CHAPTER 3

METHODOLOGY

3.1 Preparation and Validation of Bacterial Strains

The bacteria that were used in the study are probiotic *L. rhamnosus* ATCC 7469 and periodontal pathogen *P. gingivalis* ATCC 33277. The probiotic strain was grown in De Man, Rogosa, and Sharpe (MRS) nutrients (Oxoid, Basingstoke, HA, UK). The strain was streaked on MRS agar and then was incubated aerobically at 37°C for at least 18 hours before being validated by Gram staining procedures.As for the pathogen, *P. gingivalis* ATCC 33277 was grown on Wilkins-Chalgren agar and nutrient broth (Oxoid, Basingstoke, HA, UK). The strain was grown for at least 48 hours in an anaerobic chamber at 37°C. The strain was validated by Gram staining procedures for morphology confirmation.Both bacterial strains were kept as a collection for this study in 20% glycerol stock at -80°C.

3.2 Preparation of Cell-free Supernatant (CFS)

L. rhamnosus ATCC 7469 was streaked on agar and incubated for 24 hours. The probiotic colony was inoculated and grown in MRS broth aerobically at 37°C for 24 hours.The tubes containing overnight *L. rhamnosus* ATCC 7469 broth culture were then centrifuged at 4000 rpm for 15 minutes. The supernatant was filter sterilized by using 0.2 µm cellulose acetate filter sterilization. The filtered CFS was kept in sterile Eppendorf tubes until it is being used for assays.

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3.3 Antimicrobial Assay

3.3.1 Pathogens preparation

The *P. gingivalis* ATCC 33277 cultures were streaked on Wilkins-Chalgren agar and incubated for 48 hours at 37°C in an anaerobic chamber. The bacterial suspensions were prepared by transferring the bacterial colony using inoculating loop into 5 mL of sterile normal saline. The inoculums were mixed thoroughly, and the turbidity of the suspension was adjusted to 0.5 McFarland standards before the disc diffusion assay.

3.3.2 Disc Diffusion Assay

Disc diffusion assay was carried out to determine the antimicrobial activity of the *L. rhamnosus* ATCC 7469 CFS based on the guidelines from the Clinical Laboratory Standard Institute (CLSI) (Hindler et al., 2020). Sterile MRS broth and 0.2% chlorhexidine were used as negative and positive control respectively. A total of 100 µL adjusted *P. gingivalis* ATCC 33277 bacterial suspensions were spread thinly on the agar surface using a cotton swab and then three sterile filter paper discs were placed on the agar. Then, 10 µL of the *L. rhamnosus* ATCC 7469 CFS, with the negative and positive control were pipetted onto the discs (Figure 3.2). The plates were incubated for 48 hours in the anaerobic chamber at 37°C. The inhibition diameters were measured in millimetres (mm). The disc diffusion assay was carried out in three replicates.

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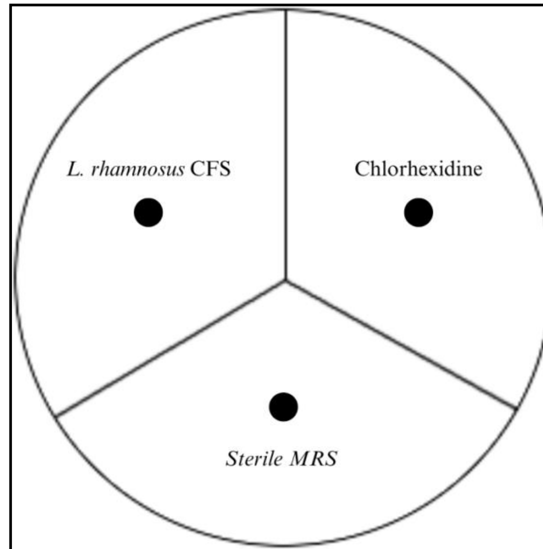

Figure 3.1: The arrangement of samples for disc diffusion assay on petri dish.

3.3.3 Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration (MIC) determination was carried out to determine the lowest concentration required to inhibit the microorganisms based on the CLSI protocol of susceptibility testing (Weinstein & Patel, 2018). MIC was carried out in a sterile 96 wells microtiter plate. Single-concentration Wilkins-Chalgren nutrient broths were used in this test. The inoculums are prepared by dilution with a ratio of 1:10. Then, 100 µL of the inoculums were aliquoted into the sterile 96 well microtiter plates accordingly. *L. rhamnosus* ATCC 7469 ATCC 7469 CFS samples ranging from 100% (v/v concentration, probiotic CFS/ sterile MRS broth) to 3.125% prepared by serial dilution were added into the well respectively. Positive control and negative control for this assay were 0.2% chlorhexidine and sterile MRS respectively following the sample concentrations. The plate was incubated for 24 hours at 37°C in an anaerobic chamber. The MIC was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Thermo Scientific, MA, USA) and values were noted as the lowest concentration of the sample that showed no changes from yellow to blue after the addition of the MTT solution.

3.3.4 Minimum Bactericidal Concentration (MBC)

Minimum bactericidal concentration (MBC) was carried out to determine the lowest concentration of CFS that can kill the microorganisms. The sample in each well from the MIC determination was streaked on the Wilkins-Chalgren agar using the streak plate method. The plates were labelled with different concentrations and were incubated for 24 hours at 37°C in an anaerobic chamber. The growth of bacteria in each plate was observed and MBC is determined by the lowest volume of free supernatant that caused 99% bacteria reduction.

3.4 Anti Biofilm Assay

Biofilm assay is carried out based on a modified method in a sterile 96-well microtiter plate and the quantification of the biofilm formation is determined by using a microplate reader (O'Toole, 2011; Brown et al., 2019). This method involves *P. gingivalis* ATCC 33277as the biofilm-forming pathogen and *L. rhamnosus* ATCC 7469CFS to determine the effect of the stated probiotic on biofilm formation by the pathogen.

The inoculums to be used for biofilm assay were prepared by transferring the overnight culture into fresh Wilkins-Chalgren broth with a ratio of 1:100 (culture: medium). Next, 100µL of the diluted culture was transferred into the 96 well microtiter plate and incubated for 24 hours. After 24 hours, the content of each well was discarded and rinsed with Phosphate Buffer Saline (PBS). Then, 100µL of sterile Wilkins-Chalgren broth was added to the well. Then, 100µL of *L. rhamnosus* ATCC 7469 CFS were added into the well with the concentration from 100% (v/v concentration, probiotic CFS/ sterile MRS broth) to 3.125% based on susceptibility concentration range respectively. Similar to other tests, 0.2% chlorhexidine and sterile MRS were tested as positive and negative controls respectively. The plate was incubated for 24 hours in anaerobic conditions and the content of each well was discarded after the incubation period.

After 24 hours, the cultures inside the well were taken out to remove the planktonic bacteria inside the well. Then, the plate was rinsed with PBS and crystal violet staining is added into each well to stain the adherent cell. The stains are then were rinsed with PBS and then aired to dry. Acetic acid was used to dissolve the dried crystal violet

stain. Quantification of biofilm formation was carried out by measuring the optical density of the stain at 600nm using a microplate reader. Biofilm reduction was calculated using the following method,

$$\text{Biofilm reduction percentage,\%} = 100 - \left(\frac{\text{Treated biofilm optical density}}{\text{Untreated biofilm optical density}} \times 100\right)$$

3.5 Evaluation and Validation of Molecular Mechanisms

3.5.1 RNA Extraction

The pellets of treated and untreated *P. gingivalis* were prepared and harvested by centrifugation at 12000 g for 1 minute prior to RNA extraction using SV Total RNA Isolation System kit (Promega, Madison, WI, USA). The cells were lysed following the kit protocol where the pellets were washed with ice cold PBS twice before RNA lysis buffer was added into the washed pellets. Then, the lysis products were diluted with RNA dilution buffer and then incubated for 3 minutes. The cleared lysate were transferred into a new centrifuge tube and 300 µL of 95% ethanol were added into the tube. The mixtures were transferred into the Spin Column assembly for extraction process.

The extraction process was carried out by centrifugation of the mixture and then 600 µL of RNA wash solution were added to the Spin Column assembly. The assembly were centrifuged again before the removal of cellular DNA by DNAse mix solution as stated in the protocol. The Spin Column assembly were incubated for 15 minutes, then DNAse stop solution were added to the column and the column were centrifuged. The column were washed again with RNA wash solution and then centrifuged. The extracted RNA were collected by adding 100 µL nuclease free water into the new assembly of the column basket and a new elution tube. The assembly were centrifuged and the eluted RNA quality were assessed and quantified using gel electrophoresis and Nanodrop quantification function available in Molecular Device SpectraMax iD3.

3.5.2 Next Generation Sequencing of *P. gingivalis* RNA

Molecular mechanism of *P. gingivalis* inhibitions was evaluated by Next Gene Sequencing (NGS) technique to investigate *P. gingivalis* ATCC 33277 gene that is disrupted by the exposure with*L. rhamnosus* ATCC 7469 CFS. The NGS technique was completed by using a genomics service provider (NovogeneAIT Genomics, Singapore).

Library preparation for the transcriptomic analysis was prepared as followed. A total amount of 1 µg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext® UltraTM RNALibrary Prep Kit for Illumina® (NEB, USA) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature to synthesis the first strand and second strand cDNA. The rest of the overhangs and ends were converted into blunt ends. Then PCR was performed withand PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system.

The clustering was performed on a cBot Cluster Generation System using PE Cluster Kit cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina platform and paired-end reads were generated.In this step, clean data (clean reads) were obtained by triming reads containing adapter and removing poly-N sequences and reads with low quality from raw data. All the downstream analyses were based on the clean data with high quality. Read mapping were done based on gene model on the genome website.

Novel genes were identified by rockhopper computational analysis. It can be used for efficient and accurate analysis of bacterial RNA-seq data, and that it can aid with elucidation of bacterial transcriptomes. Quantification of gene expression level was measured using FeatureCount then FPKM of each gene was calculated based on the length of the gene and reads count mapped to this gene. FPKM, expected number of Fragments Per Kilobase of transcript sequence per Millions base pairs sequenced, considers the effect of sequencing depth and gene length for the reads count at the same

time, and is currently the most commonly used method for estimating gene expression levels.

Differential expression analysis of two was performed using the DESeq2 R package. DESeq2 provide statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting P-values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate. Genes with an adjusted P-value <0.05 found by DESeq were assigned as differentially expressed.

Gene Ontology (GO) enrichment analysis of differentially expressed genes was implemented by the clusterProfiler R package, in which gene length bias was corrected. GO terms with corrected P-value less than 0.05 were considered significantly enriched by differential expressed genes. Kyoto Encyclopedia of Genes and Genomes (KEGG) is a database resource for understanding high-level functions and utilities of the biological system, such as the cell, the organism, and the ecosystem, from molecular level information, especially large-scale molecular datasets generated by genome sequencing and other high-through put experimental technologies (http://www.genome.jp/kegg/). We used clusterProfiler R package to test the statistical enrichment of differential expression genes in KEGG pathways.

3.5.3 Real-Time Quantitative PCR (RT - qPCR)

The validation of suppressed genes was carried out by utilising quantitative Real-Time Quantitative PCR (RT - qPCR). First, the RNA of the untreated and treated *P. gingivalis*cultures was extracted using SV Total RNA Isolation System following the procedures stated previously.

The isolated RNA was then converted into complimentary DNA based on the GoTaq RT - qPCR System protocols. The prepared cDNA for each group was then used as the template for the qPCR procedures. In addition, the genes of interest, *fimA, mfa1*, *kgp* and *rgp* primers (Table 3.4) were also designed for the qPCR step based on literature (Ishikawa et al., 2020). The cycling parameter had been optimized based on the average melt temperature of all primers as shown in Table 3.5.

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Table 3.1: List of genes of interest and the primer sequences based on literature search.

Target gene	Primer Sequence
fimA	F: TTGTTGGGACTTGCTGCTCTTG
	R: TTCGGCTGATTTGATGGCTTCC
kgp	F: GCTTGATGCTCCGACTACTC
	R: GCACAGCAATCAACTTCCTAAC
mfa1	F: ATCTTCAGCACTCTCCACAAG
	R: TTGTTGGGACTTGCTGCTCTTG
rgp	F: CCGAGCACGAAAACCAA
	R: GGGGCATCGCTGACTG
16SrRNA *P. gingivalis*	F: CCGAGCACGAAAACCAA
	R: GGGGCATCGCTGACTG

Table 3.2: Cycling parameters for RT-qPCR procedures.

Step	Cycles	Temperature	Time
GoTaq® DNA polymerase activation	1	95°C	2 minutes
Denaturation	1	95°C	15 seconds
Annealing and extension	40	57°C	1 minute

The Cycle threshold (Ct) values obtained from the RT-qCR cycles for each gene were used to calculate the relative expression of gene between treated and untreated samples in the form of fold change. The fold change value was calculated using the following formula;

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

The annotation of the formula components is as followed where where 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 while ΔCT_C refers to the gene of interest from treated samples and ΔCT_A refers to the gene of interest from untreated sample.

3.6 Statistical Analysis

Statistical analysis was done using the SPSS system (IBM, USA) wherev One-Way ANOVA was used to compare the inhibition diameter and biofilm reduction activity of *L. rhamnosus* ATCC 7469 supernatant against *P. gingivalis* ATCC 33277with negative control and positive control. Descriptive statistics were used to analyze MIC and MBC of *L. rhamnosus* ATCC 7469supernatant, 0.2% chlorhexidine, and the negative control.

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