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Influence of Photodynamic Therapy Combined with Antimicrobial Peptides on Oral Biofilm and
3D Reconstructed Human Oral Epithelium Model

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By

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ABSTRACT

This thesis explores innovative approaches to treat *Candida albicans* infections in single and mixed-species biofilms when combined also with *Streptococcus mutans*. The experimental publication 1 evaluated the antimicrobial peptides/proteins (AMPs) Hylin-a1, Temporin-SHa, and KR-12-a5, against fluconazole-resistant and susceptible *C. albicans* and identified Temporin-SHa as the most promising AMP with significant antifungal and biocompatibility abilities, inhibiting biofilm formation and reducing fungal biomass. Publication 2 assessed the AMPs DR9-RR14, RR14, Histatin 3, and Histatin 5 on mixed biofilms formed on acrylic resin, showing reduced biofilm biomass and extracellular matrix (ECM) vital components disruption. Publication 3 investigated the combined effects of His3 and His5, the most promising AMPs evaluated previously, with antimicrobial photodynamic therapy (aPDT) on mixed biofilms containing three resistant strains of *C. albicans* and *S. mutans*, showing enhanced biofilm inhibition and reduced extracellular matrix (ECM) components, also minimizing the virulence of resistant strains and highlighting the synergistic potential of this new approach. Lastly, publication 4 validated the combination of His3 and His5 with aPDT in a 3D oral epithelium model, demonstrating limited infection penetration into deeper layers, reduced hyphal invasion, and compatibility with host tissue. These findings collectively highlight the potential of combining aPDT with AMPs as a targeted approach to overcome microbial resistance and biofilm complexity. Proteomic profiling provided information about the molecular mechanisms by which this combination treatment works, proving its efficacy as a therapy. As a future investigation, the combined treatment validated in this study needs to be extended to in vivo models to evaluate tissue regeneration behavior, particularly after the remission of the lesions.

Santana LMD. Influência da terapia fotodinâmica combinada com peptídeos antimicrobianos sobre biofilme oral e modelo 3D de epitélio oral humano reconstruído [Thesis Doctor]. University of Saskatchewan, Saskatoon; São Paulo State University (UNESP), School of Dentistry, Araraquara; 2025.

RESUMO

Esta tese explora abordagens inovadoras para o tratamento de infecções por *Candida albicans* em biofilmes de espécie única e em associação com *Streptococcus mutans*. A primeira publicação experimental avaliou os peptídeos/proteínas antimicrobianos (AMPs) Hylin-a1, Temporin-SHa e KR-12-a5 contra cepas de *C. albicans* resistentes e suscetíveis ao fluconazol, identificando o Temporin-SHa como o AMP mais promissor, com significativa atividade antifúngica e biocompatibilidade, capaz de inibir a formação de biofilmes e reduzir a biomassa fúngica. A segunda publicação analisou os AMPs DR9-RR14, RR14, Histatina 3 (His3) e Histatina 5 (His5) em biofilmes mistos formados sobre resina acrílica, demonstrando redução da biomassa do biofilme e redução de componentes vitais da matriz extracelular (MEC). Na terceira publicação, foram investigados os efeitos combinados de His3 e His5, identificados anteriormente como os AMPs mais promissores, com a terapia fotodinâmica antimicrobiana (aPDT) em biofilmes mistos contendo três cepas resistentes de *C. albicans* associadas a *S. mutans*. Os resultados revelaram inibição significativa dos biofilmes, redução de componentes da MEC e diminuição da virulência das cepas resistentes, destacando o potencial sinérgico desta nova abordagem. Por fim, a quarta publicação validou a combinação de His3 e His5 com a aPDT em um modelo tridimensional (3D) de epitélio oral, evidenciando penetração limitada da infecção em camadas mais profundas, redução da invasão hifal e compatibilidade com o tecido hospedeiro. Coletivamente, esses achados ressaltam o potencial da combinação entre aPDT e AMPs como uma estratégia direcionada para superar a resistência microbiana e a complexidade dos biofilmes. A análise proteômica forneceu informações relevantes sobre os mecanismos moleculares pelos quais esse tratamento combinado atua, comprovando sua eficácia terapêutica. Como perspectiva futura, destaca-se a necessidade de expandir a investigação deste tratamento combinado para modelos *in vivo*, a fim de avaliar o comportamento de regeneração tecidual, especialmente após a remissão das lesões.

Palavras – chave: Biofilmes, *Candida albicans*, *Streptococcus mutans*

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SUMMARY

ABSTRACT.....	II
ACKNOWLEDGEMENTS	IV
LIST OF TABLES	I
LIST OF FIGURES	II
LIST OF ABBREVIATIONS.....	I
1 INTRODUCTION	2
1.1.2 Role of <i>Candida albicans</i>	3
1.1.3 Cross-Kingdom Interactions with <i>Streptococcus mutans</i>	5
1.1.4 Antimicrobial Resistance	6
1.1.6 Antimicrobial Peptides Potential	8
1.1.8 Proteomic Approaches to Study Biofilm Interactions	10
1.1.9 Clinical Potential of Combining Antimicrobial Peptides with Photodynamic Therapy	11
2 USE OF PHOTODYNAMIC THERAPY ASSOCIATED WITH ANTIMICROBIAL PEPTIDES FOR BACTERIAL CONTROL: A SYSTEMATIC REVIEW AND ME-TA-ANALYSIS	13
2.3.2 Data extraction and research question	17
2.3.5 Search strategy.....	18
2.3.6 Meta-analysis and quantitative approaches	18
2.5 DISCUSSION	33
3 HYPOTHESES AND OBJECTIVES	39
3.1 RATIONALE.....	39
3.2 HYPOTHESES	39
3.2.1 Objective 1:.....	39
3.2.2 Hypothesis 1:	40
3.2.3 Objective 2:.....	40
3.2.4 Hypothesis 2:	40
3.2.5 Objective 3:.....	40
3.2.6 Hypothesis 3:	40
3.2.7 Objective 4:.....	41
3.2.8 Hypothesis 4:	41
4 ANTIBIOFILM ACTIVITY AND BIOCOMPATIBILITY OF TEMPORIN-SHA: A PROMISING ANTIMICROBIAL PEPTIDE FOR CONTROL OF FLUCONAZOLE-RESISTANT <i>CANDIDA ALBICANS</i>	42
4.1 ABSTRACT	43
4.3 MATERIALS AND METHODS	46
4.3.1 Synthesis, Cleavage, and Purification of Peptides Hyln1, KR12-a5, and Temporin.....	46
SHa.....	46
4.3.2 Antimicrobial Activity of AMPs on Planktonic Cultures of <i>C. albicans</i>	47
4.3.2.2 Survival Curve, Minimum Inhibitory Concentration (MIC), and Minimum Fungicide	47
Concentration (MFC) of AMPs on Planktonic Cultures of <i>C. albicans</i>	47
4.3.3 Cytotoxicity Evaluation of Hylin-a, KR-12-a5 and Temporin-SHa on Oral Cell.....	51
Cultures in Normal Oral Keratinocytes (NOK-si) and Human Gingival Fibroblasts (FGH)	51
4.3.3.1 Cell Line and Cell Culture	51
4.3.3.2 Cell Viability Analysis by alamarBlue™	52

4.3.4 Antimicrobial activity of AMPs against <i>C. albicans</i> biofilm.....	52
4.3.4.1 Quantification of Total Biofilm Biomass of <i>C. albicans</i> (CaS and CaR) with Crystal Violet Dye	53
4.3.5 Efficacy of AMPs in the Inhibition of Biofilm Formation and against the Biofilm Formed.....	53
4.3.6 Data analysis	54
4.4 RESULTS	54
4.4.2 Quantification of Total Biofilm Biomass of <i>C. albicans</i> (CaS and CaR)	60
4.4.3 Efficacy of AMPs in the Inhibition of Biofilm Formation and against the Biofilm formed	62
5 UNLOCKING HISTATINS POTENTIAL AGAINST CANDIDA ALBICANS AND STREPTOCOCCUS MUTANS BIOFILMS: TARGETING EXTRACELLULAR MATRIX WHILE PRESERVING ORAL CELL INTEGRITY	73
5.1 ABSTRACT	74
5.2 INTRODUCTION	75
5.3 MATERIAL AND METHODS	76
5.3.1 Strains and cell culture condition	77
5.3.2 Biofilm inhibitory concentration (BIC-2)	77
5.3.3 Acrylic resin discs standardization and protein/peptide pellicle formation	78
5.3.4 <i>C. albicans</i> and <i>S. mutans</i> mixed biofilm formation	78
5.3.5 Culture medium pH analyses	79
5.3.6 Determination of cell viability biofilm formed on acrylic resin (CFU/mm ²).....	79
5.3.7 Quantification of Biofilm Components of Mixed Biofilms	79
5.3.8 Scanning electron microscopy (SEM)	80
5.3.9 Fluorescence images by confocal laser scanning microscope (CLSM).....	80
5.3.10 Cytotoxicity evaluation of peptides/proteins on fibroblast cells by alamarBlue	81
5.3.11 Statistical Analysis	83
5.4 RESULTS	83
5.4.1 Biofilm Inhibitory Concentration (BIC-2) by Absorbance and CFU/mL	83
5.4.2 Effect of Peptides/Proteins on Mixed Biofilm Viability by CFU/mm ²	87
5.4.3 Monitoring Acidogenesis by pH measurements	87
5.4.4 Quantification of Biofilm Components of Mixed Biofilms	89
5.4.5 Structural Analysis of Mixed Biofilms by Confocal Laser Scanning Microscopy (CLSM)	91
5.4.5.1 Morphological Changes in Mixed Biofilms Observed by SEM	92
5.4.6 Biocompatibility Assessment of Proteins/Peptides by alamarBlue.....	94
5.5 DISCUSSION.....	96
5.6 CONCLUSION	99
6 SALIVARY PROTEINS-ENHANCED ANTIMICROBIAL PHOTODYNAMIC THERAPY OVERCOMING THREE DISTICT CULTURES OF RESISTANT MIXED BIOFILMS	100
6.4.1 Acrylic resin slabs and protein adhesion:.....	104
6.4.2 Biofilm Adhesion Phase	107
6.4.3 Antimicrobial Photodynamic Therapy (aPDT).....	107
6.4.4 Biofilm Processing ECM Analysis	107
6.4.5 Confocal Scanning Laser Microscopy (CSLM)	108
6.4.6 Spectrum of absorbance with Photodithazine® and Proteins	108
6.4.7 Reactive Oxygen Species (ROS) Production in a Cell-Free Solution System.....	108
6.4.8 Photobleaching Assay.....	109
6.4.9 Data Analysis	109
6.5 RESULTS	109
6.5.1 Colony Forming Units (CFU/mm ²) and their respective CLSM images	109
6.5.3 Spectrum of absorbance with Photodithazine® and Proteins	118
6.5.4 Reactive Oxygen Species (ROS) Production in a Cell-Free Solution System.....	119

6.5.5 Photobleaching assay	119
6.6 DISCUSSION	122
6.7 CONCLUSIONS	129
7 HISTATIN-PHOTODYNAMIC THERAPY FOR POLYENE-RESISTANT BIOFILMS IN 3D MODEL ..	130
7.1 ABSTRACT	131
7.2 INTRODUCTION	132
7.3 MATERIALS AND METHODS	133
7.3.1 3D human Oral Epithelium and Protein adhesion	133
7.3.2 3D oral epithelium infection	133
7.3.3 Antimicrobial Photodynamic Therapy (aPDT)	134
7.3.5 Cell Viability Assessment (by MTT assay)	135
7.3.6 Histological Analysis	135
7.3.7 ECM protein quantification and proteomic profile	136
7.3.8 Bioinformatics and systems biology analyses from proteomic profile	136
7.3.9.1 Heatmap-Based Clustering of Protein Expression Profiles	137
7.3.9.2 Principal Component Analysis (PCA)	137
7.3.9.3 Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG)	137
7.3.9.4 Pathway Enrichment	137
7.3.9.4 Protein-Protein Interaction (PPI) Network	138
7.3.9.5 Network Topology and Hub Protein Identification	138
7.3.9.6 Nucleocytoplasmic Transport Pathway Analysis	138
7.3.9.7 Metabolic Pathway Mapping (iPath 3.0)	138
7.4 RESULTS	139
7.4.1.1 Biofilm cell viability determination (by CFU/mL)	139
7.4.1.2 MTT assay	139
7.4.1.3 Histological Analysis	139
7.4.2 ECM proteins quantification	143
7.4.3 Bioinformatics and Systems Biology Analyses from Proteomic Profile	143
7.4.3.1 Protein Overlap and Shared Expression Pattern	143
7.4.3.2 Principal Component Analysis (PCA)	143
7.4.3.3 Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG)	143
7.4.3.4 Pathway Enrichment Analysis	143
7.4.3.4 BP Enrichment	144
7.4.3.5 Molecular Function (MF) Enrichment	144
7.4.3.6 Cellular Component (CC) Enrichment	144
7.4.3.7 Subcellular Localization (COMPARTMENTS)	144
7.4.3.8 Network Clustering and Functional Associations	145
7.4.3.9 Heatmap-Based Clustering of Protein Expression Profiles	148
7.4.3.10 STRING-Based Interaction Network Analysis	151
7.4.3.11 Network Topology and Hub Proteins	154
7.4.3.12 Nucleocytoplasmic Transport Pathway Analysis	154
7.4.3.13 Metabolic Pathway Analysis	154
7.5 DISCUSSION	157
7.6 CONCLUSION	161
7.7 APPENDIX OF MANUSCRIPT	161
7.7.1 List of identified proteins in the analysis and their corresponding accession numbers	161
8 GENERAL DISCUSSION	167
8.1 STRENGTHS	167
8.1.1 Synthesis of Experimental Contributions	167
8.1.2 Integration of Findings Across Studies	169
8.1.3 Clinical Relevance	174

8.2 LIMITATIONS	175
8.3 FUTURE APPLICATIONS.....	175
9 CONCLUSION	176
10 REFERENCES	177

LIST OF TABLES

Table 1. Summary of the characteristics of the studies included.	21
Table 2: Risk of bias assessments for in vitro studies.....	31
Table 3. Classification of cytotoxicity of treatments in relation to control by ISO 10993-5:2009 guidelines.	52
Table 4. List of proteins identified through proteomic analysis, along with their respective UniProt accession numbers. Protein identification was based on MS/MS spectral matching and confirmed using a reference database.	161

LIST OF FIGURES

Figure 1. Biofilm Cycle. Created in BioRender. Lab, S. (2025) https://BioRender.com/nl247rq	2
Figure 2. Flowchart based on the PRISMA statement.....	19
Figure 3. Illustration of the results of the quantitative analysis. The experimental group (positive events) included microorganisms that received the association therapy (aPDT + AMPs), while the control group included microorganisms that received only aPDT. A) results of the meta-analysis illustrated in a forest plot. OR: odds ratio; CI: confidence interval; W: weight. B) trim-and-fill method results illustrated in a forest plot. TE: estimated mean; seTE: estimated standard deviation. OR: odds ratio; CI: confidence interval; W: weight.	33
Figure 4. (a) RP-HPLC chromatogram of pure Hylin-1; (b) RP-HPLC chromatogram of the pure KR-12-a5; (c) RP-HPLC chromatogram of the pure temporin-SHa. C18 column (25 cm × 10 mm), KR-12-a5; (c) RP-HPLC chromatogram of the pure Temporin-SHa. C18 column (25 cm X 10 mm), detection at 220 nm, using a gradient method from 5 to 95% of solvent B in 30 min with a flow rate of 1 mL min ⁻¹	49
Figure 5. Mass spectrum of pure Hylin-1. Theoretical MW = 1864.4 g/mol; obtained -1 MW/Z(a=) (b) Mass spectrum of pure KR-12-a5. Theoretical MW = 1565.1 g/mol; obtained MW/Z = 1564.7 (Z = 1); and 783.0 (Z = 2); (c) Mass 1565.1 g/mol-1; obtained MW/Z = 1564.7 (Z = 1); and 783.0 (Z = 2); (c) Mass spectrum of pure Temporin-SHa. Theoretical MW = 1380.8 g/mol; obtained MW/Z = 1380.8 (Z = 1); Temporin-SHa. Theoretical MW = 1380.8 g/mol-1; obtained MW/Z = 1380.8 (Z = 1); and 691.2 (Z = 2) and 691.2 (Z = 2).	50
Figure 6. Flowchart of treatments with Hylin-a1, KR-12-a5, and Temporin-SHa: Survivor curve, minimum inhibitory concentration (MIC), and minimum fungicidal concentration (MFC).	51
Figure 7. Mean and 95% confidence interval of survival in log ₁₀ of CaS and CaR suspensions after longitudinal exposure to predetermined concentrations (2, 4, 8, 16, 32, 64, 128, 512, and 1024 µg/mL) of Hylin-a1 (a,b), KR-12-a5 (c,d) and Temporin-SHa (e,f) at predetermined times (0; 5 min; 10 min; 20 min; 30 min; 1 h; 2 h; 3 h; 4 h; 6 h; and 24 h). Points: data averages. Error bars: minimum and maximum values. The non-intersection of the error bars denotes a statistical difference according to the 95% confidence interval (n = 12/group; p < 0.05).	56
Figure 8. Mean and 95% confidence interval of the MFC (log ₁₀) of CaS and CaR suspensions after exposure to Hylin-a1 (a,b), KR-12-a5 (c,d), and Temporin-SHa (e,f) at 64, 128, 256, 512, and 1024 µg/mL. Points: data means. Error bars: minimum and maximum values. The non-intersection of the error bars denotes a difference according to the 95% confidence interval (n = 12/group; p < 0.05).	60
Figure 9. Mean and 95% confidence interval of CaS and CaR biomass (percentage: %) measured through 570 nm of optical density (OD) after exposure to Hylin-a1 (a,b), KR-12-a5 (c,d), and Temporin-SHa (e,f) at 32, 64, 128, 256, 512, and 1024 µg/mL. CT: experimental control. Points: data averages. Error bars: minimum and maximum values. The non-intersection of the error bars denotes a statistical difference according to the 95% confidence interval (p < 0.05; n = 12/group).	61
Figure 10. Mean and 95% confidence interval of the inhibition of CaS and CaR biofilm formation after exposure to Hylin-a1 (a,b), KR-12-a5 (c,d), and Temporin-SHa (e,f). AMPs at 32, 64, 128, 256, 512, and 1024 µg/mL and experimental control (0). Points: data averages. Error bars: minimum and maximum values. The non-intersection of the error bars denotes a statistical difference according to the 95% confidence interval (p < 0.05) (n = 12/group).	63
Figure 11. Mean and 95% confidence interval of Inhibition of CaS and CaR biofilm formed after exposure to Hylin-a1 (a,b), KR-12-a5 (c,d) and Temporin-SHa (e,f). AMPs at 32, 64, 128, 256, 512 and 1024 µg/mL and experimental control (0). Points: data averages. Error bars: minimum and maximum values. The non-intersection of the error bars denotes a statistical difference according 95% confidence interval (p < 0.05) (n = 12/group).	66
Figure 12. Mean and 95% confidence interval of FGH and NOK-si cellular viability percentage (%) by alamarBlue™ assay after treatment with Hylin-a1 (a,b), KR-12-a5 (c,d), and Temporin-SHa (e,f) at 32, 64, 128, 256, 512, and 1024 µg/mL. CT: live cell control; LB: lysis buffer (dead cell control). Points: data means.	

Error bars: minimum and maximum values. The non-intersection of error bars denotes the statistical difference according to the 95% confidence interval (n = 12/group; p < 0.05).	68
Figure 13. Representative linear regression graph for calculating CC 50%. Y = percentage of living cells; X = concentration of (a) Gingival Fibroblasts (FGH) and (b) Oral Keratinocytes.	68
Figure 14. (a) Flowchart depicting the development of mixed biofilm (<i>Streptococcus mutans</i> UA159 and <i>Candida albicans</i> ATCC 10231) on acrylic resin treated with protein/peptides. (b) Flowchart of alamarBlue assay; Classification of cytotoxicity according to the cells viability reduction after treatment compared to control by ISO 10993-5:2009 guidelines (2020). Created in BioRender. Lab, S. (2025) https://BioRender.com/a33w751	83
Figure 15. Median and 95% confidence interval of BIC-2 measured by Absorbance OD 492 nm of mixed biofilm of <i>Candida albicans</i> and <i>Streptococcus mutans</i> against proteins (a) Histatin 3 and (b) Histatin 5 at predetermined concentrations: 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024, 2048 and 4096 μ M; Peptides: (c) DR9-RR14 and (d) RR14 at predetermined concentrations: 2, 4, 8, 16, 32, 64, 128, 256, 512 and 1024 μ M. CC: contamination control (vehicle only). GC: growth control (without treatment). Points: data medians. Error bars: minimum and maximum values. The non-intersection of error bars indicates statistical significance (p < 0.05), based on 95% confidence intervals. (n = 9/group).	84
Figure 16. Median and 95% confidence interval of BIC-2 measured by CFU/mL of mixed biofilm of <i>C. albicans</i> and <i>S. mutans</i> against proteins (a) Histatin 3 and (b) Histatin 5 at predetermined concentrations: 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024, 2048 and 4096 μ M; Peptides: (c) DR9-RR14 and (d) RR14 at predetermined concentrations: 2, 4, 8, 16, 32, 64, 128, 256, 512 and 1024 μ M. CC: contamination control (vehicle only). GC: growth control (without treatment). Points: data medians. Error bars: minimum and maximum values. The non-intersection of error bars indicates statistical significance (p < 0.05), based on 95% confidence intervals. (n = 9/group).	86
Figure 17. Median and 95% confidence interval of mixed biofilm (orange color: <i>Candida albicans</i> and <i>Streptococcus mutans</i> formed on acrylic resin), <i>C. albicans</i> in mixed biofilm (blue color) and <i>S. mutans</i> in mixed biofilm (green color) against peptides DR9-RR14 (2048 μ M), RR14 (4096 μ M), Histatin 3 (256 μ M) and Histatin 5 (512 μ M); NaCl: experimental control. Points: data means. Error bars: minimum and maximum values. The non-intersection of error bars denotes statistical difference according to the 95% confidence interval (p < 0.05). (b) Table with reductions (%) of the mixed biofilm formed on acrylic resin against peptides (DR9-RR14; RR14; Histatin 3; Histatin 5 and reductions of <i>C. albicans</i> and <i>S. mutans</i> in mixed biofilm (p < 0.05) (n=9/group). (c) pH measurements after 0, 12, 24, 36 and 48 h of the peptides DR-RR14, RR14, Histatin 3 and Histatin 5.	88
Figure 18. Median and 95% confidence interval of (a) Dry Weight (μ g), (b) Insoluble Dry Weight (μ g), (c) Proteins (μ g), (d) eDNA (ng), (e) ASP (Alkali-soluble polysaccharides/ μ g) and (f) WSP (Water-soluble polysaccharides/ μ g) of mixed biofilm (<i>C. albicans</i> and <i>S. mutans</i>) formed on acrylic resin against peptides DR9-RR14 (2048 μ M), RR14 (4096 μ M), Histatin 3 (256 μ M) and Histatin 5 (512 μ M); NaCl: experimental control. Points: data median. Error bars: minimum and maximum values. The non-intersection of error bars denotes statistical difference according to the 95% confidence interval (p < 0.05) (n=9/group).	90
Figure 19. Fluorescence images of the mixed biofilm containing <i>Candida albicans</i> and <i>Streptococcus mutans</i> after the most promising peptides tested in the preliminary tests: Histatin 3 (His3) and Histatin 5 (His5). Green Fluorescence (Syto 9): Represents live cells of <i>C. albicans</i> and <i>S. mutans</i> ; Red Fluorescence (Dextran): Indicates extracellular matrix (ECM) components surrounding the cells; Merged Images: Show the colocalization of live cells and the ECM in biofilms treated with NaCl, His5 and His3.	92
Figure 20. Representative Images obtained by SEM of the mixed biofilm (<i>Candida albicans</i> and <i>Streptococcus mutans</i>) treated with His3, His5, DR9-RR14, RR14 and control (NaCl). Purple color: <i>C. albicans</i> ; Green color: <i>S. mutans</i> ; Yellow color: Extracellular Matrix. Magnifications of 5, 10, 15, 20, 50X.	94
Figure 21. Median and 95% confidence interval of cellular viability through Fluorescence Intensity (Arbitrary Units-A.U.) by alamarBlue assay. CT: live cell control; LB: lysis buffer, dead cell control. Points: data medians. Error bars: minimum and maximum values. The non-intersection of error bars denotes statistical difference according to the 95% confidence interval (n=12/group) (p < 0.05) (a) DR9-RR14 peptide with 1024 μ M, 2048 μ M * (BIC-2 concentration) and 4096 μ M concentrations. (b) RR14 peptide with 2048 μ M, 4096 μ M * (BIC-2 concentration) and 8192 μ M concentrations. (c) Histatin 3 peptide with 128 μ M, 256 μ M * (BIC-2	

- concentration) and 512 μM concentrations. (d) Histatin 3 peptide with 256 μM , 512 μM * (BIC-2 concentration) and 1024 μM concentrations.95
- Figure 22. (a) Experiment overview diagram: *Candida albicans* (CaP: Polyene-resistant *Candida albicans*, CaW Wilde-type *C. albicans*; and CaF: fluconazole-resistant *C. albicans*) and *Streptococcus mutans* (Sm) were cultured and prepared for inoculation. Acrylic resin disks were also pre-treated with salivary proteins (Histatin3 or Histatin5) to assess their effect on protein and microbial adhesion. Dual-species biofilms were formed and matured on the disks. After 48 h, the biofilms were treated with antimicrobial photodynamic therapy (aPDT). Finally, post-treatment analyses were performed to assess biofilm viability and extracellular matrix composition. (b) Sequence of events for mixed biofilm grow of *Candida albicans* and *Streptococcus mutans* (UA159) (CaP+Sm, CaW+Sm or CaF+Sm) on acrylic resin disk: Acrylic disks were standardized (1) and treated with (2) His3 or His5 according to their Biofilm Inhibitory Concentration (BIC-2) before (3) biofilm adhesion and (4) maturation with both species. Next, the biofilms were subjected to (5) aPDT. (6) Detached biofilms were stored for further analysis. Created in BioRender. Lab, S. (2025) <https://BioRender.com/g09I326>. Agreement number: YW27WGA3N.106
- Figure 23. Mean and 95% confidence interval of cell viability (CFU/mm²) results of treated biofilms collected by counting on agar plates for each acrylic disk containing *Streptococcus mutans* (Sm) and (a) polyene-resistant *Candida albicans* (CaP); or (b) wild-type *C. albicans* (CaW); (c) or Fluconazole-resistant *C. albicans* (CaF). Blue color: mixed biofilm; Green color: *S. mutans*; Dot: Mean value; Error Bars: confidence interval. The non-intersection of error bars denotes statistical difference according to the 95% confidence interval. Different number of asterisks denotes statistical difference compared to CT (n=6/group) (p<0.05); The right panel shows confocal images labeled with SYTO 9 (green) of mixed biofilm with CaP+Sm, CaW+Sm or CaF+Sm under aPDT only, His3+aPDT or His5+aPDT treatments, with visible reductions in biofilm density following treatment.112
- Figure 24. Results of treatment on extracellular matrix (ECM) components of mixed biofilms containing Polyene-resistant *Candida albicans* (CaP; ATCC200955), wild-type *C. albicans* (CaW; SC5314), Fluconazole-resistant *C. albicans* (CaF; ATCC 10231) with *S. mutans* (Sm; UA159). (a) CaP+Sm biofilm alkali-soluble polysaccharides (ASP) content. (b) CaP+SM biofilm water-soluble polysaccharides (WSP) content. (c) CaP+Sm biofilm extracellular DNA (eDNA) content. (d) CaP+Sm biofilm protein content. (e) CaW+Sm biofilm ASP content. (f) CaW+Sm biofilm WSP content. (g) CaW+Sm biofilm eDNA content. (h) CaW+Sm biofilm protein content. (i) CaF+Sm biofilm ASP content. (j) CaF+Sm biofilm WSP content. (l) CaF+Sm biofilm extracellular eDNA content. (m) CaF+Sm biofilm protein content. Dot: Mean value. Error Bars: confidence interval. The non-intersection of error bars denotes statistical difference according to the 95% confidence interval One asterisk (*) indicates p<0.05; two asterisks (**) indicate; p<0.01; and three asterisks (***) indicate p<0.001.116
- Figure 25. Confocal microscopy images of the mixed biofilm most resistant strain among the *Candida albicans* evaluated (Polyene-resistant *C. albicans*; ATCC 200955) and *Streptococcus mutans* (UA159) labeled with SYTO 9 (green) and Alexa-Fluor 647 Dextran (red). The green color represents the membranes of the microbial cells in the biofilm. The red color represents polysaccharides produced by the biofilms. The first and second columns illustrate each component individually, while the third column the overlay is observed.....118
- Figure 26. (a) Histatins absorbance spectrum (with and without PDZ): The absorbance spectra of His3 (purple line), His5 (light blue line), PDZ alone (orange line), Histatin 3 + PDZ (brown line), and Histatin 5 + PDZ (dark blue line) were measured across a wavelength range of 300 to 800 nm. (b) Red light fluence response (with and without PDZ and proteins): Fluorescence intensity measurements were taken as a function of increasing red light fluence (J/cm²). Histatin 5 + PDZ (purple line) exhibits the highest fluorescence response, followed by Histatin 3 + PDZ (brown line), PDZ alone (orange line), Histatin 5 (light blue line), and Histatin 3 (green line). (c) Absorbance measurements of PDZ and its combination with Histatins over time were recorded to assess photobleaching effects: The graph shows absorbance (nm) over time (minutes) for PDZ alone (blue line), PDZ + Histatin 5 (red line), PDZ + Histatin 3 (green line), and PBS (pink line; negative control).121
- Figure 27. Mean and 95% confidence interval of (a) Dual-specie biofilm viability (CFU/mL) containing mixed biofilm of *Candida albicans* (ATCC 200955) and *Streptococcus mutans* (UA159) from 3D Oral Model

submitted to Histatin 3 (His3) or Histatin 5 (His5) followed by Antimicrobial Photodynamic Therapy (aPDT) and (b) Cell viability by MTTTM: X axis: His3+ aPDT, His5 +aPDT, His3, His5, aPDT and CT (biofilm without treatment), LB (Lyses Buffer – death control); Y axis: Log₁₀ (CFU/mL) or Fibroblasts (FGH) cell viability (%); Pink color: Mixed biofilm (*C. albicans* and *S. mutans*); Blue color: *S. mutans* biofilm; Orange color: *C. albicans* biofilm; Points: data means; Error bars: confidence interval. The non-intersection of error bars denotes statistical difference according to the 95% confidence interval. Different number of asterisks denotes statistical difference compared to CT (n = 6/group): one asterisk (*) indicates $p < 0.05$, two asterisks (**) indicate $p < 0.01$, and three asterisks (***) indicate $p < 0.001$. (c) Flowchart containing the experiment design 1-Protein Adhesion; 2- Biofilm Infection into 3D oral model; 3- antimicrobial Photodynamic Treatment (aPDT); 4- 3D oral model process. Created with BioRender.com by Agreement number PL279HY5DE. (d) Representative images of the histological sections of 3D oral model inoculated with *C. albicans* (ATCC 200955) and *S. mutans* (UA159) and submitted to His3 and His5 followed by aPDT. His3 + aPDT: His3 with aPDT; His5+aPDT: His5 with aPDT; His3: His3 only; His5: His5 only and CT: control group – only PBS; ONLY PBS- NO INFECTION (0h): tissue without any infection and treatment in the beginning of the experiments; ONLY PBS- NO INFECTION (24h): tissue without any infection and treatment after 24h. Model has stained with hematoxylin-eosin (H&E) (40X)141

Figure 28. Mean and 95% confidence interval of (a) Extracellular Matrix (ECM) proteins recovery from the 3D oral model infected with mixed biofilm of *Candida albicans* (ATCC 200955) and *Streptococcus mutans* (UA159) and submitted to Histatin 3 (His3) or Histatin 5 (His5) followed by Antimicrobial Photodynamic Therapy (aPDT); X axis: His3+ aPDT, His5 +aPDT, His3, His5, aPDT, CT (biofilm without treatment); Y axis: ECM Protein (μg). Points: data means; Error bars: confidence interval. The non-intersection of error bars denotes statistical difference according to the 95% confidence interval. Different number of asterisks denotes statistical difference compared to CT (n=9/group) ($p < 0.05$); (b) Venn diagram illustrating the number of common and unique proteins among the experimental groups. Functional Enrichment Analysis of Identified Proteins of *C. albicans* using STRING-d of (c) Principal Component Analysis (PCA) of proteomic profiles. PCA plot showing distinct clustering of samples based on treatment groups. PC1 explains 78% and PC2 explains 11.2% of the total variance, highlighting clear separation between CT, aPDT, His3+aPDT, and His5+aPDT conditions. (d) Biological Process (BP) enrichment highlights pathways related to RNA localization, RNA transport, nuclear export, and transcription regulation, suggesting a functional role in nucleocytoplasmic trafficking. (e) Molecular Function (MF) enrichment identifies key molecular functions, including structural constituents of the nuclear pore, protein-containing complex binding, and ribonucleoprotein complex binding. (f) Cellular Component (CC) enrichment reveals significant associations with nuclear structures such as the nuclear pore, nuclear envelope, nucleoplasm, and chromosomal-related structures. (g) Subcellular localization analysis (COMPARTMENTS) confirms predominant localization within the nuclear pore, nuclear lumen, and endomembrane system, reinforcing their role in nuclear transport and transcriptional regulation. (h) Local network clustering demonstrates interactions among proteins involved in nuclear-cytoplasmic transport, chromatin remodeling, and transcriptional regulation, providing a comprehensive functional context for the identified proteins of *C. albicans*.147

Figure 29. (a) The heatmap (top panel) depicts the abundance (color scale) and hierarchical clustering (dendrogram) of the 167 most abundant shared ECM extracted biofilms recovered from a 3D oral epithelium model. The x-axis represents the experimental groups and their respective biological replicates: Control (CT_a, CT_b, CT_c), Antimicrobial Photodynamic Therapy (aPDT_a, aPDT_b, aPDT_c), Histatin 3 combined with aPDT (His3+aPDT_a, His3+aPDT_b, His3+aPDT_c), and Histatin 5 combined with aPDT (His5+aPDT_a, His5+aPDT_b, His5+aPDT_c). The y-axis lists the protein names. Pearson correlation and average linkage clustering were used to generate the dendrogram. (b) Table with the top 15 most downregulated proteins based on maximum fold change, comparing the Control group (CT) versus the Histatin 5 with aPDT group (His5+aPDT) (the condition which showed the greatest reduction). Table columns include the protein name, gene name, UniProt accession number, summarized protein function, and fold change value representing the highest reduction observed among replicates.149

Figure 30 (a) STRING-db-based visualization of the protein-protein interaction (PPI) network clustered using the Markov Cluster Algorithm (MCL), revealing eight functionally distinct modules. Cluster 1 (red): nuclear pore structural constituents; Cluster 2 (orange): ribosomal large subunit biogenesis; Cluster 3 (light green): chromatin remodeling-related proteins; Cluster 4 (dark green): SAGA complex components; Cluster 5 (teal): protein transport-associated proteins; Cluster 6 (cyan): intracellular trafficking-related proteins; Cluster 7 (blue): oxidative metabolism-related proteins; Cluster 8 (purple): DNA replication. (b) Table displaying the gene count and functional annotation of each cluster, with matching colors to panel (a). Each cluster is annotated with its representative biological process, highlighting the modular organization of the identified protein network. (c) Zoomed-in region of the global STRING metabolic network showing the topologically enriched area corresponding to nucleotide and cofactor/vitamin metabolism.....152

Figure 31. Functional and Pathway Analysis of Identified Proteins. (a) Hub proteins identified in the protein-protein interaction network based on node degree, highlighting key regulators involved in nucleocytoplasmic transport, chromatin remodeling, and translation regulation. (b) KEGG Nucleocytoplasmic Transport pathway illustrating the molecular components involved in nuclear import, export, and mRNA transport. Proteins identified in this study that are mapped to this pathway include NUP85, NUP98, NUP133, NUP159, Gle1, DDX19, RanBP2, UBC9, and SUMO. (c) Global metabolic map from iPath 3.0 with pathways identified in this study highlighted in red, emphasizing nucleotide metabolism and cofactor metabolism. (d) Magnified view of the metabolic map highlighting purine and pyrimidine metabolism, revealing their involvement in nucleotide biosynthesis and transport.155

LIST OF ABBREVIATIONS

AMPs	Antimicrobial peptides
CFU	Colony-forming unit
CaF	<i>Candida albicans</i> fluconazole-resistant strain
CaP	<i>Candida albicans</i> polyene-resistant strain
CaR	<i>Candida albicans</i> fluconazole-resistant strain
CaS	<i>Candida albicans</i> susceptible strain
CaW	<i>Candida albicans</i> wild-type strain
DR9-RR14	Derived peptide from statherin DR9 sequence combined with RR14 motif
ECM	Extracellular matrix
FGH	Fibroblasts of human gingiva
GO	Gene Ontology
HPLC	High-performance liquid chromatography
His3	Histatin 3
His5	Histatin 5
KEGG	Kyoto Encyclopedia of Genes and Genomes
LED	Light-emitting diode
MFC	Minimum fungicidal concentration
MIC	Minimum inhibitory concentration
MS	Mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NOK	Normal oral keratinocytes
OHAT	Office of Health Assessment and Translation
OSF	Open Science Framework
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PPIs	Protein-protein interactions
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
PS	Photosensitizer
ROS	Reactive oxygen species
RR14	Arginine-rich peptide composed of 14 amino acids
Sm	<i>Streptococcus mutans</i>
aPDT	Antimicrobial photodynamic therapy

THESIS ORGANIZATION

This thesis adheres to the formatting guidelines outlined by the College of Graduate Studies and Postdoctoral Research, University of Saskatchewan for a manuscript-based thesis, consequently, there is some repetition of content across chapters.

- Chapter 1, the current chapter is a comprehensive introduction to the thesis topic, along with an overview of the thesis objectives.
- Chapter 2, representing literature review, is a systematic review of the literature which summarized several aspects of the thesis topic related to the application.
- Chapter 3 gives details about the study rationale, hypotheses, and objectives.
- Chapters 4 – 7, the main body of thesis, were structured as a manuscript-based format, consisting of three different research projects (objectives).
- Chapter 8 and 9 serve as general discussion section with a conclusion, summarizing the scientific gaps addressed and filled throughout this thesis and providing conclusion of the thesis. Moreover, it discusses the limitations and outlines future directions, with a potential focus on the potential translational clinical utility in oral health.
- Chapter 10 contains all the references cited in this work.

1 INTRODUCTION

1.1 Background

1.1.1 Oral Biofilms

Biofilms are complex, structured communities of microorganisms attached to a surface. Their development is coordinated through various mechanisms, including metabolic signaling, genetic exchange, and quorum sensing—an intercellular communication system. The biofilm lifecycle typically progresses through four main stages: (1) initial surface adhesion, (2) microcolony formation, (3) maturation, and (4) dispersion (Kaplan, 2010). The dispersion phase is particularly critical, as it may facilitate the spread of infection to new anatomical sites, promoting the establishment of additional biofilms. These developmental stages result in distinct cellular physiology and phenotypic traits that differ markedly from those of planktonic (free-floating) cells (Mirghani et al., 2022) (Figure 1).

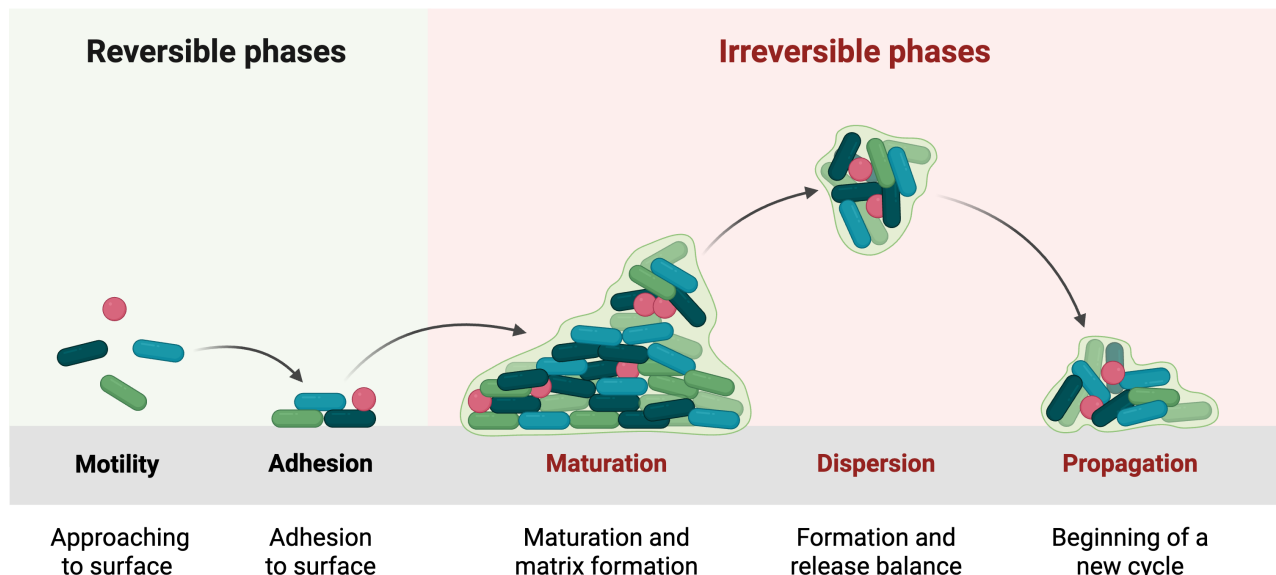


Figure 1. Biofilm Cycle. Created in BioRender. Lab, S. (2025) <https://BioRender.com/nl247rq>.

Biofilm cells are embedded within a self-produced extracellular matrix (ECM), composed of extracellular polymeric substances (EPS) synthesized by the microorganisms themselves (Flemming et al., 2007). The ECM plays a critical role in mediating cell-to-cell interactions within the biofilm and supports essential biological processes required for biofilm maintenance and stability. Its composition can dynamically adapt in response to environmental conditions, such as temperature, pH, and nutrient availability (Flemming et al., 2023). It because ECM can prevent the host's phagocytes from recognizing the fungi and activating the complement system. Additionally, it binds to antimicrobial agents, blocking their diffusion or neutralizing them, thus hindering their action (Kaplan, 2010). EPS components provide the necessary structural adhesion and cohesion, for three-dimensional organization of the biofilm (Bowen et al., 2018a).

In addition to structural functions, the ECM also serves as a recycling hub, retaining the components of lysed cells—including extracellular DNA (eDNA), which may act as a reservoir for horizontal gene transfer and contribute to the dissemination of genetic traits within the microbial community (Jiang et al., 2020). The ECM also impedes the host's immune defense mechanisms by preventing recognition by phagocytes and inhibiting activation of the complement system. Moreover, it restricts the diffusion of antimicrobial agents, either neutralizing them or reducing their access to deeper biofilm layers (Kaplan, 2010).

This three-dimensional structure creates gradients of nutrients and oxygen, which result in reduced metabolic activity in the innermost layers (Flemming et al., 2007). Consequently, antimicrobial agents that rely on active microbial metabolism for their mode of action become less effective (Kadosh & Mundodi, 2020). The ability to form such structured and protective communities makes biofilm-associated infections one of the most resilient forms of microbial persistence in clinical settings. (Thompson et al., 2011).

1.1.2 Role of *Candida albicans*

Given its highly adaptable nature, *Candida albicans* is one of the most studied fungal species in the context of oral biofilms. *Candida albicans* is the most prevalent species of *Candida*,

being a commensal microorganism typically found in the intestinal tract, genitourinary system, and the normal flora of the skin and mucous membranes(Scully et al., 1994). Immunosuppression in an individual can allow this microorganism to act as an opportunistic pathogen, penetrating the tissues and providing infections (Ishikawa et al., 2015). Individuals with conditions that weaken the immune system, such as kidney failure, AIDS, and diabetes, are more susceptible to developing oropharyngeal candidiasis. However, healthy individuals can also develop an infection due to an imbalance in the host's immune system (Scully et al., 1994). This fungus could adapt to changes in environmental pH through metabolic flexibility and robust nutrient acquisition systems, including a strong response to oxidative stress(Scully et al., 1994).

Aspects related to the virulence of *C. albicans* are mediated by factors such as substrate adherence, biofilm formation, and self-defense against the host's immune system(Thompson et al., 2011). The production of hydrolytic enzymes, such as proteases and phospholipases, can also be considered a virulence factor and is closely associated with tissue damage(Moyes & Naglik, 2011). Moreover, this fungus exhibits distinct morphologies such as blastoconidia and chlamydoconidia (yeast form), pseudohyphae, and hyphae, making it highly morphologically heterogeneous.

Biofilm formation begins with the adhesion of yeast cells to a substrate, followed by surface proliferation and the emergence of filamentous projections—comprising hyphae and, in some cases, pseudohyphae—that contribute to the maturation and structural complexity of the biofilm. Hypha formation is known to enhance virulence by enabling the invasion of epithelial cell layers, penetration of individual cells, disruption of endothelial cells, and lysis of macrophages and neutrophils. Pseudohyphae, in turn, are considered elongated yeast cells that lack the ability to separate from the mother cell during the cell cycle. Despite their different shapes, pseudohyphae more closely resemble yeast cells than true hyphae(Kadosh & Mundodi, 2020). In addition, the development of *C. albicans* biofilms can be influenced by several environmental factors, including the nature of the surface (substrate), culture medium, and pH. Study demonstrated this by evaluating biofilm formation on a polystyrene surface, revealing that both adhesion capacity and the expression of adhesion-related genes were affected under these conditions(Wu et al., 2014).

Additionally, *C. albicans* exhibits the ability to adapt to environmental pH changes through metabolic flexibility and robust nutrient acquisition systems, such as an effective response to oxidative stress (Cannon et al., 1995). Moreover, *C. albicans* expresses a variety of genes (such as CAT1 – catalase and SOD1 – superoxide dismutase) that activate pathways aiding in protection against free radicals and reactive oxygen species (Dantas et al., 2015). These features demonstrate that *C. albicans* possesses a wide range of biological tools that enable invasion, survival, and protection within host tissues.

1.1.3 Cross-Kingdom Interactions with *Streptococcus mutans*

Although *C. albicans* is the primary pathogen associated with denture stomatitis, bacteria such as *Streptococcus mutans* are also frequently found in biofilms formed under acrylic dentures, and these organisms may exhibit synergistic interactions (Bulet & Stöcklin, 2005). *C. albicans* is unable to efficiently metabolize sucrose but benefits from the free glucose and fructose generated from sucrose metabolism by *S. mutans* (Hwang et al., 2015). The synergy between these two microorganisms promotes the growth of *C. albicans* and consequently the development of polymicrobial biofilms, further enhancing the virulence potential of this model (D. Kim et al., 2017a; Rocha et al., 2018). Therefore, the biofilm of *C. albicans* and *S. mutans* exhibits a wide array of biological tools that facilitate invasion, survival, and protection within the host tissue (Pereira-Cenci et al., 2008).

Cross-kingdom metabolic cooperation promotes *C. albicans* proliferation and the development of stronger polymicrobial biofilms (D. Kim et al., 2017a). Mixed biofilm communities demonstrate greater structural intricacy and enhanced resilience against stress factors than single-species biofilms (Hwang et al., 2015). The interaction between species leads to increased virulence factor expression in *C. albicans* which subsequently boosts its ability to invade and persist in hosts (Y. Li et al., 2023). The surrounding extracellular matrix and interaction with *S. mutans* facilitate immune evasion by shielding fungal cells from phagocytosis and oxidative stress (D. Kim et al., 2017a).

Recent findings have shown that this cooperative behavior can be able to enhance the resistance of this biofilm to antimicrobial approaches. For instance, biofilms containing *C. albicans* and *S. mutans* demonstrate higher extracellular matrix (ECM) production, including increased levels of proteins, water-soluble polysaccharides (WSP), and extracellular DNA (eDNA)(Abreu-Pereira et al., 2023; Dias, Paul, et al., 2025). This process provides immune system evasion abilities and reduction drug penetration(Xiao et al., 2023).

1.1.4 Antimicrobial Resistance

The use of standardized conventional antifungal therapies may be limited due to the resistance that microorganisms can develop following drug exposure(Ho et al., 2025). Resistance to antifungal agents is considered a potential cause of treatment failure, leading to the persistence or progression of infection(Robbins et al., 2017).

The antimicrobial resistance can be classified as either primary or secondary. Intrinsic or primary resistance occurs when a microorganism exhibits low susceptibility to a given drug prior to any exposure to the agent(Brauner et al., 2016). The literature reports species in which all known isolates possess innate resistance to a specific antifungal, such as *Candida glabrata* and *Candida krusei*, which are intrinsically resistant to antifungal drugs, especially fluconazole(Silva et al., 2017a). Secondary or acquired resistance refers to resistance that may be developed by the microorganism after prolonged exposure to antifungal drugs(Brauner et al., 2016). The development of acquired resistance can occur through various mechanisms, including overexpression of the drug target, amino acid substitutions in the target that prevent binding of the inactivating enzyme, activation of stress response signaling pathways, upregulation of efflux pumps, or alterations in cellular pathways(Robbins et al., 2017). This resistance may be further accelerated by the organism's genetic plasticity or the presence of already-resistant strains. In this context, the combined effect of these contributing mechanisms leads to the selection of increasingly resistant organisms, resulting in high rates of recurrent infections (Christaki et al., 2020).

Variations in resistance phenotypes have been observed not only between different species and isolates of the same species but also across distinct growth states of the same strain(Pokharel et al., 2022). The formation of fungal biofilms can reduce susceptibility to conventional antifungal agents due to the difficulty of drug penetration through the biofilm layers to reach the target cell, thereby decreasing compound efficacy. It has been reported that in *C. albicans* biofilms, the surrounding glucan-rich matrix can act as a physical barrier, limiting the bioavailability of antifungal agents(Silva et al., 2017a).

Given the challenges related to fungal resistance, alternative strategies for inactivating *Candida* species have recently been suggested.

1.1.5 Antimicrobial Photodynamic Therapy (aPDT) as an Adjunctive Strategy

Antimicrobial photodynamic therapy (aPDT) has been employed in the inactivation of microorganisms and in the treatment of infections (Hu et al., 2018). aPDT involves the application of a photosensitizer (PS), a light source corresponding to the PS absorption band, and the presence of oxygen (Dantas et al., 2015; Demidova & Hamblin, 2005). The process of photosensitization begins with the treatment of the target cell with the PS. After, a light source with an appropriate wavelength for PS activation must be applied to illuminate the sensitized target (Donnelly et al., 2008). The interaction of light with the PS in the presence of oxygen promotes the formation of reactive oxygen species (ROS) that can induce cellular inactivation. It is suggested that this mechanism promotes the absorption of photons from the light source by the PS, which raises its electrons to an excited state. This excitation process occurs in the presence of oxygen and PS, which, when excited by light, can react with neighboring molecules through electron or hydrogen transfer (Type I reaction) or energy transfer to oxygen (Type II reaction) (Bonnett & Martínez, 2001). This therapy offers several advantages in treating infections caused by microorganisms, such as a broad spectrum of action and low mutagenic potential in exposed cells (Huang et al., 2012). However, compared to bacteria, photoinactivation of fungi appears to be more challenging

to achieve due to the larger cell size of these microorganisms, requiring a greater amount of singlet oxygen for cell death to occur (Demidova & Hamblin, 2005).

Another important aspect that has been observed is that the resistant strains of *C. albicans* are also less susceptible to aPDT alone. An application of aPDT (100 mg/L of PDZ combined with LED) led to a 4.36 log₁₀ reduction in fluconazole-susceptible *C. albicans* (CaS) in an animal model (Carmello et al., 2015). In another study, aPDT using the same photosensitizer combined with LED light for the inactivation of fluconazole-resistant *C. albicans* (CaR) resulted in a reduction of approximately 1.96 log₁₀ (Alves et al., 2019). When animals were subjected to treatment (5 sessions of aPDT), the results demonstrated that aPDT was as effective as Nystatin in inactivating CaS, achieving reductions of 3 and 3.2 log₁₀, respectively. Additionally, it was observed that animals treated with aPDT showed complete remission of oral lesions (Carmello et al., 2016). Conversely, in the same animal model contaminated with a CaR strain (ATCC 96901), animals treated with either aPDT or Nystatin alone showed similar reductions in CaR viability (1.3 and 1.1 log₁₀, for aPDT and Nystatin, respectively); however, no improvement in the macroscopic appearance of the lesion on the tongue was noted (Hidalgo et al., 2019). Therefore, treatments for infections caused by resistant strains require enhancements to improve their efficacy.

1.1.6 Antimicrobial Peptides Potential

Antimicrobial peptides (AMPs) are oligopeptides containing up to 50 amino acids with a broad spectrum of action against microorganisms, including viruses, bacteria, fungi, and parasites (Boparai & Sharma, 2020). AMPs occur naturally in both prokaryotes and eukaryotes and, in multicellular organisms, are part of the innate immune system's defense line against potentially harmful microorganisms (Bechinger & Gorr, 2017). Interestingly AMPs have grown primarily due to their low cost and their small molecular size, high biocompatibility, and antimicrobial action at low concentrations (Fosgerau & Hoffmann, 2015). AMPs with antibacterial activity have an overall positive charge and amphipathic nature, primarily targeting the lipid bilayer of the plasma membrane, along with intracellular targets (Bahar & Ren, 2013a). The action mechanism of AMPs

on bacterial plasma membranes depends on their electrostatic and hydrophobic interactions with the lipid bilayer, either intracellularly or extracellularly (Bechinger & Gorr, 2017).

Due to its significant antibacterial activity and reduced structural size, several types of AMPs have been becoming an extremely viable model for infection control assays. Peptides such as Hylin-a1 exhibit broad-spectrum antimicrobial activity against Gram-negative and Gram-positive bacteria, as well as diverse species of *Candida spp.* (*C. albicans*, *Candida krusei*, *Candida parapsilosis*, and *Cryptococcus neoformans*) (Castro et al., 2009; Vignoli Muniz et al., 2020). Another peptide that has been studied for its antimicrobial properties is KR-12-a5, a derivative of LL-37, classified as an α -helix, making it one of the smallest known peptides with antimicrobial activity (B. Mishra et al., 2013). Additionally, the peptide temporin-SHa also showed antimicrobial activity due to its secondary structure, net positive charge, hydrophobicity, helicity, and amphipathicity (Q. Chen et al., 2004). Despite numerous investigations testing the effects of various AMPs on different pathogens, there is a lack of studies in the literature addressing the antifungal potential of these AMPs in biofilm models involving resistant-*Candida* strains and *S. mutans* in the same biofilm.

Histatin 3 (His3) and Histatin 5 (His5), antimicrobial proteins with peptide sizes that come from saliva, also have distinct antimicrobial activity against oral microorganisms (Kavanagh & Dowd, 2004a). His5 particularly has an effect against *C. albicans*, inhibits its growth, and damages mitochondria and ATP production (Moffa, Mussi, et al., 2015a). Additionally, His3 also reduces *S. mutans* adhesion to hydroxyapatite (Marin et al., 2022). In addition, derived peptides like DR9, from statherin, inhibit *C. albicans* hyphal formation, reducing virulence and cell proliferation, while RR14, from His3, reduces *S. mutans* biofilm biomass (Moussa & Siqueira, 2021; Pellissari et al., 2021a). Despite their effectiveness, studies on Hist3, Hist5 and the AMP's derived (DR9-RR14 and RR14) have focused only on single-species biofilm, with limitations also in complex-biofilms involving more than one species (Gyurko et al., 2000; Kavanagh & Dowd, 2004a; Komatsu et al., 2011, 2019a; Mochon & Liu, 2008; Moffa, Machado, et al., 2015; Moussa &

9 CONCLUSION

This thesis collectively demonstrates the therapeutic potential of combining AMPs with aPDT as an innovative strategy to manage *C. albicans* and *S. mutans* biofilms, particularly those exhibiting antifungal resistance, with His3 and His5 identified as the most promising peptides through progression from simple biofilm models to mixed species and 3D oral tissue models.

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