CHAPTER 5

DISCUSSION

Periodontal disease is a pathogen-related chronic disease that can cause the degradation of gingival tissue and subsequently cause loss of teeth attachment (Kinane et al., 2017; Michaud et al., 2017). Even though the current antimicrobial treatments are sufficient to overcome colonization, it is less effective in overcoming the recolonization of putative pathogens and exhibits multiple side effects (Gerits et al., 2017; Jiao et al., 2019). In addition, the studied periodontal pathogen, *P. gingivalis*, possesses numerous virulent factors and biofilm activity that contributed to its persistent infection on the gingival site (Bostanci & Belibasakis, 2012; Jia et al., 2019; Xu et al., 2020).

Based on these factors, supportive treatments derived from various sources are investigated as a potential strategy to manage the pathogens' progression in the disease. One highlighted strategy to manage biofilm is the development of probiotics as the potential adjuvant treatment for periodontal disease management (Gerits et al., 2017). Despite the extensive research on probiotics' potential for improving oral health, the antimicrobial activity of probiotics against the periodontal pathogen is still relatively less explored.

In this study, CFS of *L. rhamnosus* ATCC 7469 was studied with for its antagonistic activity against the keystone periodontal pathogen, *P. gingivalis* as well as the effect of *L. rhamnosus* CFS treatment in the gene expression of *P. gingivalis*. Previously, *L. rhamnosus* have been investigated in periodontal health improvements in multiple studies (Alanzi et al., 2018; Gatej et al., 2018; Morales et al., 2016; Yuki et al., 2019). However, all the stated studies reported the effects of *L. rhamnosus* administration on periodontal disease in clinical trials and in *in-vivo* model. The lack of fundamental study for the underlying effect of *L. rhamnosus* on periodontal disease, especially its action against periodontal disease pathogens justified the selection as the probiotics tested in this study (Zhang et al., 2022).CFS of *L. rhamnosus* was used in this study to eliminate the possible concerns over the use of live culture as an adjunct treatment as live probiotics could produce excessive acids that might cause another oral problem which is

dental caries (Caufield et al., 2015).Previous studies proposed the use of non-viable probiotics as an alternative to prevent adverse side effects (Kataria et al., 2009). Thus, application of probiotics in CFS form can be studied with fewer concerns on the risk of live culture application.

P. gingivalis is a highly putative pathogen involved in the progression of periodontal disease. This pathogen possesses multiple virulent factors, including the LPS layer in its cell wall, fimbriae for adhesion and biofilm formation, gingipains for tissue degradation and numerous other infectious factors that contribute to its detrimental effect and persistence of survival (Jia et al., 2019; Xu et al., 2020). *P. gingivalis* has been susceptible in a few studies, particularly when treated with live probiotics from commercial strains such as *S. dentisani, L. fermentum,* and *L. salivarius* (Chen et al., 2012; Esteban-Fernández et al., 2019). In addition, the commercial strain *L. rhamnosus* live culture showed inhibitory activity against several periodontal pathogens such as *F. nucleatum, A. actinomycetemcomitans,* and *P. gingivalis* (Moman et al., 2020). The findings in this study showed that *L. rhamnosus* ATCC 7469 cell-free supernatant (CFS) exhibits antimicrobial activity against *P. gingivalis* ATCC 33277, as observed in the disc diffusion assay results. This result agrees with a previous study reporting that heat-killed *L. rhamnosus* CT-53, a clinical isolate, showed strong inhibition activity against oral pathogens such as *S. mutans, F. nucleatum* and *P. gingivalis* (Chen et al., 2020). Additionally, this study's MIC and MBC results show agreement with the inhibitory activity observed in the disc diffusion assay where *P. gingivalis* is considerably susceptible to the treatment with *L. rhamnosus* ATCC 7469 CFS treatment. Based on a previous study, probiotics live cells exhibit antagonistic activity against oral pathogens through competitive interaction and secretion of antimicrobial substances such as hydrogen peroxide, organic acids, bacteriocin substances, and other bioactive compounds (Bustamante et al., 2020).

In addition, *L. rhamnosus* ATCC 7469 CFS showed significant disruption towards the biofilm formation of *P. gingivalis* ATCC 33277, which was able to eliminate a major part of *P. gingivalis* pathogenesis. The result was in agreement with a previous study that showed the ability of *L. brevis* BBE-Y52 to inhibit biofilm formation against multiple periodontal pathogens, including *P. gingivalis* (Fang et al., 2018). The disruption of biofilm by *P. gingivalis* is critical because periodontal disease is a biofilm-

reliant disease that progresses with biofilm establishment on gingival tissue (Baek et al., 2018). Besides, the ability of LAB CFS to disrupt the biofilm formation activity at different concentrations is crucial to prevent the recolonization of *P. gingivalis* as the protective biofilm layer in the pocket is killed by treatment (Bostanci&Belibasakis, 2012). In a detailed study, the researchers explored the effect of the CFS on *L. acidophilus* LA5, *L. rhamnosus* HN001, *L. reuteri* DSM 17938, *B. breve* 110, *B. pseudolongum* 119, and *B. bifidum* 162 on the biofilm-related gene expression of *P. gingivalis.* The study concluded that the CFS from stated probiotics affected a few genes involved in biofilm formation (Ishikawa et al., 2020).

P. gingivalis treated with *L. rhamnosus* ATCC 7469 CFS showed differential expression of 327 genes where 139 genes were downregulated while 188 genes were upregulated. The downregulated genes were involved in several important functions in *P. gingivalis*survivability. Chaperone protein genes *dnaK* and *dnaJ* were significantly downregulated in the treated group of *P. gingivalis.* These genes are critical for the stress responsive machinery by the cellular central system. The chaperone proteins are also very important in cellular homeostasis both in normal and stressful conditions (Anglès et al., 2017). Downregulation of these genes indicated that a significant part of *P. gingivalis*survival was compromised by the treatment.

The treated group of *P. gingivalis* also showed downregulation of several virulent genes including mfa1, mfa3, and mfa4 genes. The *mfa1* gene, which codes for the structural components of minor fimbria, is one of the highlighted genes downregulated due to the treatment (Hasegawa & Nagano, 2021). Minor fimbriae is a short-structured, secondary fimbrial protein that contributes equally as major fimbriae in the colonization, recolonization, adhesion to the human cell, and induction of inflammatory factors (Bostanci &Belibasakis, 2012; Jia et al., 2019; Sochalska& Potempa, 2017; Xu et al., 2020). Whilst major fimbriae play vital roles in biofilm formation and virulent activities, minor fimbrial proteins are also equally damaging as the colonization and virulent factors of *P. gingivalis* involve synergistic actions (Nakayama & Ohara, 2017). In addition, the exposure to *L. rhamnosus* ATCC 7469 CFS caused the downregulation of minor fimbriae accessory genes, *mfa3* and *mfa4.* These accessory genes provide the auxiliary protein necessary for the development of fimbrial tips and are involved in mediating fimbrial functions (Hasegawa & Nagano, 2021; Sakae et al., 2021). Thus, the *L.*

rhamnosus ATCC 7469 CFS inhibit biofilm formation by suppressing the expression of fimbrial proteins in *P. gingivalis.*

Meanwhile, purine biosynthesis related genes, *purH* and *purD* were significantly downregulated in the treated *P. gingivalis.* Both genes are involved in purine biosynthesis where it is a vital process to synthesis purine nucleotides, adenine and guanine, the building blocks for DNA and RNA(Zhang et al., 2008). Other than that, metabolic activities related to vitamin B6 (pyridoxine) were also downregulated by the treatment with *L. rhamnosus*ATCC 7469 CFS. Pyridoxal-5'-phosphate (PLP) synthesis genes, *pdxH* and *pdxB* were downregulated in the treated group of *P. gingivalis.* The downregulation of the two genes subsequently downregulates the pyridoxine synthesis where it is a key element to maintain the virulent activity of pathogenic bacteria (Denise et al., 2023). An interesting relationship was discovered between the PLP pathways with another downregulated gene, *ablA,* which belong to the lysine 2, 3-aminoreductase pathway where *ablA* gene required the product of PLP pathway to start the lysine conversion. Lysine metabolism is a crucial part of cellular activity because it is a major building protein for a bacterial cell (Ruzicka et al., 2000). The downregulation of these genes was not only affecting its direct pathway but also other pathway involving in the metabolic activity of the pathogen.

Besides that, two genes related to reductase activity were also downregulated in the treated *P. gingivalis* expression. Genes *nifJ* and *nrfA*, both are in the reductase family that encodes for the enzymes involved in reduction reactionwhich is a major part of cellular biochemical activity. The *nifJ* gene is a gene coded for pyruvate-ferredoxin oxidoreductase (PFOR), a vital enzyme for anaerobic respiration metabolism (Katsyv et al., 2021). The downregulation of this gene may cause a severe damageon *P. gingivalis*growth because it is an obligate anaerobe. On the other hand, *nrfA* gene is also downregulated where it is translated as Cytochrome C nitrite reductase enzyme. An important discovery stated that Cytochrome C nitrite reductase plays vital role in maintaining the bacterial pathogenicity and inducing antibiotic resistance mechanism in the pathogen (Vázquez-Torres & Bäumler, 2016). Downregulation of this gene indicated that the exposure to *L. rhamnosus* ATCC 7469 CFS weakened the pathogenic ability of *P. gingivalis.*

On the other hand, several genes related to the metabolic pathways of *P. gingivalis*such as *galE*, *hflX*, and*folD* were also significantly downregulated. Epimerase family gene, *gale* were downregulated in the treated *P. gingivalis* where the gene is important as a NAD-dependent epimerase that holds multiple functions in cellular activity which mainly translated as the synthesis of cellular surface protein, exoenxyme production, and significantly, the virulent activity of a pathogen (Islam et al., 2019). On the other hand, *hflX* gene which codes for 50S ribosome binding GTPase was also downregulated in the treated *P. gingivalis* expression. GTPase plays crucial role in the protein translation cycle of a cell where it binds to ribosomal protein to carry out any of the transational sequence, initiation, elongation, termination or recycling (Coatham et al., 2016). Downregulation of this gene might impair the cell ability to carry out protein translation for cellular function. Additionally, a gene related to S-adenosylmethonine (SAM) metabolism which is a core metabolism in methylation process of all cells was also downregulated. *FolD* gene coding for tetrahydrofolate dehydrogenase/ cyclohydrolase was significantly downregulated in treated *P. gingivalis*. Downregulation of tetrahydrofolate dehydrogenase limits SAM regeneration which took part as cofactors in multiple cellular methylation process including the DNA, proteins and enzymes methylation metabolism (Okano et al., 2020). It is clear that the downregulation of the stated genes mainly affects the protein-building metabolism which is crucial for the repair, growth, and survivability of *P. ginigvalis*.

Additionally one gene related to cation transport system particularly magnesium transport system coded by gene *mgtE* were significantly downregulated after the treatment. Mg^{2+} ions are the most prevalent cation in the intracellular activity where combined with ATP as MgATP it plays significant role in oxidative phosphorylation, ATPase activity and glycolytic enzymes functions. Charged Mg^{2+} ion is important in synthesis of DNA/RNA polymerase, tRNA synthetase and also play a key role in protein synthesis. The downregulated gene, *mgtE* is a cation transport channel for the influx of charged Mg2+ into the cell(Franken et al., 2022).Downregulation of this gene impairs the magnesium ion transport into the cell which affected multiple cellular functions including synthesis of DNA polymerase. Interestingly, this study also discovered the donwregulation of *polA* gene, a gene coding for DNA polymerase 1 synthesis. DNA polymerase 1 is a repair enzyme in DNA replication that works as the exonuclease of 3' -5' strand, proofreading of the 3' -5' strand and also cleaving the RNA primers at the

lagging strands during DNA replication. Additionally, it works with another gene *exoR*to perform repair action at the replication fork during every replication process (Hernández-Tamayo et al., 2019). As stated previously, this gene is downregulated together with magnesium transport gene which might indicate the direct effect of impairment of cation transport system to the molecular function of *P. gingivalis*.

In addition, the exposure to *L. rhamnosus* ATCC 7469 CFS downregulated the expression of a gene related to the quorum sensing, *ftsY*. The gene *ftsY* encode signal recognition particle-docking protein which is involved in the bacterial signalling activity in the quorum sensing. It plays a role in surface protein signalling, environmental, and cellular information processing and bacterial interaction in quorum sensing (Gabarrini et al., 2020; Saier, 2006). On the other hand, a distant gene related to lipopolysaccharides (LPS) synthesis, the *kdsA* gene, 3-deoxy-8-phosphooctulonate synthase, was also downregulated upon treating *L. rhamnosus* ATCC 7469 CFS. The affected gene is involved in the molecular pathway of LPS layer formation; thus, its suppression affected the integrity of the *P. gingivalis* LPS layer (Pasala et al., 2021). As LPS are a vital part of Gram-negative bacteria, suppressing LPS synthesis activity also reduces cell survivability (Delhaye et al., 2019). In addition, *P. gingivalis* LPS acted as one of the virulent factors involved in inducing the inflammatory responses via the Toll-like receptor pathway (Jia et al., 2019; Nakayama & Ohara, 2017).

The exposure to *L. rhamnosus* ATCC 7469 CFS calso caused upregulation of multiple genes including ribosomal units genes, cellular function genes, virulent genes and also surface protein genes. Ribosomal unit genes of the 50S and 30S domain were majorly upregulated in the treated group of *P. gingivalis*. Ribosomal unit genes, *rplQ, rpmA, rplU, rplC, rplS,* and *rplX* genes encoded for 50S ribosomal subunits while genes, *rpsD, rpsN, rpsO, rpsM, rpsJ,* and *rpsG* encoded for the 30S ribosomal subunits. *rgpA* and *rgpB*. The 50S and 30S ribosomal subunits belong to small and large ribosomal subunits respectively. Large ribosomal subunit, 50S carried out peptidyl transferase center where it polymerizes amino acids into proteins while the small ribosomal subunits, 30S interprets the genetic information by decoding the tRNA and both subunits cooperates to form 70S ribosomes (Aleksashin et al., 2019). Upregulation of these genes might indicate that the cell is carrying out speedy protein formation to counter the effects of the impairments caused by the downregulated genes.

Besides ribosomal subunit genes, two genes thatencode pro-inflammatory protein of arginine-gingipains were also upregulated. Both genes are involved in the virulent activities of *P. gingivalis* against the gingival tissues (Jia et al., 2019; Takeuchi et al., 2019). Interestingly *rgpA* and *rgpB* work synergistically for the maturation of fimbrial proteins (Lee et al., 2018). The upregulation of *rgpA* and *rgpB* following exposure with *L. rhamnosus* ATCC 7469 CFS indicates that arginine gingipains genes are persistently working on the fimbrial protein formation and maturation as a counter mechanism towards the downregulation of fimbrial genes.

On the other hand, genes related to cellular metabolic activity involving glycolysis and oxidative phosphorylation were also significantly upregulated. Genes such as *gpmA, nadA, coaE,* and *serC* were the upregulated genes involved in the cellular respiration pathways of *P. gingivalis.* Firstly, *gpmA* gene which codes for putative 2, 3-bisphosphoglycerate-dependent phosphoglycerate is an important component of bacterial glycolysis metabolism. Interestingly, this gene plays a defensive role together with other gene clusters to prevent damages caused by hydrogen peroxide exposure(Roth et al., 2022). Thus, this finding might be hinting at the upregulation of protective activity of *gpmA* against hydrogen peroxide that might be present in *L. rhamnosus* ATCC 7469 CFS.

Other than that, *nadA* gene is a gene coding for quinolinate synthase which is a direct gene in the *de novo* synthesis of NAD, an important component of cellular redox reaction, especially in the iron-sulfur pathway of bacteria. Iron-sulfur pathway of bacteria is important in multiple functions such as cell growth, amino acid regulatory activity, protein transcription and translation regulators (Ollagnier-de Choudens et al., 2005). Upregulation of *nadA* gene indicates that *P. gingivalis* biological function is focused on the regeneration of damaged cells and proteins due to the damage caused by the treatment. Another cellular metabolism gene, *coaE* coding for dephospho (COA) kinase enzyme was also upregulated, a crucial enzyme involved in metabolic activity for ATP dephosphorylation to synthesis coenzyme A. Coenzyme A is took part in the majority of metabolic pathways (Shimosaka et al., 2019). Due to the major effect of *L. rhamnosus* ATCC 7469 CFS treatment on *P. gingivalis* metabolic pathways, counter mechanisms were introduced by producing more coenzyme A to re-establish the damaged metabolic pathways. The other gene involved in metabolic pathway, *serC* gene was also

upregulated in the treated group. The gene codes for putative phosphoserine transaminase which involved in L-serine biosynthesis by NAD dependent phosphorylation. L-serine is a vital amino acids involved in many biological function of all cell (Jang & Chang, 2023). Interestingly, the upregulation of this gene is in line with the upregulation of NAD synthesis gene, *nadA,* thus it might be concluded that the upregulation of NAD as a counter mechanism was able to upregulate the L-serine biosynthesis gene.

On the other hand, a few genes related to amino acid synthesis and protein maturation were also upregulated after the treatment with *L. rhamnosus* ATCC 7469 CFS. Genes including *rimP*and *panC* were also upregulated which these genes are involved in amino acids pathways and protein maturation. The *rimP* gene codes for the RIMP component that facilitates the maturation of 30S ribosomal subunits (Nord et al., 2009). Upregulation of this gene is reflected in the upregulation of various 30S ribosomal subunits as analysed in this study. On the other hand, gene related to panthotenic acid or vitamin B5, *panC* was also upregulated after the treatment with *L. rhamnosus* ATCC 7469 CFS. Pantoate-beta-alanine ligase is an enzyme involved in the metabolism of vitamin B5 for the formation of coenzyme A and multiple acyl groups (Tadi et al., 2022). Upregulation of this gene as counter mechanism was reflected in the upregulation of *coaE* gene which codes for coenzyme A synthesis.

Additionally, there are several genes that are involved in multiple functions such as cellular molecular activity, surface protein assembly, and heme uptake system. Genes involved in molecular activity are *ruvX* gene coding for Holliday junction resolvase and *miaA* gene coding for tRNA methyltransferase. Holliday junction resolvase plays an important role during mitotic recombination of bacteria where it is important to ensure proper segregation of chromosome during mitosis especially during genetic materials exchange of sister chromatids. Holliday junction resolvase also monitors and correct any DNA impairment that occurs during the exchange process (Nautiyal et al., 2016). The upregulation of this gene indicates that *P. gingivalis* is going through rapid mitosis action to produce new cells due to the damage of the treatments on its population. Besides that, *miaA,* agene coding for tRNA methyl transferase is important for translational activity by decoding the codons into proteins and also the post-translational monitoring of the tRNA (Koshla et al., 2019). Upregulation of these genes indicated that the counter mechanism of *P. gingivalis* also involved the rapid molecular activity of the pathogen.

Two more genes that are upregulated are surface protein coding gene and the heme chaperone gene, *bamD*a nd *hemW* respectively. Lipoprotein assembly gene, *bamD*was upregulated as a counter action of downregulation of lipopolysaccharides formation gene, *kdsA* (Knowles et al., 2009; Tata & Konovalova, 2019). On the other hand, upregulation of *hemW* gene, a chaperone protein in the heme uptake system indicate that *P. gingivalis* are going through major cellular regeneration that requires quick uptake of heme to be metabolized for cellular use (Haskamp et al., 2018).

Regarding the effect of differential gene expression on the gene ontology of *P. gingivalis,* gene expression under molecular functions and binding process were highly downregulated. Meanwhile, for the upregulated gene expression, highest upregulated gene belongs to the cellular component category, followed by molecular function and then binding process category. The result is reflected in the upregulated genes where most of the genes are involved in cell components building process to overcome the damage on the molecular function and binding process genes downregulation.

As for the pathways affected by the downregulated genes, quorum sensing, two-component system, purine metabolism and microbial metabolism in diverse environment were downregulated. Four genes are involved in the quorum-sensing pathways, which are PGN_1733, PGN_1733, PGN_0599, and PGN_0264. Based on the KEGG database search, the affected gene PGN_1733 is related to the large surface protein BapA in biofilm formation, PGN_0599 is involved in the type IV secretion protein pathway, and PGN_0264 is a major part of signal recognition particle docking protein. The quorum-sensing pathway is involved in the signalling and induction of signalling molecules. This pathway triggers the bacteria biofilm formation-related genes besides detecting and translating small molecule signals from neighbouring bacteria (Bramhachari, 2019; Fleitas Martínez et al., 2019).

The two-component system pathway is another vital pathway downregulated considerably in the treated group of *P. gingivalis.* Three genes were affected in this pathway which is PGN_0715, PGN_1162, and PGN_1041. Based on the KEGG database, the detected genes in the two-component system, PGN_1162, is in the short fatty acids metabolism system, while PGN_1041 is involved in the redox signal pathway. Generally, the two-component system is a mode for bacteria to sense the changes in their environment and the bacterial modulation to respond to the environmental condition.

Besides that, a two-component system is also involved in bacteria-to-bacteria cross talk throughout the biofilm formation, maturation, and cessation of biofilm (Liu et al., 2019).

In addition, purine metabolism pathway was also affected by the treatment. Several genes were affected by the treatment including PGN_0865, PGN_1948, PGN_1148, and PGN_1396. Additionally, the *purH* and *purD* genes that were downregulated belong to this pathway. Based on KEGG database search, PGN_0865 belongs to the IMP cyclohydrolase group that is involved in the initial part of purine metabolism to synthesis Guanosine monophoshate (GMP) and Adenosine monophosphate (AMP) (Wizrah et al., 2022). Meanwhile, PGN_1948 is a deoxyguanosinetriphosphatetriphosphohydrolase (dGTPase) group enzyme which is involved in DNA hydrolyzation but the specific activity is still largely unclear (Singh et al., 2015). The other two genes, PGN_1148 and PGN_1396 were not identified in the KEGG database. Other than the stated pathways, microbial metabolisms in diverse environment were also affected. KEGG database search on the genes in this pathway resulted to the downregulated genes such as *pdxH, folD, nifJ, nrfA, serC.*

On the other hand, four KEGG pathways were significantly upregulated after treating *L. rhamnosus* ATCC 7469 CFS. The three pathways are the ribosomal pathway, oxidative phosphorylation, glycine, serine, and threonine metabolism, and biosynthesis of amino acids pathway. These pathways translate genetic information, energy metabolism, and amino acid metabolism. The upregulation of the KEGG ribosome pathway indicatesan upsurge in translation activity by the bacteria (Romero-Lastra et al., 2019). The KEGG database search identified that amongst the 15 affected genes in the pathway, 12 affected genes belong to the 30S, and 50S ribosomal subunit genes that were upregulated in the treated group of *P. gingivalis.* The upregulation of the KEGG ribosomal pathway suggested that the bacteria undergo rapid translation to overcome the external stress induced by *L. rhamnosus* ATCC 7469 CFS treatment.

Furthermore, the treatment with *L. rhamnosus* ATCC 7469 cause upregulation of the oxidative phosphorylation pathway, where four genes (PGN_1761, PGN_1758, PGN_1762, and PGN_1761) were detected to be differentially expressed in the treatment group. The KEGG database search predicted all four genes to be the subunits in V/A-type ATPase (vacuolar/archaeal-type adenosine triphosphatases). The V/A-type ATPase is involved in oxidative phosphorylation, where the V-type ATPase and A-type ATPase

demonstrate coupled action to translocate protons across the membrane for energy production. Both types of ATPase work differently to generate cellular energy (Matzke et al., 2021; Zhou & Sazanov, 2019). The upregulation of this pathway suggested that the bacteria amplify their energy production for rapid cellular repair and adaptation to environmental stress.

Other than that, the upregulation of the KEGG pathway of glycine, serine, and threonine metabolism is predicted in the treated group of *P. gingivalis*. There are five genes PGN_0243, PGN_0611, PGN_1489, PGN_1495, and PGN_0612 in the pathway that were affected after the treatment with *L. rhamnosus* ATCC 7469 CFS where two of the stated genes resulted to *gpmA* and *serC* genes from the KEGG database search. The glycine, serine, and threonine metabolism pathways are vital in enhancing bacterial glycolysis activity and the production of glycine, serine, and threonine amino acids for cellular repair (Cheng et al., 2019; Chowdhury et al., 2021). The upregulation of the glycine, serine, and threonine metabolic pathway suggested that the bacteria require energy from glycolysis activity and cellular repair due to the antimicrobial activity exhibited by *L. rhamnosus* ATCC 7469 CFS.

Another pathway that was upregulated by the exposure to *L. rhamnosus* ATCC 7469 CFS is the biosynthesis of amino acids pathway where several genes including PGN_0243, PGN_1080, PGN_0351, PGN_0611, PGN_0351, PGN_1874, PGN_1996, PGN_1495, PGN_0612 phosphoserine aminotransferase, and PGN_1234 hypothetical asparagines synthase were upregulated in the pathway. KEGG database search showed that PGN_0243 and PGN_0611 were the ID for *gpmA* and *serC* gene respectively. Meanwhile, PGN_1080 codes for branched chain amino acid amintransferase which is the first step of branched chain catabolism where it synthesis and degrade valine, leucine, and isoleucine and the product of this reaction will be utilized in the further step of cellular metabolism (Nong et al., 2022). Besides, another gene that is upregulated in this pathway is PGN_0351, a gene coding for pyruvate carboxylase enzyme that catalyzes the ATP-dependent carboxylation of pyruvate into oxaloacetate which then enters Kreb's cycle for cellular metabolism (Y. Liu et al., 2018). However, KEGG database search on PGN_1874 resulted to putative 3-phosphoshikimate 1-carboxyvinyltransferase which functions in amino acids biosynthesis is largely unclear. PGN_1996 were found to be a gene coding for putative dihydrodipicolinate synthase, an enzyme that is involved in

lysine metabolism and also relevant in oxidative stress and iron homeostasis response (Olaya-Abril et al., 2020).

Another upregulated gene under the pathway is PGN_1495 which codes for putative threonine aldolase. Threonine aldolase is an enzyme that cleavage threonine into glycin and aldehyde to be utilized by the cells (Fesko, 2016). Phosphoserine aminotransferase (PSAT) coding gene PGN_0612 was also upregulated in the treated *P. gingivalis*group. PSAT is a PLP dependent enzyme that catalyzed the conversion of pyruvate isomers into phosphoserine. This metabolism is a part of phosphorylated serine biosynthesis pathway (Sekula et al., 2018). The upregulation of this pathway may have direct relationship to the upregulation of glycine, serine, and threonine metabolism pathway. Holistically, the upregulation of genes in the amino acid biosynthesis pathway is in line with the upregulation of glycine, serine, and threonine pathway.

Two genes of interest that were analyzed in the NGS outputs, which are *mfa1, rgp* with the addition of two other related genes of interest which are *fimA* and *kgp* gene, were validated for their expression after the treatment with *L. rhamnosus* ATCC 7469 CFS treatment by employing the Real-Time - Quantitative PCR (RT-qPCR) technique. In this investigation, 16s rRNA of *P. gingivalis* was used as the reference gene to determine the relative expression of the stated genes of interest (Ishikawa et al., 2020). The results for the gene expression of selected genes were in line with the results obtained from NGS procedures. The result showed slight downregulation of the *mfa1* gene and the upregulation of *fimA, kgp,* and *rgp* genes relative to the reference gene. However, the findings were not statistically significant.

The gold standard to validate the gene expression result is RT-qPCR; thus, the results are more critical and absolute (Rocha et al., 2016). The gathered result showed that the gene of interest *mfa1* gene was downregulated in the treatment group. This result agrees with the NGS screening output, where the *mfa* genes cluster was downregulated after the treatment. Additionally, the treatment group had upregulation of *fimA, kgp,* and *rgp* genes, thus validating the NGS output where *rgp* was upregulated upon treatment with *L. rhamnosus* ATCC 7469 CFS. Several possible errors occurred during the procedures that needed improvements in future studies (Taylor et al., 2019).

The study can be extended in multiple areas to understand the depth of *L. rhamnosus* ATCC 7469 CFS action in preventing periodontal pathogens colonization and improving periodontal health. One aspect that can be discovered further is assessing the antimicrobial ability of *L. rhamnosus* ATCC 7469 CFS against multiple species of periodontal pathogen, the tissue healing ability of *L. rhamnosus* ATCC 7469 CFS in oral tissue model or animal model and its mechanism, identification of bioactive material in *L. rhamnosus* ATCC 7469 CFS and the application of *L. rhamnosus* ATCC 7469 CFS in the clinical setting.

In conclusion, as shown in the results, *L. rhamnosus* ATCC 7469 CFS exhibited significant results in its antimicrobial and anti-biofilm activity. In addition, the inhibition activities were reflected in the gene expression of *P. gingivalis*, as proven by the RNA sequencing outcomes. Several important genes involved in biofilm formation and survivability were affected by the exposure to *L. rhamnosus* ATCC 7469 CFS. These results showed the potential of *L. rhamnosus* ATCC 7469 CFS to be developed as a supportive treatment to overcome periodontal pathogens colonization and biofilm formation.