

UNIVERSITI TEKNOLOGI MARA

**SELECTIVE ANTIVIRULENCE AND
ANTIBIOFILM EFFECTS OF
POMEGRANATE-DERIVED
COMPOUNDS PUNICALAGIN AND
PUNICALIN IN A DUAL-SPECIES
EXPERIMENTAL ORAL BIOFILM
MODEL**

**NURHAYATI BINTI MOHAMAD
ZAIN**

PhD

July 2026

UNIVERSITI TEKNOLOGI MARA

**SELECTIVE ANTIVIRULENCE AND
ANTIBIOFILM EFFECTS OF
POMEGRANATE-DERIVED
COMPOUNDS PUNICALAGIN AND
PUNICALIN IN A DUAL-SPECIES
EXPERIMENTAL ORAL BIOFILM
MODEL**

NURHAYATI BINTI MOHAMAD ZAIN

Thesis submitted in fulfilment
of the requirements for the degree of
**Doctor of Philosophy
(Dentistry)**

Faculty of Dentistry

July 2026

CONFIRMATION BY PANEL OF EXAMINERS

I certify that a Panel of Examiners has met on 26 May 2026 to conduct the final examination of Nurhayati Binti Mohamad Zain on her Doctors of Philosophy thesis entitled “Selective Antivirulence and Antibiofilm Effects of Pomegranate-Derived Compounds Punicalagin and Punicalin in a Dual-Species Experimental Oral Biofilm Model” in accordance with Universiti Teknologi MARA Act 1976 (Akta 173). The Panel of Examiners recommends that the student be awarded the relevant degree. The Panel of Examiners was as follows:

Gabriele R.A Fromming, PhD
Professor
Faculty of Dentistry
Universiti Teknologi MARA
(Chairman)

Evana Kamarudin, PhD
Senior Lecturer
Faculty of Health Sciences
Universiti Teknologi MARA
(Internal Examiner)

Nihal Bandara, PhD
Associate Professor
Bristol Dental School
University of Bristol
(External Examiner)

**PROFESSOR DR HJH ZURAEDA BINTI
IBRAHIM**

Dean
Institute of Postgraduates Studies
Universiti Teknologi MARA

Date: 8 July 2026

AUTHOR'S DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Teknologi MARA. It is original and is the results of my own work, unless otherwise indicated or acknowledged as referenced work. This thesis has not been submitted to any other academic institution or non-academic institution for any degree or qualification.

I, hereby, acknowledge that I have been supplied with the Academic Rules and Regulations for Post Graduate, Universiti Teknologi MARA, regulating the conduct of my study and research.

Name of Student	:	Nurhayati binti Mohamad Zain
Student ID. No.	:	2021604696
Programme	:	Doctor of Philosophy (Dentistry) – DS950
Faculty	:	Dentistry
Thesis Title	:	Selective Antivirulence and Antibiofilm Effects of Pomegranate-Derived Compounds Punicalagin and Punicalin in a Dual-Species Experimental Oral Biofilm Model
Signature of Student	:	
Date	:	July 2026

ABSTRACT

Dental caries is a biofilm-mediated disease resulting from an ecological imbalance within the oral microbiome, characterised by increased dominance of acidogenic and extracellular polysaccharide producing bacteria, particularly *Streptococcus mutans* (*S. mutans*). Although conventional antimicrobial agents such as chlorhexidine remain widely used for caries control, their non-selective activity may disrupt the commensal oral microbiota and compromise long-term ecological stability. Therefore, strategies that selectively modulate cariogenic biofilms without eliminating health-associated species are increasingly recognised as a priority in contemporary ecology-based caries management. Accordingly, this thesis aimed to systematically evaluate pomegranate - derived compounds (PDCs) as selective antibiofilm agents capable of attenuating cariogenic biofilms while preserving health-associated oral bacteria. A structured two-phase experimental framework was employed. In Phase I, seven PDCs, namely, punicalagin (PUG), punicalin (PUN), corilagin, ellagic acid, gallocatechin, kaempferol glycoside, and rutin, were comparatively screened using antimicrobial assays, biofilm inhibition and eradication assays, acidogenicity assessment, and preliminary toxicity evaluation. Among these candidates, PUG and PUN emerged as the leading candidates, demonstrating effective inhibition of early biofilm formation at sub-minimum inhibitory concentrations, attenuation of *S. mutans* virulence traits, and favourable safety profiles in preliminary toxicity screening. Phase II focused on mechanistic characterisation using optimised dual-species biofilm models comprising *S. mutans* and the commensal *Streptococcus gordonii* (*S. gordonii*). Biofilm inhibition assays revealed selective suppression of *S. mutans* biofilm biomass and viability, while largely preserving the viability of *S. gordonii*. Transcriptional analyses of *S. mutans* demonstrated significant downregulation of genes associated with extracellular polysaccharide synthesis and adhesion (*gtfB*, *gtfC*, *gbpB*, *gbpD*), quorum sensing (*comDE*), and biofilm maturation (*brpA*), with minimal effects on core glycolytic energy metabolism (*ldh*, *atpD*). In contrast, *S. gordonii* exhibited limited transcriptional perturbation, with only modest, transient changes in adhesion- and biofilm-associated genes (*gtfG*, *abpA*, *csaA*). Biofilm eradication assays targeting preformed dual-species biofilms demonstrated time-dependent destabilisation of extracellular polysaccharide rich biofilm structures without extensive bacterial killing, supported by upregulation of autolysis- and cell wall remodelling-associated genes (*atlA*, *lytF*) in *S. mutans*. Scanning electron microscopy further corroborated progressive structural destabilisation of biofilms following compound treatment, particularly with PUG. Collectively, these findings demonstrate that PUG and PUN selectively modulate biofilm behaviour within a dual-species oral biofilm model through coordinated antibiofilm and antivirulence mechanisms, reducing *S. mutans* dominance while preserving commensal species. This work provides evidence supporting the development of plant-derived antivirulence strategies aligned with ecology-based approaches for sustainable caries prevention.

ACKNOWLEDGEMENT

Alhamdulillah, all praise is due to Allah for guiding me with strength and patience through this long and winding journey of knowledge. This thesis is not merely an academic milestone, but a reflection of faith, resilience, and the grace of those who chose to walk beside me. My deepest gratitude is extended to my supervisory team, who continued to believe in me and support me during my lowest point. I am sincerely thankful to my main supervisor, Prof. Dato' Dr Mohamed Ibrahim, for his wisdom, patience, and steadfast guidance. His trust provided clarity when the path felt uncertain. I am equally grateful to my co-supervisors, Professor Dr Fathilah Abdul Razak and Associate Professor Dr Indah Mohd Amin, whose thoughtful guidance, incisive insights, and unwavering support shaped not only this research, but the scholar I have become. Their belief in me is a gift I will carry far beyond this thesis.

I am deeply thankful to my best friend, Vivi Noryati Ahmad, whose faith in me never wavered, especially during my lowest moments. Her presence reminded me that belief from others can carry us when our own feels fragile.

My greatest gratitude belongs to my beloved husband, Najmi Rahimi Muzni, a chronic myeloid leukaemia survivor since 2010 and currently recovering from a stroke in 2025. His quiet strength, patience, and unwavering support redefined my understanding of perseverance. Walking this journey beside him taught me that resilience is not loud, but enduring. Alongside him, I carried my own journey as an autoimmune fighter. Balancing health, family, and career taught me that perseverance is not measured by speed, but by the courage to keep moving forward with faith. To my precious children, Aqil Zehni, Nurdini, Durratul Syafiah, and Mardhiyah Humaira you are my purpose and my promise. Every step of this journey was taken with the hope of showing you that dreams are worth pursuing, even when the road is long.

I am forever grateful to my mother, _____, for her prayers and unwavering love. To my late father, Mohamad Zain Othman, this thesis stands as a promise fulfilled, his values continue to guide me. My heartfelt appreciation also goes to my siblings and in-laws for their prayers, patience, and understanding.

I would like to acknowledge the laboratory staff of Research Laboratory 2, Faculty of Dentistry, Universiti Teknologi MARA, for their professionalism and support throughout the experimental work. Finally, to everyone who contributed in ways seen and unseen, thank you. May Allah be the One who repays every kindness.

TABLE OF CONTENTS

	Page
CONFIRMATION BY PANEL OF EXAMINERS	ii
AUTHOR'S DECLARATION	iii
ABSTRACT	iv
ACKNOWLEDGEMENT	v
TABLE OF CONTENTS	vi
LIST OF TABLES	xii
LIST OF FIGURES	xv
LIST OF PLATES	xvii
LIST OF SYMBOLS	xviii
LIST OF ABBREVIATIONS	xix
CHAPTER 1 INTRODUCTION	1
1.1 Research Background	1
1.2 Problem Statement	2
1.3 Research Gap	3
1.4 Rationale of the Study	4
1.5 Conceptual Framework of the Study	6
1.6 Research Questions	7
1.7 Research Objectives	7
1.7.1 General Objective	7
1.7.2 Specific Objectives	8
1.8 Significance of Study	8
1.9 Hypotheses	10
1.10 Ethical Committee	10
1.11 Thesis Scope	11
1.12 Thesis Outline	12

CHAPTER 2 LITERATURE REVIEW	15
2.1 Global and Clinical Impact of Dental Caries	15
2.2 Dental Caries as a Biofilm-Mediated Disease	17
2.3 Oral Biofilm Development and Structure	19
2.3.1 Formation of the Acquired Pellicle	20
2.3.2 Bacterial Adhesion and Early Colonisation	21
2.3.3 Biofilm Maturation and Stability	23
2.3.4 Biofilm Dispersion and Clinical Implications	26
2.4 Role of Bacterial Adhesion in Cariogenic Biofilms	26
2.5 Rationale for Selection of Bacterial Species	29
2.5.1 <i>S. mutans</i> : A Cariogenic Keystone Pathogen	29
2.5.2 Virulence Determinants of <i>S. mutans</i> and Their Experimental Assessment	30
2.5.3 Biofilm-Associated Gene Regulation in <i>S. mutans</i>	31
2.5.4 <i>S. gordonii</i> as a Health-Associated Commensal	32
2.6 Limitations of Conventional Antimicrobials	34
2.7 Antibiofilm Strategies in Oral Disease Control	36
2.7.1 Modulation of Cell Surface Properties	37
2.7.2 Inhibition of Adhesion and Co-aggregation	37
2.7.3 Interference with Extracellular Polysaccharides Synthesis	38
2.7.4 Disruption of Quorum Sensing	39
2.7.5 Destabilisation of Biofilm Architecture	40
2.7.6 Biofilm Eradication and Dispersal Mechanisms	41
2.8 Plant-Derived Compounds as Selective Antibiofilm Agents	44
2.8.1 Biological Activities of Pomegranate-Derived Preparations in Oral Health	45
2.8.2 Rationale for Studying PDCs	48
2.9 Knowledge Gaps and Justification for the Present Study	51
CHAPTER 3 RESEARCH METHODOLOGY	53
3.1 Materials	53
3.1.1 Pomegranate-Derived Compounds (PDCs)	53
3.1.2 Bacterial Strains	53
3.1.3 Culture Media	53

3.1.4	Chemical Reagents	54
3.1.5	Commercial Kits	54
3.1.6	Consumables	55
3.1.7	Equipment	55
3.2	Preparation of Solution and Media	56
3.2.1	Preparation of Brain Heart Infusion (BHI) Agar	56
3.2.2	Preparation of Brain Heart Infusion (BHI) Broth	56
3.2.3	Preparation of Mitis Salivarius Agar (MSA)	57
3.2.4	Preparation of Phosphate-Buffered Saline (PBS) Solution	57
3.2.5	Preparation of Chlorhexidine (CHX)	58
3.2.6	Preparation of PDCs	58
3.3	Microorganisms Used in This Study	62
3.4	Experimental Design	62
3.5	Preparation of Standard Bacterial Suspension	65
3.6	Disc Diffusion Assay	65
3.7	Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentration (MBC)	66
3.8	Cell Surface Hydrophobicity Assay	68
3.9	Preparation of Clarified Saliva	69
3.10	Optimisation of Dual-species Biofilm Model of <i>S. mutans</i> and <i>S. gordonii</i>	70
3.11	Minimum Biofilm Inhibitory Concentration (MBIC) Using CV Assay	74
3.12	Minimum Biofilm Eradication Concentration (MBEC) Using CV Assay	76
3.13	Glycolytic pH Drop Assay	77
3.14	Brine Shrimp Lethality Assay (BSLA)	78
3.15	Selection of Compounds for Phase II	80
3.16	Phase II: Dual-Species Biofilm Evaluation of Selected Lead Compounds	82
3.16.1	Biofilm Inhibition Assay of PUG and PUN Against Dual-Species Biofilms	82
3.16.2	Biofilm Eradication Activity of PUN and PUG on Preformed Dual-Species Biofilms	92
3.17	Operational Definition of Ecological Selectivity	97
3.18	Statistical Analysis	98
3.18.1	Disc Diffusion Assay	98

3.18.2	Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)	98
3.18.3	Cell Surface Hydrophobicity Assay	98
3.18.4	Minimum Biofilm Inhibitory and Eradication Assays (MBIC and MBEC)	99
3.18.5	Glycolytic pH Drop Assay	99
3.18.6	Brine Shrimp Lethality Assay	99
3.18.7	Bacterial CFU Counts Obtained from the Dual-Species Biofilm Bead Model	99
3.18.8	Quantitative Real-Time PCR (RT-qPCR)	100
3.18.9	Scanning Electron Microscopy (SEM)	100
CHAPTER 4	RESULTS	101
4.1	Disc Diffusion Assay	101
4.2	Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)	103
4.3	Cell Surface Hydrophobicity (CSH) of <i>S. mutans</i> and <i>S. gordonii</i> Following PDCs Treatment	104
4.4	Optimisation of Dual-Species Biofilm Formation	109
4.5	Minimum Biofilm Inhibitory Concentration (MBIC) Using Crystal Violet (CV) Assay	114
4.6	Minimum Biofilm Eradication Concentration (MBEC) Using CV Assay	116
4.7	Glycolytic pH Drop Assay	118
4.8	Brine Shrimp Lethality Assay (BSLA)	120
4.9	Selection of Compounds for Phase II	123
4.10	Phase II: Dual-Species Biofilm Inhibition Assay of PUG and PUN	126
4.10.1	Biofilm Biomass Quantification	126
4.10.2	Viable Cell Enumeration	128
4.10.3	Scanning Electron Microscopy (SEM) Analysis of Dual-Species Biofilm Formation Under Inhibition Treatment with PUG and PUN	131
4.10.4	Gene Expression Analysis of Dual-Species Biofilms During Inhibition by PUG and PUN	133
4.11	Phase II: Dual-Species Biofilm Eradication Assay of PUG and PUN	138

4.11.1	Biofilm Biomass Quantification	138
4.11.2	Viable Cell Enumeration	138
4.11.3	SEM Analysis of Pre-formed Dual-Species Biofilms Following Eradication Treatment with PUG and PUN	143
4.11.4	Gene Expression Analysis of Pre-formed Dual-Species Biofilms Following Eradication Treatment with PUG and PUN	145
CHAPTER 5 DISCUSSION		149
5.1	Overview of the Key Findings	149
5.2	Ecological Rationale for Evaluating PDCs as Selective Antibiofilm and Antivirulence Agents	150
5.3	Planktonic Antimicrobial Activity via MIC/MBC Determination	152
5.4	Cell Surface Hydrophobicity as an Early Antivirulence Target	154
5.5	Formation of an Experimental Salivary Pellicle for Biofilm Development	156
5.6	Optimisation of a Reproducible Dual-Species Oral Biofilm Model	157
5.7	Model Validity and Scope of the Dual-Species <i>In vitro</i> Platform	162
5.8	Screening of PDCs for Biofilm Inhibition (MBIC Assessment)	163
5.9	Screening of PDCs for Biofilm Eradication (MBEC Assessment)	165
5.10	Glycolytic pH-Drop Assay: Effects on Acidogenic Activity	167
5.11	Preliminary Toxicity Evaluation via Brine Shrimp Lethality Assay (BSLA)	168
5.12	Rationale for Compound Selection for Phase II Evaluation	170
5.13	Phase II Dual-Species Biofilm Inhibition by PUG and PUN	171
5.14	Phase II Dual-Species Biofilm Eradication by PUG and PUN	179
5.15	Translational Relevance and Clinical Implications	185
5.16	Summary of Hypothesis Testing	187
5.17	Study Limitations	188
5.18	Future Directions	189
CHAPTER 6 CONCLUSION		193
6.1	Summary of Key Findings and Mechanistic Interpretation	193
6.2	Scientific Contribution, Translational Implications and Future Direction	194

REFERENCES	196
APPENDICES	227
AUTHOR'S PROFILE	254

LIST OF TABLES

Tables	Title	Page
Table 2.1	Key Antibiofilm and Antivirulence Mechanisms Relevant to Oral Biofilm Control and Their Experimental Assessment in the Present Study	42
Table 2.2	Summary of Studies Investigating Pomegranate-Derived Preparations for Oral Antimicrobial and Antibiofilm Activity	47
Table 2.3	Summary of Geographic Origin, Extraction Approach, and Identified Major and Minor Compounds in Pomegranate Peel Extracts Across Selected Studies	48
Table 3.1	List of Chemical Reagents Used in This Study	54
Table 3.2	List of Commercial Kits Used in This Study	54
Table 3.3	Selected PDCs with CAS Numbers, Molecular Weights (MW), Molecular Formulas (MF), Structures, And Purity	60
Table 3.4	Phase I Multi-Assay Screening Strategy Used to Prioritise Pomegranate-Derived Compounds (PDCs) for Phase II Investigation	81
Table 3.5	Composition of Reagents Used to Eliminate Genomic DNA before Reverse Transcription	87
Table 3.6	Components Used to Synthesise Complementary DNA (cDNA) from RNA Samples	88
Table 3.7	qPCR Reaction Setup Using SYBR Green Master Mix	88
Table 3.8	Thermocycling Conditions Showing Temperature and Duration for Each Step	89
Table 3.9	Target Genes and Primer Sequences Used For RT-qPCR Analysis of Biofilm- and Virulence-Associated Genes in <i>S. mutans</i> During Biofilm Inhibition Assays	90
Table 3.10	Target Genes and Primer Sequences Used for RT-qPCR Analysis of Biofilm- And Virulence-Associated Genes in <i>S. gordonii</i> During Biofilm Inhibition Assays	91

Table 3.11	Target Genes and Primer Sequences Used for RT-qPCR Analysis of Biofilm- and Virulence-Associated Genes in <i>S. mutans</i> During Biofilm Eradication Assays	95
Table 3.12	Target Genes and Primer Sequences Used for RT-qPCR Analysis of Biofilm- And Virulence-Associated Genes in <i>S. gordonii</i> During Biofilm Eradication Assays	96
Table 4.1	Zones of Inhibition (mm) of PDCs Against <i>S. gordonii</i> and <i>S. mutans</i>	102
Table 4.2	Minimum Inhibitory Concentrations (MIC), Minimum Bactericidal Concentrations (MBC), and MBC/MIC Ratio of PDCs and CHX against <i>S. gordonii</i> and <i>S. mutans</i>	103
Table 4.3	Effect of Initial Inoculum Density (CFU/mL) on Dual-Species Biofilm Biomass and Species Recovery in 96-Well and Glass-Bead Models	111
Table 4.4	Effect of Inoculum Ratio (<i>S. mutans</i> : <i>S. gordonii</i>) on Dual-Species Biofilm Biomass and Species Recovery in 96-Well and Glass-Bead Models	112
Table 4.5	Effect of Sucrose Concentration (%) on Dual-Species Biofilm Biomass and Species Recovery in 96-Well and Glass-Bead Models	113
Table 4.6	MBIC ₅₀ and MBIC ₉₀ (µg/mL) of PDCs Against Single-Species (<i>S. gordonii</i> , <i>S. mutans</i>) and Dual-Species Biofilms	115
Table 4.7	MBEC ₅₀ and MBEC ₉₀ (µg/mL) of PDCs Against Preformed Single-Species (<i>S. gordonii</i> , <i>S. mutans</i>), and Dual-Species Biofilms	117
Table 4.8	Lethal Concentration (LC ₅₀) Values, Coefficient of Determination (R ²), and Toxicity Classification of PDCs Determined Using the BSLA	123
Table 4.9	Predefined Selection Matrix Used to Prioritise PDCs for Phase II	125
Table 4.10	Statistical Comparisons of Log ₁₀ CFU/bead for <i>S. mutans</i> , <i>S. gordonii</i> , and Total Biofilm Following Inhibition Treatment with PUG, PUN, and CHX	130

Table 4.11	Statistical Comparisons of Log ₁₀ CFU/bead for <i>S. mutans</i> , <i>S. gordonii</i> , and Total Biofilm Following Eradication Treatment with PUG, PUN, and CHX	142
Table 5.1	Summary Comparison of The Ecological Antibiofilm Effects of PUG and PUN During Dual-Species Biofilm Inhibition	178
Table 5.2	Summary Comparison of The Ecological Antibiofilm Effects of PUG and PUN During Dual-Species Biofilm Eradication	184
Table 5.3	Summary of Hypothesis Testing Outcomes	188

LIST OF FIGURES

Figures	Title	Page
Figure 2.1	Conceptual Schematic Illustrating the Stepwise Development of Dental Plaque Biofilm on the Tooth Surface	19
Figure 2.2	Conceptual Illustration of Antibiofilm and Antivirulence Mechanisms Targeting Oral Biofilms	43
Figure 3.1	Workflow Diagram of Two-Phase Experimental Design for Evaluating Antibiofilm And Antivirulence Potential of PDCs	64
Figure 3.2	Schematic Diagram of Minimal Inhibitory Concentration (MIC) Plate Setup	67
Figure 3.3	Workflow for Assessment of Viable Cells in Dual-Species Biofilms by CFU Enumeration	84
Figure 3.4	Schematic Diagram Of RT-qPCR Plate Layout	92
Figure 4.1	Hydrophobicity Index (HI) of <i>S. mutans</i> After Exposure to PDCs at Sub-Minimum Inhibitory Concentrations and MIC concentration	106
Figure 4.2	Hydrophobicity Index (HI) of <i>S. gordonii</i> After Exposure to PDCs At Sub-Minimum Inhibitory Concentrations and MIC Concentration	108
Figure 4.3	Time-Dependent Glycolytic pH Drop of <i>S. mutans</i> over 120 min Following Treatment with PDCs	118
Figure 4.4	Endpoint pH at 120 min in the Glycolytic pH Drop Assay for <i>S. mutans</i> Following Treatment with PDCs	119
Figure 4.5	Dose–Response Curves of Brine Shrimp Mortality After 24 h Exposure to PDCs	121
Figure 4.6	Biomass of Dual-Species Biofilms (<i>S. mutans</i> + <i>S. gordonii</i>) Following Inhibition Treatment with PUG, PUN, and CHX	127
Figure 4.7	Effect of PUG and PUN on Inhibition of Dual-Species Biofilm Formation of <i>S. mutans</i> and <i>S. gordonii</i>	129

Figure 4.8	SEM Analysis of Dual-Species Biofilm Formation of <i>S. mutans</i> and <i>S. gordonii</i> Following Inhibition Treatment with PUG and PUN	132
Figure 4.9	Effect of PUG and PUN on <i>S. mutans</i> Gene Expression	135
Figure 4.10	Effect of PUG and PUN on <i>S. gordonii</i> Gene Expression During Inhibition of Dual-Species Biofilm Formation in a Sucrose-Dependent Environment	137
Figure 4.11	Biomass of Dual-Species Biofilms (<i>S. mutans</i> + <i>S. gordonii</i>) Following Eradication Treatment with PUG, PUN, and CHX	140
Figure 4.12	Effect of PUG and PUN on Viable Bacterial Counts of <i>S. mutans</i> and <i>S. gordonii</i> in Dual-Species Biofilms Following Eradication	141
Figure 4.13	SEM Analysis of Preformed Dual-Species Biofilms (<i>S. mutans</i> + <i>S. gordonii</i>) Following Eradication Treatment with PUG and PUN	144
Figure 4.14	Effect of PUG and PUN on <i>S. mutans</i> Gene Expression in Dual-Species Biofilms Following Eradication Treatment	146
Figure 4.15	Effect of PUG and PUN on <i>S. gordonii</i> Gene Expression in Dual-Species Biofilms Following Eradication Treatment	148
Figure 5.1	Proposed Mechanism of Biofilm Inhibition by PUG and PUN in a Dual-Species Oral Biofilm Under Sucrose-Rich Conditions	179
Figure 5.2	Proposed Mechanism of Biofilm Eradication by PUG and PUN Under Sucrose-Rich Dual-Species Biofilm Conditions	183

LIST OF PLATES

Plates	Title	Page
Plate 3.1	CV–Stained Biofilms During Solubilisation using 33% (v/v) Acetic Acid	72
Plate 3.2	Mitis–Salivarius (MS) Agar Showing Colony Differentiation of <i>S. gordonii</i> and <i>S. mutans</i>	73

LIST OF SYMBOLS

Symbols

$^{\circ}\text{C}$	Degree Celsius
%	Percentage
$\pm$	Plus–Minus (standard deviation or variation)
$\times$	Multiplication Factor (e.g., magnification, MIC multiples)
$\geq$	Greater than or Equal to
$\leq$	Less than or Equal to
$\Delta\Delta\text{CT}$	Difference in Threshold Cycle Values Used in RT-qPCR Analysis
$2^{-\Delta\Delta\text{CT}}$	Method for Relative Gene Expression Quantification
$\log_{10}$	Base-10 Logarithm
μg	Microgram
$\mu\text{g/mL}$	Microgram Per Millilitre
μL	Microlitre
OD	Optical Density
OD_{595}	Optical Density Measured at 595 nm
p	Probability Value in Statistical Tests
ns	Not Statistically Significant

LIST OF ABBREVIATIONS

Abbreviations

ATCC	American Type Culture Collection
BSLA	Brine Shrimp Lethality Assay
CFU	Colony Forming Units
CHX	Chlorhexidine
COR	Corilagin
CSH	Cell Surface Hydrophobicity
EA	Ellagic acid
EPS	Extracellular Polymeric Substances
GC	Gallocatechin
h	Hour(s)
KG	Kaempferol glucoside
LC ₅₀	Median Lethal Concentration
MATH	Microbial Adhesion to Hydrocarbon
MBC	Minimum Bactericidal Concentration
MBEC	Minimum Biofilm Eradication Concentration
MBIC	Minimum Biofilm Inhibitory Concentration
MIC	Minimum Inhibitory Concentration
min	Minute
PBS	Phosphate Buffered Saline
PDCs	Pomegranate-Derived Compounds

PUG	Punicalagin
PUN	Punicalin
RNA	Ribonucleic Acid
rpm	Revolutions Per Minute
RT-qPCR	Reverse Transcription Quantitative Polymerase Chain Reaction
RUT	Rutin
SD	Standard Deviation
SEM	Scanning Electron Microscopy

CHAPTER 1

INTRODUCTION

1.1 Research Background

The oral cavity harbours a highly diverse microbial community that predominantly exists as surface-associated biofilms rather than as free-living microorganisms (Flemming & Wingender, 2010). Dental plaque is therefore best understood as a structured microbial ecosystem, in which microorganisms interact dynamically with one another and with their local environment, giving rise to emergent properties that cannot be inferred from planktonic behaviour alone (Marsh, 2003). Within this ecosystem, oral health and disease are determined not merely by the presence of specific microbial taxa, but by shifts in collective microbial activity, metabolic output and functional balance (Marsh 2006).

Dental caries is now recognised as a biofilm-mediated disease that arises from an ecological imbalance within the oral microbiome. Under health-associated conditions, the oral biofilm remains functionally stable, with microbial activities that are compatible with enamel integrity. In contrast, sustained environmental pressures, particularly frequent exposure to fermentable carbohydrates, may drive adaptive changes in biofilm composition and behaviour, favouring acidogenic and aciduric species such as mutans streptococci and lactobacilli. This shift results in increased acid production and prolonged acid retention within the biofilm microenvironment, ultimately leading to progressive demineralisation (Marsh, 2006; Takahashi and Nyvad et al., 2011).

This ecological understanding of dental caries challenges earlier pathogen-centred models that focused primarily on eliminating specific bacterial species. Instead, contemporary research increasingly emphasises modulation of biofilm function as a therapeutic objective. Within this framework, interventions that attenuate key virulence behaviours including adhesion, extracellular polysaccharide synthesis, acidogenic metabolism, and stress tolerance may reduce the pathogenic potential of the biofilm without disrupting beneficial commensal communities. Such approaches are considered more consistent with the ecological plaque hypothesis because they target disease-

driving functions rather than attempting to sterilise the oral microbiome, which is neither feasible nor desirable (Marsh, 2003; Brookes et al., 2023).

1.2 Problem Statement

Dental caries remains one of the most prevalent chronic diseases globally, despite widespread use of chemical plaque-control agents and advances in oral hygiene practices (Peres et al., 2019). Beyond its high prevalence, dental caries imposes a substantial burden on individuals and healthcare systems, contributing to pain, impaired oral function, reduced quality of life, and significant treatment costs across the lifespan (Kassebaum et al., 2015; Listl et al., 2015; Peres et al., 2019). This persistence reflects a fundamental limitation in contemporary caries management. Most antimicrobial strategies are designed to reduce bacterial load rather than to address the underlying functional imbalance within the oral biofilm that drives disease initiation and progression (Giacaman et al., 2022). Chlorhexidine (CHX), the most widely used chemical antiseptic, exemplifies this limitation. Although CHX exhibits broad antimicrobial efficacy and is frequently employed as a clinical and experimental reference standard, its non-selective mode of action affects both cariogenic and health-associated oral bacteria. By indiscriminately suppressing commensal species that contribute to biofilm homeostasis and pH regulation, prolonged or repeated CHX use may disrupt microbial balance and promote dysbiosis rather than long-term ecological stability (Brookes et al., 2023; Dumitrel et al., 2024). In addition, adverse effects associated with extended use limit its suitability for sustained caries prevention (Poppolo Deus & Ouanounou, 2022). As a result, current chemical approaches may temporarily alleviate the microbial burden but fail to deliver durable, ecology-aligned disease control. These shortcomings underscore a clear mismatch between the ecological nature of dental caries and the mechanism of action of conventional chemical antimicrobials.

From a clinical perspective, strategies that selectively suppress cariogenic bacteria while preserving health-associated commensals may promote greater ecological stability within the oral biofilm. Commensal streptococci contribute to pH buffering, hydrogen peroxide production, and competitive inhibition of cariogenic organisms, thereby limiting dysbiosis and promoting oral health (Kreth et al., 2008; Marsh & Zaura, 2017). Interventions that disrupt these beneficial species may therefore

unintentionally reduce the ecological resilience of the oral microbiome. Selective modulation of pathogenic traits, rather than indiscriminate antimicrobial killing, is increasingly viewed as a more sustainable strategy for long-term caries prevention. By maintaining microbial balance, such approaches may reduce the risk of dysbiosis-associated relapse and support more sustainable disease control beyond short-term reductions in microbial load.

In response to these limitations, plant-derived agents have been increasingly investigated as alternative or adjunctive approaches for oral biofilm control (Lugo-Flores et al., 2021; Miclăuș et al., 2025). Pomegranate-based preparations, in particular, have demonstrated antimicrobial and antibiofilm activity against oral microorganisms in both *in vitro* and clinical studies (Umar et al., 2016; Singla et al., 2018; Kadam et al., 2019; Jacob et al., 2021). However, most of these studies rely on crude extracts with variable compositions and employ simplified experimental models that emphasise growth inhibition rather than biofilm-specific behaviour or virulence modulation.

Consequently, it remains unclear whether plant-derived agents can selectively attenuate cariogenic biofilm behaviour without adversely affecting commensal species that contribute to microbial stability. This knowledge gap limits the rational development of sustainable, long-term caries management strategies that align with the ecological plaque hypothesis. Addressing this unresolved question requires experimental approaches that move beyond non-selective antimicrobial screening and instead employ biofilm-specific, ecology-informed models capable of evaluating both pathogenic suppression and commensal preservation.

1.3 Research Gap

Despite growing interest in plant-based antibiofilm agents for oral biofilm control, several critical limitations in the existing literature limit mechanistic understanding and translational relevance. A major gap lies in the continued reliance on crude extracts rather than chemically defined phytochemicals. While plant-based studies provide preliminary evidence of antimicrobial potential, variability in compounds composition, extraction methods and dosing severely limits reproducibility and obscures identification of the specific bioactive constituents responsible for antibiofilm or antivirulence effects (Tzimas et al., 2024b). In addition, many antibiofilm studies use single-species biofilm models that do not reflect the ecological complexity

of dental plaque. Such simplified systems do not account for interspecies interactions that shape biofilm behaviour, and disease progression models are insufficient for evaluating selective effects on cariogenic bacteria in the presence of health-associated commensal species (Heersema & Smyth, 2019). The frequent omission of commensal microorganisms further limits interpretation of whether observed antibiofilm activity represents targeted virulence modulation or non-selective antimicrobial suppression. Another important gap concerns the failure to distinguish between inhibition of biofilm formation and eradication of established biofilms. Early and mature biofilms differ fundamentally in structure, physiology, and antimicrobial tolerance, yet these stages are often conflated in experimental studies. This methodological limitation risks overestimating therapeutic potential, particularly when agents effective against developing biofilms are assumed to be equally effective against mature, structured communities.

Finally, although phenotypic outcomes such as reduced biofilm biomass or bacterial viability are commonly reported, relatively few studies integrate these findings with gene-level analysis to elucidate how antibiofilm agents influence virulence regulation within structured biofilms. Without transcriptional or mechanistic insight, it remains unclear whether observed effects arise from true antivirulence activity or from non-specific growth inhibition. Collectively, these gaps underscore the need for systematic investigation of purified plant-derived compounds using ecologically relevant multispecies biofilm models and mechanistic endpoints that extend beyond bacterial growth suppression. Addressing these limitations is essential for advancing ecology-based strategies for sustainable caries prevention. Among these considerations, the absence of mechanistic insight into how chemically defined plant-derived compounds modulate virulence expression and interspecies interactions within multispecies oral biofilms represents the primary gap addressed in this thesis.

1.4 Rationale of the Study

This study adopts a structured, ecology-based experimental design to investigate selective antibiofilm strategies for caries prevention. Rather than relying on crude plant extracts, purified pomegranate-derived compounds (PDCs) were selected to ensure chemical definition and experimental consistency, thereby enabling clearer attribution of observed biological effects to specific molecular entities. This compound-focused

approach supports reproducibility and facilitates mechanistic interpretation, addressing a key limitation of extract-based antibiofilm studies. This study was designed as a hypothesis-driven investigation rather than a broad phytochemical screening exercise, with compound selection and endpoints defined by specific ecological and mechanistic questions. The investigation was conducted in two sequential phases. Phase I involved comparative screening of multiple PDCs to evaluate antibacterial, antibiofilm, antivirulence, and preliminary safety profiles, allowing rational prioritisation of candidate compounds based on biofilm-relevant performance rather than planktonic antimicrobial activity alone. Phase II focused on detailed mechanistic evaluation of selected lead compounds to elucidate their effects on biofilm behaviour under ecologically relevant conditions.

To reflect the microbial interactions that underpin dental caries, a dual-species biofilm model comprising a cariogenic pathogen (*S. mutans*) and a health-associated commensal (*S. gordonii*) was employed. This model enables direct assessment of selective antibiofilm activity, including attenuation of cariogenic virulence while preserving commensal viability, a central principle of ecology-based caries management. In this study, successful antibiofilm activity was defined as attenuation of cariogenic virulence traits while maintaining the viability of health-associated commensal bacteria, consistent with an ecology-based approach to caries management. Incorporating transcriptional analysis further enables investigation of virulence modulation at the gene-expression level, providing mechanistic insight beyond phenotypic endpoints such as biomass reduction or bacterial viability.

Through this integrated design, the study aims to advance understanding of how purified PDCs can modulate the behaviour of cariogenic biofilms without disrupting microbial homeostasis. The findings are intended to provide a robust experimental foundation for the development of sustainable, plant-inspired strategies for caries prevention that align with contemporary ecological concepts of oral biofilm management. While no *in vitro* biofilm model can fully capture the complexity of the oral ecosystem, including host factors and dynamic environmental conditions, the use of controlled multispecies systems provides a reproducible and mechanistically informative platform for studying selective virulence modulation.

1.5 Conceptual Framework of the Study

The present study is underpinned by the ecological plaque hypothesis, which conceptualises dental caries as a consequence of altered biofilm function rather than infection by a single pathogenic organism (Marsh, 2003; Marsh, 2006). Within this framework, caries progression arises from environmentally driven shifts in microbial behaviour, characterised by increased acidogenicity, enhanced biofilm stability, and persistence under acidic conditions, ultimately leading to enamel demineralisation. This environmentally driven disturbance of the normal microbial community is referred to as dysbiosis, defined as a disruption of the ecological balance of the plaque biofilm in which health-associated microbial interactions are replaced by a more cariogenic, acidogenic and acid-tolerant community.

In contrast to conventional antimicrobial approaches that aim to reduce overall bacterial load, the conceptual framework of this study emphasises selective modulation of cariogenic virulence traits while preserving health-associated members of the oral microbiota. Commensal species play an essential role in maintaining biofilm stability and ecological balance, and their indiscriminate suppression may predispose the biofilm to recurrent dysbiosis and disease relapse.

Accordingly, the framework prioritises evaluation of antibiofilm and antivirulence effects over planktonic bactericidal activity. Changes in biofilm behaviour are assessed through phenotypic endpoints related to adhesion, biofilm formation, acidogenicity, and biofilm resilience, while transcriptional analysis is employed to elucidate modulation of virulence-associated gene expression within structured biofilms. Together, these outcomes are interpreted as indicators of functional biofilm modulation rather than microbial eradication.

This conceptual framework provides the theoretical basis for the experimental design and the interpretation of the study's outcomes. By linking ecological theory to measurable biofilm-specific and molecular endpoints, it supports an ecology-based strategy for caries prevention that aligns with contemporary understanding of oral biofilm biology and guides evaluation of selective antibiofilm interventions.

1.6 Research Questions

Based on the identified research problem and gaps, the following research questions were formulated to guide the study:

1. Do selected PDCs exhibit antimicrobial and antibiofilm activity against *S. mutans* and *S. gordonii* under *in vitro* conditions?
2. What minimum concentrations of selected PDCs are required to inhibit the formation of, and eradicate, *S. mutans* and *S. gordonii* biofilms?
3. How do selected PDCs influence the cell surface hydrophobicity of *S. mutans* and *S. gordonii* under dose-dependent conditions?
4. Do selected PDCs reduce the acidogenic potential of *S. mutans* under cariogenic conditions?
5. What is the preliminary cytotoxicity profile of selected PDCs at biologically effective concentrations?
6. Do the lead compounds punicalagin (PUG) and punicalin (PUN) inhibit *S. mutans* during biofilm formation in a dual-species (*S. mutans*–*S. gordonii*) model without disproportionately suppressing *S. gordonii*?
7. Are the lead compounds PUG and PUN capable of disrupting established dual-species (*S. mutans*–*S. gordonii*) biofilms while preserving the viability of the commensal *S. gordonii*?

1.7 Research Objectives

1.7.1 General Objective

The general objective of this study is to evaluate the antimicrobial, antibiofilm, and virulence-modulating activities of PDCs against *S. mutans* and *S. gordonii*, with particular emphasis on selectively modulating cariogenic biofilms while preserving commensal bacterial viability.

1.7.2 Specific Objectives

The specific objectives of the study are to:

1. Evaluate the antibacterial activity of selected PDCs against *S. mutans* and *S. gordonii* using disc diffusion, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) assays.
2. Assess the antibiofilm activity of PDCs by determining the minimum biofilm inhibitory concentrations (MBIC₅₀ and MBIC₉₀) and minimum biofilm eradication concentrations (MBEC₅₀ and MBEC₉₀) using crystal violet-based biofilm assays.
3. Determine the effects of selected PDCs on cell surface hydrophobicity (CSH) of *S. mutans* and *S. gordonii* using the microbial adhesion to hydrocarbon assay.
4. Examine the effect of selected PDCs on the acidogenic potential of *S. mutans* using a glycolytic pH-drop model.
5. Evaluate the preliminary cytotoxicity of selected PDCs using a brine shrimp lethality assay (BSLA) and determine median lethal concentration (LC₅₀) values.
6. Investigate the effects of selected lead compounds punicalagin (PUG) and punicalin (PUN) to selectively inhibit *S. mutans* virulence during biofilm formation in a dual-species (*S. mutans*-*S. gordonii*) model while preserving *S. gordonii* viability.
7. Evaluate the capacity of selected lead compounds PUG and PUN to disrupt established dual-species (*S. mutans*-*S. gordonii*) biofilms through attenuation of *S. mutans* virulence, with concurrent assessment of their effects on the commensal *S. gordonii*.

1.8 Significance of Study

This study makes a meaningful contribution to contemporary oral microbiology and caries research by advancing ecology-based strategies for oral biofilm management. By focusing on selective modulation of cariogenic biofilm behaviour rather than indiscriminate bacterial eradication, the study aligns with and extends the ecological

plaque hypothesis, reinforcing the concept that functional balance within the oral biofilm is central to sustainable caries prevention.

From a scientific perspective, this work contributes new evidence on the antibiofilm and antivirulence potential of PDCs, specifically PUN and PUG. Unlike crude plant extracts, which are limited by compositional variability and inconsistent bioactivity, chemically defined compounds enable reproducible evaluation and clearer attribution of biological effects. This compound-focused approach strengthens mechanistic interpretation and enhances the translational relevance of plant-derived antibiofilm research.

Methodologically, the incorporation of a dual-species biofilm model comprising *S. mutans* and *S. gordonii* represents a practical and experimentally controlled improvement over single-species models by evaluating selective antibiofilm activity. By simultaneously assessing effects on a cariogenic pathogen and a health-associated commensal, the study addresses a critical limitation of single-species models and provides insight into how candidate agents influence defined interspecies interactions and functional biofilm balance under controlled conditions. However, this model does not fully reproduce the structural, taxonomic and environmental complexity of natural oral biofilms, which contain highly diverse microbial communities exposed to dynamic salivary flow, dietary substrates and host-derived factors. Therefore, the dual-species system should be interpreted as a reductionist but useful experimental platform that enables targeted evaluation of selective effects, rather than as a complete representation of the oral ecosystem.

The study further contributes to mechanistic understanding by integrating phenotypic assays with gene expression analysis of key virulence-associated pathways, including adhesion, acidogenicity, and extracellular polysaccharide synthesis. This integrated approach enables distinction between true virulence attenuation and non-specific antimicrobial effects, advancing understanding of how selective antibiofilm agents modulate biofilm behaviour at both functional and molecular levels.

Finally, the inclusion of preliminary cytotoxicity screening supports the potential suitability of the investigated compounds for long-term preventive applications. Together, the findings of this study provide a robust scientific foundation for future formulation development and translational research to produce selective, biocompatible oral care products with reduced risk of dysbiosis and antimicrobial resistance. As such, this work contributes not only to fundamental knowledge but also

to the rational development of sustainable, plant-inspired strategies for caries prevention. Notably, the originality of this work lies in its integration of chemically defined pomegranate-derived compounds, a controlled dual-species biofilm model with acknowledged ecological limitations, and mechanistic gene-expression analysis to evaluate selective antivirulence strategies for caries prevention.

1.9 Hypotheses

The hypotheses of this study are as follows:

- H1. Selected PDCs exhibit antibacterial activity against *S. mutans* and *S. gordonii* under *in vitro* conditions.
- H2. Selected PDCs exhibit antibiofilm activity against *S. mutans* and *S. gordonii* by inhibiting biofilm formation and disrupting established biofilms.
- H3. Selected PDCs modulate early virulence-associated traits of *S. mutans* and *S. gordonii*, including cell surface properties related to bacterial adhesion, in a dose-dependent manner.
- H4. Selected PDCs reduce the acidogenic potential of *S. mutans* under cariogenic conditions.
- H5. Selected PDCs exhibit acceptable preliminary safety profiles at biologically effective concentrations.
- H6. The lead compounds PUG and PUN selectively attenuate *S. mutans* virulence during biofilm formation in a dual-species (*S. mutans*–*S. gordonii*) model while preserving the viability of the commensal *S. gordonii*.
- H7. The lead compounds PUG and PUN selectively attenuate *S. mutans* virulence within established dual-species (*S. mutans*–*S. gordonii*) biofilms while preserving the viability of the commensal *S. gordonii*.

1.10 Ethical Committee

Ethical approval for the study was obtained from the Universiti Teknologi MARA (UiTM) Research Ethics Committee (REC/01/2023(PG/MR/27)) prior to commencement. Although the experimental work involved *in vitro* microbial assays,

ethical clearance was required for the collection of human saliva used to prepare experimental acquired pellicle for biofilm formation studies.

1.11 Thesis Scope

This study is confined to the *in vitro* evaluation of selected PDCs for their antibacterial, antibiofilm, and antivirulence activities against oral streptococci associated with dental caries. The investigation focuses specifically on *S. mutans* as a representative cariogenic pathogen and *S. gordonii* as a health-associated commensal and pioneer coloniser, reflecting key ecological interactions relevant to dental biofilm development.

The scope of the study encompasses a systematic assessment of antibacterial activity, virulence-associated phenotypes, and biofilm behaviour under controlled laboratory conditions. Evaluation of virulence-related traits includes modulation of bacterial adhesion-associated properties, inhibition and eradication of biofilms, and alteration of acidogenic potential. Preliminary safety assessment is limited to cytotoxicity screening to support prioritisation of candidate compounds for further investigation.

Mechanistic investigation is restricted to selected lead compounds, PUG and PUN, which were prioritised based on Phase I screening outcomes. These compounds are further examined using dual-species biofilm models to assess selective modulation of cariogenic biofilm behaviour in the presence of a health-associated commensal. Mechanistic insight is derived from integrated phenotypic and molecular analyses, including assessment of biofilm architecture, viable bacterial populations, and transcriptional regulation of predefined genes associated with adhesion, extracellular polysaccharide synthesis, stress response, and biofilm stability in *S. mutans*.

This study does not include *in vivo* experimentation, clinical trials, formulation development, pharmacokinetic and pharmacodynamic analyses, or comprehensive toxicological assessment beyond preliminary screening. Accordingly, the findings are interpreted within the context of simplified *in vitro* biofilm models and are not intended to directly predict clinical efficacy. Interactions involving additional oral microbial taxa, host immune responses, and long-term ecological dynamics within complex oral microbiomes are also beyond the scope of the present investigation.

Within these defined boundaries, the study aims to generate robust experimental evidence supporting the potential of PDCs as selective biofilm-modulating agents and to provide a scientifically grounded platform for future translational, formulation-based, and clinical research in ecology-based caries prevention.

1.12 Thesis Outline

This thesis is organised into six chapters, progressing logically from conceptual foundations and theoretical context to experimental investigation, critical interpretation, and final conclusions.

Chapter 1: Introduction

This chapter establishes the research context and theoretical basis of the study. It introduces the oral biofilm ecosystem and the role of microbial dysbiosis in dental caries, followed by a discussion of the research background and problem statement. The chapter further defines the research gap, rationale, significance, objectives, research questions, hypotheses, scope, and limitations, thereby setting clear direction and boundaries for the investigation.

Chapter 2: Literature Review

This chapter provides a critical review of current knowledge on oral biofilm biology, with particular emphasis on ecological interactions between the cariogenic pathogen *S. mutans* and the health-associated commensal *S. gordonii*. Key mechanisms of biofilm formation, extracellular polysaccharide synthesis, acidogenicity, and virulence regulation are discussed. The chapter also examines existing antimicrobial and antibiofilm strategies, highlights limitations of broad-spectrum agents such as CHX, and reviews emerging evidence on plant-derived compounds within an ecology-based framework for caries management. Relevant knowledge gaps that justify the present study are identified.

Chapter 3: Research Methodology

This chapter describes the experimental design and methodological approaches employed in the study. It outlines compound preparation, bacterial culture conditions, and Phase I screening procedures for antibacterial activity, virulence-associated traits, antibiofilm effects, acidogenicity, and preliminary cytotoxicity. Phase II mechanistic investigations using dual-species biofilm models are also detailed, including assessment of viable bacterial populations, biofilm structure, and transcriptional regulation of selected virulence-associated genes. Statistical analyses and ethical considerations are presented at the end of the chapter.

Chapter 4: Results

This chapter presents the experimental findings in a structured manner aligned with the research objectives. Phase I results describe the antibacterial, antibiofilm, antivirulence, and safety profiles of the screened PDCs. Phase II results focus on the biofilm inhibition and eradication activities of the lead compounds, PUG and PUN, in dual-species biofilms, including quantitative microbiological data, structural observations, and gene expression outcomes. Results are reported objectively without interpretation.

Chapter 5: Discussion

This chapter critically interprets the findings in the context of existing literature and the conceptual framework of the study. Phenotypic, molecular, and structural data are integrated to elucidate mechanisms underlying selective antibiofilm activity. Differences between biofilm inhibition and eradication are examined, along with the ecological implications of preserving commensal species. The chapter also discusses translational relevance, clinical implications, study limitations, and directions for future research.

Chapter 6: Conclusion

This chapter summarises the key findings of the study and their contributions to oral microbiology and antibiofilm research. It revisits the research objectives and hypotheses, highlights the novelty and significance of the work, and concludes with a concise statement on the potential of punicalagin and punicalin as selective, ecology-based agents for caries prevention.

References

A comprehensive list of all cited literature is provided in accordance with the prescribed referencing style.

Appendices

The appendices contain supplementary materials that support the thesis but are not included in the main chapters. These include relevant documentation such as intellectual property records, certificates of participation, scientific conference materials, copies of published journal articles arising from this research, and the author's profile.

CHAPTER 2

LITERATURE REVIEW

2.1 Global and Clinical Impact of Dental Caries

Dental caries is one of the most common chronic conditions worldwide, affecting people of all ages. Global epidemiological analyses consistently identify untreated carious lesions as among the most prevalent health conditions, with a substantial proportion of children, adolescents, and adults experiencing disease at some point across the lifespan (Kassebaum et al., 2015; Peres et al., 2019). Recent global estimates further emphasise the magnitude of this burden. The World Health Organization reported that oral diseases affect nearly 3.7 billion people globally, while untreated dental caries in permanent teeth remains the most common health condition according to the Global Burden of Disease 2021 estimates (WHO, 2022). In the GBD 2021 analysis, caries of permanent teeth accounted for approximately 2.37 billion incident cases and 2.24 billion prevalent cases worldwide, with an age-standardised incidence rate of 29,777.03 per 100,000 population and an age-standardised prevalence rate of 27,543.34 per 100,000 population (Li et al., 2025). In children, untreated caries in primary teeth affects approximately 514 million children globally, highlighting the early-life burden of the disease (WHO, 2022; Chen et al., 2025). The WHO Global Strategy and Action Plan on Oral Health 2023–2030 further recognises oral diseases as a major public health challenge and calls for stronger integration of oral health into universal health coverage and noncommunicable disease prevention strategies (WHO, 2024).

Despite substantial advances in preventive dentistry, including fluoride-based interventions and improved oral hygiene awareness, the global burden of dental caries has remained persistently high over recent decades (Peres et al., 2019). Recent GBD 2021 data also indicate that the overall burden of oral conditions has shown only limited improvement over the past three decades, suggesting that current strategies have not produced sufficient population-level reduction in disease burden (Bernabe et al., 2025). This continued prevalence suggests that current preventive strategies may not sufficiently address the underlying biological mechanisms responsible for disease initiation and progression. Traditional interventions primarily focus on controlling

mineral loss or reducing bacterial load, whereas the ecological processes that drive biofilm dysbiosis and sustained acid production are often insufficiently targeted. This mismatch between disease pathogenesis and therapeutic strategies highlights the need for preventive approaches that specifically target biofilm function and microbial behaviour.

Clinically, dental caries is characterised by progressive demineralisation and destruction of enamel and dentine, often resulting in pain, hypersensitivity, pulpal involvement, and increased susceptibility to secondary infection. When left untreated, caries may lead to tooth loss, compromised mastication, and impaired speech, thereby negatively affecting oral function and overall well-being. Progression of caries into the dental pulp may result in pulpitis, periapical infection and odontogenic abscess formation. Although many dental infections remain localised, severe or untreated odontogenic infections may spread through fascial spaces of the head and neck, leading to cellulitis, deep neck space infection, Ludwig's angina, airway compromise, osteomyelitis, mediastinitis, sepsis and, in rare cases, life-threatening complications (Bali et al., 2015; Neal et al., 2022). These systemic complications demonstrate that dental caries is not merely a localised tooth-related condition, but may progress into a medically significant infection with consequences for general health.

In children, dental caries has been associated with difficulties in eating, sleeping disturbances, and reduced school performance, while in adults, it may interfere with daily activities and limit occupational productivity (Jackson et al., 2011; Peres et al., 2019). Pain, infection, emergency dental visits and hospital-based management of severe odontogenic infections may further increase school absenteeism, work absenteeism, caregiver burden and healthcare utilisation, thereby negatively affecting quality of life and socioeconomic productivity.

From a public health perspective, the management of dental caries imposes a considerable economic burden on healthcare systems. Restorative and rehabilitative dental treatments account for a substantial proportion of oral healthcare expenditure in many countries, often exceeding the costs associated with the management of other chronic conditions (Peres et al., 2019). The economic burden of oral diseases is substantial. The WHO Global Oral Health Status Report estimated that direct expenditure for oral diseases across 194 countries amounted to approximately US\$387 billion in 2019, representing about 4.8% of global direct health expenditure, while productivity losses were estimated at approximately US\$323 billion globally (WHO,

2022). More recent economic analyses similarly estimated the total worldwide economic impact of oral conditions at approximately US\$710 billion, comprising direct treatment costs and indirect productivity losses (Jevdjevic et al., 2024).

These costs are further exacerbated by the recurrent nature of the disease, which often necessitates repeated clinical interventions. In addition, out-of-pocket dental expenditure may act as a financial barrier to care, particularly in low- and middle-income settings, increasing the risk of delayed treatment, disease progression and more costly emergency management.

The pervasive clinical and societal impact of dental caries underscores the importance of developing preventive strategies that address not only disease manifestation but also the biological processes underpinning lesion initiation and progression. Accordingly, an improved understanding of caries pathogenesis at the level of the oral biofilm is essential for developing more effective and sustainable approaches to disease control. Given the high global prevalence, recurrent treatment costs, risk of systemic infectious complications and negative effects on quality of life, ecology-based approaches that modulate cariogenic biofilm function may provide a more biologically targeted and sustainable direction for caries prevention.

2.2 Dental Caries as a Biofilm-Mediated Disease

Dental caries is now widely understood as a dysbiosis-driven disease resulting from ecological shifts within the oral biofilm rather than from the action of a single infectious agent (Marsh, 2003; Takahashi & Nyvad, 2011). Dental plaque is a biofilm, a structured microbial community attached to the tooth surface and embedded in a self-produced extracellular matrix (Bowen et al., 2018; Diaz & Valm, 2020). Within this environment, bacteria interact closely with one another and with their environment. These interactions give rise to emergent biological properties that differ fundamentally from those of planktonic cells (Diaz & Valm, 2020). A defining feature of biofilms is their enhanced tolerance to antimicrobial agents and host defence mechanisms. The extracellular matrix restricts diffusion, buffers environmental stress, and supports metabolic cooperation, while biofilm-associated bacteria exhibit distinct transcriptional profiles linked to adhesion, stress adaptation, and persistence (Donlan & Costerton, 2002; Hall-Stoodley et al., 2004; Flemming et al., 2016; Karygianni et al., 2020). These

properties help explain the chronic and recurrent nature of dental caries despite routine mechanical and chemical plaque-control measures.

The ecological plaque hypothesis provides a conceptual framework for understanding caries development as a consequence of environmentally driven changes in biofilm behaviour rather than the introduction of exogenous pathogens. Under health-associated conditions, the oral biofilm maintains functional stability and a near-neutral pH at the tooth surface. However, frequent exposure to fermentable carbohydrates, particularly sucrose, induces repeated episodes of biofilm acidification, selectively favouring acidogenic and aciduric organisms while disadvantaging less tolerant species. Importantly, this shift reflects changes in the activity and relative abundance of resident microorganisms rather than microbial replacement.

Sustained acid production and retention within the biofilm microenvironment ultimately drive enamel demineralisation and lesion formation. From this perspective, dental caries is best regarded as a disease of biofilm function, in which metabolic outputs, especially acidogenicity, are central determinants of pathogenicity. This ecological view challenges earlier pathogen-centric models and has significant implications for caries prevention strategies.

Historically, *Streptococcus mutans* (*S. mutans*) was regarded as the primary causative agent of dental caries due to its well-characterised virulence traits, including extracellular polysaccharide synthesis, rapid acid production, and survival under acidic conditions. While these attributes remain highly relevant, contemporary microbiome studies show that caries-associated biofilms are not exclusively dominated by *S. mutans*. Instead, caries is increasingly understood as a dysbiotic state in which collective microbial behaviour shifts toward disease-promoting functions.

This understanding highlights the limitations of strategies focused solely on eliminating individual species. Removal of specific bacteria without addressing the ecological drivers of dysbiosis may permit recolonisation or replacement by other acidogenic organisms, undermining long-term disease control (Brookes et al., 2023; Dumitrel et al., 2024). Consequently, effective caries management requires approaches that target biofilm behaviour and virulence rather than indiscriminate microbial killing.

Recognition of caries as an ecology-driven disease has therefore shifted attention toward selective antibiofilm strategies that modulate key virulence-associated processes such as adhesion, extracellular matrix formation, and acid production while preserving commensal species that contribute to biofilm stability. This conceptual shift

underpins the present study, which prioritises antibiofilm and antivirulence effects over bactericidal activity alone.

2.3 Oral Biofilm Development and Structure

Oral biofilm development is a dynamic, multistage process involving acquired pellicle formation, bacterial adhesion, biofilm maturation, and dispersion (Jakubovics & Kolenbrander, 2010; Sterzenbach et al., 2020). These overlapping stages collectively determine the structural organisation, metabolic activity, and pathogenic potential of dental plaque (Figure 2.1). Understanding these processes is essential for distinguishing between strategies that prevent biofilm formation and those required to disrupt established biofilms. Clinically, this distinction is critical. Early biofilms are relatively sparse and metabolically adaptable, whereas mature biofilms exhibit complex architecture, dense extracellular matrices, and increased tolerance to antimicrobial intervention. These biological differences underpin the rationale for separately evaluating biofilm inhibition and biofilm eradication.

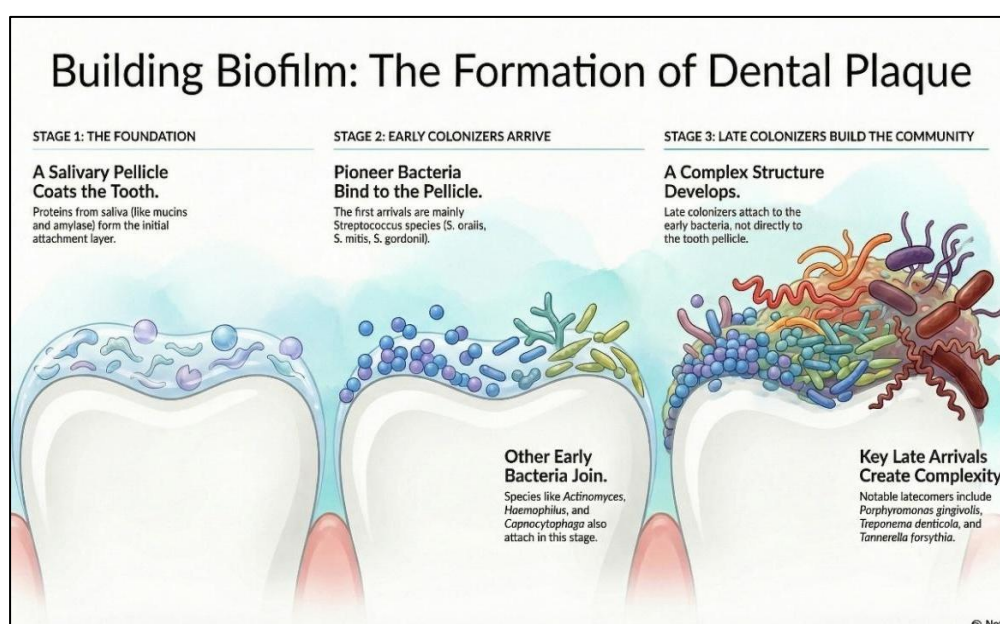


Figure 2.1 Conceptual Schematic Illustrating the Stepwise Development of Dental Plaque Biofilm on the Tooth Surface

The diagram depicts the formation of the acquired salivary pellicle, initial adhesion of pioneer oral bacteria, co-aggregation and recruitment of early colonisers, and the progressive maturation of a multispecies biofilm in which late colonisers attach to pre-existing bacterial communities rather than directly to the enamel surface. This conceptual illustration was created by the author based on established models of oral biofilm development described in the literature (Jakubovics & Kolenbrander, 2010)

2.3.1 Formation of the Acquired Pellicle

The first step in oral biofilm development occurs immediately after tooth cleaning, when salivary components begin to adsorb onto the enamel surface. This process leads to the formation of the acquired pellicle, a thin, protein-rich layer covering the tooth surface that is composed primarily of salivary glycoproteins, phosphoproteins, enzymes, and peptides, including acidic proline-rich proteins, α -amylase, cystatins, immunoglobulins, lysozyme, and mucins (Enax et al., 2023). Although acellular, the pellicle is biologically active and plays a central role in shaping subsequent microbial colonisation.

The pellicle serves two seemingly opposing functions. On the one hand, it acts as a lubricant and a protective barrier that limits enamel demineralisation by modulating mineral exchange between the tooth surface and saliva. Previous studies have demonstrated that the acquired dental pellicle protects tooth surfaces against acidic challenges by acting as a selectively permeable barrier that can partially limit the diffusion of erosive acids toward the enamel surface (Lubarsky et al., 2012). Despite its protective role, the acquired enamel pellicle does not provide complete or long-term resistance to acid-mediated demineralisation. Short-term pellicle formation can transiently delay enamel dissolution during brief erosive challenges; however, this protective effect diminishes with prolonged or repeated acid exposure. For example, a pellicle formed over 2 hours was shown to reduce enamel demineralisation during a 10-minute erosive challenge but failed to confer sustained protection during longer exposure periods (Hara et al., 2006). Extending this concept, Mutahar et al. (2017) demonstrated that the presence of an acquired enamel pellicle fundamentally alters the nature of acid-induced erosion, shifting it from bulk mineral dissolution to a process dominated by surface softening. Consequently, pellicle-covered enamel exhibited reduced tissue loss despite increased surface softening, highlighting that the pellicle modifies erosion dynamics rather than providing absolute protection. Although pellicle formation initiates rapidly upon saliva exposure and approaches functional maturation within approximately 30 minutes to 2 hours, the use of a 24 hour pellicle by Mutahar et al. ensured the development of a stable and well-formed layer prior to repeated erosive cycling (Mutahar et al., 2017).

On the other hand, acquired pellicle provides a conditioned surface containing specific receptors that facilitate bacterial attachment (Lynge Pedersen & Belstrøm,

2019). The composition and conformation of pellicle proteins determine which bacterial species can adhere efficiently, thereby influencing the early microbial composition of the biofilm (Vukosavljevic et al., 2011). Importantly, the pellicle is not static. Its structure and binding properties change over time due to enzymatic modification, bacterial activity, and continual interaction with saliva. These dynamic changes create a surface that increasingly favours microbial colonisation and biofilm development (Chawhuaveang et al., 2021; Enax et al., 2023).

2.3.2 Bacterial Adhesion and Early Colonisation

Following pellicle formation, bacteria from saliva encounter the conditioned tooth surface and initiate adhesion. Bacterial adhesion occurs through a sequence of coordinated steps that ultimately determine whether bacterial attachment remains transient or progresses toward stable biofilm formation. Initially, microorganisms are transported to the surface, where they undergo non-specific, reversible adhesion mediated by physicochemical interactions. This is followed by a transition to specific, irreversible adhesion, driven by molecular interactions between bacterial adhesins and complementary receptors on the conditioned surface. Together, these two main phases, non-specific adhesion followed by specific adhesion, govern the shift from transient attachment to stable colonisation and the subsequent emergence of a structured biofilm.

2.3.2.1 Non-Specific Adhesion

Non-specific adhesion represents the initial, reversible interaction between bacterial cells and the pellicle-coated surface. At this stage, attachment is governed primarily by physicochemical forces, including van der Waals interactions, electrostatic attraction, and the hydrophobic effect (Bos et al., 1999; Busscher & van der Mei, 2012; Tu et al., 2023). These forces allow bacteria to approach and weakly associate with the surface without the involvement of specialised binding molecules.

Cell surface hydrophobicity plays a particularly important role in this phase. Bacteria with more hydrophobic cell surfaces tend to associate with hydrophobic regions of the pellicle, whereas surface charge characteristics may either facilitate or hinder attachment, depending on local conditions (Weiss et al., 1982; Krasowska & Sigler, 2014). These physicochemical interactions are relatively weak and reversible,

allowing bacteria to detach under the influence of salivary flow or mechanical forces. Non-specific adhesion may therefore be regarded as a transitional or exploratory stage, during which microorganisms assess the surface environment before progressing to irreversible, receptor-mediated attachment.

2.3.2.2 Specific Adhesion (Adhesins and Pellicle Receptors)

If favourable conditions persist, bacteria progress from initial reversible attachment to specific adhesion, a stage characterised by highly selective interactions between bacterial surface adhesins and complementary receptors within the acquired pellicle. On tooth surfaces, primary colonisers such as oral streptococci and *Actinomyces* spp. mediate this process through adhesins that recognise specific salivary pellicle components, including proline-rich proteins, MG2 mucin, and α -amylase (Whittaker et al., 1996; Ruhl et al., 2004). The acquired pellicle is composed mainly of salivary proteins and glycoproteins, including amylase, acidic proline-rich proteins, mucins, cystatins, lysozyme and immunoglobulins, many of which provide binding sites for early colonising bacteria. Thus, bacterial adhesion to the tooth surface is not random, but is shaped by both the physicochemical properties of bacterial cells and specific molecular recognition of pellicle-associated receptors.

Cell surface hydrophobicity is particularly relevant during this early transition because it influences the ability of bacterial cells to approach, remain near, and establish close contact with the pellicle-coated surface. Hydrophobic interactions can reduce the energy barrier between bacterial cells and the conditioning film, thereby facilitating short-range adhesin–receptor interactions (Gibbons & Etherden, 1983; Gibbons et al., 1983; Sterzenbach et al., 2020). Comparative studies have shown that several early plaque colonisers, including *Actinomyces* spp. and oral streptococci, display marked cell surface hydrophobicity and adhere efficiently to salivary pellicles (Gibbons & Etherden, 1983). Furthermore, non-hydrophobic mutants of oral streptococci demonstrate reduced adherence to experimental salivary pellicles, indicating that hydrophobicity is not merely an associated phenotype but contributes functionally to stable attachment (Gibbons et al., 1983). In *S. mutans*, reductions in hydrophobicity have also been associated with decreased adherence and biofilm formation, further supporting the relevance of this parameter in evaluating anti-adhesion activity (Liu et al., 2020).

This transition typically occurs within the first few hours following initial surface contact, once bacteria have moved sufficiently close to the pellicle to enable short-range molecular interactions. These adhesin-receptor bindings stabilise attachment and mark the shift from reversible to irreversible colonisation. *In vivo* evidence indicates that firm bacterial adherence to the acquired enamel pellicle is established within 2–6 hours after tooth cleaning, with the microbial profile becoming distinct from saliva during this period, reflecting selective and irreversible attachment rather than transient deposition (Li et al., 2004). In addition to stabilising surface attachment, these molecular interactions facilitate co-adhesion, whereby additional microorganisms attach to cells already adhered. Through this process, early colonisers establish a microbial scaffold during the early hours of plaque development, which supports the subsequent recruitment of secondary and later colonising species.

Once specific adhesion is established, bacterial cells undergo progressive physiological adaptation to a sessile lifestyle, typically within the first 4–8 hours of attachment to the surface. During this period, gene expression shifts toward pathways associated with adhesion, stress tolerance, and extracellular polymeric substance production, initiating the transition from isolated adherent cells to structured microcolonies and setting the stage for biofilm maturation (Fitri et al., 2025).

Collectively, these early adhesion processes underscore the importance of physicochemical surface properties and molecular interactions in determining whether initial bacterial contact progresses toward stable colonisation. Given the contribution of cell surface hydrophobicity to reversible attachment and early surface sampling, assessment of hydrophobicity provides a relevant functional indicator of adhesion potential. Therefore, the present study incorporated evaluation of cell surface hydrophobicity using the microbial adhesion to hydrocarbons (MATH) method to examine whether the tested compounds influence early colonisation behaviour at the level of initial surface interaction.

2.3.3 Biofilm Maturation and Stability

Biofilm maturation involves the organisation of adherent cells into three-dimensional, structured communities embedded within an EPS matrix. This matrix, composed mainly of polysaccharides, proteins, lipids, and extracellular DNA, provides mechanical stability and creates diffusion-limited microenvironments within the

biofilm (Flemming & Wingender, 2010). As such, mature biofilms represent organised multicellular structures rather than simple accumulations of surface-attached planktonic cells.

As the biofilm thickens, spatial heterogeneity emerges. Channels form within the matrix, allowing the transport of nutrients and waste products, while gradients of oxygen, pH, and metabolites develop across different regions of the biofilm (Wimpenny et al., 2000; Røder et al., 2020). These gradients drive phenotypic diversity, with bacterial subpopulations adopting distinct metabolic states, including actively growing, slow-growing, and stress-adapted forms.

Mature biofilms are markedly more tolerant to antimicrobial agents than early biofilms. This tolerance is mediated by several interrelated mechanisms, including reduced antimicrobial penetration through the EPS matrix, binding or sequestration of antimicrobial agents by matrix components, altered bacterial metabolic activity, nutrient and oxygen limitation, stress-response activation, and the presence of slow-growing or dormant subpopulations (Azeredo et al., 2017; Fu et al., 2021). These characteristics explain why concentrations that are effective in preventing early biofilm formation may not necessarily eradicate or structurally disrupt established biofilms. Therefore, inhibition of biofilm formation and disruption of mature biofilms represent biologically distinct outcomes and must be interpreted according to the timing of compound exposure within the biofilm life cycle.

In the present study, crystal violet (CV) staining was employed as a quantitative assay for total adherent biofilm biomass. CV is widely used in microtiter plate-based biofilm studies because it provides a simple, reproducible, and high-throughput measure of the total material that remains attached to a surface after washing (Peeters et al., 2008; Azeredo et al., 2017). In this context, CV staining was useful for assessing net biofilm accumulation following exposure to the test compounds. However, CV does not specifically measure bacterial viability, extracellular matrix production, or EPS degradation. Rather, the assay stains the biofilm as a composite structure comprising viable cells, non-viable cells, cellular debris, and extracellular matrix components. Therefore, CV absorbance was interpreted only as a measure of total adherent biomass and not as a standalone indicator of bacterial killing or matrix-specific disruption (Skogman et al., 2012; Stiefel et al., 2016; Welch et al., 2012).

This limitation is particularly important when evaluating compounds with potential antibiofilm activity. A reduction in CV absorbance may indicate reduced cell

attachment, decreased biofilm accumulation, loss of matrix-associated material, detachment of biofilm from the surface, or a combination of these effects. Conversely, an antimicrobial compound may substantially reduce the number of viable bacteria within an established biofilm without causing a proportional reduction in CV-detected biomass if the extracellular scaffold remains attached to the surface. In such cases, the biological integrity of the biofilm may be compromised despite the persistence of visible or stainable biomass. Therefore, reliance on CV staining alone may underestimate the antimicrobial potential of a compound that kills biofilm-embedded cells without physically removing the residual matrix structure (Stiefel et al., 2016; Welch et al., 2012).

For this reason, CV staining was not used in isolation in this study. Instead, it was integrated with viable cell enumeration by colony-forming unit (CFU) counts and structural assessment by scanning electron microscopy (SEM). CFU analysis provided information on recoverable viable bacteria and allowed species-specific assessment of *S. mutans* and *S. gordonii* within the dual-species biofilm. This was essential because changes in total biomass do not necessarily reflect changes in viable cell density or species composition. By comparing CV-derived biomass data with CFU counts, it was possible to distinguish between reductions in total attached material and reductions in viable bacterial recovery. This combined interpretation of biomass and viability data is recommended because biofilm susceptibility cannot be adequately assessed using a single endpoint alone (Skogman et al., 2012; Stiefel et al., 2016). For example, a concurrent decrease in CV absorbance and CFU counts would suggest reduced biofilm accumulation accompanied by reduced viable bacterial burden. In contrast, reduced CFU counts without a marked reduction in CV absorbance would suggest that the compound compromised bacterial viability while leaving residual extracellular or cellular material attached.

SEM further complemented CV and CFU analyses by providing visual evidence of biofilm architecture, surface coverage, cellular organisation, and matrix-associated structural alterations. This was important because biomass assays provide quantitative absorbance values but do not reveal how the biofilm structure is physically arranged or altered after treatment. SEM therefore enabled qualitative assessment of whether treated biofilms showed reduced surface coverage, disrupted architecture, exposed substratum, altered cell clustering, or reduced matrix-like material. Together, CV, CFU, and SEM

provided a more comprehensive interpretation of biofilm modulation than any single method alone (Azeredo et al., 2017; Stiefel et al., 2016).

2.3.4 Biofilm Dispersion and Clinical Implications

Biofilm dispersion represents the final stage of the biofilm life cycle, allowing microorganisms to detach and colonise new surfaces. Dispersion can occur passively through mechanical forces, such as salivary flow and tooth brushing or actively through regulated bacterial processes that degrade components of the extracellular matrix (Lv et al., 2025). Dispersed cells may exhibit phenotypes distinct from both planktonic and biofilm-associated states, often displaying enhanced capacity for reattachment and colonisation (Lv et al., 2025; Yaghmoor et al., 2024). Clinically, this process contributes to the persistence and spread of biofilms across different oral sites, including natural tooth surfaces and dental materials.

From a therapeutic standpoint, dispersion has dual implications. While controlled biofilm disruption may facilitate mechanical removal and reduce biofilm burden, uncontrolled dispersion can promote recolonisation and disease recurrence. This underscores the challenge of eradicating mature biofilms without creating conditions that favour rapid re-establishment. Taken together, the staged development and structural complexity of oral biofilms explain why early intervention strategies aimed at preventing adhesion and maturation differ fundamentally from those required to disrupt established biofilms. This distinction provides a critical biological basis for evaluating antibiofilm agents in terms of both inhibitory and eradication potential.

2.4 Role of Bacterial Adhesion in Cariogenic Biofilms

Bacterial adhesion constitutes the critical gateway between transient oral colonisation and the establishment of cariogenic biofilms. In the absence of stable attachment to oral surfaces, bacteria are readily cleared by salivary flow, mastication, and routine oral hygiene measures. Once adhesion is achieved, however, microorganisms can proliferate, interact with neighbouring species, and initiate the formation of a structured biofilm community capable of sustained metabolic activity.

Adhesion is not merely a physical anchoring process but is intimately linked to the expression of key virulence traits associated with cariogenesis. Stable attachment

facilitates extracellular polysaccharide production, enhances acid retention at the biofilm–surface interface, and promotes tolerance to environmental stressors such as low pH. Collectively, these changes create a localised microenvironment that favours persistent acidogenic and aciduric activity, ultimately driving enamel demineralisation and lesion progression (Wang et al., 2023; Spatafora et al., 2024). As such, adhesion represents a decisive early determinant of cariogenic potential rather than a passive preliminary step.

At the molecular level, early bacterial attachment is governed predominantly by physicochemical interactions between the bacterial cell surface and oral substrates (Bos et al., 1999). Among these, CSH plays a central role by influencing the strength and stability of initial, reversible adhesion. CSH reflects the balance of hydrophobic and hydrophilic components on the bacterial envelope, including surface proteins, lipoteichoic acids, and polysaccharides (Krasowska & Sigler, 2014). Bacteria with higher surface hydrophobicity exhibit greater affinity for hydrophobic domains within the acquired salivary pellicle, facilitating closer surface contact and promoting transition to irreversible adhesion. In contrast, reduced hydrophobicity weakens these interactions, rendering bacterial attachment more susceptible to removal by shear forces (Hsu et al., 2013). Importantly, CSH is a dynamic property rather than a fixed bacterial trait. It can be modulated by environmental conditions such as nutrient availability, pH fluctuations, and exposure to external agents (Stoderegger & Herndl, 2005; Azeem et al., 2025; Zhu et al., 2025). In cariogenic species such as *S. mutans*, increased CSH has been consistently associated with enhanced adhesion and biofilm-forming capacity, linking physicochemical surface properties directly to virulence expression. This dynamic nature positions CSH as an attractive antivirulence target, as modest alterations at this early stage can substantially influence downstream biofilm development without necessitating bacterial killing.

While enamel represents the primary natural substrate for cariogenic biofilms, oral bacteria encounter a wide range of artificial surfaces introduced through restorative and prosthetic dentistry. Dental biomaterials, including orthodontic brackets, composite resins, acrylic denture bases, ceramics, and implant components, vary considerably in surface roughness, surface free energy, and chemical composition, all of which modulate bacterial adhesion and retention (Tzimas et al., 2024a). Once exposed to the oral environment, these materials rapidly acquire a conditioning film derived from

saliva, rendering them susceptible to colonisation through mechanisms broadly similar to those governing adhesion to tooth surfaces.

The clinical consequences of bacterial adhesion to dental biomaterials closely parallel those observed on natural enamel surfaces. At orthodontic brackets and restoration margins, persistent biofilms are frequently associated with *S. mutans*. Its strong ability to adhere to dental materials, together with pronounced acidogenic metabolism, promotes localised acid accumulation at the tooth–material interface, thereby increasing susceptibility to enamel demineralisation, often manifesting clinically as white spot lesions and secondary caries (Bisht et al., 2022; Sardana et al., 2023). This phenomenon is well recognised in orthodontic patients, where plaque retention around bracket bases and archwire interfaces creates sheltered niches that favour the persistence of *S. mutans* and sustained acid production (Jing et al., 2019).

Similarly, restorative materials, such as composite resins and glass ionomer cement, may exhibit surface irregularities that enhance bacterial retention. The capacity of *S. mutans* to adhere to these surfaces and remain metabolically active within surface-associated biofilms supports prolonged acid exposure in regions that are less accessible to routine oral hygiene measures, contributing to the development of secondary caries (Askar et al., 2021).

On prosthetic and implant-related surfaces, biofilms are typically polymicrobial. However, the presence of *S. mutans* during early colonisation may contribute to biofilm establishment through its strong surface adherence and extracellular polysaccharide production, particularly under cariogenic conditions such as frequent sugar exposure. Denture base materials and implant abutments can serve as initial attachment sites, where *S. mutans* promotes microbial retention and matrix development, thereby facilitating biofilm maturation on artificial substrates. Beyond their role in biofilm formation, oral microbial communities have been implicated in the deterioration of metallic biomaterials through microbiologically influenced corrosion, a process that leads to surface degradation, compromised mechanical integrity, and reduced service life of dental devices (Xu et al., 2024). Notably, a substantial proportion of implant failures has been associated with corrosion-related mechanisms. In addition, corrosion processes may release metal ions (Gopalakrishnan et al., 2021), which can elicit adverse biological responses, including local tissue inflammation, infection, and hypersensitivity reactions (Biguetti et al., 2021; Di Spirito et al., 2022). Collectively, these findings indicate that adhesion-mediated biofilm activity involving *S. mutans* is

not confined to natural tooth surfaces but may also have broader implications for the stability and biocompatibility of dental biomaterials within the oral environment.

2.5 Rationale for Selection of Bacterial Species

The selection of bacterial species for experimental biofilm studies is a critical determinant of biological relevance and interpretability. Dental plaque is inherently polymicrobial, and disease outcomes arise from interactions among multiple organisms rather than from the activity of a single species (Marsh & Zaura, 2017). Consequently, experimental models that incorporate both cariogenic and health-associated bacteria provide a more realistic representation of oral biofilm behaviour than single-species systems (Arthur et al., 2013; Salli & Ouwehand, 2015; Heersema & Smyth, 2019).

In the present study, *S. mutans* and *S. gordonii* were selected to represent two functionally distinct yet ecologically connected members of the oral microbiota. Their inclusion reflects not only their contrasting roles in health and disease but also the interspecies interactions that shape biofilm development, virulence expression, and cariogenic potential. This pairing provides a biologically relevant platform for evaluating selective antibiofilm strategies that aim to attenuate pathogenic traits while preserving commensal function.

2.5.1 *S. mutans*: A Cariogenic Keystone Pathogen

S. mutans is widely regarded as a cariogenic keystone pathogen due to its ability to exert a disproportionate influence on biofilm pathogenicity relative to its abundance within the microbial community (Takahashi & Nyvad, 2011; Lemos et al., 2019). Its cariogenic potential is primarily attributed to three interrelated virulence traits, which include its ability to synthesise extracellular polysaccharides (EPS), rapidly ferment carbohydrates into organic acids, and survive under prolonged acidic conditions (Lemos et al., 2019). Although it is not always numerically dominant in dental plaque, *S. mutans* plays a critical role in driving ecological changes that favour cariogenic biofilm development.

EPS synthesis is a defining feature of *S. mutans* virulence. Through sucrose-dependent metabolism mediated by glucosyltransferase enzymes, *S. mutans* synthesises large quantities of extracellular glucan that form the structural backbone of cariogenic

biofilms. These glucans enhance bacterial adhesion to the tooth surface, promote cell-cell aggregation, and contribute to the formation of a dense extracellular matrix that retains organic acids within the biofilm microenvironment (Koo et al., 2010; Bowen & Koo, 2011; Lemos et al., 2019).

In the present study, EPS-associated biofilm biomass and structural integrity are evaluated using CV biofilm assays and scanning electron microscopy, providing complementary quantitative and morphological assessment of biofilm architecture. Closely linked to EPS production is *S. mutans*'s pronounced acidogenicity. The organism rapidly ferments dietary carbohydrates, particularly sucrose, into organic acids, predominantly lactic acid. Sustained acid production results in repeated and prolonged reductions in local pH at the tooth surface, creating an acidic microenvironment that favours acidogenic and acid-tolerant species while promoting mineral demineralisation (Guo et al., 2013; Ikäläinen et al., 2024). In this study, acidogenic potential is assessed using a glycolytic pH-drop assay, which reflects the functional metabolic activity of *S. mutans* under cariogenic conditions.

Equally important is the organism's aciduricity, which enables *S. mutans* to survive and remain metabolically active under acidic conditions that inhibit or eliminate less acid-tolerant species. Adaptive responses such as proton extrusion, membrane modifications, and stress-response pathways allow the bacterium to maintain intracellular pH homeostasis and metabolic activity under acidic conditions. Together these properties enable *S. mutans* to remodel the biofilm microenvironment in ways that promote ecological dysbiosis and drive caries progression.

2.5.2 Virulence Determinants of *S. mutans* and Their Experimental Assessment

The cariogenicity of *S. mutans* arises from the coordinated expression of multiple virulence determinants that operate at different stages of biofilm development rather than from a single pathogenic trait. Adhesion represents a fundamental virulence property, enabling initial attachment to the salivary pellicle and resistance to mechanical removal (Bos et al., 1999; Busscher & van der Mei, 2012). In the present study, adhesion-related behaviour is indirectly evaluated by assessing CSH, which reflects physicochemical interactions governing early, non-specific attachment.

EPS synthesis further amplifies adhesion and biofilm stability. Through the action of glucosyltransferase enzymes, *S. mutans* converts sucrose into water-soluble

and insoluble glucans that strengthen biofilm cohesion and facilitate microcolony formation. EPS-related biofilm formation and persistence are assessed using CV assays to determine minimum biofilm inhibitory and eradication concentrations, complemented by scanning electron microscopy to visualise structural disruption.

Acid stress response mechanisms allow *S. mutans* to tolerate and adapt to low-pH environments. These include proton extrusion systems, alterations in membrane composition, and induction of stress-response pathways that preserve cellular function under acidic conditions. The functional consequences of these adaptations are reflected in sustained acid production, as assessed via glycolytic pH-drop analysis in this study.

By evaluating these virulence-associated traits using complementary phenotypic assays, the present study adopts a functional approach to cariogenicity that extends beyond growth inhibition and provides mechanistic insight into selective virulence attenuation. However, phenotypic alterations in biofilm behaviour alone cannot fully explain the molecular mechanisms responsible for these effects. Consequently, transcriptional analysis of key virulence-associated genes is necessary to determine whether the observed changes in adhesion, EPS synthesis, and acidogenicity are associated with modulation of gene expression.

2.5.3 Biofilm-Associated Gene Regulation in *S. mutans*

In biofilm-associated cells, virulence in *S. mutans* is regulated at the transcriptional level in response to environmental cues, including carbohydrate availability, pH fluctuations, and population density. Gene expression patterns in biofilms differ markedly from those observed in planktonic cultures, reflecting the unique physiological state of sessile bacteria (Shemesh et al., 2007).

Following biofilm establishment, genes involved in adhesion, extracellular polysaccharide synthesis, acid tolerance, and stress adaptation are frequently upregulated, reflecting the physiological adaptations required for bacterial survival within structured biofilm communities (Bowen et al., 2018; Koo et al., 2017). Key examples include glucosyltransferases (*gtfB*, *gtfC*, *gtfD*), glucan-binding proteins (*gcbB*, *gcbD*), and regulatory systems such as *vicRK* and *liaSR*, which collectively contribute to biofilm stability and environmental adaptation (Fitri et al., 2025). Importantly, modulation of gene expression does not necessarily correlate with immediate changes in bacterial viability, highlighting the distinction between virulence

attenuation and bacterial killing. This distinction underscores the importance of transcriptional analysis in identifying antivirulence effects that may not be captured by conventional antimicrobial assays. In the present study, quantitative gene-expression analysis is employed to elucidate the molecular mechanisms underlying the observed phenotypic changes in biofilm behaviour. Specifically, transcriptional responses of key virulence-associated genes involved in adhesion, extracellular polysaccharide synthesis, and stress adaptation are examined to determine whether the tested compounds modulate regulatory pathways that contribute to cariogenic biofilm development. Understanding how these virulence-associated genes are regulated within biofilms is therefore essential for identifying strategies that selectively disrupt cariogenic biofilm development. Accordingly, this study integrates phenotypic and transcriptional analyses to determine whether the tested compounds modulate key virulence pathways in *S. mutans*.

2.5.4 *S. gordonii* as a Health-Associated Commensal

In contrast to the highly cariogenic phenotype of *S. mutans*, *S. gordonii* is widely regarded as a health-associated commensal and a dominant early coloniser of the tooth surface. As a member of the mitis group streptococci, *S. gordonii* plays a critical role in the initial stages of biofilm formation by adhering to the acquired salivary pellicle and contributing to the establishment of a relatively stable and health-compatible microbial community. Its early presence can influence subsequent microbial succession by shaping local environmental conditions and interspecies interactions.

Beyond surface colonisation, *S. gordonii* actively contributes to the ecological balance within oral biofilms through metabolic and antagonistic mechanisms. It produces hydrogen peroxide, which can inhibit the growth and competitiveness of acidogenic species, including *S. mutans* (Barnard & Stinson, 1999; Zheng et al., 2011; Redanz et al., 2018). In addition, arginine metabolism by *S. gordonii* produces ammonia, which buffers local pH and counteracts acidification within the biofilm (Del Rey et al., 2025). These activities support a biofilm environment that is less conducive to enamel demineralisation and caries progression.

The relationship between *S. gordonii* and *S. mutans* within oral biofilms is therefore best described as competitive yet context-dependent. Under non-cariogenic conditions, particularly in the absence of frequent exposure to fermentable

carbohydrates, *S. gordonii* can restrict the growth and dominance of *S. mutans* by competing for adhesion sites and nutrients, producing hydrogen peroxide, and contributing to environmental pH buffering (Kreth et al., 2008; Rostami et al., 2022). These activities collectively limit acid accumulation and reduce the ecological advantage of acidogenic species during early biofilm development. However, this antagonistic balance is not fixed. In the presence of frequent sugar exposure, sustained acidification of the local environment selectively favours the aciduric and acidogenic traits of *S. mutans*, enabling it to outcompete or suppress *S. gordonii* within mixed-species biofilms (Liu et al., 2011). Transcriptomic and ecological studies demonstrate that under such conditions, *S. mutans* adapts its carbohydrate metabolism and stress-response pathways to persist despite competitive pressure, while the inhibitory activities of *S. gordonii*, including hydrogen peroxide production and extracellular DNase-mediated interference, are diminished at low pH (Liu et al., 2011; Rostami et al., 2022). As a result, cariogenic biofilms are characterised not by the complete exclusion of commensal species, but by a functional shift in which the protective influence of *S. gordonii* is overridden by the metabolic dominance of *S. mutans*. Importantly, *S. gordonii* does not disappear entirely in cariogenic biofilms. Both species can coexist within mixed-species biofilms, particularly during early or transitional stages of dysbiosis (Kreth et al., 2008). In such settings, *S. gordonii* may persist at lower abundance but with diminished functional influence, as the acidic environment compromises its metabolic activity and competitive capacity. Thus, cariogenic biofilms are not defined by the complete absence of commensal species, but rather by a functional imbalance in which the protective activities of organisms such as *S. gordonii* are overwhelmed by the virulence of *S. mutans*. Although several oral streptococci such as *Streptococcus mitis* and *Streptococcus sanguinis* also contribute to oral microbial homeostasis, *S. gordonii* was selected in this study because its ecological and molecular interactions with *S. mutans* are among the most extensively characterised in oral microbiology. Numerous studies have mapped the competitive and regulatory mechanisms operating between these species, including hydrogen peroxide-mediated antagonism, competition for adhesion sites, and modulation of extracellular polysaccharide-dependent biofilm architecture (Kreth et al., 2008 ; Rostami et al., 2022). Consequently, the *S. mutans*–*S. gordonii* pairing has been widely used as a simplified yet biologically relevant model for investigating ecological shifts in cariogenic biofilms. The availability of well-defined molecular pathways and

established dual-species biofilm models therefore allows clearer mechanistic interpretation of how candidate antibiofilm compounds influence interspecies interactions and cariogenic virulence.

Given this dynamic interplay, contemporary ecological models of caries emphasise the importance of preserving or restoring commensal species rather than indiscriminately eliminating oral bacteria. Maintenance of *S. gordonii* and similar health-associated streptococci is considered beneficial for sustaining biofilm buffering capacity and resisting cariogenic shifts. Consequently, selective antibiofilm strategies that suppress *S. mutans* virulence while sparing or supporting *S. gordonii* align with modern, ecology-based approaches to caries management.

2.6 Limitations of Conventional Antimicrobials

Conventional chemical plaque-control agents used in dentistry, particularly non-specific oral antiseptics and biocidal mouthrinses, are commonly broad-spectrum in activity and are often intended to reduce the overall microbial burden rather than selectively target specific pathogenic mechanisms. This limitation should be distinguished from narrow-spectrum or targeted antimicrobial therapies, which are designed to act against particular organisms, pathways, or virulence mechanisms. In the context of routine oral biofilm control, however, commonly used agents such as chlorhexidine (CHX) act largely as non-selective antimicrobials and do not specifically discriminate between pathogenic and health-associated members of the oral microbiota. Although such agents can reduce overall bacterial load, their activity is generally non-selective and may disrupt ecological balance within the oral biofilm, thereby contributing to unintended consequences.

Chlorhexidine (CHX) is widely regarded as the gold standard chemical antiseptic in oral care and is commonly used as a positive control in antibiofilm studies. Its antimicrobial activity is attributed to disruption of bacterial cell membranes and precipitation of intracellular components, leading to rapid cell death (Poppolo Deus & Ouanounou, 2022). In clinical settings, CHX has demonstrated effectiveness in reducing plaque accumulation and gingival inflammation when used as an adjunct to mechanical cleaning (James et al., 2017; Singaraju et al., 2024)

In addition to its bactericidal effects, CHX exhibits high substantivity within the oral cavity. Owing to its cationic nature, CHX readily binds to the negatively charged

surfaces of teeth, oral mucosa, and salivary glycoproteins, thereby enabling prolonged retention at the site of application. This strong surface adherence allows CHX to interfere with bacterial adhesion and colonisation and contributes to sustained antimicrobial activity for up to approximately 12 hours following use (Poppolo Deus & Ouanounou, 2022).

Despite these benefits, several limitations of CHX have been recognised. Its activity is non-selective, affecting both commensal and pathogenic bacteria. This is particularly relevant in oral biofilms, where health is associated with a balanced and functionally stable microbial community rather than a sterile surface (Marsh, 2006; Nyvad et al., 2020). Repeated exposure to broad-spectrum antiseptics may therefore alter community composition and reduce microbial diversity, potentially disturbing ecological homeostasis (Brookes et al., 2021). Prolonged or repeated use has been associated with adverse effects, including tooth staining, taste alterations, and mucosal irritation, which limit patient compliance (Poppolo Deus & Ouanounou, 2022). In addition, CHX does not specifically target the virulence mechanisms that drive cariogenic biofilm behaviour and may fail to provide sustained ecological control once treatment is discontinued. Suppression of the overall microbial population without addressing the underlying environmental drivers of dysbiosis may allow rapid recolonisation by acidogenic and acid-tolerant species. This highlights the fundamental limitations of antimicrobial strategies that rely primarily on bacterial killing rather than functional modulation of the biofilm.

Consequently, there is growing recognition that broad-spectrum oral antiseptics are most appropriate when used as preventive or adjunctive therapies rather than as stand-alone long-term interventions. In this context, the goal is not complete microbial eradication, but reduction of pathogenic pressure while minimising adverse effects associated with prolonged broad-spectrum antimicrobial use. Such approaches aim to complement mechanical plaque control and host-mediated defences, thereby reducing reliance on repeated bactericidal exposure.

In response to these limitations, there has been a conceptual shift toward ecology-based strategies for controlling oral disease. These approaches are grounded in the recognition that dental caries and other biofilm-associated oral diseases arise from imbalances in microbial activity rather than from the presence of specific pathogens alone. Ecology-based interventions aim to restore or maintain a biofilm environment compatible with health by targeting key pathogenic behaviours. Rather than

indiscriminately eliminating bacteria, these approaches seek to attenuate virulence traits, including adhesion, extracellular matrix production, acid production, and stress tolerance (Marsh, 2003). By reducing the expression or activity of these traits, the pathogenic potential of the biofilm can be diminished without disrupting beneficial microbial populations.

The rationale for antivirulence strategies is rooted in the observation that killing bacteria does not necessarily translate into long-term disease control. In contrast to non-specific biocidal approaches, targeted or narrow-spectrum strategies aim to reduce pathogenic function while limiting collateral effects on the resident microbiota (Alm et al., 2020). This distinction is important because the objective of ecological biofilm management is not simply to eliminate microorganisms, but to suppress disease-associated activities while preserving health-associated community functions. Broad-spectrum antimicrobial pressure may create ecological voids that favour recolonisation by organisms equally or more pathogenic, perpetuating cycles of dysbiosis. In contrast, modulating virulence reduces selective pressure for resistance, allowing commensal species to compete more effectively within the biofilm community. Such approaches are particularly relevant in the oral cavity, where complete microbial eradication is neither achievable nor desirable. The preservation of early colonisers and health-associated species supports biofilm resilience and functional stability, both of which are essential for maintaining oral homeostasis. This conceptual distinction between non-selective bactericidal antiseptics, targeted antimicrobial therapy, and antivirulence modulation provides the foundation for evaluating antibiofilm agents that inhibit biofilm formation or weaken established biofilms through targeted modulation rather than indiscriminate killing.

2.7 Antibiofilm Strategies in Oral Disease Control

Antibiofilm activity does not arise from a single mechanism but reflects the combined effects of multiple biological and physicochemical processes acting at different stages of biofilm development. Unlike planktonic inhibition, which often correlates directly with growth suppression, antibiofilm and antivirulence effects frequently involve modulation of bacterial behaviour, surface interactions, and community organisation.

Understanding these mechanisms is essential for interpreting experimental outcomes, particularly when reductions in biofilm biomass or virulence are not accompanied by complete bacterial elimination. Such effects are characteristic of virulence attenuation rather than bactericidal action and have important implications for sustainable biofilm control. The principal antibiofilm and antivirulence mechanisms relevant to oral biofilms are summarised in Table 2.1 and illustrated schematically in Figure 2.2. Figure 2.2 integrates current understanding of antibiofilm and antivirulence mechanisms, providing a conceptual framework for interpreting the experimental findings in subsequent subchapters.

2.7.1 Modulation of Cell Surface Properties

The bacterial cell surface represents the primary interface between microorganisms and their environment. Alterations in surface charge (zeta potential), hydrophobicity, and macromolecular composition can significantly influence bacterial adhesion, aggregation, and susceptibility to environmental stress. Agents that modulate cell-surface properties weaken early attachment forces, reducing bacteria's ability to establish stable contact with the salivary pellicle or neighbouring cells. As a result, interference at the bacterial surface can disrupt biofilm initiation and limit subsequent biofilm maturation without necessarily compromising bacterial viability.

Consistent with this mechanistic framework, numerous previous studies have included assessment of cell surface hydrophobicity (CSH) as a key experimental parameter when evaluating the antibiofilm or antivirulence effects of compounds and extracts (Shafiei et al., 2020; Karnjana et al., 2023; Chonsut et al., 2024). Measurement of CSH is therefore widely accepted as a relevant method for probing early adhesion-related mechanisms, rather than merely assessing bactericidal activity.

2.7.2 Inhibition of Adhesion and Co-aggregation

Stable adhesion to surfaces and co-aggregation with other microorganisms are essential for biofilm establishment and expansion. In oral biofilms, early colonisers attach to the pellicle-coated tooth surface and subsequently provide binding sites for secondary colonisers, leading to increasingly complex microbial communities (Marsh & Zaura, 2017; Sterzenbach et al., 2020).

Inhibition of bacterial adhesion may occur through several mechanisms, including interference with bacterial adhesins, modification of pellicle–bacteria interactions, or disruption of interbacterial binding processes (Kreve & Reis, 2021; Li et al., 2023). In addition, inhibition of coaggregation restricts bacteria's ability to organise into densely packed multicellular communities, thereby reducing biofilm cohesion and structural integrity (Peterson et al., 2015).

Blocking adhesion has been achieved through strategies targeting bacterial adhesins, such as fimbriae, surface-associated proteins, and members of the antigen I/II (Li et al., 2023). Alteration of pellicle or surface bacterial interactions, achieved by modifying surface energy, chemistry, or topography, including PEGylation, superhydrophilic or superhydrophobic surfaces, and nano-textured coatings, has also been shown to weaken or delay initial bacterial attachment (Song et al., 2015; Sterzenbach et al., 2020). Furthermore, disruption of interbacterial binding mechanisms, including coaggregation adhesin–receptor interactions and extracellular polymer- or protein-mediated bridging, has been shown to limit the formation of compact bacterial clusters and reduce biofilm cohesion, highlighting the central role of cell–cell adhesion dynamics in determining biofilm architecture (Quispe Haro et al., 2024). When adhesion and co-aggregation are impaired, biofilms tend to remain sparse, loosely organised, and more susceptible to mechanical and chemical challenges. Such effects are consistent with simplified biofilm structures and reduced viable bacterial burden, even in the absence of strong bactericidal activity.

2.7.3 Interference with Extracellular Polysaccharides Synthesis

Extracellular polysaccharides are major structural components of the biofilm matrix, often accounting for a large fraction of matrix mass and determining viscoelasticity and mechanical stability (Pandit et al., 2020; Lu et al., 2024). In cariogenic biofilms, *S. mutans*-derived glucans surround and hold bacterial microcolonies together, create compartmentalised acidic microenvironments at the tooth surface, and enhance tolerance to antimicrobials and environmental stress (Karygianni et al., 2020; Deng et al., 2021; Shi et al., 2025). The EPS matrix can impede the penetration of antimicrobial agents or sequester these compounds, contributing to drug tolerance and the protection of embedded cells (Karygianni et al., 2020; Lu et al., 2024).

Targeting extracellular polysaccharides synthesis enzymes (e.g., *S. mutans* glucosyltransferases) or regulatory system governing EPS production (vicK-regulated pathways) reduces EPS quantity or alters its structural properties, yielding thinner, sparser, and less cohesive biofilms with diminished cariogenicity and virulence, even when planktonic viability is relatively preserved (Xiao et al., 2012; Zhang et al., 2021). Small molecules such as asiatic acid, cannabidiol (a compound derived from *Cannabis Sativa*), 6-shogaol (a compound derived from ginger), and others selectively reduce EPS content and biofilm biomass while only modestly affecting planktonic growth, consistent with primary antivirulence rather than bactericidal action (Barak et al., 2022; Jung et al., 2025; Shi et al., 2025).

2.7.4 Disruption of Quorum Sensing

Quorum sensing (QS) is a cell-density-dependent communication system that allows bacteria to coordinate gene expression at the community level. In biofilms, QS regulates processes such as adhesion, EPS synthesis, virulence factor production, stress tolerance, and metabolic adaptation (Wright & Ramachandra, 2022; Nagi et al., 2023; Juszczuk-Kubiak, 2024). Quorum quenching (QQ) refers to strategies that interfere with QS, the bacterial cell–cell communication system that coordinates collective behaviours such as virulence expression, biofilm development, and stress adaptation. QQ can be achieved through degradation of signalling molecules, antagonism of QS receptors, or inhibition of signal synthesis, and primarily disrupts synchronised virulence behaviours rather than directly inhibiting bacterial growth (Grandclément et al., 2015; Khan et al., 2023; Juszczuk-Kubiak, 2024). Evidence indicates that QS inhibitors reduce biofilm biomass, thickness, and structural organisation while strongly suppressing QS-regulated virulence factors at sub-MIC concentrations, with minimal effects on planktonic growth. Interventions down-regulate key QS and virulence-associated gene networks, luxS/AI-2 signalling pathways, and genes involved in efflux and extracellular polymeric substance production, resulting in marked transcriptional shifts without substantial loss of bacterial viability (Grandclément et al., 2015; Basavaraju et al., 2016; Muras et al., 2018; Polizzi et al., 2022). By weakening coordinated stress responses and collective defence mechanisms, quorum quenching further enhances biofilm susceptibility to disinfectants, antibiotics, and host immune

clearance, and frequently acts synergistically with conventional antimicrobial agents (Cella et al., 2023; Patel et al., 2025).

2.7.5 Destabilisation of Biofilm Architecture

The three-dimensional architecture of a biofilm is essential for its protective function. Mature biofilms develop into a highly heterogeneous three-dimensional architecture characterised by microcolonies, water channels, and steep nutrient and oxygen gradients (Bridier & Briandet, 2022; Lobo-Cabrera et al., 2024). These microcolonies and channel networks function analogously to a circulatory system, facilitating nutrient distribution and waste removal while simultaneously protecting cells in deeper layers from antibiotics and host immune factors (Bridier & Briandet, 2022; Lobo-Cabrera et al., 2024; Serra & Hengge, 2014). The EPS matrix, composed primarily of polysaccharides, proteins, extracellular DNA, and lipids, provides structural cohesion and mechanical stability, enabling biofilms to withstand shear forces, neutrophil-mediated attack, and chemical stress (Yan & Bassler, 2019; Karygianni et al., 2020; Almatroudi, 2025). In addition, restricted diffusion and binding of antimicrobial agents within the EPS matrix reduce effective drug penetration, further diminishing antimicrobial efficacy in the inner biofilm layers.

In cariogenic plaque, *S. mutans*–derived glucan-rich EPS play a central role in organising biofilm architecture at the tooth surface. By forming diffusion-limiting microcolonies at the biofilm–enamel interface, this matrix creates localised acidic niches that are critical for enamel demineralisation and the development of dental caries (Xiao et al., 2012). Increasingly, the literature indicates that the EPS matrix itself constitutes a key virulence determinant in cariogenic biofilms, often exerting a greater influence on pathogenicity than the absolute abundance of *S. mutans* cells (Kim et al., 2015; Klein et al., 2015).

Accordingly, strategies aimed at destabilising the EPS matrix consistently induce pronounced changes in biofilm structure and function. Approaches that degrade glucans or interfere with their synthesis, including glucanohydrolases such as dextranase and mutanase, cranberry-extract, usnic acid, and inhibitors of bacterial signalling, have been shown to flatten or fragment *S. mutans* microcolonies, reduce EPS biovolume, weaken mechanical stability, and increase pH at the biofilm–enamel interface (Philip et al., 2019; Ren et al., 2019; Lin et al., 2022; Pandit et al., 2024). These

structural effects are further amplified when EPS-targeting approaches are combined with conventional antimicrobials, with dual targeting leading to physical collapse of *S. mutans* clusters and substantially enhanced antimicrobial efficacy compared with antimicrobials used alone (Ren et al., 2019).

Collectively, these findings indicate that disruption of the EPS scaffold undermines the protected acidic microenvironments that sustain cariogenic activity and exposes bacteria previously shielded within the biofilm. More broadly, oral biofilms rely on an EPS-rich three-dimensional framework to maintain cohesion, resist environmental and host-derived stresses, and support dysbiosis. When this framework is disrupted, biofilms become thinner, more permeable, and more susceptible to antimicrobial agents and host defences. In the context of cariogenic plaque, EPS-focused destabilisation therefore not only increases bacterial exposure but also directly attenuates cariogenic potential by disrupting the localised acidic conditions that drive tooth demineralisation, with architectural changes often preceding measurable reductions in viable *S. mutans*.

2.7.6 Biofilm Eradication and Dispersal Mechanisms

Eradication of established biofilms is substantially more challenging than inhibiting biofilm formation. Mature biofilms contain metabolically diverse bacterial populations, including slow-growing and stress-adapted cells that exhibit heightened tolerance to antimicrobial agents (Jiao et al., 2019; Rath et al., 2021). Biofilm eradication may involve partial killing of exposed cells, disruption of the extracellular matrix, and induction of biofilm dispersal processes. Dispersal can occur through degradation of matrix components or through active bacterial responses that promote detachment from the biofilm structure (Wille & Coenye, 2020; Wang et al., 2023; Bharathi et al., 2025). However, while dispersal can reduce biofilm biomass and structural integrity, it also carries the risk of releasing viable cells capable of recolonisation or dissemination. For this reason, effective eradication strategies increasingly emphasise combined approaches that couple matrix destabilisation with reduction of bacterial viability, rather than relying on bactericidal activity alone.

Within an oral-ecological framework, it is also important to recognise that complete biofilm dispersal is not always the desired outcome. For oral health maintenance, partial thinning or remodelling of biofilms achieved through mechanical

cleaning, sublethal antimicrobial exposure, selective rinses, or probiotic interventions may be preferable, as this allows a protective, low-pathogenic community to remain attached and continue contributing to immune priming and haemostatic balance (Marsh, 2017; Marsh & Zaura, 2017; Hernández et al., 2022). In contrast, in the context of overt infection, such as deep carious lesions or peri-implantitis, more aggressive strategies that combine biofilm destabilisation with bacterial killing become necessary. Even in these settings, however, such approaches must be carefully balanced to minimise the unintended release of virulent cells and disruption of the broader oral microbiome (Wang et al., 2020; Chen et al., 2022; Hawas et al., 2022; Lv et al., 2025)

Collectively, these observations illustrate that antibiofilm agents can exert biologically meaningful effects without complete microbial elimination. Modulation of surface properties, interference with adhesion and EPS synthesis, disruption of quorum sensing, and destabilisation of biofilm architecture act in concert to weaken biofilm integrity and pathogenic potential. This conceptual framework provides a basis for interpreting changes in biofilm structure, viable cell counts, and gene expression observed in experimental models.

Table 2.1
Key Antibiofilm and Antivirulence Mechanisms Relevant to Oral Biofilm Control and Their Experimental Assessment in The Present Study

Biofilm target	Role in cariogenic biofilms	Functional consequence of modulation	Assays used in this study
Cell surface hydrophobicity (CSH)	Governs early, non-specific bacterial attachment to pellicle-coated surfaces	Reduced initial adhesion and weaker surface association	Microbial adhesion to hydrocarbon (MATH) assay
Bacterial adhesion and co-aggregation	Enables irreversible attachment and microcolony formation	Impaired biofilm initiation and reduced biofilm density	CSH assay; dual-species biofilm model
Extracellular polysaccharide (EPS) synthesis	Provides structural integrity, cohesion, and acid retention within biofilms	Destabilised biofilm matrix and reduced structural resilience	CV biofilm assay (MBIC/MBEC); SEM
Acidogenicity	Drives local pH reduction and enamel demineralisation	Reduced cariogenic potential	Glycolytic pH-drop assay
Quorum sensing and virulence regulation	Coordinates biofilm-associated gene expression and community behaviour	Altered virulence gene expression without bacterial killing	RT-qPCR

Biofilm target	Role in cariogenic biofilms	Functional consequence of modulation	Assays used in this study
Biofilm architecture	Confers protection and tolerance to antimicrobials	Increased permeability and susceptibility	SEM; CFU enumeration
Biofilm eradication and dispersal	Determines persistence versus removal of established biofilms	Partial disruption and reduced viable burden	MBEC; CFU enumeration; SEM

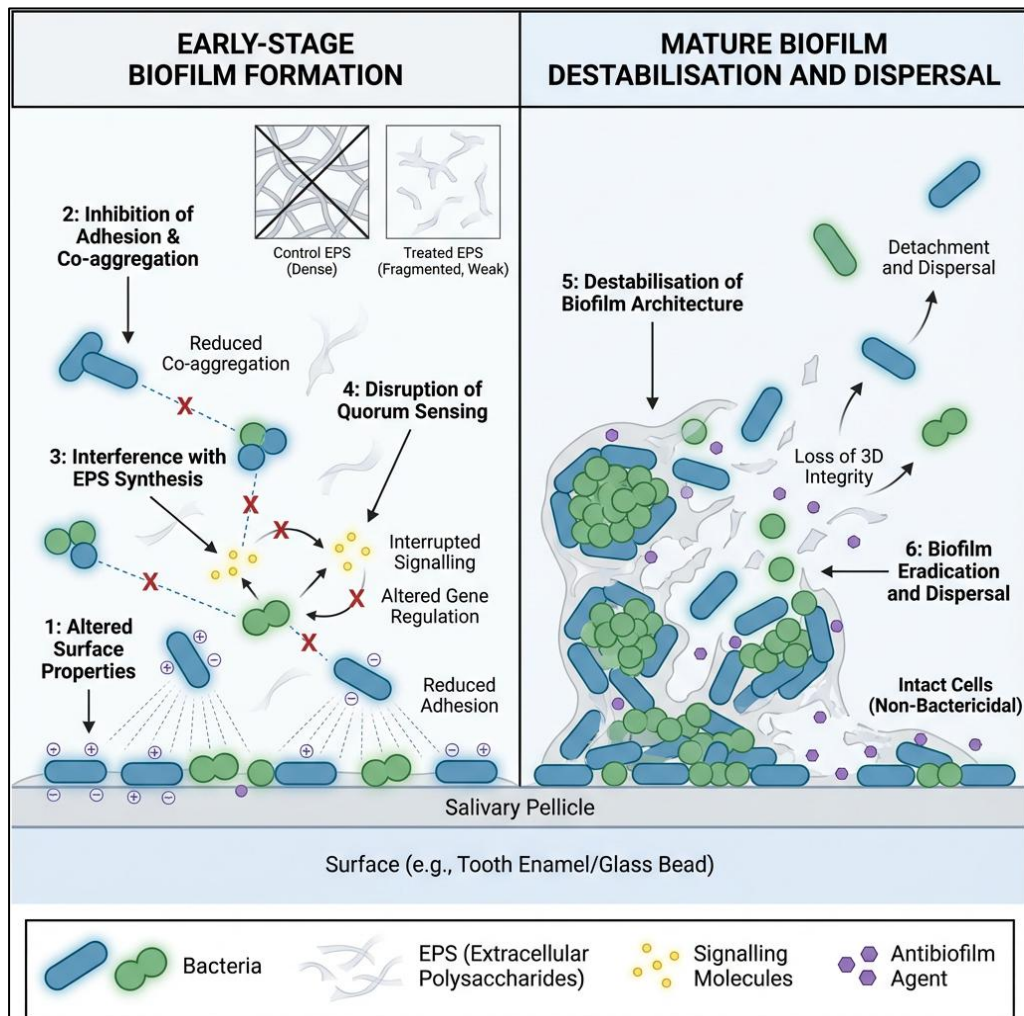


Figure 2.2 Conceptual Illustration of Antibiofilm and Antivirulence Mechanisms Targeting Oral Biofilms

The schematic depicts inhibition of early biofilm formation through interference with bacterial adhesion, co-aggregation, EPS synthesis, and quorum sensing, as well as destabilisation of mature biofilms through disruption of biofilm architecture and promotion of detachment and dispersion. The diagram represents biofilm development on a salivary pellicle covering a solid surface such as tooth enamel or glass bead. The illustration was developed by the author based on published literature (Jakubovics & Kolenbrander, 2010; Kashi et al., 2025)

2.8 Plant-Derived Compounds as Selective Antibiofilm Agents

Plant secondary metabolites, including polyphenols, flavonoids, essential oils, and alkaloids, are widely recognised as part of plant chemical defence systems and frequently influence microbial behaviour rather than exerting direct bactericidal effects (Milho et al., 2021; Silva et al., 2023; Mulat et al., 2025). Rather than eliminating microorganisms outright, many phytochemicals have been reported to interfere with processes central to biofilm development and pathogenicity, such as microbial adhesion, quorum sensing, EPS synthesis, acid production, and the activity of specific virulence-associated enzymes or transport mechanisms (Koo et al., 2017; Sateriale et al., 2020; Milho et al., 2021; Tzimas et al., 2024b; Mulat et al., 2025). Through these mechanisms, biofilm integrity and virulence expression may be attenuated while a proportion of the microbial community remains viable.

This emphasis on antivirulence activity has attracted increasing interest in ecology-based biofilm management. By targeting pathogenic traits rather than microbial survival, such approaches are thought to exert lower selective pressure for resistance compared with conventional bactericidal antibiotics, while also offering flexibility for combination with established antimicrobials or emerging delivery strategies, including nanocarrier-based systems (Bonincontro et al., 2023).

Within this framework, several plant-derived compounds have been selected in previous studies specifically for their antibiofilm properties rather than their antibacterial potency. Individual phytochemicals, including flavonoids, phenolic acids, and essential-oil constituents, have been shown to reduce biofilm biomass, suppress EPS production or acidogenicity, disrupt quorum-sensing pathways, or promote biofilm destabilisation at sublethal concentrations. Notably, compounds such as cyanidin, catechin, curcumin, quercetin, cinnamic acid and vanillic acid have demonstrated inhibitory effects on oral or oral-related biofilms without complete microbial eradication, supporting their potential role as antivirulence or biofilm-modulating agents (Li et al., 2018; Mashal et al., 2024; Rudin et al., 2025).

2.8.1 Biological Activities of Pomegranate-Derived Preparations in Oral Health

Among the medicinal plants investigated for oral applications, pomegranate (*Punica granatum*) has received considerable attention. Systematic reviews and experimental studies consistently report that pomegranate-derived preparations exhibit antimicrobial, antibiofilm, antioxidant, and anti-inflammatory activities against key oral pathogens and inflamed oral tissues (Celiksoy et al., 2020; Sateriale et al., 2020; Gallas et al., 2023; Potra Cicalău et al., 2024; Silla et al., 2025). Polyphenol-rich pomegranate extracts, particularly those containing ellagitannins such as punicalagin, demonstrate strong radical-scavenging capacity and enhance antioxidant responses in host tissues. *In vitro* studies using human gingival epithelial cells report upregulation of antioxidant defence mechanisms (e.g., superoxide dismutase 1) following exposure to inflammatory stimuli (Celiksoy et al., 2020; Syahputra et al., 2025). In parallel, pomegranate-derived preparations have been shown to reduce pro-inflammatory mediators, including tumour necrosis factor-alpha (TNF- α), interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1), and to reduce inflammatory cell infiltration in both *in vitro* and *in vivo* models of gingivitis and periodontitis, supporting their anti-inflammatory potential in the oral cavity (Celiksoy et al., 2020; Syahputra et al., 2025).

In parallel with these host-modulatory effects, pomegranate peel and fruit extracts demonstrate antimicrobial and antibiofilm activity against key oral pathogens. These include oral microorganisms, including *Candida albicans*, *Fusobacterium nucleatum*, *Enterococcus faecalis*, *Lactobacillus*, *S. mutans*, *Streptococcus oralis*, *Porphyromonas gingivalis* and *Rothia dentocariosa* to disrupt both developing and established dental plaque biofilms (Sateriale et al., 2020; Gallas et al., 2023; Potra Cicalău et al., 2024; Kumar et al., 2025).

At the microbiological level, studies employing crude pomegranate extracts, particularly peel-derived preparations, further report suppression of virulence-associated traits, including reduced biofilm biomass, diminished acidogenicity, and impaired extracellular polysaccharide synthesis. As summarised in Table 2.2, most investigations focus on crude extracts formulated as mouthwashes or topical agents and assess their effects predominantly against *S. mutans* in *in vitro* and clinical settings. Despite these promising findings, the existing literature is subject to several methodological and conceptual limitations. Many studies rely predominantly on growth-based endpoints, short-term exposure models, and single-species or saliva-

based systems, which provide limited insight into biofilm behaviour under ecologically relevant conditions. Few investigations clearly distinguish between biofilm inhibition and biofilm eradication, or examine transcriptional regulation of key virulence traits. Moreover, the impact of pomegranate-derived agents on health-associated commensal species remains largely unexplored.

A further limitation of extract-based studies is the variability of chemical composition and the lack of standardisation. The composition of crude pomegranate extracts varies markedly with cultivar, plant part, extraction solvent, extraction technology, temperature, pressure, and drying or processing conditions, leading to wide ranges in total phenolics, tannins, flavonoids, anthocyanins, and individual ellagitannins such as punicalagin (Mphahlele et al., 2016; Magangana et al., 2020; Campos et al., 2022; Hasikić et al., 2025). Such variability complicates reproducibility and makes it difficult to compare antimicrobial or antibiofilm outcomes across studies using differently prepared extracts.

Moreover, crude extracts contain complex mixtures of phenolics, flavonoids, tannins, organic acids, and volatiles, alongside inactive or even antagonistic components, which can mask or modulate the activity of specific molecules (Toledo-Merma et al., 2022; Salim et al., 2023). Although fractionation and bioassay-guided approaches have begun to identify key actives such as punicalagin as major contributors to the antimicrobial properties of pomegranate peel, the relative contribution of individual constituents to antibiofilm effects remains only partly resolved (Gosset-Errard et al., 2021; Salim et al., 2025).

These compositional and mechanistic uncertainties hinder the establishment of robust quality specifications, such as the identification of marker compounds and the definition of minimal phenolic or tannin content, thereby restricting the development of standardised, clinically translatable formulations with predictable efficacy and safety profiles (Magangana et al., 2020; Campos et al., 2022; Toledo-Merma et al., 2022; Hasikić et al., 2025). To address these issues, a complementary compound focused analysis was undertaken (Table 2.3) to systematically identify major and minor compounds present in pomegranate peel. This analysis informed the rational selection of compounds for the present study and provided a foundation for compound-specific investigation beyond extract-based approaches.

Table 2.2

Summary of Studies Investigating Pomegranate-Derived Preparations for Oral Antimicrobial and Antibiofilm Activity

Author (Year)	Study Type	Part	Preparation Type	Extraction Method	Target Microorganisms	Biofilm / Assay Model	Virulence / Functional Endpoints	Key Outcomes
Gulube & Patel, (2016)	<i>In vitro</i>	Peel	Crude extract	Aqueous	<i>S. mutans</i>	Biofilm inhibition	Acid production, EPS	reduced biofilm, reduced acidogenicity
Ferrazzano et al., (2017)	<i>In vitro</i>	Peel, juice	Crude extract	Hydroalcoholic	<i>S. mutans</i>	MIC/MBC	None	Growth inhibition
(Abd-El-Aziz & A Sallam, 2020)	<i>In vitro</i>	Peel	Crude extract	Ethanol / water	<i>S. mutans</i>	Adhesion assay	Adhesion	Reduced adhesion of <i>S. mutans</i>
(Fernandes et al., 2022)	<i>In vitro</i> / <i>ex vivo</i>	Peel	Extract formulation	Methanol	<i>S. mutans</i> , <i>C. albicans</i>	Dual-species biofilm	Biomass	Reduced biofilm, reduced demineralisation
(Mahd et al., 2023)	<i>In vitro</i> / clinical	Whole fruit	Crude extract	Hydroalcoholic	<i>S. mutans</i>	Disc diffusion, plaque index	None	Reduced plaque score
(Pinni et al., 2018)	<i>In vitro</i> + clinical	Peel	Mouthwash	Aqueous	<i>S. mutans</i>	Salivary CFU	None	Comparable to CHX
(Singla et al., 2018)	Clinical	Seed	Mouthwash	Aqueous (Soxhlet)	Oral streptococci	Salivary CFU	None	decreased CFU over time
(Umar et al., 2016)	Clinical	Peel	Mouthwash	Aqueous	<i>S. mutans</i>	Salivary CFU, pH	Acidogenicity (indirect)	decreased CFU, increased pH
(Jacob et al., 2021)	Clinical	Peel	Mouthwash	Aqueous	Mixed oral flora	Saliva qPCR	None	Reduced bacterial load
(Kadam et al., 2019)	Clinical	Peel	Mouthwash	Aqueous	<i>S. mutans</i>	Saliva enzymatic assays	Acidogenic enzymes	increased pH, decreased enzyme activity

Table 2.3

Summary of Geographic Origin, Extraction Approach, and Identified Major and Minor Compounds in Pomegranate Peel Extracts Across Selected Studies

Geographic origin	Identified compounds (Major + Minor)	Types of extract	References
India (Bhagwa cultivar)	punicalagin (α , β), punicalin, corilagin, ellagic acid, gallic acid, granatin a/b, catechin, quercetin	Ethanollic peel extract	Foujdar et al. (2022)
South Africa	ellagic acid, gallic acid, catechin epicatechin, rutin, anthocyanins	methanollic peel extract	(Fawole et al., 2012)
China (multiple cultivars)	punicalagin (α , β), punicalin, corilagin, granatin a/b, kaempferol-3-O-glucoside, pedunculagin, ellagic acid, ellagic acid glycosides, gallic acid, galloyl glucoses, catechin, gallo catechin, epicatechin, quercetin, rutin	Methanollic extract	(Man et al., 2022)
China	punicalagin, ellagic acid, ellagic acid glycosides, gallic acid, catechin, rutin	Methanollic extract	(Li et al., 2015)
China	punicalagin, ellagic acid, gallic acid, catechin, quercetin, rutin, minor phenolic acids	Mechanochemical-assisted extraction	(Bao et al., 2026)
Pakistan	punicalagin, punicalin, corilagin, granatin a/b, pedunculagin, ellagic acid, ellagic acid derivatives, gallic acid, galloyl glucoses, catechin, epicatechin, quercetin, rutin, trace anthocyanins	Hydroalcoholic extract	(Badshah et al., 2025)
India	punicalagin, ellagic acid, gallic acid, catechin, quercetin, punicalcortein, anthocyanins, granatin, threonic acid	Aqueous	(More & Arya, 2025)

Taken together, these considerations underscore the need for studies that move beyond crude extract-based approaches toward chemically defined phytochemicals, ecologically relevant biofilm models, and mechanistic evaluation of virulence modulation. The present study was therefore designed to address these gaps using purified PDCs, a dual-species biofilm system, and integrated phenotypic and gene-expression analyses.

2.8.2 Rationale for Studying PDCs

Pomegranate contains a diverse range of polyphenolic constituents that have been associated with antimicrobial and antibiofilm activity. These include ellagitannins such as punicalagin, punicalin, and corilagin; phenolic acids such as ellagic acid; and

flavonoids and flavanols, including rutin, gallic acid, and kaempferol-7-O-glucoside. Structural variation among these compounds, including differences in molecular weight, hydroxyl group density, and glycosylation patterns, may influence their solubility, protein-binding affinity, and interaction with bacterial cell surfaces or extracellular matrix components (Ivanov et al., 2022; Suručić et al., 2025). Polyphenols have been reported to interfere with adhesion, extracellular polysaccharide synthesis, membrane integrity, and quorum-sensing pathways in various microbial systems (Yang et al., 2021; Ivanov et al., 2022), suggesting potential relevance to biofilm-targeted modulation.

Given the reported antibiofilm activity of pomegranate extracts, further investigation at the compound level is warranted. Evaluation of purified compounds enables more precise attribution of biological effects than extract-based studies, particularly when the objective is to elucidate how specific molecules influence bacterial behaviour within structured biofilms. The use of purified compounds allows direct association between specific molecules and measurable outcomes such as adhesion potential, extracellular matrix formation, acidogenicity, and virulence gene expression (Salim et al., 2025; Suručić et al., 2025). In contrast, crude extracts contain multiple constituents that may exert overlapping, synergistic, or antagonistic effects, potentially obscuring compound-specific mechanisms (Caesar & Cech, 2019; Vaou et al., 2022).

Published studies on pomegranate in dentistry have predominantly evaluated crude extracts, peel extracts, juice preparations, or mouthrinse formulations, with outcomes commonly focused on growth inhibition, plaque reduction, or reduction of cariogenic bacterial counts, particularly against *S. mutans* (Ferrazzano et al., 2017; Mahd et al., 2023; Rafeie et al., 2025; Umar et al., 2016). While these studies support the general anticariogenic potential of pomegranate-derived preparations, they provide limited information on which individual compounds are responsible for the observed effects, whether these compounds act selectively within mixed-species biofilms, and whether their antibiofilm activity reflects bacterial killing, inhibition of adhesion, disruption of extracellular matrix-associated biomass, or modulation of virulence expression. Therefore, the present study differs from many previous extract-based investigations by evaluating purified pomegranate-derived compounds individually rather than testing pomegranate preparations as chemically complex mixtures.

This study also differs from earlier work by focusing on selective antibiofilm and antivirulence activity within an oral dual-species biofilm model rather than assessing antimicrobial activity against a single organism alone. The inclusion of both *S. mutans* and *S. gordonii* allowed assessment of whether the compounds could suppress a cariogenic species while preserving a health-associated early coloniser. This distinction is important because an ideal anticaries strategy should not simply reduce total bacterial burden, but should shift biofilm behaviour away from cariogenicity while maintaining ecological compatibility with oral health. Accordingly, the present study integrated multiple endpoints, including planktonic antimicrobial activity, bacterial cell-surface hydrophobicity, glycolytic pH reduction, biofilm biomass, species-specific viable counts, SEM-based architecture, and virulence gene expression. This multi-endpoint approach enabled evaluation of PDC activity beyond simple growth inhibition and allowed a more mechanistic assessment of biofilm modulation.

Furthermore, the present study examined both biofilm inhibition and eradication/disruption phases. Early compound exposure was used to determine whether selected PDCs could prevent or reduce biofilm development, whereas treatment of established biofilms was used to assess whether these compounds could compromise mature biofilm integrity. This temporal distinction is important because compounds that inhibit early attachment or accumulation may not necessarily disrupt established biofilms. By comparing total biomass, viable bacterial recovery, structural organisation, and gene expression across these phases, the present study provides a more comprehensive evaluation of PDC effects on biofilm formation, stability, and biological function.

Importantly, compound selection in the present study was not restricted to the most abundant constituents of pomegranate peel. While major compounds such as punicalagin and ellagic acid are frequently emphasised in the literature, abundance alone does not necessarily predict biological relevance or antibiofilm potency (Rongai et al., 2019). Inclusion of both major and minor compounds, therefore, permits a broader and less assumption-driven evaluation of pomegranate-derived compound bioactivity.

From a translational perspective, purified plant compounds offer advantages in dose standardisation, reproducibility, safety profiling, and formulation development compared with heterogeneous extracts (Calixto, 2000; Nasim et al., 2022). Clinical and pharmacological studies of individual polyphenols such as curcumin, resveratrol, and silymarin demonstrate the feasibility of compound-specific approaches in translational

research (Huang et al., 2024). Collectively, these considerations support a compound-focused investigative strategy to elucidate selective antibiofilm and antivirulence mechanisms under controlled experimental conditions.

2.9 Knowledge Gaps and Justification for the Present Study

Despite substantial progress in understanding oral biofilm biology and the cariogenic process, several important knowledge gaps remain. These gaps limit the translation of current findings into effective, sustainable strategies for caries control and provide the rationale for the present investigation.

First, although many studies reported antibiofilm effects of plant-derived agents against oral microorganisms, the majority rely on crude extracts rather than chemically defined compounds (Umar et al., 2016; Singla et al., 2018; Jacob et al., 2021). While such studies provide valuable preliminary insights, variability in extract composition, extraction methods, and phytochemical content compromises reproducibility and meaningful comparison across studies. Consequently, the specific bioactive constituents responsible for observed antibiofilm or antivirulence effects often remain unidentified, limiting mechanistic interpretation and translational relevance.

Second, a critical gap remains regarding effective dosing and concentration in studies of pomegranate-based oral formulations. Most clinical trials evaluate a single, empirically selected concentration without dose–response comparison, and employ heterogeneous units of measurement (e.g. % w/v, mg/mL), and extraction methods (Nóbrega et al., 2015; Umar et al., 2016; Singla et al., 2018; Abd-El-Aziz & A Sallam, 2020; Jacob et al., 2021; Fernandes et al., 2022; Mahd et al., 2023). Consequently, the minimal effective concentration required to achieve sustained antibiofilm or anticariogenic effects remains undefined. Furthermore, crude extract concentrations are rarely standardised to quantified bioactive constituents, preventing direct linkage between clinical efficacy and specific phytochemicals. This limitation restricts reproducibility, mechanistic interpretation, and translational relevance of existing clinical evidence.

Third, many antibiofilm studies continue to employ single-species biofilm models, typically focusing on a single cariogenic organism. While experimentally convenient, such models do not reflect the ecological complexity of dental plaque, where disease emerges from interactions among multiple microbial species. The

absence of commensal bacteria in these systems limits interpretation of whether observed effects represent selective modulation of pathogenic traits or non-selective antimicrobial activity.

Fourth, relatively few studies distinguish clearly between biofilm inhibition and biofilm eradication. Early-stage biofilms and mature biofilms differ fundamentally in structure, physiology, and antimicrobial tolerance. Failure to differentiate these stages can lead to overestimation of therapeutic potential, particularly when agents effective against developing biofilms are assumed to be equally effective against established communities.

Fifth, although phenotypic outcomes, such as reduced biofilm biomass or viable cell counts, are frequently reported, mechanistic insight at the gene-expression level remains limited, particularly in multispecies biofilms. Virulence attenuation may occur through transcriptional modulation without immediate effects on bacterial viability, and such changes cannot be inferred from growth-based assays alone (Li et al., 2025)

Finally, the impact of antibiofilm agents on health-associated commensal species is often overlooked. Many studies focus exclusively on pathogenic organisms, with little consideration of whether treatment disrupts beneficial early colonisers that contribute to ecological stability. Preservation of commensal species is increasingly recognised as a key requirement for ecology-based disease control, yet experimental evidence addressing this balance remains scarce (Hoare et al., 2017)

Collectively, these gaps highlight the need for studies that integrate purified phytochemicals, ecologically relevant multispecies biofilm models, and mechanistic analyses to comprehensively evaluate selective antibiofilm and antivirulence activity. Although pomegranate-derived preparations have demonstrated promising antimicrobial and antibiofilm effects against oral pathogens, most existing studies rely on crude extracts, which vary substantially in chemical composition and provide limited mechanistic insight. Consequently, the specific contributions of individual phytochemicals to antibiofilm activity remain insufficiently defined.

To address these limitations, the present study investigates purified pomegranate-derived compounds using a dual-species biofilm model consisting of *S. mutans* and *S. gordonii*. By integrating phenotypic biofilm assays with gene expression analysis, this study aims to evaluate how selected compounds influence biofilm formation and virulence-related traits within an ecologically relevant oral biofilm system.

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Materials

3.1.1 Pomegranate-Derived Compounds (PDCs)

Corilagin (COR) - CAS 23094-69-1

Ellagic acid (EA) - CAS 476-66-4

Gallocatechin (GC) - CAS 3371-27-5

Kaempferol-glucoside (KG) - 16290-07-6

Punicalagin (PUG) - CAS 65995-63-3

Punicalin (PUN) - CAS 65995-64-4

Rutin (RUT) - CAS 153-18-4

3.1.2 Bacterial Strains

Streptococcus mutans (ATCC 700610)

Streptococcus gordonii (ATCC 51656)

3.1.3 Culture Media

Brain Heart Infusion (BHI) agar - Thermo Fisher (USA)

Brain Heart Infusion (BHI) broth - Thermo Fisher (USA)

Mitis-Salivarius Agar (MSA) - Sigma-Aldrich (Germany)

3.1.4 Chemical Reagents

Table 3.1
List of Chemical Reagents Used in This Study

No	Reagent	Description	Manufacturer
1.	Crystal violet (CV)	1%, aqueous solution	Sigma-Aldrich (Germany)
2.	Dimethylsulfoxide (DMSO)	ACS reagent, $\geq 99.9\%$	Sigma-Aldrich (Germany)
3.	Chlorhexidine digluconate solution	20% in H ₂ O	Sigma-Aldrich (Germany)
4.	Phosphate-buffered saline	Tablet, pH 7.2-7.6. Tablet/200 mL	Sigma-Aldrich (Germany)
5.	Acetic acid	Glacial, ACS reagent, $\geq 99.7\%$. 30 mL of glacial acetic acid was added to deionised water to make up to 100 mL, resulting in a 30% (v/v) acetic acid solution.	Sigma-Aldrich (Germany)
6.	Glycerol	ACS reagent, $\geq 99.5\%$	Sigma-Aldrich (Germany)
7.	n-hexadecane	analytical standard	Sigma-Aldrich (Germany)
8.	Osmium tetroxide	ACS reagent, $\geq 98.0\%$	Sigma-Aldrich (Germany)
9.	Glutaraldehyde solution	Grade I, 25% in H ₂ O, specially purified for use as an electron microscopy fixative	Sigma-Aldrich (Germany)
10.	TE buffer, 1x, molecular biology grade	Buffer composed of 10mM Tris-HCl containing 1mM EDTA•Na ₂	Promega

3.1.5 Commercial Kits

Table 3.2
List of Commercial Kits Used in This Study

No	Kit	Description	Manufacturer
1.	RNeasy Mini Kit	RNeasy Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free Reagents and Buffers	Qiagen (Germany)
2.	RNAprotect Bacteria Reagent		Qiagen (Germany)
3.	QuantiTect Reverse Transcription Kit	100 μ l 7x gDNA Wipeout Buffer, 50 μ l Quantiscript Reverse Transcriptase, 200 μ l 5x Quantiscript RT Buffer, 50 μ l RT Primer Mix, 1.9 ml RNase-Free Water	Qiagen (Germany)
4.	DNase I RNase Free (supplied with MnCl ₂)	1 U/ μ L	Thermo Fisher Scientific (USA)

No	Kit	Description	Manufacturer
5.	iQ SYBR Green Supermix	2.5 ml (2 x 1.25 ml), 2x qPCR mix, contains dNTPs, iTaq™ DNA Polymerase, MgCl ₂ , SYBR® Green I, enhancers, stabilizers, fluorescein, for 100 x 50 µl reactions	BioRad (USA)

3.1.6 Consumables

1. Millex™ polyethersulfone (PES) syringe filters - MERCK (USA)
2. Millex™-FG Filter Unit - MERCK (USA)
3. Falcon Tube 15 mL
4. Corning® 96-well Clear Flat Bottom Polystyrene Not Treated Microplates - Corning(USA)
5. NEST ® Petri dishes – Nest Scientific USA
6. Qorpak™ PYREX™ Screw Cap Culture Tubes with PTFE Lined Cap – Fisher Scientific (USA)
7. 5 mm glass beads – MERCK (USA)
8. Cuvettes – Kartell
9. Nylon syringe filter (0.22 µm) - Bioflow
10. Syringe – Tarumo Japan
11. 96 well plates -MicroAmp; Applied Biosystems; Cat No. N801-0560)
12. Optical adhesive covers -Applied Biosystems; Cat No. 4313663)

3.1.7 Equipment

1. Autoclave - Hirayama (Japan)
2. Esco | Class II Biological Safety Cabinets, Gen 3 (S-Series)
3. Analytical balance – Denver xl-1810 USA
4. Centrifuge – Jouan A14 Franc3
5. Chiller (4°C)
6. Electric dry cabinet – Weifo KD-112
7. Freezer (-20°C)- Zanussi Germany
8. Freezer (-80°C) – Hetofrig CL410 Denmark
9. Incubators – Memmert Germany
10. Hot plate/ stirrer – Thermolyne

11. Light microscope – Nikon Japan
12. pH meter – Hanna HI-8417 instrument
13. Micropipettes – Eppendorf
14. Spectrophotometer – Shimadzu UV160A Japan
15. Microplate reader - Infinite® 200 PRO
16. Ultrasonic sonicator – Selecta CE95
17. Vortex mixer – Snijder Scientific Holland
18. Autoclave
19. Analytical balance – Denver xl-1810 USA
20. Centrifuge – Jouan A14 France
21. Chiller (4°C)
22. Electric dry cabinet – Weifo KD-112
23. Fume cupboard – Ductless Labcaire 4850 England
24. Scanning electron microscope (SEM)- FEI Quanta™ FEG 450
25. NanoDrop spectrophotometer -Thermo Fisher Scientific
26. StepOne Plus Real-Time PCR System (Applied Biosystem USA)

3.2 Preparation of Solution and Media

3.2.1 Preparation of Brain Heart Infusion (BHI) Agar

Brain Heart Infusion (BHI) agar was used as the growth medium for bacterial cultivation. The BHI agar was prepared according to the manufacturer's instructions (Thermo Fisher, USA). The prepared medium was sterilised by autoclaving at 121 °C for 15 minutes. Once cooled and solidified in petri dishes, the agar plates were stored at 4 °C until further use.

3.2.2 Preparation of Brain Heart Infusion (BHI) Broth

Two types of Brain Heart Infusion (BHI) broth were prepared for bacterial culture: standard BHI broth and BHI broth supplemented with 1% (w/v) sucrose.

3.2.2.1 Standard BHI Broth

BHI broth was prepared according to the manufacturer's instructions (Thermo Fisher, USA). The powder was dissolved in distilled water and mixed thoroughly. The solution was then sterilised by autoclaving at 121 °C for 15 minutes.

3.2.2.2 BHI Broth with Sucrose

To prepare the sucrose-supplemented broth, BHI powder was first dissolved in distilled water as described for the standard broth. To optimise sucrose concentration for biofilm formation, sucrose was added to sterile BHI broth to yield final concentrations of 0.25%, 0.5%, 1%, and 2% (w/v). Each formulation was sterilised by autoclaving at 121 °C for 15 minutes. After sterilisation, the broths were allowed to cool to room temperature and stored at 4 °C until further use.

3.2.3 Preparation of Mitis Salivarius Agar (MSA)

Mitis Salivarius Agar (MSA) was prepared according to the manufacturer's instructions (Sigma-Aldrich, Germany). Briefly, 90 g of dehydrated MSA powder was suspended in 1 L of distilled water and gently heated with continuous stirring until completely dissolved. The medium was then sterilised by autoclaving at 121 °C for 15 minutes. After autoclaving, the medium was cooled to approximately 45–50 °C before being poured aseptically into sterile Petri dishes. The plates were left to solidify at room temperature and subsequently stored at 4 °C until use. Prior to inoculation, the agar plates were equilibrated to room temperature. The MSA was used for the selective isolation and differentiation of oral streptococci, including *S. mutans* and *S. gordonii*, based on distinct colony morphology.

3.2.4 Preparation of Phosphate-Buffered Saline (PBS) Solution

Phosphate-buffered saline (PBS) was used as the buffer for serial dilutions. The PBS was prepared according to the manufacturer's instructions (Sigma-Aldrich, USA). One PBS tablet was dissolved in 200 mL of deionised water, yielding a solution containing 0.01 M phosphate buffer, 0.137 M sodium chloride, and 0.0027 M potassium

chloride, with a final pH of 7.4 at 25 °C. The solution was subsequently sterilised by autoclaving. The solution was stored at 4 °C for short-term use.

3.2.5 Preparation of Chlorhexidine (CHX)

Chlorhexidine digluconate (CHX) was used as the positive control in all antimicrobial and antibiofilm assays. A 0.12% (w/v) working solution was prepared by diluting a 20% (w/v) CHX stock solution (Sigma-Aldrich, USA) in sterile distilled water. Unless otherwise stated, all references to CHX in this study refer to the 0.12% (w/v) concentration. The solution was freshly prepared prior to each experiment and stored in a sterile, light-protected container at room temperature to preserve its stability and antimicrobial activity.

3.2.6 Preparation of PDCs

Table 3.3 presents the details of the pomegranate-derived compounds (PDCs) utilised in this study. The selection of these compounds was guided by the findings of Man et al. (2022), who reported the compound profiles of nine pomegranate cultivars and identified several polyphenolic constituents commonly present in pomegranate peel. Based on this report, representative compounds were selected to capture the structural diversity of major polyphenolic classes associated with *Punica granatum* (*P. granatum*), including ellagitannins, phenolic acids, flavonoids, and related derivatives. This strategy enabled preliminary exploration of potential structure–activity relationships among chemically distinct pomegranate polyphenols with respect to antibiofilm and antivirulence effects.

In this study, the term PDCs denotes polyphenolic compounds naturally present in *P. granatum* and commonly discussed in the literature in relation to pomegranate. Both major and relatively less emphasised constituents were included in the screening panel to allow comparative evaluation of their biological activity, recognising that compound abundance in plant extracts does not necessarily predict antimicrobial or antibiofilm potency.

For experimental purposes, the compounds were obtained as commercially available, chemically defined standards, some of which are produced by suppliers using plant sources other than pomegranate. This approach was adopted to ensure compound

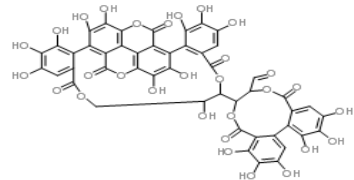
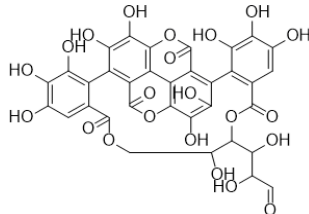
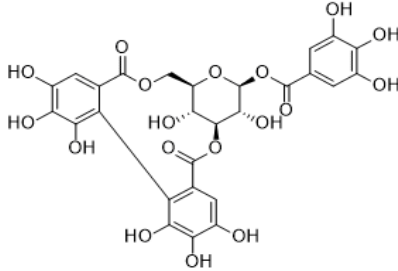
purity, consistent quality, and reproducible concentration-dependent evaluation under controlled experimental conditions, without altering the chemical identity or biological relevance of the compounds investigated. The use of compounds rather than crude extracts also facilitated systematic screening in Phase I of the study, enabling identification of lead candidates for subsequent mechanistic investigation in dual-species biofilm models.

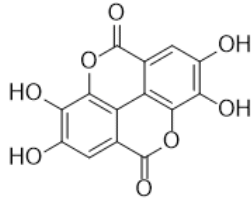
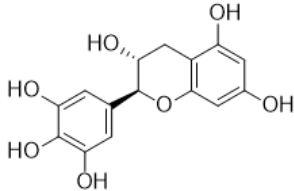
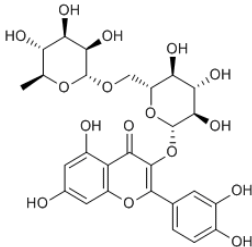
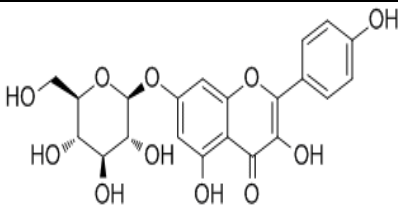
Stock solutions (20 mg/mL) were prepared by dissolving each compound in 50% DMSO. Working solutions were prepared by further diluting the stock solutions with sterile distilled water to achieve the desired concentrations for experimental use. The final DMSO concentration in all working solutions was maintained between 0.1% and 0.5%, depending on the required concentration of the compound. Before application, all stock solutions were filter-sterilised using Millex™-FG Filter Unit (0.22 µm) syringe filters (Merck, USA) that were validated for use with organic solvents such as DMSO. This step was performed under aseptic conditions to ensure the compounds remained sterile for subsequent experimental procedures.

Table 3.3

Selected PDCs with CAS Numbers, Molecular Weights (MW), Molecular Formulas (MF), Structures, and Purity

The compounds were identified as major constituents of pomegranate peel based on the study by Man et al. (2022). Molecular information was retrieved from the supplier's website, BOC Sciences (<https://www.bocsci.com>).

Compound	CAS No	Molecular weight (MW) Molecular formula (MF)	Purity (HPLC)	Molecular structure
Punicalagin (PUG)	65995-63-3	MW:1084.7 MF: C ₄₈ H ₂₈ O ₃₀	98%	
Punicalin (PUN)	65995-64-4	MW: 782.53 MF: C ₃₄ H ₂₂ O ₂₂	98%	
Corilagin (COR)	23094-69-1	MW: 634.45 MF: C ₂₇ H ₂₂ O ₁₈	>98%	

Compound	CAS No	Molecular weight (MW) Molecular formula (MF)	Purity (HPLC)	Molecular structure
Ellagic acid (EA)	476-66-4	MW: 302.19 MF: C ₁₄ H ₆ O ₈	>98%	
Gallocatechin (GC)	3371-27-5	MW: 306.27 MF: C ₁₅ H ₁₄ O ₇	>98%	
Rutin (RUT)	153-18-4	MW: 610.53 MF: C ₂₇ H ₃₀ O ₁₆	95%	
Kaempferol-glucoside (KG)	16290-07-6	MW : 448.38 MF: C ₂₁ H ₂₀ O ₁₁	> 98%	

3.3 Microorganisms Used in This Study

Two bacterial strains, *S. mutans* ATCC 700610 and *S. gordonii* ATCC 51656, were selected to represent the oral microbiota in this study. The selection was based on the origin of isolation: *S. mutans* (ATCC 700610) was initially isolated from a child with active dental caries, while *S. gordonii* (ATCC 51656) was originally isolated from the human oral cavity. These species were selected based on their distinct roles in oral biofilm ecology: *S. gordonii* is a pioneer coloniser involved in initial surface attachment and coaggregation with other microbes. At the same time, *S. mutans* is a key late-stage coloniser with high acidogenic and aciduric potential, making it a major contributor to cariogenic biofilms. Their inclusion enables the development of a realistic, clinically relevant dual-species biofilm model to evaluate the antibiofilm activity of the test compounds.

Lyophilised bacterial cultures were rehydrated in appropriate broth media and inoculated onto culture media as recommended by the American Type Culture Collection (ATCC). After incubation at 37 °C for 24 hours, the resulting colonies were subcultured onto fresh brain heart infusion (BHI) agar slants, stored at 4 °C, and used within two weeks. Bacterial stocks were prepared in 50% (v/v) glycerol for long-term preservation and stored at –80 °C. Subcultures were performed biweekly to maintain culture viability.

3.4 Experimental Design

This study employed a two-phase experimental design to identify and characterise pomegranate-derived phytochemicals with selective antibiofilm activity against cariogenic biofilms. In Phase I, compound prioritisation was primarily driven by antibiofilm efficacy, with minimum biofilm inhibitory concentration (MBIC) and minimum biofilm eradication concentration (MBEC) serving as the key determinants for progression. Preference was given to compounds demonstrating strong activity against *S. mutans* relative to the commensal partner, consistent with a selective targeting strategy rather than broad-spectrum microbial suppression. Functional modulation of virulence-associated traits, including acidogenicity and cell-surface hydrophobicity, and acceptable safety profiles were considered supportive factors.

Phase II focused on the mechanistic evaluation of the leading compounds using a dual-species biofilm model. Antibiofilm efficacy was quantified by colony-forming unit (CFU) enumeration following treatment of established biofilms. Molecular responses were assessed through reverse transcription quantitative PCR (RT-qPCR) analysis of selected virulence- and biofilm-associated genes. Structural and morphological effects on biofilm architecture were examined using scanning electron microscopy (SEM).

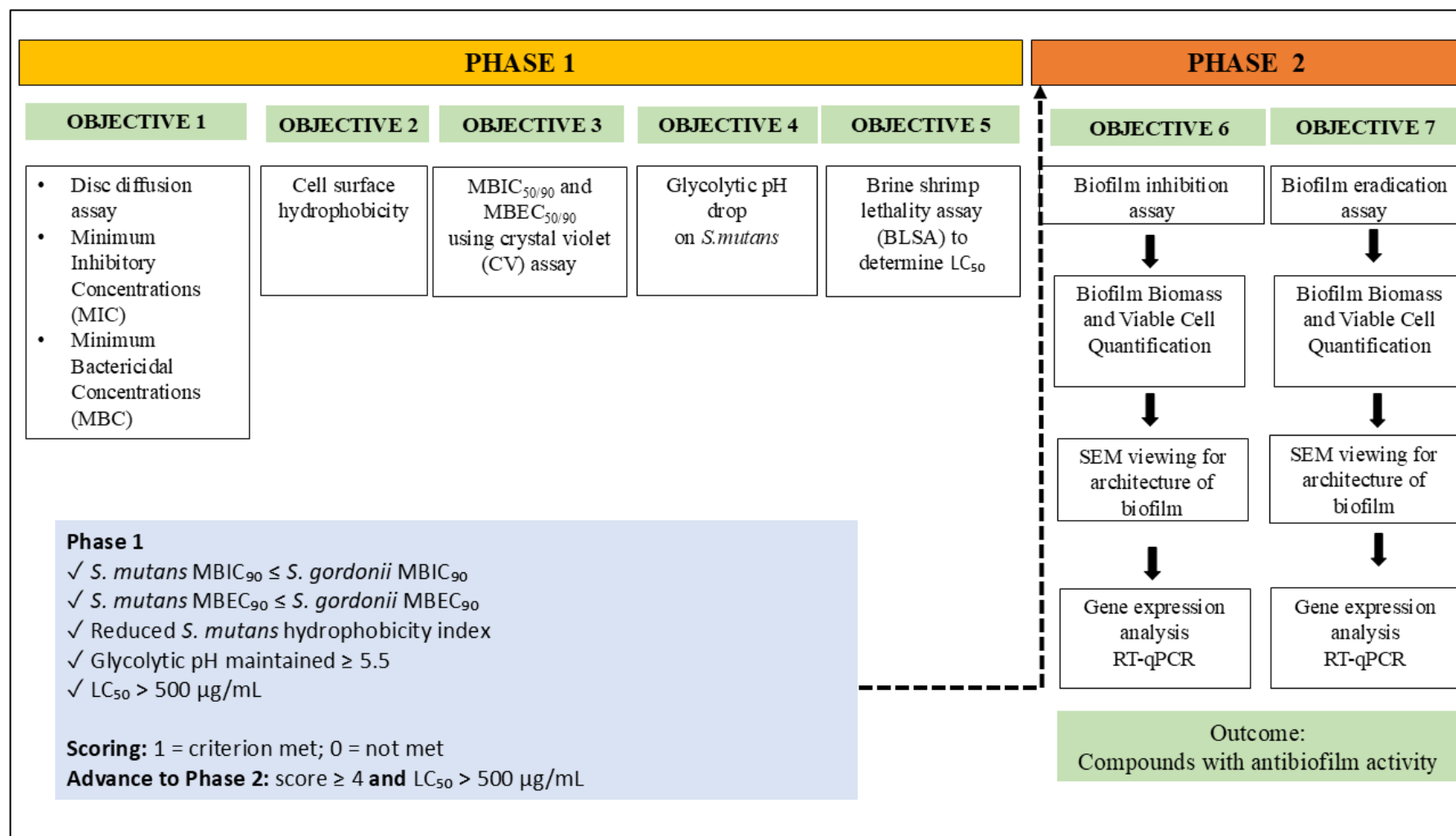


Figure 3.1 Workflow Diagram of Two-Phase Experimental Design for Evaluating Antibiofilm And Antivirulence Potential of PDCs

3.5 Preparation of Standard Bacterial Suspension

Fresh bacterial cultures were prepared before each experiment to ensure consistency and viability. Three to five well-isolated colonies were aseptically picked from an 18- to 24-hour agar plate using a sterile inoculation loop and transferred into a sterile 15 ml tube containing 10 ml of BHI broth. The turbidity of the bacterial suspension was adjusted in sterile BHI broth to match the optical density of a 0.5 McFarland standard, corresponding to an approximate bacterial concentration of $1-2 \times 10^8$ CFU/mL. Spectrophotometric confirmation was performed at 600 nm; an absorbance reading between 0.08 and 0.10 indicates the appropriate density. Following turbidity adjustment to the 0.5 McFarland standard, the suspension was immediately used for plate inoculation to minimise variation in bacterial density and ensure consistency in results. Standardisation of the inoculum to the 0.5 McFarland standard was essential to ensure reproducibility across antimicrobial, biofilm formation, and mechanistic assays by minimising variability in initial bacterial load.

3.6 Disc Diffusion Assay

Screening for antibacterial activity of the compounds was performed according to the Clinical and Laboratory Standards Institute (CLSI) guidelines, with minor modifications. (CLSI, 2012). This assay was conducted as an initial qualitative screening method to assess the inhibitory potential of the compounds against bacterial growth.

Sterile blank paper discs, each 6 mm in diameter, were impregnated with 25 μ L of each phytochemical stock solution at a concentration of 20 mg/mL, resulting in a final loading of 0.5 mg per disc. A standardised bacterial suspension, adjusted to approximately 10^8 CFU/mL, was evenly spread over brain heart infusion (BHI) agar plates using a sterile swab to ensure uniform coverage. After inoculation, the petri dish lids were briefly left ajar for 3–5 minutes to allow excess moisture to evaporate before placing the discs.

For quality control, a disc treated with sterile distilled water served as the negative control, and a disc containing 0.12% w/v CHX served as the positive control. This procedure was repeated for all bacterial strains tested. The plates were inverted and incubated at 37 °C for 16 to 18 hours. Following incubation, antibacterial activity was

assessed by measuring the diameter of the inhibition zones surrounding each disc. Measurements were recorded in millimetres using a digital sliding calliper positioned beneath the inverted plates for accuracy. All assays were performed in triplicate (technical replicates) and independently repeated five times (biological replicates). Data are presented as mean $\pm$ standard deviation ($n = 5$).

3.7 Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentration (MBC)

MIC and MBC assays were conducted primarily to establish baseline antimicrobial activity and to determine sub-inhibitory concentrations used for subsequent antivirulence and biofilm assays. The minimum inhibitory concentrations (MICs) of the compounds against planktonic bacterial cells were determined using the broth microdilution method, following CLSI (2012) guidelines with minor modifications. This assay was performed to evaluate the inhibitory and bactericidal effects of the compounds against planktonic bacterial growth.

A standardised bacterial suspension of approximately 10^8 CFU/mL was prepared as described in Section 3.5 and then diluted 1:20 in sterile broth to yield 5×10^6 CFU/mL.

Working solutions of each compound were freshly prepared at a concentration of 12800 μ g/mL. Twofold serial dilutions were prepared in sterile broth across a 96-well microtiter plate to yield final concentrations of 12.5, 25, 50, 100, 200, 400, 800, 1600, 3200, and 6400 μ g/mL. Then, 10 μ L of the diluted bacterial suspension was added to each well containing 90 μ L of broth, resulting in a final volume of 100 μ L per well. Consequently, the final bacterial concentration in each well was approximately 5×10^5 CFU/mL (or 5×10^4 CFU/well), consistent with standard microdilution assay conditions.

All treatments were performed in triplicate (technical replicates), and the entire experiment was independently repeated five times (biological replicates).

For each assay, growth control (GC) wells containing only bacterial suspension and BHI broth were included to confirm bacterial viability. Sterility control wells consisting solely of BHI broth were used to confirm the absence of contamination and to validate the sterility of the media. In order to address any potential interference from the inherent colouration of phytochemicals, additional wells containing only the PDCs

and sterile BHI broth were prepared as blank controls. These blank controls were serially diluted in the same manner as the test wells. For example, PUG, which exhibits a yellow to brown hue, required such controls to eliminate background absorbance when measuring optical density (OD) during MIC determination.

After incubation at 37 °C for 24 hours, bacterial growth was assessed by measuring optical density at 600 nm using a microtiter plate reader (Tecan Infinite® 200 PRO). The MIC was the lowest concentration with an OD600 difference of <0.05 compared to the blank control (Wan et al., 2023). Following MIC determination, the MBC was assessed to identify the lowest concentration of compound required to achieve bacterial killing. From the wells showing no visible growth, 10 µL of each sample was plated onto BHI agar and incubated at 37 °C for 36 to 48 hours. The MBC was the lowest concentration at which no colony formation occurred, indicating a 99.9% reduction in viable bacteria.

MIC and MBC values represent categorical endpoint concentrations and were recorded descriptively across five independent biological experiments.

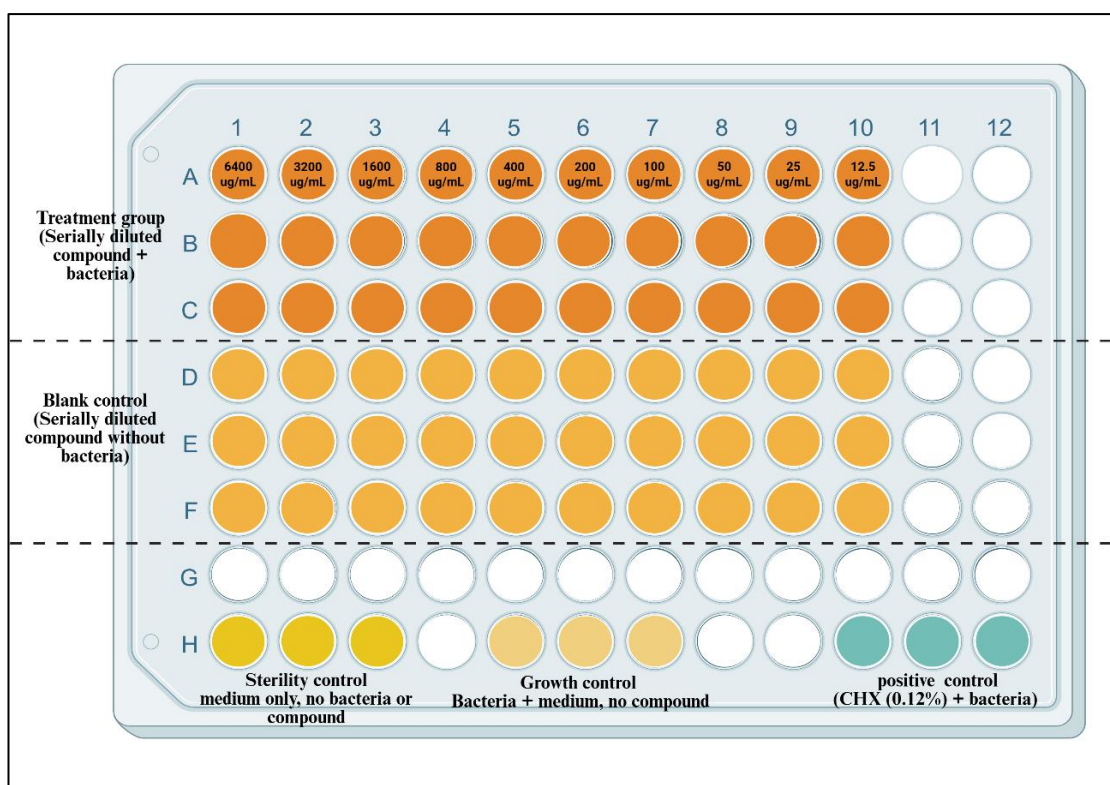


Figure 3.2 Schematic Diagram of Minimal Inhibitory Concentration (MIC) Plate Setup

3.8 Cell Surface Hydrophobicity Assay

The effects of compounds on the cell surface hydrophobicity (CSH) of *S. gordonii* and *S. mutans* were assessed using microbial adhesion to hydrocarbon (MATH) assay as originally described by Rosenberg (1984), with reference to later methodological refinements and contemporary applications in *S. mutans* studies (He et al., 2019). This assay was performed to evaluate changes in bacterial surface hydrophobicity, which influence adhesion and early biofilm formation.

The PDCs were tested at the MIC and at subinhibitory concentrations ($0.5\times$ MIC, $0.25\times$ MIC, and $0.125\times$ MIC). Bacterial cultures were incubated with the compounds at 37 °C for 24 hours. Following incubation, the cells were harvested by centrifugation at $5000 \times g$ for 5 minutes at 4 °C, washed twice with sterile distilled water. They were resuspended in PUM buffer to an optical density of 0.8 at 600 nm (OD_0). The PUM buffer was prepared by dissolving 17.4 g of $K_2HPO_4 \cdot 3H_2O$, 7.26 g of KH_2PO_4 , 1.8 g of urea, and 0.2 g of $MgSO_4 \cdot 7H_2O$ in distilled water, then adjusting the volume to 1000 mL and the final pH to 7.1. A 3 mL aliquot of the bacterial suspension was transferred into a clean tube, followed by the addition of 0.2 mL hexadecane (Sigma-Aldrich). The mixture was vortexed vigorously for 60 seconds, then allowed to stand undisturbed for 15 minutes to facilitate phase separation. The absorbance of the aqueous phase was measured at 600 nm (OD_1). The percentage of cell surface hydrophobicity was calculated using the following formula (3.1).

$$\text{Hydrophobicity (\%)} = \frac{OD_0 - OD_1}{OD_0} \times 100 \quad (3.1)$$

OD_0 = OD_{600} of the bacterial suspension before adding hexadecane

OD_1 = OD_{600} of the bacterial suspension after adding hexadecane.

Cells incubated with 1% DMSO and added with hexadecane without compounds served as the negative control. The cell surface hydrophobicity assays were performed independently in five determinations, each in triplicate. Data were expressed as the percentage reduction in cell surface hydrophobicity (CSH) calculated using equation (3.2)

$$\text{Percentage of reduction in CSH: } \left(1 - \left(\frac{T}{C}\right)\right) \times 100 \quad (3.2)$$

Where C represents the mean CSH value of the negative control group (bacteria cultured without test compounds) and T represents the mean CSH value of the treated group (bacteria exposed to the respective compound concentration). This calculation allowed for the quantification of the inhibitory effect of each compound on bacterial surface hydrophobicity relative to untreated controls.

3.9 Preparation of Clarified Saliva

This study involved human saliva and was conducted in accordance with institutional and international ethical guidelines, with approval obtained from the Research Ethics Committee, Universiti Teknologi MARA (Reference No: REC/01/2023 [PG/MR/27]). To obtain a biologically relevant source of salivary enzymes and host factors for the study, stimulated whole saliva was collected from a single healthy adult female volunteer (40 years old). The subject met all inclusion and exclusion criteria established for the study, including the absence of systemic illness, no antibiotic use within the past 3 months, and no signs of active oral disease or inflammation. Written informed consent was obtained prior to sample collection.

To stimulate saliva, the subject chewed sterile Parafilm M® (approximately 1 cm²) to promote consistent salivary flow. Whole saliva was collected in a sterile container over a defined period until a total of 50 mL was obtained. Immediately after collection, the saliva was transferred to a prechilled tube containing an equal volume (1:1 ratio, v/v) of adsorption buffer supplemented with 50 µL 0.1 M phenylmethyl sulfonyl fluoride (PMSF) to inhibit proteolytic degradation (Lemos et al., 2010; Rahmani-Badi et al., 2015). The mixture was centrifuged at 5500× g for 10 minutes at 4 °C to remove cellular debris and particulate matter. Following centrifugation, the clear supernatant was carefully transferred to a sterile container and passed through a Millex™ Polyethersulfone (PES) syringe filter with a 0.22 µm pore size to ensure sterility and eliminate residual microbial contamination. This PES membrane filter type was selected due to its low protein-binding properties, thereby minimising loss of salivary proteins during filtration while ensuring sterility and removing residual

microbial contamination. This filtration step also helps maintain enzymatic integrity by removing microbial enzymes that may interfere with downstream assays (Lemos et al., 2010). The processed salivary filtrate was used immediately to preserve enzymatic activity and protein integrity for subsequent experimental pellicle formation.

3.10 Optimisation of Dual-Species Biofilm Model of *S. mutans* and *S. gordonii*

Optimisation experiments were conducted to establish a reproducible dual-species biofilm model comprising *S. mutans* and *S. gordonii* for subsequent antibiofilm and mechanistic studies. The optimisation strategy was designed to ensure consistent biofilm formation while permitting reliable recovery and quantification of both bacterial species. In addition, optimisation aimed to establish a biofilm model that reflects key features of cariogenic biofilms, including sucrose-dependent biofilm formation, acidogenic activity, and stable coexistence of both species under conditions that favour cariogenic growth. Optimisation was performed in two stages: initial parameter screening using a 96-well microtiter plate platform, followed by validation in a glass-bead biofilm model established in 24-well plates for Phase 2 assays.

An experimental acquired pellicle was established using clarified human saliva to simulate the initial proteinaceous layer that naturally coats oral surfaces *in vivo*. This saliva-derived pellicle provides a biologically relevant interface for bacterial adhesion and subsequent biofilm development, thereby enhancing the physiological relevance of the *in vitro* model.

Briefly, wells of sterile polystyrene microtiter plates were treated with clarified human saliva, prepared as described previously, to allow adsorption of salivary proteins and glycoproteins onto the surface. Each well was incubated with 200 µL of clarified saliva at 37 °C for 2 hours to facilitate pellicle formation. To preserve the integrity and activity of salivary components throughout the incubation period, the saliva was gently aspirated and replenished with fresh aliquots every 30 minutes. This approach ensured a continuous supply of pellicle-forming proteins while minimising degradation of labile salivary constituents, following the method described by Pelá et al. (2020). After the incubation period, wells were gently rinsed with sterile phosphate-buffered saline (PBS) to remove unbound components, leaving a uniform experimental pellicle firmly adhered to the well surfaces. These saliva-conditioned wells were used immediately for subsequent biofilm formation experiments.

Standardised overnight cultures of *S. mutans* and *S. gordonii* were adjusted based on established OD–CFU calibration curves and combined immediately before inoculation to generate mixed bacterial suspensions with defined total cell densities. The total starting inoculum was systematically varied from 1×10^6 to 1×10^8 CFU/mL to identify a density that promoted consistent surface attachment and biofilm maturation without excessive planktonic overgrowth or premature biofilm detachment. (Heersema & Smyth, 2019). This range allowed evaluation of density-dependent effects on interspecies interactions and biofilm stability. In parallel, the relative inoculum ratio of *S. mutans* to *S. gordonii* was assessed at 1:1, 2:1, and 1:2 while maintaining a constant total inoculum density, to compensate for known differences in growth rate, acidogenicity, and competitive fitness between the two species.

Biofilm formation was further optimised by varying the sucrose concentration in the culture medium, given its critical role in extracellular polysaccharide synthesis and biofilm matrix development. Final sucrose concentrations of 0.5%, 1%, and 2% (w/v) were evaluated to identify a condition that supported structured biofilm formation while preserving species balance. Higher sucrose concentrations were carefully assessed, as excessive sucrose availability is known to preferentially enhance glucan production and rapid biofilm accumulation by *S. mutans*, potentially leading to competitive dominance and reduced retention of *S. gordonii* within the adherent biofilm.

After 24 hours of incubation, planktonic cells were gently aspirated, and wells were carefully washed with sterile PBS to remove non-adherent bacteria. Adherent biofilms were stained with 100 μ L 0.1% (w/v) CV solution for 30 min at room temperature. Excess stain was removed by thorough washing with PBS, and the plates were air-dried. The bound dye was subsequently solubilised with 33% acetic acid (200 μ L) (Plate 3.1). Following solubilisation, the eluate was carefully transferred to a fresh 96-well microtiter plate, and biofilm biomass was quantified by measuring absorbance at 595 nm using a Tecan Infinite® 200 PRO microplate reader. Absorbance values reflected total attached biofilm biomass and were used as a primary screening metric to identify conditions that produced robust, reproducible biofilms with minimal well-to-well variability. In addition to biomass quantification, the acidogenic potential of the developing biofilm was evaluated by measuring the final pH of the culture medium after incubation. The pH of the spent culture medium was measured using a calibrated microelectrode pH meter immediately after the incubation period. Lower final pH values were interpreted as indicative of increased acidogenic activity under sucrose-

supplemented growth conditions and were considered supportive of a cariogenic-like biofilm environment.



Plate 3.1 CV–Stained Biofilms During Solubilisation using 33% (v/v) Acetic Acid

To confirm that CV–measured biomass represented a true dual-species biofilm, viable cell recovery and colony-forming unit enumeration were performed under selected optimisation conditions. Following removal of planktonic cells and washing, adherent biofilms were mechanically detached by scraping the well base with the addition of 100 μ L of PBS to ensure efficient recovery of firmly attached cells. The resulting biofilm suspensions were serially diluted and plated onto Mitis–Salivarius (MS) agar. Plates containing between 30 and 300 colonies were selected for enumeration.

Distinct colony morphologies on MSA enabled differentiation between *S. mutans* and *S. gordonii*. *S. mutans* colonies were identified by their raised, convex morphology with undulate edges, opaque pale-blue colouration, and granular frosted glass appearance, occasionally displaying a glistening surface. *S. gordonii* colonies were generally larger, smooth, firm, and flatter, with darker pigmentation (Plate 3.2). Species identity was further confirmed using commercial biochemical identification systems, namely the Rapid ID 32A system (bioMérieux, France) for *S. gordonii* and the API 20 Strep system (bioMérieux, France) for *S. mutans*, according to the manufacturers' instructions. Viable counts were expressed as CFU/mL and calculated as the number of colonies divided by the product of the dilution factor and the plated volume. Viable counts were expressed as CFU/mL and calculated using the following formula:(Equation 3.3):

$$\text{Total CFU/mL} = \frac{\text{Number of colonies on plate}}{\text{Dilution factor} \times \text{Volume plated (mL)}} \quad (3.3)$$

Optimised conditions were defined as those that consistently produced reproducible CV biomass measurements and recoverable CFU counts for both *S. mutans* and *S. gordonii* across independent experiments. Conditions showing pronounced dominance of one species and reduced recovery of the other were excluded.

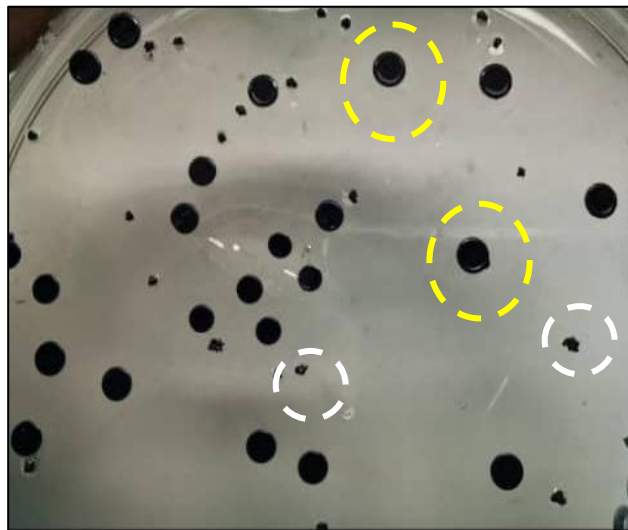


Plate 3.2 Mitis–Salivarius (MS) Agar Showing Colony Differentiation of *S. gordonii* and *S. mutans*

Colonies were recovered from dual-species biofilms grown in 96-well plates after 48 h of incubation. Larger, smooth, and more regularly circular colonies (yellow circle) correspond to *S. gordonii*, whereas smaller colonies with a raised, convex morphology, slightly irregular edges, and a granular surface appearance (white circle) correspond to *S. mutans*, as determined by characteristic colony morphology on selective agar. Colony identification was initially determined by comparison with observations from corresponding single-species control plates and was further confirmed using the Rapid ID 32A system and the API 20 Strep identification kit. This combined morphological and biochemical approach was employed to ensure accurate species-specific enumeration in dual-species CFU analysis

Following optimisation in the 96-well screening platform, the selected parameters were translated to a glass-bead biofilm model established in 24-well plates for Phase 2 analyses. This additional optimisation step was necessary because the Phase 2 platform differed from the screening platform in both substrate material and culture volume. Unlike the polystyrene surface of 96-well plates, the Phase 2 model utilised glass beads, which possess distinct physicochemical surface properties that can influence bacterial adhesion, extracellular matrix development, and biofilm

architecture. In addition, the larger working volume of the 24-well format alters nutrient availability, diffusion dynamics, and biofilm maturation. Therefore, optimisation was performed to confirm that the selected inoculum density, species ratio, sucrose concentration, and incubation time supported reproducible dual-species biofilm formation under these modified conditions.

For the glass-bead model optimisation, sterile 5-mm glass beads were placed in individual wells of 24-well plates and coated with clarified saliva using the same pellicle-formation protocol. Pellicle-coated beads were inoculated with dual-species suspensions prepared using the selected inoculum density and species ratio and incubated in biofilm growth medium supplemented with the selected sucrose concentration for 24 hours or 48 hours. For biofilm recovery, each bead was transferred into a 1-mL microcentrifuge tube containing PBS, then sonicated and vortexed to detach the biofilm. The resulting suspension was serially diluted and plated onto MSA for viable cell enumeration. Separate bead sets were used in the CV assay to quantify total biofilm biomass.

Based on reproducibility of biomass measurements and consistent recovery of both species across both platforms, an inoculum density of 1×10^6 CFU/mL per species, a 1:1 inoculum ratio, and a sucrose concentration of 1% (w/v) were selected. These parameters were applied uniformly in all subsequent dual-species antibiofilm assays.

3.11 Minimum Biofilm Inhibitory Concentration (MBIC) Using CV Assay

Both single-species and dual-species biofilm models involving *S. gordonii* and *S. mutans* were established and treated with varying concentrations of selected compounds to investigate their antibiofilm effects. This assay was performed to assess the ability of the compounds to inhibit early biofilm formation. Pure cultures were grown overnight in BHI broth at 37°C under microaerophilic conditions. The bacterial cultures were then adjusted to $\sim 1 \times 10^6$ CFU/mL using a spectrophotometer. Biofilm inhibition assays were performed in sterile 96-well polystyrene microtiter plates (Corning® Costar®) following a modified protocol by (Sun et al., 2019). Compound solutions prepared in BHI were first dispensed into each well at 100 µL, with final concentrations ranging from 12.5 to 1600 µg/mL. The final DMSO concentration was maintained at 1% (v/v) across all wells. Wells containing 1% DMSO in BHI without

compounds served as negative controls, while wells treated with 0.12% CHX served as positive controls.

Following compound dispensing, bacterial suspension was added to each well to initiate biofilm formation. For the single-species biofilm model, 100 μ L of either *S. mutans* or *S. gordonii* suspension was added to the wells containing the compound. For the dual-species model, equal volumes (50 μ L each) of *S. mutans* and *S. gordonii* suspensions were added to achieve a 1:1 inoculum ratio. The total volume per well was maintained at 200 μ L of bacterial inoculum. Plates were incubated at 37 °C for 24 hours to allow biofilm development in the continuous presence of test compounds.

Following incubation, biofilm biomass was quantified using CV staining and spectrophotometric measurements as described in Section 3.10. All experiments were performed in triplicate (technical replicates) and repeated independently five times (biological replicates). The percentage of biofilm inhibition was calculated relative to the untreated control using Equation (3.4), and the primary outcome measure was the reduction in biofilm biomass expressed as percentage inhibition.

$$\text{Biofilm inhibition \%} = \left[1 - \left(\frac{OD \text{ negative control} - OD \text{ test}}{OD \text{ negative control}} \right) \right] \times 100 \quad (3.4)$$

The minimum biofilm inhibitory concentration (MBIC) was defined as the lowest concentration at which biofilm formation was substantially inhibited relative to the untreated control. MBIC₅₀ and MBIC₉₀ values were derived from five independent biological experiments. As MBIC represents a discrete concentration threshold rather than a continuous quantitative variable, values were recorded descriptively and were not subjected to inferential statistical comparison. A concentration was assigned as the MBIC endpoint only when the predefined inhibition criterion ($\geq 50\%$ or $\geq 90\%$) was consistently achieved in the majority of independent biological repeats. Where variability occurred between experiments, the modal concentration was selected. In cases of discordance between adjacent concentrations, the higher concentration was conservatively reported to prevent overestimation of antibiofilm efficacy.

As this assay was designed to determine threshold inhibition concentrations rather than to model dose–response relationships, nonlinear curve fitting was not

performed. MBIC₅₀ and MBIC₉₀ were therefore identified as the lowest tested concentrations achieving the predefined inhibition criteria.

3.12 Minimum Biofilm Eradication Concentration (MBEC) Using CV Assay

The capacity of the selected compounds to disrupt and eradicate preformed biofilms was assessed using the CV assay. This assay was performed to evaluate compound efficacy against mature biofilm biomass, providing an indicator of biofilm structural disruption. Standardised overnight cultures of *S. mutans* and *S. gordonii* were adjusted to 1×10^6 CFU/mL in brain heart infusion (BHI) broth supplemented with 1% (w/v) sucrose. Equal volumes of each bacterial suspension were combined immediately prior to inoculation to obtain a 1:1 inoculum ratio. A 100 μ L aliquot of the bacterial culture was dispensed into the wells of a sterile 96-well microtiter plate, followed by incubation at 37 °C for 48 hours under static conditions, with the culture medium replenished every 24 hours to maintain nutrient availability. After incubation, nonadherent cells were carefully removed by washing twice with sterile phosphate-buffered saline (PBS, 1 $\times$). Test compounds at increasing concentrations (12.5–1600 μ g/mL) were then applied to the wells containing the established biofilms. Treatment lasted 4 hours. Wells treated with 1% DMSO served as the negative control, while those treated with 0.12% CHX acted as the positive control. All treatments were performed in triplicate (technical replicates), and the entire experiment was independently repeated five times (biological replicates).

Following treatment, the wells were gently washed with PBS to remove residual compounds and planktonic cells. Biofilm biomass was stained by adding 100 μ L of 0.1% (w/v) CV solution to each well and incubating at room temperature for 30 minutes. Excess dye was discarded, and the wells were rewashed with PBS. The bound dye was solubilised with 30% (v/v) acetic acid, and the absorbance was measured at 595 nm using a microplate reader. The percentage of biofilm eradication was calculated relative to the untreated control using Equation (3.5). The primary outcome measure was the reduction in biofilm biomass, expressed as percentage eradication compared with the negative control.

$$\text{Biofilm eradication \%} = 1 - \left(\frac{\text{OD test}}{\text{OD negative control}} \right) \times 100 \quad (3.5)$$

The minimum biofilm eradication concentration (MBEC) was defined as the lowest concentration at which a substantial reduction in biofilm biomass relative to the untreated control was observed. MBEC₅₀ and MBEC₉₀ values were derived from at least three independent biological experiments. As MBEC represents a categorical endpoint concentration threshold rather than a continuous distribution, results were analysed descriptively. A concentration was assigned as the MBEC endpoint only when the predefined eradication criterion ($\geq 50\%$ or $\geq 90\%$ biomass reduction) was consistently achieved in the majority of independent biological repeats. Where variability occurred between experiments, the modal concentration was selected. In cases of discordance between adjacent concentrations, the higher concentration was conservatively reported to prevent overestimation of biofilm eradication efficacy.

As this assay was designed to determine threshold eradication concentrations rather than to model dose–response relationships, nonlinear curve fitting was not performed. MBEC₅₀ and MBEC₉₀ were therefore identified as the lowest concentrations tested that met the predefined eradication criteria.

3.13 Glycolytic pH Drop Assay

The influence of PDCs on acid production by *S. mutans* was assessed using the glycolytic pH drop method and quantification of acidity (He et al., 2019). This assay was performed to evaluate the effect of selected compounds on acidogenicity, serving as a functional proxy for glycolytic activity and cariogenic virulence potential. This assay is essential because it provides insight into the compound's ability to inhibit *S. mutans* acidogenic activity, a key factor in dental caries development. In this assay, *S. mutans* cultures were collected during the mid-log phase, rinsed with phosphate-buffered saline (PBS), and then suspended in a salt solution containing 50 mM potassium chloride and 1 mM magnesium chloride, with or without varying concentrations of compounds (12.5 to 400 $\mu\text{g/mL}$). The concentration range was selected based on prior MIC and MBIC findings to include sub-inhibitory and inhibitory levels for functional comparison. Glucose was added to reach a final concentration of

1% (w/v), and the pH was adjusted to 7.2–7.4 using 0.2 M potassium hydroxide. The extent of acid production, as indicated by the drop in pH during glycolysis, was measured at 15-minute intervals for 120 minutes. The pH value measured at 120 min was defined as the final pH and used for endpoint comparison of acidogenic activity among treatment groups. The primary outcome measure was the change in pH (Δ pH) over time relative to the untreated control group. All experiments were performed in triplicate and repeated independently at least three times to ensure reproducibility.

Statistical analysis was performed using two-way ANOVA to evaluate the effects of treatment and time, followed by Tukey's multiple comparison test where appropriate. Data were assessed for normality using the Shapiro–Wilk test, and homogeneity of variance was evaluated prior to analysis. A p-value < 0.05 was considered statistically significant.

3.14 Brine Shrimp Lethality Assay (BSLA)

The preliminary biological evaluation of the pomegranate-derived compounds was conducted using the brine shrimp (*Artemia salina*) lethality assay, based on the method described by Sarah et al., (2017), with slight modifications. This assay was employed as an initial screening tool to estimate general cytotoxic potential and to support early-stage safety prioritisation of candidate compounds. The modifications included the use of 1% DMSO to aid compound solubility, adjustment of the concentration range to suit purified compounds, and the use of 20 nauplii per test condition. Brine shrimp eggs were incubated in artificial seawater prepared by dissolving 27 g of marine salt in 3 L of distilled water. Hatching was carried out in a well-aerated glass container under constant illumination at room temperature for 48 hours to obtain free-swimming larvae (nauplii). After incubation, the nauplii were drawn to one side of the container by a directed light source. Active nauplii were carefully separated from unhatched eggs using a micropipette and transferred into a clean petri dish containing fresh artificial seawater. These nauplii were used for the toxicity assay. A stock solution of the compound was prepared by dissolving 20 mg of the phytocompound in one mL of distilled water containing 1% DMSO yielding a concentration of 20,000 μ g/mL. Due to the limited aqueous solubility of some compounds, 1% DMSO was used as a co-solvent to aid dissolution. From this stock, a series of twofold dilutions were prepared in distilled water to obtain concentrations that

were double the desired final test concentrations. The working concentrations prepared were 25, 50, 100, 200, 400, 800, 1600, 3200, 6400, and 12,800 µg/mL. In 10 clean, labelled test tubes, one mL of each prepared solution was mixed with one mL of seawater containing 20 brine shrimp (*Artemia nauplii*). This 1:1 dilution step yielded final test concentrations of 12.5, 25, 50, 100, 200, 400, 800, 1600, 3200, and 6400 µg/mL. All tubes were incubated at room temperature under illumination for 24 hours. After incubation, the number of dead nauplii in each tube was counted using a magnifying glass to assess the compound's toxicity at different concentrations. Appropriate control groups were also included: a negative control with seawater only and a solvent control with seawater and 1% DMSO. Each concentration was tested in triplicate to ensure reproducibility. The percentage of deaths was calculated using the following formula, as shown at (3.6)

$$\text{Percentage of Death (\%)} = \left(\frac{\text{No of dead nauplii}}{\text{initial number of nauplii}} \right) \times 100\% \quad (3.6)$$

The median lethal concentration (LC₅₀), defined as the concentration causing 50% mortality of brine shrimp nauplii, was determined using nonlinear regression analysis with a sigmoidal dose–response (variable slope) model in GraphPad Prism version 10 (GraphPad Software, USA). Nonlinear regression was selected because it allows direct modelling of concentration–response relationships without requiring prior data transformation and provides reliable estimation of LC₅₀ values with confidence intervals. The software calculated LC₅₀ values and their corresponding 95% confidence intervals from the fitted dose–response curves. Toxicity results were interpreted using Clarkson’s toxicity classification criteria. This system was selected because it provides a graded toxicity assessment suitable for screening and phase-based compound selection. According to Clarkson’s classification, compounds with LC₅₀ values above 1000 µg/mL are considered non-toxic, values between 500–1000 µg/mL indicate low toxicity, values between 100–500 µg/mL indicate moderate toxicity, and values below 100 µg/mL are classified as highly toxic. The BSLA was employed as a rapid and cost-effective preliminary toxicity screening method to support early-stage compound prioritisation. As this assay reflects general acute toxicity, it does not replace

mammalian cell-based cytocompatibility testing; therefore, further evaluation using relevant oral cell models will be required to confirm clinical biocompatibility.

3.15 Selection of Compounds for Phase II

A multi-assay screening strategy was employed in Phase I to systematically prioritise pomegranate-derived phytochemicals for advancement to Phase II (Table 3.4). Each assay was designed to interrogate a distinct biological property relevant to cariogenicity, antibiofilm efficacy, selectivity, functional activity, and safety, with predefined decision rules applied to minimise selection bias and ensure reproducibility. Preliminary antibacterial activity was assessed using the disc diffusion assay, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) assays. These assays served as supportive evidence of antibacterial susceptibility and were used to guide the concentration ranges employed in subsequent biofilm-based assays; however, they were not used as primary selection criteria.

Antibiofilm activity formed the core basis for compound prioritisation. Compounds were required to demonstrate measurable MBIC and minimum biofilm eradication concentration (MBEC) activity against *S. mutans*, reflecting efficacy against both biofilm formation and established biofilms. To favour selective suppression of the cariogenic species while preserving biofilm ecological balance, only compounds whose MBIC₉₀ and MBEC₅₀ values against *S. mutans* were lower than or equal to those observed for *S. gordonii* were considered favourable. This criterion was applied to prioritise selective inhibition of the pathogenic species without disproportionate suppression of the commensal streptococcus.

Additional functional assays were incorporated to refine prioritisation. The microbial adhesion to hydrocarbon (MATH) assay was used to assess bacterial cell-surface hydrophobicity, with compounds that produced a marked reduction in the *S. mutans* hydrophobicity index being prioritised. Acidogenic potential was evaluated using the glycolytic pH-drop assay, and compounds that maintained the glycolytic pH of *S. mutans* suspensions at or above pH 5.5 were considered favourable, reflecting attenuation of acidogenicity.

Safety profiling was conducted using the BSLA. Compounds exhibiting an LC₅₀ value greater than 500 µg/mL, indicative of low toxicity according to established criteria, were considered acceptable for further evaluation. To ensure objective and

transparent compound selection, each compound was evaluated using a five-parameter scoring system comprising:

- (i) *S. mutans* MBIC₉₀ ≤ *S. gordonii* MBIC₉₀;
- (ii) *S. mutans* MBEC₅₀ ≤ *S. gordonii* MBEC₅₀;
- (iii) reduction in *S. mutans* hydrophobicity index;
- (iv) maintenance of glycolytic pH-drop at ≥ 5.5; and
- (v) LC₅₀ > 500 µg/mL.

For each parameter met, the compound was assigned a score of 1; otherwise, 0, yielding a maximum possible score of 5. An LC₅₀ value exceeding 500 µg/mL was applied as a compulsory safety prerequisite, and only compounds meeting this requirement were eligible for scoring. Compounds achieving a total score of ≥4/5 were subsequently shortlisted for Phase II mechanistic investigations using a dual-species biofilm bead model. Based on this predefined and integrated selection framework, PUN and PUG were advanced for continued investigation.

Table 3.4

Phase I Multi-Assay Screening Strategy Used to Prioritise Pomegranate-Derived Compounds (PDCs) For Phase II Investigation

The strategy integrates antibacterial reference assays, antibiofilm selectivity criteria, virulence-related functional assays, and preliminary toxicity assessment.

Assay	Purpose	Selection Criterion for Phase II
Disc Diffusion Assay	Preliminary screening of antibacterial activity	Supportive evidence only (zone of inhibition indicating susceptibility). Not used as a primary selection criterion.
Minimum Inhibitory Concentration (MIC)	Determination of the lowest concentration that inhibits visible bacterial growth	Used for reference and to guide concentration ranges for MBIC/MBEC assays.
Minimum Bactericidal Concentration (MBC)	Determination of the lowest concentration that kills bacteria	Used for reference and to guide concentration ranges for MBIC/MBEC assays.
Cell Surface Hydrophobicity Assay	Evaluation of bacterial cell surface hydrophobicity	Compounds showing substantial reduction in hydrophobicity were prioritised when antibiofilm potency (MBIC/MBEC) was comparable.

Assay	Purpose	Selection Criterion for Phase II
Minimum Biofilm Inhibitory Concentration (MBIC)	Assessment of the ability to prevent biofilm formation	Compounds with MBIC ₉₀ values against <i>S. mutans</i> ≤ those for <i>S. gordonii</i> were advanced to Phase II; MBIC ₅₀ values were reported for reference only.
Minimum Biofilm Eradication Concentration (MBEC)	Evaluation of the ability to disrupt established biofilms	Compounds with MBEC ₅₀ values against <i>S. mutans</i> ≤ those for <i>S. gordonii</i> were advanced to Phase II; MBEC ₉₀ values were reported for reference only.
Glycolytic pH Drop Assay	Measurement of acid production through glycolysis (acidogenicity)	Compounds were prioritised if they prevented the glycolytic pH of <i>S. mutans</i> suspensions from falling below pH 5.5
Brine Shrimp Lethality Assay (BSLA)	General screening for cytotoxicity using <i>Artemia salina</i> nauplii to determine LC ₅₀ = Lethal concentration to 50% of brine shrimp nauplii.	Ideal cut-off: LC ₅₀ > 500 µg/mL (low toxicity).

3.16 Phase II: Dual-Species Biofilm Evaluation of Selected Lead Compounds

3.16.1 Biofilm Inhibition Assay of PUG and PUN Against Dual-Species Biofilms

3.16.1.1 Biofilm Formation and Treatment

The acquired pellicle was formed on glass beads (5 mm diameter), which were individually placed in 24-well plates and inoculated with *S. mutans* and *S. gordonii* at a 1:1 ratio (1×10^6 CFU/mL). For biofilm inhibition assays, test compounds were added at time zero (bacterial inoculation) and remained present throughout the biofilm development period. Dual-species biofilms were incubated under optimised conditions as described in Section 3.10. The effects of PUN and PUG at 50 µg/mL (MBIC₉₀) were evaluated on biofilm biomass, microcolony architecture, bacterial viability, and gene expression. The concentration of 50 µg/mL was selected based on the MBIC₉₀ values determined for *S. mutans*, given its dominant role in cariogenic biofilm formation. Targeting the MBIC₉₀ of *S. mutans* allows evaluation of compound effects on the primary cariogenic pathogen while maintaining the ecological context of the dual-species biofilm model.

For each treatment group, a total of twenty-four biofilm-coated beads were prepared in twenty-four independent wells (one bead per well) to ensure biological

independence across two time points. Twelve beads were designated for the early biofilm stage (8 hours), and twelve beads for the established biofilm stage (24 hours). These time points were selected to capture transcriptional responses associated with initial bacterial adhesion and extracellular polysaccharide synthesis during early biofilm development, as well as gene regulation associated with mature biofilm architecture and metabolic activity in established biofilms (Bowen & Koo, 2011; Lemos et al., 2019; Zhou et al., 2025).

Following incubation, beads from each time point were randomly allocated to downstream assays: three beads for biomass quantification, three for CFU enumeration, three for RT-qPCR, and three for scanning electron microscopy (SEM). Each bead represented a separate technical replicate for the respective downstream assay, as beads within the same treatment group were generated from the same bacterial inoculum, treatment preparation, and incubation run. No bead was reused across time points or analytical procedures. This design enabled parallel qualitative and quantitative assessment of biofilm responses across multiple analytical endpoints while avoiding repeated measurement of the same biofilm-coated bead.

Where independent experimental runs were performed, biological replication was defined at the level of separately prepared bacterial cultures and independently repeated biofilm experiments, rather than at the level of individual beads. Technical replicate values from beads within the same experimental run were therefore interpreted as within-run replicate measurements, whereas independent experimental repeats represented biological replicates.

3.16.1.2 Assessment of Biomass Following Biofilm Inhibition Treatment

Biofilm biomass was quantified using CV assay. Following incubation, beads were gently washed by sequential dipping into sterile phosphate-buffered saline (PBS, pH 7.2) three times to remove loosely attached and planktonic cells, taking care to minimise disruption of the adherent biofilm. The washed beads were transferred to a fresh 24-well plate and air-dried at room temperature. Biofilm biomass was stained by immersing each bead in 0.1% (w/v) CV solution for 15 minutes at room temperature. Beads were then rinsed thoroughly with distilled water until the washings were clear and air-dried completely. The bound CV was solubilised by transferring each bead to a new well containing 33% (v/v) acetic acid and incubating for 15 minutes with gentle

agitation. The resulting eluate was mixed, and an aliquot of 200 μL from each well was transferred to a 96-well microplate for absorbance measurement at 595 nm (OD_{595}) using a microplate reader. Biomass was expressed as OD_{595} values per bead.

3.16.1.3 Assessment of Viable Cells Following Biofilm Inhibition Treatment (CFU Enumeration)

The bacterial population in the biofilm was determined by gently dipping the individual bead in 10 ml of sterile distilled water to remove planktonic cells, then transferring the bead to a sterile microcentrifuge tube containing 1 ml of sterile PBS. The tubes were sonicated for a few seconds and vortexed for 1 min to dislodge attached cells. The suspension was then serially diluted in PBS. 100 μL of bacterial suspension from each tube was pipetted onto three separate MSA plates (triplicate). The plates were incubated in 5% CO_2 at 37 $^{\circ}\text{C}$. The CFU were counted after 24-48 hours of incubation, and the distinct colony morphologies allowed for differentiation between the two species as described in section

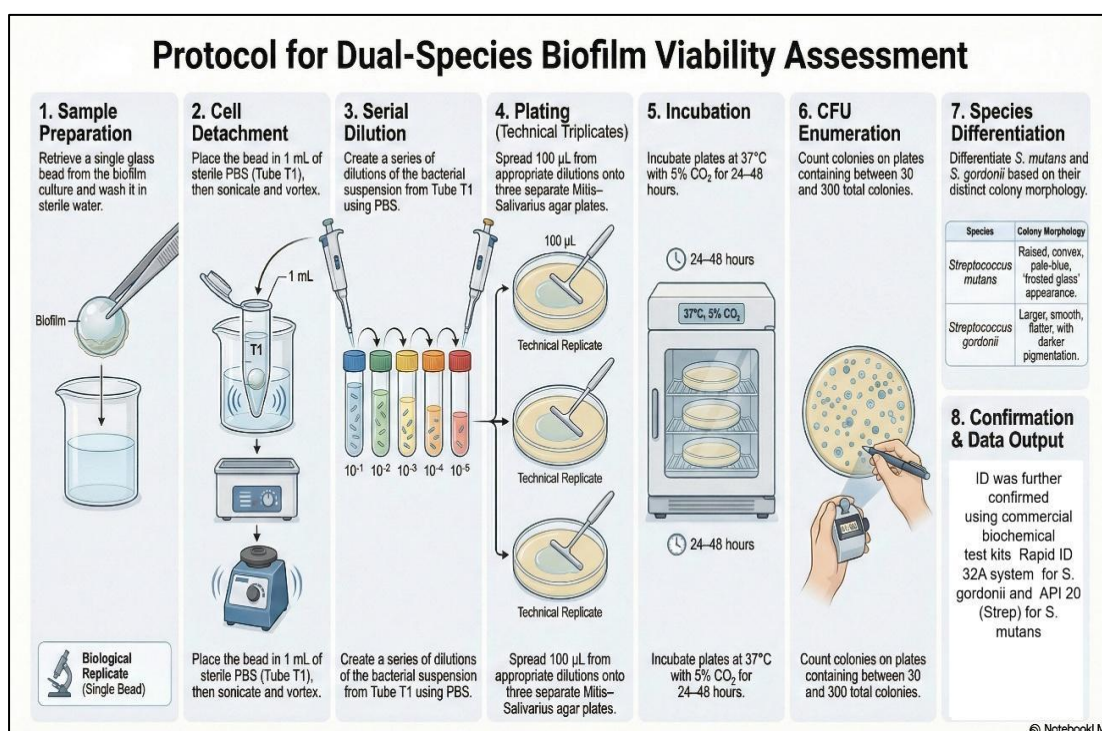


Figure 3.3 Workflow for Assessment of Viable Cells in Dual-Species Biofilms by CFU Enumeration

Biofilm-associated cells were detached from individual glass beads, serially diluted, and plated in technical triplicate on Mitis–Salivarius agar to determine species-specific CFU counts.

3.16.1.4 Scanning Electron Microscope (SEM) Analysis

To observe the surface architecture and structural morphology of dual-species biofilms formed on saliva-coated glass beads, scanning electron microscopy (SEM) was performed on samples collected 24 hours post-treatment.

Three representative beads from each treatment group ($n = 3$) were fixed overnight in 2.5% glutaraldehyde at 4 °C to preserve biofilm structure. The following day, beads were washed with 0.1 M sodium cacodylate buffer (pH 7.4) and post-fixed in 2% (w/v) osmium tetroxide prepared in the same buffer. After fixation, samples were rinsed twice with distilled water (15 minutes per wash) and dehydrated through a graded ethanol series (10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 95%), each for 15 minutes. Complete dehydration was achieved by two treatments with 100% ethanol, followed by incubation in ethanol–acetone mixtures at 3:1, 1:1, and 1:3 ratios (15 minutes per step). Final dehydration was carried out in 100% acetone, repeated three times for 15 minutes each. Samples were subjected to critical-point drying (CPD) for approximately 2 hours to preserve ultrastructural integrity.

Once dried, beads were mounted onto aluminium stubs using copper tape and sputter-coated with gold under low vacuum using an ion sputter coater. Field emission scanning electron microscopy (FESEM) analysis was performed using an FEI Quanta™ FEG 450 electron microscope at magnifications of 1,000× and 5,000×.

Although *S. mutans* and *S. gordonii* differ in ultrastructural characteristics, reliable species differentiation cannot be achieved by SEM alone due to their similar coccoid morphology and overlapping size ranges. Therefore, SEM was used solely to evaluate overall biofilm morphology, including structural density, extracellular matrix distribution, and surface attachment characteristics, rather than species-specific identification within the mixed biofilm.

3.16.1.5 Gene Expression Analysis Following Treatment with PUG and PUN

RNA was extracted from bacterial cells attached to saliva-coated glass beads at 8 hours and 24 hours post-inoculation, representing early and established biofilm stages, to evaluate gene expression in dual-species biofilms. At each time point (8 hours and 24 hours), biofilm-coated beads were removed from the wells using sterile forceps and placed into 1.5 mL microcentrifuge tubes containing 1 mL of cold PBS. Beads were

vortexed vigorously for 1 minute to dislodge adherent bacterial cells into suspension. Immediately after vortexing, the suspension was transferred into a new tube containing two volumes of RNeasy Protect Bacteria Reagent (i.e., 1 mL bacterial suspension + 2 mL RNeasy Protect) to stabilise RNA and prevent post-sampling transcriptional changes. The mixture was vortexed briefly and incubated at room temperature for 5 minutes. Samples were centrifuged at $5,000 \times g$ for 10 minutes at 4°C to pellet the stabilised bacteria. The supernatant was discarded, and the pellet was used for RNA extraction or stored at -80°C until further processing. The bacterial pellet was resuspended in 200 μL of TE buffer (Promega) containing lysozyme (15 mg/mL) and 20 μL of Qiagen proteinase K, and incubated at room temperature for 10 minutes to weaken the cell walls. RNA extraction was then performed using the RNeasy Minikit (Qiagen, Germany) according to the manufacturer's instructions. Seven hundred microlitres (700 μL) of RNeasy lysis buffer, RLT (with 10 μl β -mercaptoethanol per ml), was added, and the mixture was vortexed vigorously. Then, 96-100% ethanol (500 μl) was added and mixed immediately by pipetting. Half of the mixture (700 μl) was transferred to an RNeasy spin column and centrifuged for 15 s at $8000 \times g$. The flow-through was discarded after centrifugation and reinserted in the collection tube. This step was repeated twice to collect all the bacterial lysate. Bacterial lysate was washed with 350 μl of buffer RW1, centrifuged for 15 seconds at $8000 \times g$, and the flow-through was discarded.

DNase 1 (Qiagen, Germany) was prepared according to the manufacturer's instructions. Ten μl DNase 1 was added to 70 μl Buffer RDD and added directly onto the RNeasy spin column membrane before incubation at room temperature for 15 minutes. gDNA was removed from the mini spin column by buffer RW1 (350 μl) by centrifuging for 15 s at $\geq 8000 \times g$. The flow-through was discarded, and the mini spin columns were placed in new tubes. RPE buffer (500 μl) was added twice to the same RNeasy spin column in two separate steps, and the column was centrifuged at $8000 \times g$ for 15 seconds and 2 minutes, respectively, before discarding the flow-through from the collection tube. RNA was eluted into a new 1.5 ml collection tube by adding 30 μl RNase-free water directly to the spin column and centrifuging at $\geq 8000 \times g$ for 1 minute. This step was repeated twice to elute all RNA. A Nano-Drop spectrophotometer was used to determine RNA concentration and purity. Three μl of eluted RNA was used to determine the concentration by measuring the A_{260} absorbance for each sample. Three independent measurements of the same sample were performed. Purity was assessed by calculating the A_{260}/A_{280} ratio. A_{260}/A_{280} ratios between 1.8 and 2.0 were used in the

gene expression study. Nuclease-free water was used as blanks. All RNA samples were diluted to 10 ng μl^{-1} in nuclease-free water and subjected to cDNA synthesis. cDNA synthesis was performed using the Quantitect reverse transcription kit (Qiagen, Germany). It consisted of three steps, as described below. In the first step, 1 $\times$ gDNA wipeout buffer (2 μl) was added to 12 μl RNA (10 ng/ μL), as shown in Table 3.5, and incubated at 42 °C for 2 minutes.

Table 3.5

Composition of Reagents Used to Eliminate Genomic DNA before Reverse Transcription

Component	Volume/reaction
gDNA Wipeout Buffer, 7 $\times$	2 μl
RNA (10 ng μl^{-1})	12 μl
Total volume	14 μl

In the second step, a 2 $\times$ Reverse Transcription (RT) master mix was prepared using the components provided in the kit, to which 14 μl of template RNA was added, as outlined in Table 3.6. A reverse transcriptase-negative control (RT control) was also included, containing all reaction components except that water was used instead of Quantiscript reverse transcriptase. This control was used to verify the absence of genomic DNA contamination in the RNA sample.

The third step involved synthesising complementary DNA (cDNA) from the extracted RNA through reverse transcription. This was carried out using a thermal cycler, with cycling conditions optimised according to the primers' specific annealing and extension requirements. The reverse transcription process consisted of an initial incubation at 42 °C for 15 minutes to enable cDNA synthesis, followed by a 3-minute incubation at 85 °C to inactivate the enzyme and halt the reaction. The resulting cDNA was stored at –20 °C for subsequent analyses.

Quantitative PCR (qPCR) was then performed using 96-well MicroAmp® plates (Applied Biosystems, Cat. No. N801-0560), sealed with optical adhesive films (Applied Biosystems, Cat. No. 4313663). Amplification was carried out on a StepOnePlus™ Real-Time PCR System (Applied Biosystems) in accordance with the manufacturer's instructions. The plate layout, including the arrangement of samples and controls, is shown in Figure 3.4.

Table 3.6

Components Used to Synthesise Complementary DNA (cDNA) from RNA Samples

Component	Volume/ reaction
Reverse-transcription master mix	
Quantiscript Reverse Transcriptase	1 μ l
Quantiscript RT buffer, 5x	4 μ l
RT primer MIX	1 μ l
Template RNA	14 μ l
Total volume	20 μ l

Note: The kit components were thawed on ice, and the RT master mix prepared on ice.

Quantitative real-time PCR (RT-qPCR) was performed using iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions, gene-specific primers (Table 3.9 and Table 3.10), and nuclease-free water. The final reaction volume was 20 μ l per well. A no-template control was included by substituting cDNA with water. The reactions were set up in triplicate as outlined in Table 3.7. Plates were centrifuged at 1500 rpm for 3 minutes at 20 °C to ensure all components settled at the bottom of the wells.

Table 3.7

qPCR Reaction Setup Using SYBR Green Master Mix

Component	Volume/ Reaction (μ l)
IQ SYBR Green Supermix	10
Forward primer (10 μ M)	0.4
Reverse primer (10 μ M)	0.4
RNAse and DNAse –free water	8.2
cDNA (10ng/ μ l)	1
Total volume/well	20

The PCR machine thermocycling profile was configured as shown in Table 3.8. qPCR was performed on a real-time PCR system, using the standard amplification conditions recommended for SYBR Green–based detection. The thermal protocol included initial enzyme activation, denaturation, annealing, and extension steps. A melt curve analysis was performed at the end of the amplification cycles to verify the specificity of the PCR products.

Table 3.8
Thermocycling Conditions Showing Temperature and Duration for Each Step

Temperature (°C)	Time (min: sec)
50	2.00
95	10.00
95	00:15 denaturation
59 (T _m value for each primer)	1:00 min annealing and extension

Reactions were run for 40 cycles, and amplified PCR products were subjected to melting curve analysis (65–95 °C) to confirm amplification specificity. Threshold cycle (C_t) values were obtained for each reaction. Relative gene expression was calculated using the comparative cycle threshold ($2^{-\Delta\Delta C_t}$) method (Livak & Schmittgen, 2001). C_t values of target genes were normalised to the endogenous reference gene (16S rRNA) to obtain ΔC_t , followed by comparison with the untreated control group (cells grown under the same conditions without compound) to determine $\Delta\Delta C_t$. The reference gene exhibited stable C_t values across all experimental groups. Each bead represented a technical replicate for the respective treatment group within the same experimental run. For RT-qPCR analysis, RNA extracted from each bead-derived biofilm sample was analysed in technical triplicates. Fold changes in gene expression were expressed relative to the untreated control group.

Table 3.9

Target Genes and Primer Sequences Used For RT-qPCR Analysis of Biofilm- and Virulence-Associated Genes in *S. mutans* During Biofilm Inhibition Assays

Gene	Description		Primer sequence (5'-3')	Reference
<i>16S rRNA</i>	Housekeeping gene	F	CCTACGGGAGGCAGCAGTAG	(Kim et al., 2015)
		R	CAACAGAGCTTTACGATCCGAAA	
<i>gtfB</i>	Synthesise insoluble glucans for biofilm structure	F	AGCAATGCAGCCAATCTACAAAT	
		R	ACGAACCTTGCCGTTATTGTCA	
<i>gtfC</i>	Produces soluble and insoluble glucans for adhesion	F	GGTTTAACGTCAAAATTAGCTGTATTAGC	
		R	CTCAACCAACCGCCACTGTT	
<i>gtfD</i>	Binds extracellular glucans, supporting biofilm stability.	F	ACAGCAGACAGCAGCCAAGA	
		R	ACTGGGTTTGCTGCGTTTG	
<i>gbpB</i>	Involved in cell surface integrity and biofilm accumulation	F	ATGGCGGTTATGGACACGTT	
		R	TTTGGCCACCTTGAACACCT	
<i>brpA</i>	Regulates biofilm maturation and stress tolerance.	F	GGAGGAGCTGCATCAGGATTC	(Hasan et al., 2014)
		R	AACTCCAGCACATCCAGCAAG	
<i>comDE</i>	Controls quorum sensing and biofilm-related gene regulation	F	ACAATTCCTTGAGTTCCATCCAAG	
		R	TGGTCTGCTGCCTGTTGC	
<i>ldh</i>	Catalyses lactic acid production during fermentation	F	TAT GAA GAC TGT GCG GAT GC	(Yamashita et al., 2019)
		R	GGT TAG CAG CAA CGA GGA AG-	
<i>atpD</i>	Maintains intracellular pH via proton extrusion.	F	TGT TGA TGG TCT GGG TGA AA	
		R	TTT GAC GGT CTC CGA TAA CC	

Note: F = forward primer; R = reverse primer

Table 3.10

Target Genes and Primer Sequences Used for RT-qPCR Analysis of Biofilm- And Virulence-Associated Genes in *S. gordonii* During Biofilm Inhibition Assays

Gene	Description		Primer sequence (5'-3')	Reference
<i>16S rRNA</i>	Housekeeping gene	F	AAGCAACGCGAAGAACCTTA	Huang et al., 2014)
		R	GTCTCGCTAGAGTGCCCAAC	
<i>gtfG</i>	Synthesises glucans contributing to biofilm matrix formation.	F	CCAAGACTTTGCAACCCGTG	
		R	GACACTGTGGAGAGCACGAA	
<i>abpA</i>	Encodes amylase-binding protein A, mediating adhesion to salivary amylase.	F	TGATGCAGTTGAAGGTGGAA	
		R	TAGCTGCACCAACACGTTTC	
<i>cshA</i>	Encodes a surface protein regulating cell surface hydrophobicity and adhesion.	F	CAGACGATGCAACCCCTATT	
		R	TAACGGTCAAGGTCACCACA	
<i>sfpA</i>	Encodes an antigen I/II family adhesin involved in surface attachment and co-adhesion.	F	GGTTGTCCAGCCAGCCTTAT	
		R	CTGCCTTAATTTGGGCCTGC	

Note: F = forward primer; R = reverse primer

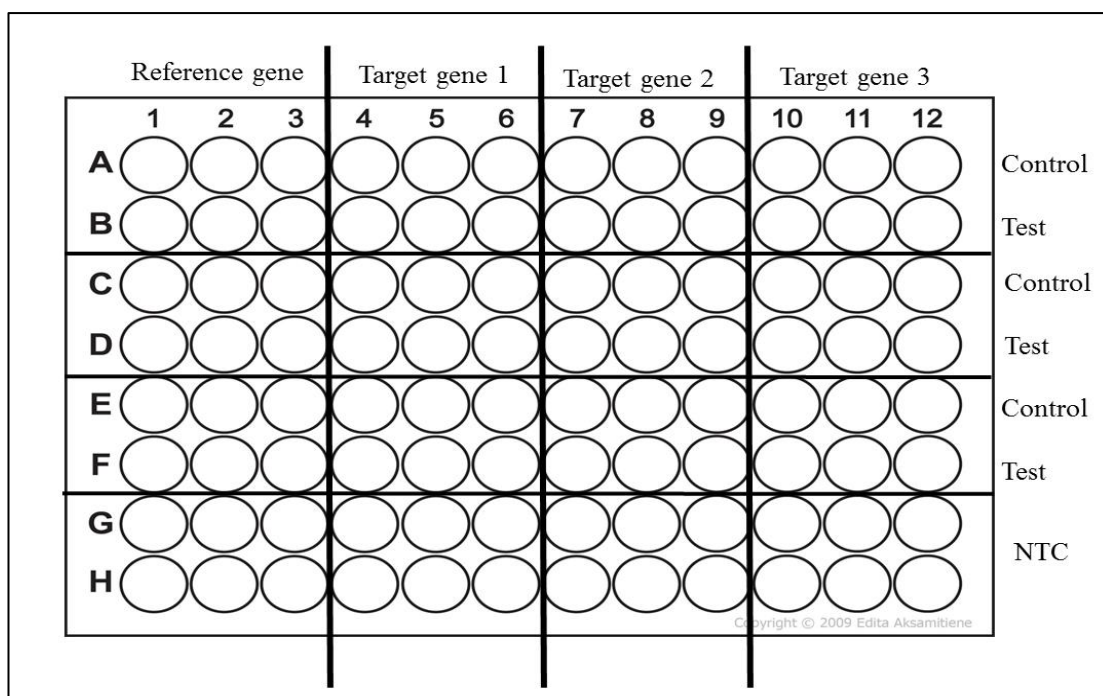


Figure 3.4 Schematic Diagram Of RT-qPCR Plate Layout

Each gene was analysed in technical triplicates. Columns 1–3 represent the reference gene, while columns 4–6, 7–9, and 10–12 represent target genes 1, 2, and 3, respectively. Rows A–F correspond to control and treated samples across three technical replicates, while rows G–H represent no-template controls (NTC).

3.16.2 Biofilm Eradication Activity of PUN and PUG on Preformed Dual-Species Biofilms

3.16.2.1 Formation of Preformed Biofilms and Treatment

The acquired pellicle was formed on sterile glass beads (5 mm diameter), which were individually placed into the wells of a 24-well tissue culture plate. A dual-species suspension of *S. mutans* and *S. gordonii* was prepared at a 1:1 ratio (1×10^6 CFU/mL per strain). Each well received one bead and 1 mL of the inoculum in BHI broth supplemented with 1% (w/v) sucrose. The plate was incubated statically at 37 °C for 48 hours to allow the formation of mature biofilms on the glass beads, with the culture medium replenished every 24 hours to maintain nutrient availability. During this 48-hour incubation period, no test compounds were present, and this duration represents biofilm growth (incubation) time rather than treatment exposure.

After incubation, nonadherent cells were removed by washing the beads twice with sterile PBS. For biofilm eradication assays, treatments were applied only after biofilm maturation, and the defined treatment periods (5 minutes or 4 hours) represent

compound exposure times rather than biofilm incubation durations. Mature biofilms were divided into two treatment groups: one exposed to PUG and PUN at 100 µg/mL (MBEC₅₀) for 5 minutes, and the other for 4 hours, both at 37 °C. The two treatment durations (5 minutes and 4 hours) were designed to differentiate between extrinsic effects (immediate, surface-level disruption) and intrinsic mechanisms (time-dependent intracellular or metabolic effects) contributing to biofilm eradication. In oral biofilm models involving *S. mutans*, very short exposure periods (approximately 1–5 minutes) primarily evaluate rapid surface interactions between antimicrobial agents and the biofilm, including disruption of the EPS matrix (Pandit et al., 2024). In contrast, longer exposure periods allow assessment of time-dependent antimicrobial responses within established biofilms, including metabolic inhibition, stress responses, and modulation of biofilm-associated gene expression. Accordingly, 5-minute and 4-hour exposure periods were selected to distinguish rapid surface-level biofilm disruption from deeper physiological responses within mature dual-species biofilms. For each treatment duration, beads treated with 1% DMSO served as the negative control, and those treated with 0.12% CHX as the positive control. For each treatment group and time point, three independent beads (technical replicates, n = 3) were analysed.

3.16.2.2 Assessment of Biomass Within Established Dual-Species Biofilms Following Eradication Treatment

Biomass following eradication treatment was quantified using the CV assay as described for the biofilm inhibition experiments (Section 3.16.1.2), with compound exposure applied to established dual-species biofilms. Each bead represented one independent technical replicate (n = 3) per treatment condition.

3.16.2.3 Assessment of Viable Cells Following Biofilm Eradication Treatment (CFU Enumeration)

Viable cell enumeration following biofilm eradication treatment was performed using the same CFU counting protocol described for the biofilm inhibition assay. CFU values were calculated for each independent bead (n = 3 per group) and expressed as CFU/mL following adjustment for dilution factor and plated volume.

3.16.2.4 Scanning Electron Microscopy (SEM) Analysis

Biofilm-coated beads were prepared and processed according to the protocol described in Section 3.16.1.5. Three independent beads per treatment group ($n = 3$) were examined to evaluate structural alterations following eradication treatment. SEM analysis was performed only for the 4 hours treatment condition, as structural changes in biofilm architecture such as extracellular matrix disruption and microcolony destabilisation typically require longer exposure to antimicrobial agents and are unlikely to be detectable following very short treatment durations such as 5 minutes.

3.16.2.5 Gene Expression Analysis Following Treatment with PUG and PUN

The procedures for RNA extraction, cDNA synthesis, and RT-qPCR followed the protocol in Section 3.16.1.4, with minor modifications to the primers. Specifically, certain primers were retained from the previous set, whereas others were replaced to target different genes under investigation. The primer sequences used for gene expression analysis in this assay are listed in Table 3.11 and Table 3.12. Each treatment group included three independent technical replicates ($n = 3$), with qPCR reactions performed in technical triplicate.

Table 3.11

Target Genes and Primer Sequences Used for RT-qPCR Analysis of Biofilm- and Virulence-Associated Genes in *S. mutans* During Biofilm Eradication Assays

Gene	Description		Primer sequence (5'-3')	Reference
<i>16S rRNA</i>	Housekeeping gene	F	CCTACGGGAGGCAGCAGTAG	(Kim et al., 2015)
		R	CAACAGAGCTTTACGATCCGAAA	
<i>gtfB</i>	Synthesises insoluble glucans for biofilm structure	F	AGCAATGCAGCCAATCTACAAAT	
		R	ACGAACTTTGCCGTTATTGTCA	
<i>gtfC</i>	Produces soluble and insoluble glucans for adhesion	F	GGTTTAACGTCAAAATTAGCTGTATTAGC	
		R	CTCAACCAACCGCCACTGTT	
<i>gbpD</i>	Binds extracellular glucans, supporting biofilm stability.	F	ACAGCAGACAGCAGCCAAGA	
		R	ACTGGGTTTGCTGCGTTTG	
<i>gbpB</i>	Involved in cell surface integrity and biofilm accumulation	F	ATGGCGGTTATGGACACGTT	
		R	TTTGGCCACCTTGAACACCT	
<i>altA</i>	Involved in autolysis and cell wall turnover	F	CAGCAAACACAGGAACAGTCTG	(Nagasawa et al., 2020)
		R	TCAGGAGTCTGCACAGAACTAG	
<i>lytF</i>	Encodes an autolysin contributing to cell separation and biofilm development.	F	GAATGCTGGCTGTTAGTTCTTATAC	Nagasawa et al., 2023
		R	CTGTACAGCGTTGACATCAAAAG	

Note: F = forward primer; R = reverse primer

Table 3.12

Target Genes and Primer Sequences Used For RT-qPCR Analysis of Biofilm- And Virulence-Associated Genes in *S. gordonii* During Biofilm Eradication Assays

Gene	Description		Primer sequence (5'-3')	Reference
<i>16S rRNA</i>	Housekeeping gene	F	AAGCAACGCGAAGAACCTTA	(Huang et al., 2014)
		R	GTCTCGCTAGAGTGCCCAAC	
<i>cshA</i>	Encodes a surface protein regulating cell surface hydrophobicity and adhesion.	F	CAGACGATGCAACCCCTATT	
		R	TAACGGTCAAGGTCACCACA	
<i>sfpA</i>	Encodes an antigen I/II family adhesin involved in surface attachment and co-adhesion.	F	GGTTGTCCAGCCAGCCTTAT	
		R	CTGCCTTAATTTGGGCCTGC	
<i>scaA</i>	Encodes a metal ion (Mn ²⁺ /Fe ²⁺) uptake component involved in oxidative stress tolerance and biofilm persistence	F	GGGAATATC TTGGCGGTACAA	(Veerapandian & Vedyappan, 2019)
		R	GGTCTTGAGACTCTTGGCATAG	
<i>scaR</i>	Encodes a regulatory protein controlling metal ion homeostasis and adaptive stress responses	F	TAGTCCACCATCTGGGCTATAC	
		R	GCCAACTTGAAGGCCATTTC	

Note: F = forward primer; R = reverse primer

3.17 Operational Definition of Ecological Selectivity

In this study, ecological selectivity was defined before data analysis to ensure consistent interpretation of the dual-species biofilm findings. Selectivity was considered to reflect preferential suppression of *S. mutans* while maintaining the viability and functional stability of the commensal species *S. gordonii* within the same biofilm system.

A compound was interpreted as selective when a consistent pattern was observed across microbiological and molecular parameters. This included a statistically significant reduction ($p < 0.05$) in the $\log_{10}$ CFU/mL of *S. mutans* in treated dual-species biofilms compared with untreated controls, together with no statistically significant reduction, or only a comparatively smaller reduction, in the $\log_{10}$ CFU/mL of *S. gordonii* under the same treatment conditions.

At the molecular level, selectivity was further supported by significant downregulation ($p < 0.05$) of *S. mutans* genes associated with virulence, including those involved in extracellular polysaccharide production and acid tolerance. In contrast, expression of *S. gordonii* genes related to adhesion and ecological function was expected to remain unaffected or show no significant suppression.

Scanning electron microscopy (SEM) was used to examine overall biofilm architecture. As species-level differentiation was not possible using conventional SEM, morphological observations were interpreted cautiously. Structural changes such as reduced matrix density or altered microcolony organisation were considered supportive of the microbiological findings but were not used as standalone evidence of selectivity.

Selectivity was therefore determined through integrated interpretation of viable counts, gene expression profiles, and structural observations rather than reliance on a single outcome measure. This predefined integrative framework was applied consistently across all dual-species analyses to minimise interpretative bias. This operational definition is aligned with the ecological plaque hypothesis, which emphasises modulation of pathogenic dominance while preserving beneficial members of the microbial community, rather than indiscriminate biofilm eradication. All dual-species results presented in Chapter 4 were interpreted according to these predefined criteria.

3.18 Statistical Analysis

All quantitative experiments were performed using biological replicates as specified for each experimental phase. Phase I screening assays were conducted using five independent biological replicates ($n = 5$), whereas Phase II glass-bead biofilm assays utilised three independent biological replicates ($n = 3$). Each biological replicate was measured in technical triplicate. Data are presented as mean $\pm$ standard deviation (SD). Normality of data distribution was assessed using the Shapiro–Wilk test prior to statistical analysis. Data sets that satisfied normality assumptions were analysed using appropriate parametric statistical tests. All statistical analyses and graphical visualisations were performed using GraphPad Prism version 10 (GraphPad Software, USA). A p -value < 0.05 was considered statistically significant.

3.18.1 Disc Diffusion Assay

Zone of inhibition (mm) was analysed using one-way ANOVA, followed by Tukey's post hoc test for multiple group comparisons.

3.18.2 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

MIC and MBC values were determined based on visual assessment of turbidity (MIC) and colony growth on agar plates (MBC). No statistical test was applied because these determinations were based on defined endpoints.

3.18.3 Cell Surface Hydrophobicity Assay

Hydrophobicity percentages obtained via the microbial adhesion to hydrocarbons (MATH) method were analysed using one-way ANOVA, with Tukey's post hoc test for group comparison.

3.18.4 Minimum Biofilm Inhibitory and Eradication Assays (MBIC and MBEC)

No inferential statistical analysis was applied to MBIC or MBEC values. These parameters were determined descriptively using a threshold-based approach, defined as the lowest tested concentrations achieving the specified percentage (50% and 90%) reduction in biofilm biomass relative to untreated controls. MBIC and MBEC values are therefore presented as observational outcomes rather than statistically derived estimates.

3.18.5 Glycolytic pH Drop Assay

Changes in pH over time were analysed using two-way ANOVA, with treatment and time as fixed factors. Tukey's post hoc test was applied to assess differences at individual time points across treatments.

3.18.6 Brine Shrimp Lethality Assay

The median lethal concentration (LC_{50}), the concentration required to kill 50% of the brine shrimp nauplii, was determined by plotting mortality data against the logarithm of the compound concentration. The data were fitted using a nonlinear regression model (sigmoidal dose-response curve with variable slope) in GraphPad Prism version 10 (GraphPad Software, USA). The LC_{50} values and corresponding 95% confidence intervals (CI) were extrapolated from the curve. A goodness-of-fit (R^2 value) was reported to assess the accuracy of the fitted model.

3.18.7 Bacterial CFU Counts Obtained from the Dual-Species Biofilm Bead Model

CFU values from biofilm inhibition and eradication assays were $\log_{10}$ transformed to normalise the data. A two-way ANOVA was performed to evaluate the effects of treatment (PUG, PUN, positive control) and sampling time, including their interaction. Tukey's post hoc test was applied for pairwise comparison.

3.18.8 Quantitative Real-Time PCR (RT-qPCR)

Gene expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method, normalised to 16S rRNA as the reference gene. Group differences were assessed using one-way ANOVA followed by Tukey's post hoc test. The target genes were selected in advance based on their known biological relevance; therefore, additional correction beyond Tukey's post hoc test was not applied.

3.18.9 Scanning Electron Microscopy (SEM)

SEM images were assessed qualitatively to observe structural changes in the biofilm between the treated and control groups. No statistical test was applied.

CHAPTER 4

RESULTS

4.1 Disc Diffusion Assay

The antimicrobial activity of selected pomegranate-derived compounds (PDCs), namely corilagin (COR), ellagic acid (EA), gallic acid (GA), kaempferol glucoside (KG), punicalin (PUN), punicalagin (PUG) and rutin (RUT) were evaluated against *Streptococcus gordonii* (*S. gordonii*) and *Streptococcus mutans* (*S. mutans*) using the agar diffusion method, and the results are presented as mean zones of inhibition with corresponding standard deviations (Table 4.1). DMSO (1% v/v) served as the vehicle control and was used as the baseline to confirm that any observed inhibition was attributable to the tested compounds rather than the solvent. CHX (0.12% w/v) served as the positive control and was used as the reference antimicrobial agent for comparison of relative inhibitory activity.

For each bacterial species, inhibition-zone diameters produced by the PDCs and CHX were compared using one-way ANOVA followed by Tukey's post hoc test. Statistical comparisons were therefore performed within each bacterial species, with CHX included as the positive reference control. The DMSO vehicle control was not included in the letter-based comparison because it produced no measurable inhibition zone.

Overall, all tested compounds exhibited measurable inhibitory activity against both microorganisms when compared with the DMSO vehicle control, which showed no inhibition. Among the compounds, PUG produced the largest inhibition zones for both *S. gordonii* (17.5 ± 2.0 mm) and *S. mutans* (18.5 ± 2.0 mm). The inhibition produced by PUG was statistically comparable to that of CHX (0.12%), indicating that PUG exhibited inhibitory activity similar to the positive control under the conditions of this assay ($p > 0.05$). EA also demonstrated potent antimicrobial activity, yielding inhibition zones of 16.0 ± 1.7 mm against *S. gordonii* and 17.0 ± 1.7 mm against *S. mutans*. However, EA produced significantly smaller inhibition zones than PUG and CHX, while remaining significantly more active than several lower-performing compounds ($p < 0.05$).

COR and PUN showed moderate activity against both species, with inhibition zones of 14.0–15.0 mm. Their inhibitory effects were greater than the DMSO vehicle control but significantly lower than those of PUG and CHX ($p < 0.05$). In contrast, GC and KG exhibited comparatively smaller inhibition zones against both bacteria, indicating lower antimicrobial efficacy within the tested group. RUT demonstrated intermediate activity, with inhibition zones significantly lower than those of PUG and CHX, but higher than those observed for GC and KG.

The negative control, DMSO (1% v/v), showed no observable zones of inhibition against either bacterial species. This confirmed that the inhibitory effects observed for the PDCs were compound-associated and not attributable to the solvent vehicle.

Table 4.1

Zones of Inhibition (mm) of PDCs Against *S. gordonii* and *S. mutans*

Values are expressed as mean $\pm$ SD from five independent experiments ($n = 5$). DMSO (1% v/v) was used as the vehicle control to determine whether the solvent contributed to bacterial inhibition, while CHX (0.12% w/v) was used as the positive antimicrobial control. Statistical comparisons of inhibition-zone diameters were performed separately for each bacterial species using one-way ANOVA followed by Tukey's post hoc test. Superscript letters indicate comparisons among treatments with measurable inhibition zones within the same column. Values sharing the same superscript letter are not significantly different ($p > 0.05$), while values with different letters are significantly different ($p < 0.05$). "ni" denotes no inhibition zone.

Compounds	Zone of inhibition (mm $\pm$ SD)	
	<i>S. gordonii</i>	<i>S. mutans</i>
COR	14.0 $\pm$ 1.7 ^c	15.0 $\pm$ 1.7 ^d
EA	16.0 $\pm$ 1.7 ^b	17.0 $\pm$ 1.7 ^c
GC	11.5 $\pm$ 1.4 ^d	12.5 $\pm$ 1.4 ^e
KG	11.0 $\pm$ 1.2 ^d	12.0 $\pm$ 1.2 ^e
PUN	14.0 $\pm$ 1.2 ^c	15.0 $\pm$ 1.2 ^d
PUG	17.5 $\pm$ 2.0 ^a	18.5 $\pm$ 2.0 ^a
RUT	13.0 $\pm$ 1.2 ^{cd}	14.0 $\pm$ 1.2 ^d
CHX (0.12%)	17.0 $\pm$ 1.7 ^a	18.5 $\pm$ 2.0 ^a
DMSO (1%)	ni	ni

Note: Values sharing the same superscript letter within each column are not significantly different ($p > 0.05$), while values with different letters indicate significant differences ($p < 0.05$).

4.2 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of the selected PDCs against *S. gordonii* and *S. mutans* are presented in Table 4.2. CHX was included as a positive control.

For *S. gordonii*, MIC values ranged from 25 to 200 µg/mL. GC exhibited an MIC of 25 µg/mL, whereas COR, EA, KG, and PUG each had an MIC of 100 µg/mL. Higher MIC values of 200 µg/mL were recorded for PUN and RUT. Corresponding MBC values for *S. gordonii* ranged from 100 to 800 µg/mL. The lowest MBC (100 µg/mL) was observed for GC, whereas COR exhibited the highest MBC (800 µg/mL). The MBC/MIC ratios for *S. gordonii* ranged from 2 to 8.

For *S. mutans*, MIC values ranged from 100 to 200 µg/mL. GC, EA, KG, and PUG each exhibited an MIC of 100 µg/mL, whereas COR, PUN, and RUT exhibited an MIC of 200 µg/mL. MBC values for *S. mutans* ranged from 200 to 400 µg/mL. The calculated MBC/MIC ratios for *S. mutans* ranged from 2 to 4. CHX demonstrated MIC values of 0.015% against *S. gordonii* and 0.03% against *S. mutans*. The corresponding MBC values were 0.015% and 0.06%, respectively. The MBC/MIC ratios for CHX were 1 for *S. gordonii* and 2 for *S. mutans*.

Table 4.2

Minimum Inhibitory Concentrations (MIC), Minimum Bactericidal Concentrations (MBC), and MBC/MIC Ratio of PDCs and CHX against *S. gordonii* and *S. mutans*. MIC and MBC were defined as endpoint values (no inferential statistics). Lower MBC/MIC ratios indicate stronger bactericidal activity. CHX was included as a positive control.

Compound	<i>S. gordonii</i>			<i>S. mutans</i>		
	MIC µg/mL	MBC µg/mL	MBC/MIC ratio	MIC µg/mL	MBC µg/mL	MBC/MIC ratio
GC	25	100	4	100	400	4
COR	100	800	8	200	400	2
EA	100	400	4	100	200	2
KG	100	200	2	100	400	4
PUN	200	400	2	200	400	2
PUG	100	400	4	100	200	2
RUT	200	400	2	200	400	2
CHX	0.015	0.015	1	0.03	0.06	2

4.3 Cell Surface Hydrophobicity (CSH) of *S. mutans* and *S. gordonii* Following PDCs Treatment

The effect of compounds on the CSH of *S. mutans* was evaluated by measuring the hydrophobicity index (HI, %) following exposure to subinhibitory concentrations (0.125×MIC, 0.25×MIC, 0.5×MIC) and to the MIC. The results are shown in Figure 4.1. Statistical differences among groups were analysed using one-way ANOVA followed by post hoc multiple comparisons, with significance indicated by different superscripts ($p < 0.05$).

Untreated control cells consistently exhibited high hydrophobicity (HI = 71%), reflecting the inherent hydrophobicity of *S. mutans* under baseline conditions. Among the PDCs, PUG, PUN and RUT produced significant reductions in HI at sub-MIC concentrations. For PUG, HI values were comparable to the control at 0.125×MIC ($p > 0.05$), but a significant reduction was observed at 0.25×MIC, with further significant decreases at 0.5×MIC and MIC ($p < 0.05$), demonstrating a clear concentration-dependent trend. A similar pattern was observed for PUN, where HI was significantly reduced beginning at 0.25×MIC, followed by progressively lower values at 0.5×MIC and MIC ($p < 0.05$). RUT also induced a statistically significant, graded decrease in hydrophobicity at sub-inhibitory concentrations, with HI values not significantly different from the control at 0.125×MIC ($p > 0.05$), but significantly reduced at 0.25×MIC and further decreased at 0.5×MIC and MIC ($p < 0.05$), indicating a dose-dependent response.

In contrast, COR and EA did not significantly alter HI at lower sub-MIC concentrations (0.125×MIC and 0.25×MIC; $p > 0.05$). However, significant reductions in hydrophobicity were observed at 0.5×MIC, with additional decreases at MIC ($p < 0.05$), indicating that their effects on CSH were evident only at higher concentrations.

GC showed no significant changes in HI at any sub-MIC concentration tested ($p > 0.05$), with a significant reduction observed only at MIC. Similarly, KG did not induce significant alterations in hydrophobicity across all concentrations tested, including MIC ($p > 0.05$).

Overall, the results demonstrate compound-specific and concentration-dependent modulation of *S. mutans* cell surface hydrophobicity, with PUG, PUN, and RUT exhibiting significant effects at sub-MIC concentrations ($\geq 0.25 \times \text{MIC}$), COR and

EA showing reductions only at higher concentrations ($0.5\times\text{MIC}$), and GC and KG displaying minimal or no effect.

The hydrophobicity index (HI) of *S. gordonii* following exposure to increasing concentrations of PDCs is presented in Figure 4.2. Overall, the compounds exhibited compound-specific, concentration-dependent effects on CSH, with varying degrees of statistical significance relative to the untreated control. Treatment with COR resulted in no significant change in HI at sub-MIC concentrations ($0.125\times$, $0.25\times$, and $0.5\times\text{MIC}$), as values remained comparable to the control group ($p > 0.05$). A significant reduction in HI was observed only at the MIC ($p < 0.05$), indicating that COR predominantly affected the CSH of *S. gordonii* at higher concentrations. In contrast, EA exhibited a clear and progressive reduction in HI with increasing concentration. Significant decreases were observed beginning at $0.125\times\text{MIC}$ and continued through MIC, with each concentration showing statistically distinct groupings ($p < 0.05$). This compound demonstrated the most pronounced concentration-dependent effect among all tested compounds. Similarly, KG produced a stepwise and significant decline in HI, with reductions becoming significant from $0.25\times\text{MIC}$ onward ($p < 0.05$). The HI values at $0.5\times\text{MIC}$ and MIC were markedly lower than the control, indicating a consistent downward trend. For GC, HI values remained comparable to the control at sub-MIC concentrations, with a significant reduction observed only at MIC ($p < 0.05$). This pattern suggests a threshold-dependent response, where hydrophobicity changes were evident only at the highest tested concentration.

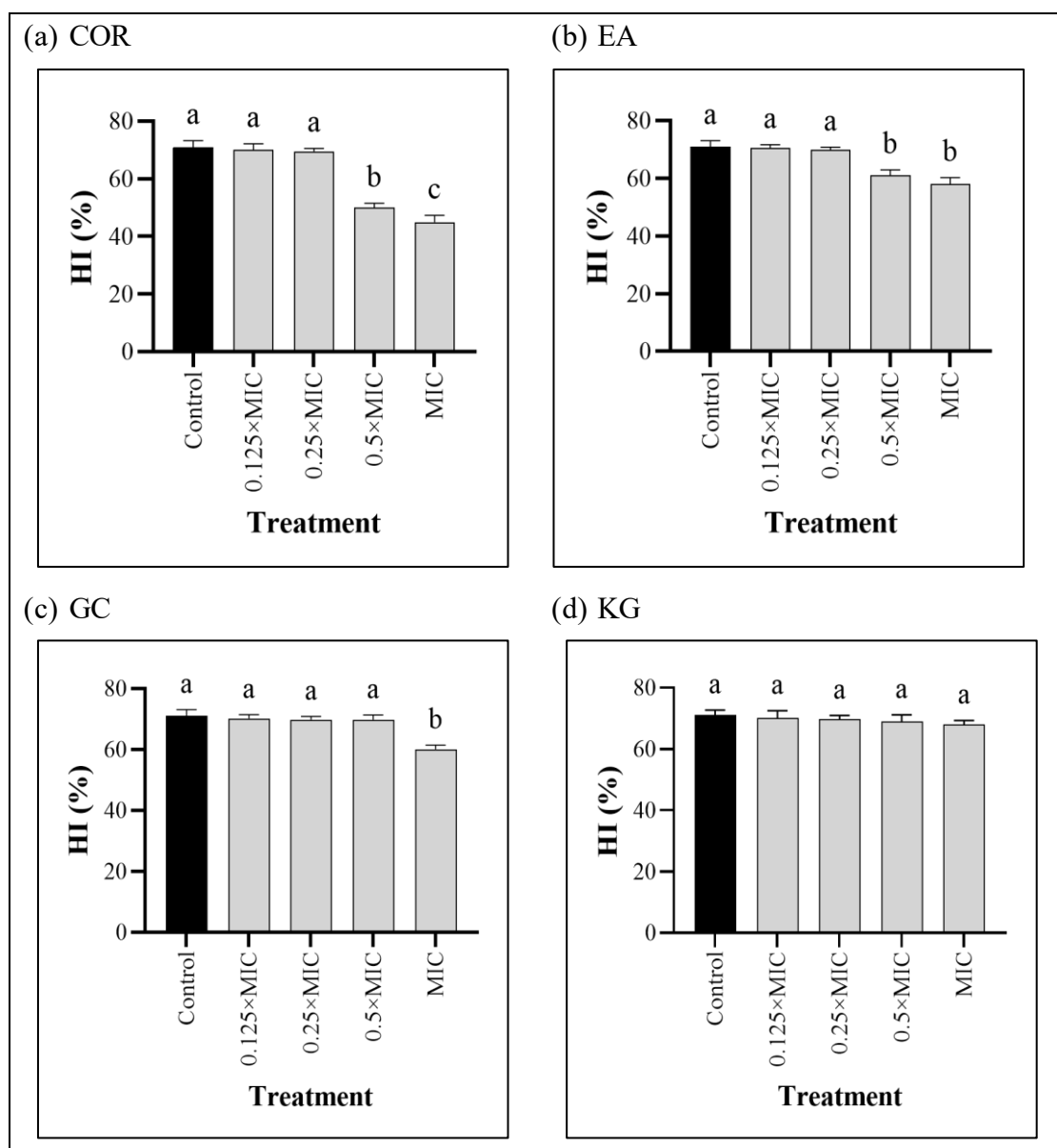


Figure 4.1 Hydrophobicity Index (HI) of *S. mutans* After Exposure to PDCs at Sub-Minimum Inhibitory Concentrations and MIC concentration
Panels represent individual compounds: (a) COR, (b) EA, (c) GC, (d) KG, (e) PUN, (f) PUG, and (g) RUT. Data are presented as mean $\pm$ SD ($n = 5$). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test within each compound panel. Different letters above the bars indicate statistically significant differences ($p < 0.05$).

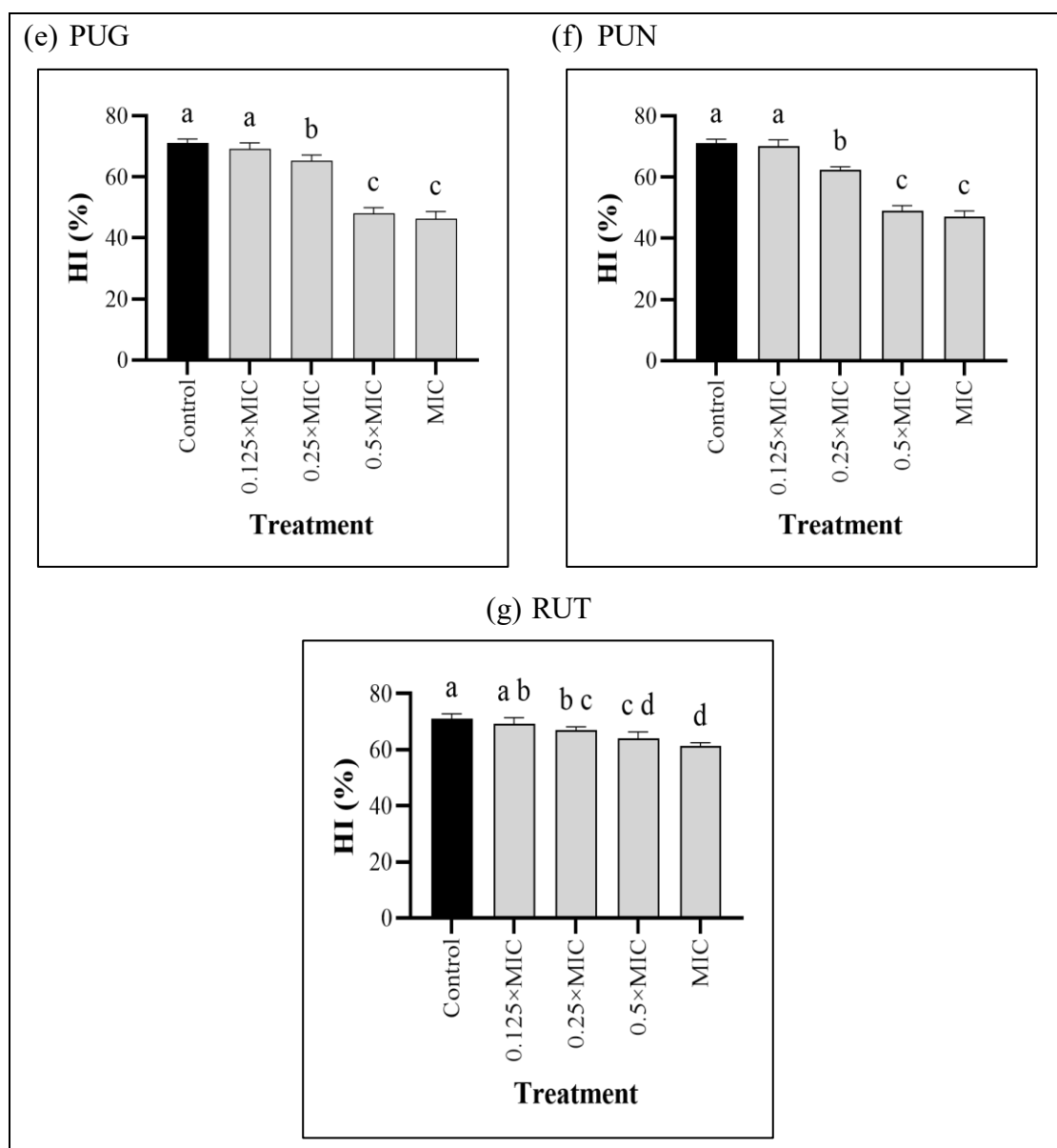


Figure 4.1 (continue)

Treatment with PUG did not result in any statistically significant changes in HI across all tested concentrations ($p > 0.05$). HI values remained stable and comparable to the untreated control, indicating minimal impact on the surface hydrophobicity of *S. gordonii*. A similar trend was observed for PUN, where HI values were maintained at sub-MIC levels, with a significant reduction detected only at MIC ($p < 0.05$). This indicates limited influence on hydrophobicity except at higher concentrations. Finally, RUT demonstrated a significant and concentration-dependent reduction in HI, with statistically significant decreases observed from 0.25× MIC onward. The lowest HI was recorded at MIC, representing the greatest reduction among the tested concentrations for this compound.

Collectively, these findings demonstrate that *S. gordonii* exhibits differential sensitivity of surface hydrophobicity to phytochemical exposure, with EA, KG, and RUT showing clear concentration-dependent reductions, while PUG displayed no detectable effect across the tested range. Other compounds, including COR, GC, and PUN, primarily affected hydrophobicity at MIC, indicating a threshold-dependent response.

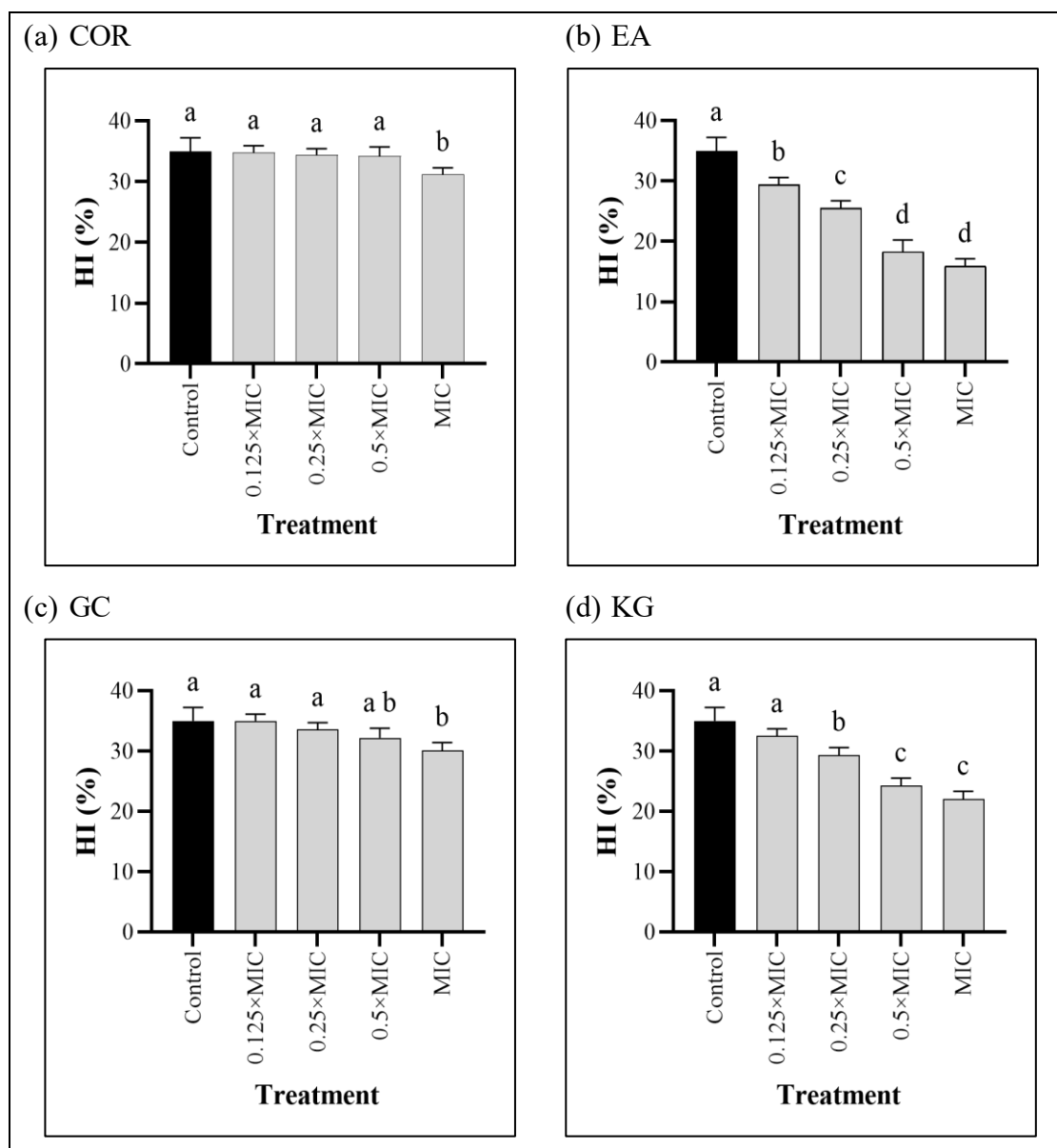


Figure 4.2 Hydrophobicity Index (HI) of *S. gordonii* After Exposure to PDCs at Sub-Minimum Inhibitory Concentrations and MIC Concentration
Panels represent individual compounds: (a) COR, (b) EA, (c) GC, (d) KG, (e) PUN, (f) PUG, and (g) RUT. Data are presented as mean $\pm$ SD (n = 5). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test within each compound panel. Different letters above the bars indicate statistically significant differences ($p < 0.05$).

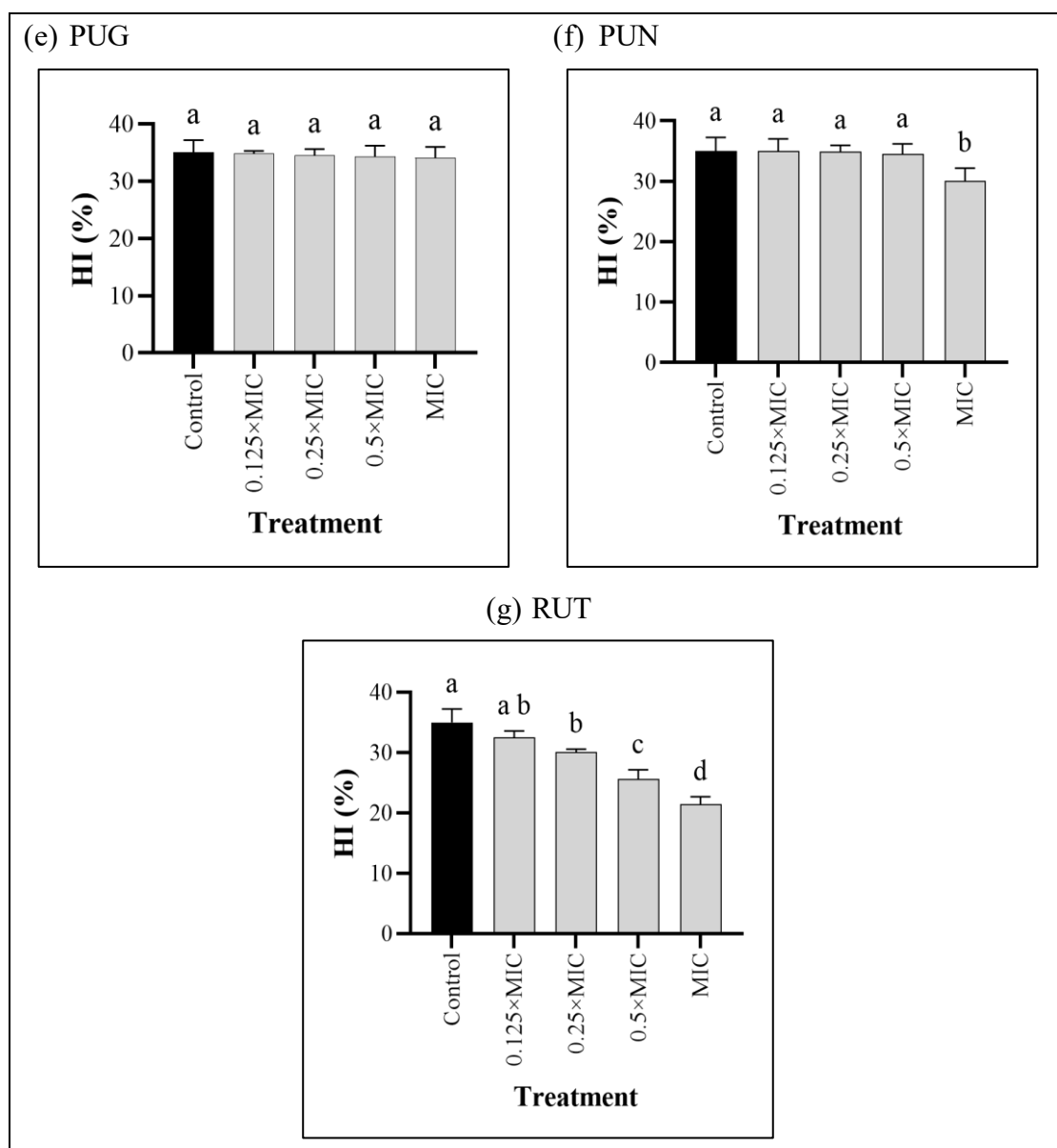


Figure 4.2 (continue)

4.4 Optimisation of Dual-Species Biofilm Formation

Optimisation experiments were conducted to assess the effects of three key parameters initial inoculum density, species inoculum ratio and sucrose concentration, on dual-species biofilm formation by *S. mutans* and *S. gordonii*.

Variation in the initial inoculum density from 1×10^6 to 1×10^8 CFU/mL per species produced distinct differences in biofilm formation and species recovery. Table 4.3 summarises the optimisation results obtained using both 96-well plate and glass-bead models. Differences in biomass and absolute CFU values were expected due to differences in assay configuration and culture volume. However, both experimental

systems showed comparable trends in biomass development and species dominance. At a starting inoculum density of 1×10^6 CFU/mL per species, reproducible biofilm biomass, measured by CV staining was observed at both 24 hours and 48 hours, and viable recovery of both *S. mutans* and *S. gordonii* was consistently detected. Increasing the inoculum density to 1×10^7 CFU/mL resulted in greater total biomass but this was accompanied by a disproportionate increase in the recovery of *S. mutans* relative to *S. gordonii*. At the highest inoculum density tested (1×10^8 CFU/mL), biofilm biomass was markedly increased. However, recovery of *S. gordonii* was substantially reduced or absent, indicating pronounced species dominance by *S. mutans*.

The influence of inoculum ratio on dual-species biofilm composition was evaluated using *S. mutans*:*S. gordonii* ratios of 1:2, 1:1, and 2:1. Biofilms initiated with a 1:2 ratio demonstrated greater recovery of *S. gordonii*, particularly at 24 hours, although overall biomass was relatively lower. In contrast, biofilms formed at a 2:1 ratio exhibited increased biomass but were characterised by numerical dominance of *S. mutans*, with reduced recovery of *S. gordonii*, especially at 48 hours. Among the ratio, the 1:1 inoculum ratio produced reproducible biomass measurements and consistent recovery of both species at both incubation time points, indicating stable coexistence within the dual-species biofilm models (Table 4.4).

Sucrose concentration exerted a strong modulatory effect on both biofilm biomass and species composition. Increasing sucrose concentration from 0.5% to 4% (w/v) resulted in a progressive increase in CV-measured biomass. At 0.5% sucrose, both species were recovered at comparable levels, indicating a relatively balanced biofilm community at 1% sucrose, robust biofilm formation was observed, accompanied by increased recovery of *S. mutans* while maintaining detectable levels of *S. gordonii*. In contrast, sucrose concentrations of 2% and above resulted in marked *S. mutans* dominance with substantially reduced or absent recovery of *S. gordonii*. Higher sucrose concentrations (2% and 4%) promoted excessive biofilm accumulation and more pronounced acidification, but these conditions also disrupted species balance by favouring *S. mutans* dominance and suppressing *S. gordonii*. These conditions were therefore considered less suitable for a stable and interpretable dual-species cariogenic biofilm model.

Table 4.3

Effect of Initial Inoculum Density (CFU/mL) on Dual-Species Biofilm Biomass and Species Recovery in 96-Well and Glass-Bead Models

Initial inoculum (CFU/mL per species)	96-well plate model			Glass bead model			Interpretation	Decision
	CV biomass Mean $\pm$ SD	<i>S. mutans</i> (log10 CFU/mL $\pm$ SD)	<i>S. gordonii</i> (log10 CFU/mL $\pm$ SD)	CV biomass Mean $\pm$ SD	<i>S. mutans</i> (log10 CFU/mL $\pm$ SD)	<i>S. gordonii</i> (log10 CFU/mL $\pm$ SD)		
1×10^6	1.35 ± 0.05	6.01 ± 0.10	6.05 ± 0.12	1.45 ± 0.06	6.0 ± 0.12	6.25 ± 0.15	Balanced recovery; reproducible	Included
1×10^7	1.75 ± 0.08	5.10 ± 0.12	1.60 ± 0.18	1.95 ± 0.10	6.30 ± 0.15	1.10 ± 0.22	Increased dominance of <i>S.</i> <i>mutans</i>	Excluded
1×10^8	1.35 ± 0.12	6.00 ± 0.10	3.80 ± 0.12	1.60 ± 0.15	6.20 ± 0.12	0.40 ± 0.22	Loss of <i>S. gordonii</i>	Excluded

Note: Biomass is expressed as CV absorbance (OD₅₉₅), and viable counts as log₁₀ CFU/mL (mean $\pm$ SD). Data were analysed by one-way ANOVA with Tukey's post hoc test; different letters indicate $p < 0.05$.

Table 4.4

Effect of Inoculum Ratio (*S. mutans*:*S. gordonii*) on Dual-Species Biofilm Biomass and Species Recovery in 96-Well and Glass-Bead Models

Ratio (<i>S. gordonii</i> : <i>S. mutans</i>)	96-well plate model			Glass bead model			Interpretation	Decision
	CV biomass Mean $\pm$ SD	<i>S. mutans</i> (log10 CFU/mL $\pm$ SD)	<i>S. gordonii</i> (log10 CFU/mL $\pm$ SD)	CV biomass Mean $\pm$ SD	<i>S. mutans</i> (log10 CFU/mL $\pm$ SD)	<i>S. gordonii</i> (log10 CFU/mL $\pm$ SD)		
1:2	1.72 $\pm$ 0.09	5.60 $\pm$ 0.14	1.70 $\pm$ 0.20	1.95 $\pm$ 0.11	6.85 $\pm$ 0.16	5.55 $\pm$ 0.25	<i>S. mutans</i> dominance; reduced <i>S. gordonii</i> recovery	Excluded
1:1	1.46 $\pm$ 0.05	5.25 $\pm$ 0.10	3.15 $\pm$ 0.12	1.61 $\pm$ 0.07	5.45 $\pm$ 0.12	3.30 $\pm$ 0.14	Balanced recovery; reproducible	Included
2:1	1.60 $\pm$ 1.35	6.15 $\pm$ 0.55	4.50 $\pm$ 0.60	1.78 $\pm$ 0.42	6.25 $\pm$ 1.65	3.60 $\pm$ 1.70	High variability; poor reproducibility	Excluded

Note: Biomass is expressed as CV absorbance (OD₅₉₅), and viable counts as log₁₀ CFU/mL (mean $\pm$ SD). Data were analysed by one-way ANOVA with Tukey's post hoc test; different letters indicate $p < 0.05$.

Table 4.5

Effect of Sucrose Concentration (%) on Dual-Species Biofilm Biomass and Species Recovery in 96-Well and Glass-Bead Models

Sucrose concentration (%)	96-well plate model				Glass bead model			Interpretation	Decision
	CV biomass Mean $\pm$ SD	<i>S. mutans</i> (log ₁₀ CFU/mL $\pm$ SD)	<i>S. gordonii</i> (log ₁₀ CFU/mL $\pm$ SD)	Final pH (mean $\pm$ SD)	CV biomass Mean $\pm$ SD	<i>S. mutans</i> (log ₁₀ CFU/mL $\pm$ SD)	<i>S. gordonii</i> (log ₁₀ CFU/mL $\pm$ SD)		
0.5	0.28 $\pm$ 0.06	5.90 $\pm$ 0.15	6.10 $\pm$ 0.18	6.35 $\pm$ 0.1	0.35 $\pm$ 0.08	6.05 $\pm$ 0.20	6.20 $\pm$ 0.22	Low biomass with limited acidification; insufficient matrix development	Excluded
1.0	1.55 $\pm$ 0.06	5.30 $\pm$ 0.12	3.20 $\pm$ 0.14	5.48 $\pm$ 0.10	4.89 $\pm$ 0.09	5.50 $\pm$ 0.15	3.35 $\pm$ 0.16	Reproducible biomass with balanced species recovery and moderate acidogenic activity	Included
2.0	2.12 $\pm$ 0.18	6.85 $\pm$ 0.30	1.80 $\pm$ 0.40	4.82 $\pm$ 0.20	8.05 $\pm$ 0.22	7.00 $\pm$ 0.35	1.60 $\pm$ 0.45	Excessive biomass with increased acidification and <i>S. mutans</i> overgrowth	Excluded
4.0	2.20 $\pm$ 0.40	7.60 $\pm$ 0.60	ND	4.55 $\pm$ 0.15	8.45 $\pm$ 0.55	7.90 $\pm$ 0.62	ND	Excessive biomass with strong acidification and complete suppression of <i>S. gordonii</i> suppression of <i>S. gordonii</i>	Excluded

Note

ND : No colonies detected (below the detection limit of approximately 10 CFU/mL; log₁₀ = 1).Biomass is expressed as CV absorbance (OD₅₉₅), and viable counts as log₁₀ CFU/mL (mean $\pm$ SD). Data were analysed by one-way ANOVA with Tukey's post hoc test; different letters indicate p < 0.05.

4.5 Minimum Biofilm Inhibitory Concentration (MBIC) Using Crystal Violet (CV) Assay

Table 4.6 presents the MBIC₅₀ and MBIC₉₀ values of the tested compounds against *S. gordonii*, *S. mutans*, and dual-species biofilms. Overall, the data demonstrate compound- and species-dependent variability in biofilm inhibitory activity. However, dual-species biofilms did not consistently require higher inhibitory concentrations than single-species biofilms for all compounds. Instead, dual-species MBIC values were compound-dependent, with some compounds showing values comparable to one or both single-species biofilms, while a marked increase in the dual-species model was most evident for KG.

Among the tested compounds, PUG and PUN consistently exhibited the lowest MBIC values, particularly against *S. mutans* and dual-species biofilms. PUG showed an MBIC₅₀ of 50 µg/mL and an MBIC₉₀ of 50 µg/mL against *S. mutans*, while the corresponding values in the dual-species model were 25 µg/mL and 50 µg/mL, respectively. These findings indicate that PUG retained strong biofilm-inhibitory activity in the dual-species model, with no increase in MBIC₉₀ compared with the *S. mutans* biofilm. Similarly, PUN demonstrated low MBIC₅₀ and MBIC₉₀ values across all biofilm models, with identical MBIC₉₀ values of 50 µg/mL observed for *S. gordonii*, *S. mutans*, and dual-species biofilms. Thus, PUN showed stable inhibitory activity across both single-species and dual-species conditions.

GC and EA displayed moderate inhibitory activity. For GC, the dual-species MBIC₅₀ and MBIC₉₀ values were 50 µg/mL and 100 µg/mL, respectively, which were identical to those observed for *S. mutans* biofilms. This suggests that the dual-species condition did not further reduce the inhibitory activity of GC beyond that observed against *S. mutans*. EA showed MBIC₅₀ values of 25 µg/mL against *S. gordonii* and 100 µg/mL against both *S. mutans* and dual-species biofilms, while the MBIC₉₀ value was 200 µg/mL across all three biofilm models. Therefore, EA demonstrated similar MBIC₉₀ values across the single- and dual-species models, although a higher MBIC₅₀ was observed for *S. mutans* and dual-species biofilms compared with *S. gordonii*.

In contrast, KG and COR exhibited reduced biofilm-inhibitory activity, particularly at the MBIC₉₀ level. KG showed the clearest increase in inhibitory concentration in the dual-species model, with the MBIC₅₀ increasing to 400 µg/mL and the MBIC₉₀ exceeding the highest tested concentration (>1600 µg/mL). This indicates

reduced KG activity against the dual-species biofilm compared with both single-species biofilms. COR exhibited high MBIC values against *S. mutans* and dual-species biofilms, with MBIC₅₀ and MBIC₉₀ values of 200 µg/mL and 400 µg/mL, respectively, in both models. Thus, COR showed reduced activity against *S. mutans* and the dual-species biofilm compared with *S. gordonii*, but the dual-species values were not higher than those observed for *S. mutans* alone. RUT showed poor biofilm-inhibitory activity within the tested concentration range, with MBIC₉₀ values exceeding 1600 µg/mL for all biofilm conditions. Although the MBIC₅₀ increased from 100 µg/mL against *S. gordonii* to 200 µg/mL against *S. mutans* and dual-species biofilms, RUT remained weakly active overall, and its MBIC₉₀ endpoint was not achieved in any model.

Across compounds, MBIC₉₀ values were generally higher than MBIC₅₀ values, indicating that greater concentrations were required to achieve ≥90% inhibition of biofilm formation than ≥50% inhibition. However, the relationship between single-species and dual-species biofilm inhibition was not uniform across compounds. PUN, PUG, GC, EA, and COR showed dual-species MBIC values that were comparable to either *S. mutans* or both single-species models, whereas KG showed a pronounced loss of activity in the dual-species biofilm. These findings indicate that increased tolerance in the dual-species model was compound-specific rather than a general feature observed across all tested PDCs.

Table 4.6
MBIC₅₀ and MBIC₉₀ (µg/mL) of PDCs Against Single-Species (*S. gordonii*, *S. mutans*) and Dual-Species Biofilms

	<i>S. gordonii</i>		<i>S. mutans</i>		Dual-species	
	MBIC ₅₀ µg/mL	MBIC ₉₀ µg/mL	MBIC ₅₀ µg/mL	MBIC ₉₀ µg/mL	MBIC ₅₀ µg/mL	MBIC ₉₀ µg/mL
COR	50	100	200	400	200	400
EA	25	200	100	200	100	200
GC	12.5	50	50	100	50	100
KG	50	200	200	400	400	>1600
PUN	50	50	25	50	25	50
PUG	12.5	100	50	50	25	50
RUT	100	>1600	200	>1600	200	>1600

Note: Compounds with *S. mutans* MBIC₉₀ ≤ *S. gordonii* MBIC₉₀

MBIC₅₀ and MBIC₉₀ denote the minimum concentrations required to achieve ≥50% and ≥90% inhibition of biofilm biomass, respectively, relative to the untreated control. Values were derived from ≥5 independent experiments. “>1600” indicates that the inhibitory endpoint was not achieved within the tested concentration range.

4.6 Minimum Biofilm Eradication Concentration (MBEC) Using CV Assay

Table 4.7 summarises the MBEC₅₀ and MBEC₉₀ values of the tested compounds against pre-formed biofilms of *S. gordonii*, *S. mutans*, and dual-species biofilms. Overall, substantially higher concentrations were required to reduce established biofilm biomass than to inhibit biofilm formation, with marked compound- and species-dependent differences observed. However, the dual-species biofilm did not consistently show higher MBEC values than the corresponding single-species biofilms. Instead, the eradication profile was compound-dependent, with most dual-species MBEC values being comparable to or lower than those observed in at least one of the single-species biofilm models.

Across all biofilm models, COR and KG showed no detectable biofilm eradication at the tested concentrations, with MBEC₅₀ and MBEC₉₀ values exceeding 1600 µg/mL for all species and biofilm conditions.

Among the remaining compounds, GC demonstrated the lowest and most consistent MBEC values across single-species and dual-species biofilms, with identical MBEC₅₀ values of 100 µg/mL and MBEC₉₀ values of 200 µg/mL for *S. gordonii*, *S. mutans*, and dual-species biofilms. This indicates that the dual-species condition did not increase the concentration required for GC-mediated reduction of established biofilm biomass. EA exhibited higher MBEC values, with MBEC₅₀ values of 200 µg/mL against *S. gordonii* and 100 µg/mL against both *S. mutans* and dual-species biofilms, while MBEC₉₀ values were uniformly 800 µg/mL across all biofilm models. Thus, EA showed similar MBEC₉₀ values across all models, and the dual-species MBEC₅₀ was not higher than that observed for the single-species biofilms.

PUG and PUN displayed variable eradication profiles depending on biofilm composition. PUG showed an MBEC₅₀ of 200 µg/mL against *S. gordonii* and 100 µg/mL against both *S. mutans* and dual-species biofilms. The MBEC₉₀ for PUG exceeded 1600 µg/mL against *S. gordonii*, whereas lower MBEC₉₀ values of 400 µg/mL were observed for both *S. mutans* and dual-species biofilms. Therefore, the dual-species MBEC values for PUG were comparable to those of *S. mutans* and lower than those observed for *S. gordonii*. In contrast, PUN exhibited a low MBEC₅₀ of 50 µg/mL against *S. mutans*, increasing to 100 µg/mL in the dual-species model and 200 µg/mL for *S. gordonii*. Its MBEC₉₀ values ranged from 800 µg/mL against *S. mutans* to >1600 µg/mL against *S. gordonii*, with the dual-species MBEC₉₀ recorded at 1600 µg/mL. Although

the dual-species MBEC values for PUN were higher than those for *S. mutans*, they were lower than or did not exceed those observed for *S. gordonii*.

RUT exhibited measurable eradication activity, with MBEC₅₀ values ranging from 50 to 100 µg/mL and MBEC₉₀ values ranging from 100 to 200 µg/mL across all biofilm conditions. For RUT, the dual-species MBEC₅₀ was 100 µg/mL, which was equal to that of *S. mutans* but higher than that of *S. gordonii*. The dual-species MBEC₉₀ was 200 µg/mL, which was equal to that of *S. mutans* and higher than that of *S. gordonii*. Therefore, RUT was the only compound for which the dual-species MBEC₉₀ showed an increase relative to one of the single-species biofilms, specifically *S. gordonii*.

Across compounds, MBEC₉₀ values were consistently higher than MBEC₅₀ values, indicating that greater concentrations were required to achieve ≥90% reduction of established biofilm biomass than ≥50% reduction. However, the data do not support a general conclusion that dual-species biofilms exhibited higher MBEC values than single-species biofilms. Instead, dual-species MBEC values were largely comparable to the values observed for *S. mutans* or lower than those observed for *S. gordonii*, depending on the compound. The only notable increase in the dual-species model was observed for RUT MBEC₉₀ when compared with *S. gordonii*. These findings indicate that enhanced tolerance in the dual-species biofilm was not a universal pattern in this CV-based MBEC assay.

Table 4.7

MBEC₅₀ and MBEC₉₀ (µg/mL) of PDCs Against Preformed Single-Species (*S. gordonii*, *S. mutans*), and Dual-Species Biofilms

	<i>S. gordonii</i>		<i>S. mutans</i>		Dual-species	
	MBEC ₅₀ µg/mL	MBEC ₉₀ µg/mL	MBEC ₅₀ µg/mL	MBEC ₉₀ µg/mL	MBEC ₅₀ µg/mL	MBEC ₉₀ µg/mL
COR	>1600	>1600	>1600	>1600	>1600	>1600
EA	200	800	100	800	100	800
GC	100	200	100	200	100	200
KG	>1600	>1600	>1600	>1600	>1600	>1600
PUN	200	>1600	50	800	100	1600
PUG	200	>1600	100	400	100	400
RUT	50	100	100	200	100	200

Note: Compounds with MBEC₅₀ (*S. mutans*) ≤ MBEC₅₀ (*S. gordonii*)

MBEC₅₀ and MBEC₉₀ denote the minimum concentrations required to achieve ≥50% and ≥90% reduction of pre-formed biofilm biomass, respectively, relative to the untreated control. Values were derived from ≥5 independent experiments. “>1600” indicates that the endpoint was not achieved within the tested concentration range.

4.7 Glycolytic pH Drop Assay

The glycolytic pH drop assay demonstrated marked differences in the acidogenic potential of *S. mutans* following PDC treatment over a 120-min period (Figure 4.3). In the untreated control (1% DMSO), the addition of glucose induced a rapid and sustained decrease in pH, with values falling below the critical demineralisation threshold (pH 5.5) within 30 min. The pH declined progressively throughout the incubation period, reaching a final value of 3.5 ± 0.2 at 120 min, indicative of high glycolytic activity and substantial acid production by *S. mutans*.

In contrast, treatment with the PDCs attenuated both the rate and extent of pH reduction to varying degrees. Cultures treated with GC, COR, EA, KG, PUN, PUG, and RUT exhibited a slower decline in pH compared with the untreated control, maintaining higher pH values throughout the time course. Among these compounds, PUN and PUG exhibited the most pronounced suppression of acid production, as evidenced by a more gradual pH decline and consistently higher pH values throughout the 120-min period. Notably, treatment with PUG, PUN, and CHX maintained the pH above the critical threshold of 5.5 at 120 min, indicating effective inhibition of glycolytic acidification.

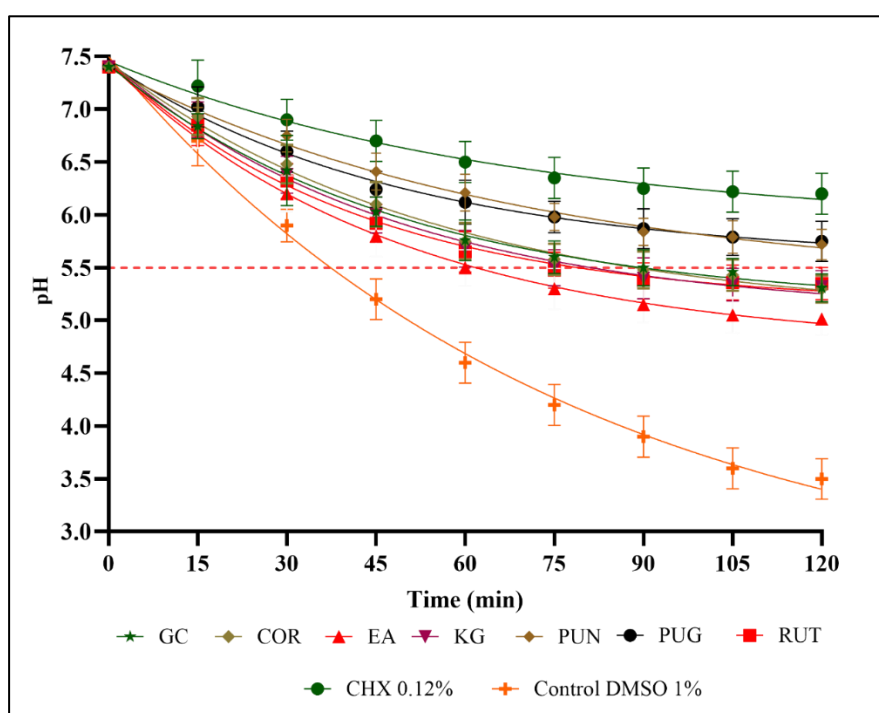


Figure 4.3 Time-Dependent Glycolytic pH Drop of *S. mutans* over 120 min Following Treatment with PDCs

The dashed line indicates the critical demineralisation threshold (pH 5.5). DMSO (1%) served as the negative control and CHX as the positive control.

The endpoint analysis of final pH values at 120 minutes is shown in Figure 4.4. The untreated control (1% DMSO) exhibited the lowest final pH, indicating maximal acidification over the assay period. In contrast, all compound-treated groups showed significantly higher final pH values than the control ($p < 0.05$). Among the tested compounds, PUN and PUG produced the highest final pH values (approximately pH 5.72–5.75), approaching those observed for the CHX control (pH $\approx$ 6.2). GC, COR, EA, KG, and RUT showed intermediate effects, with final pH values consistently exceeding the critical demineralisation threshold of pH 5.5, as indicated by the dashed reference line. Statistical analysis confirmed significant differences among treatment groups, as indicated by distinct superscripts ($p < 0.05$).

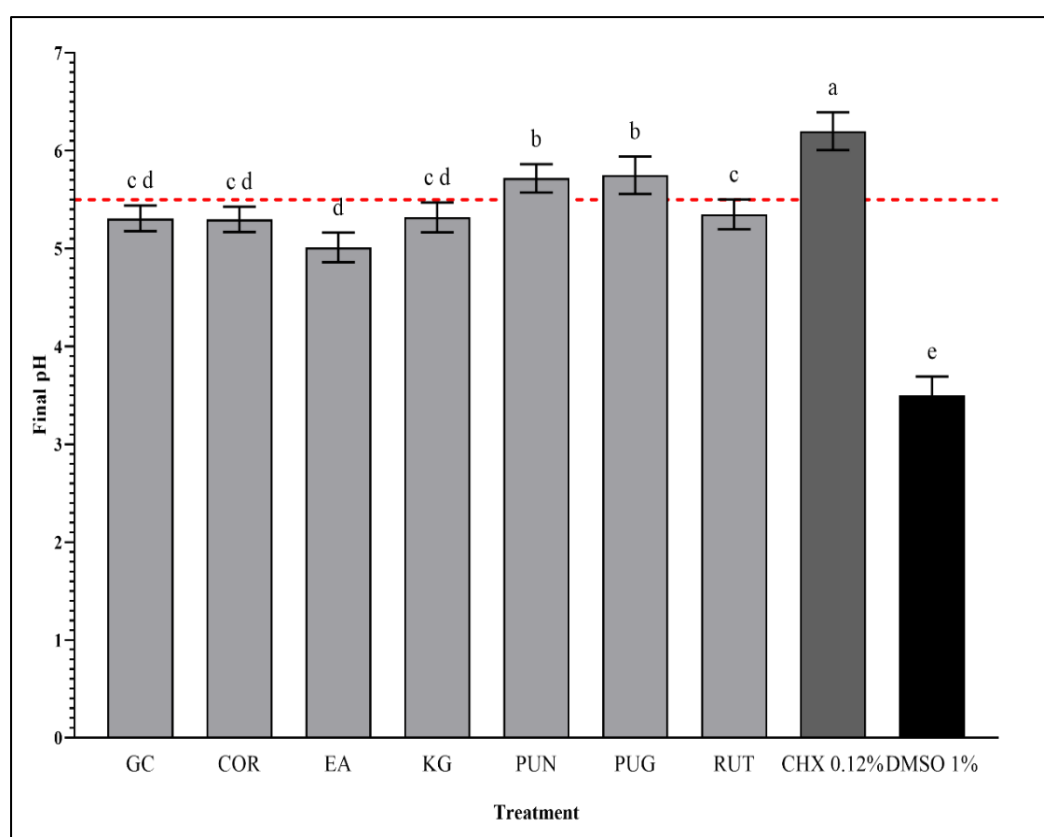


Figure 4.4 Endpoint pH at 120 min in the Glycolytic pH Drop Assay for *S. mutans* Following Treatment with PDCs.

Bars show mean $\pm$ SD ($n = 5$). The dashed red line indicates the critical demineralisation threshold (pH 5.5). Different superscript letters indicate statistically significant differences ($p < 0.05$).

4.8 Brine Shrimp Lethality Assay (BSLA)

The acute toxicity of selected PDCs was evaluated using the BSLA. The median lethal concentration (LC_{50}), coefficients of determination (R^2), and corresponding toxicity classifications are summarised in Table 4.8. Dose–response relationships for each compound are illustrated in Figure 4.6, which shows a concentration-dependent increase in brine shrimp mortality for COR, EA, CG, KG, PUG, PUN, and RUT. Nonlinear regression analysis of percentage mortality against $\log_{10}$ -transformed concentrations demonstrated good model fitting across all compounds, as reflected by high R^2 values. Based on the derived LC_{50} values, the compounds were categorised according to established toxicity criteria, as reported in Table 4.8. Among the compounds assessed, EA demonstrated the highest toxicity, with an LC_{50} value of 159.2 $\mu\text{g/mL}$, and was therefore categorised as moderately toxic according to Clarkson's criteria. In contrast, all other compounds showed LC_{50} values exceeding 500 $\mu\text{g/mL}$, indicating low toxicity. The hydrolysable tannins, COR, PUG and PUN exhibited LC_{50} values of 588.9 $\mu\text{g/mL}$, 819.7 $\mu\text{g/mL}$, and 941.2 $\mu\text{g/mL}$, respectively, placing them consistently within the low-toxicity category. Similarly, the flavonoid compounds GC, KG and RUT demonstrated low toxicity, with LC_{50} values ranging from 594.0 $\mu\text{g/mL}$ to 998.9 $\mu\text{g/mL}$.

Regression analysis of the dose–response curves revealed high coefficients of determination ($R^2 = 0.9879\text{--}0.9956$) across all compounds, indicating strong linearity and reliable LC_{50} estimates from the assay.

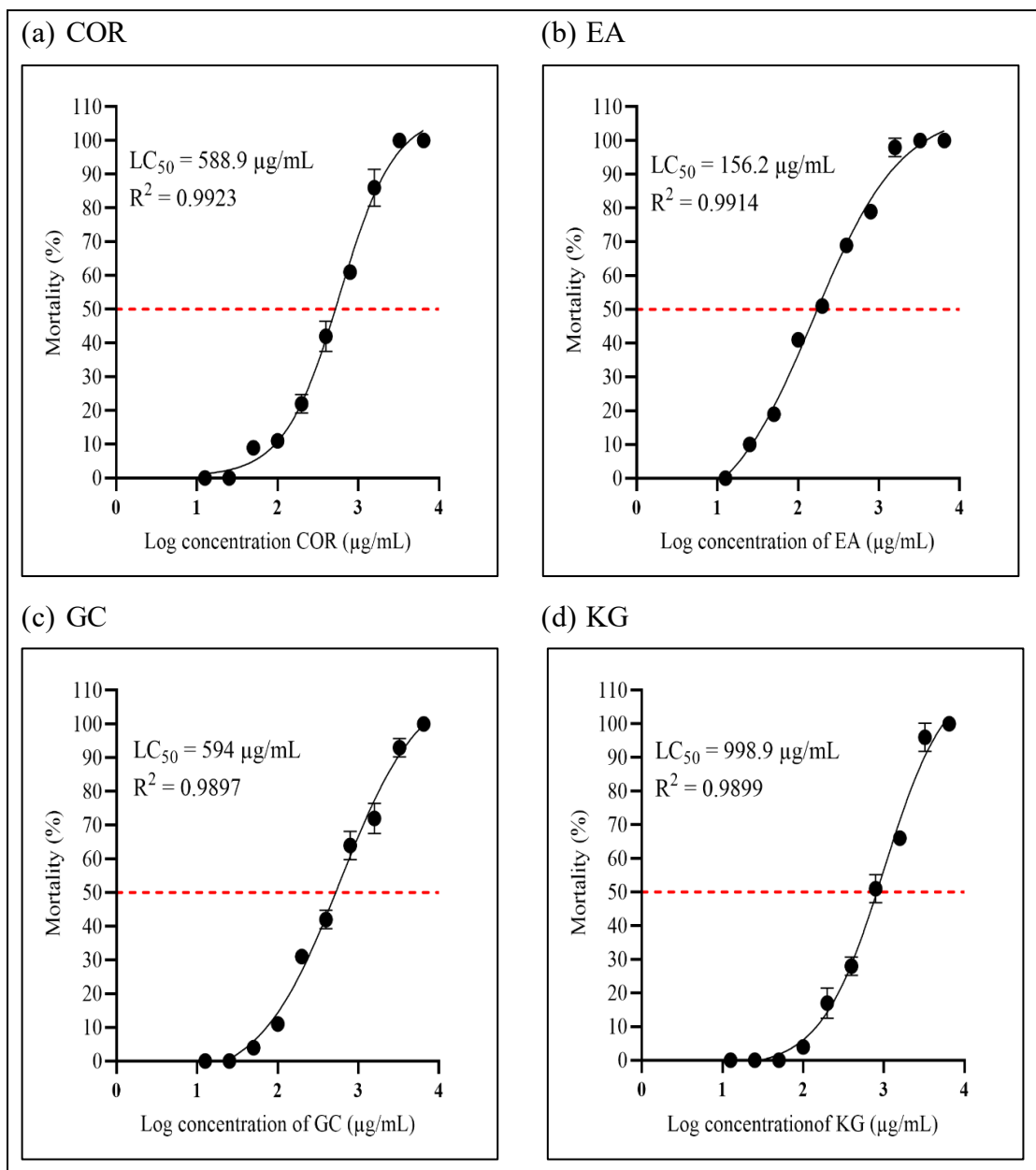


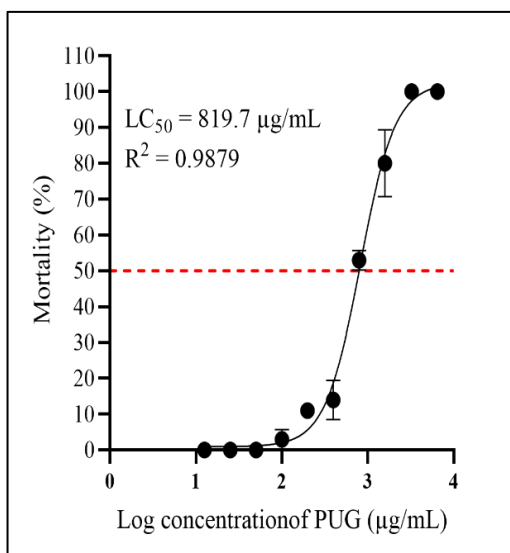
Figure 4.5 Dose–Response Curves of Brine Shrimp Mortality After 24 h Exposure to PDCs

(a) COR, (b) EA, (c) GC, (d) KG, (e) PUG, (f) PUN, and (g) RUT.

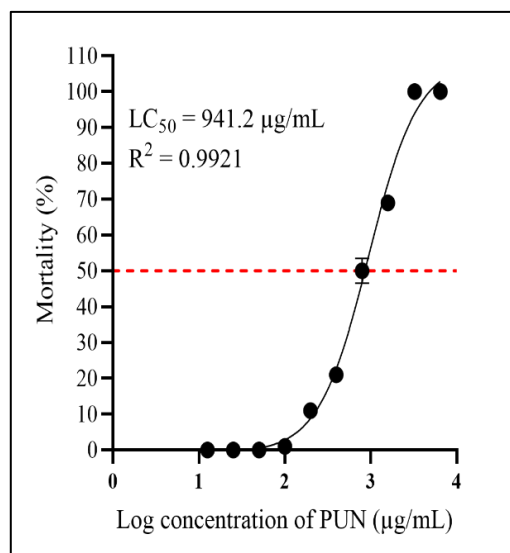
Mortality (%) was plotted against $\log_{10}$ concentration ($\mu\text{g/mL}$) and fitted using nonlinear regression (sigmoidal dose–response with variable slope) to estimate LC_{50} .

The red dashed line indicates the 50% mortality threshold used for LC_{50} determination. Data represent mean $\pm$ SD ($n = 5$).

(e) PUG



(f) PUN



(g) RUT

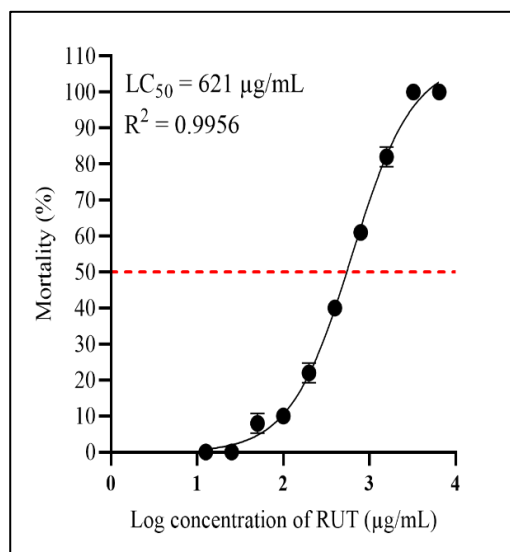


Figure 4.5 (continue)

Table 4.8

Lethal Concentration (LC₅₀) Values, Coefficient of Determination (R²), and Toxicity Classification of PDCs Determined Using the BSLA

PDCs	Class	LC ₅₀ (µg/mL)	R ²	Toxicity category
COR	Hydrolysable tannin	588.9	0.9923	Low toxicity
EA	Polyphenol	159.2	0.9927	moderate toxicity
GC	Flavonoid	594	0.9897	Low toxicity
KG	Flavonoid	998.9	0.9899	Low toxicity
PUG	Hydrolysable tannin	819.7	0.9879	Low toxicity
PUN	Hydrolysable tannin	941.2	0.9921	Low toxicity
RUT	Flavonoid	621	0.9956	Low toxicity

Note: Classification followed Clarkson's criteria (LC₅₀ < 250 µg/mL: moderate toxicity; LC₅₀ > 500 µg/mL: low toxicity)

4.9 Selection of Compounds for Phase II

Compound prioritisation for Phase II was guided by a predefined five-parameter scoring matrix integrating antibiofilm efficacy, virulence modulation, and cytotoxicity. The selection matrix, together with the individual binary scores assigned to each compound for each evaluation criterion and the resulting cumulative scores, is presented in Table 4.9. Each parameter was scored as 1 (criterion met) or 0 (criterion not met), yielding a maximum possible score of 5. Preliminary safety was treated as a compulsory prerequisite rather than a weighted scoring criterion; compounds exhibiting LC₅₀ values below 500 µg/mL were automatically excluded from further consideration, irrespective of their antibacterial or antibiofilm performance. Compounds achieving a total score of $\geq 4/5$ and meeting the predefined safety threshold were considered eligible for advancement to Phase II.

Among the seven compounds evaluated, PUG and PUN achieved the highest total scores (5/5), meeting all predefined criteria. Both compounds demonstrated preferential antibiofilm activity against *S. mutans*, as evidenced by MBIC₉₀ and MBEC₉₀ values that were lower than or equal to those observed against *S. gordonii*. In addition, these compounds produced measurable reductions in *S. mutans* cell-surface hydrophobicity and maintained glycolytic pH values at or above pH 5.5, indicating attenuation of key virulence-associated traits. Notably, both compounds exhibited low cytotoxicity, with LC₅₀ values exceeding 500 µg/mL.

In contrast, EA met three of the five criteria but failed to maintain glycolytic pH above the threshold and did not meet the cytotoxicity requirement, resulting in exclusion despite favourable antibiofilm and hydrophobicity profiles. The remaining compounds COR, GC, KG, and RUT achieved low overall scores (1–2/5), primarily due to a lack of selective antibiofilm activity against *S. mutans* and minimal effects on virulence-associated functional parameters.

Changes in *S. gordonii* hydrophobicity were not considered in the selection process, in line with the study objective of preferentially suppressing cariogenic traits while preserving commensal streptococci. Based on the integrated scoring outcomes, PUG and PUN were shortlisted for Phase II mechanistic investigations using a dual-species biofilm model.

Table 4.9

Predefined Selection Matrix Used to Prioritise PDCs for Phase II.

PDC	Biofilm selectivity		Virulence modulation		Safety	Score	Decision
	MBIC ₉₀ selectivity	MBEC ₅₀ selectivity	HI reduction ($\leq 0.5 \times \text{MIC}$)	Glycolytic pH-drop ≥ 5.5	LC ₅₀ >500 $\mu\text{g/mL}$		
COR	0	0	1	0	1	2	Excluded
EA	1	1	1	0	0	3	Excluded
GC	0	1	0	0	1	2	Excluded
KG	0	0	0	1	1	2	Excluded
PUG	1	1	1	1	1	5	Included
PUN	1	1	1	1	1	5	Included
RUT	0	0	1	0	1	2	Excluded

Note: Selection was based on antibiofilm selectivity against *S. mutans*, virulence-related functional outcomes, and preliminary cytotoxicity. Criteria were scored as 1 (met) or 0 (not met). LC₅₀ > 500 $\mu\text{g/mL}$ was required for advancement. Compounds scoring $\geq 4/5$ and meeting this prerequisite were shortlisted.

Abbreviations:

MBIC₉₀ selectivity: MBIC₉₀ of *S. mutans* $\leq$ MBIC₉₀ of *S. gordonii*;

MBEC₅₀ selectivity: MBEC₅₀ of *S. mutans* $\leq$ MBEC₅₀ of *S. gordonii*;

HI: hydrophobicity index;

LC₅₀: median lethal concentration from brine shrimp lethality assay.

4.10 Phase II: Dual-Species Biofilm Inhibition Assay of PUG and PUN

The inhibitory effects of PUG and PUN on dual-species biofilm formation by *S. mutans* and *S. gordonii* were evaluated using a bead-based biofilm model at early (8 hours) and established (24 hours) stages of biofilm development to assess compound activity during different phases of biofilm model.

4.10.1 Biofilm Biomass Quantification

Biofilm biomass was quantified following treatment with PUG, PUN, and 0.12% CHX at two stages of biofilm development, early (8 hours) and established (24 hours) (Figure 4.6). Untreated biofilms exhibited the highest biomass at both time points, confirming active biofilm development under control conditions. At 8 hours, treatment with PUG and PUN resulted in a significant reduction in biofilm biomass compared with the untreated control ($p < 0.05$), indicating effective inhibition of early biofilm formation. PUG demonstrated a greater inhibitory effect than PUN, suggesting stronger interference with initial biofilm development. In contrast, no detectable biofilm biomass was observed in the CHX-treated group at 8 hours, indicating that biofilm formation was not initiated under these conditions. At 24 hours, biofilm biomass remained significantly lower in all treatment groups than in the control ($p < 0.05$). Biofilms treated with PUG and PUN continued to exhibit suppressed biomass, with PUG maintaining a more pronounced inhibitory effect than PUN. Although minimal biomass formation was observed in the CHX-treated group at 24 hours, levels remained markedly lower than those of untreated controls, indicating sustained inhibition of biofilm development.

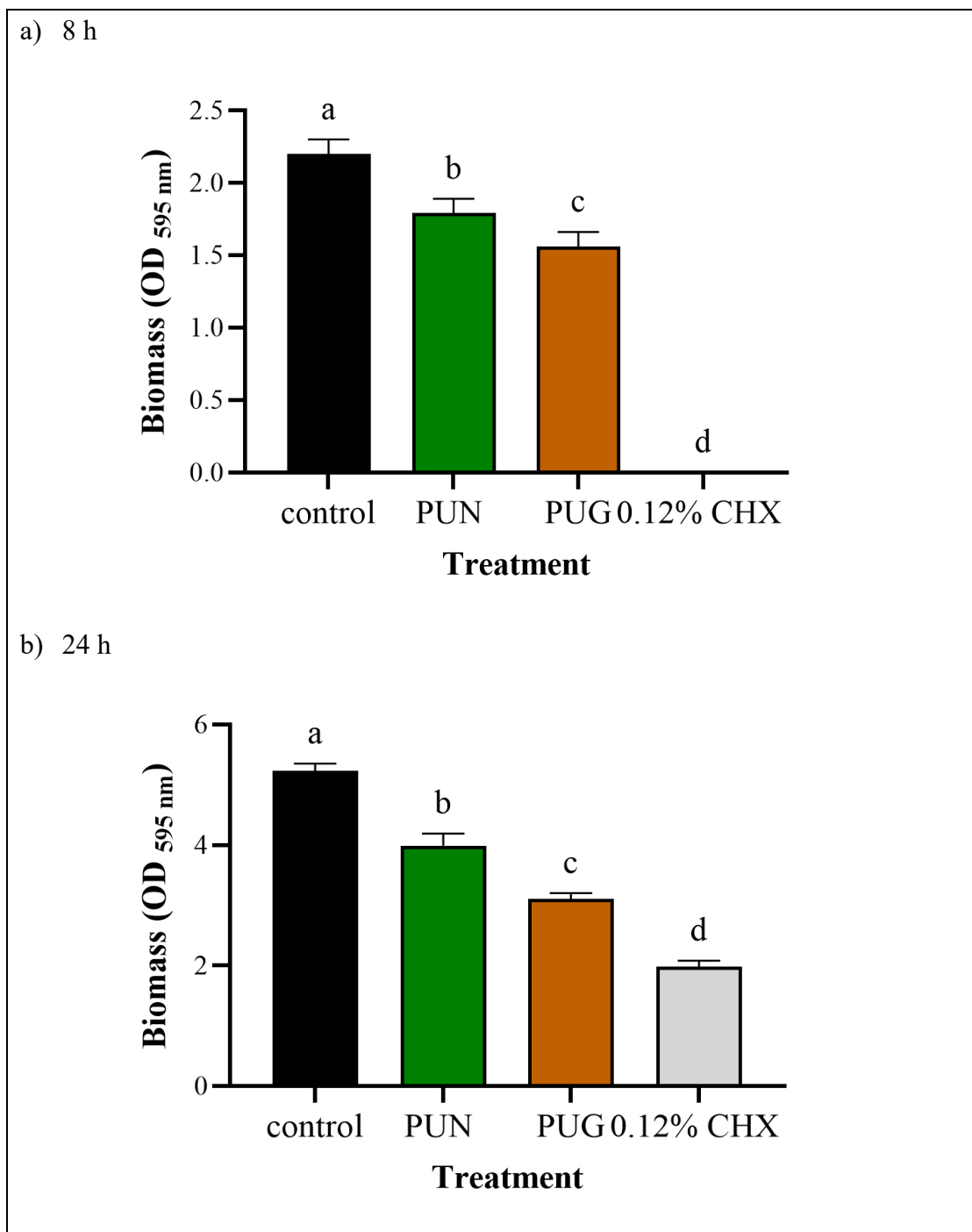


Figure 4.6 Biomass of Dual-Species Biofilms (*S. mutans* + *S. gordonii*) Following Inhibition Treatment with PUG, PUN, and CHX

Biofilms were formed on saliva-coated glass beads and treated with PUG and PUN (50 µg/mL) and CHX (0.12%). Biofilm biomass was assessed at (a) 8 h and (b) 24 h using CV staining (OD₅₉₅). Bars represent mean ± SD of five independent experiments, each performed using three beads per treatment condition. Statistical analysis was conducted using one-way ANOVA followed by Tukey's post hoc test. Different letters indicate statistically significant differences ($p < 0.05$).

4.10.2 Viable Cell Enumeration

Figure 4.7 illustrates the effects of PUG and PUN on the viability of *S. mutans* and *S. gordonii* within dual-species biofilms at early (8 h) and established (24 h) stages of biofilm development, as determined by colony-forming unit (CFU) enumeration. The corresponding statistical comparisons between treatment groups for *S. mutans* and *S. gordonii* at both time points are summarised in Table 4.10.

At 8 hours, untreated dual-species biofilms exhibited mean viable counts of *S. mutans* of approximately $1.51 \log_{10}$ CFU/bead and *S. gordonii* of $\sim 1.91 \log_{10}$ CFU/bead. Treatment with PUG (50 μ g/mL) significantly reduced *S. mutans* viability to a mean of $\sim 0.95 \log_{10}$ CFU/bead, corresponding to an approximate 0.6-log reduction relative to the control ($p < 0.0001$). In contrast, *S. gordonii* counts remained largely unchanged ($\sim 1.90 \log_{10}$ CFU/bead, ns). Similarly, PUN (50 μ g/mL) reduced *S. mutans* counts to $\sim 1.11 \log_{10}$ CFU/bead, representing a ~ 0.4 -log reduction compared with the control ($p < 0.0001$), while *S. gordonii* viability was preserved ($\sim 1.89 \log_{10}$ CFU/bead, ns). The positive control, 0.12% chlorhexidine (CHX), completely suppressed both species, with no CFU detected.

At 24 hours, untreated biofilms demonstrated increased bacterial burden, with *S. mutans* counts of approximately $5.0 \log_{10}$ CFU/bead and *S. gordonii* counts of $\sim 2.7 \log_{10}$ CFU/bead. Exposure to PUG resulted in a marked reduction in *S. mutans* viability to approximately $1.0 \log_{10}$ CFU/bead, corresponding to a ~ 4 -log reduction relative to the untreated control ($p < 0.0001$). *S. gordonii* counts remained largely unaffected (~ 2.6 – $2.8 \log_{10}$ CFU/bead, ns). Treatment with PUN also significantly suppressed *S. mutans* within the dual-species biofilm, reducing viable counts to approximately 2.8 – $3.0 \log_{10}$ CFU/bead, representing a ~ 2 -log reduction relative to the control ($p < 0.0001$). As observed at 8 hours, *S. gordonii* viability was maintained. CHX treatment again resulted in near-complete eradication of both species at 24 hours.

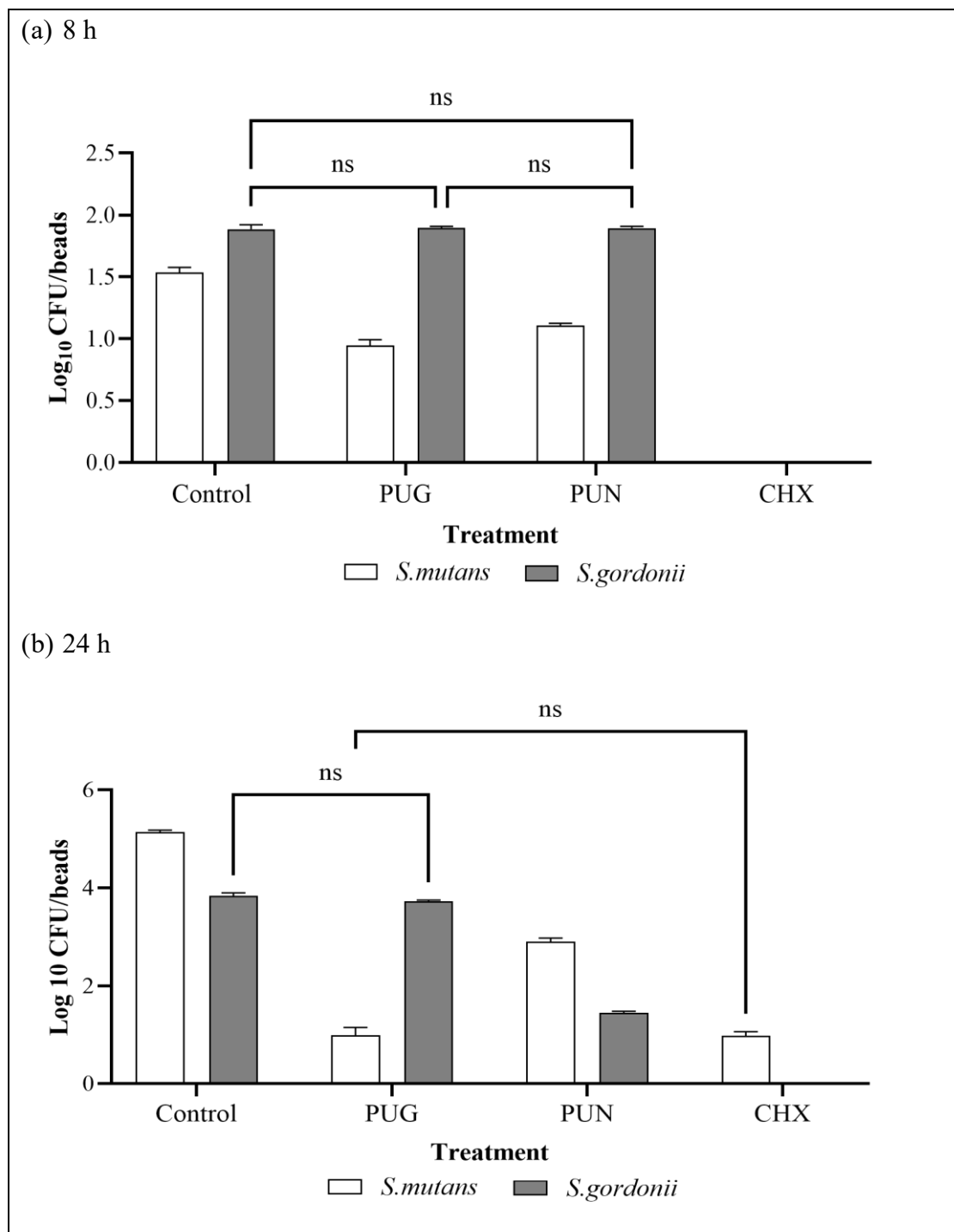


Figure 4.7 Effect of PUG and PUN on Inhibition of Dual-Species Biofilm Formation of *S. mutans* and *S. gordonii*

Log₁₀ CFU/bead counts at (a) 8 h and (b) 24 h for biofilms treated with PUG (50 µg/mL), PUN (50 µg/mL), CHX (0.12%, positive control), and DMSO (1%, negative control). Bars represent mean ± standard deviation (SD) of five independent experiments, each performed using three beads per treatment condition. Statistical analysis was conducted using one-way ANOVA followed by Tukey's post hoc test. For clarity, only non-significant comparisons (ns, $p > 0.05$) are indicated in the figure. Significant differences are summarised Table 4.10.

Table 4.10

Statistical Comparisons of Log₁₀ CFU/bead for *S. mutans*, *S. gordonii*, and Total Biofilm Following Inhibition Treatment with PUG, PUN, and CHX

Pairwise comparisons were performed for biofilms assessed at 8 h and 24 h. Data were analysed using one-way ANOVA followed by Tukey's HSD post hoc test. Statistical significance was set at $p < 0.05$.

Time	Biofilm population	Comparison	p-value	Interpretation
8 h	<i>S. mutans</i>	Control vs PUG	< 0.0001	Significant
		Control vs PUN	< 0.0001	Significant
		Control vs CHX	< 0.0001	Significant
		PUG vs PUN	< 0.0001	Significant
		PUG vs CHX	< 0.0001	Significant
		PUN vs CHX	< 0.0001	Significant
	<i>S. gordonii</i>	Control vs PUG	> 0.05	Not significant
		Control vs PUN	> 0.05	Not significant
		Control vs CHX	< 0.0001	Significant
		PUG vs PUN	> 0.05	Not significant
		PUG vs CHX	< 0.0001	Significant
		PUN vs CHX	< 0.0001	Significant
24 h	<i>S. mutans</i>	Control vs PUG	< 0.0001	Significant
		Control vs PUN	< 0.0001	Significant
		Control vs CHX	< 0.0001	Significant
		PUG vs PUN	< 0.0001	Significant
		PUG vs CHX	< 0.0001	Significant
		PUN vs CHX	< 0.0001	Significant
	<i>S. gordonii</i>	Control vs PUG	> 0.05	Not significant
		Control vs PUN	< 0.0001	Significant
		Control vs CHX	< 0.0001	Significant
		PUG vs PUN	< 0.0001	Significant
		PUG vs CHX	< 0.0001	Significant
		PUN vs CHX	< 0.0001	Significant

4.10.3 Scanning Electron Microscopy (SEM) Analysis of Dual-Species Biofilm Formation Under Inhibition Treatment with PUG and PUN

Scanning electron microscopy (SEM) was employed to visualise the structural organisation and surface morphology of *S. mutans*–*S. gordonii* dual-species biofilms after 24 hours treatment with PUG and PUN. Representative micrographs were captured at 1000× and 5000× magnifications to assess both overall biofilm architecture and individual cell-level organisation (Figure 4.8).

In the untreated control (1% DMSO), SEM images at 1000× magnification (A-I) revealed a dense, continuous biofilm layer covering the substratum, characterised by extensive surface colonisation and irregular topography. The biofilm appeared highly cohesive, with abundant extracellular material bridging bacterial aggregates. At higher magnification (5000×; A-II), bacterial cells were observed to be tightly packed into multilayered microcolonies, embedded within a thick extracellular matrix, forming a compact, well-organised biofilm.

Following PUG treatment (50 µg/mL), notable alterations in biofilm morphology were observed. At 1000× magnification (B-I), the biofilm exhibited reduced surface coverage with visible discontinuities and exposed substratum areas compared with the control. Biofilm clusters appeared less extensive and more spatially separated. At 5000× magnification (B-II), bacterial cells were predominantly organised into smaller, less compact aggregates, with reduced intercellular cohesion. The extracellular matrix appeared diminished, and individual cells or short chains were more readily distinguishable than in the untreated control.

Biofilms treated with PUN (50 µg/mL) displayed further morphological disruption. At low magnification (C-I), SEM images showed a marked reduction in biofilm biomass, with sparse bacterial attachment and large areas of exposed substratum. Biofilm formation was limited to isolated clusters rather than continuous coverage. At higher magnification (C-II), bacterial cells were loosely distributed, with minimal extracellular material and reduced clustering, indicating substantial disruption of biofilm integrity.

Treatment with 0.12% CHX resulted in the most pronounced morphological alterations. At 1000× magnification (D-I), only scattered bacterial remnants and debris were visible, with extensive exposure of the underlying surface. At 5000× magnification (D-II), very few intact bacterial aggregates were observed, and remaining

cells appeared isolated and irregularly distributed, indicating near-complete disruption of the dual-species biofilm structure.

Overall, SEM analysis demonstrated progressive disruption of dual-species biofilm architecture across treatment groups, with a clear morphological transition from dense, matrix-rich biofilms in the untreated control to increasingly fragmented and sparse bacterial structures following PUG and PUN exposure. Among the compounds tested, PUN produced greater biofilm disruption than PUG, whereas CHX exhibited the most significant overall reduction in biofilm presence.

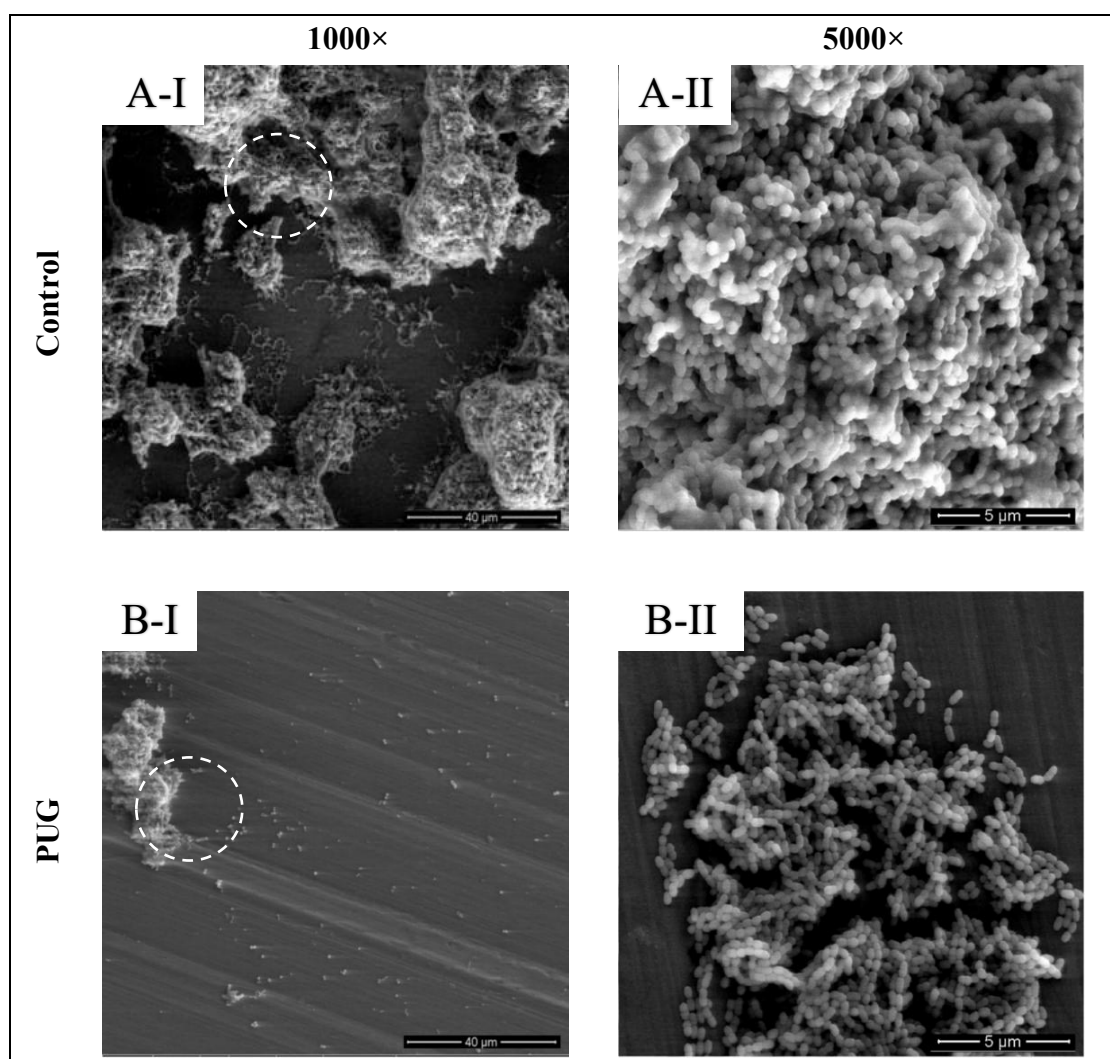


Figure 4.8 SEM Analysis of Dual-Species Biofilm Formation of *S. mutans* and *S. gordonii* Following Inhibition Treatment with PUG and PUN
SEM micrographs at 1000× magnification showing biofilm architecture after 24 h of biofilm formation in the presence of different treatments: (A) DMSO (1% negative control), (B) PUG 50 μg/mL, (C) PUN 50 μg/mL, and (D) CHX (0.12% positive control). The white circle indicates the region selected for higher-magnification imaging (5000×).

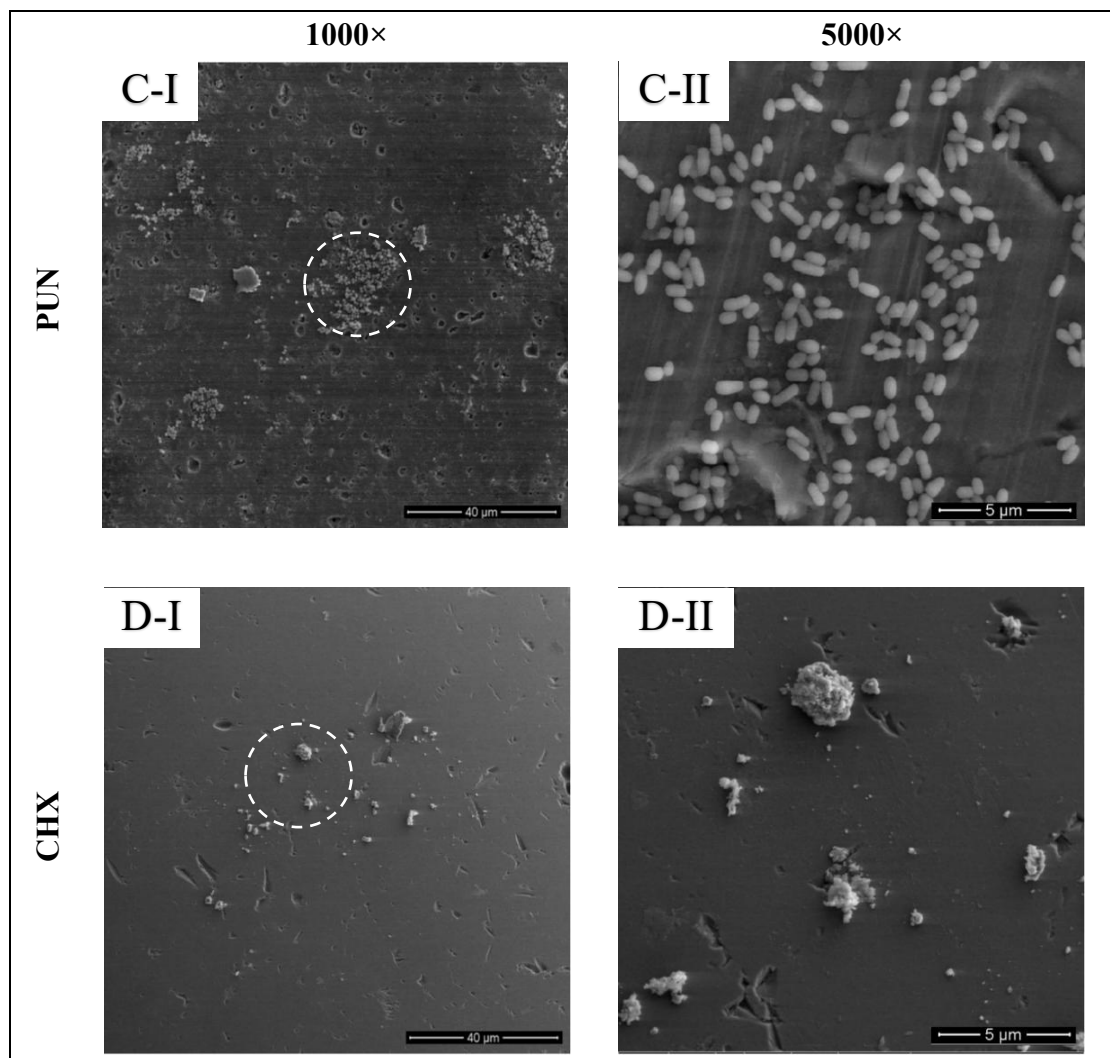


Figure 4.8 (continued)

4.10.4 Gene Expression Analysis of Dual-Species Biofilms During Inhibition by PUG and PUN

4.10.4.1 Gene Expression of *S. mutans*

The transcriptional effects of PUN and PUG on selected virulence-associated and metabolic genes of *S. mutans* were evaluated at 8 hours and 24 hours using RT-qPCR. Gene expression levels were normalised to the untreated control, which was set to a fold change of 1.0 (Figure 4.9). At 8 hours, both PUN and PUG significantly downregulated genes involved in glucan synthesis and surface adhesion, namely *gtfC*, *gtfB*, *gbpD*, and *gbpB*, compared with the untreated control ($p < 0.05$ to $p < 0.0001$). Among these genes, *gtfC* and *gtfB* showed the most pronounced early suppression, particularly following PUG treatment. Across all adhesion-related genes, PUG

consistently produced a greater reduction in transcript levels than PUN. Notably, *gbpB* exhibited a smaller magnitude of downregulation at 8 hours compared with the other glucan-binding and glucosyltransferase genes, indicating a comparatively delayed transcriptional response.

The quorum-sensing regulatory gene *comDE* was also significantly downregulated at 8 hours following treatment with both compounds ($p < 0.05$), with PUG again exerting a stronger inhibitory effect. In contrast, *brpA* expression showed only moderate suppression at this time point, with no significant difference observed between PUN and PUG, suggesting reduced early sensitivity of this stress-response-associated gene.

At 24 hours, transcriptional suppression was more extensive and uniform across virulence-associated genes. Both PUN and PUG significantly reduced the expression of *gtfC*, *gtfB*, *gbpD*, *gbpB*, *comDE*, and *brpA* relative to the untreated control ($p < 0.0001$). The magnitude of downregulation at 24 hours exceeded that observed at 8 hours for all virulence genes, demonstrating a clear time-dependent inhibitory trend. At this later time point, *gbpB* and *brpA* exhibited stronger suppression than at 8 hours, indicating delayed but sustained transcriptional inhibition. PUG consistently produced a greater reduction in gene expression than PUN across all virulence-associated targets.

With respect to genes involved in acidogenicity and energy metabolism, *ldh*, which mediates lactic acid generation from pyruvic acid, showed no significant change at 8 hours but exhibited a modest yet statistically significant reduction in expression at 24 hours following treatment with both compounds ($p < 0.05$), with PUG again showing a slightly greater effect. Similarly, *atpD*, a component of the F_1F_0 -ATPase operon involved in proton translocation and acid tolerance, showed no significant change at 8 hours but was significantly down-regulated at 24 hours compared with the untreated control ($p < 0.05$). This indicates that prolonged exposure to PUG and PUN may affect energy metabolism and acid-adaptive mechanisms in the 24-hour biofilm. Overall, the results indicate that PUN and PUG preferentially and progressively suppress genes associated with adhesion, extracellular polysaccharide synthesis, quorum sensing, and stress response, while genes involved in core metabolic maintenance and proton pumping are comparatively less affected. Across all time points and gene categories, PUG consistently demonstrated stronger transcriptional inhibitory activity than PUN.

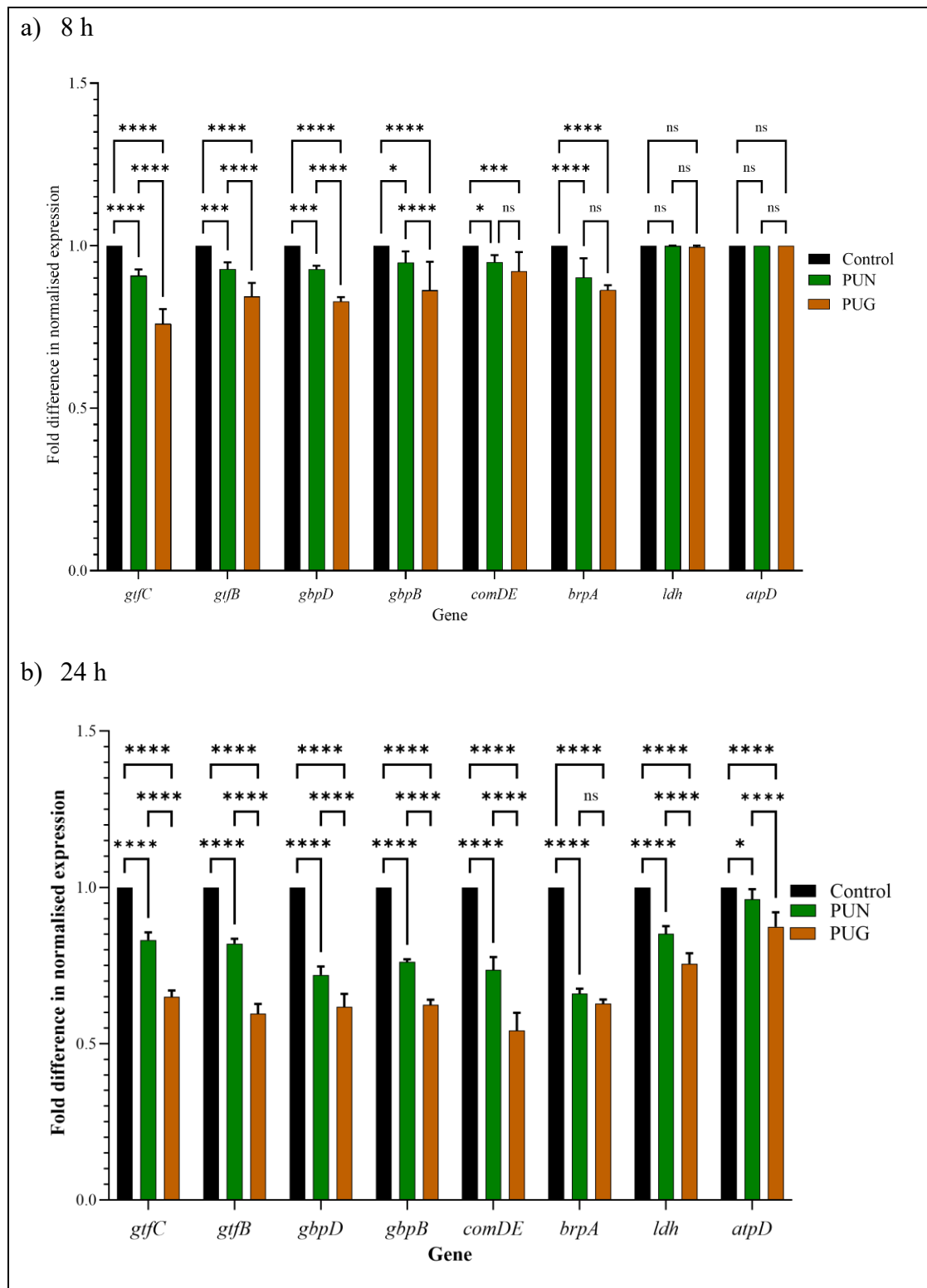


Figure 4.9 Effect of PUG and PUN on *S. mutans* Gene Expression During Inhibition of Dual-Species Biofilm Formation in a Sucrose-Dependent Environment

Gene expression was analysed at (a) 8 h and (b) 24 h in biofilms formed in the presence of PUG and PUN. Expression levels of target genes were normalised to 16S rRNA, and fold changes relative to the control were calculated using the $2^{-\Delta\Delta CT}$ method. Data are presented as mean $\pm$ SD. Statistical significance was denoted as ** ($p < 0.05$), *** ($p < 0.001$), and **** ($p < 0.0001$), while “ns” indicates non-significant differences.

4.10.4.2 Gene Expression of *S. gordonii*

The transcriptional response of *S. gordonii* in dual-species biofilms following treatment with PUN and PUG was assessed at 8 hours and 24 hours under sucrose-dependent conditions using RT-qPCR. Gene expression levels were normalised to 16S rRNA and expressed as fold changes relative to the untreated control (Figure 4.10).

At 8 hours, *gtfG* expression was significantly reduced by both compounds compared with the control ($p < 0.0001$), with PUN producing a greater reduction than PUG. In contrast, *abpA* expression showed a significant decrease following PUN treatment ($p < 0.0001$), while no significant change was observed following PUG exposure. For *csaA* and *sspA*, modest but statistically significant reductions were detected following PUN treatment ($p < 0.05$), whereas PUG treatment did not result in significant differences relative to the control. Across all genes at this time point, PUG-treated samples generally exhibited expression levels comparable to the untreated control.

At 24 hours, a distinct temporal pattern was observed. Expression of *gtfG* remained significantly reduced following treatment with both PUN and PUG compared with the control ($p < 0.0001$), with PUN again demonstrating a more pronounced inhibitory effect. For *abpA*, *csaA*, and *sspA*, significant downregulation was observed following PUN treatment ($p < 0.0001$), whereas expression levels in PUG-treated samples did not differ significantly from the untreated control. The magnitude of suppression for these genes was greater at 24 hours than at 8 hours, indicating a time-dependent response to PUN exposure.

Overall, the results indicate that PUN induces significant, time-dependent transcriptional suppression of multiple adhesion- and surface-associated genes in *S. gordonii* within dual-species biofilms. In contrast, PUG exerts minimal or no effect on most *S. gordonii* genes, except for *gtfG*. This differential transcriptional response was consistently observed at both 8 hours and 24 hours.

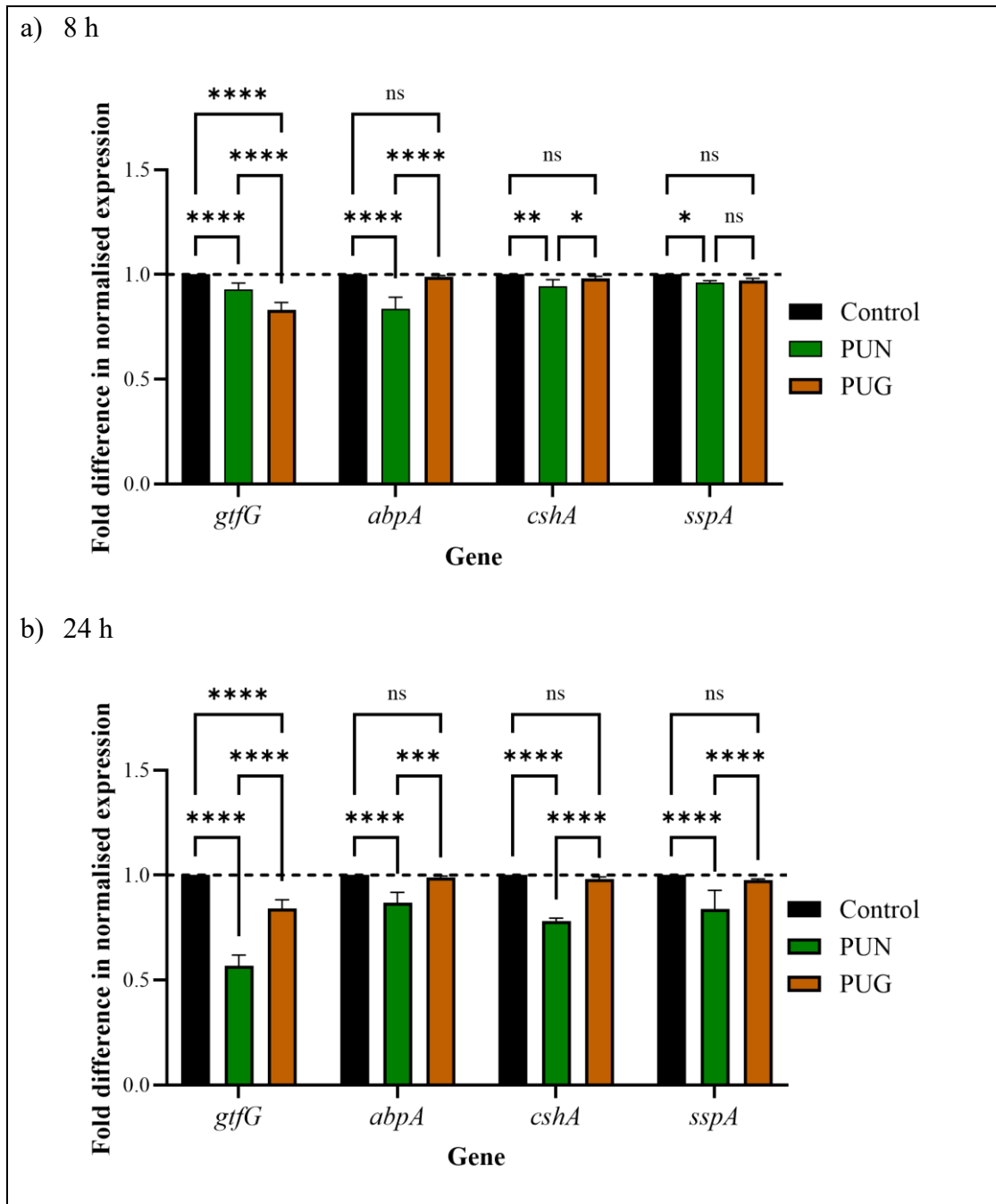


Figure 4.10 Effect of PUG and PUN on *S. gordonii* Gene Expression During Inhibition of Dual-Species Biofilm Formation in a Sucrose-Dependent Environment

Gene expression was analysed at (a) 8 h and (b) 24 h in biofilms formed in the presence of PUG and PUN. Expression levels of target genes were normalised to 16S rRNA, and fold changes relative to the control were calculated using the $2^{-\Delta\Delta CT}$ method. Data are presented as mean $\pm$ SD. Statistical significance was denoted as ** ($p < 0.05$), *** ($p < 0.001$), and **** ($p < 0.0001$), while “ns” indicates non-significant differences.

4.11 Phase II: Dual-Species Biofilm Eradication Assay of PUG and PUN

The effects of PUG, PUN, and CHX on established *S. mutans*–*S. gordonii* dual-species biofilms were evaluated by quantification of total biofilm biomass and viable bacterial counts following 5 minutes and 4 hours treatments.

4.11.1 Biofilm Biomass Quantification

Figure 4.11 illustrates the biomass of *S. mutans*–*S. gordonii* dual-species biofilms following short (5 minutes) and prolonged (4 hours) exposure to PUN, PUG, and CHX, quantified using CV staining (OD₅₉₅ nm).

At 5 minutes exposure (Figure 4.11a), no significant differences in biofilm biomass were observed between the untreated control, PUN-treated, and PUG-treated groups ($p > 0.05$), as indicated by shared statistical grouping. In contrast, CHX treatment resulted in significantly lower biomass than in the control and compound-treated groups ($p < 0.05$).

At 4 hours exposure (Figure 4.11b), biofilm biomass differed significantly among all treatment groups ($p < 0.05$). The untreated control exhibited the highest biomass, followed by PUN-treated biofilms. A further reduction was observed in biofilms treated with PUG, while CHX treatment produced the lowest biomass. Each treatment group was statistically distinct, as indicated by different superscripts. Data are presented as mean $\pm$ SD, and statistical significance was assessed using one-way ANOVA with appropriate post hoc analysis.

4.11.2 Viable Cell Enumeration

Viable bacterial counts were determined by CFU enumeration and expressed as log₁₀ CFU per bead for *S. mutans*, *S. gordonii*, and total bacterial counts (Figure 4.12). The corresponding results of Tukey's post hoc pairwise comparisons for *S. mutans* and *S. gordonii* at both 5 min and 4 h treatment intervals are summarised in Table 4.11. In the untreated control, both species were recovered at high levels, resulting in the highest total CFU values. Following treatment with PUG and PUN, viable counts were reduced relative to the untreated control, but the statistical significance of this reduction differed according to bacterial species and treatment duration. For *S. mutans*, both PUG

and PUN produced significant reductions in CFU counts at 5 min and 4 h compared with the untreated control ($p < 0.05$). This indicates that both compounds exerted an early and sustained inhibitory effect on viable *S. mutans* within the pre-formed dual-species biofilm.

For *S. gordonii*, reductions in viable counts following PUG and PUN treatment were not statistically significant after 5 min of exposure ($p > 0.05$), indicating that short-term treatment did not markedly compromise the recovery of this species. However, after 4 h of treatment, *S. gordonii* CFU counts were significantly reduced compared with the untreated control ($p < 0.05$). Therefore, although *S. gordonii* was relatively preserved during short-term exposure, longer exposure resulted in a measurable reduction in viable cell recovery.

In contrast, CHX treatment resulted in a marked reduction in viable cell counts for both *S. mutans* and *S. gordonii*, leading to a substantial decrease in total CFU values relative to the untreated control and compound-treated biofilms. This confirms the broad bactericidal effect of CHX against both species within the established dual-species biofilm.

Collectively, the results demonstrate that PUG and PUN reduced overall biofilm biomass and viable bacterial recovery in a species- and time-dependent manner. The reduction in *S. mutans* CFU counts at both 5 min and 4 h suggests that the compounds affected the cariogenic species more consistently than *S. gordonii*. In contrast, *S. gordonii* viability was not significantly affected after 5 min but was significantly reduced after 4 h, indicating that selectivity was more evident during short-term exposure. Among the compounds tested, PUG produced a greater reduction in biofilm biomass than PUN, while CHX produced the strongest reduction in both biomass and viable bacterial counts.

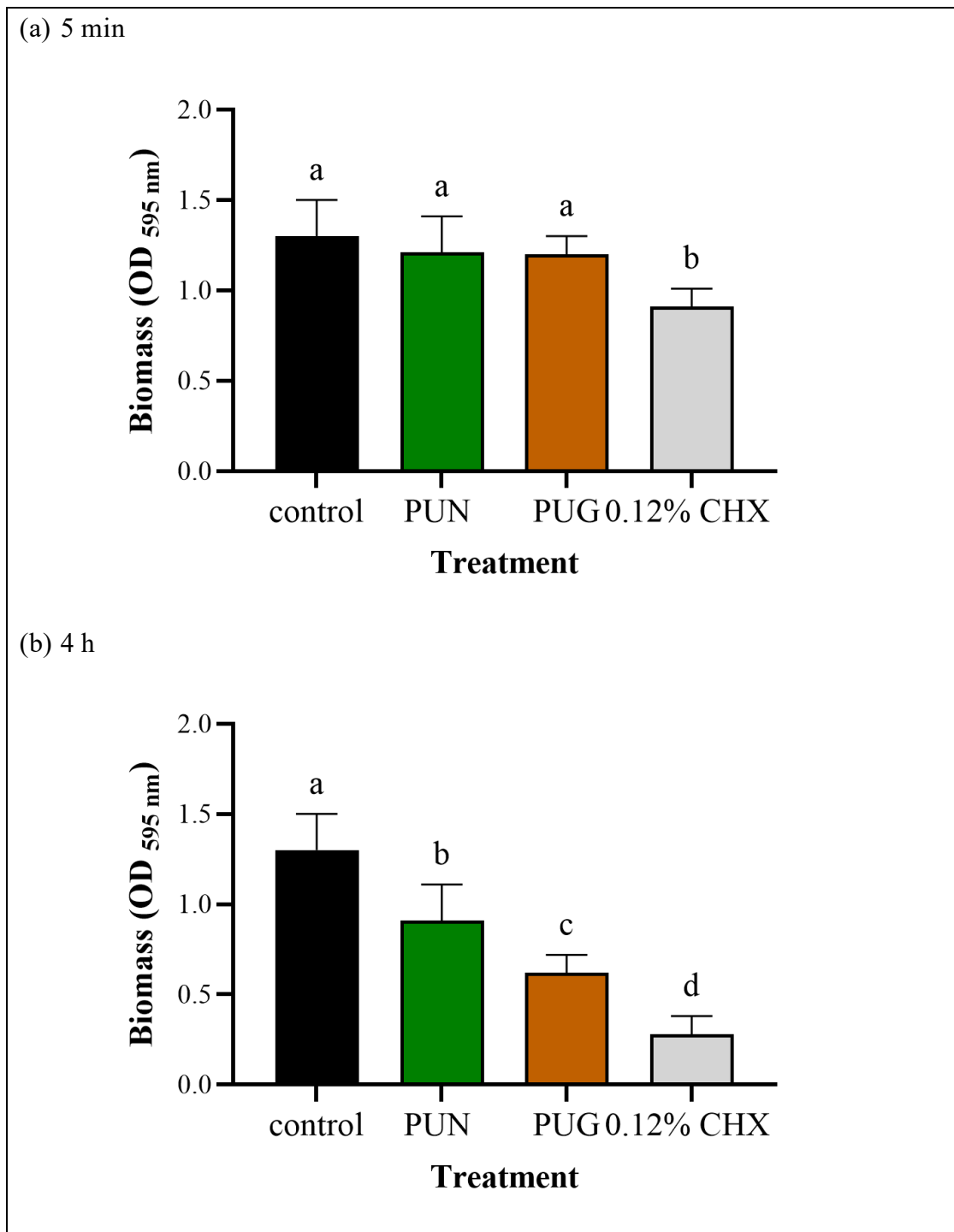


Figure 4.11 Biomass of Dual-Species Biofilms (*S. mutans* + *S. gordonii*) Following Eradication Treatment with PUG, PUN, and CHX

Biofilms were formed on saliva-coated glass beads for 48 h and subsequently treated with PUG and PUN (50 $\mu\text{g/mL}$) and CHX (0.12%). Biofilm biomass was assessed after (a) 5 min and (b) 4 h of treatment using CV staining (OD₅₉₅). Bars represent mean $\pm$ SD of five independent experiments, each performed using three beads per treatment condition. Statistical analysis was conducted using one-way ANOVA followed by Tukey's post hoc test. Different letters indicate statistically significant differences ($p < 0.05$).

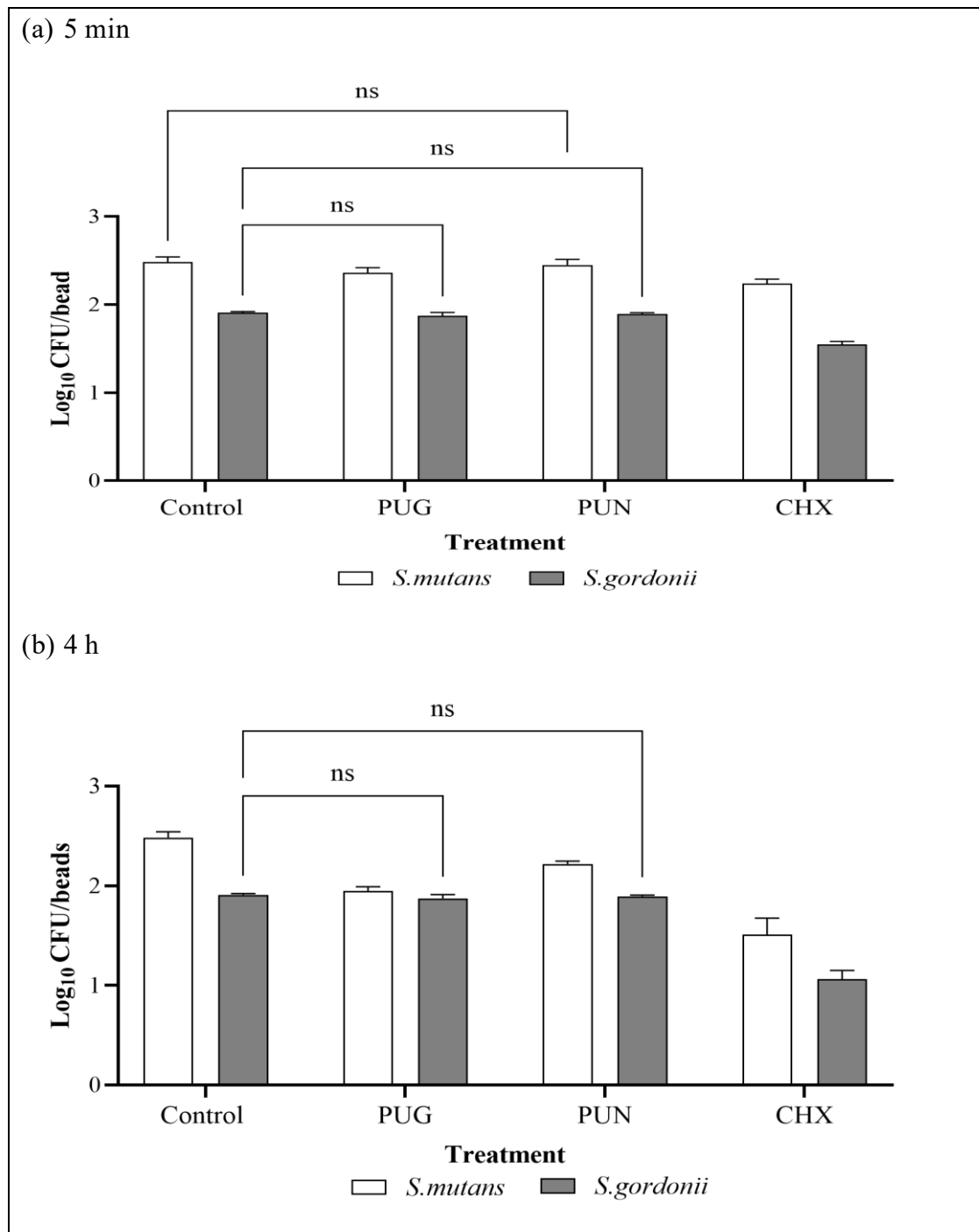


Figure 4.12 Effect of PUG and PUN on Viable Bacterial Counts of *S. mutans* and *S. gordonii* in Dual-Species Biofilms Following Eradication Treatment Log₁₀ CFU/bead at (a) 5 min and (b) 4 h for pre-formed dual-species biofilms treated with PUG (50 µg/mL), PUN (50 µg/mL), and CHX (0.12%). Bars represent mean ± SD of five independent experiments, each performed using three beads per treatment condition. Statistical analysis was conducted using one-way ANOVA followed by Tukey's post hoc test. For clarity, only non-significant comparisons (ns, $p > 0.05$) are indicated in the figure. Significant differences are summarised in Table 4.11.

Table 4.11

Statistical Comparisons of Log₁₀ CFU/bead for *S. mutans*, *S. gordonii*, and Total Biofilm Following Eradication Treatment with PUG, PUN, and CHX

Pairwise comparisons were performed for pre-formed dual-species biofilms assessed after 5 min and 4 h of treatment. Data were analysed using one-way ANOVA followed by Tukey's HSD post hoc test. Statistical significance was set at $p < 0.05$.

Time	Biofilm population	Comparison	p-value	Interpretation
5 min	<i>S. mutans</i>	Control vs PUG	< 0.0001	Significant
		Control vs PUN	< 0.0001	Significant
		Control vs CHX	< 0.0001	Significant
		PUG vs PUN	> 0.05	Not significant
		PUG vs CHX	< 0.0001	Significant
		PUN vs CHX	< 0.0001	Significant
	<i>S. gordonii</i>	Control vs PUG	> 0.05	Not significant
		Control vs PUN	> 0.05	Not significant
		Control vs CHX	< 0.0001	Significant
		PUG vs PUN	> 0.05	Not significant
		PUG vs CHX	< 0.0001	Significant
		PUN vs CHX	< 0.0001	Significant
4 h	<i>S. mutans</i>	Control vs PUG	< 0.0001	Significant
		Control vs PUN	< 0.0001	Significant
		Control vs CHX	< 0.0001	Significant
		PUG vs PUN	< 0.0001	Significant
		PUG vs CHX	< 0.0001	Significant
		PUN vs CHX	< 0.0001	Significant
	<i>S. gordonii</i>	Control vs PUG	> 0.05	Not significant
		Control vs PUN	> 0.05	Not significant
		Control vs CHX	< 0.0001	Significant
		PUG vs PUN	> 0.05	Not significant
		PUG vs CHX	< 0.0001	Significant
		PUN vs CHX	< 0.0001	Significant

4.11.3 SEM Analysis of Pre-formed Dual-Species Biofilms Following Eradication Treatment with PUG and PUN

SEM examination of the untreated (negative control) 48-hour dual-species biofilm revealed a dense, mature structure, characterised by extensive accumulation of coccal cells embedded within a thick, continuous extracellular matrix (Figure 4.13A). The biofilm surface appeared compact and heterogeneous, with cells displaying both rounded and slightly ovoid morphologies distributed throughout the matrix. Due to morphological overlap between species and the observation that both species exhibited similar ovoid morphology in single-species biofilms, species-level identification based on cell shape was not feasible. Accordingly, SEM observations were interpreted qualitatively, while species-specific quantification was obtained through CFU enumeration.

Treatment with PUG resulted in pronounced disruption of the dual-species biofilm architecture (Figure 4.13B). The PUG-treated biofilm exhibited marked loss of extracellular matrix continuity, with extensive regions of biofilm thinning and surface exposure. Numerous areas of void formation and reduced cellular cohesion were observed (boxed regions), and coccus cells appeared less densely packed compared with the untreated control. These morphological features indicate substantial weakening of the biofilm structural integrity following PUG treatment.

In contrast, PUN treatment produced moderate biofilm disruption relative to the untreated control (Figure 4.13C). Although partial disorganisation of the extracellular matrix was evident, the biofilm largely retained a cohesive architecture, with clustered coccal cells embedded within residual matrix material. Localised areas of matrix disruption and surface exposure were observed (arrows), but these were less extensive than those seen in the PUG-treated biofilm.

Treatment with CHX resulted in the most extensive biofilm disruption among all groups (Figure 4.13D). The biofilm structure was markedly diminished, with substantial loss of extracellular matrix and sparse, scattered coccal cell aggregates remaining on the surface. Large exposed areas of the underlying substrate were visible, indicating near-complete biofilm removal. Notably, the pronounced architectural disruption observed in PUG- and PUN-treated biofilms was not accompanied by a significant reduction in viable cell counts at the 4 hours eradication time point, as

determined by CFU enumeration. This indicates a dissociation between biofilm structural destabilisation and immediate loss of bacterial viability.

Collectively, SEM observations demonstrated a treatment-dependent reduction in biofilm integrity, with the untreated control exhibiting a dense, cohesive structure, PUG inducing greater structural disruption than PUN, and CHX producing the most pronounced biofilm removal. As SEM analysis was used for qualitative assessment of biofilm morphology and architecture, quantitative evaluation of treatment effects was supported by CFU enumeration data.

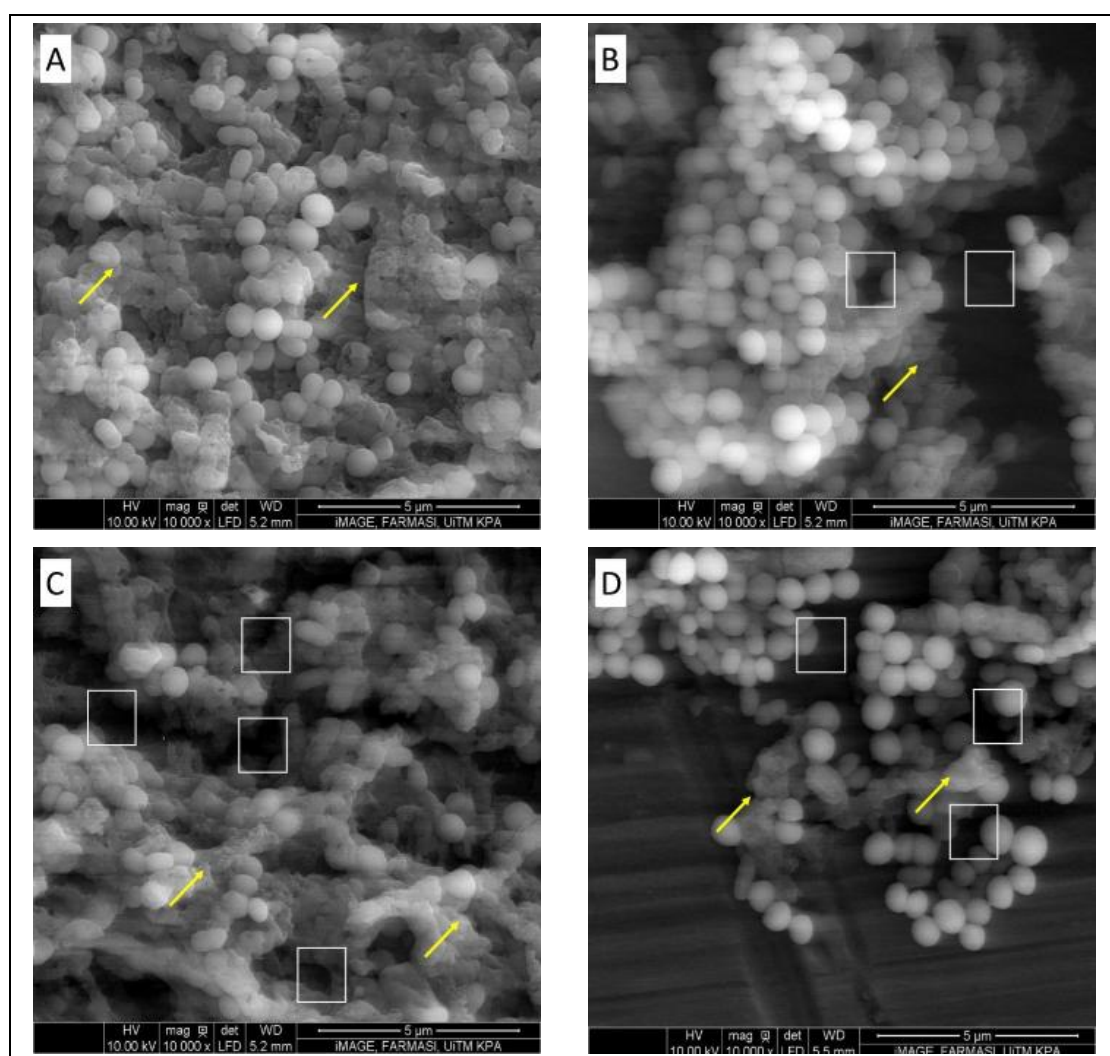


Figure 4.13 SEM Analysis of Preformed Dual-Species Biofilms (*S. mutans* + *S. gordonii*) Following Eradication Treatment with PUG and PUN
SEM micrographs showing biofilm morphology after 48 h of biofilm formation on saliva-coated glass beads and subsequent 4 h treatment with (a) 1% DMSO (negative control), (b) PUG (50 µg/mL), (c) PUN (50 µg/mL), and (d) CHX (0.12%, positive control). Yellow arrows indicate residual extracellular polymeric substance (EPS), and white boxes highlight regions of matrix disruption or bacterial clearance.
Magnification: 10,000×.

4.11.4 Gene Expression Analysis of Pre-formed Dual-Species Biofilms Following Eradication Treatment with PUG and PUN

4.11.4.1 Gene Expression of *S. mutans*

The effects of PUN and PUG on the expression of selected biofilm-associated genes in *S. mutans* within preformed dual-species biofilms were evaluated following 5 minute and 4 hours exposures using RT-qPCR. Gene expression levels were normalised to the housekeeping gene and expressed as fold change relative to the untreated control (Figure 4.14)

Following 5 minutes of exposure, no significant changes in the expression of *gtfC*, *gtfB*, *gbpD*, and *gbpB* were observed in biofilms treated with either PUN or PUG compared with the untreated control ($p > 0.05$) (Figure 4.14a). Expression levels for these genes remained near baseline, with fold changes of approximately 1.0 across all groups. In contrast, *atlA* expression was significantly upregulated in PUG-treated biofilms compared with both the control and PUN-treated groups ($p < 0.0001$). No significant difference in *atlA* expression was observed between the control and PUN-treated biofilms ($p > 0.05$). Expression of *lytF* did not differ significantly among the control, PUN-, and PUG-treated groups following 5 minutes of exposure ($p > 0.05$). Overall, short-term exposure to PUN and PUG induced minimal transcriptional changes in most target genes, with the notable exception of *atlA* following PUG treatment.

Following 4 hours of exposure, significant alterations in gene expression were observed for multiple targets (Figure 4.14b). Both PUN and PUG treatments resulted in significant downregulation of *gtfC*, *gtfB*, *gbpD*, and *gbpB* compared with the untreated control ($p < 0.0001$). The magnitude of downregulation varied across compounds, with PUN generally exhibiting lower fold changes than PUG for these genes. Expression of *atlA* was significantly upregulated following PUG treatment compared with both the control and PUN-treated groups ($p < 0.0001$). In contrast, *atlA* expression in PUN-treated biofilms did not differ significantly from that in untreated controls ($p > 0.05$). Similarly, *lytF* expression was significantly higher in PUG-treated biofilms than in the control and PUN-treated groups ($p < 0.0001$). A modest but significant increase in *lytF* expression was also observed following PUN treatment compared with control ($p < 0.05$). Collectively, prolonged exposure to PUN and PUG induced time-dependent

transcriptional changes in preformed biofilms, with more pronounced effects at 4 hours than at 5 minutes.

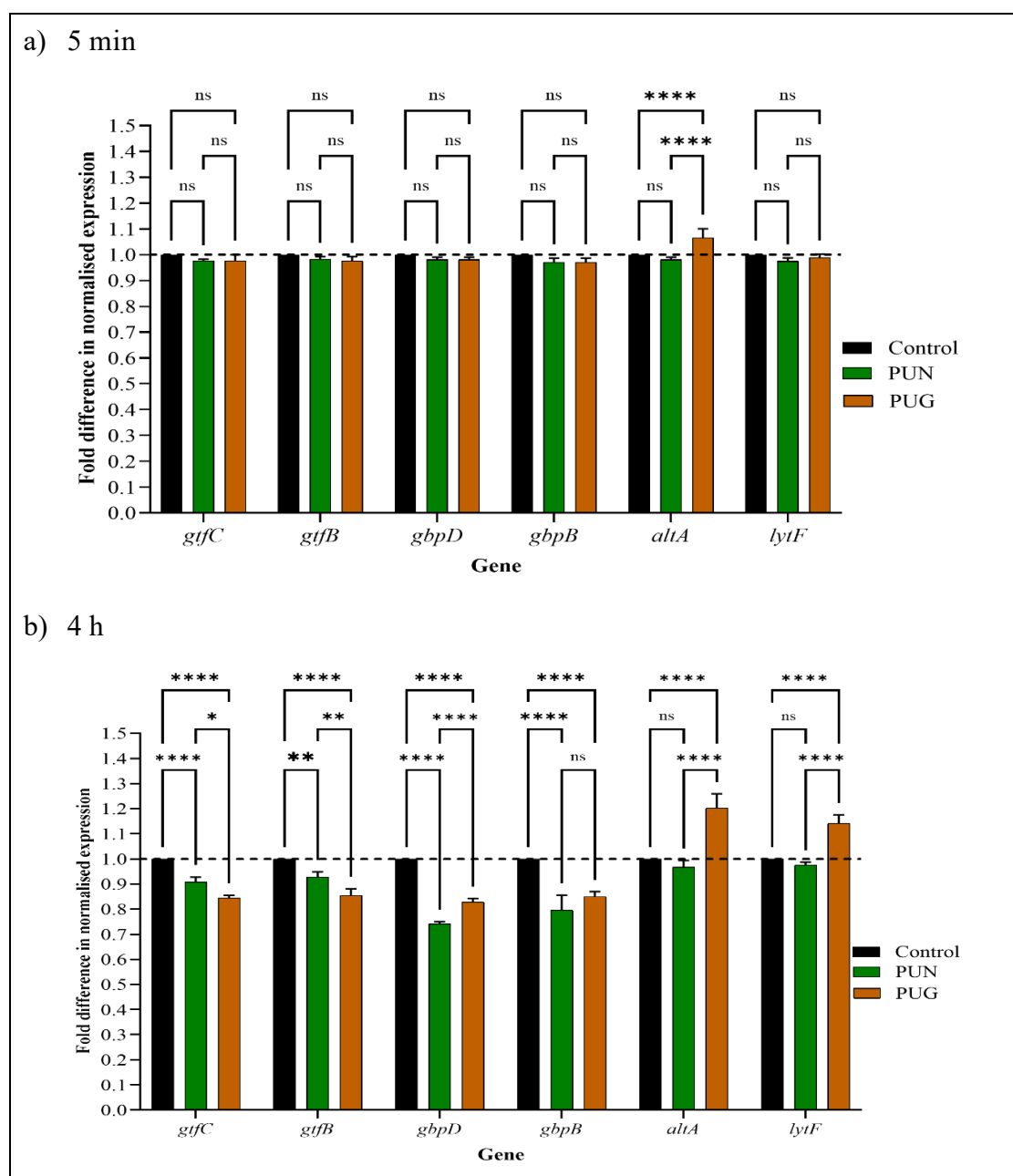


Figure 4.14 Effect of PUG and PUN on *S. mutans* Gene Expression in Dual-Species Biofilms Following Eradication Treatment

Gene expression was analysed at (a) 5 min and (b) 4 h after treatment of pre-formed dual-species biofilms with PUG and PUN (100 $\mu\text{g/mL}$; MBEC₅₀). Expression levels of target genes were normalised to the 16S rRNA reference gene, and fold changes relative to the untreated control were calculated using the $2^{-\Delta\Delta\text{CT}}$ method. The dashed horizontal line indicates the baseline expression level of the control (fold change = 1.0). Bars represent mean $\pm$ SD of five independent determinations performed in triplicate. Statistical analysis was conducted using one-way ANOVA followed by Tukey's post hoc test. Statistical significance is indicated as * ($p < 0.05$), ** ($p < 0.01$), and **** ($p < 0.0001$), while "ns" denotes no significant difference.

4.11.4.2 Gene Expression of *S. gordonii*

The transcriptional response of *S. gordonii* to PUN and PUG was examined at 5 minutes and 4 hours to determine whether these compounds elicited immediate or sustained changes in gene expression in the commensal species. Gene expression levels were normalised to the reference gene and expressed as fold change relative to the untreated control (Figure 4.15)

At 5 minutes, expression of the adhesion-associated genes *csaA* and *sspA* remained near baseline following exposure to both PUN and PUG, with no statistically significant differences relative to the control (Figure 4.15a). In contrast, the metal homeostasis-associated genes *scaA* and *scaR* showed a slight increase in expression for both compounds, although the magnitude of these changes was modest.

At 4 hours, expression levels of *csaA*, *sspA*, and *scaR* were not significantly different from those of the untreated control for either compound (Figure 4.15b). However, *scaA* expression was significantly upregulated following PUN exposure, whereas no significant change was observed with PUG. Notably, this increase in *scaA* was not accompanied by parallel upregulation of *scaR* or alterations in adhesion-associated genes.

Overall, gene expression in *S. gordonii* remained largely stable following exposure to PUN and PUG at both 5 minutes and 4 hours, with only minor, gene-specific changes observed and no consistent pattern of widespread transcriptional alteration.

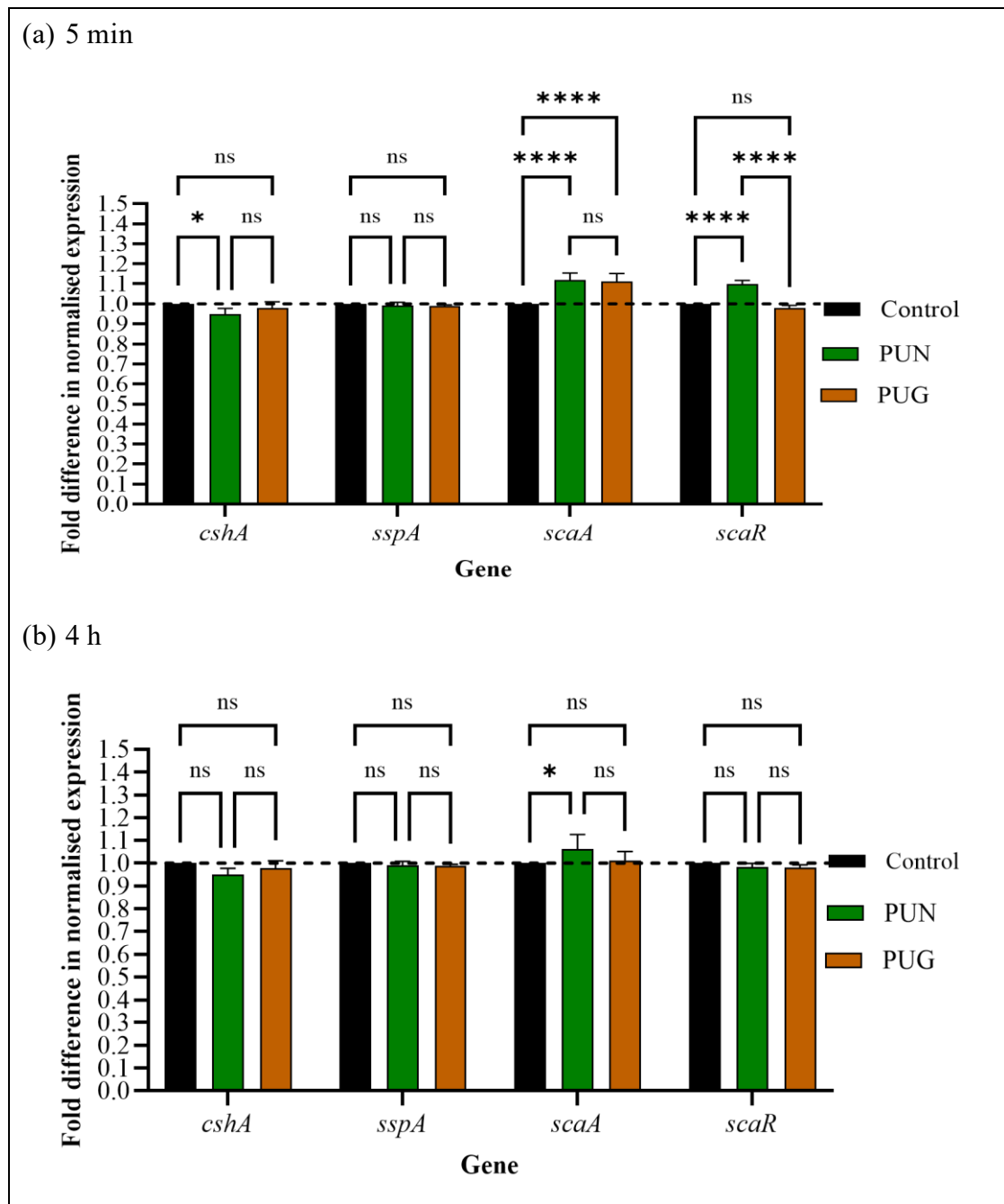


Figure 4.15 Effect of PUG and PUN on *S. gordonii* Gene Expression in Dual-Species Biofilms Following Eradication Treatment

Gene expression was analysed at (a) 5 min and (b) 4 h after treatment of pre-formed dual-species biofilms with PUG and PUN (100 $\mu\text{g/mL}$; MBEC₅₀). Expression levels of *cshA*, *sspA*, *scaA*, and *scaR* were normalised to the 16S rRNA reference gene, and fold changes relative to the untreated control were calculated using the $2^{-\Delta\Delta\text{CT}}$ method. The dashed horizontal line indicates the baseline expression level of the control (fold change = 1.0). Bars represent mean $\pm$ SD of five independent determinations performed in triplicate. Statistical analysis was conducted using one-way ANOVA followed by Tukey's post hoc test. Statistical significance is indicated as * ($p < 0.05$) and **** ($p < 0.0001$), while "ns" denotes no significant difference.

CHAPTER 5

DISCUSSION

5.1 Overview of the Key Findings

This study investigated the antibiofilm and antivirulence potential of purified pomegranate-derived compounds (PDCs) using an ecology-based experimental framework designed to evaluate selective modulation of cariogenic biofilms rather than indiscriminate bacterial killing. This ecology-centred approach reflects the contemporary understanding that dental caries arises from functional dysbiosis within the oral biofilm rather than infection by a single pathogen, thereby emphasising targeted attenuation of virulence traits while preserving health-associated microbial communities. The investigation was structured into two sequential experimental phases. Phase I involved comparative screening of multiple PDCs to evaluate antibacterial activity, modulation of virulence-associated traits, antibiofilm effects, acidogenicity, and preliminary cytotoxicity. Phase II focused on detailed mechanistic evaluation of the lead compounds punicalagin (PUG) and punicalin (PUN) using an ecologically relevant dual-species biofilm model comprising *Streptococcus mutans* (*S. mutans*) and *Streptococcus gordonii* (*S. gordonii*).

Overall, the findings demonstrate that selected PDCs exhibit multiple antivirulence effects relevant to cariogenic biofilm development, including modulation of cell surface hydrophobicity, inhibition of biofilm formation, reduction of acidogenic potential, and selective effects on bacterial viability. Among the screened compounds, PUG and PUN were prioritised for further investigation based on their consistent antibiofilm performance across multiple phenotypic assays. These compounds demonstrated the capacity to attenuate key virulence traits associated with *S. mutans*, while exerting comparatively limited effects on the health-associated commensal *S. gordonii*.

In the dual-species biofilm model, both compounds inhibited biofilm formation and partially disrupted established biofilm architecture. Structural and microbiological analyses indicated that the antibiofilm effects were associated with reduced extracellular polysaccharide accumulation and decreased recovery of *S. mutans* cells, while *S. gordonii* populations remained comparatively preserved.

Gene expression analysis further revealed downregulation of several virulence-associated pathways involved in adhesion, extracellular polysaccharide synthesis, and stress adaptation in *S. mutans*, supporting the hypothesis that PUG and PUN act primarily through antivirulence and biofilm-modulating mechanisms rather than through broad bactericidal activity.

To the best of current knowledge, this study represents the first systematic evaluation of multiple purified pomegranate-derived compounds within a dual-species oral biofilm framework. Most previous investigations have relied on crude plant extracts, and even when purified compounds were examined (Li et al., 2020; Zeng et al., 2019), studies have largely employed simplified single-species biofilm models, limiting their relevance to the ecological complexity of caries-associated oral biofilms.

By incorporating both cariogenic and commensal bacteria, the present study was designed to more closely reflect clinically relevant biofilm dynamics, in which microbial persistence and virulence are shaped by competitive and cooperative interspecies interactions. This experimental framework therefore enables a more nuanced interpretation of antimicrobial efficacy, including the potential for selective suppression of cariogenic species while preserving commensal microorganisms that contribute to oral microbial homeostasis. Accordingly, the following sections discuss the findings of each experimental phase in relation to the study objectives and research questions, while integrating current scientific literature to contextualise the results and explore the underlying biological and ecological mechanisms. Particular emphasis is placed on interpreting results across successive experimental tiers, ranging from antibiofilm inhibition and eradication to modulation of early virulence determinants and preliminary cellular safety, thereby providing a coherent and mechanistically informed interpretation of the study outcomes.

5.2 Ecological Rationale for Evaluating PDCs as Selective Antibiofilm and Antivirulence Agents

Caries prevention should aim not at indiscriminate bacterial elimination, but at restoring environmental conditions that favour commensal competitiveness while suppressing cariogenic dominance. This paradigm shift has driven the development of ecological approaches to oral biofilm management, which prioritise selective suppression of pathogenic traits while preserving or enhancing beneficial microbial

functions. Collectively, these concepts support a caries management strategy focused on maintaining microbial balance rather than achieving sterility.

Within this paradigm, the indiscriminate bactericidal activity of conventional antimicrobials, particularly chlorhexidine (CHX), is increasingly regarded as biologically suboptimal because of its potential to disrupt microbial homeostasis and compromise ecological stability. While CHX remains highly effective for short-term plaque and gingivitis control, its broad-spectrum, non-selective mode of action does not differentiate between pathogenic taxa and health-associated commensals that contribute to colonisation resistance, immune modulation, and metabolic balance within the oral ecosystem (Angjelova et al., 2025).

At the level of microbial life forms, CHX has been shown to exert differential effects on planktonic versus surface-associated bacteria. Early mechanistic evidence demonstrated that initially enamel-attached *Streptococcus sanguinis*, a key early coloniser associated with oral health, is markedly more susceptible to CHX than its planktonic counterpart during early biofilm formation (Decker et al., 2003). This heightened vulnerability is attributed to the strong substantivity of CHX and its affinity for oral surfaces, which results in increased antimicrobial activity at the enamel–biofilm interface. Although such activity may enhance immediate plaque suppression, preferential elimination of early commensal colonisers may destabilise the foundational biofilm architecture, potentially facilitating recolonisation by more acidogenic or dysbiosis-associated species. At the community level, clinical microbiome studies further demonstrate that CHX induces substantial ecological perturbations. Prolonged use has been associated with reduced microbial diversity, suppression of species richness, and broad alterations in community structure across oral niches (do Amaral et al., 2023).

More recent analyses indicate that even short-term CHX exposure can significantly disrupt health-associated taxa, including nitrate-reducing genera, and induce widespread perturbations of functional pathways indicative of microbial stress rather than ecological recovery (Wattanawongwan et al., 2025). In response to this challenge, the present study was designed to evaluate pomegranate-derived phytochemicals not merely as antimicrobial agents, but as selective antivirulence modulators capable of attenuating cariogenic traits while preserving commensal viability. By integrating assessments of planktonic susceptibility, biofilm inhibition and eradication, virulence-associated functional assays, transcriptional profiling, and

toxicity screening, this work adopts a mechanism-guided framework that emphasises functional specificity over indiscriminate microbial suppression.

5.3 Planktonic Antimicrobial Activity via MIC/MBC Determination

Initial planktonic antimicrobial screening using disc diffusion assays, together with minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) analyses, demonstrated that all tested compounds possessed measurable antibacterial activity against *S. mutans* and *S. gordonii*. Disc diffusion testing served as a rapid, qualitative method to visualise relative antimicrobial potency among compounds, revealing clear zones of inhibition for all agents tested. Among these, punicalagin (PUG) consistently produced the largest inhibition zones, comparable to those observed for CHX, indicating strong intrinsic activity against planktonic oral streptococci. The MIC and MBC determinations further substantiated these findings by defining the concentration-dependent inhibitory and bactericidal thresholds for each compound.

The compounds evaluated in this study represent multiple biologically relevant classes of plant polyphenols, including hydrolysable tannins which include punicalagin (PUG), punicalin (PUN), corilagin (COR), flavonoid that include gallic acid (GA), kaempferol-glucoside (KG) and rutin (RUT), and phenolic acid, ellagic acid (EA). The pronounced planktonic efficacy observed for punicalagin highlights the antimicrobial potential of hydrolysable tannins, which are characterised by high molecular weight, multiple phenolic hydroxyl groups, and strong capacity to interact with bacterial cell surfaces, membrane-associated proteins, and extracellular enzymes.

The pronounced planktonic efficacy observed for PUG highlights the antimicrobial potential of hydrolysable tannins, which are characterised by high molecular weight, a high density of phenolic hydroxyl groups, and a strong capacity to engage in multivalent interactions with bacterial cell surfaces, membrane-associated proteins, and extracellular enzymes. Mechanistic studies have shown that hydrolysable tannins exert their antibacterial activity through a combination of membrane-targeting and protein-binding effects, including disruption of membrane integrity, alteration of membrane fluidity, and interference with surface-exposed enzymes critical for bacterial growth and metabolism. For instance, Villanueva et al. demonstrated that unmodified hydrolysable tannins exhibit broad antibacterial and antibiofilm activity against both

Gram-positive and Gram-negative bacteria, and that their biological activity is closely linked to structural features such as molecular size, charge distribution, and capacity for surface interactions rather than simple growth inhibition alone (Villanueva et al., 2023).

At the cellular level, hydrolysable tannins have been shown to induce pronounced physicochemical changes in bacterial membranes. Olchowik-Grabarek et al. reported that ellagitannins and gallotannins interact directly with membrane lipids and proteins, leading to increased membrane permeability, altered membrane fluidity, and destabilisation of barrier function in *Staphylococcus aureus*. These membranotropic effects were closely correlated with antibacterial potency and were influenced by tannin molecular flexibility, hydrophobicity, and dipole moment, providing a structural basis for the strong planktonic activity observed for larger, more flexible tannin molecules (Olchowik-Grabarek et al., 2022).

Despite their utility, planktonic susceptibility assays provide only limited insight into antimicrobial performance under biofilm-associated conditions. In the oral cavity, *Streptococcus* species predominantly exist within structured biofilms, where cells are embedded in an extracellular polymeric matrix and exhibit altered metabolic states, reduced growth rates, and enhanced tolerance to environmental stressors. These physiological adaptations substantially diminish susceptibility to agents that are otherwise effective against free-living bacteria. Consequently, large inhibition zones or low MIC values obtained under planktonic conditions do not reliably predict antibiofilm efficacy. The present findings reinforce this limitation. Certain compounds exhibiting relatively low MIC values in planktonic assays, such as GC, did not demonstrate correspondingly strong antibiofilm or antivirulence effects. This divergence shows that conclusions drawn from planktonic assays alone are insufficient for evaluating therapeutic relevance in biofilm-associated diseases, and emphasises the importance of assessment approaches that better reflect biofilm biology. Accordingly, MIC and MBC values were used in this study as reference benchmarks to guide the selection of concentrations for subsequent biofilm inhibition and eradication assays. This approach enabled interpretation of antibiofilm outcomes in relation to established planktonic inhibitory and bactericidal thresholds. Antibiofilm activity is increasingly recognised as concentration-dependent and may arise through distinct mechanisms that encompass both bactericidal effects and targeted antivirulence modulation. At concentrations at or above the MBC, biofilm reduction is predominantly driven by reduced bacterial viability or direct killing of biofilm-embedded cells. This requirement reflects the

intrinsic tolerance of biofilm-associated bacteria, which exhibit reduced metabolic activity, physiological heterogeneity, and persister subpopulations that require bactericidal concentrations to achieve cell death (Hall & Mah, 2017). In this context, antibiofilm effects observed at bactericidal levels should be interpreted primarily as killing-dominated outcomes rather than selective disruption of biofilm regulatory pathways (Coenye, 2023).

In contrast, biofilm inhibition observed at sub-MIC or sub-MBC concentrations is more consistent with non-lethal mechanisms. Sub-inhibitory antimicrobial exposure has been shown to alter biofilm biomass and architecture without substantial reductions in viable cell counts, indicating functional modulation of biofilm development rather than bactericidal action (Elawady et al., 2024). In oral streptococci, (Goc et al., 2019). demonstrated that while biofilm eradication at MBC-equivalent concentrations was associated with loss of bacterial viability, sub-MBC exposure selectively attenuated adhesion, acidogenicity, and aciduricity, thereby reducing cariogenic potential without eliminating bacterial survival (Goc et al., 2019). Complementing this concentration-dependent framework, Xu et al. showed that tannic acid suppresses *S mutans* biofilm formation through inhibition of glucosyltransferase activity and extracellular polysaccharide synthesis without impairing bacterial growth, exemplifying a targeted antivirulence mechanism independent of killing (Xu et al., 2025).

Together, these studies establish that antibiofilm efficacy reflects a mechanistic spectrum ranging from killing-driven biofilm eradication at high concentrations to selective modulation of adhesion, matrix production, and virulence-associated pathways at sub-inhibitory levels. This integrated framework supports the use of MIC/MBC-guided concentration selection to distinguish bactericidal effects from true antivirulence activity in biofilm studies.

5.4 Cell Surface Hydrophobicity as an Early Antivirulence Target

Cell surface hydrophobicity (CSH) plays a central role in the earliest stages of bacterial attachment to surfaces and is therefore a critical determinant of initial biofilm establishment. Hydrophobic interactions enable bacteria to adhere to the tooth surface and to neighbouring cells even before sucrose-dependent mechanisms become dominant. Bacterial cells with low hydrophobicity generally exhibit weak surface adhesion, which translates into reduced surface retention and impaired biofilm

initiation. For this reason, alterations in CSH are increasingly recognised as an important early event influencing downstream biofilm development.

In cariogenic streptococci, particularly *S. mutans*, high baseline hydrophobicity is a well-documented characteristic that contributes to its strong adhesive capacity and competitive advantage during early colonisation. Numerous studies investigating natural products and plant-derived compounds with anticariogenic potential have therefore incorporated CSH assays as an established parameter for assessing virulence-associated behaviour. In untreated controls, the hydrophobicity index of *S. mutans* is consistently reported to exceed 70%, highlighting its inherent tendency for surface attachment and biofilm initiation (Vijayakumar et al., 2021; Liu et al., 2023; Sangavi et al., 2024)

In contrast, comparatively few studies have examined CSH in oral commensal streptococci. This limited focus is largely attributable to the biological role of these species in maintaining oral microbial homeostasis. Early colonisers such as *S. gordonii*, *Streptococcus mitis*, *Streptococcus intermedius*, and *Streptococcus oralis* rely predominantly on specific adhesin–receptor interactions with salivary components rather than nonspecific hydrophobic forces for surface attachment. Consequently, their baseline hydrophobicity is typically low, often reported to be below 10%, reflecting an ecological strategy geared towards stable colonisation rather than aggressive surface dominance (Christian et al., 2024). As a result, CSH has not traditionally been considered a primary virulence-associated trait in commensal species.

The findings of the present study are in agreement with this established pathogenic–commensal distinction. The baseline CSH of untreated *S. mutans* was approximately 70%, whereas *S. gordonii* exhibited a markedly lower value of around 35%. Although the baseline hydrophobicity of *S. gordonii* observed here was higher than that reported in some previous studies, this variation may be attributed to strain differences, growth conditions, or methodological factors inherent to the assay. Importantly, the relative contrast between the cariogenic and commensal species remained clear, reinforcing the concept that elevated hydrophobicity is a defining virulence-associated feature of *S. mutans*.

From a mechanistic standpoint, early interference with CSH is likely to compromise the initial physical anchoring of *S. mutans* to the tooth surface, thereby limiting subsequent microcolony formation, extracellular polysaccharide accumulation, and establishment of an acidic microenvironment. Previous studies have demonstrated

that reducing the hydrophobic nature of *S. mutans* diminishes sucrose-independent adherence, delays biofilm maturation, and ultimately suppresses cariogenic potential without exerting bactericidal pressure (Sangavi et al., 2024; Vijayakumar et al., 2021). This positions CSH modulation as an early-stage antivirulence intervention acting upstream of irreversible biofilm architecture.

5.5 Formation of an Experimental Salivary Pellicle for Biofilm Development

In the present study, an experimental salivary pellicle was incorporated during both the initial screening phase and Phase II biofilm assays, and was applied prior to bacterial inoculation for single-species and dual-species biofilm models. This approach was adopted to more accurately simulate *in vivo* oral conditions, as initial bacterial adhesion and subsequent biofilm development occur on saliva-conditioned surfaces rather than on bare abiotic substrates. Saliva contains a range of proteins, including proline-rich proteins (PRPs), mucins, cystatins, histatins, statherin, host defence peptides, and α -amylase, which collectively form the acquired pellicle on oral surfaces. Among these components, salivary α -amylase plays a key role in the adhesion of pioneer bacteria such as *S. sanguinis*, *S. mitis*, and *S. gordonii* by acting as a pellicle-associated receptor for early-colonising streptococci, thereby supporting initial attachment, co-adhesion, and stable microbial colonisation (Lynge Pedersen & Belstrøm, 2019). Saliva processing is a critical methodological consideration in experimental pellicle formation, as inappropriate handling may compromise the integrity and functionality of pellicle-forming proteins. In this study, clarified whole saliva was filtered to remove debris and microorganisms, with minimal trapping or substantial alteration of salivary proteins involved in bacterial adhesion. Polyethersulfone (PES) low-protein-binding filters were selected to preserve key pellicle components, including proline-rich proteins, mucins, and glycoproteins, which mediate receptor–ligand interactions essential for early coloniser attachment and interspecies interactions. The use of PES membranes for saliva filtration has been widely reported in previous studies, supporting this methodological choice for maintaining salivary protein integrity while ensuring sterility (Ansari et al., 2017; Dang et al., 2018). To minimise biological variability, saliva was obtained from a single healthy donor, with exclusion criteria for recent antibiotic use and oral disease. Although quantitative analysis of individual salivary proteins was not performed, this

controlled approach ensured consistency across experimental phases and enabled reliable comparison of compound effects. While alternative models employing artificial saliva with defined media or Mucin-based Media have been reported (Ketagoda et al., 2024; Zhang et al., 2025). In the present study, the use of carefully processed, clarified whole saliva was considered an appropriate balance between biological authenticity and experimental reproducibility.

The importance of salivary pellicle incorporation was further underscored by the use of different abiotic substrates in this study. Glass beads, which possess surface properties more closely resembling dental enamel, support the formation of a tooth-like salivary pellicle that facilitates physiologically relevant bacterial adhesion and early biofilm development. In contrast, polystyrene surfaces are intrinsically hydrophobic and may introduce substrate-driven bias in microbial attachment. Salivary conditioning of polystyrene surfaces was therefore essential to reduce hydrophobic bias, given that the salivary pellicle is one of the most important factors influencing bacterial adhesion and is known to homogenise surface contact angles while increasing surface hydrophilicity. (Sardin, 2004). This conditioning promotes adhesion mediated by pellicle–bacteria interactions rather than nonspecific physicochemical surface effects.

Collectively, incorporating a well-processed experimental salivary pellicle across substrates and experimental phases strengthened the translational relevance of the employed biofilm models. This approach ensured that observed differences in biofilm inhibition, eradication, and virulence modulation could be attributed to the effects of pomegranate-derived compounds rather than artefacts arising from substrate properties or saliva handling, thereby supporting more meaningful interpretation within a clinically relevant oral biofilm context.

5.6 Optimisation of a Reproducible Dual-Species Oral Biofilm Model

Establishing a reproducible, biologically relevant dual-species biofilm model is a critical prerequisite for meaningful evaluation of antibiofilm efficacy, particularly in systems involving functionally distinct oral streptococci, such as *S. mutans* and *S. gordonii*. In the present study, optimisation was undertaken to balance robust biofilm formation with reliable retention of both species within the adherent community, thereby avoiding experimental artefacts arising from single-species dominance. The combined evaluation of total inoculum density, sucrose concentration, and initial

inoculum ratio allowed identification of conditions that supported stable coexistence while maintaining assay reproducibility.

Total starting inoculum density emerged as a key determinant of biofilm stability and experimental consistency. Although higher inoculum densities increased total biomass, they were frequently associated with increased heterogeneity and surface crowding, which can obscure subtle antibiofilm effects and reduce reproducibility. In contrast, a lower total inoculum of 1×10^6 CFU/mL consistently produced well-defined, adherent biofilms with minimal variability. Importantly, this inoculum density and a 1:1 inoculum ratio has been widely adopted in established dual-species cariogenic biofilm models involving *S. mutans* and commensal species such as *Streptococcus sanguinis* and *S. gordonii*, where each species was inoculated at 1×10^6 CFU/mL to achieve a balanced and reproducible baseline community (Guo et al., 2019; Sun et al., 2019; Hu et al., 2022)

The convergence of our selected inoculum parameters with these independent studies supports the biological relevance and methodological robustness of the optimised model. Critically, establishing a stable and reproducible untreated (baseline) biofilm is essential before the application of any antibiofilm intervention, as it ensures that observed treatment-associated effects can be attributed to true biological modulation rather than variability arising from uneven initial colonisation (Heersema & Smyth, 2019). The deliberate selection of this inoculum density therefore reflects a balance between biological realism and experimental control, providing a reliable platform for mechanistic evaluation of antibiofilm activity.

Sucrose concentration played a central role in shaping biofilm accumulation and matrix development. Increasing sucrose levels from 0.5% to 4.0% (w/v) resulted in progressively higher biofilm biomass, consistent with sucrose's role as a key substrate for extracellular polysaccharide synthesis. However, although 2.0-4.0 % sucrose produced the greater biomass, this condition was associated with increased variability and excessive matrix accumulation, suggesting the formation of an overly dense biofilm architecture that may restrict nutrient diffusion and complicate downstream analyses. In contrast, 1.0% (w/v) sucrose consistently supported robust and reproducible biofilm formation while maintaining structural stability. Importantly, CFU analysis confirmed that this concentration did not compromise *S. gordonii* retention when combined with an appropriate inoculum density and a balanced inoculum ratio. These findings indicate

that sucrose concentration alone does not dictate species dominance; rather, its impact is modulated by initial community composition and overall cell density.

The selection of 1.0% (w/v) sucrose in the present study is further supported by the work of Cai et al., who reported that the turning concentration for cariogenic biofilm formation lies within a functional range of approximately 0.6% to 2.4% sucrose, underscoring the strong and non-linear influence of sucrose on biofilm accumulation and caries development (Cai et al., 2018). Consistent with this functional window, biofilms formed at 1.0% sucrose in this study exhibited reproducible biomass together with stable dual-species retention under optimised inoculum conditions. In contrast, higher sucrose levels led to disproportionately dense biofilms with greater variability. Collectively, these observations support the use of 1.0% sucrose as a biologically relevant compromise, balancing effective biofilm matrix formation with experimental stability and reproducibility.

The initial inoculum ratio was particularly influential in maintaining species balance within the dual-species biofilm. A 1:1 (*S. mutans*:*S. gordonii*) ratio consistently supported coexistence of both organisms across independent experiments, whereas biased ratios resulted in less reliable recovery of the non-dominant species. This finding underscores the importance of controlling early competitive interactions during biofilm initiation, especially in sucrose-rich environments that can favour rapid glucan-mediated attachment by *S. mutans*. *S. mutans* can outcompete other oral streptococci by rapidly synthesising extracellular polysaccharides that enhance surface adhesion, biofilm accumulation, and ecological dominance, as demonstrated in both *in vitro* and *in vivo* studies (Díaz-Garrido et al., 2020; Tanzer et al., 2012). By equalising the starting abundance of both species, the present model allowed subsequent biofilm development to reflect genuine interspecies interactions rather than initial numerical advantage, thereby enhancing the biological relevance and ecological validity of the system. The combined use of CV and CFU enumeration proved essential for comprehensive assessment of optimisation outcomes. The CV assay was employed as it remains the most widely used method for initial biofilm screening (Borowicz et al., 2023). Its low cost and technical simplicity enable extensive biological and technical replication, thereby improving statistical robustness and reducing variability. Importantly, the assay's scalability enables testing across multiple concentrations, bacterial species, and biofilm models. Beyond its practical advantages, the CV assay is also highly standardised and extensively reported in the biofilm literature. Its widespread adoption

across laboratories and bacterial species allows direct comparison with previous studies and facilitates contextualisation of the present findings within the broader antibiofilm field. This methodological consistency supports reproducibility and strengthens the relevance of the results. However, because this assay does not differentiate species composition, CFU enumeration of adherent biofilms was critical for confirming true dual-species retention. Mechanical scraping of the base of the 96-well plates ensured recovery of firmly attached cells, reducing the risk of underestimating viable biofilm populations. The limitations of the CV assay should also be acknowledged. The assay measures total biofilm biomass, including viable and non-viable cells as well as the extracellular polymeric substance (EPS) matrix, and does not distinguish between these components (Latka & Drulis-Kawa, 2020). However, this characteristic is appropriate for the present study, which focuses on early biofilm development. During biofilm initiation, EPS production occurs alongside bacterial attachment and plays a central role in microcolony formation and biofilm stability. This is particularly relevant for *S. mutans*, which produces substantial amounts of EPS in the presence of sucrose, promoting rapid biofilm accumulation from the earliest stages. Because the test compounds were present throughout the biofilm-formation period, reductions in CV-measured biomass likely reflect suppression of EPS synthesis and early matrix assembly rather than effects on established biofilms or direct bacterial killing. Accordingly, CV staining provides a meaningful measure of impaired biofilm formation and supports an antivirulence-focused interpretation centred on disruption of EPS production and biofilm architecture.

The consistent recovery of mixed *S. mutans* and *S. gordonii* colonies from Mitis–Salivarius agar (MSA) plates further validated the effectiveness of the optimised conditions in sustaining a stable dual-species community. MSA is a commonly used selective and differential medium in cariology research for isolating and enumerating oral streptococci, enabling reliable species differentiation within mixed cariogenic biofilms. (Kim & Young Lee, 2015; Bansal et al., 2024). Distinct colony morphologies on MSA enabled reliable differentiation between *S. mutans* and *S. gordonii* during CFU analysis, reinforcing the robustness of the enumeration strategy. The characteristic frosted glass appearance and occasional glistening of *S. mutans* colonies under sucrose-rich conditions reflected active glucan production, whereas the flatter, darker colonies of *S. gordonii* remained clearly distinguishable. Biochemical confirmation of

representative colonies further strengthened confidence in species identification during optimisation, ensuring that observed trends were not artefacts of misclassification.

The final optimised condition of 1×10^6 CFU/mL total inoculum at a 1:1 (*S. mutans. gordonii*) ratio in the presence of 1.0% (w/v) sucrose represented a controlled experimental system that supported reproducible biofilm formation and retention of both bacterial species. This ratio was not intended to reproduce the natural proportional abundance of *S. mutans* and *S. gordonii* in dental plaque, as natural supragingival plaque is a complex multispecies community with microbial composition that varies according to plaque age, host factors, diet, sucrose exposure, pH, and caries status. Instead, the 1:1 inoculum was selected as a standardised starting condition to ensure that both the cariogenic species (*S. mutans*) and the health-associated early coloniser (*S. gordonii*) were present at comparable initial levels, allowing direct evaluation of species-selective antibiofilm effects.

The 24-hour biofilm generated in this study should therefore be interpreted as an established experimental dual-species biofilm rather than as a direct representation of mature natural dental plaque. This distinction is important because early dental plaque formation in vivo involves sequential colonisation by diverse pioneer organisms, including oral streptococci and *Actinomyces* spp., followed by progressive ecological succession toward more complex communities (Kolenbrander et al., 2002; Palmer et al., 2003; Heller et al., 2016). Moreover, *S. mutans* is not necessarily abundant in all natural plaque samples but is typically associated with cariogenic conditions and ecological shifts favouring acidogenic and aciduric organisms (Marsh, 2003; Zhang et al., 2022).

Importantly, applying the same optimised parameters to the bead-based dual-species biofilm assay ensured methodological consistency across surface models, allowing comparison of antibiofilm outcomes without confounding from differences in inoculum density, sucrose concentration, or biofilm initiation conditions. Thus, the model provides a simplified and reproducible platform for assessing selective modulation of a cariogenic–commensal interaction, while acknowledging that it does not replicate the full microbial diversity or species proportions of natural dental plaque.

5.7 Model Validity and Scope of the Dual-Species *In vitro* Platform

In the present thesis, the dual-species *S. mutans*–*S. gordonii* biofilm system was implemented across two complementary *in vitro* platforms, a 96-well polystyrene microtiter plate model for Phase I high-throughput screening and glass bead model for Phase II mechanistic validation. The 96-well format enabled systematic comparison of multiple compounds under controlled and reproducible conditions, consistent with standard biofilm screening methodologies widely adopted in antimicrobial research (Haney et al., 2018; Kragh et al., 2019). The subsequent use of saliva-conditioned glass beads, which more closely approximate enamel surface characteristics, provided a structurally and biologically relevant substrate for confirmation of biomass, viability, transcriptional, and ultrastructural outcomes ((Abdul Razak et al., 2017; Gilmore et al., 2024)). Together, these platforms allowed efficient compound prioritisation while strengthening biological relevance through cross-model validation. The dual-species configuration captures critical ecological features of cariogenic dysbiosis, particularly competitive and antagonistic interactions between an acidogenic, EPS-producing pathogen (*S. mutans*) and a health-associated early coloniser (*S. gordonii*). This ecological contrast enables assessment of selective antibiofilm activity, species-specific viability effects, and preservation of commensal-associated functions within a controlled environment. Unlike single-species systems, which cannot account for interspecies modulation of virulence or matrix formation, the dual-species framework reflects essential elements of biofilm competition, colonisation resistance, and metabolic interplay that are central to the ecological plaque hypothesis (de Oliveira et al., 2020; Luo et al., 2022). As such, it provides a more biologically meaningful representation of early cariogenic biofilm dynamics while maintaining analytical clarity.

Nevertheless, the model does not replicate the full complexity of the *in vivo* oral ecosystem. Natural dental plaque comprises highly diverse, multi-taxa microbial communities embedded within dynamic salivary flow and influenced by host immune responses, dietary fluctuations, and mechanical shear forces (Lamont et al., 2018; Li et al., 2022)). The present *in vitro* system lacks continuous saliva turnover, immune-mediated modulation, and the spatial heterogeneity characteristic of mature multispecies biofilms. Furthermore, while inclusion of two functionally distinct streptococci introduces ecological interaction, it does not fully capture the network-

level cooperation and functional redundancy observed within natural plaque communities.

Despite these limitations, the dual-species platform represents an appropriate and necessary mechanistic step within a staged translational framework. *In vitro* reductionist models are essential for isolating specific biological interactions and resolving compound-specific effects before introducing higher ecological complexity. By maintaining experimental control over inoculum density, sucrose concentration, and species ratio, the system enables precise evaluation of selective antibiofilm and antivirulence mechanisms without confounding variability inherent to complex microcosm models. Importantly, the consistent findings across both the polystyrene and saliva-conditioned bead models indicate that the observed effects are likely due to true biofilm modulation rather than differences related to the surface used. Accordingly, the present model serves as a rational bridge between preliminary compound screening and future evaluation in more complex multispecies, microcosm, or *in situ* biofilm systems. Such progression will be necessary to confirm ecological stability, long-term community effects, and host-relevant performance under clinically dynamic conditions. However, mechanistic clarity at the dual-species level remains a critical prerequisite for meaningful advancement to higher-order biological models.

5.8 Screening of PDCs for Biofilm Inhibition (MBIC Assessment)

Biofilm inhibition targets the earliest stage of biofilm development, beginning with bacterial adhesion and the formation of initial microcolonies. At this stage, extracellular polymeric substance (EPS) production remains limited, and the biofilm has not yet developed the complex three-dimensional architecture associated with heightened tolerance to antimicrobial agents and host defences. Intervention at this point therefore represents a preventive strategy aimed at disrupting biofilm establishment before irreversible structural and physiological adaptations occur.

In this study, early biofilm inhibition was assessed using the minimum biofilm inhibitory concentration (MBIC) approach, with biofilm biomass quantified by the crystal violet (CV) assay. MBIC was selected as it enables direct evaluation of interference with biofilm initiation under controlled conditions and is suitable for comparative screening of multiple compounds. As a Phase-1 strategy, this approach facilitates prioritisation of candidates based on their ability to suppress biofilm

formation rather than solely on planktonic growth inhibition. However, because CV staining measures total adherent biomass as a composite of bacterial cells and extracellular material, MBIC values were interpreted as indicators of reduced biofilm accumulation rather than as direct measures of cell viability or EPS-specific inhibition.

Interpretation of MBIC values alongside MIC data was essential to determine whether reductions in biofilm biomass occurred at concentrations associated with planktonic growth inhibition or at concentrations below those required to inhibit planktonic growth. Reduced bacterial viability was not disregarded, as the cellular component is an integral part of the biofilm and loss of viable biofilm-associated cells represents a valid mechanism of antibiofilm activity. Therefore, biofilm reduction observed at or near MIC levels was interpreted as antibiofilm activity that may involve reduced bacterial growth, decreased viable cell accumulation, and impaired biofilm development. In contrast, biofilm inhibition observed at sub-MIC concentrations suggested additional biofilm-modulatory or antivirulence effects, such as interference with surface attachment, cell–cell interactions, early microcolony development, and matrix organisation, without requiring overt planktonic growth inhibition.

Together, this dual-level evaluation enabled discrimination between compounds whose antibiofilm effects were likely driven mainly by antimicrobial pressure on the cellular component and those that also demonstrated non-lethal modulation of early biofilm-associated processes. Both mechanisms were considered biologically relevant; however, they imply different modes of action and therefore required interpretation in relation to MIC values (Haney et al., 2018).

The inclusion of both single- and dual-species biofilm models enabled a structured evaluation of antibiofilm efficacy across increasing biological complexity and allowed assessment of compound selectivity and robustness. From a cariology perspective, the desired activity profile is selective inhibition of *S. mutans* biofilm formation with minimal impact on *S. gordonii*, a health-associated commensal. Such selectivity is most readily identified under single-species conditions, where direct compound–bacterium interactions can be examined without confounding interspecies dynamics. Accordingly, single-species assays were an essential first step in identifying compounds with targeted activity against cariogenic biofilms.

However, dental biofilms are inherently polymicrobial, and interspecies interactions, including coaggregation, metabolic cooperation, competition, and the development of a shared extracellular matrix, can substantially modify biofilm

structure, tolerance, and susceptibility to intervention. Dual-species biofilm models were therefore incorporated to determine whether the selective antibiofilm effects observed in single-species systems could be preserved under conditions that more closely reflect interspecies interactions within the oral biofilm environment. Rather than assuming that dual-species biofilms are uniformly more tolerant than single-species biofilms, the present study used the dual-species model to determine whether compound efficacy was maintained, reduced, or enhanced in the presence of a second species. This was important because mixed-species interactions may alter compound susceptibility in a compound-specific and species-dependent manner.

Within this framework, compounds for which the MBIC₉₀ against *S. mutans* was less than or equal to that against *S. gordonii* were explicitly highlighted, as this pattern reflects a desirable selectivity profile in cariogenic biofilm control. Preferential suppression of *S. mutans* biofilm formation, with comparatively lower impact on *S. gordonii*, aligns with an ecological approach to caries prevention rather than indiscriminate inhibition of oral streptococci. Compounds meeting this criterion were therefore awarded one point in the scoring framework, as they demonstrate targeted antibiofilm activity consistent with the intended therapeutic strategy.

While selectivity could be clearly identified from single-species MBIC data, progression to Phase II evaluation required confirmation of efficacy under more biologically relevant conditions. Dual-species biofilms incorporate interspecies interactions and shared matrix components that are not captured in single-species systems and are known to increase biofilm tolerance. The use of dual-species MBIC₉₀ as the selection criterion for Phase II thus identified compounds capable of achieving near-complete suppression of biofilm formation in a mixed-species environment. This stringent endpoint ensured that only compounds with robust, reproducible antibiofilm activity under increased biological complexity were advanced to downstream mechanistic and functional analyses.

5.9 Screening of PDCs for Biofilm Eradication (MBEC Assessment)

In contrast to biofilm inhibition, eradication of established biofilms represents a fundamentally more stringent biological challenge. In this study, selected PDCs demonstrated measurable minimum biofilm eradication concentrations (MBEC), with the exception of COR and KG, for which 90% eradication of mature biofilms was not

achieved within the tested concentration range. This observation is consistent with increasing evidence that eradication of established biofilms represents an exceptionally stringent benchmark and that MBEC values may lie well beyond experimentally or clinically practical limits (Tuan et al., 2024)

For compounds that achieved MBEC, eradication occurred at concentrations substantially higher than their corresponding MBIC values. This pronounced divergence reflects the fundamental biological distinction between preventing biofilm formation and disrupting an established biofilm community. As biofilms mature, structural entrenchment driven by extracellular polymeric substances (EPS), extracellular DNA (eDNA), and spatial heterogeneity imposes severe diffusion limitations and protects metabolically diverse bacterial subpopulations from antimicrobial exposure (Rostami et al., 2022). Consequently, concentrations sufficient to inhibit early adhesion and microcolony formation are often inadequate to penetrate and destabilise the mature biofilm matrix.

The inability of COR and KG to reach MBEC within the tested concentration range likely reflects these intrinsic biofilm defences rather than a lack of antibiofilm potential. Both compounds exhibited inhibitory activity during early stages of biofilm development, suggesting that their primary effects may be directed towards interference with adhesion, early extracellular matrix assembly, or surface-associated virulence traits rather than penetration of a fully mature biofilm. In cariogenic systems, this challenge is further amplified under sucrose-rich conditions, where *S. mutans* rapidly synthesises a dense, glucan-rich EPS matrix that stabilises biofilm architecture and diminishes susceptibility to eradication-based interventions.

In polymicrobial oral biofilms, ecological interactions further modulate susceptibility to eradication. Although *S. gordonii* exhibits antagonistic effects against *S. mutans* through hydrogen peroxide production and arginine metabolism via the arginine deiminase pathway (Kreth et al., 2008), such antagonism is most effective during early biofilm development. Arginine-mediated alkali generation can buffer local acidity and weaken biofilm cohesion, and has also been shown to reduce biofilm mechanical stability under shear stress (Gloag et al., 2021). However, once a sucrose-stabilised biofilm is established, *S. mutans* adapts its spatial organisation and gene expression to persist within structured microcolonies, even in the presence of commensal species (Rose et al., 2023). At this stage, biofilm architecture and metabolic

adaptation outweigh interspecies antagonism, rendering eradication increasingly difficult.

5.10 Glycolytic pH-Drop Assay: Effects on Acidogenic Activity

The glycolytic pH drop assay used in this study assesses acidogenicity at the cellular level and reflects the combined effects of glucose uptake, glycolytic metabolism, acid production, and proton extrusion by *S. mutans*. Because this assay was used as an initial screening tool in Phase I, the results reflect functional metabolic outcomes rather than direct evidence of gene-level regulation. Suppression of pH reduction in this assay may occur via kinetically distinguishable mechanisms. Direct interference with glycolytic enzymes or acid-handling systems would be expected to affect acid production immediately after glucose addition, thereby reducing the rate of pH decline in the early phase of the assay. In contrast, down-regulation of genes involved in glycolysis, such as *ldh*, would be expected to produce a delayed effect, as pre-existing enzyme pools remain active during the early stages of the assay before reduced synthesis becomes functionally relevant.

In the present study, treatment with PDCs resulted in a slower decline in pH over the 120-min period than in the untreated control. The early divergence of treated groups from the control suggests that acidogenicity was reduced at the functional metabolic level rather than solely through delayed transcriptional effects. However, because Phase I of the study did not include enzyme activity or gene expression analyses, it is not possible to determine whether this effect resulted from direct enzyme inhibition, reduced metabolic flux, or altered proton handling.

Similar multilevel effects have been reported in previous antivirulence studies targeting *S. mutans*, in which reductions in glycolytic pH drop were accompanied by selective modulation of acidogenic and aciduric pathways rather than by complete metabolic arrest. For example, Wang et al. demonstrated that sub-inhibitory exposure to an antimicrobial peptide significantly attenuated glycolytic acid production and LDH activity, while transcriptional analysis revealed downregulation of *ldh* and variable regulation of *atpD*, highlighting that impairment of proton extrusion may arise from both transcriptional and membrane-associated mechanisms rather than gene suppression alone (Wang et al., 2018).

More recently, Liu et al. further substantiated this approach by integrating glycolytic pH-drop assays with quantitative PCR, showing that suppression of acid production was associated with coordinated downregulation of *ldh* and *atpD* under sub-MIC conditions, thereby reinforcing the value of combining functional assays with targeted gene expression analysis (Liu et al., 2025). Collectively, these findings support the present study's phased design, in which functional screening was intentionally separated from molecular validation to avoid overinterpretation of early phenotypic outcomes and to establish a more robust mechanistic link between reduced acidogenicity and modulation of metabolic and stress-response pathways.

5.11 Preliminary Toxicity Evaluation via Brine Shrimp Lethality Assay (BSLA)

The BSLA was employed as a preliminary screening tool to evaluate the acute toxicity of selected PDCs prior to further biological investigation. In natural product research, the BSLA is widely used as a rapid and cost-effective assay that provides an initial indication of general cytotoxic potential. Its principal value lies in enabling early toxicity classification and supporting the prioritisation of candidates for subsequent phytochemical and biological evaluation, while identifying compounds that may pose toxicological concern (Maulydia et al., 2025). Although the BSLA does not replace mammalian toxicity testing, its use in early screening can help exclude overtly toxic compounds and inform rational decision-making within a multi-phase development framework.

In the present study, all PDCs exhibited clear concentration-dependent mortality of brine shrimp nauplii, as evidenced by sigmoidal dose–response relationships and high coefficient of determination values from nonlinear regression analysis. The consistently high R^2 values indicate good model fitting and support the reliability of the derived LC_{50} estimates, confirming that the assay conditions were appropriate and that the observed responses were reproducible and concentration-related.

According to Clarkson's toxicity classification, most compounds evaluated were categorised as low toxicity, indicating a favourable preliminary safety profile in this model. The hydrolysable tannins (COR, PUG, and PUN) and flavonoid compounds (GC, KG, and RUT) all exhibited LC_{50} values exceeding 500 $\mu\text{g/mL}$, suggesting limited acute cytotoxicity at the concentrations tested. When considered alongside their demonstrated antimicrobial and antibiofilm activities, these findings imply that their

biological effects are unlikely to be driven by nonspecific cytotoxic mechanisms, an important consideration for compounds intended for oral applications.

Notably, the moderate toxicity observed for EA contrasts with reports of negligible acute toxicity in *in vivo* studies using pomegranate peel extracts. For instance, Elkoraichi et al., (2023) reported no mortality, behavioural abnormalities, or histopathological alterations in rats following 21 days of oral administration of aqueous pomegranate peel extract. Similarly, Jahromi et al., (2015) observed no mortality or toxic effects in BALB/c mice. Taken together, these studies indicate that pomegranate peel extracts were well tolerated under the specific *in vivo* conditions evaluated. However, direct comparisons with isolated EA should be interpreted with caution, given differences in composition, dosing, and biological context between purified compounds and complex plant extracts. Pomegranate peel extracts comprise a mixture of polyphenols, including hydrolysable tannins such as PUG and PUN, as well as other phenolic constituents, which may be present at concentrations that differ substantially from those tested for individual compounds. It is therefore plausible that the concentration of EA in the extract was below the threshold required to elicit acute toxicity, or that interactions among coexisting phytochemicals mitigated its toxic effects. Synergistic or antagonistic interactions within complex extracts may modulate bioavailability, cellular uptake, or target engagement, resulting in an overall toxicity profile that differs from that of isolated constituents (Chen et al., 2022). These observations reinforce the importance of compound-level toxicity assessment, as conducted in the present study, to identify constituents with narrower safety margins that may be masked within whole-extract formulations. Accordingly, while extract-based studies support the general safety of pomegranate peel preparations, the moderate toxicity observed for EA in the BSLA underscores the need for cautious dose consideration and further validation in mammalian cell-based models when advancing isolated compounds for targeted oral applications. This may be related to its lower molecular weight and simpler structure, which could facilitate greater cellular uptake and stronger interactions with intracellular targets compared with larger, more complex polyphenolic compounds. These observations emphasise the need to consider toxicity profiles alongside biological efficacy during compound selection.

It is important to note that the BSLA reflects general acute toxicity rather than tissue-specific or long-term effects. The observed lethality is likely attributable to broad physiological disturbances in brine shrimp nauplii, including membrane and metabolic

disruption, which may not directly correspond to responses in mammalian or oral cell systems. Accordingly, the BSLA is interpreted here as an initial safety screen rather than a definitive assessment of biocompatibility, and further evaluation using relevant mammalian cell-based models is required to refine safety margins.

Overall, the BSLA findings indicate that most PDCs exhibit low acute toxicity while retaining biological activity, supporting their suitability for continued investigation as antibiofilm and antivirulence agents. Based on the predefined scoring framework, only compounds with LC_{50} values exceeding 500 $\mu\text{g/mL}$ met the toxicity criterion and were advanced to Phase II, whereas EA, with an LC_{50} below this threshold, did not meet the criterion and was assigned a score of 0. Although a positive association between BSLA-derived LC_{50} values and *in vivo* LD_{50} data has been reported, this relationship is indicative rather than predictive; therefore, in this study, the BSLA was used to guide early compound prioritisation rather than to infer *in vivo* safety.

5.12 Rationale for Compound Selection for Phase II Evaluation

Selection of compounds for Phase II followed a stepwise, biofilm-centred screening strategy rather than reliance on planktonic antimicrobial activity alone. This approach was adopted because conventional susceptibility measures, such as minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), do not reliably reflect compound performance against biofilms, which are structurally complex and metabolically heterogeneous. Current reviews of biofilm antimicrobial susceptibility testing have highlighted that exclusive reliance on planktonic endpoints can be misleading and have emphasised the importance of incorporating biofilm-specific parameters when advancing candidate compounds (Coenye, 2023).

Accordingly, compound prioritisation was based on outcomes from biofilm-focused screening assays, including biofilm inhibition results, to identify agents demonstrating consistent and meaningful activity under biofilm-relevant conditions. The use of integrated antibiofilm screening frameworks has been recommended in natural product research as a rational strategy for down-selecting lead compounds and for distinguishing true antibiofilm activity from nonspecific antimicrobial effects (Skogman et al., 2016). Compounds that exhibited weak, inconsistent, or concentration-dependent biofilm inhibition were not progressed further, ensuring that subsequent investigations focused on candidates with robust biofilm-relevant activity.

Based on this screening strategy, PUG and PUN were advanced to Phase II for further investigation. While Phase I established their inhibitory effects during early biofilm formation, it did not address the underlying biological mechanisms or structural and cellular changes associated with these effects. Phase II was therefore designed to provide deeper insight by examining viable cell populations, biofilm architecture, and gene regulation using complementary approaches, including colony-forming unit (CFU) enumeration, scanning electron microscopy (SEM), and gene expression analysis.

5.13 Phase II Dual-Species Biofilm Inhibition by PUG and PUN

Phase II investigations were undertaken to elucidate the biological mechanisms underlying the antibiofilm activity of PUG and PUN within a dual-species oral biofilm comprising *S. mutans* and *S. gordonii*. The use of a multispecies or ecologically representative biofilm model is essential for oral microbiology research, as cariogenic biofilms arise from ecological interactions rather than the behaviour of a single organism (Li et al., 2024). This phase therefore aimed to determine whether the lead compounds identified during Phase I could selectively modulate cariogenic biofilm behaviour within a biologically relevant microbial community.

The significant reduction in biofilm biomass at both 8 hours and 24 hours indicates that PUG and PUN interfere with biofilm formation from the initial stages and that these effects persist during biofilm progression (Fu et al., 2021). Sustained suppression of biomass at 24 hours suggests that early disruption was not compensated by delayed biofilm growth, a phenomenon commonly observed when interference occurs at the level of adhesion or EPS synthesis rather than transient metabolic inhibition. In contrast, CHX completely prevented biofilm formation at 8 hours and maintained near-total suppression at 24 hours, consistent with its broad-spectrum bactericidal activity and limited specificity toward biofilm-associated pathways. At the concentration used (0.12%), CHX exerts strong bactericidal effects during the initial adhesion phase, effectively eliminating or severely damaging bacterial cells before a protective extracellular biofilm matrix can be established. However, this early inhibition does not indicate permanent sterilisation of the system. Over prolonged incubation, the antimicrobial activity of CHX may decline due to dilution, adsorption to surfaces, or interaction with organic components of the growth medium. As a result, a small

subpopulation of surviving or recovering cells may resume growth once inhibitory pressure diminishes, leading to detectable biofilm formation at later time points.

To determine whether the reduction in biomass reflected selective ecological modulation rather than uniform bacterial killing, viable cell enumeration was performed for each species within the dual-species biofilm. Viable cell enumeration demonstrated that the reduction in total biofilm burden following PUG and PUN treatment was driven primarily by suppression of *S. mutans*, while *S. gordonii* viability was largely preserved. This selective effect became more pronounced at 24 hours, particularly for PUG. Importantly, cariogenic dysbiosis is characterised by a shift in species dominance toward acidogenic and EPS-producing species rather than an overall increase in total bacterial load. From this perspective, selective suppression of *S. mutans* without elimination of commensal species represents a biologically relevant strategy for modulating cariogenic biofilms.

The preservation of *S. gordonii*, an early coloniser associated with oral health and biofilm homeostasis, further supports the ecological relevance of this selective inhibition. In contrast, CHX eliminated both species, consistent with previous reports demonstrating its non-selective antimicrobial action and potential to disrupt microbial balance.

To further investigate the molecular basis underlying these ecological effects, transcriptional profiling was performed to evaluate the response of key virulence-associated genes during early and later stages of biofilm development. The transcriptional profiles observed at 8 hours and 24 hours provide mechanistic insight into the temporal sequence underlying the antibiofilm and anti-acidogenic effects of PUG and PUN. At 8 hours, both compounds markedly downregulated genes involved in extracellular polysaccharide synthesis (*gtfB*, *gtfC*) and glucan binding (*gbpB*, *gbpD*), as well as regulatory genes associated with quorum sensing and biofilm stress adaptation (*comDE* and *brpA*). These changes are consistent with the disruption of early matrix development and biofilm organisation, which would reduce cell retention and limit the formation of diffusion-restricted microenvironments that favour acid accumulation (Atazhanova et al., 2024).

Notably, despite maintaining biofilm pH above the critical demineralisation threshold (pH 5.5) following 120 min of exposure, transcription of key metabolic genes (*ldh* and *atpD*) was not significantly reduced at 8 hours. This apparent discrepancy may indicate that the early pH changes observed following PUG and PUN exposure in

planktonic *S. mutans* cannot be fully explained by immediate transcriptional suppression of glycolytic pathways or proton extrusion systems alone. It is possible that these effects arise, at least in part, from rapid functional influences occurring upstream or post-transcriptionally, including reduced substrate utilisation, subtle perturbations in intracellular redox balance, or partial inhibition of key metabolic or proton-handling enzymes, which may collectively modulate acid production prior to the emergence of detectable gene expression changes (Baker et al., 2015; Hasona et al., 2007; J. Li et al., 2024).

In a dual-species context, the preservation of biofilm pH may be influenced by metabolic buffering from *S. gordonii*, which possesses alkali-generating pathways, such as the arginine deiminase system, that can counteract local acidification within mixed biofilms (Huang et al., 2018). As a consequence of this buffered microenvironment, *S. mutans* may not experience sufficient acid stress at early stages to necessitate immediate transcriptional reprogramming of acidogenic or acid-tolerance pathways, with broader metabolic and stress-related gene modulation emerging only as the biofilm matures and environmental pressures become sustained (Y Li et al., 2025). By 24 hours, broader transcriptional modulation was observed, including effects on genes linked to metabolic activity and stress tolerance, indicating a secondary adaptive response as the biofilm matures and experiences sustained environmental pressure (Pisithkul et al., 2019; Stewart et al., 2019). Collectively, these findings support a model in which PUG and PUN primarily disrupt early biofilm architecture and associated regulatory pathways, resulting in functional suppression of acidification at initial stages, while broader transcriptional adjustments in metabolic genes emerge later as the biofilm matures and undergoes adaptive responses to sustained environmental pressure. This staged mechanism explains the maintenance of pH despite minimal early changes in *ldh* and *atpD* expression. More broadly, it highlights the potential advantage of targeting biofilm virulence and regulatory processes rather than directly inhibiting core metabolic pathways (Koo et al., 2017; Martínez et al., 2019). Investigating *S. gordonii* transcriptional responses is essential for interpreting dual-species biofilm behaviour, as commensal streptococci actively shape biofilm architecture, competitiveness, and ecological stability rather than serving as passive bystanders. Inclusion of *S. gordonii* gene expression data enables differentiation between selective antivirulence effects and nonspecific antimicrobial activity, a distinction that cannot be made when analysis is restricted to *S. mutans* alone. Mixed-species and dual-transcriptome studies have shown

that commensal streptococci mount highly specific, partner-dependent transcriptional responses, particularly in genes linked to adhesion, transport, and metabolism (Pandey et al., 2023; Rose et al., 2023). Accordingly, a pattern in which PUG and PUN primarily perturb *S. mutans* virulence-associated pathways while leaving *S. gordonii* growth and key colonisation functions largely intact is consistent with a selective antivirulence mode of action rather than nonspecific toxicity (Fitri et al., 2025; Kolenbrander, 2000). Moreover, the *S. gordonii* genes examined (*gtfG*, *abpA*, *csaA*, and *sspA*) encode critical determinants of adhesion, surface properties, and interspecies interactions (Kuboniwa et al., 2006; Choo et al., 2021), enabling assessment of whether commensal functionality is preserved while pathogenic traits are selectively attenuated.

At 8 hours, limited or non-significant modulation of *csaA* and *sspA*, together with modest changes in *gtfG* and *abpA*, suggests that early exposure to PUG and PUN does not markedly disrupt core commensal adhesion mechanisms. This preservation of *S. gordonii* surface properties at an early stage is consistent with a selective antivirulence action of PUG and PUN, in which pathogenic biofilm architecture is weakened without broadly impairing beneficial colonisers. Importantly, the observed changes did not reflect uniform suppression across all commensal genes; rather, they indicated compound-induced, selective transcriptional adjustments, supporting the notion that PUG and PUN modulate interspecies interactions rather than exert indiscriminate antimicrobial toxicity. Consistent with this interpretation, studies of *S. gordonii* interactions have shown that key adhesin genes such as *csaA* and *sspB* are often not acutely upregulated during early dual-species encounters, even when interspecies or interkingdom signalling is already underway, indicating that basal adhesion machinery can remain stable while other pathways adjust (Dutton et al., 2016; Jesionowski et al., 2016). Likewise, early transcriptional responses to partner species or host-derived cues frequently involve metabolic, stress, or signalling pathways rather than wholesale disruption of surface adhesins (Liu et al., 2022; Mutha et al., 2018). Within this framework, the modest 8 h effects of PUG and PUN on *gtfG*, *abpA*, *csaA*, and *sspA* are compatible with preservation of core adhesion and surface properties in a commensal coloniser.

By 24 hours, more pronounced transcriptional modulation was observed, particularly for *gtfG* and *abpA*, indicating that prolonged exposure influences glucan-mediated interactions and salivary pellicle binding under competitive biofilm conditions (Ricker et al., 2014; Chi et al., 2022). Assessing commensal gene expression

therefore provides critical evidence that the observed antibiofilm and pH-stabilising effects arise from ecological rebalancing within the biofilm rather than wholesale microbial suppression. This approach strengthens the translational relevance of the findings, as effective oral antibiofilm strategies are increasingly recognised to attenuate cariogenic virulence while preserving, or minimally perturbing, beneficial oral commensals.

Because transcriptional responses alone cannot fully capture the structural consequences of biofilm disruption, morphological analysis was conducted to visualise architectural alterations within the treated biofilms. The SEM findings provide structural corroboration for the inhibitory effects observed in the biomass, viability, and transcriptional analyses. The dense, matrix-rich architecture of untreated dual-species biofilms is characteristic of sucrose-driven cariogenic communities, in which extensive EPS production supports tight microcolony packing, surface continuity, and diffusion-limited microenvironments that favour acid retention and virulence expression (Xiao et al., 2012; Klein et al., 2015). In contrast, treatment with PUG and PUN resulted in a progressive loss of biofilm organisation, reflected by reduced surface coverage, fragmented microcolonies, and diminished extracellular matrix. These architectural changes are consistent with the early downregulation of *gtf* and *gbp* genes and support the conclusion that both compounds interfere with EPS-dependent biofilm assembly rather than merely suppressing planktonic growth. Similar antibiofilm mechanisms have also been reported for several plant-derived polyphenols investigated in oral biofilm systems. Cranberry-derived proanthocyanidins have been shown to inhibit the adhesion of *S. mutans* and reduce its tolerance to acid, thereby limiting biofilm accumulation (Gregoire et al., 2007). Likewise, tea catechins, particularly epigallocatechin gallate (EGCG), interfere with bacterial adhesion and glucan-dependent biofilm development through suppression of glucosyltransferase enzymes and modification of bacterial cell surface properties (Xu et al., 2011; Wu et al., 2018). Curcumin has also demonstrated antibiofilm activity against cariogenic streptococci through interference with carbohydrate metabolism and disruption of biofilm structural integrity (Li et al., 2018). Similarly, resveratrol has been reported to reduce *S. mutans* biofilm formation by modulating genes for acidogenic metabolism, acid tolerance and extracellular polysaccharide synthesis (Li et al., 2020).

In comparison with these compounds, the present findings indicate that PUG and PUN exert coordinated antibiofilm and antivirulence effects within a dual-species

oral biofilm model, including suppression of extracellular polysaccharide synthesis, adhesion-related traits, and quorum-sensing pathways. Importantly, unlike many previous phytochemical studies conducted using single-species systems, the current work demonstrates selective modulation of *S. mutans* virulence while largely preserving the commensal organism *S. gordonii*, highlighting the ecological selectivity of pomegranate-derived polyphenols. These findings are consistent with reports describing matrix-targeting effects of several plant polyphenols, which weaken EPS-dependent biofilm cohesion without necessarily eliminating all bacterial cells. Structural evidence from the present study provides further support for this mechanism.

SEM observations further revealed the persistence of bacterial cells arranged in smaller, loosely associated clusters, aligning with CFU data demonstrating selective suppression of *S. mutans* while largely preserving *S. gordonii* viability. This preservation of cellular presence within a weakened matrix provides a structural basis for the observed maintenance of biofilm pH above the critical threshold despite limited early changes in metabolic gene expression, as reduced matrix density would limit acid retention and enhance diffusion. Although SEM images suggested noticeable structural disruption following PUN treatment, the combined biomass, viability, and transcriptional analyses indicate that PUG produced the more consistent and selective antibiofilm effects overall. This difference in performance may also be related to structural distinctions between the two ellagitannins. PUG possesses a larger molecular structure (MW 1084.7 g/mol) compared with PUN (MW 782.53 g/mol), resulting in a greater number of hydroxyl-rich polyphenolic moieties capable of participating in hydrogen bonding and hydrophobic interactions with bacterial surface proteins. The higher molecular weight and extended ellagitannin framework of PUG may therefore enable multivalent interactions with adhesion-associated proteins of *S. mutans*, including glucan-binding proteins and other surface adhesins involved in early biofilm establishment. Such multivalent binding could interfere more effectively with bacterial attachment and extracellular polysaccharide (EPS) assembly, thereby weakening biofilm structural cohesion. In contrast, the comparatively smaller structure of PUN may provide fewer interaction sites, potentially resulting in less efficient disruption of adhesion-related processes. Ellagitannins and other hydrolysable tannins are well known for their ability to form complexes with and precipitate proteins, a property strongly influenced by molecular size, structural flexibility, and the number and density of galloyl or hexahydroxydiphenoyl (HHDP) groups (Karonen et al., 2019). Larger and

more structurally flexible ellagitannins generally exhibit greater protein-precipitation capacity than smaller molecules, particularly when they contain multiple galloyl or HHDP substituents and higher degrees of oligomerisation (Marsh et al., 2020). These structural characteristics may therefore enhance the ability of PUG to interact with bacterial adhesins and glucosyltransferases involved in EPS synthesis, providing a mechanistic basis for its more consistent antibiofilm activity compared with PUN.

By contrast, SEM analysis of CHX-treated biofilms revealed near-complete architectural collapse, characterised by minimal surface coverage and abundant cellular debris. Such extensive disruption is consistent with the established broad-spectrum, bactericidal mode of action of CHX, which lacks selectivity between pathogenic and commensal oral microorganisms (Bescos et al., 2020; Chatzigiannidou et al., 2020). Together, these SEM observations reinforce the interpretation that PUG and PUN act primarily as biofilm-modulating agents that destabilise pathogenic architecture and intercellular cohesion, rather than indiscriminately eradicating biofilm cells. This mode of action is coherent with the selective, antivirulence-driven inhibition profile observed across Phase II assays and aligns with contemporary ecological approaches to cariogenic biofilm management that emphasise preservation of beneficial oral commensals. Collectively, these findings indicate that PUG and PUN primarily act as biofilm-modulating agents that weaken pathogenic biofilm architecture and virulence regulation rather than functioning as non-selective bactericidal agents

The combined biomass, viability, transcriptional, and structural findings indicate that PUG and PUN exert distinct ecological effects within the dual-species biofilm. To facilitate integrated interpretation of these outcomes, the principal functional effects of both compounds during dual-species biofilm inhibition are summarised in Table 5.1.

Table 5.1
Summary Comparison of the Ecological Antibiofilm Effects of PUG and PUN During Dual-Species Biofilm Inhibition

Feature	PUG	PUN
Anti-adhesion effect on <i>S. mutans</i>	Strong	Strong
Effect on <i>S. gordonii</i> during biofilm formation	Minimal / selective	Broader
Acidogenicity suppression	Present	Present
Virulence gene modulation (<i>gtfB</i> , <i>gtfC</i> , <i>gbpB</i> , <i>gbpD</i>)	Strong downregulation	Moderate downregulation
Metabolic gene modulation (<i>ldh</i> , <i>atpD</i>)	Downregulated during biofilm maturation	Downregulated but less pronounced
Ecological selectivity	Higher	Lower

Overall, PUG appears to exhibit greater ecological selectivity against *S. mutans* during biofilm inhibition, whereas PUN demonstrates broader antibiofilm activity across the dual-species biofilm.

As summarised in Table 5.1, Phase II findings demonstrate that PUG and PUN inhibit dual-species biofilm formation through coordinated effects on EPS-dependent biomass accumulation, selective suppression of *S. mutans* viability, downregulation of virulence-associated gene expression, and disruption of biofilm architecture. Among the two compounds, PUG consistently exhibited greater potency and selectivity toward cariogenic pathways. Importantly, the preservation of *S. gordonii* viability and limited disruption of commensal gene expression distinguish these compounds from broad-spectrum antimicrobials and align with contemporary oral microbiology strategies that aim to restore ecological balance rather than eradicate biofilms indiscriminately (Marsh et al., 2016).

To further conceptualise these integrated findings, a mechanistic model illustrating dual-species biofilm inhibition by PUG and PUN is proposed in Figure 5.1. Figure 5.1 integrates the phenotypic, structural, and transcriptional findings to illustrate the proposed mechanism of selective antibiofilm activity, linking reduced biomass and viability of *S. mutans* with suppression of virulence-associated gene expression and corresponding alterations in biofilm architecture

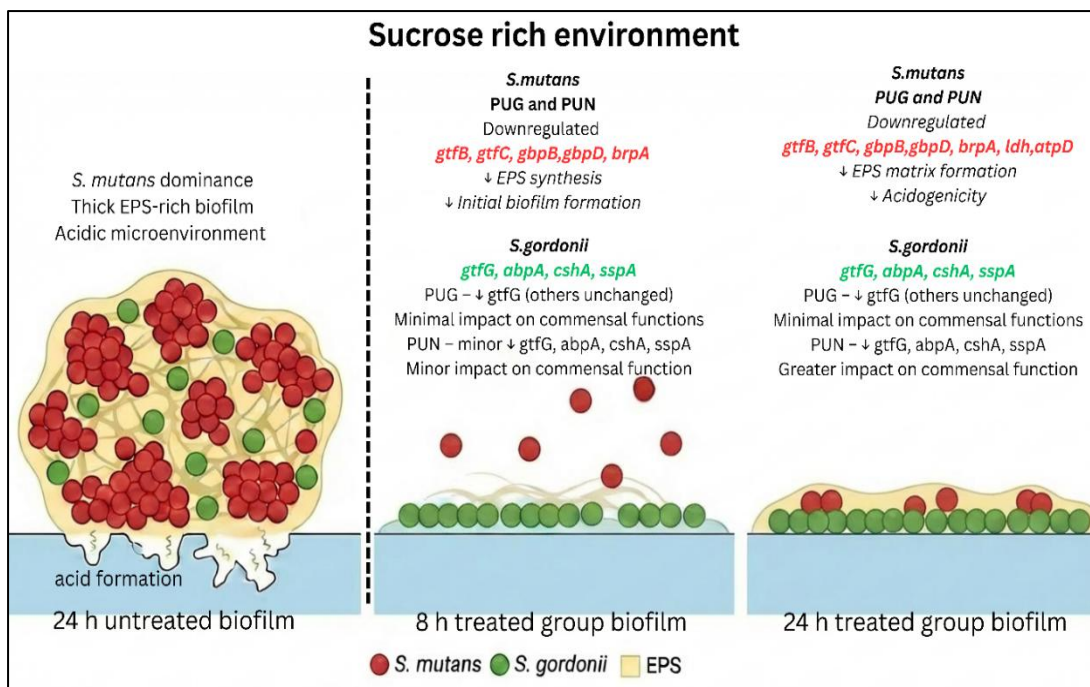


Figure 5.1 Proposed Mechanism of Biofilm Inhibition by PUG and PUN in a Dual-Species Oral Biofilm Under Sucrose-Rich Conditions

In untreated conditions (left), sucrose promotes *S. mutans* dominance within a dense EPS-rich biofilm, creating an acidic microenvironment characteristic of cariogenic biofilms. When PUG and PUN are present during incubation, early-stage biofilm development (8 h; middle) is disrupted through downregulation of key *S. mutans* virulence genes (*gtfB*, *gtfC*, *gbpB*, *gbpD*, and *brpA*), resulting in reduced EPS synthesis and impaired initial biofilm formation. At 24 h (right), additional suppression of metabolic genes (*ldh* and *atpD*) further limits EPS matrix development and acidogenicity. In contrast, adhesion-associated genes of *S. gordonii* (*gtfG*, *abpA*, *cshA*, and *sspA*) remain largely preserved following PUG exposure, while PUN causes broader downregulation. These effects collectively reduce *S. mutans* dominance and weaken biofilm architecture while largely maintaining commensal functions supporting an ecology-based mechanism for selective modulation of cariogenic biofilms.

5.14 Phase II Dual-Species Biofilm Eradication by PUG and PUN

While Section 5.13 focused on the inhibition of biofilm formation, the present section evaluates whether PUG and PUN can disrupt established dual-species biofilms formed by *S. mutans* and *S. gordonii*, which represent a more resilient and clinically relevant stage of biofilm development. Such mature biofilms possess complex architecture, extensive EPS matrices, and physiological heterogeneity, that substantially increase tolerance to antimicrobial agents (Verderosa et al., 2019; Hindieh et al., 2025).

This distinction between biofilm inhibition and biofilm eradication is critical when evaluating candidate antibiofilm agents, as compounds effective against early

biofilm formation may exhibit reduced activity against structurally mature communities. The lack of biomass reduction after 5-minute exposure suggests that brief contact is insufficient to disrupt the dense EPS matrix characteristic of mature oral biofilms. EPS acts as a physical and chemical barrier, limiting compound penetration and delaying antimicrobial action (Magoro et al., 2025; Xiao et al., 2012). In contrast, CHX produced an immediate and significant reduction in biomass, reflecting its strong, non-specific membrane-disruptive activity and rapid killing kinetics (McDonnell & Russell, 1999; Cieplik et al., 2019).

The significant biomass reduction observed after 4 hours of exposure to PUG and PUN suggests that prolonged contact allows for gradual destabilisation of the biofilm matrix. In the present eradication experiments, higher concentrations of PUG and PUN were applied compared with those used during the biofilm inhibition assays. This adjustment reflects the well-recognised tolerance of mature biofilms, in which the dense EPS matrix, reduced metabolic activity of interior cells, and spatial heterogeneity collectively limit antimicrobial penetration and efficacy. Consequently, concentrations that are sufficient to inhibit early biofilm formation are often inadequate to disrupt established biofilms. The requirement for higher compound concentrations in the eradication phase therefore aligns with the intrinsic protective properties of mature biofilm communities and supports the interpretation that the observed reductions in biomass and *S. mutans* viability resulted from gradual matrix destabilisation rather than immediate bactericidal activity. These findings are consistent with previous studies demonstrating that higher antimicrobial concentrations are required to penetrate EPS-rich biofilms compared with planktonic or early-stage biofilm cells (Stewart & Costerton, 2001; Koo et al., 2013; Flemming et al., 2016).

The greater reduction achieved by PUG compared with PUN indicates compound-specific differences in interaction with matrix components or biofilm-associated cells. Importantly, despite measurable biomass loss, eradication was incomplete, underscoring the intrinsic tolerance of mature dual-species biofilms and distinguishing matrix disruption from complete biofilm removal.

Although complete eradication of mature biofilms was not achieved, this outcome is consistent with well-recognised resilience of structured oral biofilms. Mature oral biofilms are intrinsically resilient, limiting antimicrobial penetration and increase tolerance to antimicrobial agents. In the present study, mature biofilms were modelled using 48 hours dual-species culture grown in the presence of 1% sucrose, as

sucrose promotes glucan synthesis via the *S. mutans* glucosyltransferase (*gtfB* and *gtfC*) leading to the formation of EPS-rich matrices and organised microcolonies characteristic of established cariogenic oral biofilms (Xiao & Koo, 2010; Bowen & Koo, 2011; Koo et al., 2013).

Contemporary ecological models of caries management emphasise modulation of biofilm behaviour rather than complete microbial eradication. Commensal organisms contribute to ecological stability, pH buffering, and colonisation resistance (Takahashi & Nyvad, 2011; Marsh et al., 2016). In this context, the ability of PUG and PUN to weaken EPS architecture, suppress *S. mutans* virulence gene expression, and preserve *S. gordonii* represents a desirable ecological outcome that may facilitate mechanical plaque removal and reduce cariogenic pressure. Therefore, the limited eradication observed in this study is consistent with an ecology-based strategy in which selective biofilm modulation, rather than indiscriminate bacterial killing, is prioritised for sustainable oral biofilm management.

Consistent with the observed biomass reduction, viable cell enumeration indicates that the decrease in total biofilm burden was primarily associated with suppression of *S. mutans* viability after 4 hours of exposure, whereas no statistically significant reduction was observed for *S. gordonii*, supporting a selective ecological modulation of the dual-species biofilm. In mature biofilms, removal or loosening of EPS can occur without immediate bacterial death, particularly over short exposure periods (Koo et al., 2013).

In contrast, CHX caused a marked and broad reduction in CFU for both *S. mutans* and *S. gordonii*, consistent with its bactericidal action and ability to penetrate biofilms given sufficient concentration and exposure time (Cieplik et al., 2019). While effective, this non-selective killing also eliminates commensal species, raising concerns regarding long-term ecological disruption when used repeatedly in the oral cavity (Marsh et al., 2016). The preferential reduction of *S. mutans* alongside preservation of *S. gordonii* indicates that PUG and PUN may not act primarily through rapid broad-spectrum bactericidal mechanisms. Instead, they appear to selectively weaken cariogenic biofilm populations while maintaining commensal viability, a profile that aligns with ecological plaque control strategies rather than conventional antimicrobial eradication. This selective reduction in *S. mutans* viability following prolonged exposure is consistent with the transcriptional suppression of key EPS-associated virulence genes, including *gtfB*, *gtfC*, and *gbpD*, suggesting that disruption of glucan

synthesis and adhesive stability contributes to weakening of the cariogenic biofilm matrix. Gene expression analysis supports the interpretation that PUG and PUN exert delayed, regulatory effects in established biofilms. The absence of significant transcriptional changes at 5 minutes suggests that short-term exposure does not produce immediate changes in gene expression in protected biofilm-associated cells. However, following 4 hours of treatment, both compounds significantly downregulated key virulence-associated genes in *S. mutans*, including *gtfB*, *gtfC*, and *gbpD*, which are central to EPS synthesis, adhesion, and biofilm maintenance (Bowen & Koo, 2011). Importantly, the upregulation of *altA* and *lytF*, particularly following PUG exposure, suggests activation of autolysin-mediated cell wall remodelling pathways. Autolysins have been implicated in cell separation, biofilm restructuring, and controlled cell lysis, processes that can contribute to matrix destabilisation without immediate loss of viability (Ahn & Burne, 2006). This transcriptional pattern indicates that PUG, and to a lesser extent PUN, may promote internal biofilm weakening through modulation of cell wall turnover and EPS-associated gene networks rather than inducing acute cellular damage.

In contrast to *S. mutans*, *S. gordonii* exhibited only minor and transient transcriptional responses to PUG and PUN. The modest upregulation of *scaA*, which is involved in metal homeostasis, suggests a limited adaptive response, with no clear evidence of antimicrobial induced stress (Jakubovics et al., 2014). Importantly, adhesion-associated genes such as *cshA* and *sspA* remained largely unchanged, and no significant viability loss was observed. This stability supports the concept that *S. gordonii*, a health-associated commensal and early coloniser, is largely preserved during treatment. Such selectivity is desirable in oral biofilm management, as commensal streptococci contribute to microbial homeostasis and can antagonise cariogenic species through competitive and metabolic interactions (Kreth et al., 2008). These transcriptional and structural changes underlying the destabilisation of mature biofilms are summarised schematically in Figure 5.2. Figure 5.2 integrates phenotypic, structural, and transcriptional evidence to illustrate the proposed mechanism of biofilm destabilisation, whereby extracellular polysaccharide disruption and modulation of autolysis-related genes correspond with the observed structural disintegration without extensive bactericidal effects.

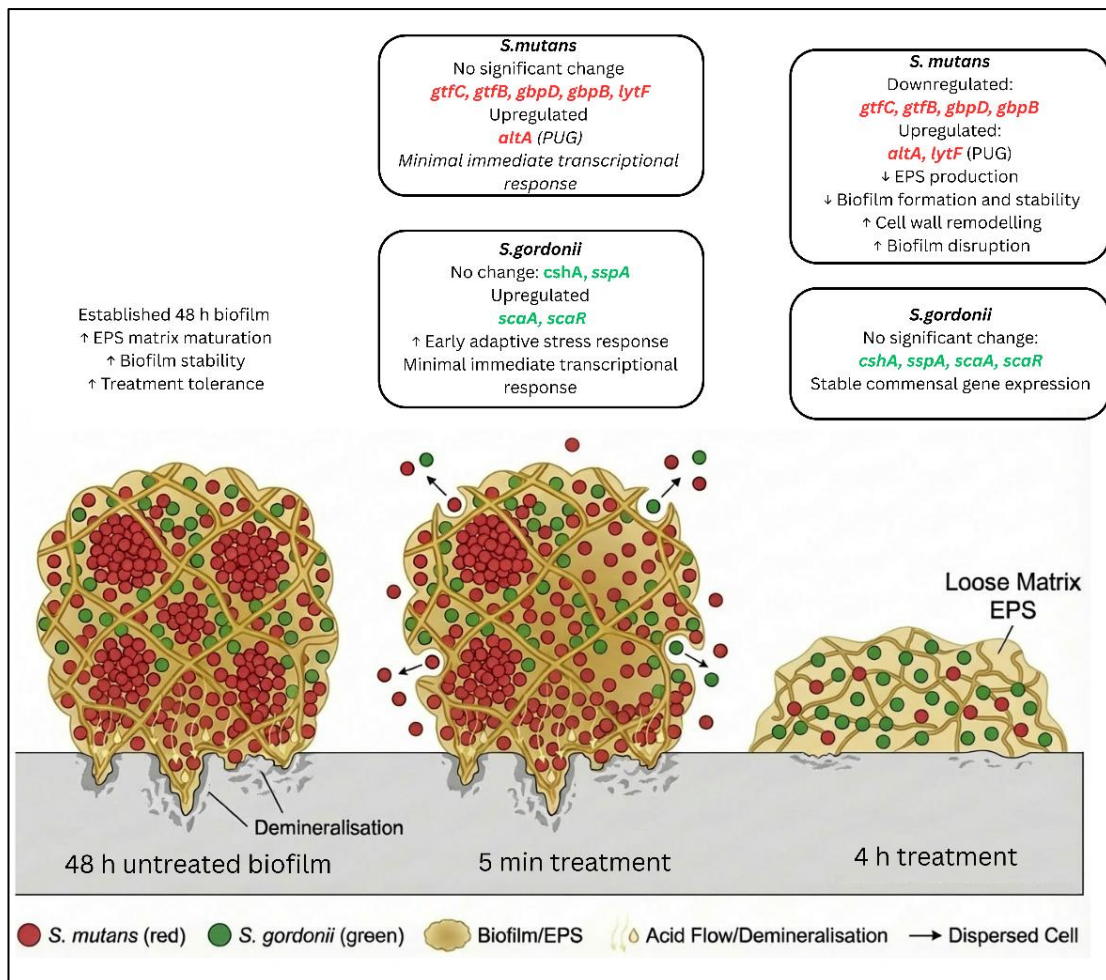


Figure 5.2 Proposed Mechanism of Biofilm Eradication by PUG and PUN Under Sucrose-Rich Dual-Species Biofilm Conditions.

A mature 48-h biofilm composed of *S. mutans* (red) and *S. gordonii* (green) is characterised by a dense extracellular polymeric substance (EPS) matrix, increased structural stability, and acid-mediated enamel demineralisation. Following short-term exposure (5 min), minimal immediate transcriptional responses are observed in *S. mutans*, with only *atlA* upregulated after PUG treatment, while *S. gordonii* exhibits a limited adaptive stress response marked by upregulation of *scaA* and *scaR*. After prolonged exposure (4 h), PUG and PUN suppress EPS-associated virulence genes (*gtfC*, *gtfB*, *gbpD*, and *gbpB*) in *S. mutans*, accompanied by upregulation of autolysis-related genes (*atlA* and *lytF*), resulting in reduced EPS production, biofilm destabilisation, and partial dispersal of the mature biofilm structure. In contrast, *S. gordonii* gene expression remains largely unchanged, indicating preservation of commensal bacterial stability. These findings illustrate a selective ecology-modulating mechanism underlying the eradication of established cariogenic biofilms by pomegranate-derived compounds.

SEM analysis provided qualitative support of the biochemical and transcriptional findings. PUG-treated biofilms showed pronounced disruption of EPS continuity, reduced cellular cohesion, and formation of voids within the biofilm matrix. PUN treatment resulted in more moderate structural changes, consistent with its weaker

transcriptional and biomass effects. These observations support the concept that natural compounds often induce matrix destabilisation rather than bactericidal eradication (Flemming et al., 2016). The extensive clearance observed following CHX treatment reflects its ability to cause widespread cell death and matrix collapse, highlighting the non-selective nature of its antimicrobial activity. A comparative summary of the ecological antibiofilm effects of PUG and PUN observed during the eradication phase is summarised in Table 5.2.

Table 5.2
Summary Comparison of the Ecological Antibiofilm Effects of PUG and PUN During Dual-Species Biofilm Eradication

Feature	PUG	PUN
Immediate effect (5 min exposure)	Minimal	Minimal
Biomass reduction after prolonged exposure (4 h)	Greater reduction	Moderate reduction
Effect on <i>S. mutans</i> viability	Significant reduction	Moderate reduction
Effect on <i>S. gordonii</i> viability	Minimal / preserved	Minimal / preserved
EPS matrix disruption (SEM)	Pronounced disruption and void formation	Moderate structural disruption
Virulence gene modulation	Strong downregulation of <i>gtfB</i> , <i>gtfC</i> , <i>gbpD</i> with increased <i>atfA</i> and <i>lytF</i> expression	Downregulation of <i>gtfB</i> , <i>gtfC</i> , <i>gbpD</i> with weaker autolysin response
Overall eradication profile	Partial but stronger biofilm destabilisation	Partial but weaker destabilisation

Overall, both compounds exhibited limited ability to eradicate mature dual-species biofilms; however, PUG demonstrated greater capacity to destabilise EPS architecture and reduce *S. mutans* viability, whereas PUN produced more moderate structural and transcriptional effects.

Taken together, the eradication data demonstrate that PUG and PUN exhibit limited yet biologically meaningful activity against mature dual-species biofilms. Their effects are characterised by time-dependent reduction in biofilm biomass, destabilisation of EPS architecture, selective transcriptional suppression of *S. mutans* virulence pathways, and preservation of commensal viability. Among the two compounds, PUG consistently showed greater capacity to weaken established biofilms. These findings are consistent with current understanding that complete eradication of mature oral biofilms using non-toxic agents is inherently challenging. Instead, strategies

that attenuate virulence while preserving microbial balance are increasingly favoured in oral microbiology (Takahashi & Nyvad, 2011; Marsh et al., 2016). In this context, PUG emerges as a promising biofilm-modulating agent with potential utility as an adjunctive or preventive intervention rather than a standalone biofilm eradicator. These observations highlight the potential clinical relevance of selective biofilm-modulating compounds and provide a rationale for considering PUG and related phytochemicals in preventive or adjunctive strategies aimed at ecological management of cariogenic biofilms.

5.15 Translational Relevance and Clinical Implications

From a translational standpoint, the findings of this study highlight important distinctions between biofilm inhibition and biofilm eradication that are directly relevant to clinical caries management. While conventional antimicrobial strategies have largely focused on rapid bacterial killing, increasing evidence indicates that indiscriminate eradication of oral biofilms may disrupt microbial homeostasis and contribute to recurrent dysbiosis rather than long-term disease control (Takahashi & Nyvad, 2011; Marsh et al., 2016). The present results demonstrate that pomegranate-derived compounds, particularly PUG, exert biologically meaningful effects on cariogenic biofilms without inducing extensive loss of commensal viability, supporting their potential role as biofilm-modulating rather than biofilm-eliminating agents.

From a researcher's perspective, the limited ability of PUG and PUN to eradicate mature dual-species biofilms underscores the intrinsic resilience of established oral biofilms. Mature biofilms are characterised by dense extracellular polymeric substance (EPS) matrices, metabolic heterogeneity, and stress-adapted subpopulations that collectively reduce susceptibility to antimicrobial agents (Flemming et al., 2016; Hall & Mah, 2017). The observed reduction in biomass without corresponding large-scale bacterial killing suggests that these compounds primarily destabilise biofilm architecture and virulence expression rather than inducing acute cytotoxicity. This profile is consistent with an antivirulence or ecological intervention strategy, which aims to weaken pathogenic traits while preserving overall microbial function (Rasko & Sperandio, 2010).

An additional advantage of antivirulence-oriented approaches lies in their potential to impose lower selective pressure for antimicrobial resistance compared with

conventional bactericidal agents. Traditional antimicrobial therapies act by inhibiting bacterial growth or inducing cell death, thereby exerting strong evolutionary pressure that favours the emergence and persistence of resistant strains (Munita & Arias, 2016; Galgano et al., 2025). In contrast, antivirulence strategies aim to attenuate pathogenic behaviours without necessarily compromising bacterial viability. By targeting processes such as adhesion, quorum sensing, or extracellular matrix synthesis, these approaches interfere with the ability of bacteria to cause disease rather than their ability to survive (Rasko & Sperandio, 2010). Because bacterial survival is not directly threatened, the selective advantage conferred by resistance mutations may be reduced, potentially slowing the evolutionary development of resistance mechanisms.

Clinically, such a mechanism is particularly relevant in the oral cavity, where complete biofilm removal is neither feasible nor desirable. Dental plaque is a normal biological structure, and disease arises when ecological balance shifts in favour of acidogenic and aciduric species such as *S. mutans*. The selective suppression of *S. mutans* virulence pathways and preservation of *S. gordonii* viability observed in this study align with the ecological plaque hypothesis, which emphasises restoration of microbial balance rather than sterilisation (Marsh, 2003; Marsh et al., 2016). From a clinical perspective, agents that reduce EPS integrity and cariogenic dominance without eliminating commensal bacteria may help stabilise plaque composition and reduce caries risk over time.

The differential performance of PUG and PUN in eradication assays also has important practical implications for formulation and clinical use. Specifically, the absence of significant biofilm eradication following short-term exposure indicates that these compounds are not suitable for rapid-action applications such as conventional mouthrinses, where contact times are typically limited to 30–60 seconds. The modest effects observed following brief treatment further suggest that these compounds do not function as immediate bactericidal agents comparable to CHX. Instead, their activity profile supports greater suitability for sustained-contact formulations, including varnishes, gels, dentifrices, or other delivery systems that promote prolonged retention at the tooth–biofilm interface. In such contexts, extended exposure is expected to facilitate the cumulative weakening of biofilm structure and virulence expression while minimising adverse effects associated with long-term use of broad-spectrum antimicrobials, including staining, taste alteration, and disruption of the oral microbiome (Brookes et al., 2023).

From a clinical management perspective, these findings support the potential role of PUG-containing formulations as adjuncts rather than replacements for mechanical plaque control. Mechanical disruption remains essential for the removal of mature biofilms; however, agents that weaken EPS cohesion and downregulate virulence gene expression may enhance the effectiveness of brushing and professional debridement. This is particularly relevant for high-risk patient groups, such as individuals with xerostomia, those wearing orthodontic appliances, or those with limited manual dexterity, in whom complete plaque removal is challenging.

Finally, the selective and non-cytotoxic nature of the observed effects supports further development of PUG as a candidate for preventive rather than curative applications. From a translational research perspective, these data justify progression toward formulation studies, bioavailability assessment, and evaluation in more complex multispecies or in situ biofilm models. From a clinical standpoint, they reinforce the shift toward biologically informed caries prevention strategies that prioritise modulation of biofilm behaviour over aggressive antimicrobial eradication.

5.16 Summary of Hypothesis Testing

To provide a structured evaluation of the study outcomes, each proposed hypothesis was assessed in relation to the experimental findings obtained across both phases of the study. The overall outcomes are summarised in Table 5.3.

In general, the findings supported the majority of the proposed hypotheses, although the extent of support varied depending on the biological endpoint and experimental conditions. Hypotheses H1, H4, and H6 were accepted, confirming the antibacterial activity, attenuation of acidogenicity, and selective antivirulence effects of the selected compounds. In contrast, H2, H3, H5, and H7 were partially accepted, reflecting variability in responses across bacterial species, biofilm developmental stages, and compound-specific characteristics. Notably, the antibiofilm findings (H3) indicated that while inhibition of biofilm formation was consistently observed, disruption of established biofilms was less pronounced and influenced by exposure conditions. Similarly, selective effects in dual-species biofilms (H7) were dependent on treatment duration, highlighting the increased resilience of mature biofilm structures. Variability in preliminary safety profiles (H5), particularly the higher toxicity observed for ellagic acid, further emphasises the importance of compound-specific evaluation.

Taken together, these findings support the overall premise that selected PDCs, particularly PUG and PUN, function primarily as biofilm-modulating and antivirulence agents rather than broad-spectrum bactericidal agents, aligning with an ecological approach to cariogenic biofilm control.

Table 5.3
Summary of Hypothesis Testing Outcomes

Hypothesis	Outcome	Justification
H1	Accepted	Selected PDCs demonstrated antibacterial activity against <i>S. mutans</i> and <i>S. gordonii</i> as evidenced by disc diffusion, MIC, and MBC assays.
H2	Partially accepted	Modulation of cell surface hydrophobicity was observed, with more pronounced effects in <i>S. mutans</i> compared to <i>S. gordonii</i> , indicating selective antivirulence activity.
H3	Partially accepted	PDCs effectively inhibited biofilm formation, while disruption of established biofilms was less pronounced and dependent on exposure conditions, as supported by MBIC and MBEC findings.
H4	Accepted	Glycolytic acidification in <i>S. mutans</i> was attenuated, with a reduced rate and extent of pH decline. PUG and PUN maintained pH above the critical threshold of 5.5.
H5	Partially accepted	Most PDCs exhibited acceptable safety ($LC_{50} > 500 \mu\text{g/mL}$), whereas ellagic acid showed higher toxicity ($LC_{50} = 159 \mu\text{g/mL}$), indicating compound-dependent variation.
H6	Accepted	PUG and PUN selectively attenuated <i>S. mutans</i> virulence during biofilm formation, with minimal impact on <i>S. gordonii</i> , supported by CFU and gene expression data.
H7	Partially accepted	Selective effects were observed in established biofilms, particularly at longer exposure (4 h), whereas short exposure (5 min) showed limited impact.

5.17 Study Limitations

Although the dual-species *in vitro* model in this study provides a controlled platform for mechanistic investigation, several limitations should be acknowledged. First, despite its suitability for examining defined interspecies interactions, the model does not capture the full taxonomic diversity and functional redundancy of natural dental plaque communities. *In vivo*, additional early colonisers such as *Streptococcus sanguinis* and *Actinomyces* spp. contribute to biofilm development through competitive adhesion, hydrogen peroxide production, and alkali-generating pathways. The presence of these species may alter ecological competitiveness and influence the efficacy and selectivity of PUG and PUN observed in the simplified system. Future studies

incorporating multispecies or microcosm biofilm models will be necessary to determine whether the selective effects observed here are preserved under greater ecological complexity. Integration of such models with meta transcriptomic approaches would enable comprehensive characterisation of community-wide functional responses, including shifts in metabolic activity, stress adaptation, and interspecies signalling under compound exposure.

Second, the *in vitro* design limits direct extrapolation to clinical settings. The absence of continuous salivary flow, immune modulation, dietary cycling, and mechanical shear means that the antibiofilm and antivirulence effects demonstrated in this thesis require confirmation under *in vivo* or *in situ* conditions. Third, toxicity assessment was limited to preliminary screening using the BSLA. While appropriate for early compound prioritisation, BSLA does not provide tissue-specific or long-term safety information. Comprehensive cytocompatibility testing using relevant oral epithelial and fibroblast models will be required to refine therapeutic windows and support translational development. Finally, mechanistic interpretations were primarily derived from transcriptional and phenotypic outcomes. Although gene expression analysis provides important insight into regulatory responses, it does not directly confirm protein-level activity or compound–target interactions. Future investigations employing proteomic, metabolomic, or molecular interaction approaches would further strengthen mechanistic understanding and clarify the mode of action of PUG and PUN.

5.18 Future Directions

Building upon the findings of this study, several directions for future research are warranted to strengthen biological understanding and support the translational progression of PUG and PUN as biofilm-modulating agents.

First, evaluation of these compounds in more complex multispecies oral biofilm models is necessary. While the present dual-species system captures key interactions between a cariogenic pathogen and a commensal species, natural dental plaque comprises highly diverse microbial communities with functional redundancy and competitive dynamics. Inclusion of additional acidogenic, aciduric, and health-associated species would allow assessment of whether the selective antibiofilm effects observed here are maintained within a more ecologically representative environment. In addition, future studies may benefit from applying metatranscriptomic or

metagenomic approaches to characterise global community responses to PUG and PUN exposure within multispecies oral biofilms. Whereas the present study examined expression of selected virulence-associated genes using RT-qPCR, high-throughput transcriptomic profiling would enable comprehensive analysis of metabolic pathways, stress responses, and interspecies signalling networks across the entire microbial community. Integration of metatranscriptomic data with microbial composition analysis would therefore provide deeper insight into the ecological mechanisms underlying biofilm modulation and help determine whether PUG and PUN promote a stable, health-associated oral microbiome.

Second, long-term investigations of ecological stability are required to determine how repeated exposure to these compounds influences oral microbiome composition. Although short-term assays provide valuable information on immediate antibiofilm effects, prolonged exposure may lead to microbial adaptation or community shifts. Longitudinal studies using repeated-treatment biofilm models or microcosm systems would help clarify whether PUG and PUN support sustained ecological modulation or inadvertently promote dysbiosis. Such studies are particularly relevant given the increasing emphasis on preserving commensal microbial populations in contemporary caries prevention strategies.

Third, investigation of potential synergistic or additive effects with established anticariogenic agents, such as fluoride or arginine-based formulations, represents a logical extension of this work. Fluoride enhances enamel resistance and inhibits bacterial metabolism, whereas arginine promotes alkali generation and ecological balance within dental biofilms. Combining these agents with compounds that disrupt extracellular polysaccharide (EPS) integrity and virulence regulation may produce complementary effects that enhance overall caries control. Systematic evaluation of such combinations could identify formulations that achieve improved efficacy at lower concentrations while minimising potential adverse effects.

Fourth, further investigation into the structure–activity relationships (SAR) of pomegranate-derived ellagitannins would provide valuable mechanistic insight. The present findings indicate that PUG demonstrated greater ecological selectivity and antibiofilm activity than PUN, suggesting that molecular features such as molecular size, hydroxyl group density, and the number and arrangement of galloyl or hexahydroxydiphenoyl (HHDP) groups may influence antibiofilm potency. Systematic comparison of structurally related ellagitannins and their derivatives could therefore

help identify the structural determinants responsible for optimal interactions with bacterial adhesins, glucosyltransferases, and extracellular polymeric substances. Such studies may involve evaluation of additional purified ellagitannins, semi-synthetic derivatives, or structurally simplified analogues to determine how specific functional groups contribute to antibiofilm activity and ecological selectivity.

Fifth, development and optimisation of delivery systems to enhance compound retention within the oral cavity will be critical for clinical translation. The limited eradication observed following short exposure highlights the importance of sustained contact for maximal biofilm modulation. Encapsulation strategies, bioadhesive carriers, or slow-release systems incorporated into dentifrices, varnishes, or gels could improve compound stability and prolong interaction with biofilms. Future studies should therefore evaluate how formulation influences compound stability, release kinetics, and antibiofilm performance under simulated oral conditions.

Although the brine shrimp lethality assay (BSLA) used in this study provided a rapid preliminary indication of the general toxicity profile of the tested compounds, it represents a non-specific screening model and does not directly reflect the biological responses of human oral tissues. Future investigations should therefore include cytocompatibility assessment using mammalian cell models relevant to the oral environment. Human gingival fibroblasts (HGF-1) may be used to evaluate potential effects on gingival connective tissues, while human oral keratinocytes (HOK) or immortalised oral epithelial cell lines such as OKF6/TERT-2 could be employed to assess epithelial tolerance to the compounds. These models would allow evaluation of cell viability, proliferation, and potential inflammatory responses following exposure to PUG and PUN, thereby providing a more clinically relevant safety assessment prior to *in vivo* studies. Establishing these cytocompatibility margins is essential to define the therapeutic window of the compounds and represents the immediate next translational hurdle prior to formulation development and clinical application.

Following confirmation of cytocompatibility in mammalian cell models, subsequent *in vivo* validation of both anticariogenic efficacy and safety will be required before clinical application can be considered. Animal models and, ultimately, human studies are necessary to determine whether the antibiofilm and antivirulence effects observed *in vitro* translate into measurable reductions in caries development without compromising oral tissue health or microbial homeostasis. Such investigations should

integrate microbiological, clinical, and safety endpoints to provide a comprehensive and clinically meaningful evaluation.

Collectively, these future investigations will address key knowledge gaps related to ecological impact, molecular mechanism, formulation, and in vivo performance. Advancing along these lines will not only clarify the clinical applicability of PUG and PUN but also contribute to the broader development of biofilm-modulating strategies that align with contemporary ecology-based approaches to caries prevention.

More broadly, this line of research supports the emerging paradigm of precision modulation of the oral microbiome, where targeted antivirulence strategies selectively attenuate pathogenic behaviours while preserving beneficial microbial functions. By integrating ecological microbiology, natural product chemistry, and translational formulation research, future studies may facilitate the development of next-generation therapeutics that promote microbial homeostasis rather than indiscriminate antimicrobial eradication.

CHAPTER 6

CONCLUSION

6.1 Summary of Key Findings and Mechanistic Interpretation

This thesis provides a comprehensive and mechanistically informed evaluation of pomegranate-derived compounds (PDC) as selective antibiofilm agents targeting cariogenic oral biofilms. Using a structured two-phase experimental framework, the study systematically progressed from broad compound screening to in-depth mechanistic analysis within biologically relevant dual-species biofilm models comprising *Streptococcus mutans* (*S. mutans*) and *Streptococcus gordonii* (*S. gordonii*). Through this integrated experimental approach, the study successfully addressed the research objectives by identifying biofilm-active PDCs and elucidating the mechanisms underlying their selective effects on cariogenic biofilms.

In Phase I, comparative screening of multiple PDCs demonstrated marked heterogeneity in antibacterial and antibiofilm performance. While several compounds exhibited planktonic antimicrobial activity, punicalagin (PUG) and punicalin (PUN) emerged as the most promising candidates based on their ability to inhibit biofilm formation at sub-MIC concentrations, attenuate virulence-associated traits such as cell surface hydrophobicity and acidogenicity, and maintain favourable preliminary safety profiles. Importantly, prioritisation was guided by biofilm-specific endpoints rather than planktonic susceptibility alone, addressing a key limitation of many previous phytochemical studies.

Phase II provided mechanistic insight into how PUG and PUN interact with multispecies oral biofilms under both inhibition and eradication conditions. In dual-species biofilm inhibition assays, both compounds suppressed biofilm biomass and selectively reduced *S. mutans* viability, while largely preserving *S. gordonii*. This selective activity was supported at the molecular level by significant downregulation of *S. mutans* genes involved in extracellular polysaccharide synthesis, adhesion, quorum sensing, and stress response, including *gtfB*, *gtfC*, *gbpB*, *gbpD*, *comDE*, and *brpA*. The preservation of core metabolic gene expression further suggests that these compounds act primarily through antivirulence and antibiofilm mechanisms rather than indiscriminate bactericidal effects.

In biofilm eradication assays targeting mature, structurally complex biofilms, PUG and PUN exhibited a more limited but biologically meaningful activity profile. While short-term exposure did not substantially reduce biomass or viability, prolonged treatment resulted in significant disruption of the biofilm matrix without extensive bacterial killing. This dissociation between biomass reduction and viable cell counts reflects the intrinsic tolerance of mature biofilms and underscores the difficulty of complete eradication using non-toxic agents. Transcriptional upregulation of autolysis- and cell wall remodelling-associated genes (*atlA* and *lytF*) following prolonged exposure, particularly with PUG, suggests internal structural destabilisation of the biofilm architecture rather than direct cytotoxicity. In contrast, CHX produced rapid and extensive killing of both species, reinforcing the mechanistic distinction between ecological biofilm modulation and broad-spectrum antisepsis.

Across all experimental parameters, PUG consistently demonstrated superior antibiofilm potency and ecological selectivity compared to PUN, particularly in suppressing *S. mutans* virulence pathways and destabilising extracellular matrix structure. At the same time, the limited transcriptional and viability effects observed in *S. gordonii* support the ecological selectivity of these compounds. This is a critical finding, as preservation of commensal pioneer species is increasingly recognised as essential for maintaining oral microbial homeostasis and preventing recurrent dysbiosis.

6.2 Scientific Contribution, Translational Implications and Future Direction

The findings of this thesis advance current understanding of how plant-derived phytochemicals can be rationally evaluated and prioritised as biofilm-modulating agents rather than conventional antimicrobials. By integrating phenotypic, molecular, and structural analyses within a dual-species model, this work moves beyond reductionist single-species approaches and more closely reflects the biological complexity of dental plaque. The results support the concept that effective caries prevention does not require complete biofilm eradication, but rather targeted attenuation of cariogenic dominance and virulence within the biofilm ecosystem.

From a translational perspective, while both compounds represent significant advancements in plant-derived antivirulence strategies, the distinct activity profile of PUG positions it as a primary candidate for further translational development. Its ability to selectively attenuate *S. mutans* dominance while preserving the viability of the

commensal *S. gordonii* supports its potential incorporation into sustained-contact oral care formulations, such as varnishes or bioadhesive gels. This work provides the scientific foundation necessary to advance PUG toward mammalian cytocompatibility testing and subsequent *in vivo* validation in the pursuit of sustainable, ecology-based caries prevention. In conclusion, this thesis provides original mechanistic insight into the selective modulation of cariogenic biofilms by PDCs. The findings demonstrate that these compounds can attenuate *S. mutans*–driven cariogenic biofilm development through coordinated effects on biofilm architecture, regulation of virulence-associated gene expression, and preservation of ecological balance within the microbial community. By integrating microbiological, structural, and molecular evidence, this study proposes a mechanistically informed framework describing how plant-derived polyphenols can modulate biofilm pathogenicity without indiscriminate bacterial eradication. This framework provides a scientific basis for the future development of targeted ecology-based antibiofilm strategies and plant-based therapeutics for sustainable prevention and management of dental caries.

REFERENCES

- Abd-El-Aziz, A. B. E.-D., & A Sallam, R. (2020). Antibacterial effect of green tea and pomegranate peel extracts on *Streptococcus mutans* of orthodontic treated patients. *Journal of Radiation Research and Applied Sciences*, 13(1), 132–143.
- Abdul Razak, F., Baharuddin, B. A., Akbar, E. F. M., Norizan, A. H., Ibrahim, N. F., & Musa, M. Y. (2017). Alternative sweeteners influence the biomass of oral biofilm. *Archives of Oral Biology*, 80, 180–184.
- Ahn, S. J., & Burne, R. A. (2006). The *atla* operon of *Streptococcus mutans*: role in autolysin maturation and cell surface biogenesis. *Journal of bacteriology*, 188(19), 6877-6888.
- Alm, R. A., & Lahiri, S. D. (2020). Narrow-spectrum antibacterial agents - benefits and challenges. *Antibiotics*, 9(7), 418.
- Almatroudi, A. (2025). Biofilm Resilience: Molecular Mechanisms Driving Antibiotic Resistance in Clinical Contexts. *Biology*, 14(2). 165
- Angelova, A., Jovanova, E., Polizzi, A., Leonardi, R., & Isola, G. (2025). Effects of antiseptic formulations on oral microbiota and related systemic diseases: A scoping review. *Antibiotics*, 14(8), 815.
- Ansari, J. M., Abraham, N. M., Massaro, J., Murphy, K., Smith-Carpenter, J., & Fikrig, E. (2017). Anti-biofilm activity of a self-aggregating peptide against *Streptococcus mutans*. *Frontiers in Microbiology*, 8, 488.
- Arthur, R. A., Waeiss, R. A., Hara, A. T., Lippert, F., Eckert, G. J., & Zero, D. T. (2013). A defined-multispecies microbial model for studying enamel caries development. *Caries Research*, 47(4), 318–324.
- Askar, H., Krois, J., Göstemeyer, G., & Schwendicke, F. (2021). Secondary caries risk of different adhesive strategies and restorative materials in permanent teeth: Systematic review and network meta-analysis. *Journal of Dentistry*, 104, 103541
- Atazhanova, G. A., Levaya, Y. K., Badekova, K. Z., Ishmuratova, M. Y., Smagulov, M. K., Ospanova, Z. O., & Smagulova, E. M. (2024). Inhibition of the Biofilm Formation of Plant *Streptococcus mutans*. *Pharmaceuticals*, 17(12), 1613
- Azeem, K., Fatima, S., Ali, A., Ubaid, A., Husain, F. M., & Abid, M. (2025). Biochemistry of Bacterial Biofilm: Insights into Antibiotic Resistance Mechanisms and Therapeutic Intervention. *Life*, 15(1), 49.

- Azeredo, J., Azevedo, N. F., Briandet, R., Cerca, N., Coenye, T., Costa, A. R., ... & Sternberg, C. (2017). Critical review on biofilm methods. *Critical reviews in microbiology*, 43(3), 313-351.
- Badshah, S. M., Sultana, N., Al-Rawi, M. B. A., Tariq, Z., Lin, K. S., Hussain, A., Syed, W., Ahmed, D., Hussain, J., Khan, B., & Rehman, F. U. (2025). Phytochemical profiling, biological potential and *in silico* identification of anticancerous compound from Pakistani pomegranate Peel. *Scientific Reports*, 15(1), 26689.
- Baker, J. L., Abranches, J., Faustoferri, R. C., Hubbard, C. J., Lemos, J. A., Courtney, M. A., & Quivey, R. G. (2015). Transcriptional profile of glucose-shocked and acid-adapted strains of *Streptococcus mutans*. *Molecular Oral Microbiology*, 30(6), 496–517.
- Bali, R. K., Sharma, P., Gaba, S., Kaur, A., & Ghanghas, P. (2015). A review of complications of odontogenic infections. *National journal of maxillofacial surgery*, 6(2), 136-143.
- Bansal, K., Shamoo, A., Atif, M., Batra, P., & Chaudhry, R. (2024). Isolation and detection of bacterial species on mitis salivarius bacitracin agar from the plaque samples of caries active children. *Journal of Indian Society of Pedodontics and Preventive Dentistry*, 42(1), 15–21.
- Bao, N., Li, M., Chen, Z., Zhou, W., Rashed, M. M. A., Han, F., Zhai, K., & Jiang, B. (2026). Efficient Recovery of Phenolic Compounds from Pomegranate (*Punica granatum L.*) Peel Using Mechanochemical-Assisted Extraction for *In vitro* Biological Activities. *Food and Bioprocess Technology*, 19(1), 13.
- Barak, T., Sharon, E., Steinberg, D., Feldman, M., Sionov, R. V., & Shalish, M. (2022). Anti-Bacterial Effect of Cannabidiol against the Cariogenic *Streptococcus mutans* Bacterium: An *In vitro* Study. *International Journal of Molecular Sciences*, 23(24), 15878.
- Barnard, J. P., & Stinson, M. W. (1999). Influence of Environmental Conditions on Hydrogen Peroxide Formation by *Streptococcus gordonii*. *Infection and immunity*, 67(12), 6558-6564
- Basavaraju, M., Sisnity, V. S., Palaparthi, R., & Addanki, P. K. (2016). Quorum quenching: Signal jamming in dental plaque biofilms. *Journal of Dental Sciences*, 11(4), 349–352.
- Bernabe, E., Marcenes, W., Abdulkader, R. S., Abreu, L. G., Afzal, S., Alhalaiqa, F. N., & Kassebaum, N. J. (2025). Trends in the global, regional, and national

- burden of oral conditions from 1990 to 2021: a systematic analysis for the Global Burden of Disease Study 2021. *The Lancet*, 405(10482), 897-910.
- Bescos, R., Ashworth, A., Cutler, C., Brookes, Z. L., Belfield, L., Rodiles, A., Casas-Agustench, P., Farnham, G., Liddle, L., Burleigh, M., White, D., Easton, C., & Hickson, M. (2020). Effects of Chlorhexidine mouthwash on the oral microbiome. *Scientific Reports*, 10(1), 5254.
- Bharathi, S., Dhanraj, G., Sundramurthy, V. P., & Mohanasundaram, S. (2025). Comprehensive strategies for overcoming dental biofilms: Microbial dynamics and innovative methods. *Microbial Pathogenesis*, 205, 107690.
- Bigueti, C. C., Cavalla, F., Fonseca, A. C., Tabanez, A. P., Siddiqui, D. A., Wheelis, S. E., Taga, R., Fakhouri, W. D., Silva, R. M., Rodrigues, D. C., & Garlet, G. P. (2021). Effects of Titanium Corrosion Products on In Vivo Biological Response: A Basis for the Understanding of Osseointegration Failures Mechanisms. *Frontiers in Materials*, 8, 651970.
- Bisht, S., Khera, A., & Raghav, P. (2022). White spot lesions during orthodontic clear aligner therapy: A scoping review. *Journal of Orthodontic Science*, 11(1), 9.
- Bonincontro, G., Scuderi, S. A., Marino, A., & Simonetti, G. (2023). Synergistic Effect of Plant Compounds in Combination with Conventional Antimicrobials against Biofilm of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Candida* spp. *Pharmaceuticals*, 16(11), 1531.
- Borowicz, M., Krzyżanowska, D. M., & Jafra, S. (2023). Crystal violet-based assay for the assessment of bacterial biofilm formation in medical tubing. *Journal of Microbiological Methods*, 204, 106656.
- Bos, R., Van Der Mei, H. C., & Busscher, H. J. (1999). Physico-chemistry of initial microbial adhesive interactions—its mechanisms and methods for study. *FEMS microbiology reviews*, 23(2), 179-230.
- Bowen, W. H., & Koo, H. J. C. R. (2011). Biology of *Streptococcus mutans*-derived glucosyltransferases: role in extracellular matrix formation of cariogenic biofilms. *Caries research*, 45(1), 69-86.
- Bowen, W. H., Burne, R. A., Wu, H., & Koo, H. (2018). Oral biofilms: pathogens, matrix, and polymicrobial interactions in microenvironments. *Trends in microbiology*, 26(3), 229-242.
- Bridier, A., & Briandet, R. (2022). Microbial Biofilms: Structural Plasticity and Emerging Properties. *Microorganisms*, 10(1), 138.

- Brookes, Z. L., Belfield, L. A., Ashworth, A., Casas-Agustench, P., Raja, M., Pollard, A. J., & Bescos, R. (2021). Effects of chlorhexidine mouthwash on the oral microbiome. *Journal of dentistry*, 113, 103768.
- Brookes, Z., Teoh, L., Cieplik, F., & Kumar, P. (2023). Mouthwash Effects on the Oral Microbiome: Are They Good, Bad, or Balanced?. *International Dental Journal*, 73, S74–S81.
- Busscher, H. J., & van der Mei, H. C. (2012). How do bacteria know they are on a surface and regulate their response to an adhering state? *PLoS Pathogens*, 8(1). e1002440.
- Caesar, L. K., & Cech, N. B. (2019). Synergy and antagonism in natural product extracts: When 1 + 1 does not equal 2. *Natural Product Reports*, 36(6), 869–888.
- Cai, J. N., Jung, J. E., Lee, M. H., Choi, H. M., & Jeon, J. G. (2018). Sucrose challenges to *Streptococcus mutans* biofilms and the curve fitting for the biofilm changes. *FEMS Microbiology Ecology*, 94(7), 1j.
- Calixto, J. B. (2000). Efficacy, safety, quality control, marketing and regulatory guidelines for herbal medicines (phytotherapeutic agents). *Brazilian Journal of Medical and Biological Research*, 33(2), 179-189.
- Campos, L., Seixas, L., Henriques, M. H. F., Peres, A. M., & Veloso, A. C. A. (2022). Pomegranate Peels and Seeds as a Source of Phenolic Compounds: Effect of Cultivar, By-Product, and Extraction Solvent. *International Journal of Food Science*, 2022(1), 9189575.
- Celiksoy, V., Moses, R. L., Sloan, A. J., Moseley, R., & Heard, C. M. (2020). Article evaluation of the *in vitro* oral wound healing effects of pomegranate (*Punica granatum*) rind extract and punicalagin, in combination with zn (ii). *Biomolecules*, 10(9), 1–15.
- Cella, M. A., Coulson, T., MacEachern, S., Badr, S., Ahmadi, A., Tabatabaei, M. S., Labbe, A., & Griffiths, M. W. (2023). Probiotic disruption of quorum sensing reduces virulence and increases cefoxitin sensitivity in methicillin-resistant *Staphylococcus aureus*. *Scientific Reports*, 13(1), 4373.
- Chatzigiannidou, I., Teughels, W., Van de Wiele, T., & Boon, N. (2020). Oral biofilms exposure to chlorhexidine results in altered microbial composition and metabolic profile. *Npj Biofilms and Microbiomes*, 6(1), 13.

- Chawhuaveang, D. D., Yu, O. Y., Yin, I. X., Lam, W. Y. H., Mei, M. L., & Chu, C. H. (2021). Acquired salivary pellicle and oral diseases: A literature review. In *Journal of Dental Sciences* 16(1), 523–529.
- Chen, R., Du, M., & Liu, C. (2022). Strategies for dispersion of cariogenic biofilms: applications and mechanisms. *Frontiers in Microbiology*, 13, 981203.
- Chen, X., Li, H., Zhang, B., & Deng, Z. (2022). The synergistic and antagonistic antioxidant interactions of dietary phytochemical combinations. *Critical Reviews in Food Science and Nutrition*, 62(20), 5658–5677.
- Chi, Y., Wang, Y., Ji, M., Li, Y., Zhu, H., Yan, Y., Fu, D., Zou, L., & Ren, B. (2022). Natural products from traditional medicine as promising agents targeting at different stages of oral biofilm development. *Frontiers in Microbiology*, 13, 955459.
- Chonsut, P., Romyasamit, C., Konyanee, A., Niyomtham, N., Goodla, L., & Mordmuang, A. (2024). Potential Activities of *Centella asiatica* Leaf Extract against Pathogenic Bacteria-Associated Biofilms and Its Anti-Inflammatory Effects. *Advances in Pharmacological and Pharmaceutical Sciences*, 2024 (1), 5959077.
- Choo, S. W., Mohammed, W. K., Mutha, N. V. R., Rostami, N., Ahmed, H., Krasnogor, N., Tan, G. Y. A., & Jakubovics, N. S. (2021). Transcriptomic Responses to Coaggregation between *Streptococcus gordonii* and *Streptococcus oralis*. *Applied and Environmental Microbiology*, 87(22), e01558-21.
- Christian, N., Burden, D., Emam, A., Brenk, A., Sperber, S., Kalu, M., Cuadra, G., & Palazzolo, D. (2024). Effects of E-Liquids and Their Aerosols on Biofilm Formation and Growth of Oral Commensal Streptococcal Communities: Effect of Cinnamon and Menthol Flavors. *Dentistry Journal*, 12(8), 232.
- Cieplik, F., Jakubovics, N. S., Buchalla, W., Maisch, T., Hellwig, E., & Al-Ahmad, A. (2019). Resistance toward chlorhexidine in oral bacteria—is there cause for concern?. *Frontiers in microbiology*, 10, 587.
- Clinical and Laboratory Standards Institute. (2012). *Performance standards for antimicrobial disk susceptibility tests* (11th ed.; CLSI standard M02-A11). Clinical and Laboratory Standards Institute.
- Coenye, T. (2023). Biofilm antimicrobial susceptibility testing: where are we and where could we be going?. *Clinical Microbiology Reviews*, 36(4), e00024-23.

- Dang, M. H., Jung, J. E., Choi, H. M., & Jeon, J. G. (2018). Difference in virulence and composition of a cariogenic biofilm according to substratum direction. *Scientific Reports*, 8(1), 6244.
- de Oliveira, R. V. D., Bonafé, F. S. S., Spolidorio, D. M. P., Koga-ito, C. Y., de Farias, A. L., Kirker, K. R., James, G. A., & Brighenti, F. L. (2020). *Streptococcus mutans* and *Actinomyces naeslundii* interaction in dual-species biofilm. *Microorganisms*, 8(2), 194.
- Del Rey, Y. C., Rikvold, P. D., Lund, M. B., Raittio, E. J., Schramm, A., Meyer, R. L., & Schlafer, S. (2025). Arginine modulates the pH, microbial composition, and matrix architecture of biofilms from caries-active patients. *International Journal of Oral Science*, 17(1), 70.
- Deng, Y., Yang, Y., Zhang, B., Chen, H., Lu, Y., Ren, S., Lei, L., & Hu, T. (2021). The vicK gene of *Streptococcus mutans* mediates its cariogenicity via exopolysaccharides metabolism. *International Journal of Oral Science*, 13(1), 45.
- Di Spirito, F., Lo Giudice, R., Amato, M., Di Palo, M. P., D'Ambrosio, F., Amato, A., & Martina, S. (2022). Inflammatory, Reactive, and Hypersensitivity Lesions Potentially Due to Metal Nanoparticles from Dental Implants and Supported Restorations: An Umbrella Review. *Applied Sciences*, 12(21), 11208.
- Diaz, P. I., & Valm, A. M. (2020). Microbial Interactions in Oral Communities Mediate Emergent Biofilm Properties. *Journal of Dental Research*, 99(1), 18–25.
- Díaz-Garrido, N., Lozano, C. P., Kreth, J., & Giacaman, R. A. (2020). Competition and Caries on Enamel of a Dual-Species Biofilm Model with *Streptococcus mutans* and *Streptococcus sanguinis*. *Applied and Environmental Microbiology*, 86(21), e01262-20.
- do Amaral, G. C. L. S., Hassan, M. A., Sloniak, M. C., Pannuti, C. M., Romito, G. A., & Villar, C. C. (2023). Effects of antimicrobial mouthwashes on the human oral microbiome: Systematic review of controlled clinical trials. *International Journal of Dental Hygiene*, 21(1), 128-140.
- Donlan, R. M., & Costerton, J. W. (2002). Biofilms: Survival mechanisms of clinically relevant microorganisms. *Clinical Microbiology Reviews*, 15(2), 167–193.
- Dumitrel, S. I., Matichescu, A., Dinu, S., Buzatu, R., Popovici, R., Dinu, D. C., & Bratu, D. C. (2024). New Insights Regarding the Use of Relevant Synthetic Compounds in Dentistry. *Molecules*, 29(16), 3802.

- Dutton, L. C., Paszkiewicz, K. H., Silverman, R. J., Splatt, P. R., Shaw, S., Nobbs, A. H., Lamont, R. J., Jenkinson, H. F., & Ramsdale, M. (2016). Transcriptional landscape of trans-kingdom communication between *Candida albicans* and *Streptococcus gordonii*. *Molecular Oral Microbiology*, 31(2), 136–161.
- Elawady, R., Aboulela, A. G., Gaballah, A., Ghazal, A. A., & Amer, A. N. (2024). Antimicrobial Sub-MIC induces *Staphylococcus aureus* biofilm formation without affecting the bacterial count. *BMC Infectious Diseases*, 24(1), 1065.
- Elkoraichi, I., Latif, M., El Faqer, O., Elaje, R., Thoume, A., Mtairag, E. M., Rais, S., & Daoudi, F. (2023). In vivo and in vitro evaluation of antioxidant, antihemolytic, acute toxicity effects and high-performance liquid chromatography analysis of an aqueous extract of Moroccan *Punica granatum* L. *J Herbmed Pharmacol* 12: 168–175.
- Enax, J., Ganss, B., Amaechi, B. T., Schulze zur Wiesche, E., & Meyer, F. (2023). The composition of the dental pellicle: an updated literature review. *Frontiers in Oral Health* , 4, 1260442.
- Foujdar, R., Chopra, H. K., Bera, M. B., & Batra, K. (2022). Isolation, characterization, bio-accessibility and cytotoxic effect of ellagitannins purified from peels of *Punica granatum* Indian var. Bhagwa. *Journal of Food Measurement and Characterization*, 16(2), 1733-1743.
- Fawole, O. A., Makunga, N. P., & Opara, U. L. (2012). Antibacterial, antioxidant and tyrosinase-inhibition activities of pomegranate fruit peel methanolic extract. *BMC Complementary and Alternative Medicine*, 12(1), 200.
- Fernandes, G. L., Vieira, A. P. M., Danelon, M., Emerenciano, N. G., Berretta, A. A., Buszinski, A. F. M., Hori, J. I., Lima, M. H. F. de, Reis, T. F. dos, Lima, J. A. de, Delbem, A. C. B., Silva, S. C. M. da, & Barbosa, D. B. (2022). Pomegranate Extract Potentiates the Anti-Demineralizing, Anti-Biofilm, and Anti-Inflammatory Actions of Non-Alcoholic Mouthwash When Associated with Sodium-Fluoride Trimetaphosphate. *Antibiotics*, 11(11), 1477.
- Ferrazzano, G. F., Scioscia, E., Sateriale, D., Pastore, G., Colicchio, R., Pagliuca, C., Cantile, T., Alcidi, B., Coda, M., Ingenito, A., Scaglione, E., Cicatiello, A. G., Volpe, M. G., Di Stasio, M., Salvatore, P., & Pagliarulo, C. (2017). In vitro antibacterial activity of pomegranate juice and peel extracts on cariogenic bacteria. *BioMed Research International*, 2017(1), 2152749.

- Fitri, D. K., Tuygunov, N., Wan Harun, W. H. A., Purwasena, I. A., Cahyanto, A., & Zakaria, M. N. (2025). Key virulence genes associated with *Streptococcus mutans* biofilm formation: a systematic review. *Frontiers in Oral Health*, 6, 1654428.
- Flemming, H. C., & Wingender, J. (2010). The biofilm matrix. *Nature Reviews Microbiology*, 8(9), 623–633.
- Flemming, H. C., Wingender, J., Szewzyk, U., Steinberg, P., Rice, S. A., & Kjelleberg, S. (2016). Biofilms: an emergent form of bacterial life. *Nature Reviews Microbiology*, 14(9), 563-575.
- Fu, J., Zhang, Y., Lin, S., Zhang, W., Shu, G., Lin, J., Li, H., Xu, F., Tang, H., Peng, G., Zhao, L., Chen, S., & Fu, H. (2021). Strategies for Interfering With Bacterial Early Stage Biofilms. *Frontiers in Microbiology*, 12, 675843.
- Galgano, M., Pellegrini, F., Catalano, E., Capozzi, L., Del Sambro, L., Sposato, A., Lucente, M. S., Vasinioti, V. I., Catella, C., Odigie, A. E., Tempesta, M., Pratelli, A., & Capozza, P. (2025). Acquired Bacterial Resistance to Antibiotics and Resistance Genes: From Past to Future. *Antibiotics*, 14(3), 222.
- Gallas, J. A., Pelozo, L. L., Oliveira, W. P., Salvador, S. L., Corona, S. M., & Souza-Gabriel, A. E. (2023). Characterization, Antimicrobial Activity, and Antioxidant Efficacy of a Pomegranate Peel Solution Against Persistent Root Canal Pathogens. *Cureus*, 15(8), e43142.
- Giacaman, R. A., Fernández, C. E., Muñoz-Sandoval, C., León, S., García-Manríquez, N., Echeverría, C., Valdes, S., Castro, R.J., & Gambetta-Tessini, K. (2022). Understanding dental caries as a non-communicable and behavioral disease: Management implications. *Frontiers in oral health*, 3, 764479.
- Gibbons, R. J., & Etherden, I. (1983). Comparative hydrophobicities of oral bacteria and their adherence to salivary pellicles. *Infection and immunity*, 41(3), 1190-1196.
- Gibbons, R. J., Etherden, I., & Skobe, Z. (1983). Association of fimbriae with the hydrophobicity of *Streptococcus sanguis* FC-1 and adherence to salivary pellicles. *Infection and Immunity*, 41(1), 414-417.
- Gilmore, A., Badham, M., Rudisin, W., Ashton, N., & Williams, D. (2024). A Bead Biofilm Reactor for High-Throughput Growth and Translational Applications. *Microorganisms*, 12(8), 1588.

- Gloag, E. S., Wozniak, D. J., Wolf, K. L., Masters, J. G., Daep, C. A., & Stoodley, P. (2021). Arginine Induced *Streptococcus gordonii* Biofilm Detachment Using a Novel Rotating-Disc Rheometry Method. *Frontiers in Cellular and Infection Microbiology*, *11*, 784388.
- Goc, A., Sumera, W., Niedzwiecki, A., & Rath, M. (2019). 10-undecynoic acid is a new anti-adherent agent killing biofilm of oral *Streptococcus* spp. *PloS one*, *14*(4), e0214763.
- Gopalakrishnan, U., Felicita, A. S., Mahendra, L., Kanji, M. A., Varadarajan, S., Raj, A. T., Feroz, S. M. A., Mehta, D., Baeshen, H. A., & Patil, S. (2021). Assessing the Potential Association Between Microbes and Corrosion of Intra-Oral Metallic Alloy-Based Dental Appliances Through a Systematic Review of the Literature. *Frontiers in Bioengineering and Biotechnology*, *9*, 631103.
- Gosset-Erard, C., Zhao, M., Lordel-Madeleine, S., & Ennahar, S. (2021). Identification of punicalagin as the bioactive compound behind the antimicrobial activity of pomegranate (*Punica granatum* L.) peels. *Food Chemistry*, *352*, 129396.
- Grandclément, C., Tannières, M., Moréra, S., Dessaux, Y., & Faure, D. (2015). Quorum quenching: Role in nature and applied developments. *FEMS Microbiology Reviews*, *40*(1), 86–116.
- Gregoire, S., Singh, A. P., Vorsa, N., & Koo, H. (2007). Influence of cranberry phenolics on glucan synthesis by glucosyltransferases and *Streptococcus mutans* acidogenicity. *Journal of applied microbiology*, *103*(5), 1960-1968.
- Gulube, Z., & Patel, M. (2016). Effect of *Punica granatum* on the virulence factors of cariogenic bacteria *Streptococcus mutans*. *Microbial Pathogenesis*, *98*, 45–49.
- Guo, L., Hu, W., He, X., Lux, R., McLean, J., & Shi, W. (2013). Investigating Acid Production by *Streptococcus mutans* with a Surface-Displayed pH-Sensitive Green Fluorescent Protein. *PLoS one*, *8*(2), e57182.
- Guo, X., Liu, S., Zhou, X., Hu, H., Zhang, K., Du, X., Peng, X., Ren, B., Cheng, L., & Li, M. (2019). Effect of D-cysteine on dual-species biofilms of *Streptococcus mutans* and *Streptococcus sanguinis*. *Scientific Reports*, *9*(1), 6689.
- Hall, C. W., & Mah, T. F. (2017). Molecular mechanisms of biofilm-based antibiotic resistance and tolerance in pathogenic bacteria. *FEMS Microbiology Reviews*, *(3)*, 276–301.

- Hall-Stoodley, L., Costerton, J. W., & Stoodley, P. (2004). Bacterial biofilms: from the natural environment to infectious diseases. *Nature reviews microbiology*, 2(2), 95-108.
- Haney, E. F., Trimble, M. J., Cheng, J. T., Vallé, Q., & Hancock, R. E. W. (2018). Critical assessment of methods to quantify biofilm growth and evaluate antibiofilm activity of host defence peptides. *Biomolecules*, 8(2), 29.
- Hara, A. T., Ando, M., González-Cabezas, C., Cury, J. A., Serra, M. C., & Zero, D. T. (2006). Protective effect of the dental pellicle against erosive challenges in situ. *Journal of Dental Research*, 85(7), 612–616.
- Hasan, S., Singh, K., Danisuddin, M., Verma, P. K., & Khan, A. U. (2014). Inhibition of major virulence pathways of *Streptococcus mutans* by quercitrin and deoxynojirimycin: a synergistic approach of infection control. *PloS One*, 9(3), e91736.
- Hasikić, A., Divković, L., Džafić, A., Starčević, H., Ćurić, S., Ahmetović, E., Djedović, B., Haušić, S., Kolarević, L., Ibišević, M., Kozarević, E. C., & Smajlagić, A. (2025). Antioxidant Activity of Pomegranate Peel Extracts (*Punica granatum L.*): Effects of Extraction Solvent and Technique on Antioxidant Activity. *International Research Journal of Pure and Applied Chemistry*, 26(4), 153–159.
- Hasona, A., Zuobi-Hasona, K., Crowley, P. J., Abranches, J., Ruelf, M. A., Bleiweis, A. S., & Brady, L. J. (2007). Membrane composition changes and physiological adaptation by *Streptococcus mutans* signal recognition particle pathway mutants. *Journal of Bacteriology*, 189(4), 1219–1230.
- Hawas, S., Verderosa, A. D., & Totsika, M. (2022). Combination Therapies for Biofilm Inhibition and Eradication: A Comparative Review of Laboratory and Preclinical Studies. *Frontiers in Cellular and Infection Microbiology*, 12, 850030.
- He, Z., Huang, Z., Jiang, W., & Zhou, W. (2019). Antimicrobial Activity of Cinnamaldehyde on *Streptococcus mutans* Biofilms. *Frontiers in Microbiology*, 10, 2241.
- Heersema, L. A., & Smyth, H. D. C. (2019). A multispecies biofilm *in vitro* screening model of dental caries for high-throughput susceptibility testing. *High-Throughput*, 8(2), 14.

- Heller, D., Helmerhorst, E. J., Gower, A. C., Siqueira, W. L., Paster, B. J., & Oppenheim, F. G. (2016). Microbial diversity in the early in vivo-formed dental biofilm. *Applied and environmental microbiology*, 82(6), 1881-1888.
- Hernández, P., Sánchez, M. C., Llama-Palacios, A., Ciudad, M. J., & Collado, L. (2022). Strategies to Combat Caries by Maintaining the Integrity of Biofilm and Homeostasis during the Rapid Phase of Supragingival Plaque Formation. *Antibiotics*, 11(7), 880.
- Hindieh, P., Yaghi, J., Assaf, J. C., Chokr, A., Atoui, A., Tzenios, N., Louka, N., & Khoury, A. El. (2025). Emerging Multimodal Strategies for Bacterial Biofilm Eradication: A Comprehensive Review. *Microorganisms*, 13(12), 2796.
- Hoare, A., Marsh, P. D., & Diaz, P. I. (2017). Ecological Therapeutic Opportunities for Oral Diseases. *Microbiology Spectrum*, 5(4), 10-1128.
- Hsu, L. C., Fang, J., Borca-Tasciuc, D. A., Worobo, R. W., & Moraru, C. I. (2013). Effect of micro- and nanoscale topography on the adhesion of bacterial cells to solid surfaces. *Applied and Environmental Microbiology*, 79(8), 2703–2712.
- Hu, X., Wang, M., Shen, Y., Zhang, L., Pan, Y., Sun, Y., & Zhang, K. (2022). Regulatory Effect of Irresistin-16 on Competitive Dual-Species Biofilms Composed of *Streptococcus mutans* and *Streptococcus sanguinis*. *Pathogens*, 11(1), 70.
- Huang, Q., An, Z., Xin, X., Gou, X., Tian, X., Hu, Y., Mei, Z., & Feng, Q. (2024). The Effectiveness of Curcumin, Resveratrol, and Silymarin on MASLD: A Systematic Review and Meta-Analysis. *Food Science and Nutrition*, 12(12), 10010–10029.
- Huang, R., Li, M., Ye, M., Yang, K., Xu, X., & Gregory, R. L. (2014). Effects of nicotine on *Streptococcus gordonii* growth, biofilm formation, and cell aggregation. *Applied and Environmental Microbiology*, 80(23), 7212–7218.
- Huang, X., Browngardt, C. M., Jiang, M., Ahn, S. J., Burne, R. A., & Nascimento, M. M. (2018). Diversity in Antagonistic Interactions between Commensal Oral Streptococci and *Streptococcus mutans*. *Caries Research*, 52(1–2), 88–101.
- Ikäläinen, H., Guzman, C., Saari, M., Söderling, E., & Loimaranta, V. (2024). Real-time acid production and extracellular matrix formation in mature biofilms of three *Streptococcus mutans* strains with special reference to xylitol. *Biofilm*, 8, 100219.

- Ivanov, M., Novović, K., Malešević, M., Dinić, M., Stojković, D., Jovčić, B., & Soković, M. (2022). Polyphenols as Inhibitors of Antibiotic Resistant Bacteria—Mechanisms Underlying Rutin Interference with Bacterial Virulence. *Pharmaceuticals*, 15(3), 385.
- Jackson, S. L., Vann, W. F., Kotch, J. B., Pahel, B. T., & Lee, J. Y. (2011). Impact of poor oral health on children's school attendance and performance. *American Journal of Public Health*, 101(10), 1900–1906.
- Jacob, B., Malli Sureshababu, N., Ranjan, M., Ranganath, A., & Siddique, R. (2021). The Antimicrobial Effect of Pomegranate Peel Extract versus Chlorhexidine in High Caries Risk Individuals Using Quantitative Real-Time Polymerase Chain Reaction: A Randomized Triple-Blind Controlled Clinical Trial. *International Journal of Dentistry*, 2021(1), 5563945.
- Jahromi, S. B., Pourshafie, M. R., Mirabzadeh, E., Tavasoli, A., Katiraei, F., Mostafavi, E., & Abbasian, S. (2015). Punica granatum peel extract toxicity in mice. *Jundishapur Journal of Natural Pharmaceutical Products*, 10(4), e23770.
- Jakubovics, N. S., & Kolenbrander, P. E. (2010). The road to ruin: The formation of disease-associated oral biofilms. *Oral Diseases*, 16(8), 729–739.
- James, P., Worthington, H. V., Parnell, C., Harding, M., Lamont, T., Cheung, A., Whelton, H., & Riley, P. (2017). Chlorhexidine mouthrinse as an adjunctive treatment for gingival health. *Cochrane Database of Systematic Reviews*, 2017(3).
- Jesionowski, A. M., Mansfield, J. M., Brittan, J. L., Jenkinson, H. F., & Vickerman, M. M. (2016). Transcriptome analysis of *Streptococcus gordonii* Challis DL1 indicates a role for the biofilm-associated fruRBA operon in response to *Candida albicans*. *Molecular Oral Microbiology*, 31(4), 314–328.
- Jevdjovic, M., & Listl, S. (2025). Global, regional, and country-level economic impacts of oral conditions in 2019. *Journal of Dental Research*, 104(1), 17-21.
- Jiao, Y., Tay, F. R., Niu, L. na, & Chen, J. hua. (2019). Advancing antimicrobial strategies for managing oral biofilm infections. *International Journal of Oral Science*, 11(3), 28.
- Jing, D., Hao, J., Shen, Y., Tang, G., Lei, L., & Zhao, Z. (2019). Effect of fixed orthodontic treatment on oral microbiota and salivary proteins. *Experimental and Therapeutic Medicine*, 17(5), 4237-4243.

- Jung, E. H., Hwang, G., & Kim, K. R. (2025). Effect of 6-Shogaol Derived from Ginger (*Zingiber officinale*) on Dual-Species Biofilm Formation by *Streptococcus mutans* and *Candida albicans*. *Nutrients*, 17(18), 2999.
- Juszczuk-Kubiak, E. (2024). Molecular Aspects of the Functioning of Pathogenic Bacteria Biofilm Based on Quorum Sensing (QS) Signal-Response System and Innovative Non-Antibiotic Strategies for Their Elimination. *International Journal of Molecular Sciences*, 25(5), 2655.
- Karnjana, K., Jewboonchu, J., Niyomtham, N., Tangngamsakul, P., Bunluepuech, K., Goodla, L., & Mordmuang, A. (2023). The potency of herbal extracts and its green synthesized nanoparticle formulation as antibacterial agents against *Streptococcus mutans* associated biofilms. *Biotechnology Reports*, 37, e00777.
- Karonen, M., Oraviita, M., Mueller-Harvey, I., Salminen, J. P., & Green, R. J. (2019). Ellagitannins with glucopyranose cores have higher affinities to proteins than acyclic ellagitannins by isothermal titration calorimetry. *Journal of Agricultural and Food Chemistry*, 67(46), 12730-12740.
- Karygianni, L., Ren, Z., Koo, H., & Thurnheer, T. (2020). Biofilm Matrixome: Extracellular Components in Structured Microbial Communities. *Trends in Microbiology*, 28(8), 668–681.
- Kashi, M., Varseh, M., Hariri, Y., Chegini, Z., & Shariati, A. (2025). Natural compounds: new therapeutic approach for inhibition of *Streptococcus mutans* and dental caries. *Frontiers in Pharmacology*, 16, 1548117.
- Kassebaum, N. J., Bernabé, E., Dahiya, M., Bhandari, B., Murray, C. J. L., & Marcenes, W. (2015). Global burden of untreated caries: A systematic review and metaregression. *Journal of Dental Research*, 94(5), 650–658.
- Ketagoda, D. H. K., Varga, P., Fitzsimmons, T. R., Moore, N. E., Weyrich, L. S., & Zilm, P. S. (2024). Development of an *in vitro* biofilm model of the human supra-gingival microbiome for Oral microbiome transplantation. *Journal of Microbiological Methods*, 223, 106961.
- Khan, M. A., Celik, I., Khan, H. M., Shahid, M., Shahzad, A., Kumar, S., & Ahmed, B. (2023). Antibiofilm and anti-quorum sensing activity of *Psidium guajava* L. leaf extract: *In vitro* and in silico approach. *PLOS One*, 18(12), e0295524.
- Kim, B. S., Park, S. J., Kim, M. K., Kim, Y. H., Lee, S. B., Lee, K. H., Choi, N. Y., Lee, Y. R., Lee, Y. E., & You, Y. O. (2015). Inhibitory effects of chrysanthemum boreale essential oil on biofilm formation and virulence factor

- expression of *Streptococcus mutans*. *Evidence-Based Complementary and Alternative Medicine*, 2015(1), 616309.
- Kim, D., Hwang, G., Liu, Y., Wang, Y., Singh, A. P., Vorsa, N., & Koo, H. (2015). Cranberry flavonoids modulate cariogenic properties of mixed-species biofilm through exopolysaccharides-matrix disruption. *PLoS ONE*, 10(12), e0145844.
- Kim, Y. H., & Young Lee, S. (2015). Identification of non-streptococcal organisms from human dental plaque grown on the Streptococcus-selective medium mitis-salivarius agar. *Archives of Oral Biology*, 60(2), 267–271.
- Klein, M. I., Hwang, G., Santos, P. H. S., Campanella, O. H., & Koo, H. (2015). *Streptococcus mutans*-derived extracellular matrix in cariogenic oral biofilms. *Frontiers in Cellular and Infection Microbiology* 5, 10.
- Kolenbrander, P. E. (2000). Oral microbial communities: Biofilms, Interactions, and Genetic Systems 1. *Annual Review in Microbiology*, 54 (1), 413-437.
- Koo, H., Allan, R. N., Howlin, R. P., Stoodley, P., & Hall-Stoodley, L. (2017). Targeting microbial biofilms: Current and prospective therapeutic strategies. *Nature Reviews Microbiology*, 15(12), 740–755.
- Koo, H., Xiao, J., Klein, M. I., & Jeon, J. G. (2010). Exopolysaccharides produced by *Streptococcus mutans* glucosyltransferases modulate the establishment of microcolonies within multispecies biofilms. *Journal of Bacteriology*, 192(12), 3024–3032.
- Kragh, K. N., Alhede, M., Kvich, L., & Bjarnsholt, T. (2019). Into the well—A close look at the complex structures of a microtiter biofilm and the crystal violet assay. *Biofilm*, 1, 100006.
- Krasowska, A., & Sigler, K. (2014). How microorganisms use hydrophobicity and what does this mean for human needs? *Frontiers in Cellular and Infection Microbiology*, 4, 112.
- Kreth, J., Zhang, Y., & Herzberg, M. C. (2008). Streptococcal antagonism in oral biofilms: *Streptococcus sanguinis* and *Streptococcus gordonii* interference with *Streptococcus mutans*. *Journal of Bacteriology*, 190(13), 4632–4640.
- Kreve, S., & Reis, A. C. D. (2021). Bacterial adhesion to biomaterials: What regulates this attachment? A review. *Japanese Dental Science Review*, 57, 85–96.
- Kuboniwa, M., Tribble, G. D., James, C. E., Kilic, A. O., Tao, L., Herzberg, M. C., Shizukuishi, S., & Lamont, R. J. (2006). *Streptococcus gordonii* utilizes several

- distinct gene functions to recruit *Porphyromonas gingivalis* into a mixed community. *Molecular Microbiology*, 60(1), 121–139.
- Kumar, K. H., Prabakar, J., & Shanmugam, R. (2025). Evaluating the Effectiveness of Punica granatum as a Natural Dental Plaque Disclosing Agent Against *Streptococcus mutans*, *Lactobacillus* and *Enterococcus faecalis*: An In-vitro Study. *Journal of Pioneering Medical Sciences*, 14.
- Kadam, N. S., Kunte, S. S., Patel, A. R., Shah, P. P., Lodaya, R. R., & Lakade, L. S. (2019). Comparative Evaluation of the Effect of Pomegranate Peel Extract and Chlorhexidine 0.2% Mouthwash on Salivary pH in Children between 6 and 8 Years of Age: An In vivo Study. *Journal of International Oral Health*, 11(1), 40-44.
- Lamont, R. J., Koo, H., & Hajishengallis, G. (2018). The oral microbiota: dynamic communities and host interactions. *Nature Reviews Microbiology*, 16(12), 745–759.
- Latka, A., & Drulis-Kawa, Z. (2020). Advantages and limitations of microtiter biofilm assays in the model of antibiofilm activity of Klebsiella phage KP34 and its depolymerase. *Scientific Reports*, 10(1), 20338.
- Lemos, J. A., Abranches, J., Koo, H., Marquis, R. E., & Burne, R. A. (2010). Protocols to study the physiology of oral biofilms. In *Oral Biology: Molecular Techniques and Applications* (pp. 87-102). Totowa, NJ: Humana Press.
- Lemos, J. A., Palmer, S. R., Zeng, L., Wen, Z. T., Kajfasz, J. K., Freires, I. A., Abranches, J., & Brady, L. J. (2019). The Biology of *Streptococcus mutans*. *Microbiology Spectrum*, 7(1), 10-1128
- Li, B., Li, X., Lin, H., & Zhou, Y. (2018). Curcumin as a Promising Antibacterial Agent: Effects on Metabolism and Biofilm Formation in *S. mutans*. *BioMed Research International*, 2018(1), 4508709.
- Li, B., Pan, T., Lin, H., & Zhou, Y. (2020). The enhancing antibiofilm activity of curcumin on *Streptococcus mutans* strains from severe early childhood caries. *BMC Microbiology*, 20(1), 286.
- Li, J., Helmerhorst, E. J., Leone, C. W., Troxler, R. F., Yaskell, T., Haffajee, A. D., Socransky, S. S., & Oppenheim, F. G. (2004). Identification of early microbial colonizers in human dental biofilm. *Journal of Applied Microbiology*, 97(6), 1311–1318.

- Li, J., He, X., Li, M., Zhao, W., Liu, L., & Kong, X. (2015). Chemical fingerprint and quantitative analysis for quality control of polyphenols extracted from pomegranate peel by HPLC. *Food Chemistry*, 176, 7–11.
- Li, J., Wu, T., Peng, W., & Zhu, Y. (2020). Effects of resveratrol on cariogenic virulence properties of *Streptococcus mutans*. *BMC microbiology*, 20(1), 99.
- Li, J., Ma, Q., Huang, J., Liu, Y., Zhou, J., Yu, S., Zhang, Q., Lin, Y., Wang, L., Zou, J., & Li, Y. (2024). Small RNA SmsR1 modulates acidogenicity and cariogenic virulence by affecting protein acetylation in *Streptococcus mutans*. *PLoS Pathogens*, 20(4), e1012147.
- Li, J., Jia, T., & Yang, L. (2025). Targeting antivirulence factor strategies of bacterial pathogens. *Biosafety and Health*, 7(1), 1–4.
- Li, P., Yin, R., Cheng, J., & Lin, J. (2023). Bacterial Biofilm Formation on Biomaterials and Approaches to Its Treatment and Prevention. *International Journal of Molecular Sciences*, 24(4), 11680.
- Li, P., Zhang, Y., Chen, D., & Lin, H. (2024). Investigation of a novel biofilm model close to the original oral microbiome. *Applied Microbiology and Biotechnology*, 108(1), 330.
- Li, X., Liu, Y., Yang, X., Li, C., & Song, Z. (2022). The Oral Microbiota: Community Composition, Influencing Factors, Pathogenesis, and Interventions. *Frontiers in Microbiology*, 13, 895537.
- Liu, Y., Han, L., Yang, H., Liu, S., & Huang, C. (2020). Effect of apigenin on surface-associated characteristics and adherence of *Streptococcus mutans*. *Dental materials journal*, 39(6), 933-940.
- Li, Y., Huang, S., Du, J., Huang, J., & Huang, X. (2025). *In vitro* evaluation of tolerance ability of cross-kingdom biofilm towards oral dynamic fluctuations. *BMC Oral Health*, 25(1), 1216.
- Li, Z., Yu, C., & Chen, H. (2025). Global, regional, and national caries of permanent teeth incidence, prevalence, and disability-adjusted life years, 1990-2021: analysis for the global burden of disease study. *BMC Oral Health*, 25(1), 715.
- Lin, Y., Zhou, X., & Li, Y. (2022). Strategies for *Streptococcus mutans* biofilm dispersal through extracellular polymeric substances disruption. *Molecular Oral Microbiology*, 37(1), 1–8.
- Listl, S., Galloway, J., Mossey, P. A., & Marcenes, W. (2015). Global economic impact of dental diseases. *Journal of Dental Research*, 94(10), 1355–1361.

- Liu, J., Wu, C., Huang, I. H., Merritt, J., & Qi, F. (2011). Differential response of *Streptococcus mutans* towards friend and foe in mixed-species cultures. *Microbiology*, 157(9), 2433–2444.
- Liu, Q., Zhou, L., Peng, S., Li, Q. L., & Wong, H. M. (2025). Antimicrobial Peptide P-113-DPS Suppresses the Cariogenic Virulence of *Streptococcus mutans*. *ACS Applied Bio Materials*, 8(6), 4973–4980.
- Liu, S., Sun, Y., Liu, Y., Hu, F., Xu, L., Zheng, Q., Wang, Q., Zeng, G., & Zhang, K. (2022). Genomic and phenotypic characterization of *Streptococcus mutans* isolates suggests key gene clusters in regulating its interaction with *Streptococcus gordonii*. *Frontiers in Microbiology*, 13, 945108.
- Liu, S., Zhang, T., Li, Z., Wang, Y., Liu, L., & Song, Z. (2023). Antibacterial mechanism of areca nut essential oils against *Streptococcus mutans* by targeting the biofilm and the cell membrane. *Frontiers in Cellular and Infection Microbiology*, 13, 1140689.
- Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods*, 25(4), 402–408.
- Lobo-Cabrera, F. J., Herrero, M. D. R., Govantes, F., & Cuetos, A. (2024). Computer simulation study of nutrient-driven bacterial biofilm stratification. *Journal of the Royal Society Interface*, 21(215).
- Lu, L., Zhao, Y., Li, M., Wang, X., Zhu, J., Liao, L., & Wang, J. (2024). Contemporary strategies and approaches for characterizing composition and enhancing biofilm penetration targeting bacterial extracellular polymeric substances. *Journal of Pharmaceutical Analysis*, 14(4), 100906.
- Lubarsky, G. V., D'Sa, R. A., Deb, S., Meenan, B. J., & Lemoine, P. (2012). The role of enamel proteins in protecting mature human enamel against acidic environments: A double layer force spectroscopy study. *Biointerphases*, 7(1–4), 1–8.
- Lugo-Flores, M. A., Quintero-Cabello, K. P., Palafox-Rivera, P., Silva-Espinoza, B. A., Cruz-Valenzuela, M. R., Ortega-Ramirez, L. A., Gonzalez-Aguilar, G. A., & Ayala-Zavala, J. F. (2021). Plant-derived substances with antibacterial, antioxidant, and flavoring potential to formulate oral health care products. *Biomedicines*, 9(11), 1669.

- Luo, A., Wang, F., Sun, D., Liu, X., & Xin, B. (2022). Formation, Development, and Cross-Species Interactions in Biofilms. *Frontiers in Microbiology*, *12*, 757327.
- LV, C., Wang, Z., Li, Z., Shi, X., Xiao, M., & Xu, Y. (2025). Formation, architecture, and persistence of oral biofilms: recent scientific discoveries and new strategies for their regulation. *Frontiers in Microbiology*, *16*, 1602962.
- Lynge Pedersen, A. M., & Belstrøm, D. (2019). The role of natural salivary defences in maintaining a healthy oral microbiota. *Journal of Dentistry*, *80*, S3–S12.
- Magangana, T. P., Makunga, N. P., Fawole, O. A., & Opara, U. L. (2020). Processing factors affecting the phytochemical and nutritional properties of pomegranate (*Punica granatum L.*) peel waste: A review. *Molecules*, *25*(20), 4690.
- Magoro, K., Mnisi, N. M. L., Musekene, E., & Manganyi, M. C. (2025). Biofilm battles: confronting stubborn resistance in dental healthcare by harnessing the antimicrobial potential of South African medicinal plants. *Clinical Phytoscience*, *11*(1), 25.
- Mahd, M. A., Aref, P., Emadi, F., Javadi, F., Javad, M., Fard, K., & Tavassoli-Hojjati, S. (2023). Effect of hydroalcoholic extract of whole pomegranate fruit on cariogenic bacteria and its clinical effect on dental plaque formation in 8-10-year-old children. *Dental Research Journal*, *20*(1), 116.
- Man, G., Xu, L., Wang, Y., Liao, X., & Xu, Z. (2022). Profiling Phenolic Composition in Pomegranate Peel From Nine Selected Cultivars Using UHPLC-QTOF-MS and UPLC-QQQ-MS. *Frontiers in Nutrition*, *8*, 807447.
- Marsh, P. D. (2003). Are dental diseases examples of ecological catastrophes? *Microbiology*, *149*(2), 279–294.
- Marsh, P. D. (2006). Dental plaque as a biofilm and a microbial community—implications for health and disease. *BMC Oral health*, *6*(Suppl 1), S14.
- Marsh, P. D. (2017). Ecological Events in Oral Health and Disease: New Opportunities for Prevention and Disease Control? *Journal of the California Dental Association*, *45*(10), 525–537.
- Marsh, P. D., & Zaura, E. (2017). Dental biofilm: ecological interactions in health and disease. *Journal of Clinical Periodontology*, *44*, S12–S22.
- Marsh, K. J., Wallis, I. R., Kulheim, C., Clark, R., Nicolle, D., Foley, W. J., & Salminen, J. P. (2020). New approaches to tannin analysis of leaves can be used to explain in vitro biological activities associated with herbivore defence. *New Phytologist*, *225*(1), 488–498.

- Martínez, O. F., Cardoso, M. H., Ribeiro, S. M., & Franco, O. L. (2019). Recent advances in antivirulence therapeutic strategies with a focus on dismantling bacterial membrane microdomains, toxin neutralization, quorum-sensing interference and biofilm inhibition. *Frontiers in Cellular and Infection Microbiology*, 9(74).
- Mashal, S., Siddiqua, A., Ullah, N., Baloch, R., Khan, M., Hasnain, S. Z. U., Imran Aziz, M., Huseynov, E., Selakovic, D., Rosic, G., Makhkamov, T., Yuldashev, A., Islamov, S., Abdullayeva, N., Khujanazarov, U., & Amin, A. (2024). Bioactive plant waste components targeting oral bacterial pathogens as a promising strategy for biofilm eradication. *Frontiers in Chemistry*, 12, 1406869.
- Matsui, R., & Cvitkovitch, D. (2010). Acid tolerance mechanisms utilized by *Streptococcus mutans*. *Future Microbiology*, 5(3), 403–417.
- Mauliydia, N. B., Khairan, K., Idroes, R., & Tallei, T. E. (2025, April). Cytotoxicity assessment of medicinal plants from three geothermal areas using the brine shrimp lethality assay. In *IOP Conference Series: Earth and Environmental Science* (Vol. 1476, No. 1, p. 012018). IOP Publishing.
- McDonnell, G., & Russell, A. D. (1999). Antiseptics and disinfectants: activity, action, and resistance. *Clinical microbiology reviews*, 12(1), 147-179.
- Miclăuș, Z. E., Moca, R. T., Matei, R. I., Moca, A. E., Țenț, A., & Porumb, A. (2025). Phytotherapy in Pediatric Dentistry: A Narrative Review of Clinical Applications and Evidence. *Children*, 12(11), 1559.
- Milho, C., Silva, J., Guimarães, R., Ferreira, I. C. F. R., Barros, L., & Alves, M. J. (2021). Antimicrobials from medicinal plants: An emergent strategy to control oral biofilms. *Applied Sciences*, 11(9), 4020.
- More, P. R., & Arya, S. S. (2025). Extraction of bioactives from pomegranate peels using aqueous biphasic separation (ABPS): An optimization and bioactive profiling. *Biomass Conversion and Biorefinery*, 15(21), 28223–28237.
- Mphahlele, R. R., Fawole, O. A., Mokwena, L. M., & Opara, U. L. (2016). Effect of extraction method on chemical, volatile composition and antioxidant properties of pomegranate juice. *South African Journal of Botany*, 103, 135–144.
- Mulat, M., Banicod, R. J. S., Tabassum, N., Javaid, A., Karthikeyan, A., Jeong, G. J., Kim, Y. M., Jung, W. K., & Khan, F. (2025). Multiple Strategies for the Application of Medicinal Plant-Derived Bioactive Compounds in Controlling Microbial Biofilm and Virulence Properties. *Antibiotics*, 14(6), 555.

- Munita, J. M., & Arias, C. A. (2016). Mechanisms of Antibiotic Resistance. *Microbiology Spectrum*, 4(2), 481-511.
- Muras, A., Mayer, C., Romero, M., Camino, T., Ferrer, M. D., Mira, A., & Otero, A. (2018). Inhibition of *Streptococcus mutans* biofilm formation by extracts of *Tenacibaculum* sp. 20J, a bacterium with wide-spectrum quorum quenching activity. *Journal of Oral Microbiology*, 10(1), 1429788.
- Mutahar, M., Carpenter, G., Bartlett, D., German, M., & Moazzez, R. (2017). The presence of acquired enamel pellicle changes acid-induced erosion from dissolution to a softening process. *Scientific Reports*, 7(1), 10920.
- Mutha, N. V. R., Mohammed, W. K., Krasnogor, N., Tan, G. Y. A., Choo, S. W., & Jakubovics, N. S. (2018). Transcriptional responses of *Streptococcus gordonii* and *Fusobacterium nucleatum* to coaggregation. *Molecular Oral Microbiology*, 33(6), 450–464.
- Nagasawa, R., Yamamoto, T., Utada, A. S., Nomura, N., & Obana, N. (2020). Competence-Stimulating-Peptide-Dependent Localized Cell Death and Extracellular DNA Production in *Streptococcus mutans* Biofilms. *Applied and Environmental Microbiology*, 86(23), e02080-20.
- Nagasawa, R., Nomura, N., & Obana, N. (2023). Identification of a novel gene involved in cell-to-cell communication-induced cell death and eDNA production in *Streptococcus mutans*. *Microbes and environments*, 38(2), ME22085.
- Nagi, M., Chapple, I. L. C., Sharma, P., Kuehne, S. A., & Hirschfeld, J. (2023). Quorum Sensing in Oral Biofilms: Influence on Host Cells. *Microorganisms*, 11(7), 1688.
- Nasim, N., Sandeep, I. S., & Mohanty, S. (2022). Plant-derived natural products for drug discovery: current approaches and prospects. *Nucleus*, 65(3), 399–411.
- Neal, T. W., & Schlieve, T. (2022). Complications of severe odontogenic infections: a review. *Biology*, 11(12), 1784.
- Nóbrega, R. de M., Santos, R. L., Coelho Soares, R. de S., Muniz Alves, P., Medeiros, A. C. D., & Pereira, J. V. (2015). A randomized, controlled clinical trial on the clinical and microbiological efficacy of *Punica granatum* Linn mouthwash. *Pesquisa Brasileira Em Odontopediatria e Clinica Integrada*, 15(1), 301–308.
- Nyvad, B., & Takahashi, N. (2020). Integrated hypothesis of dental caries and periodontal diseases. *Journal of oral microbiology*, 12(1), 1710953.

- Olchowik-Grabarek, E., Sękowski, S., Kwiatek, A., Płaczkiewicz, J., Abdulladjanova, N., Shlyonsky, V., Swiecicka, I., & Zamaraeva, M. (2022). The Structural Changes in the Membranes of *Staphylococcus aureus* Caused by Hydrolysable Tannins Witness Their Antibacterial Activity. *Membranes*, 12(11), 1124.
- Pandey, S. D., Perpich, J. D., Stocke, K. S., Mansfield, J. M., Kikuchi, Y., Yakoumatos, L., Muszyński, A., Azadi, P., Tettelin, H., Whiteley, M., Uriarte, S. M., Bagaitkar, J., Vickerman, M., & Lamont, R. J. (2023). Impact of Polymicrobial Infection on Fitness of *Streptococcus gordonii* *In Vivo*. *MBio*, 14(3), e00658-23.
- Pandit, S., Fazilati, M., Gaska, K., Derouiche, A., Nypelö, T., Mijakovic, I., & Kádár, R. (2020). The exo-polysaccharide component of extracellular matrix is essential for the viscoelastic properties of *Bacillus subtilis* biofilms. *International Journal of Molecular Sciences*, 21(18), 1–17.
- Pandit, S., Kim, M. A., Jung, J. E., Choi, H. M., & Jeon, J. G. (2024). Usnic acid brief exposure suppresses cariogenic properties and complexity of *Streptococcus mutans* biofilms. *Biofilm*, 8, 100241.
- Patel, K., Panchal, R., Sakariya, B., Gevariya, M., Raiyani, R., Soni, R., & Goswami, D. (2025). Combatting antibiotic resistance by exploring the promise of Quorum Quenching in targeting bacterial virulence. *The Microbe*, 6, 100224.
- Peeters, E., Nelis, H. J., & Coenye, T. (2008). Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. *Journal of microbiological methods*, 72(2), 157-165.
- Pela, V. T., Ventura, T. M. O., & Buzalaf, M. A. R. (2020). Optimizing the formation of the acquired enamel pellicle in vitro for proteomic analysis. *Journal of Applied Oral Science*, 28, e20200189.
- Peres, M. A., D Macpherson, L. M., Weyant, R. J., Daly, B., Venturelli, R., Mathur, M. R., Listl, S., Keller Celeste, R., Guarnizo-Herreño, C. C., Kearns, C., Benzian, H., Allison, P., & Watt, R. G. (2019). Oral diseases: a global public health challenge. *The Lancet*, 394(10194), 249-260.
- Peterson, B. W., He, Y., Ren, Y., Zerdoum, A., Libera, M. R., Sharma, P. K., van Winkelhoff, A. J., Neut, D., Stoodley, P., van der Mei, H. C., & Busscher, H. J. (2015). Viscoelasticity of biofilms and their recalcitrance to mechanical and chemical challenges. *FEMS Microbiology Reviews*, 39(2), 234–245.

- Philip, N., Bandara, H. M. H. N., Leishman, S. J., & Walsh, L. J. (2019). Inhibitory effects of fruit berry extracts on *Streptococcus mutans* biofilms. *European Journal of Oral Sciences*, 127(2), 122–129.
- Pinni, J., Sankar Avula, J. S., Mukthineni, S., Bandi, S., & Gokul, T. (2018). Antimicrobial activity of pomegranate (*Punica granatum*) pericarp extract against *Streptococcus mutans*- A source for natural mouth rinse: An in-vitro and in-vivo study. *Biomedical and Pharmacology Journal*, 11(4), 2025–2030.
- Pisithkul, T., Schroeder, J. W., Trujillo, E. A., Yeesin, P., Stevenson, D. M., Chaiamarit, T., Coon, J. J., Wang, J. D., & Amador-Noguez, D. (2019). Metabolic remodeling during biofilm development of bacillus subtilis. *MBio*, 10(3), 10-1128.
- Polizzi, A., Donzella, M., Nicolosi, G., Santonocito, S., Pesce, P., & Isola, G. (2022). Drugs for the Quorum Sensing Inhibition of Oral Biofilm: New Frontiers and Insights in the Treatment of Periodontitis. *Pharmaceutics*, 14(12), 2740.
- Poppolo Deus, F., & Ouanounou, A. (2022). Chlorhexidine in Dentistry: Pharmacology, Uses, and Adverse Effects. *International Dental Journal*, 72(3), 269–277.
- Potra Cicalău, G. I., Vicaș, L. G., Ciavoi, G., Ghitea, T. C., Csaba, N., Cristea, R. A., Miere, F., & Ganea, M. (2024). A Natural Approach to the Prevention and Treatment of Gingivitis and Periodontitis: A Review of Pomegranate's Bioactive Properties. *Life*, 14 (10), 1298.
- Quispe Haro, J. J., Chen, F., Los, R., Shi, S., Sun, W., Chen, Y., Idema, T., & Wegner, S. V. (2024). Optogenetic Control of Bacterial Cell-Cell Adhesion Dynamics: Unraveling the Influence on Biofilm Architecture and Functionality. *Advanced Science*, 11(23), 2310079.
- Rafeie, N., Salimi, Y., Aghamir, Z. S., Amini, A., Taheri, H., Sadreddini, S., Kamali, F., Akhbarian, G., Azizi, N., Bagherianlemraski, M., Valizadeh, M., Alimohammadi, F., Sedighnia, N., Qadirifard, M., & Naziri, M. (2025). Effects of pomegranate extract on preventing dental caries: a systematic review. *Frontiers in Oral Health*, 6, 1484364.
- Rahmani-Badi, A., Sepehr, S., & Babaie-Naiej, H. (2015). A combination of cis-2-decenoic acid and chlorhexidine removes dental plaque. *Archives of Oral Biology*, 60(11), 1655–1661.
- Rasko, D. A., & Sperandio, V. (2010). Anti-virulence strategies to combat bacteria-mediated disease. *Nature reviews Drug discovery*, 9(2), 117-128.

- Rath, S., Bal, S. C. B., & Dubey, D. (2021). Oral Biofilm: Development Mechanism, Multidrug Resistance, and Their Effective Management with Novel Techniques. *Rambam Maimonides Medical Journal*, 12(1), e0004.
- Redanz, S., Cheng, X., Giacaman, R. A., Pfeifer, C. S., Merritt, J., & Kreth, J. (2018). Live and let die: Hydrogen peroxide production by the commensal flora and its role in maintaining a symbiotic microbiome. *Molecular Oral Microbiology*, 33(5), 337–352.
- Ren, Z., Kim, D., Paula, A. J., Hwang, G., Liu, Y., Li, J., Daniell, H., & Koo, H. (2019). Dual-Targeting Approach Degrades Biofilm Matrix and Enhances Bacterial Killing. *Journal of Dental Research*, 98(3), 322–330.
- Ricker, A., Vickerman, M., & Dongari-Bagtzoglou, A. (2014). *Streptococcus gordonii* glucosyltransferase promotes biofilm interactions with *Candida albicans*. *Journal of Oral Microbiology*, 6(1), 23419.
- Røder, H. L., Olsen, N. M. C., Whiteley, M., & Burmølle, M. (2020). Unravelling interspecies interactions across heterogeneities in complex biofilm communities. *Environmental Microbiology*, 22(1), 5–16.
- Rongai, D., Pulcini, P., Di Lernia, G., Nota, P., Preka, P., & Milano, F. (2019). Punicalagin content and antifungal activity of different pomegranate (*Punica granatum L.*) genotypes. *Horticulturae*, 5(3), 52.
- Rose, M., Wilson, N., Williams, E., Letner, H., Bettinger, R., Bouchendouka, A., Batagower, J., & Kaspar, J. R. (2023). Growth with Commensal Streptococci Alters *Streptococcus mutans* Behaviors. *Journal of Dental Research*, 102(4), 450–458.
- Rosenberg, M. (1984). Bacterial adherence to hydrocarbons: a useful technique for studying cell surface hydrophobicity. *FEMS Microbiology Letters*, 22(3), 289–295.
- Rostami, N., Shields, R. C., Serrage, H. J., Lawler, C., Brittan, J. L., Yassin, S., Ahmed, H., Treumann, A., Thompson, P., Waldron, K. J., Nobbs, A. H., & Jakubovics, N. S. (2022). Interspecies competition in oral biofilms mediated by *Streptococcus gordonii* extracellular deoxyribonuclease *SsnA*. *NPJ Biofilms and Microbiomes*, 8(1), 96.
- Rudin, L., Kneubühler, J., Dubey, B. N., Ahmad, S., Bornstein, M. M., & Shyp, V. (2025). Inhibitory effect of plant flavonoid cyanidin on oral microbial biofilm. *Microbiology Spectrum*, 13(6), e02848-24.

- Ruhl, S, Sandberg, A. L., & Cisar, J. O. (2004). Salivary receptors for the proline-rich protein-binding and lectin-like adhesins of oral actinomyces and streptococci. *Journal of dental research*, 83(6), 505-510.
- Salim, A., Deiana, P., Fancello, F., Molinu, M. G., Santona, M., & Zara, S. (2023). Antimicrobial and antibiofilm activities of pomegranate peel phenolic compounds: Varietal screening through a multivariate approach. *Journal of Bioresources and Bioproducts*, 8(2), 146–161.
- Salim, A., Marquez, L., Jakkala, K., Woo, S., Caputo, M., Fancello, F., Deiana, P., Santona, M., Molinu, M. G., Quave, C. L., & Zara, S. (2025). Identification of Punicalagin as a Key Bioactive Compound Responsible for the Antimicrobial Properties of *Punica granatum* L. Peel Extract against *Staphylococcus aureus*. *Journal of Agricultural and Food Chemistry*, 73(29), 18280–18290.
- Salli, K. M., & Ouwehand, A. C. (2015). The use of *in vitro* model systems to study dental biofilms associated with caries: A short review. *Journal of Oral Microbiology*, 7(1), 1–7.
- Sangavi, R., Malligarjunan, N., Satish, L., Raja, V., Pandian, S. K., & Gowrishankar, S. (2024). Anticariogenic activity of marine brown algae *Padina boergeresii* and its active components towards *Streptococcus mutans*. *Frontiers in Cellular and Infection Microbiology*, 14, 1458825.
- Sarah, Q. S., Anny, F. C., & Misbahuddin, M. (2017). Brine shrimp lethality assay. *Bangladesh Journal of Pharmacology*, 12(2), 186–189.
- Sardana, D., Schwendicke, F., Kosan, E., & Tüfekçi, E. (2023). White spot lesions in orthodontics: consensus statements for prevention and management. *Angle Orthodontist*, 93(6), 621–628.
- Sardin, S., M. J. J., B. G., & B. O. (2004). *In vitro* streptococcal adherence on prosthetic and implant materials. Interactions with physicochemical surface properties. *Journal of Oral Rehabilitation*, 31(2), 140-148.
- Sateriale, D., Imperatore, R., Colicchio, R., Pagliuca, C., Varricchio, E., Volpe, M. G., Salvatore, P., Paolucci, M., & Pagliarulo, C. (2020). Phytocompounds vs. Dental Plaque Bacteria: *In vitro* Effects of Myrtle and Pomegranate Polyphenolic Extracts Against Single-Species and Multispecies Oral Biofilms. *Frontiers in Microbiology*, 11, 592265.

- Serra, D. O., & Hengge, R. (2014). Stress responses go three dimensional - The spatial order of physiological differentiation in bacterial macrocolony biofilms. *Environmental Microbiology*, 16(6), 1455–1471.
- Shafiei, Z., Rahim, Z. H. A., Philip, K., Thurairajah, N., & Yaacob, H. (2020). Potential effects of Psidium sp., Mangifera sp., Mentha sp. and its mixture (PEM) in reducing bacterial populations in biofilms, adherence and acid production of *S. sanguinis* and *S. mutans*. *Archives of Oral Biology*, 109, 104554.
- Shemesh, M., Tam, A., & Steinberg, D. (2007). Differential gene expression profiling of *Streptococcus mutans* cultured under biofilm and planktonic conditions. *Microbiology*, 153(5), 1307–1317.
- Shi, Q., Li, F., Peng, Y., Sun, Q., Zhao, Hong, Lu, F., & Zhao, Huabing. (2025). Asiatic Acid Disrupts the Biofilm Virulence of *Streptococcus mutans* by Transcriptional Reprogramming of Quorum Sensing System. *International Journal of Molecular Sciences*, 26(19), 9510.
- Silla, A., Punzo, A., Bonvicini, F., Perillo, M., Malaguti, M., Lorenzini, A., Foltran, I., Mercatante, D., Mandrioli, M., Rodriguez-Estrada, M. T., Hrelia, S., & Caliceti, C. (2025). Anti-Inflammatory, Antioxidant and Antibacterial Properties of Tomato Skin and Pomegranate Peel Extracts: A Sustainable Approach for Oral Health Care. *Antioxidants*, 14(1), 54.
- Silva, E., Teixeira, J. A., Pereira, M. O., Rocha, C. M. R., & Sousa, A. M. (2023). Evolving biofilm inhibition and eradication in clinical settings through plant-based antibiofilm agents. *Phytomedicine*, 119, 154973.
- Singaraju, A., Nuvvula, S., Rudravaram, V. R. K., Anchala, K., Venkata Saikiran, K., & Mallineni, S. K. (2024). Efficacy of Triphala and Chlorhexidine Mouthwashes on Gingival Inflammation and Dental Plaque in Children: A Systematic Review. *Oral*, 4(4), 567–577.
- Singla, S., Malhotra, R., Shashikiran, N. D., & Saxena, S. (2018). Antibacterial efficacy of mouthwash prepared from pomegranate, grape seed and guava extracts against oral streptococci: An *in vivo* study. *Journal of Clinical Pediatric Dentistry*, 42(2), 109–113.
- Skogman, M. E., Vuorela, P. M., & Fallarero, A. (2012). Combining biofilm matrix measurements with biomass and viability assays in susceptibility assessments of antimicrobials against *Staphylococcus aureus* biofilms. *The Journal of antibiotics*, 65(9), 453-459.

- Skogman, M. E., Vuorela, P. M., & Fallarero, A. (2016). A platform of anti-biofilm assays suited to the exploration of natural compound libraries. *Journal of Visualized Experiments*, (118), 54829.
- Song, F., Koo, H., & Ren, D. (2015). Effects of material properties on bacterial adhesion and biofilm formation. *Journal of Dental Research*, 94(8), 1027–1034.
- Spatafora, G., Li, Y., He, X., Cowan, A., & Tanner, A. C. R. (2024). The Evolving Microbiome of Dental Caries. *Microorganisms*, 12(1), 121.
- Sterzenbach, T., Helbig, R., Hannig, C., & Hannig, M. (2020). Bioadhesion in the oral cavity and approaches for biofilm management by surface modifications. *Clinical Oral Investigations*, 24(12), 4237–4260.
- Stewart, P. S., White, B., Boegli, L., Hamerly, T., Williamson, K. S., Franklin, M. J., Bothner, B., James, G. A., Fisher, S., Vital-Lopez, F. G., & Wallqvist, A. (2019). Conceptual model of biofilm antibiotic tolerance that integrates phenomena of diffusion, metabolism, gene expression, and physiology. *Journal of Bacteriology*, 201(22), 10-1128.
- Stewart, P. S., & Costerton, J. W. (2001). Antibiotic resistance of bacteria in biofilms. *The lancet*, 358(9276), 135-138.
- Stiefel, P., Rosenberg, U., Schneider, J., Mauerhofer, S., Maniura-Weber, K., & Ren, Q. (2016). Is biofilm removal properly assessed? Comparison of different quantification methods in a 96-well plate system. *Applied microbiology and biotechnology*, 100(9), 4135-4145.
- Stoderegger, K. E., & Herndl, G. J. (2005). Dynamics in bacterial surface properties of a natural bacterial community in the coastal North Sea during a spring phytoplankton bloom. *FEMS Microbiology Ecology*, 53(2), 285–294.
- Sun, Yan, Pan, Y., Sun, Yu, Li, M., Huang, S., Qiu, W., Tu, H., & Zhang, K. (2019). Effects of Norspermidine on Dual-Species Biofilms Composed of *Streptococcus mutans* and *Streptococcus sanguinis*. *BioMed Research International*, 2019(1), 1950790.
- Suručić, R., Travar, M., Kundaković Vasović, T., Radović Selgrad, J., Suručić, L., Momčilović, M., Stojiljković, M. P., & Škrbić, R. (2025). *In vitro* and *In Silico* Analysis of Differential Antibacterial Activity of Pomegranate Polyphenols Against Gram-Positive and Gram-Negative Bacteria. *Antibiotics*, 14(9), 912.
- Syahputra, A., Ervina, I., Darwis, A. F., Syahputra, H., Marpaung, E. S. N., Sitorus, I. J., & Aditya, R. (2025). *In vitro* Antimicrobial Activity of *Punica granatum L.*

- Extract and Antiinflammatory Activity of Nano Gel In Vivo In wistar Rats Gingiva. *International Journal of Applied Pharmaceutics*, 17(3), 189–199.
- Takahashi, N., & Nyvad, B. (2011). The role of bacteria in the caries process: ecological perspectives. *Journal of dental research*, 90(3), 294–303.
- Tanzer, J. M., Thompson, A., Sharma, K., Vickerman, M. M., Haase, E. M., & Scannapieco, F. A. (2012). *Streptococcus mutans* out-competes *Streptococcus gordonii* in vivo. *Journal of Dental Research*, 91(5), 513–519.
- Toledo-Merma, P. R., Cornejo-Figueroa, M. H., Crisosto-Fuster, A. D. R., Strieder, M. M., Chañi-Paucar, L. O., Náthia-Neves, G., Rodríguez-Papuico, H., Rostagno, M. A., Meireles, M. A. A., & Alcázar-Alay, S. C. (2022). Phenolic Compounds Recovery from Pomegranate (*Punica granatum L.*) By-Products of Pressurized Liquid Extraction. *Foods*, 11(8), 1070.
- Tu, Y., Ren, H., He, Y., Ying, J., & Chen, Y. (2023). Interaction between microorganisms and dental material surfaces: general concepts and research progress. *Journal of Oral Microbiology*, 15(1), 2196897.
- Tuan, D. A., Uyen, P. V. N., Khuon, N. Van, Binh, L. A., & Masak, J. (2024). Innovative antifungal strategies: enhanced biofilm inhibition of *Candida albicans* by a modified tea tree oil formulation. *Frontiers in Microbiology*, 15, 1518598.
- Tzimas, K., Rahiotis, C., & Pappa, E. (2024a). Biofilm Formation on Hybrid, Resin-Based CAD/CAM Materials for Indirect Restorations: A Comprehensive Review. *Materials*, 17(7), 1474.
- Tzimas, K., Antoniadou, M., Varzakas, T., & Voidarou, C. (2024b). Plant-Derived Compounds: A Promising Tool for Dental Caries Prevention. *Current Issues in Molecular Biology*, 46(6), 5257–5290.
- Umar, D., Dilshad, B., Farhan, M., Ali, A., & Baroudi, K. (2016). The effect of pomegranate mouthrinse on *Streptococcus mutans* count and salivary pH: An in vivo study. *Journal of Advanced Pharmaceutical Technology and Research*, 7(1), 13–16.
- Vaou, N., Stavropoulou, E., Voidarou, C., Tsakris, Z., Rozos, G., Tsigalou, C., & Bezirtzoglou, E. (2022). Interactions between Medical Plant-Derived Bioactive Compounds: Focus on Antimicrobial Combination Effects. *Antibiotics*, 11(8), 1014.

- Veerapandian, R., & Vedyappan, G. (2019). Inhibition of adhesive nanofibrillar mediated *Streptococcus gordonii* - *Candida albicans* mono- and dual-species biofilms. *bioRxiv*, 689661.
- Verderosa, A. D., Totsika, M., & Fairfull-Smith, K. E. (2019). Bacterial Biofilm Eradication Agents: A Current Review. *Frontiers in Chemistry*, 7, 824.
- Vijayakumar, A., Sarveswari, H. B., Vasudevan, S., Shanmugam, K., Solomon, A. P., & Neelakantan, P. (2021). Antibiotics Baicalein Inhibits *Streptococcus mutans* Biofilms and Dental Caries-Related Virulence Phenotypes. *Antibiotics*, 10(2), 215.
- Villanueva, X., Zhen, L., Ares, J. N., Vackier, T., Lange, H., Crestini, C., & Steenackers, H. P. (2023). Effect of chemical modifications of tannins on their antimicrobial and antibiofilm effect against Gram-negative and Gram-positive bacteria. *Frontiers in Microbiology*, 13, 987164.
- Vukosavljevic, D., Custodio, W., & Siqueira, W. L. (2011). Salivary proteins as predictors and controls for oral health. *Journal of Cell Communication and Signaling*, 5(4), 271–275.
- Wan, Y., Wang, Xiaowen, Yang, L., Li, Q., Zheng, X., Bai, T., & Wang, Xin. (2023). Antibacterial Activity of Juglone Revealed in a Wound Model of *Staphylococcus aureus* Infection. *International Journal of Molecular Sciences*, 24(4), 3931.
- Wang, X., Li, J., Zhang, S., Zhou, W., Zhang, L., & Huang, X. (2023). pH-activated antibiofilm strategies for controlling dental caries. *Frontiers in Cellular and Infection Microbiology*, 13, 1130506.
- Wang, Y., Wang, X., Jiang, W., Wang, K., Luo, J., Li, W., Zhou, X., & Zhang, L. (2018). Antimicrobial peptide GH12 suppresses cariogenic virulence factors of *Streptococcus mutans*. *Journal of Oral Microbiology*, 10(1), 1442089.
- Wang, Z., Shen, Y., & Haapasalo, M. (2020). Dynamics of Dissolution, Killing, and Inhibition of Dental Plaque Biofilm. *Frontiers in Microbiology*, 11, 964.
- Weiss, E., Rosenberg, M., Judes, H., & Rosenberg, E. (1982). Current Microbiology Cell-Surface Hydrophobicity of Adherent Oral Bacteria. *Current Microbiology*, 7(2), 125-128.
- Welch, K., Cai, Y., & Strømme, M. (2012). A method for quantitative determination of biofilm viability. *Journal of Functional Biomaterials*, 3(2), 418-431.

- Whittaker, C. J., Klier, C. M., & Kolenbrander, P. E. (1996). Mechanisms Of Adhesion By Oral Bacteria. *Annual review of microbiology*, 50(1), 513-552.
- World Health Organization. (2022). *Global oral health status report: towards universal health coverage for oral health by 2030*. World Health Organization.
- World Health Organization. (2024). *Global strategy and action plan on oral health 2023–2030*. World Health Organization.
- Wille, J., & Coenye, T. (2020). Biofilm dispersion: The key to biofilm eradication or opening Pandora's box? *Biofilm*, 2, 100027.
- Wimpenny, J., Manz, W., & Szewzyk, U. (2000). Heterogeneity in biofilms. *FEMS microbiology reviews*, 24(5), 661-671.
- Wu, C. Y., Su, T. Y., Wang, M. Y., Yang, S. F., Mar, K., & Hung, S. L. (2018). Inhibitory effects of tea catechin epigallocatechin-3-gallate against biofilms formed from *Streptococcus mutans* and a probiotic lactobacillus strain. *Archives of oral biology*, 94, 69-77.
- Wright, P. P., & Ramachandra, S. S. (2022). Quorum Sensing and Quorum Quenching with a Focus on Cariogenic and Periodontopathic Oral Biofilms. *Microorganisms*, 10(9), 1783.
- Xiao, J., Klein, M. I., Falsetta, M. L., Lu, B., Delahunty, C. M., Yates, J. R., Heydorn, A., & Koo, H. (2012). The exopolysaccharide matrix modulates the interaction between 3D architecture and virulence of a mixed-species oral biofilm. *PLoS Pathogens*, 8(4), e1002623.
- Xiao, J., & Koo, H. (2010). Structural organization and dynamics of exopolysaccharide matrix and microcolonies formation by *Streptococcus mutans* in biofilms. *Journal of applied microbiology*, 108(6), 2103-2113.
- Xu, M., Wang, X., Gong, T., Yang, Z., Zhang, D., Ma, Q., & Li, Y. (2025). Glucosyltransferase activity-based screening identifies tannic acid as an inhibitor of *Streptococcus mutans* biofilm. *Frontiers in Microbiology*, 16, 1555497.
- Xu, W., Yu, F., Addison, O., Zhang, B., Guan, F., Zhang, R., Hou, B., & Sand, W. (2024). Microbial corrosion of metallic biomaterials in the oral environment. *Acta Biomaterialia*, 184, 22–36.
- Xu, X., Zhou, X. D., & Wu, C. D. (2011). The tea catechin epigallocatechin gallate suppresses cariogenic virulence factors of *Streptococcus mutans*. *Antimicrobial agents and chemotherapy*, 55(3), 1229-1236.

- Yaghmoor, R. B., Abdel-Hadi, M., Petridis, H., Allan, E., & Young, A. M. (2024). Effects of Novel Dental Composites on *Streptococcus mutans* Biofilms. *Journal of Functional Biomaterials*, 15(1), 13.
- Yamashita, T., Kawada-Matsuo, M., Katsumata, T., Watanabe, A., Oogai, Y., Nishitani, Y., Miyawaki, S., & Komatsuzawa, H. (2019). Antibacterial activity of disodium succinoyl glycyrrhetinate, a derivative of glycyrrhetic acid against *Streptococcus mutans*. *Microbiology and Immunology*, 63(7), 251–260.
- Yan, J., & Bassler, B. L. (2019). Surviving as a Community: Antibiotic Tolerance and Persistence in Bacterial Biofilms. *Cell Host and Microbe*, 26(1), 15–21.
- Yang, D., Hao, S., Zhao, L., Shi, F., Ye, G., Zou, Y., Song, X., Li, L., Yin, Z., He, X., Feng, S., Chen, H., Zhang, Y., Gao, Y., Li, Y., & Tang, H. (2021). Paeonol Attenuates Quorum-Sensing Regulated Virulence and Biofilm Formation in *Pseudomonas aeruginosa*. *Frontiers in Microbiology*, 12, 692474.
- Ye, D., Liu, Y., Li, J., Zhou, J., Cao, J., Wu, Y., Wang, X., Fang, Y., Ye, X., Zou, J., & Ma, Q. (2025). Competitive dynamics and balance between *Streptococcus mutans* and commensal streptococci in oral microecology. *Critical Reviews in Microbiology*, 51(3), 532–543.
- Zeng, Y., Nikitkova, A., Abdelsalam, H., Li, J., & Xiao, J. (2019). Activity of quercetin and kaempferol against *Streptococcus mutans* biofilm. *Archives of Oral Biology*, 98, 9–16.
- Zhang, Q., Ma, Q., Wang, Y., Wu, H., & Zou, J. (2021). Molecular mechanisms of inhibiting glucosyltransferases for biofilm formation in *Streptococcus mutans*. *International Journal of Oral Science*, 13(1), 30.
- Zhang, Y., Fang, J., Yang, J., Gao, X., Dong, L., Zheng, X., Sun, L., Xia, B., Zhao, N., Ma, Z., & Wang, Y. (2022). *Streptococcus mutans*-associated bacteria in dental plaque of severe early childhood caries. *Journal of oral microbiology*, 14(1), 2046309.
- Zhang, X., Liu, Y., Chen, G., Bai, S., Li, Z., Wang, F., & Xu, D. (2025). Long-term continuous corrosion of 316L stainless steel by *Streptococcus mutans* in simulated oral environment. *Frontiers in Bioengineering and Biotechnology*, 13, 1725414.
- Zheng, L., Itzek, A., Chen, Z., & Kreth, J. (2011). Environmental influences on competitive hydrogen peroxide production in *Streptococcus gordonii*. *Applied and Environmental Microbiology*, 77(13), 4318–4328.

- Zhou, J., Ma, Q., Liang, J., Pan, Y., Chen, Y., Yu, S., Liu, Y., Zhang, Q., Li, Y., & Zou, J. (2025). smu_1558c-mediated regulation of growth and biofilm formation in *Streptococcus mutans*. *Frontiers in Microbiology*, *15*, 1507928.
- Zhu, S., Li, M., Qian, T., Chen, J., & Pan, T. (2025). Influence of Surfactants on Interfacial Microbial Degradation of Hydrophobic Organic Compounds. *Catalysts*, *15*(2), 187.

APPENDICES

APPENDIX 1

Ethics approval letter for the present study issued by the Universiti Teknologi MARA Research Ethics Committee

www.uitm.edu.my	
	Pejabat Timbalan Naib Canselor (Penyelidikan dan Inovasi)
Reference : 600-TNCPI (5/1/6) Our reference : REC/01/2023 (PG/MR/27) Date : 31 January 2023	
Professor Dato' Dr Mohamed Ibrahim Abu Hassan (Nurhayati binti Mohamad Zain – 2021604696) Faculty of Dentistry UiTM Sungai Buloh Campus Jalan Hospital 47000 Sungai Buloh SELANGOR	
Dear Professor Dato' Dr Mohamed Ibrahim,	
APPROVAL LETTER - UiTM RESEARCH ETHICS COMMITTEE	
Thank you for submitting your research proposal to the Research Ethics Committee (REC). After considering your application, the Committee approved your proposal titled "Antibiofilm Effect of Punicalagin and Punicalin on Oral Streptococci in Experimental Oral Biofilm" at the Microbiology Lab Level 4, Dental Faculty, Universiti Teknologi MARA Sungai Buloh.	
Details of the approval are as follows:	
Ref. number:	REC/01/2023 (PG/MR/27)
Approval Period:	31 January 2023 until 30 September 2024
Authorised personnel:	1. Professor Dato' Dr Mohamed Ibrahim Abu Hassan 2. Professor Dr Fathilah Abdul Razak 3. Assoc. Prof. Ts Dr Indah Mohd Amin 4. Nurhayati binti Mohamad Zain
The UiTM Research Ethics Committee operates in accordance to the ICH Good Clinical Practice Guidelines, Malaysian Good Clinical Practice Guidelines and the Declaration of Helsinki. The approval of this project is conditional upon your continuing compliance with these guidelines and declaration.	
We draw to your attention the requirement that a report on this research, must be submitted every 12 months from the date of the approval or on the completion of the project, whichever occurs first. Failure to submit reports will result in withdrawal of consent for the project to proceed. Amendments, if any, to the study documents are to be submitted to the REC for approval.	
If you require further information, please contact the REC Secretariat at 03-55448069/03-55442794 or email at recsecretariat@uitm.edu.my .	
Yours sincerely,	
EMERITUS PROFESSOR DATO' DR RAYMOND AZMAN ALI Chairman UiTM Research Ethics Committee	
c.c.: Dean, Faculty of Dentistry, UiTM	
Universiti Teknologi MARA Aras 3, Bangunan Wawasan 40450 Shah Alam, Selangor, MALAYSIA Tel: (+603) 5544 2004/2255 Faks: (+603) 5544 2070	
	

APPENDIX 2

3rd Prize for oral presentation at 5th International Conference on Oral Microbiology and Oral Immunology (ICOMOI) 2022



APPENDIX 3

Certificate of participation at 17th International Dental Collaboration of the Mekong River Region Congress 2023



APPENDIX 4

Certificate of participation at 8th International Conference on Molecular Biology and Biotechnology (ICMBB 2025)



APPENDIX 5

Certificate of Copyright Notification issued under the Copyright Act 1987 (Malaysia) for the work titled “Experimental Formulation and Protocol for Selective Cariogenic Biofilm Eradication Using Pomegranate-Derived Punicalagin.”



COPYRIGHT ACT 1987
COPYRIGHT (VOLUNTARY NOTIFICATION) REGULATIONS 2012
CERTIFICATE OF COPYRIGHT NOTIFICATION
[Subregulation 8(2)]

Notification Number : CRLY2025W03866

Title of Work : EXPERIMENTAL FORMULATION AND
PROTOCOL FOR SELECTIVE CARIOGENIC
BIOFILM ERADICATION USING POMEGRANATE-
DERIVED PUNICALAGIN

Category of Work : LITERARY

Date of Notification : 11 JUNE 2025

Date of Creation : 01 JANUARY 2025

This is to certify, under the Copyright Act 1987 [Act 332] and the Copyright (Voluntary Notification) Regulations 2012 that the copyrighted work bearing the Notification No. above for the applicant **UNIVERSITI TEKNOLOGI MARA (UiTM)** as the **OWNER** and **NURHAYATI BINTI MOHAMAD ZAIN** (), **MOHAMED IBRAHIM BIN ABU HASSAN** (), **FATHILAH BINTI ABDUL RAZAK** (), **INDAH BINTI MOHD AMIN** (), **VIVI NORYATI BINTI AHMAD** () as the **AUTHOR** have been recorded in the Register of Copyright, in accordance with section 26B of the Copyright Act 1987 [Act 332].


YUSNIEZA SYARMILA BINTI YUSOFF
DEPUTY CONTROLLER OF COPYRIGHT
MALAYSIA



(Agency under the Ministry of Domestic Trade and Cost of Living)

APPENDIX 6

Published article titled “Preliminary screening of pomegranate-derived compounds for antimicrobial and antivirulence effects against cariogenic streptococci” in The Saudi Dental Journal (2025)

The Saudi Dental Journal (2025) 37:84
<https://doi.org/10.1007/s44445-025-00083-2>

RESEARCH ARTICLE



Preliminary screening of pomegranate-derived compounds for antimicrobial and anti-virulence effects against cariogenic streptococci

Nurhayati Mohamad Zain¹ · Indah Mohd Amin¹ · Fathilah Abdul Razak^{2,3} · Mohamed Ibrahim Abu Hassan^{4,5}

Received: 15 July 2025 / Accepted: 28 September 2025
© The Author(s) 2025

Abstract

As a biofilm-mediated disease, dental caries is primarily attributed to the activity of *Streptococcus mutans* and *Streptococcus sobrinus*, key contributors to enamel mineral loss under acidic conditions. Current broad-spectrum antimicrobials disrupt the oral microbiota and carry undesirable side effects, prompting interest in targeted, microbiome-friendly alternatives. This study evaluated the antimicrobial, anti-cariogenic, and cytotoxic properties of pomegranate (*Punica granatum*) derived compounds, corilagin, ellagic acid, gallic acid, kaempferol-7-O-glucoside, punicalagin, punicalin, and rutin against cariogenic *S. mutans* and *S. sobrinus*, and the commensal *S. gordonii*. Antibacterial activity was assessed using disc diffusion, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) methods. Anti-virulence effects were evaluated through glycolytic pH drop and cell surface hydrophobicity assays. Cytotoxicity was determined using the brine shrimp lethality assay. Punicalagin, punicalin, and ellagic acid showed strong, selective bactericidal activity against *S. mutans* and *S. sobrinus*, with low MICs and MBC/MIC ratios, while sparing *S. gordonii*. These compounds significantly suppressed acid production, maintaining pH above the critical demineralisation threshold, and reduced surface hydrophobicity in cariogenic strains without affecting the commensal. Most compounds exhibited low toxicity ($LC_{50} > 500 \mu\text{g/mL}$), indicating a favourable safety profile. Overall, punicalagin and punicalin demonstrated dual antimicrobial and anti-virulence activity with selective targeting of cariogenic pathogens. These findings support the potential inclusion of punicalagin and punicalin in oral care formulations aimed at preventing dental caries while preserving beneficial oral microbiota. As this is a preliminary screening study, the results should be interpreted cautiously, and further biofilm and host-cell assays are needed to confirm translational potential.

Keywords Anticaries · Oral streptococci · Pomegranate-derived compound

1 Introduction

Dental caries ranks among the most widespread chronic conditions worldwide, impacting more than a billion individuals and playing a major role in global oral health inequalities. Untreated caries leads to pain, infection, tooth loss, school absenteeism, and reduced quality of life, especially among vulnerable populations such as children, the elderly, and individuals with limited access to dental care (Eid et al. 2020; Lima et al. 2018).

Streptococcus mutans (*S. mutans*) and *Streptococcus sobrinus* (*S. sobrinus*), belonging to the mutans streptococci group, are recognised as key contributors to dental caries because of their ability to produce acid, tolerate acidic environments and synthesise glucans. These traits enable them to colonise the tooth surface, form biofilms, and drive

✉ Nurhayati Mohamad Zain
nurhayati8370@uitm.edu.my

¹ Centre of Preclinical Science Studies, Faculty of Dentistry, Universiti Teknologi MARA, Sungai Buloh Campus, Sungai Buloh, Selangor, Malaysia

² Faculty of Dentistry, Universiti Malaya, Kuala Lumpur, Malaysia

³ School of Dentistry, Management and Science University, Selangor, Shah Alam, Malaysia

⁴ Faculty of Dentistry, MAHSA University, Jenjarom, Selangor, Malaysia

⁵ Centre of Restorative Dentistry Studies, Faculty of Dentistry, Universiti Teknologi MARA, Sungai Buloh Campus, Sungai Buloh, Selangor, Malaysia

Published online: 20 November 2025

Springer

enamel demineralisation. Importantly, the co-presence of *S. mutans* and *S. sobrinus* has been linked to more severe caries outcomes. For example, children harbouring both species show higher DMFT scores than those with *S. mutans* alone (Korona-Glowniak et al. 2022; Okada et al. 2012). Despite preventive advances, including fluoride-based therapies and oral hygiene measures, current interventions often fall short in completely preventing caries.

The use of broad-spectrum antimicrobials, such as chlorhexidine, while effective, is associated with side effects including tooth staining, mucosal irritation, altered taste, and disruption of the commensal oral flora (Boulares et al. 2025; James et al. 2017). This may result in microbial dysbiosis and reduced ecological resilience of the oral microbiome. According to the ecological plaque hypothesis, disease occurs when environmental changes in the oral cavity promote the overgrowth of pathogenic bacteria. This has led to growing interest in strategies that modulate the oral ecosystem rather than eradicate bacteria indiscriminately.

Natural products, particularly those derived from medicinal plants, are being explored as selective antimicrobial agents with the potential to suppress oral pathogens while preserving beneficial microbes (Tzimas et al. 2024). Pomegranate (*Punica granatum*) has attracted attention for its broad pharmacological properties, including antimicrobial, antioxidant, and anticancer activities (Sweidan et al. 2023; Chaves et al. 2020). While crude pomegranate extracts have shown effectiveness across various applications, the results have been inconsistent due to variations in composition influenced by factors such as cultivar, extraction method, and solvent polarity (Man et al. 2022; Young et al. 2017). Modern drug discovery increasingly prioritizes purified phytochemicals over complex plant extracts due to their superior selectivity, consistent potency, and suitability for high-throughput screening (Nemmani 2021). Isolated phytochemicals not only serve as therapeutic agents themselves but also act as valuable chemical leads for the synthesis of bioactive analogs. The enduring pharmaceutical relevance of phytochemicals is underscored by data showing that nearly 50% of all approved drugs within the 25 years leading up to 2007 were derived from plant-based natural products or their synthetic counterparts (Alam et al. 2022).

The present study conducted a preliminary screening of seven purified pomegranate-derived compounds—corilagin, ellagic acid, gallic acid, kaempferol-7-O-glucoside, punicalagin, punicalin, and rutin for their antimicrobial, cytotoxic, and anti-virulence properties. The compounds were tested against the cariogenic streptococci *S. mutans* and *S. sobrinus*, alongside the commensal *Streptococcus gordonii*. The evaluation included antibacterial activity, cytotoxicity using the brine shrimp lethality assay, and inhibition of two key virulence traits, glycolytic acid production and cell surface hydrophobicity. This approach provides preliminary insights

into the potential of pomegranate-derived agents as microbiome-friendly oral therapeutics for targeted caries prevention.

2 Materials and methods

2.1 Phytochemicals

The compounds used in this study corilagin (CG), ellagic acid (EA), gallic acid (GA), kaempferol-7-O-glucoside (KPG), punicalagin (PUG), punicalin (PUN) and rutin (RT) were selected based on their identification as major bioactive constituents of pomegranate peel, as reported by Man et al. (2022). To ensure consistency, purity, and traceability of the test materials, all compounds were procured from a reputable commercial supplier, BOC Sciences (Shirley, NY, USA).

2.2 Bacterial strain

The bacterial strain, *Streptococcus mutans* (ATCC 700610), *Streptococcus sobrinus* (ATCC 33478) and *Streptococcus gordonii* (ATCC 51656) were obtained from the American Type Culture Collection (ATCC). *Streptococcus mutans* (*S. mutans*) and *Streptococcus sobrinus* (*S. sobrinus*) were selected as the primary cariogenic pathogens due to their well-established role in dental plaque formation, acid production, and enamel demineralisation. In contrast, *S. gordonii* represents a common commensal species in the oral cavity that contributes to microbial homeostasis and early colonisation without significant cariogenic potential. The inclusion of both groups enables the assessment of compound selectivity, allowing differentiation between broad-spectrum antimicrobial effects and targeted inhibition of pathogenic species.

2.3 Disc diffusion assay

The antibacterial activity of the phytochemicals was assessed using the disc diffusion assay following the CLSI guidelines (CLSI 2012) with minor modifications. A volume of 25 µL from each compound stock solution (20 mg/mL) was applied onto sterile filter paper discs (6 mm diameter), corresponding to 0.5 mg per disc. A standardised bacterial suspension, approximately 10⁸ CFU/mL, was evenly spread on brain heart infusion (BHI) agar plates using sterile cotton swabs. After allowing the surface to dry for 5 min, the treated discs were placed on the agar. Sterile distilled water and 0.12% chlorhexidine (CHX) discs served as negative and positive controls, respectively. The plates were then incubated at 37 °C for 16–18 h. Following incubation, inhibition zones were recorded in millimetres with a digital calliper under inverted plates. All experiments were conducted in triplicate and repeated on three separate occasions to confirm reproducibility.

2.4 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MICs of the compounds were determined using the broth microdilution method, as outlined in CLSI guidelines (CLSI 2018), with minor adjustments. A bacterial inoculum of $\sim 10^6$ CFU/mL was diluted 1:20 in sterile BHI broth to achieve a concentration of 5×10^5 CFU/mL. Serial twofold dilutions of each compound (starting from 12,800 $\mu\text{g/mL}$) were prepared in 96-well microtiter plates to obtain final concentrations ranging from 12.5 to 6400 $\mu\text{g/mL}$. Each well received 90 μL of the diluted compound and 10 μL of bacterial suspension, resulting in a final concentration of $\sim 5 \times 10^5$ CFU/mL per well. Each test was conducted in triplicate. Growth control wells (bacteria + broth), sterility controls (broth only), and compound blanks (compound + broth without bacteria) were included to verify viability, sterility, and exclude background absorbance. After 24 h of incubation at 37 °C, bacterial growth was quantified by measuring the optical density at 600 nm (OD₆₀₀) using a microplate reader (Tecan Infinite 200 PRO). The MIC was defined as the lowest concentration at which the OD₆₀₀ differed by less than 0.05 compared to the corresponding blank control. To determine MBC, 10 μL from wells showing no visible turbidity were streaked onto BHI agar plates and incubated at 37 °C for 36–48 h. The MBC was defined as the lowest concentration that completely prevented colony formation, indicating $\geq 99.9\%$ bacterial killing.

2.5 Brine shrimp lethality assay (BSLA)

The cytotoxicity of the pomegranate-derived compounds was assessed using the brine shrimp (*Artemia salina*) lethality assay, a widely accepted preliminary screening tool for evaluating general bioactivity and toxicity. Although not directly representative of mammalian systems, this assay provides a simple, rapid, and cost-effective method for identifying potentially harmful compounds before more advanced cytotoxicity models. Its use is especially valuable in natural product research where high-throughput, early-stage screening of multiple compounds is needed to inform further safety testing relevant to oral applications.

The assay was performed with minor modifications based on the method by Sarah et al. (2017). Artificial seawater was prepared by dissolving 27 g of marine salt in 3 L of distilled water. Brine shrimp eggs were incubated at room temperature for 48 h under continuous aeration and illumination. The hatched nauplii were then attracted to a light source, collected and transferred into fresh seawater. Stock solution of the test compounds (20,000 $\mu\text{g/mL}$) was prepared in distilled water containing 1% DMSO, followed by serial twofold dilutions to achieve concentrations ranging from 12.5 to 6400 $\mu\text{g/mL}$. For each concentration, 20 nauplii were exposed to 1 mL of the compound solution combined with 1

mL of seawater. After 24 h of incubation at room temperature, mortality was assessed by counting the dead nauplii. Negative (seawater) and solvent (1% DMSO) controls were included. Each concentration was tested in triplicate. The mortality percentage was calculated, and LC₅₀ values were estimated by nonlinear regression (variable slope) using GraphPad Prism v10. Toxicity was categorised using Meyer's and Clarkson's criteria: LC₅₀ > 1000 $\mu\text{g/mL}$ (non-toxic), 500–1000 $\mu\text{g/mL}$ (low toxicity), 100–500 $\mu\text{g/mL}$ (moderate toxicity) and < 100 $\mu\text{g/mL}$ (high toxicity).

2.6 Glycolytic pH drop assay

The effect of pomegranate-derived compounds on the acidogenic activity of *S. mutans* was assessed using a glycolytic pH drop assay, with modifications from the method of He et al. (2019). Bacterial cultures at the mid-logarithmic phase were collected, rinsed twice with phosphate-buffered saline (PBS), and resuspended in a saline solution containing 50 mM potassium chloride and 1 mM magnesium chloride. Each compound was evaluated at its previously established minimum inhibitory concentration (MIC), ensuring a standardised sub-inhibitory level relevant for assessing anti-virulence activity. This strategy allowed the bacteria to remain viable while enabling measurement of reductions in pathogenic traits. Chlorhexidine (0.12%) and 1% DMSO were included as positive and negative controls, respectively. The suspension was supplemented with glucose to a final concentration of 1% (w/v), and its initial pH was adjusted to 7.2–7.4 using 0.2 M potassium hydroxide (KOH). Measurements were taken at 15-min intervals for 120 min with a calibrated pH meter. The acidogenic response was expressed as the overall decrease in pH during the assay.

2.7 Cell surface hydrophobicity (CSH) assay

The influence of the test compounds on the cell surface hydrophobicity of *S. mutans* and *S. gordonii* was evaluated using the microbial adhesion to hydrocarbon (MATH) method, as described by Rosenberg (2006), with modifications. To assess anti-adhesive properties independent of bactericidal effects, all compounds were tested at their respective MICs, which represent biologically relevant sub-inhibitory concentrations. This allows the evaluation of virulence modulation under non-lethal exposure conditions. Untreated cells were used as the negative control (NC), whereas a 1% DMSO vehicle control (VC) was included to verify that the solvent did not influence cell surface characteristics. Bacterial suspensions were exposed to the test compounds at 37 °C for 1 h. Following exposure, the cells were harvested by centrifugation (5000 $\times g$, 5 min, 4 °C), rinsed twice with sterile distilled water, and resuspended in PUM buffer (potassium phosphate, urea, and magnesium sulfate; pH 7.1) to achieve

an optical density of 0.8 at 600 nm (OD_0). For each measurement, 3 mL of the bacterial suspension was combined with 0.2 mL of hexadecane, vortexed for 60 s, and allowed to separate for 15 min. The absorbance of the aqueous layer was then recorded at 600 nm (OD_1), and cell surface hydrophobicity was determined using the following formula:

$$\text{Hydrophobicity (\%)} = \frac{OD_0 - OD_1}{OD_0} \times 100$$

The assay was performed in five independent determinations, each in triplicate.

2.8 Statistical analysis

All experiments were carried out in at least three independent replicates. Results are expressed as mean values $\pm$ standard deviation (SD). Statistical analyses were performed using GraphPad Prism (version 10). For comparison among multiple groups, one-way ANOVA followed by Tukey's post hoc test was applied to determine significant differences in both disc diffusion and cell surface hydrophobicity assays. A threshold of $p < 0.05$ was considered statistically significant, and distinct superscript letters were used to indicate differences between groups.

For the brine shrimp lethality assay, the lethal concentration (LC_{50}) values were calculated using nonlinear regression analysis with a variable slope model. Goodness of fit was assessed using the coefficient of determination (R^2) to confirm the reliability of dose–response relationships.

3 Results

3.1 Disc diffusion assay

All pomegranate-derived compounds exhibited measurable antibacterial activity against *S. gordonii*, *S. mutans*, and *S. sobrinus*, while no inhibition was observed with the negative control (Table 1). Among them, PUG demonstrated the most potent effect, with inhibition zones comparable to CHX, 0.12%, particularly against *S. mutans* (18.5 ± 2.0 mm) and *S. sobrinus* (19.0 ± 1.1 mm). EA followed closely, showing consistent activity across all species. In contrast, GC and KPG showed significantly lower inhibition ($p < 0.05$). These results indicate that different compounds exert distinct effects on oral streptococcal species, reflecting species- and compound-specific antimicrobial activities.

3.2 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The antimicrobial activity of selected pomegranate-derived compounds was assessed against *S. gordonii*, *S. mutans*,

Table 1 Zone of inhibition (mean diameter in mm $\pm$ standard deviation) of selected pomegranate-derived compounds and controls against *S. gordonii*, *S. mutans* and *S. sobrinus*

Compounds	Zone of inhibition (mm $\pm$ SD)		
	<i>S. gordonii</i>	<i>S. mutans</i>	<i>S. sobrinus</i>
GC	11.6 ± 1.4^d	12.5 ± 1.4^e	13.0 ± 1.2^e
CG	14.0 ± 1.7^c	15.0 ± 1.7^d	15.5 ± 1.5^d
EA	16.0 ± 1.7^b	17.0 ± 1.7^c	17.2 ± 1.3^c
KPG	11.0 ± 1.2^d	12.0 ± 1.2^e	11.8 ± 1.3^e
PUN	14.0 ± 1.2^c	15.0 ± 1.2^d	15.4 ± 1.4^d
PUG	14.2 ± 2.0^c	18.5 ± 2.0^a	19.0 ± 1.1^a
RT	13.0 ± 1.2^{cd}	14.0 ± 1.2^d	14.5 ± 1.5^d
CHX (0.12%)	17.0 ± 1.7^a	18.5 ± 2.0^a	19.3 ± 1.2^a
DMSO (1%)	ni	ni	ni

Different superscript letters (a–e) within the same column indicate statistically significant differences ($p < 0.05$). “ni” indicates no inhibition

Chlorhexidine (CHX, 0.12%) was used as a positive control, while dimethyl sulfoxide (DMSO, 1%) served as the negative control

and *S. sobrinus* using MIC, MBC, and MBC/MIC ratios (Table 2). PUG, PUN and EA exhibited the most potent and selective bactericidal activity against cariogenic *S. mutans* and *S. sobrinus*, with low MIC values (100 μ g/mL) and favourable MBC/MIC ratios (2), while showing higher MICs and MBCs against *S. gordonii*. CG displayed moderate activity, with a higher MBC/MIC ratio (8) against *S. gordonii*, indicating reduced bactericidal efficacy. KPG and RT showed weaker overall activity with higher MIC values and MBC/MIC ratios of 2–4 across all species. CHX demonstrated potent but non-selective antimicrobial activity across all tested species. It showed identical or similar MIC and MBC values for both cariogenic (*S. mutans*, *S. sobrinus*) and commensal (*S. gordonii*) bacteria, with MBC/MIC ratios ranging from 1 to 2. These findings highlight CHX's broad-spectrum action.

3.3 Brine shrimp lethality assay (BSLA)

The brine shrimp lethality assay results are summarised in Table 3. Most of the tested pomegranate-derived compounds demonstrated low toxicity, with LC_{50} values exceeding 500 μ g/mL and strong dose–response correlations ($R^2 > 0.98$), indicating a favourable safety profile. PUN (941.2 μ g/mL) and KPG (998.9 μ g/mL) exhibited the highest LC_{50} values. In contrast, EA was the only compound classified under moderate toxicity, with an LC_{50} of 159.2 μ g/mL, according to Clarkson's toxicity criteria. All other compounds were categorised as low toxicity. Notably, the LC_{50} values for all compounds were substantially higher than their respective MIC values, indicating a wide therapeutic margin and supporting their potential for safe antimicrobial application.

Table 2 Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and MBC/MIC ratio of selected pomegranate-derived compounds against *S. gordonii*, *S. mutans* and *S. sobrinus*

Compound	<i>S. gordonii</i>			<i>S. mutans</i>			<i>S. sobrinus</i>		
	MIC μg/mL	MBC μg/mL	MBC/MIC ratio	MIC μg/mL	MBC μg/mL	MBC/MIC ratio	MIC μg/mL	MBC μg/mL	MBC/MIC ratio
GC	25	100	4	100	400	4	100	400	4
CG	100	800	8	200	400	2	200	400	2
EA	100	400	4	100	200	2	100	200	2
KPG	100	200	2	100	400	4	100	400	4
PUN	200	400	2	200	400	2	100	200	2
PUG	100	400	4	100	200	2	100	200	2
RT	200	400	2	200	400	2	200	400	2
	MIC %	MBC %	MBC/MIC ratio	MIC %	MBC %	MBC/MIC ratio	MIC %	MBC %	MBC/MIC ratio
CHX	0.015	0.015	1	0.03	0.06	2	0.03	0.06	2

CHX was included as a positive control. The MBC/MIC ratio was used to indicate bactericidal efficacy, with lower ratios suggesting stronger bactericidal activity

Table 3 Lethal concentration (LC₅₀), coefficient of determination (R²), and toxicity classification of selected pomegranate-derived phytochemicals as determined by the brine shrimp lethality assay

Compounds	Class	LC ₅₀ (μg/mL)	R ²	Toxicity category
RT	Flavonoid	621	0.9956	Low toxicity
CG	Hydrolysable tannin	588.9	0.9923	Low toxicity
EA	Polyphenol	159.2	0.9927	moderate toxicity
PUG	Hydrolysable tannin	819.7	0.9879	Low toxicity
PUN	Hydrolysable tannin	941.2	0.9921	Low toxicity
KPG	Flavonoid	998.9	0.9899	Low toxicity
GC	Flavonoid	594	0.9897	Low toxicity

Compounds were categorised based on Clarkson's criteria, where LC₅₀ < 250 μg/mL indicates moderate toxicity and LC₅₀ > 500 μg/mL indicates low toxicity

3.4 Glycolytic pH drop

The glycolytic pH drop assay (Fig. 1) demonstrated clear differences in the acidogenic potential of *S. mutans* and *S. sobrinus* following treatment with the tested compounds over 120 min period. In the untreated control (1% DMSO), both species exhibited a rapid and sustained decrease in pH, dropping below the critical demineralisation threshold (pH 5.5) within 30 min. By the end of the experiment, the final pH reached 3.5 ± 0.2 for *S. mutans* and 4.0 ± 0.3 for *S. sobrinus*, indicating high glycolytic activity. In contrast, selected compounds notably suppressed acid production. For *S. mutans* PUG, PUN, and CHX, the final pH values were maintained above 5.5 at 120 min, indicating effective suppression of acid production. For *S. sobrinus*, PUG, PUN, GT,

and CHX achieved similar suppression, suggesting broader inhibitory effects in this species. To further assess early acidogenic activity, the initial rate of pH drop during the first 15 min was calculated for each treatment (Table 4). The control group exhibited the highest pH drop rates, 0.0467 pH units/min for *S. mutans* and 0.0600 pH units/min for *S. sobrinus*. Among the tested compounds, PUG most effectively reduced initial acid production, with corresponding rates of 0.025 and 0.013 pH units/min, respectively. These results indicate that specific pomegranate-derived compounds can attenuate the acidogenic activity of cariogenic bacteria in a time-dependent manner.

3.5 Cell surface hydrophobicity (CSH) assay

The hydrophobicity index of *S. mutans*, *S. sobrinus*, and *S. gordonii* was significantly influenced by treatment with the tested compounds (Fig. 2a–c). For *S. mutans* (Fig. 2a), untreated control cells showed the highest hydrophobicity (~75%). Treatment with CHX 0.12% significantly reduced hydrophobicity ($p < 0.05$) to below 40%, showing the most substantial reduction among all treatments. Among the compounds, PUN, PUG, and CG caused significant decreases in hydrophobicity compared to the control, with PUG and PUN showing the lowest values among natural compounds. EA and RT also showed moderate but statistically significant reductions ($p < 0.05$). In contrast, KPG and NC (DMSO control) maintained high hydrophobicity levels similar to the untreated control (denoted by shared superscript 'a'), indicating minimal effect. For *S. sobrinus* (Fig. 2b), a similar pattern was observed. The control group maintained a high hydrophobicity index (~55%), whereas CHX significantly decreased the hydrophobicity ($p < 0.05$). Natural compounds, such as PUN, PUG, and CG, caused significant

Fig. 1 Effect of compounds on glycolytic pH drop by (a) *S. mutans* and (b) *S. sobrinus* over a 120 min incubation period. The initial pH was 7.4, and pH was recorded at regular intervals 15 min. The horizontal black dashed line at pH 5.5 indicates the critical pH threshold below which enamel demineralization is likely to occur. Data are presented as mean $\pm$ SD ($n=5$)

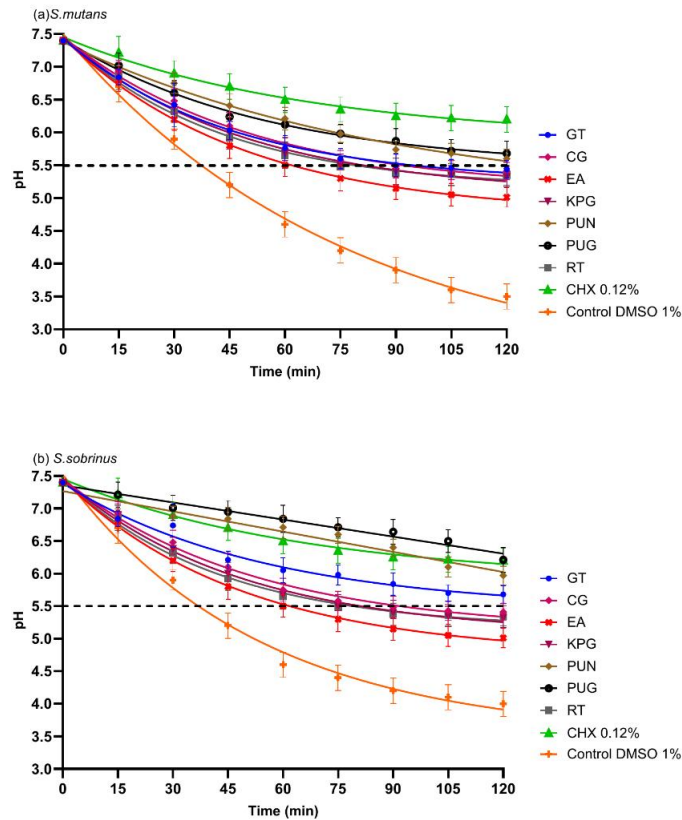


Table 4 Initial glycolytic pH drop rates (pH unit/min) of *S. mutans* and *S. sobrinus* within the first 15 min following treatment with pomegranate-derived compounds and controls

Treatment	Initial Rate (pH unit/min)	
	<i>S. mutans</i>	<i>S. sobrinus</i>
GT	0.037	0.041
CG	0.031	0.033
EA	0.04	0.057
KPG	0.033	0.055
PUG	0.025	0.013
PUN	0.032	0.033
RT	0.037	0.031
CHX 0.12%	0.012	0.018
Control DMSO 1%	0.0467	0.06

reductions ($p < 0.05$) compared to the untreated group, with PUN showing one of the most potent effects. Interestingly, KPG, EA, and RT retained hydrophobicity values statistically comparable to those of the control, suggesting less pronounced anti-hydrophobicity activity against *S. sobrinus*. For *S. gordonii* (Fig. 2c), the baseline hydrophobicity was lower (~35%) compared to the mutans group. CHX 0.12% again showed the most drastic reduction ($p < 0.05$). Among the natural compounds, only EA significantly reduced hydrophobicity ($p < 0.05$), while PUN, PUG, CG, and KPG did not produce a statistically significant effect, maintaining values comparable to the control. This suggests a degree of selectivity, where some compounds reduced hydrophobicity in cariogenic strains while sparing the commensal *S. gordonii*.

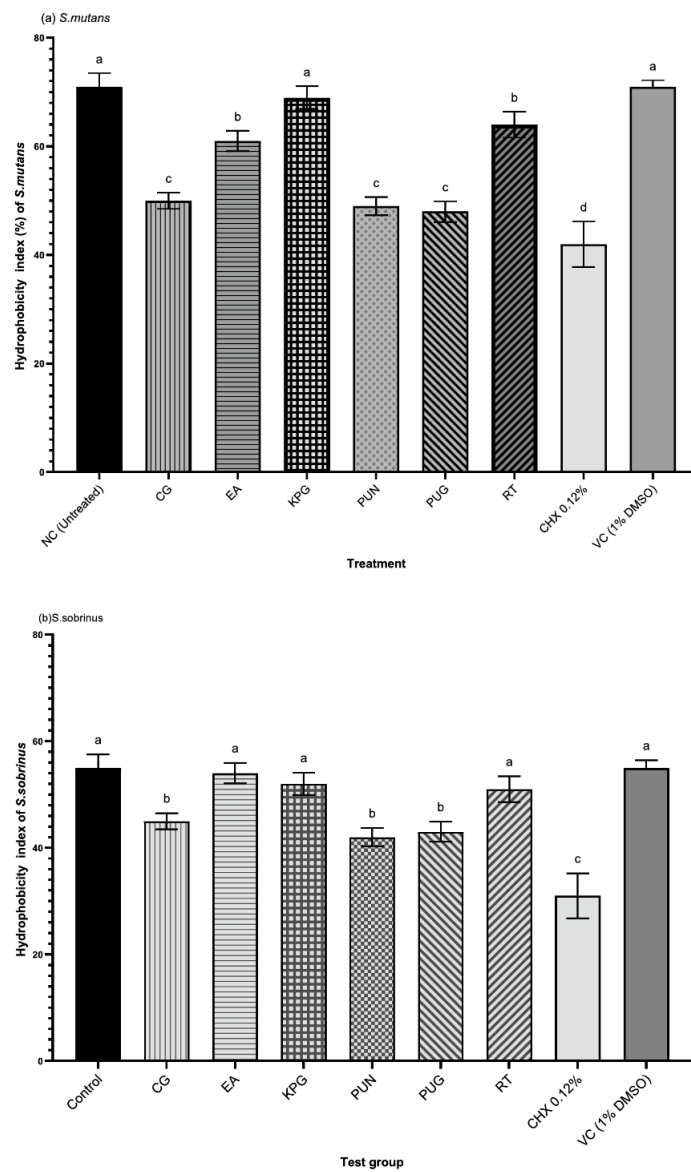


Fig. 2 The hydrophobicity index of (a) *S. mutans* (b) *S. sobrinus* and (c) *S. gordonii* after treatment with different test compounds. An untreated control was used as a baseline. Data are presented as

mean $\pm$ standard deviation ($n=5$). Bars with different letters (a–d) indicate statistically significant differences ($p < 0.05$, ANOVA followed by post-hoc test)

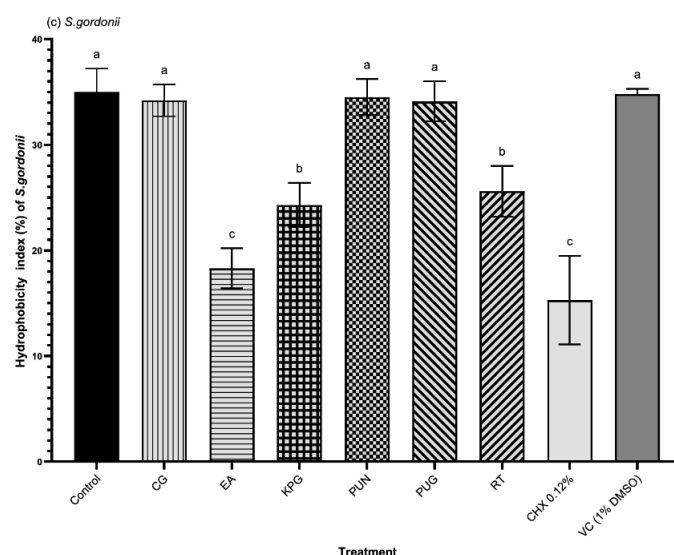


Fig. 2 (continued)

4 Discussion

This study demonstrated the selective antimicrobial and anti-virulence effects of purified pomegranate-derived phytochemicals against cariogenic mutans streptococci, while sparing the beneficial oral commensal *S. gordonii*. These findings align with the ecological approach to caries prevention, which favours microbiome-preserving strategies over broad-spectrum eradication.

The inclusion of both *S. mutans* and *S. sobrinus* was guided by increasing evidence of their synergistic role in cariogenesis. While *S. mutans* is well-established as a key etiological agent, *S. sobrinus* is often associated with increased caries severity, particularly in early childhood caries (ECC) and severe ECC. Studies have shown that individuals harboring both species exhibited significantly higher DMFT scores than those colonised by *S. mutans* alone (Korona-Glowniak et al. 2022; Veena and Nagarathna 2020; Gotouda et al. 2017). Thus evaluating both pathogens provides a more comprehensive and clinically relevant assessment of compound efficacy. To assess selectivity, *S. gordonii* was included as a representative oral commensal. *Streptococcus gordonii* an early coloniser of dental surfaces, supports oral microbial balance by competing with *S. mutans* for binding sites and nutrients while also generating alkaline by-products and antimicrobial substances (Ye et al. 2025).

The cytotoxicity profiles of the tested compounds were first evaluated using the brine shrimp lethality assay (BSLA), a widely accepted screening tool in natural product research. Most compounds exhibited low toxicity, with LC_{50} values exceeding 500 $\mu\text{g/mL}$. Notably, PUN and KPG demonstrated the highest LC_{50} values, while EA was the only compound classified under moderate toxicity ($LC_{50} = 159.2 \mu\text{g/mL}$). Nevertheless, this moderate cytotoxicity observed in vitro contrasts with findings from in vivo model. Oral administration of EA at doses up to 2000 mg/kg showed no sign of toxicity in animal (Hurtado-Núñez et al. 2022). Likewise, Sprague–Dawley rats receiving a punicalagin-rich diet ($\sim 4.8 \text{ g/kg/day}$) for 37 days exhibited no harmful effects (Cerdá et al. 2003). Collectively, these results indicate that although BSLA provides useful preliminary safety information, in vitro cytotoxicity outcomes may not fully predict in vivo tolerance, highlighting the importance of confirming safety through mammalian-based cytocompatibility assays in future work.

In addition to their antimicrobial selectivity, PUG and PUN significantly suppressed acidogenic activity in *S. mutans* and *S. sobrinus* as shown by the glycolytic pH drop assay in Sect. 3.4. The glycolytic pH drop assay reflects the capacity of cariogenic bacteria to ferment dietary sugars into organic acids, primarily lactic acid, leading to a rapid decrease in environmental pH and promoting enamel

demineralisation. *S. mutans* and *S. sobrinus* can persist and continue glycolytic activity at low-pH environments, which gives them an advantage over oral bacteria that are less tolerant to low pH. This adaptation is considered a key factor in caries progression (Nascimento et al. 2004). Marsh (2017) has also noted that oral microbial dysbiosis often arises from ecological changes, such as sustained acidity or inflammation, which favour the growth of acidogenic, aciduric, or proteolytic species. In the present study, treatment with PUG and PUN significantly reduced the glycolytic pH drop in both *S. mutans* and *S. sobrinus*, showing effective suppression of acid production. Lower acidogenicity implies interference with important virulence mechanisms and could help promote a shift towards a less cariogenic microbial balance. These findings are consistent with the ecological plaque hypothesis, which stresses modifying environmental conditions to influence microbial composition rather than relying solely on broad-spectrum antimicrobials (Rajasekaran et al. 2024).

The cell surface hydrophobicity (CSH) as demonstrated in Sect. 3.5 plays an important role in the initial adhesion of oral bacteria to tooth surfaces, which is the first step in biofilm formation. According to Spatafora et al. (2024), the adhesion to oral surfaces involves specific adhesins and non-specific forces such as hydrophobic interactions and van der Waals forces. Consequently, reducing the hydrophobicity index (HI) of bacterial cells can weaken their ability to adhere, coaggregate with other species, and establish biofilms. Previous studies have shown that several plant-derived compounds are capable of lowering the HI of *S. mutans*, thereby reducing its prevalence within treated groups (Karnjana et al. 2023; Hassan et al., 2015). However, little is known about how *S. sobrinus* responds to such interventions, and direct comparisons with *S. mutans* remain scarce. In the present study, treatment with pomegranate-derived compounds significantly reduced the HI of *S. mutans* and *S. sobrinus*. In contrast, *S. gordonii*, a beneficial early coloniser that contributes to microbial homeostasis, exhibited no significant changes in HI after treatment. This selective action suggests that the tested compounds can target pathogenic species while preserving commensal adhesion, an attribute that is crucial for maintaining a balanced oral microbiome.

PUG and PUN consistently outperformed other pomegranate-derived phytochemicals in their antimicrobial and anti-virulence activities against *S. mutans* and *S. sobrinus*. Their enhanced efficacy is likely attributed to their unique molecular structures and physicochemical properties. PUG (MW: 1084.7 g/mol) and PUN (MW: 782.5 g/mol) are large, highly hydroxylated ellagitannins containing multiple galloyl moieties. These features enable strong interactions with bacterial surface proteins, glucosyltransferases, and membrane components, contributing to their broad antimicrobial

potential. As hydrolysable tannins, PUG and PUN exert antibacterial effects through multiple, well-documented mechanisms. Tannins exert their antibacterial effects through multiple mechanisms, such as sequestering iron, disrupting cell membrane integrity, interfering with cell wall synthesis, and inhibiting fatty acid biosynthesis. Additionally, they can suppress the expression of key virulence factors, including those involved in biofilm development, enzymatic activity, adhesion, motility, and toxin production by acting as quorum-sensing antagonists (Farha et al. 2020). In contrast, EA, although structurally related as a degradation product of ellagitannins, exhibits low absorption primarily due to its poor solubility (less than 10 µg/mL) and low lipophilicity, which together limit its membrane permeability and stability in aqueous environments (Nyamba et al. 2021). The CG (molecular weight: 634.45 g/mol), while also an ellagitannin, is structurally smaller and shows moderate activity, possibly due to fewer reactive hydroxyl groups available for hydrogen bonding. Flavonoids such as GC, KPG, and RT are known for their antioxidant properties but generally possess less antimicrobial potency, which may be attributed to their limited structural complexity and fewer functional groups relevant to disrupting bacterial virulence mechanisms. These findings suggest that larger, highly polar, and polyphenol-rich compounds may exert stronger selective pressure on cariogenic bacteria.

Despite promising results, this work should be interpreted as a preliminary screening study. Only three bacterial species were evaluated, and virulence testing was restricted to acidogenicity and cell surface hydrophobicity. While these assays provide valuable first insights, other key virulence traits such as biofilm formation, extracellular polysaccharide production, and acidurance were not included. Moreover, the use of planktonic monospecies cultures does not fully capture the complex dynamics of multispecies oral biofilms. All tested strains, as described in Sect. 2.2, were laboratory reference strains, which may not reflect the diversity and resistance profiles of clinical isolates.

The cytotoxicity assessment was also limited to the brine shrimp lethality assay as described in Sect. 2.5, which, although widely used for preliminary toxicity screening, does not predict mammalian cytocompatibility. Importantly, no host-cell assays were conducted to evaluate the compatibility of the compounds with oral epithelial or keratinocyte cells. These limitations highlight the need for further studies incorporating dual- or multispecies biofilm models, clinical isolates, and host cell cytocompatibility assays to fully validate the selective anti-virulence potential of punicalagin and punicalin. In addition, integrating these compounds into prototype oral care formulations such as varnishes, rinses, or gels and testing their stability and performance under simulated oral conditions will be critical steps toward translational application.

5 Conclusion

This study highlights the selective antimicrobial and anti-virulence potential of purified pomegranate-derived compounds against cariogenic *Streptococcus* species. Punicalagin (PUG) and punicalin (PUN) demonstrated the most promising activity, combining selective bactericidal effects with inhibition of acidogenicity and surface hydrophobicity, two key traits in caries development. Their ability to suppress pathogenic species while sparing beneficial commensals underscores their relevance to microbiome-friendly and ecological approaches in oral health management. Overall, these findings provide an essential first step toward the translational development of phytochemical-based therapeutics in preventive dentistry. In particular, their incorporation into oral care formulations such as mouth rinse, gels or tooth-pastes represents a promising direction for future development, offering a strong foundation for more advanced studies and future formulation work.

Acknowledgements This study was carried out in Research Laboratory 1, Faculty of Dentistry, Universiti Teknologi MARA (UiTM), Sungai Buloh, Selangor, Malaysia. The authors sincerely acknowledge the invaluable support and contributions of the research team throughout the project.

Authors contributions Conceptualisation, NMZ FAR, IMA and MIAB; methodology, NMZ and FAR; investigation, NMZ; writing—original draft preparation, NMZ; writing—review and editing, NMZ, FAR, MIAB, IMA.

Funding None.

Data availability All data generated or analysed during this study are included in the manuscript. Further inquiries can be directed to the corresponding author.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

Open Access This article is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License, which permits any non-commercial use, sharing, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if you modified the licensed material. You do not have permission under this licence to share adapted material derived from this article or parts of it. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by-nc-nd/4.0/>.

References

- Alam S, Sarker MMR, Sultana TN, Chowdhury MNR, Rashid MA, Chaity NI et al (2022) Antidiabetic phytochemicals from medicinal plants: prospective candidates for new drug discovery and development. *Front Endocrinol* 13:800714. <https://doi.org/10.3389/fendo.2022.800714>
- Boulares A, Jdidi H, Bragazzi NL (2025) Impact of mouthwash-induced oral microbiome disruption on Alzheimer's disease risk: a perspective review. *Int Dent J* 75(1):45–50
- Cerdá B, Cerón JJ, Tomás-Barberán FA, Espín JC (2003) Repeated oral administration of high doses of the pomegranate ellagitannin punicalagin to rats for 37 days is not toxic. *J Agric Food Chem* 51(11):3493–3501
- Chaves FM, Pavan IC, da Silva LG, de Freitas LB, Rostagno MA, Antunes AE et al (2020) Pomegranate juice and peel extracts are able to inhibit proliferation, migration and colony formation of prostate cancer cell lines and modulate the Akt/mTOR/S6K signaling pathway. *Plant Foods Human Nutr* 75(1):54–62. <https://doi.org/10.1007/s11130-019-00776-0>
- Clinical and Laboratory Standards Institute (2012) Performance standards for antimicrobial disk susceptibility tests; approved standard—eleventh edition. CLSI document M02-A11. Wayne, PA
- Clinical and Laboratory Standards Institute (2018) Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. 11th ed. CLSI standard M07. Wayne, PA
- Eid SA, Khattab NMA, Elheeny AA (2020) Untreated dental caries prevalence and impact on the quality of life among 11 to 14-year-old Egyptian schoolchildren: a cross-sectional study. *BMC Oral Health* 20:83. <https://doi.org/10.1186/s12903-020-01077-8>
- Farha AK, Yang QQ, Kim G, Li HB, Zhu F, Liu HY et al (2020) Tannins as an alternative to antibiotics. *Food Biosci* 38:100751. <https://doi.org/10.1016/j.fbio.2020.100751>
- Gotouda H, Shinozaki-Kuwahara N, Taguchi C, Ohta M, Shimosaka M, Ito T et al (2017) Evaluation of dental caries and concomitant infection based on the ratio of cariogenic bacteria using plaque samples in adults. *Dentistry* 7(461):2161–1122. <https://doi.org/10.4172/2161-1122.1000461>
- He Z, Huang Z, Jiang W, Zhou W (2019) Antimicrobial activity of cinnamaldehyde on *Streptococcus mutans* biofilms. *Front Microbiol* 10:2241. <https://doi.org/10.3389/fmicb.2019.02241>
- Hurtado-Núñez GE, Cortés-Rojas C, Sánchez-Ceja SG, Martínez-Flores HE, Salgado-Garciglia R, Bartolomé-Camacho MC et al (2022) Gallic, ellagic acids and their oral combined administration induce kidney, lung, and heart injury after acute exposure in Wistar rats. *Food Chem Toxicol* 170:113492. <https://doi.org/10.1016/j.fct.2022.113492>
- James P, Worthington HV, Parnell C, Harding M, Lamont T, Cheung A, Whelton H, Riley P (2017) Chlorhexidine mouthrinse as an adjunctive treatment for gingival health. *Cochrane Database Syst Rev* 3(3):CD008676. <https://doi.org/10.1002/14651858.CD008676>
- Karnjana K, Jewboonchu J, Niyomtham N, Tangngamsakul P, Bunluepuech K, Goodla L et al (2023) The potency of herbal extracts and its green synthesized nanoparticle formulation as antibacterial agents against *Streptococcus mutans* associated biofilms. *Biotechnol Rep* 37:e00777. <https://doi.org/10.1016/j.btre.2022.e00777>
- Korona-Glowniak I, Skawinska-Bednarczyk A, Wrobel R, Pietrak J, Tkacz-Ciebia I, Maslanko-Switala M et al (2022) *Streptococcus sobrinus* as a predominant oral bacteria related to the occurrence of dental caries in Polish children at 12 years old. *Int J Environ Res Public Health* 15(22):15005. <https://doi.org/10.3390/ijerph152215005>
- Lima SLDA, Santana CCP, Paschoal MAB, Paiva SM, Ferreira MC (2018) Impact of untreated dental caries on the quality of life of

- Brazilian children: population-based study. *Int J Paediatr Dent* 28(4):390–399
- Man G, Xu L, Wang Y, Liao X, Xu Z (2022) Profiling phenolic composition in pomegranate peel from nine selected cultivars using UHPLC-QTOF-MS and UPLC-QQQ-MS. *Front Nutr* 8:807447. <https://doi.org/10.3389/fnut.2021.807447>
- Marsh PD (2017) Ecological events in oral health and disease: new opportunities for prevention and disease control? *J Calif Dent Assoc* 45(10):525–537. <https://doi.org/10.1177/0022034517735295>
- Nascimento MM, Lemos JA, Abranches J, Gonçalves RB, Burne RA (2004) Adaptive acid tolerance response of *Streptococcus sobrinus*. *J Bacteriol* 186(19):6383–6390. <https://doi.org/10.1128/jb.186.19.6383-6390.2004>
- Nemmani KVS (2021) Pharmacological Screening: Drug Discovery. In: Poduri R (ed) *Drug discovery and development: from targets and molecules to medicines*. Springer, Singapore, pp 211–233. https://doi.org/10.1007/978-981-15-5534-3_7
- Nyamba I, Lechanteur A, Semdé R, Evrard B (2021) Physical formulation approaches for improving aqueous solubility and bioavailability of ellagic acid: a review. *Eur J Pharm Biopharm* 159:198–210. <https://doi.org/10.1016/j.ejpb.2020.11.004>
- Okada M, Kawamura M, Oda Y, Yasuda RIE, Kojima T, Kurihara H (2012) Caries prevalence associated with *Streptococcus mutans* and *Streptococcus sobrinus* in Japanese schoolchildren. *Int J Paediatr Dent* 22(5):342–348
- Rajasekaran JJ, Krishnamurthy HK, Bosco J, Jayaraman V, Krishna K, Wang T, Bei K (2024) Oral microbiome: a review of its impact on oral and systemic health. *Microorganisms*. <https://doi.org/10.3390/microorganisms12091797>
- Rosenberg M (2006) Microbial adhesion to hydrocarbons: twenty-five years of doing MATH. *FEMS Microbiol Lett* 262(2):129–134. <https://doi.org/10.1111/j.1574-6968.2006.00291.x>
- Sarah QS, Anny FC, Misbahuddin M (2017) Brine shrimp lethality assay. *Bangladesh J Pharmacol* 12(2):186–189. <https://doi.org/10.3329/bjp.v12i2.32796>
- Spatafora G, Li Y, He X, Cowan A, Tanner ACR (2024) The evolving microbiome of dental caries. *Microorganisms* 12(1):121. <https://doi.org/10.3390/microorganisms12010121>
- Sweidan N, Abu Rayyan W, Mahmoud I, Ali L (2023) Phytochemical analysis, antioxidant, and antimicrobial activities of Jordanian pomegranate peels. *PLoS ONE* 18(11):e0295129. <https://doi.org/10.1371/journal.pone.0295129>
- Tzimas K, Antoniadou M, Varzakas T, Voidarou C (2024) Plant-derived compounds: a promising tool for dental caries prevention. *Curr Issues Mol Biol* 46(6):5257–5290. <https://doi.org/10.3390/cimb46060315>
- Veena RL, Nagarathna C (2020) Correlation of *Streptococcus mutans* and *Streptococcus sobrinus* colonization with and without caries experience in preschool children. *Indian J Dent Res* 31(1):73–79
- Ye D, Liu Y, Li J, Zhou J, Cao J, Wu Y et al (2025) Competitive dynamics and balance between *Streptococcus mutans* and commensal streptococci in oral microecology. *Crit Rev Microbiol* 51(3):532–543. <https://doi.org/10.1080/1040841X.2024.2389386>
- Young JE, Pan Z, Teh HE, Menon V, Moderegger B, Pesek JJ et al (2017) Phenolic composition of pomegranate peel extracts using an liquid chromatography-mass spectrometry approach with silica hydride columns. *J Sep Sci* 40(7):1449–1456

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

APPENDIX 7

Published article titled “Antibiofilm activity of punicalin and punicalagin against dual-species *Streptococcus* biofilms: Molecular and microbiological evaluation” in the Asia-Pacific Journal of Molecular Biology and Biotechnology (2025)



AsPac J. Mol. Biol. Biotechnol. 2025
ISSN: 0128-7451, eISSN: 2672-7277
Vol. 33 (4): 128-137
<https://doi.org/10.35118/apjmbb.2025.033.4.12>

RESEARCH ARTICLE

Antibiofilm activity of Punicalin and Punicalagin against dual-species *Streptococcus* biofilms: Molecular and microbiological evaluation

Nurhayati Mohamad Zain^{a*}, Indah Amin^a, Fathilah Abdul Razak^{b,c}, Mohamed Ibrahim Abu Hassan^{d,e*}

^aCentre of Preclinical Science Studies, Faculty of Dentistry, Universiti Teknologi MARA, Sungai Buloh Campus, Selangor, Malaysia

^bFaculty of Dentistry, Universiti Malaya, Kuala Lumpur, Malaysia

^cSchool of Dentistry, Management and Science University, Shah Alam, Malaysia

^dFaculty of Dentistry, MAHSA University, Jalan SP 2, Bandar Saujana, 42610 Jenjarom, Selangor, Malaysia

^eCentre of Restorative Dentistry Studies, Faculty of Dentistry, Universiti Teknologi MARA, Sungai Buloh Campus, Selangor, Malaysia

*Correspondence email: nurhayati8370@uitm.edu.my; mibrahim@uitm.edu.my

Abstract

Dental caries is a biofilm-associated disease primarily caused by *Streptococcus mutans* (*S. mutans*), while *Streptococcus gordonii* (*S. gordonii*) influences the early stages of biofilm formation. Natural compounds with selective antibiofilm activity offer the potential for alternative caries prevention. This study investigates the antibiofilm effects of punicalin (PUN) and punicalagin (PUG) from pomegranate against *S. mutans* and *S. gordonii* in single- and dual-species biofilms. The minimum biofilm inhibitory concentration (MBIC) of phytocompounds against both single and dual-species biofilms was evaluated using the crystal violet assay. The MBIC₉₀ concentration, determined as 50 µg/mL, was selected and further examined using a glass bead assay to assess the impact on gene expression in *S. mutans* and *S. gordonii* through RT-qPCR analysis. Additionally, we prepared PUN- and PUG-treated biofilms for morphological examination using scanning electron microscopy. Our results demonstrated that PUN and PUG exhibited significant antibiofilm activities against single- and dual-species biofilms. Particularly, PUN showed a notable reduction in bacterial adherence and biofilm formation ($P < 0.05$) across all tested species. Conversely, PUG displayed selective antibiofilm activity, primarily targeting *S. mutans*. The RT-qPCR data confirmed reduced expression of virulence genes in *S. mutans*, which may contribute to the antibiofilm activity. Our findings highlight the potential of PUN and PUG as effective inhibitors of biofilm formation and their ability to influence virulence factors in *S. mutans* dual-species biofilms. These compounds have potential to be incorporated into clinical applications as antibiofilm agents, thereby aiding in the prevention of dental caries development.

Keywords: antibiofilm, dual-species, punicalagin, punicalin, *Streptococcus gordonii*, *Streptococcus mutans*

INTRODUCTION

Dental caries remains one of the most prevalent oral diseases, affecting approximately 2 billion individuals with permanent teeth and 514 million children with primary tooth decay (Global Oral Health Status Report: Towards Universal Health Coverage for Oral Health by 2030, 2022). The biofilm-associated disease arises from an imbalance in the oral microbiome, favouring acidogenic and aciduric bacteria that promote enamel demineralisation and subsequent tooth decay (Zhan, 2018). As dysbiosis progresses, cariogenic bacteria such as *Streptococcus mutans* (*S. mutans*) dominate the biofilm, producing

extracellular polysaccharides (EPS) that enhance adhesion and acid retention. Conversely, *Streptococcus gordonii* (*S. gordonii*), a commensal oral species, is involved in early biofilm formation and microbial homeostasis, potentially inhibiting the overgrowth of pathogenic species (Abranches et al., 2018). Traditional caries prevention primarily focuses on mechanical plaque removal through toothbrushing and flossing. However, complete biofilm elimination is challenging, particularly for individuals with limited manual dexterity, such as children and elderly patients. Dental professionals often recommend mouthwashes containing chlorhexidine (CHX), the gold-standard antiseptic, to improve oral hygiene (Brookes et al.,

Received: 11 March 2025

Accepted: 1 October 2025

Published: 5 December 2025

Published by: Malaysian Society for Molecular Biology and Biotechnology (MSMBB)



2020). Chlorhexidine reduces biofilm by disrupting bacterial cell membranes and causing cell lysis (Łukomska-Szymańska et al., 2017). However, prolonged use is associated with undesirable side effects, including tooth staining (Brookes et al., 2020), antimicrobial resistance (Kampf, 2016), and hypersensitivity reaction, including severe anaphylaxis (Fontaine et al., 2024).

Numerous *in vitro* studies have successfully identified pure compounds exhibiting antimicrobial and antibiofilm activity against *S. mutans* (Gabe et al., 2019; Zeng et al., 2019). However, extending efficacy assessment to dual-species and multispecies biofilms is essential to reflect better actual oral conditions, where *S. mutans* interacts with other microbes, influencing biofilm structure, resilience, and antimicrobial response. Previous *in vitro* and clinical studies have shown that pomegranate peel extract effectively reduces oral plaque by targeting oral microorganisms (Jacob et al., 2021; Abd-El-Aziz & A Sallam, 2020; Ferrazzano et al., 2017; Umar et al., 2016; Subramaniam et al., 2012). However, the effective concentrations reported in studies vary due to differences in pomegranate cultivars, countries of origin, and extraction solvents, leading to inconsistencies that make standardisation and commercialization difficult. A promising approach is to investigate purified bioactive compounds from pomegranate for a more comprehensive analysis. While pomegranate peel extracts contain various polyphenols including punicalagin, punicalin, ellagic acid, catechin and gallic acid (Man et al., 2022; Li et al., 2015), studies suggest that punicalagin (PUG) and punicalin (PUN) play a more prominent role. Therefore, this study evaluates the antibiofilm effects of purified PUG and PUN to determine their targeted effects on *S. mutans* and *S. gordonii* in dual-species biofilms. A particular focus is placed on their effects at the molecular level, including alterations in virulence gene expression in both species.

MATERIALS AND METHODS

Compound and preparation

PUN (CAS 65995-64-4) and PUG (CAS 65995-63-3) from *Punica granatum* L. ($\geq 98\%$ purity, HPLC) were purchased from Chengdu Biopurify Phytochemicals Ltd (China). They were dissolved in 10% DMSO (Sigma-Aldrich) and stored as 20 mg/mL stock at -20°C . For assays, compounds were diluted in DMSO, ensuring a working concentration of $\leq 1\%$ to prevent any antimicrobial effects from DMSO.

Bacterial strains

S. gordonii ATCC 51656 and *S. mutans* UA 159 (ATCC)

were obtained from the Microb Bank, Research Lab 1, Dentistry, UiTM, and stored in 25% glycerol.

Saliva collection and preparation of experimental pellicle

Written consent was obtained from a healthy 40-year-old female subject who met the exclusion criteria. The study was approved by the UiTM Research Ethics Committee (REC/01/2023 (PG/MR/27)). Stimulated whole saliva (50 mL) was collected using Parafilm M stimulation, mixed 1:1 with adsorption buffer, supplemented with 0.1 M phenylmethylsulfonyl fluoride (PMSF), and centrifuged at 5500 g for 10 min at 4°C (Rahmani-Badi et al., 2015). The supernatant was filtered through a $0.22\ \mu\text{m}$ polyethersulfone (PES) filter and used immediately to preserve enzymatic activity (Lemos et al., 2010).

Determination of minimum biofilm inhibition concentration (MBIC₉₀) using the crystal violet assay

This study employed two types of biofilm assays: (1) a microtiter plate-based biofilm inhibition assay to determine MBIC₉₀ using the crystal violet assay and (2) a biofilm bead assay using MBIC₉₀ concentrations from the previous assay to assess biofilm formation, microcolony architecture, and gene expression. The MBIC₉₀ was defined as the lowest compound concentration that reduces biofilm formation by at least 90% compared to untreated controls. In this study, MBIC₉₀ was chosen over MBIC₅₀ for several reasons. A 90% reduction threshold ensures more potent and more clinically relevant inhibition of biofilm formation, aligning with the expected efficacy of antibiofilm agents used in oral health applications.

Single- and dual-species biofilms were grown in 96-well polystyrene plates (Corning® Costar®) following a modified protocol by Sun et al. (2019). An experimental pellicle was formed by treating wells with clarified human saliva for 120 minutes at 37°C , with the saliva replaced every 30 minutes (Pelá et al., 2020). *S. mutans* and *S. gordonii* suspensions (10^6 CFU/mL) were mixed with PUG and PUN, resulting in 25–400 $\mu\text{g/mL}$ final concentrations. Dual-species biofilms were established using a 1:1 ratio by mixing equal volumes of each bacterial suspension, 100 μL of *S. mutans* and 100 μL of *S. gordonii*. Cultures in 1% DMSO served as the negative control (solvent control), while 0.12% CHX, a widely used oral antiseptic with established antimicrobial activity, was used as the positive control. After 24 hours of incubation at 37°C , biofilms were washed, gently tapped to remove excess water and stained with 0.1% crystal violet for 30 min, and destained with 30% glacial acetic acid. Biofilm biomass was quantified by

measuring absorbance at 595 nm using a Tecan Infinite® 200 PRO. Each experiment was conducted in triplicate with five independent replicates. Results were expressed as biofilm inhibition percentages using the following equation (Teanpaisan et al., 2017).

$$\text{Biofilm inhibition \%} = \left[1 - \left(\frac{\text{OD test}}{\text{OD negative control}} \right) \right] \times 100$$

We aimed to determine the minimum biofilm inhibition concentration (MBIC₉₀), the lowest compound concentration that reduces biofilm formation by at least 90% compared to the untreated control. The MBIC₉₀ concentration was then used in the subsequent biofilm bead assay.

Biofilm bead assay

The biofilm bead assay was optimised based on Konrat et al. (2016) to determine the ideal bacterial ratio and concentration for stable biofilm formation. The acquired pellicle was formed on glass beads (5 mm radius), which were individually placed in 24-well plates and inoculated with *S. mutans* and *S. gordonii* at a 1:1 ratio (1×10^6 CFU/mL). The assay evaluated the effects of 50 µg/mL (MBIC₉₀) of PUN and PUG on biofilm formation, microcolony architecture, and expression of virulence genes. The concentration of 50 µg/mL for PUN and PUG was chosen based on its MBIC₉₀ value for *S. mutans*, as this bacterium plays a primary role in cariogenic biofilms. Beads were collected at 8 and 24 hours for CFU counting (bacterial adherence), SEM analysis (microcolony architecture), and RNA extraction (expression of virulence genes).

Gene expression analysis (RT-qPCR)

Gene expression analysis was conducted using reverse transcription quantitative PCR (RT-qPCR) with SYBR Green dye as the fluorescent reporter, in accordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009). For *S. mutans*, the primer sequences for the reference gene (16S rRNA) and biofilm-associated genes were adapted from Kim et al. (2015). These genes include *gfb* (water-insoluble glucan production), *gfc* (soluble and insoluble glucans synthesis), *gfd* (water-soluble glucan production), and *glpB* (encoding a glucan-binding protein). Additionally, the primers for *brpA*, a biofilm regulatory protein, and *comDE*, which plays a role in competence-stimulating peptide production, were adapted from Hasan et al. 2015. For *S. gordonii*, the 16S rRNA gene served as an internal reference. The biofilm-associated genes analysed included *gfb*

(glucosyltransferase production); *abpA* (amylase-binding protein synthesis); *eshA*, (encoding a high-molecular-weight adhesin for biofilm formation); and *sspA*, (encoding a surface protein crucial for adherence to salivary agglutinin glycoproteins and coaggregation with other oral bacteria). All primers were adapted from Huang et al. 2014. The complete primer sequences for all genes are provided in Supplementary Table 1. The relative expression levels were analysed via the 2^{-ΔΔCT} method.

Scanning electron microscopy (SEM)

Beads were fixed in 4% glutaraldehyde overnight, washed with 0.1 M sodium cacodylate buffer (pH 7.4), and fixed in 2% osmium tetroxide overnight. The samples underwent stepwise ethanol dehydration, followed by immersion in acetone and critical point drying (~2 hours). Dried samples were mounted on aluminium stubs, gold-coated, and examined at 1,000× and 5,000× magnification using a Quanta™ FEG 450 SEM (Netherlands).

Statistical analysis

Statistical analysis was carried out using IBM SPSS Statistics 27. All experiments were conducted in triplicate with five independent replicates, and data were expressed as a mean ± standard deviation (SD). The normality of the data was assessed using the Shapiro-Wilk test, and Levene's test was used to evaluate the homogeneity of variances. For the comparison of multiple groups, a one-way analysis of variance (ANOVA) was conducted, followed by Tukey's Honestly Significant Difference (HSD) test for post hoc analysis. Statistical significance was set at $p < 0.05$, with highly significant differences at $p < 0.01$.

RESULTS

Minimum biofilm inhibition concentration (MBIC₉₀)

The difference in MBIC₉₀ values between PUG and PUN suggests variations in antibiofilm activity. The MBIC₉₀ values for PUG were 50 µg/mL for *S. mutans*, 100 µg/mL for *S. gordonii*, and 100 µg/mL for the mixed biofilm. The MBIC₉₀ values for PUN were 50 µg/mL for *S. mutans*, *S. gordonii*, and the mixed biofilm. The MBIC₉₀ for CHX was consistently 0.12% across all tested biofilms.

Biofilm bead assay

Figure 1 shows log₁₀ CFU/bead values, illustrating inhibition of dual-species biofilm after exposure to 50 µg/mL PUG and PUN, 1% DMSO (negative control), and 0.12% CHX (positive control) at 8 and

24 hours. In the negative control group, the *S. gordonii* population was higher at 8 hours ($1.9 \log_{10}$ CFU/bead) than *S. mutans* ($1.51 \log_{10}$ CFU/bead). However, by 24 hours, *S. mutans* became dominant, reaching $5.14 \log_{10}$ CFU/bead, while *S. gordonii* was $2.84 \log_{10}$ CFU/bead. In the PUG- and PUN-treated groups, both compounds inhibited *S. mutans*

adherence at 8 hours compared to the negative control, while not affecting *S. gordonii*. By 24 hours, both compounds reduced total bacterial counts, but their effects differed: PUN inhibited both species, while PUG selectively reduced *S. mutans*. Notably, *S. mutans* levels in the PUG-treated group at 24 hours were similar to those in the CHX group.

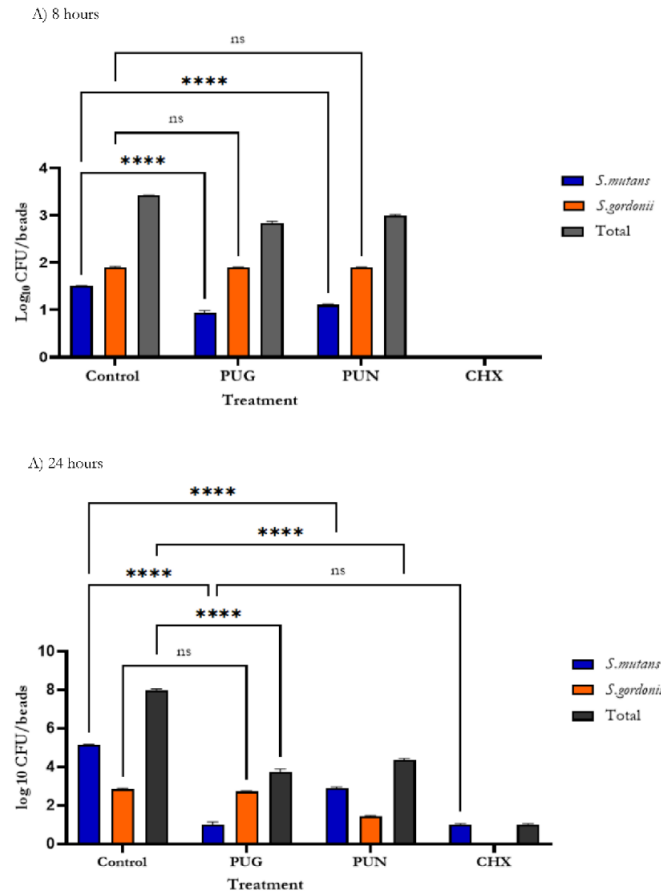


Figure 1. Effect of PUG and PUN on dual-species biofilm formation of *S. mutans* and *S. gordonii* at 8 and 24 hours. (A) Log CFU/bead values at 8 hours and B) at 24 hours for biofilm treated with 50 μ g/mL PUN, 50 μ g/mL PUG, 0.12% CHX (positive control) and 1% DMSO (negative control). The bar represents mean values $\pm$ standard deviations obtained from triplicate measurements conducted in five independent experiments. Asterisks indicate statistical significance, while "ns" denotes non-significant differences. The statistical analysis employed ANOVA followed by Tukey's HSD test, with a significance level set at $p = 0.05$.

Biofilm architecture by SEM

Figure 2 presents SEM micrographs of beads treated with 1% DMSO, PUN, PUG, and 0.12% CHX for 24 hours. The negative control biofilms (Figure 2-A-I and Figure 2A-II) exhibited multilayered, confluent, and thick biofilm masses showing extensive streptococcal interactions. The PUG-treated group showed a cluster of cells with scattered cells (Figure 2B-I and Figure 2B-II). Although SEM cannot differentiate between *S. mutans* and *S. gordonii*, viable cell count

analysis confirmed a higher colonisation of *S. gordonii* in the PUG-treated group. The PUN-treated group exhibited fewer cells and microcolonies via SEM (Figure 2C-I and Figure 2C-II). Scanning electron micrograph (SEM) of CHX-treated biofilm (Figure 2D-I and Figure 2D-II) showing disrupted bacterial structures with visible debris agglutination. The large, clustered formations may represent agglutinated cellular debris resulting from membrane lysis, a characteristic effect of chlorhexidine treatment.

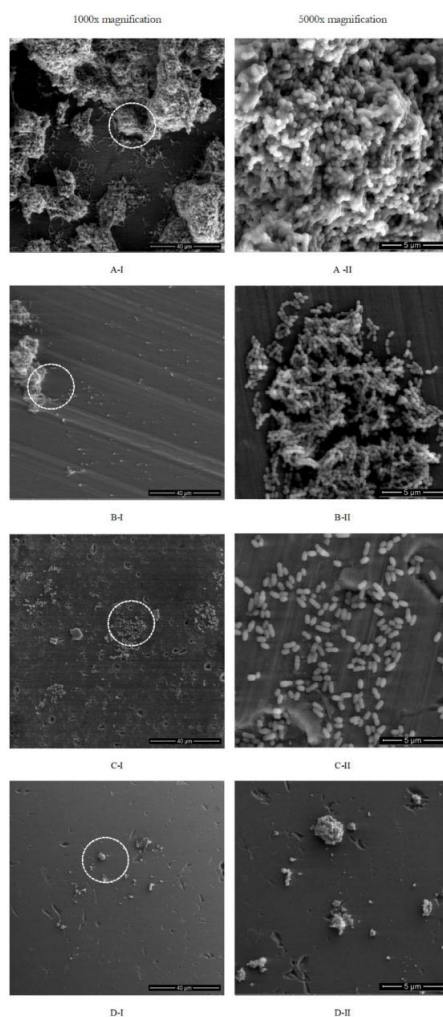


Figure 2. Morphological alteration of dual-species biofilm after 24 hours of treatment with PUG and PUN. SEM micrograph at 1000x (left column) and 5000x magnifications (right column) showing biofilm architecture for (A-I and A-II) 1% DMSO (negative control), (B-I and B-II) PUG 50 µg/mL, (C-I and C-II) PUN 50 µg/mL, (D-I and D-II) 0.12% CHX. The white circle indicates the region where the picture was captured for 5000x magnification.

Gene expression analysis

Figure 3 illustrates the gene expression fold changes for *S. mutans* within dual-species biofilm treated with PUG and PUN. Choosing 8 hours allows for assessing whether PUN and PUG interfere with the initial attachment and extracellular matrix production, and 24 hours reflects a mature biofilm, where key virulence genes regulate stability and structural integrity. At the 8-hour time, both PUG and PUN resulted in the downregulation of the tested virulence genes (*gffC*, *gffB*, *gffD*, *glpB*, *comDE* and *brpA*) in *S. mutans*. However, the suppressive effect was more pronounced with PUG, indicating a more significant

impact on gene expression. At 24 hours, a similar downregulation pattern was observed, with both PUG and PUN suppressing the tested virulence genes of *S. mutans*, although the effect was more substantial with PUG. Figure 4 shows the gene expression fold changes for *S. gordonii* within the same biofilm during treatment with PUG and PUN. Treatment with PUN downregulated all the tested genes (*gffG*, *abpA*, *csbA* and *sspA*). However, no gene downregulation was detected at the 8-hour sampling for the PUG-treated group, whereas at 24 hours, only *gffG* was downregulated in *S. gordonii*.

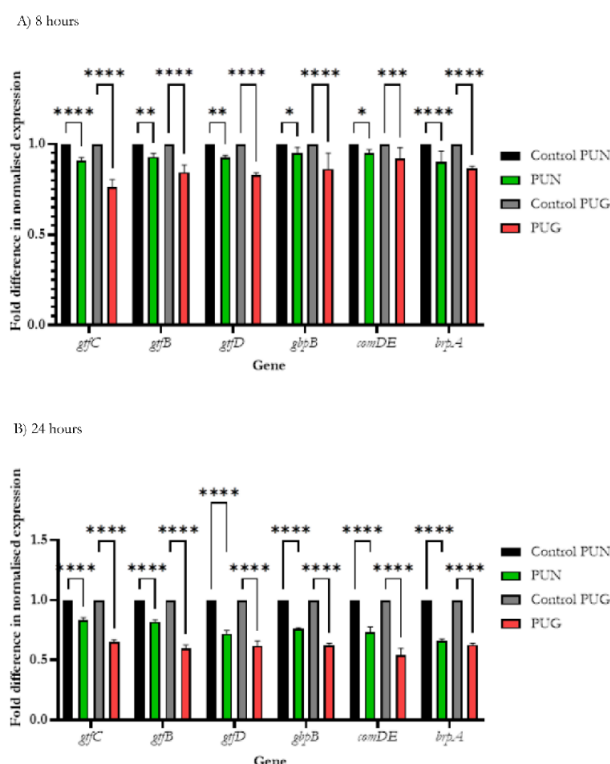


Figure 3. Effect of PUG and PUN on *S. mutans* gene expression in dual-species biofilm at (A) 8 and (B) 24 hours in a sucrose-dependent environment. The expression level of each gene was normalised to 16S rRNA expression, and the fold change relative to the control was determined using the $2^{-\Delta\Delta CT}$ method. The results are presented as the mean $\pm$ SD with the error bars indicating standard deviation. Statistical significance was denoted by **, $P < 0.05$; ***, $P < 0.0005$; ****, $P < 0.0001$, while “ns” indicates non-significant differences.

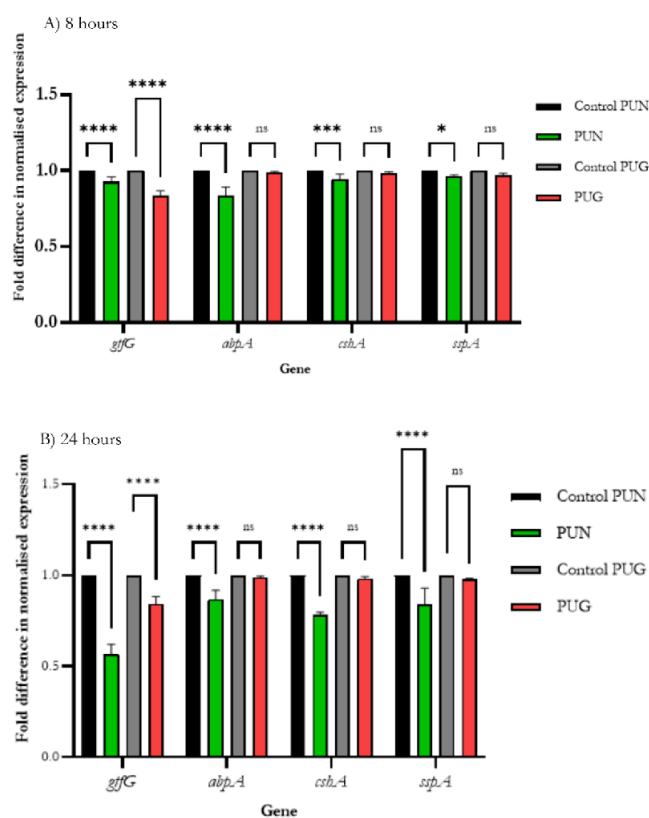


Figure 4. Effect of PUG and PUN *S. gordonii* gene expression in dual-species biofilm at (A) 8 hours and (B) 24 hours in a sucrose-dependent environment. The expression level of each gene was normalised to 16S rRNA expression, and the fold change relative to the control was determined using the $2^{-\Delta\Delta CT}$ method. The results are presented as the mean $\pm$ SD with the error bars indicating standard deviation. Statistical significance was denoted by **, $P < 0.05$; ***, $P < 0.0005$; ****, $P < 0.0001$, while “ns” indicates non-significant differences.

DISCUSSION

This study assessed the in vitro antibiofilm activity of pomegranate-derived PUN and PUG against cariogenic bacteria *S. mutans* and commensal species *S. gordonii* in a dual-species model. Unlike conventional antimicrobial agents that indiscriminately disrupt the oral microbiome, potentially leading to dysbiosis and antimicrobial resistance, our approach focuses on selectively inhibiting *S. mutans* while preserving beneficial commensals such as *S. gordonii*. The findings suggest that PUG exhibits more selective

antibiofilm activity, while PUN has a broader inhibitory effect on both species.

The ability of *S. mutans* to form a robust biofilm is heavily dependent on its glucosyltransferase enzymes (Gtfs), which synthesise extracellular polysaccharides (EPS) to facilitate adhesion and bacterial aggregation (Kim et al., 2015). Our results show that PUG and PUN significantly downregulate key biofilm-associated genes in *S. mutans*, particularly *gffB* (responsible for water-insoluble glucan synthesis) and *glpB* (a glucan-binding protein that stabilises the biofilm matrix). Suppression of these genes leads to a

weaker biofilm structure, making bacterial detachment easier and reducing overall pathogenicity. This aligns with previous studies showing that phytochemicals inhibit *S. mutans* biofilm via virulence genes suppression (Kaur et al., 2017; Hasan et al., 2015; Kim et al., 2015). However, most of these studies focus on single-species biofilms, which fail to capture the synergistic and competitive interactions within oral microbial communities. In contrast, *S. gordonii* biofilms are primarily influenced by *gffG*, which facilitates interbacterial interactions and glucan-mediated adhesion. While PUN broadly inhibited adhesion-associated genes (*csbA*, *sspA*) and *gffG*, PUG selectively downregulated *gffG* at 24 hours without affecting other genes, preserving commensal biofilm integrity. This suggests that PUG preferentially inhibits *S. mutans* while sparing *S. gordonii*, making it a promising candidate for microbiome-friendly antibiofilm therapy.

One key finding is the differential effects of PUG and PUN on dual-species biofilms. PUG selectively inhibits *S. mutans*, likely due to its strong affinity for *S. mutans* adhesins and EPS-regulating pathways, making it a more targeted inhibitor of cariogenic biofilms. In contrast, PUN exhibits broader antibiofilm activity, suppressing both *S. mutans* and *S. gordonii*. This difference may be attributed to structural variations between the two compounds. PUG, as a larger ellagitannin, may interact more selectively with *S. mutans* surface adhesins, potentially blocking key biofilm-forming pathways. PUN, with a simpler structure, may disrupt biofilm integrity across both species, making it a more general antibiofilm agent. This apparent species-dependent activity observed in our dual-species model suggests a potential role in maintaining microbial balance. This balance is particularly important in preventing caries progression while preserving commensal bacteria, as oral homeostasis depends on a delicate balance between cariogenic and non-cariogenic species (Abbranches et al., 2018). Sucrose is a key environmental factor influencing cariogenic biofilm development.

In a sucrose-supplemented environment, *S. mutans* typically outcompete commensal species, forming a highly structured acidogenic biofilm (Díaz-Garrido et al., 2020). Our findings show that PUG and PUN remained effective against *S. mutans* despite the presence of sucrose, suggesting their potential to counteract sucrose-driven biofilm formation. Clinically, this is significant because PUG selectivity for *S. mutans* may preserve oral commensals, whereas PUN could provide broader antibiofilm coverage.

To our knowledge, this is the first in vitro study specifically evaluating isolated PUN and PUG against dual-species *S. mutans*-*S. gordonii* biofilms. Clinical

studies on pomegranate peel extract have reported a reduction in oral plaque, salivary *S. mutans* and improved oral hygiene in patients (Marrone et al., 2024; Mahd et al., 2023; Pinni et al., 2018), but these studies used whole extracts rather than purified compounds. In general, whole extracts contain multiple bioactive compounds, which may vary in concentration due to differences in plant source, extraction method, and environmental factors (Khanna et al., 2025; Haido et al., 2024; Vázquez-León et al., 2017). This variability affects reproducibility and makes it difficult to determine which specific compounds are responsible for the observed effects. Selective isolation allows for dose optimisation and toxicity assessment, supporting safer translation into clinical use. A common challenge in using isolated phytochemicals is the potential loss of efficacy that can occur when they are separated from their natural matrix. However, our results demonstrate that both PUG and PUN exhibit significant antibiofilm activity independently, aligning with other studies on the efficacy of single compounds, such as thymol isolated from the essential oil of *Thymus* spp. (Utami et al., 2021; Miladi et al., 2017). This suggests that PUG and PUN are capable of acting independently, making them promising candidates for targeted caries prevention strategies.

Future studies should investigate the impact of PUG and PUN on other commensal species, such as *S. sanguinis* and *S. mitis*, to assess their broader microbiome selectivity and potential effects on maintaining microbial balance. To complement the gene expression data, advanced bioinformatics and molecular docking analyses could provide insight into how PUG and PUN interact with bacterial surface proteins involved in biofilm formation. Understanding these interactions could refine their formulation and optimise their effectiveness as targeted oral health therapies.

CONCLUSION

Exposure to PUN and PUG significantly affected the formation of dual-species biofilm involving *S. gordonii* and *S. mutans*. PUN exhibited broad-spectrum antibiofilm activity, targeting both bacterial species rather than acting selectively on *S. mutans*. In contrast, PUG selectively regulated the composition of dual-species biofilms by reducing the *S. mutans* population, reducing EPS synthesis and disrupting biofilm formation while sparing *S. gordonii*. This selective targeting of *S. mutans*, even in the presence of commensal *S. gordonii*, highlights PUG's potential as a targeted antibiofilm agent. Furthermore, PUG may

serve as a valuable tool for investigating the molecular mechanisms by which *S. mutans* responds to biofilm inhibitors, while commensal streptococci evade its inhibitory effects, maintaining microbial balance in the oral environment.

ACKNOWLEDGEMENTS

This research was carried out in Research Laboratory 1, Faculty of Dentistry, UiTM Sungai Buloh, Selangor, Malaysia. We extend our appreciation to all research team members for their foundational contributions and unwavering support throughout the study. The study was approved by the UiTM Research Ethics Committee (REC/01/2023 (PG/MR/27))

CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

REFERENCES

- Abd-El-Aziz, A. B., & A Sallam, R. (2020). Antibacterial effect of green tea and pomegranate peel extracts on *Streptococcus mutans* of orthodontic treated patients. *Journal of Radiation Research and Applied Sciences*, 13(1), 132–143. <https://doi.org/10.1080/16878507.2019.1693733>
- Abranches, J., Zeng, L., Kajfasz, J. K., Palmer, S. R., Chakraborty, B., Wen, Z. T., Richards, V. P., Brady, L. J., & Lemos, J. A. (2018). Biology of oral streptococci. *Microbiology Spectrum*, 6(5). <https://doi.org/10.1128/microbiolspec.gpp3-0042-2018>
- Brookes, Z. L. S., Bescos, R., Belfield, L. A., Ali, K., & Roberts, A. (2020). Current uses of chlorhexidine for management of Oral Disease: A Narrative Review. *Journal of Dentistry*, 103, 103497. <https://doi.org/10.1016/j.jdent.2020.103497>
- Bustin, S. A., Benes, V., Garson, J. A., Hellems, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J. & Wittwer, C. T. (2009). The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry*, 55, (4)611-622. <https://doi.org/10.1373/clinchem.2008.112797>
- Díaz-Garrido, N., Lozano, C. P., Kreth, J., & Giacaman, R. A. (2020). Competition and caries on enamel of a dual-species biofilm model with *Streptococcus mutans* and *Streptococcus sanguinis*. *Applied and Environmental Microbiology*, 86(21), e01262-20. <https://doi.org/10.1128/AEM.01262-20>
- Utami, D. T., Pratiwi, S. U. T., Haniastuti, T., & Hertiani, T. (2021). Eugenol and Thymol as Potential Inhibitors for Polymicrobial Oral Biofilms: An in vitro Study. *Journal of International Oral Health*, 13(1), 45-52. https://doi.org/10.4103/jioh.jioh_247_20
- Ferrazzano, G. F., Scioscia, E., Sateriale, D., Pastore, G., Colicchio, R., Pagliuca, C., Cantile, T., Alcidi, B., Coda, M., Ingenito, A., Scaglione, E., Cicatiello, A.G., Volpe, M.G Staso, M.D., & Pagliarulo, C. (2017). In vitro antibacterial activity of pomegranate juice and peel extracts on cariogenic bacteria. *BioMed Research International*, 2017(1), 2152749. <https://doi.org/10.1155/2017/2152749>
- Fontaine, C., Rochefort-Morel, C., Veyrac, G., Polard, E., Scailteux, L. M., & Network, F. P. (2024). Immediate allergies to chlorhexidine: A series of pediatric cases from the French pharmacovigilance database: Immediate allergies to chlorhexidine: series of pediatric cases from the French pharmacovigilance database. *Archives de Pédiatrie*, 31(7), 419-425. <https://doi.org/10.1016/j.arcped.2024.06.004>
- Gabe, V., Kacergius, T., Abu-Lafi, S., Kalesinskas, P., Masalha, M., Falah, M., Abu-Farich, B., Melnikaitis, A., Zeidan, M. & Rayan, A. (2019). Inhibitory effects of ethyl gallate on *Streptococcus mutans* biofilm formation by optical profilometry and gene expression analysis. *Molecules*, 24(3), 529. <https://doi.org/10.3390/molecules24030529>
- Haido, M. H., Matti, A. H., Taher, S. M., & Haido, M. (2024). Optimization of extraction conditions of bioactive compounds from Kurdistan species *Urtica dioica*. *Cureus*, 16(5): e61146. <https://doi.org/10.7759/cureus.61146>
- Hasan, S., Danishuddin, M., & Khan, A. U. (2015). Inhibitory effect of *Zingiber officinale* towards *Streptococcus mutans* virulence and caries development: in vitro and in vivo studies. *BMC Microbiology*, 15, 1-14. <https://doi.org/10.1186/s12866-014-0320-5>
- Huang, R., Li, M., Ye, M., Yang, K., Xu, X., & Gregory, R. L. (2014). Effects of nicotine on *Streptococcus gordonii* growth, biofilm formation, and cell aggregation. *Applied and Environmental Microbiology*, 80(23), 7212-7218. <https://doi.org/10.1128/AEM.02395-14>
- Jacob, B., Malli Sureshbabu, N., Ranjan, M., Ranganath, A., & Siddique, R. (2021). The antimicrobial effect of pomegranate peel extract versus chlorhexidine in high caries risk individuals using quantitative real-time polymerase chain reaction: A randomized triple-blind controlled clinical trial. *International Journal of Dentistry*, 2021(1), 5563945. <https://doi.org/10.1155/2021/5563945>
- Kampf, G. (2016). Acquired resistance to chlorhexidine—is it time to establish an 'antiseptic stewardship' initiative? *Journal of Hospital Infection*, 94(3),213-227. <https://doi.org/10.1016/j.jhin.2016.08.018>
- Khanna, R., Pandey, G., Barthwal, S., Ginwal, H. S., & Varshney, V. K. (2025). Phytochemical Profiling, Isolation, Characterization and Quantification of Triterpenoids from *Terminalia Arjuna*: A Multi-Location Study Using HPLC and Statistical Analyses. *Journal of Chromatographic Science*, 63(6), bmaf042. <https://doi.org/10.1093/chromsci/bmaf042>
- Kaur, G., Balamurugan, P., & Princy, S. A. (2017). Inhibition of the quorum sensing system (ComDE Pathway) by aromatic 1, 3-di-m-tolylurea (DMTU): cariostatic effect with fluoride in wistar rats. *Frontiers in Cellular and Infection Microbiology*, 7, 313. <https://doi.org/10.3389/fcimb.2017.00313>
- Kim, B. S., Park, S. J., Kim, M. K., Kim, Y. H., Lee, S. B., Lee, K. H., Choi, N.Y., Lee, Y.R., Lee, Y.E. & You, Y. O. (2015). Inhibitory effects of *Chrysanthemum boreale* essential oil on biofilm formation and virulence factor expression of *Streptococcus mutans*. *Evidence-Based Complementary and Alternative Medicine*, 2015(1), 616309. <https://doi.org/10.1155/2015/616309>

- Konrat, K., Schwabke, I., Laue, M., Dittmann, C., Levin, K., Andrich, R., Arvand, M. & Schaudinn, C. (2016). The bead assay for biofilms: a quick, easy and robust method for testing disinfectants. *PLoS One*, 11(6), e0157663. <https://doi.org/10.1371/journal.pone.0157663>
- Lemos, J. A., Abranches, J., Koo, H., Marquis, R. E., & Burne, R. A. (2010). Protocols to study the physiology of oral biofilms. *Oral Biology: Molecular Techniques and Applications*, 87-102. https://doi.org/10.1007/978-1-60761-820-1_7
- Li, J., He, X., Li, M., Zhao, W., Liu, L., & Kong, X. (2015). Chemical fingerprint and quantitative analysis for quality control of polyphenols extracted from pomegranate peel by HPLC. *Food Chemistry*, 176, 7-11. <https://doi.org/10.1016/j.foodchem.2014.12.040>
- Lukomska-Szymanska, M., Sokolowski, J., & Lapińska, B. (2017). Chlorhexidine—mechanism of action and its application to dentistry. *Journal of Stomatology*, 70(4), 405-417. <https://doi.org/10.5604/01.3001.0010.5698>
- Nijampatnam, B., Casals, L., Zheng, R., Wu, H., & Velu, S. E. (2016). Hydroxychalcone inhibitors of *Streptococcus mutans* glucosyl transferases and biofilms as potential anticaries agents. *Bioorganic and Medicinal Chemistry Letters*, 26(15), 3508-3513. <https://doi.org/10.1016/j.bmcl.2016.06.033>
- Mahdi, M. A., Aref, P., Emadi, F., Javadi, F., Fard, M. J. K., & Tavassoli-Hojjati, S. (2023). Effect of hydroalcoholic extract of whole pomegranate fruit on cariogenic bacteria and its clinical effect on dental plaque formation in 8–10-year-old children. *Dental Research Journal*, 20(1), 116. https://doi.org/10.4103/drj.drj_173_23
- Man, G., Xu, L., Wang, Y., Liao, X., & Xu, Z. (2022). Profiling phenolic composition in pomegranate peel from nine selected cultivars using UHPLC-QTOF-MS and UPLC-QQQ-MS. *Frontiers in Nutrition*, 8, 807447. <https://doi.org/10.3389/fnut.2021.807447>
- Marrone, G., Basilicata, M., Di Lauro, M., Vita, C., Masci, C., Klinger, F. G., Cornali, K., Maddaloni, G., Bollero, P., De Lorenzo, A. & Noce, A. (2024). Healthy Effects of Pomegranate (*Punica granatum* L.) in Internal Medicine and Dentistry. *Applied Sciences*, 14(4), 1570. <https://doi.org/10.3390/app14041570>
- Miladi, H., Zmantar, T., Kouidhi, B., Al Qurashi, Y. M. A., Bakhrouf, A., Chaabouni, Y., Mahdouani, K. & Chaieb, K. (2017). Synergistic effect of eugenol, carvacrol, thymol, p-cymene and γ -terpinene on inhibition of drug resistance and biofilm formation of oral bacteria. *Microbial pathogenesis*, 112, 156-163. <https://doi.org/10.1016/j.micpath.2017.09.057>
- Pelá, V. T., Ventura, T. M. O., & Buzalaf, M. A. R. (2020). Optimizing the formation of the acquired enamel pellicle in vitro for proteomic analysis. *Journal of Applied Oral Science*, 28, e20200189. <https://doi.org/10.1590/1678-7757-2020-0189>
- Pinni, J., Avula, J. S., Mukthineni, S., Bandi, S., & Gokul, T. (2018). Antimicrobial activity of pomegranate (*Punica granatum*) pericarp extract against *Streptococcus mutans*—A source for natural mouth rinse: An in-vitro and in-vivo study. *Biomed Pharmacol J*, 11(4), 2025-30. <http://dx.doi.org/10.13005/bpj/1578>
- Rahmani-Badi, A., Sepehr, S., & Babaie-Najef, H. (2015). A combination of cis-2-decenoic acid and chlorhexidine removes dental plaque. *Archives of Oral Biology*, 60(11), 1655-1661. <https://doi.org/10.1016/j.archoralbio.2015.08.006>
- Subramaniam, P., Dwivedi, S., Uma, E., & Babu, K. G. (2012). Effect of pomegranate and aloe vera extract on *Streptococcus mutans*: An in vitro study. *Dental Hypotheses*, 3(3), 99-105. <https://doi.org/10.4103/2155-8213.103920>
- Sun, Y., Pan, Y., Sun, Y., Li, M., Huang, S., Qiu, W., Tu, H. & Zhang, K. (2019). Effects of norspermidine on dual-species biofilms composed of *Streptococcus mutans* and *Streptococcus sanguinis*. *BioMed Research International*, 2019(1), 1950790. <https://doi.org/10.1155/2019/1950790>
- Teaupaisan, R., Kawsud, P., Pahunmunto, N., & Puripattanavong, J. (2017). Screening for antibacterial and antibiofilm activity in Thai medicinal plant extracts against oral microorganisms. *Journal of Traditional and Complementary Medicine*, 7(2), 172-177. <https://doi.org/10.1016/j.jtcme.2016.06.007>
- Umar, D., Dilshad, B., Farhan, M., Ali, A., & Baroudi, K. (2016). The effect of pomegranate mouthrinse on *Streptococcus mutans* count and salivary pH: an in vivo study. *Journal of Advanced Pharmaceutical Technology & Research*, 7(1), 13-16. <https://doi.org/10.4103/2231-4040.173266>
- Vázquez-León, L. A., Páramo-Calderón, D. E., Robles-Olvera, V. J., Valdés-Rodríguez, O. A., Pérez-Vázquez, A., García-Alvarado, M. A., & Rodríguez-Jimenes, G. C. (2017). Variation in bioactive compounds and antiradical activity of *Moringa oleifera* leaves: influence of climatic factors, tree age, and soil parameters. *European Food Research and Technology*, 243(9), 1593-1608. <https://doi.org/10.1007/s00217-017-2868-4>
- World Health Organization, 2022. *Global oral health status report: towards universal health coverage for oral health by 2030*. World Health Organization.
- Zeng, Y., Nikitkova, A., Abdelsalam, H., Li, J., & Xiao, J. (2019). Activity of quercetin and kaempferol against *Streptococcus mutans* biofilm. *Archives of Oral Biology*, 98, 9-16. <https://doi.org/10.1016/j.archoralbio.2018.11.005>

AUTHOR'S PROFILE



Nurhayati Mohamad Zain is a Senior Lecturer at the Faculty of Dentistry, Universiti Teknologi MARA (UiTM) Sungai Buloh. Her research interests are in oral microbiology and oral biofilm research, with a particular focus on natural products for the prevention and management of oral infections. She is also interested in the incorporation of natural product–derived compounds into dental materials to enhance their antimicrobial and antibiofilm properties for oral healthcare applications.

LIST OF PUBLICATION:

- Mohamad Zain, N., Mohd Amin, I., Abdul Razak, F., & Abu Hassan, M. I. (2025). Preliminary screening of pomegranate-derived compounds for antimicrobial and antivirulence effects against cariogenic streptococci. *The Saudi Dental Journal*, 37(10), 84.
- Mohamad Zain, N. Mohd Amin, I., Abdul Razak, F., & Abu Hassan, M. I. (2025). Antibiofilm activity of punicalin and punicalagin against dual-species *Streptococcus* biofilms: Molecular and microbiological evaluation. *Asia Pacific Journal of Molecular Biology and Biotechnology*, 33(4), 128–137.

Zain, N. M., Amin, I. M., Razak, F. A., & Hassan, M. I. A. (2026). Eradication of Dual-Species Cariogenic Biofilms by Punicalin and Punicalagin: Microbiological and Gene Expression Analyses. *Malaysian Applied Biology*, 55(2), 11-21.

COPYRIGHT

Experimental formulation and protocol for selective cariogenic biofilm eradication using pomegranate-derived punicalagin. CRLY2025W03866

AWARD

3rd Prize for oral presentation at 5th International Conference on Oral Microbiology and Oral Immunology (ICOMOI) 2022