosynthesis of amino acids pathways. The detailed information of each pathway is as shown in Table 4.6.



(a)



(b)

Figure 4.10: KEGG enrichment analysis (a) the distribution of downregulated genes involved in KEGG pathways. (b) The distribution of upregulated genes involved in stated KEGG pathways. Gene count indicated the number of differentially expressed genes under the pathways, gene ratio indicated the ratio of gene counts in the pathway over overall genes while multi-coloured padj represents the adjusted p-value for each pathways.

Table 4.6: Detailed information on each affected KEGG pathways, significantly affected pathways are marked with asterisk.

KEGG Pathway ID	Pathway Description	Related Genes ID	Differentially Expressed Genes in Pathways
pgn02024	Quorum sensing (downregulated)	PGN_1733, PGN_1733, PGN_0599, PGN_0264.	*ftsY*
pgn02020	Two-component system (downregulated)	PGN_0715, PGN_1162, PGN_1041.	-
pgn00230	Purine metabolism (downregulated)	PGN_0865, PGN_1948, PGN_1148, PGN_1396.	*purH, purD*
pgn01120	Microbial metabolism in diverse environments (downregulated)	PGN_0403, :PGN_1206, PGN_1418, PGN_0351, PGN_1746, PGN_1162.	*gpmA, pdxH, folD, nifJ, nrfA, serC*
pgn03010*	Ribosome (upregulated)	PGN_1840, PGN_1842, PGN_1647, PGN_1648, PGN_1855, PGN_1698, PGN_1844, PGN_0167, PGN_1868, PGN_0035, PGN_0279, PGN_1869, PGN_1855, PGN_1871, PGN_1857.	*rplQ, rpsD, rpmA, rplU, rpsN, rpsO, rpsM, rplC, rplS, rpsJ*
pgn00190*	Oxidative phosphorylation (upregulated)	PGN_1761, PGN_1758, PGN_1762, PGN_1761.	-
pgn00260	Glycine, serine and threonine metabolism (upregulated)	PGN_0243, PGN_0611, PGN_1489, PGN_1495, PGN_0612.	*gpmA, serC*

pgn00260	Biosynthesis of amino acids (upregulated)	PGN_0243, PGN_1080, PGN_0351, PGN_0611, PGN_0351, PGN_1874, PGN_1996, PGN_1495, PGN_0612, PGN_1234.	*gpmA, serC*

4.3.6 Validation of Gene of Interest by Real-Time Quantitative PCR (RT - QPCR)

The genes of interest detected in the NGS procedure are validated using RT - qPCR technique. The gene of interest includes *fimA*, *mfa1*, *kgp*, and *rgp* with 16s rRNA acted as a reference gene. The concentration and purity of the extracted RNA were determined before RT - qPCR procedure. The purity of RNA was determined based on the ratio of absorbance at 260nm to absorbance at 280nm (A260/A280). The concentration of RNA from untreated sample was 1206.40 ng/µl with the purity of 2.038 (Table 4.5). Meanwhile, the RNA concentration of *P. gingivalis* treated with *L. rhamnosus* ATCC 7469 CFS was 1776.24 ng/µl with the purity of 2.026. The quality of RNA was also assessed by gel electrophoresis method (Figure 4.9). The extracted RNA was observed to be intact and pure with the presence of singular intense band for each sample. The treated and untreated RNA were then converted into cDNA to be used as template for RT-qPCR.

Table 4.7: The quantification of RNA concentration and RNA purity determined by molecular spectrophotometer.

Samples	**RNA concentration (ng/µl)**	**RNA purity (A260:A280)**
Untreated *P. gingivalis*RNA	1206.40	2.038
Treated *P. gingivalis*RNA	1776.24	2.026

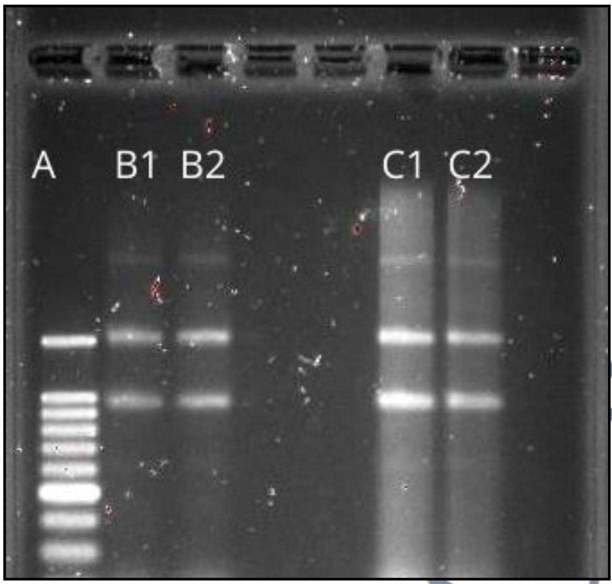


Figure 4.11: The visualization of gel electrophoresis to assess the quality of extracted RNA (A: 1000bp DNA ladder, B1: Untreated *P.gingivalis* RNA sample 1, B2: Untreated *P.gingivalis* RNA sample 2, C1: Treated *P.gingivalis* RNA sample 1, C2: Treated *P.gingivalis* RNA sample 2).

Then, the detected Ct value from the qPCR procedures were translated as fold changes by the following calculations:

$$\text{Fold change}=(\Delta CT_D-\Delta CT_B)-(\Delta CT_C-\Delta CT_A)$$

ΔCT_D represents reference gene 16s rRNA from untreated samples,

ΔCT_B represents reference gene 16s rRNA from sample treated with *L. rhamnosus* ATCC 7469 CFS,

ΔCT_C refers to the gene of interest from treated samples,

ΔCT_A refers to the gene of interest from untreated sample.

The relative expression is the fold change of target genes in *P. gingivalis* treated with *L. rhamnosus* ATCC 7469 CFS relative to untreated *P. gingivalis* sample with the normalization to the reference gene, 16s rRNA. The relative expression of selected genes in treated samples, *fimA*, *mfa1*, *kgp*, and *rgp* were recorded as 3.77± 1.78, 1.66± 2.00, -1.46± 4.07, and -1.01± 3.79 respectively (Figure 4.10) where *mfa1* gene was downregulated while *fimA*, *kgp*, and *rgp*genes were upregulated.

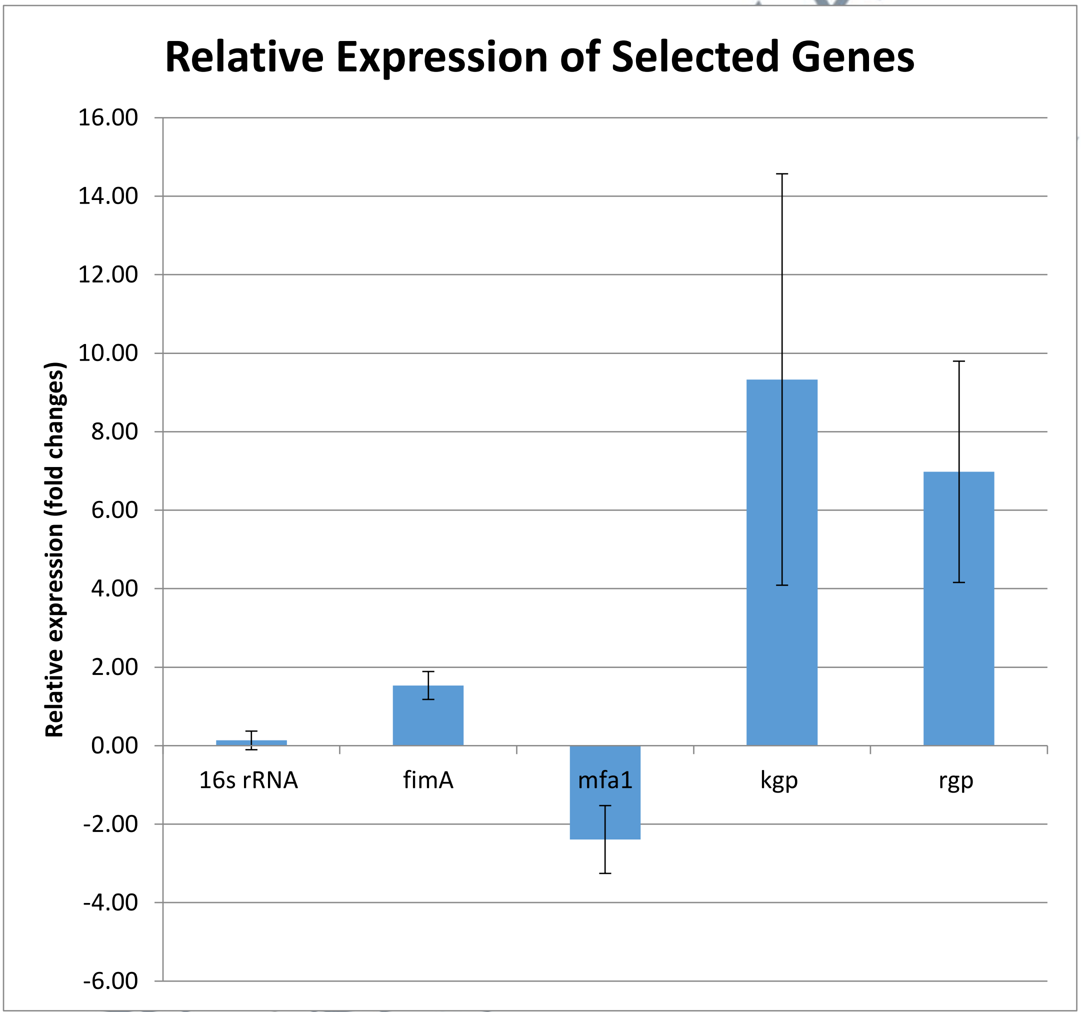


Figure 4.12: The relative expression of the selected genes in *P. gingivalis* treated with *L. rhamnosus* ATCC 7469 CFS.