

Effect of environmental signals on the development of oral biofilm

Kanchana Nandanan Chathoth

▶ To cite this version:

Kanchana Nandanan Chathoth. Effect of environmental signals on the development of oral biofilm. Human health and pathology. Université de Rennes, 2021. English. NNT: 2021REN1B007 . tel-03412828

HAL Id: tel-03412828 https://theses.hal.science/tel-03412828

Submitted on 3 Nov 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers. L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



THESE DE DOCTORAT DE

L'UNIVERSITE DE RENNES 1

COMUE UNIVERSITE BRETAGNE LOIRE

ECOLE DOCTORALE N° 605 *Biologie Santé* Spécialité : Microbiologie, Virologie, Parasitologie

Par

Kanchana Nandanan CHATHOTH

Effect of environmental signals on the development of oral biofilm

Thèse présentée et soutenue à Rennes, le 22 Février 2021 Unité de recherche : U-1241 Nutrition Metabolisms and Cancer, U.F.R. d'Odontologie, Université de Rennes 1, 2 Avenue du Pr. Léon Bernard, 35043 RENNES

Composition du Jury :

Rapporteurs:

Pr. Alain DUFOUR Pr. Olivier LESOUHAITIER Professeur d'université, Université de Bretagne-Sud, Lorient Professeur d'université, Université de Rouen, Evreux

Président :

Pr. Vincent MEURIC Professeur d'université - Praticien hospitalier, Université de Rennes 1, Rennes

Examinateurs :

- Pr. Sylvie BOISRAME Professeur d'université Praticien hospitalier, Université de Bretagne Occidentale, Brest
- Dr. Nabila HADDAD Maître de conférences, ONIRIS Ecole Nationale Vétérinaire, Nantes
- Dr. Fabrice MAHE Maître de conférences, Université de Rennes 1, Rennes

Directeur de thèse :

Dr. Christine BAYSSE Maître de conférences, Université de Rennes 1, Rennes, France

Co-directeur de thèse :

Dr. Bénédicte MARTIN	Project Manager, SLS France,	Saint Jacques de la Lande
----------------------	------------------------------	---------------------------

Abstract	Х
Résumé	XI
Abbreviations	XII
Acknowledgements	XIV
1. INTRODUCTION	1
1.1. Aetiology of Periodontal disease	1
1.1.1. The disease	1
1.1.2. Oral microbiome of healthy individuals	3
1.1.3. Oral microbiome of periodontitis patients	4
1.1.4. Oral biofilm formation and dysbiosis	6
1.2. Iron and the three-species oral biofilm model	8
1.2.1. Iron and its biological significance	8
1.2.2. Iron in diseases	9
1.2.3. Iron in the oral cavity	10
1.2.4. The three-species model and their relation with iron	11
1.2.4.1. Selected oral bacteria: Streptococcus gordonii, Porphyromonas gingivalis and Treponema denticola	11
1.2.4.2. The iron transport systems of the selected oral bacteria	14
1.2.4.2.1. Iron acquisition strategies employed by <i>P. gingivalis</i>	15
1.2.4.2.2. Iron acquisition strategies employed by oral anaerobic spirochetes	18
1.2.4.2.3. Iron acquisition by streptococcal species	19
1.3. Hypothesis: The impact of iron and oxygen in the three-species oral biofilm model	21

TABLE OF CONTENTS

1.4. Objectives of the thesis	32
2. GROWTH MEDIA FOR ORAL BACTERIA	35
2.1. Introduction	35
2.2. New growth media for oral bacteria	37
2.3. Complementary data	41
2.3.1. Determination of optimal PPIX concentrations for <i>P. gingivalis</i> and <i>T. denticola</i> growth	41
2.3.2. H ₂ S quantification in MMBC medium	43
2.4. Discussion and perspectives	46
3. DEVELOPMENT OF <i>IN VITRO</i> MODELS AND PROTOCOLS FOR TESTING ANTIMICROBIAL AGENTS AGAINST ORAL BIOFILM	47
3.1. Introduction	47
3.2. Detachment of oral bacteria in biofilms is induced by a solution containing the haemoglobin of <i>Arenicola marina</i>	49
3.3. Test of antibacterial gels against a multispecies oral biofilm with an adapted <i>in vitro</i> assay (Publication under review in the Journal of Applied Microbiology)	68
3.4. Discussion and perspectives	92
4. EFFECT OF IRON ON THE DEVELOPMENT OF ORAL BIOFILMS	94
4.1. Introduction	94
4.2. Effect of iron and interspecies associations on biofilm initiation by three oral bacteria	95
4.3. Effect of iron and interspecies associations on biofilm growth and development	119
4.4. Mathematical modelling as a tool to study the interspecies associations and effect of iron on oral biofilm development	147

4.5. Complementary data	177
4.6. Discussion and perspectives	180
5. CONCLUSION AND PERSPECTIVES	182
6. ANNEXURE	186
6.1. Poster communications	186
6.1.1 New growth media for oral bacteria	186
6.1.2. <i>Arenicola marina</i> extracellular hemoglobin: a new promising treatment in periodontal disease	188
6.1.3. A simple method derived from the Calgary biofilm device for testing antimicrobial gels against oral biofilms	190
6.1.4. Effect of iron on the development of oral biofilm	192
6.2. Oral communications	194
6.2.1. New growth media for oral bacteria	194
6.2.2. A simple method derived from the Calgary biofilm device for testing antimicrobial gels against oral biofilms	195
6.2.3. Effect of iron on the development of oral biofilm	196
6.2.4. The events that may contribute to subgingival dysbiosis: a focus on the interplay between iron, sulfide and oxygen	197
7. REFERENCES	198

LIST OF FIGURES

Figure number	Title	Page number
	Chapter 1	
1	Comparison of the effect of microbial colonization on the tooth in health and in disease	2
2	Association of chronic periodontitis with several systemic disorders	3
3	Bacterial make-up of the subgingival biofilm and its organization into different microbial complexes	5
4	The steps involved in the oral biofilm formation.	7
5	Interaction between periodontal pathogens and other external factors	8
6	Inter-species association between <i>P. gingivalis</i> and <i>S. gordonii</i> via FimA–GAPDH and Mfa1–SspA/B adhesion-receptor pairs on the surfaces of the organisms	12
7	Metabolic cooperation between P. gingivalis and T. denticola	13
8	Schematic representation of the various heme acquisition systems in <i>P. gingivalis</i>	16
9	A schematic overview of the major iron, heme and siderophore acquisition systems in <i>S. pyogenes</i> .	20
10	Cascade of events leading to dysbiosis in periodontitis: the possible role of oxygen, iron and H_2S in the multifactorial process.	22
	Chapter 2.3	
1	Minimal concentration of PPIX required for <i>P. gingivalis</i> growth in absence of hemin.	42
2	Growth of <i>T. denticola</i> in MMBC-2 media with different iron sources and in NOS medium.	43

3	H ₂ S production in MMBC-3 medium.	45
	Chapter 3.2	
1	The oxygenation solution containing M101 induced detachment of oral bacteria from biofilm.	64
2	The oxygenation solution containing M101 increased cell staining by membrane impermeant PI dye	65
3	The oxygenation solution containing M101 induced reduction of each species in the biofilm.	66
S1	Antimicrobial activity of the oxygenation solution containing M101 on planktonic cultures.	67
	Chapter 3.3	
1	The process of testing antimicrobial gels on oral biofilms established on the surface of the pegs of the MBEC TM apparatus	87
2	Representative microscopic images of the three-species oral biofilm on the surface of the pegs.	88
3	qPCR quantification of the number of bacteria (CFU ml ⁻¹) in the biofilm (collected from the pegs) and in planktonic form (collected from the 96-well microtiter plate).	89
	Chapter 4.2	
1	Effect of iron and inter-species association on attachment of each species in either mono- or multi-species sessile cells.	112
2	Effect of iron on the microscopic parameters obtained for various mono-, dual- and three-species biofilms.	113
3	Representative microscopic images of bacteria attached at 2 hours in mono-, two- and three-species biofilms.	114
4	Biofilm initiation in the subgingival niche - effect of iron and interspecies associations.	115

S1	Comparison between mono-, dual- and three-specie 2-hour biofilms.	116
	Chapter 4.3	
1	Growth of mono-species biofilms at various iron concentrations.	135
2	Microscopic parameters for mono-species biofilms.	136
3	Effect of interspecies interactions at optimal iron concentration for multi-species biofilms.	137
4	The microscopic parameters obtained for various mono-, dual- and three-species biofilms grown at optimal iron concentration.	138
5	Representative 3-dimentional images obtained for mono and multi- species 72-hour biofilms at optimal iron concentration.	139
6	Growth of individual species in <i>S. gordonii-P. gingivalis-T. denticola</i> biofilms at various iron concentrations.	140
7	Microscopic parameters for <i>S. gordonii-P. gingivalis-T. denticola</i> biofilms.	141
8	Representative 3-dimentional images obtained for three-species 144-hour biofilms at various iron concentration.	142
S1	Representative microscopic images of <i>T. denticola</i> biofilms at 24 and 144 hours grown at 8 μ M of iron.	144
S2	Percentages of each species in dual- and three-species biofilms.	144
S3	The total bacterial concentration in mono-, dual- and three-species biofilms grown at optimal iron concentration.	145
S4	The average thickness of various mono-, dual- and three-species biofilms grown at optimal iron concentration.	145
S5	Average thickness of <i>S. gordonii-P. gingivalis-T. denticola</i> biofilms.	146
	Chapter 4.4	

1	Schematic of the modelling domain and grid of the two-	151
	dimensional cellular automaton.	
2	Biofilm growth simulation for <i>S. gordonii</i> mono-bacterial biofilm.	158
3	Biofilm growth simulation for <i>P. gingivalis</i> mono-bacterial biofilm.	159
4	Biofilm growth simulation for <i>T. denticola</i> mono-bacterial biofilm.	160
5	Simulated image for mono-bacterial biofilm.	162
6	Biofilm growth simulation for dual-species biofilm.	165
	Chapter 4.5	
1	Growth of individual species in S. gordonii-T. denticola biofilms at	178
	various iron concentrations.	
2	Microscopic parameters for S. gordonii-T. denticola biofilms.	178
3	Representative 3-dimentional images obtained for 72-hour S.	179
	gordonii-T. denticola biofilms at various iron concentration.	

LIST OF TABLES

Table number	Title	Page number
	Chapter 3.3	
1	Species-specific primer sequences used in this study	90
2	<i>In vitro</i> inhibition of Hyalugel®-ADO and blue®m oral gel against <i>S. gordonii</i> and <i>P. gingivalis</i> using an agar diffusion assay	91
	Chapter 4.2	
1	List of primers used for the study.	117
1 S	Concentration of each species (CFU/ml) in mono-species and multispecies biofilms at 0.8, 8 and 80 μ M of iron.	118
	Chapter 4.3	
1	List of primers used for the study.	143
	Chapter 4.4	
1	Model parameters and variables	172
2	Damage parameters and variables	173
3	Size parameters of <i>S. gordonii</i> , <i>P. gingivalis</i> and <i>T. denticola</i> estimated from SEM experiments and previous report (Martin <i>et al.</i> , 2017).	174
4	Estimation of yield coefficients of <i>S. gordonii</i> , <i>P. gingivalis</i> and <i>T. denticola</i> .	175
5	Experimental growth parameters for <i>S. gordonii</i> , <i>P. gingivalis</i> and <i>T. denticola</i>	176

ABSTRACT

The oral bacteria form a complex community and their pathogenic switch can be influenced by host-related factors such as systemic iron and oxygen levels. Previous studies have suggested that genetic hemochromatosis, characterized by systemic iron overload, favours the occurrence of severe periodontitis but the mechanisms involved are unclear. Therefore, this thesis aimed to analyse the behaviour of multispecies oral biofilms at different iron levels composed of periodontal bacteria selected for their importance in the development of periodontal diseases: Streptococcus gordonii, Porphyromonas gingivalis and Treponema denticola. To realize this multispecies biofilm, an iron-controlled growth medium (Mixed Medium for Bacterial Community) was developed. This work includes applied research work and a fundamental part. In the framework of the development of a system for evaluating the anti-biofilm activity of new products, methods for testing the effectiveness of antimicrobial liquids or gels were developed and used to challenge the 3-species biofilm. To better understand the effect of iron on the behaviour of three-species oral biofilm, the interspecies associations in biofilm initiation/attachment and followed by biofilm growth and development in response to iron levels (0.8, 8, 80 µM FeSO₄) were investigated. This study, for the first time, showed the interactions between S. gordonii and T. denticola in biofilms and the effect of iron on these biofilms. The most striking result was the lower initial attachment and growth of this dual species biofilm at the lower iron concentrations. Moreover, the presence of the three-species altogether favoured the attachment of both pathogens P. gingivalis and T. denticola compared to mono-species biofilms and displayed a preferential growth of all the three species at 80 µM iron at 144 hours. Such preference for high iron levels for biofilm maturation may lead to oral dysbiosis and the progression of periodontitis during ironoverloaded diseases. To further evaluate the inter-bacterial associations, the effect of iron in the evolution of the oral biofilm is being studied in the form of a new mathematical model with T. denticola in a previously established model for P. gingivalis and S. gordonii. The parameters were calibrated by measurements made from biological experiments in monospecies biofilms which will be used to test different hypotheses for either independence or competition for substrate between species, mutual benefit/syntrophy or production of toxic molecules for dual- and three-species biofilms. Finally, the validation of the model will be done by comparing the simulated results with those of the experimental results obtained for dualand three-species biofilms. Altogether this study will enable a better understanding of the irondriven pathogenesis of the oral microbiota.

RÉSUMÉ

Le microbiote buccal forme une communauté complexe. La dysbiose de ce microbiote est à l'origine des maladies parodontales. Parmi de nombreux facteurs pouvant induire cette dysbiose, les niveaux systémiques de fer ont récemment été identifiés comme possibles signaux. Des études antérieures ont suggéré que l'hémochromatose génétique, caractérisée par une surcharge systémique en fer, favorisait le développement de parodontites sévères, mais les mécanismes impliqués ne sont pas élucidés. Par conséquent, ces travaux de thèse ont eu pour objectif d'analyser le comportement de biofilms buccaux à différentes concentrations en fer. La complexité du biofilm buccal a été réduite à trois espèces clefs afin de pouvoir analyser leurs interactions. Les espèces bactériennes ont été sélectionnées pour leur importance dans le développement des maladies parodontales et leurs coopérations métaboliques : *Streptococcus* gordonii, Porphyromonas gingivalis et Treponema denticola. Pour réaliser ce biofilm multiespèces, un milieu de croissance permettant de contrôler la concentration en fer et la croissance des trois espèces bactériennes (Mixed Medium for Bacterial Community) a été développé. Ce modèle de biofilm a servi de base pour mener des travaux de recherche appliquée et fondamentale. Des méthodes pour tester l'efficacité de liquides ou gels antimicrobiens sur le biofilm buccal ont été développées. En parallèle, l'effet de différentes concentrations en fer sur l'attachement, l'initiation du biofilm et son développement a été étudié. Cette étude a montré pour la première fois les interactions entre S. gordonii et T. denticola dans les biofilms. La carence en fer s'est révélée délétère pour l'attachement et la croissance d'un biofilm composé uniquement de ces deux espèces bactériennes. De plus, la présence des trois espèces bactériennes favorise l'attachement des deux pathogènes P. gingivalis et T. denticola par rapport aux biofilms mono-espèces. Enfin, une forte concentration en fer permet une meilleure croissance du biofilm total et de chacune des trois espèces au sein du complexe. Une telle préférence pour des niveaux élevés de fer pour la maturation du biofilm peut expliquer la dysbiose buccale et les parodontites sévères observées lors des maladies de surcharge en fer. Pour optimiser l'analyse des associations entre les espèces bactériennes et l'effet du fer sur ces interactions, un modèle mathématique est en cours d'élaboration. Les premières étapes de ce projet sont présentées dans cette thèse. Les paramètres mathématiques ont été calibrés par des mesures biologiques obtenues sur des biofilms à une espèce bactérienne. Ces paramètres seront utilisés pour tester différentes hypothèses d'interactions entre les espèces : indépendance ou compétition pour le substrat, bénéfice mutuel/syntrophie et/ou production de molécules toxiques. Enfin, la validation du modèle obtenu mathématiquement se fera en comparant les résultats simulés avec les résultats expérimentaux obtenus pour les biofilms bi et tri-espèces. Dans l'ensemble, cette étude permettra une meilleure compréhension du comportement du biofilm buccal en fonction de la concentration en fer.

ABBREVIATIONS

- PSD Polymicrobial Synergy and Dysbiosis
- IMPEDE Inflammation-Mediated Polymicrobial-Emergence and Dysbiotic-Exacerbation
- TCA Tricarboxylic acid
- Fe-S Iron-sulfur clusters
- **ROS** Reactive Oxygen Species
- LPS Lipopolysaccharides
- DNA Deoxyribonucleic acid
- IDA Iron Deficiency Anemia
- SOD Superoxide dismutase
- TSAT Transferrin saturation
- GCF Gingival Crevicular Fluid
- ICP-MS Inductively Coupled Plasma Mass-Spectrometry
- GAPDH Glyceraldehyde-3-phosphate dehydrogenase
- FimA Long fimbriae of P. gingivalis
- Mfa Short fimbriae of *P. gingivalis*
- H₂S Hydrogen Sulfide
- RgpB Arg-gingipain B
- RgpA Arg-gingipain A
- Kgp Lys-gingipain
- HagA Hemagglutinin A
- metHb Methemoglobin
- HusA Heme Uptake System protein A
- OMP Outer Membrane Protein
- pO₂ Partial pressure of Oxygen
- SOR Superoxide reductase
- LctO Lactate Oxidase
- LAAO L-Amino Acid Oxidase
- H₂O₂ Hydrogen Peroxide
- VSCs Volatile Sulfur Compounds
- GSH Glutathione
- BSA Bovine Serum Albumin
- CAA Casamino acids

MMBC - Medium for Mixed Bacterial Community

PPIX - Protoporphyrin IX

PLP - Pyridoxal-5'-phosphate

LCD - L -Cysteine Desulfhydrase

BHI - Brain Heart Infusion

OMIZ-M - Oral Microbiology and Immunology Zurich Medium

OBGM - Oral Bacterial Growth Medium

BM - Biofilm Medium

PBS - Phosphate Buffered Saline

qPCR - quantitative Polymerase Chain Reaction

Ct - Threshold Cycles

MBECTM - Minimum Biofilm Elimination Concentration

CA - Cellular Automaton

IbMs - Individual-based Models

ACKNOWLEDGEMENTS

I want to express my heartfelt gratitude towards my jury members Pr. Alain Dufour, Pr. Sylvie Boisrame, Pr. Olivier Lesouhaitier, Dr. Nabila Haddad, and Pr. Vincent Meuric for agreeing to evaluate my thesis and to come for my defence. I thank them for investing their precious time and patience for the same.

I express my sincere thanks to my thesis director Dr. Christine Baysse for her patience, guidance, help and encouragement throughout my thesis. My sincere thanks to Dr. Bénédicte Martin, my thesis co-director, for her guidance, valuable inputs and suggestions, technical help with experiments and troubleshooting. I thank both of you from the bottom of my heart, for believing in my capabilities and giving me this opportunity. I am extremely thankful for your timely help, suggestions, and patience, especially during the proofreading of my thesis and papers.

I express my sincere gratitude to Pr. Martine Bonnaure-Mallet for allowing me to be a part of her team. Thank you so much for your tremendous help and support in providing the opportunity to register as a PhD student and in acquiring the contract of associate assistant at the Odontology department. I express my gratitude to the previous and present Deans of Odontology, Pr. Anne Dautel and Pr. Sylvie Jeanne for their support in the realization of this thesis.

I wish to express my gratitude to our collaborator, Dr. Fabrice Mahé, IRMAR for this valuable collaboration. It was indeed an enriching experience and a great opportunity to learn. I am extremely thankful to him for being so kind and patient in explaining the intricacies in mathematical modelling. My project and thesis would not have been the same without his help and guidance. I am very thankful to you for having added value to this thesis.

My heartfelt gratitude to Dr. Zohreh Shacoori for being an extremely understanding, co-operative, friendly and humble colleague. Thank you for making all the Microbiology TPs at the Odontology fun and fulfilling. Thank you for always being considerate, patient with me and for always helping and supporting me so that I could maintain the balance between my work as an associate assistant and as a PhD student. It was a pleasure to assist you and it was a wonderful learning experience too. Thank you very much!

I am very thankful to Dr. Olivier Loréal for his immense support, kindness, suggestions and help during these years. Thank you so much for always being there and for helping me in the difficult times. I would also like to thank Dr. Bruno Clement for his support and encouragement. I also express my sincere gratitude to my thesis committee members, Dr. Alain Sarniguet and Dr. Yves Le-Loir for their regular feedback, encouragement and valuable suggestions during my thesis.

I am very thankful to Mme. Ludivine Malherbe, Mme. Nolwenn Oliviero, Mme. Patricia Leroyer, Mme. Isabelle Cannie and Mme. Catherine Le Lann for all the support and help you offered. Thank you so much for always attending to all my queries and requests with a pleasant smile. You all are the people that made it possible for me to achieve my goals. Thank you so much!

I would like to express my heartfelt thanks to Pr. Vincent Meuric and Dr. Emile Boyer for giving me space in their office and for giving me such good company. Thank you for always being kind, friendly and helpful. Thank you very much for your suggestions, advice and ideas. You helped me retain my calm during the most difficult times of my PhD. Thank you so much!

My heartfelt thanks to Emilie Saucisse (M2), Amel Saim-Mamoun (M2), Olga Pacios Santamaria (M1) and Stéven Yvenou (M1) for their help during their internship. Thank you for the friendly discussions and good time spent in the laboratory. I sincerely wish you all tremendous success for all your future endeavours.

I want to express my gratitude to the MRic photonics platform and their engineers Mme. Stephanie Dutertre and Mr. Xavier Pinson for readily helping me with the use of the facilities in the platform and also for always readily answering my questions and concerns regarding the same. I want to express my special thanks to Dr. Claude Bendavid for providing help with glucose estimations and Dr. Martine Ropert for ICP-MS analysis in the measurement of iron concentration. My sincere thanks to Dr. Latifa Bousarghin for providing me with the Pierce[™] BCA Protein Assay Kit for protein estimation and Dr. Pascal Loyer for providing propidium iodide solution. My sincere thanks to Dr. Sandrine David-Le Gall for her help and support during these years. I would also like to thank Dr. Alain Fautrel for the initial tests with Raman spectroscopy. Unfortunately, we could not proceed with the work but I am very thankful for the learning experience.

My sincere thanks to Mme. Ionescu Corinne (HR), Mme. Josée Massart and Mme. Élodie Le Coz (Odontology), Mme. Emilie Gesnys (CMI Rennes), Mme. Badia M'ssassi and Mme. Nathalie Theret (Biologie Santé), Mme. Adina Pascu, and Mme. Patricia Jouas (NuMeCan) for being kind, thoughtful, friendly and always doing their best to help me in every administrative work. Without your help, I would have got lost amidst the administrative work/papers. Thank you for being warm, welcoming and a true saviour during the time of need! I want to thank the DSI department and especially our dear Mr. Thomas Poussou for readily helping me during the various times when the software of my computer failed to co-operate. You have saved my data/results and thus also my thesis. Thank you so much for being so humble, friendly and kind!

I want to express my gratitude to the entire team of blue®m for the generous gift of their product, blue®m oral gel and for their immense support, co-operation and timely help in providing the product-related information. I would like to specially mention Mme. Denise Leusink, Mr. Fokke Jan Middendorp and Mme. Cristel Malherbe-Bredius for their friendly nature, support, encouragement and for being readily available for addressing my queries regarding the product. It was a pleasure discussing and associating with you.

I would like to thank the friends I have made during these years, Yoann, Elise, Mélanie, Kevin, Julien, Moussa, Yann, Thomas and Julie for helping me at various stages. I would like to especially thank Saba, Luis and Gentiana for always being kind, for enquiring about my progress and for always motivating and encouraging me to deliver my best.

I want to express my sincere gratitude to my teachers, Dr. K. Balakrishnan, Dr. N. S. Vasanthi and Dr. K. Subramanian for always being an inspiration. Thank you for your guidance, support, encouragement and blessings. I want to express my sincere thanks to Dr. Savitri Sharma, Dr. S. Shivaji, Dr. Swapna Reddy, Dr. Joveeta Joseph and Dr. R. Ravikumar who have guided me in research projects at different time points.

I would like to especially mention my friends for their best wishes, love, support and prayers. I want to express my heartfelt thanks to Sibi di for being that elder sister I never had. Thank you so much for having my back! Thank you so much for always checking on me and making sure that I was doing good. I want to thank my dearest friends who are now family to me, Bhuvanesh, Pramoda, Karthik, Meenakshi and Apurba for your constant encouragement, love and care. Thank you for always being there. I also want to thank Nithiya, Aswani, Rini, Vasantha Krishnan, Balveer Singh, Sunitha, Shakti, Vaniji and Shahna for their constant encouragement, motivational chats, support and help. My special thanks to my dearest friend

Divya Lakshmi for always being supportive, encouraging and loving. I would also like to thank Divya Sreevalsan for being an inspiration and for always sending me positive vibes.

I wish to express my heartfelt gratitude to my parents Bhargavi Nandanan and C. K. Nandanan for being my backbone and driving force. Your love and support mean a lot to me. Thank you so much for believing in me and for supporting my decisions. My special thanks to my brother, Suraj Chathoth for always being excited about my work and progress. Thank you for making me believe in my worth. I would also like to thank my dearest sister-in-law Shereena Tamburankandiyil and my nephews Rohit and Vihaan for the love, encouragement and support. I also thank my husband's parents, Lilly P and Damodaran N, sister Poornima D, her husband, Mageshwar R and little Haasini M for all the love, support, good wishes and prayers.

One person I cannot thank enough is my husband, Dr. Arun Prasath Damodaran. Thank you for being by my side in all ups and downs. Thank you for celebrating every small success, helping me believe in myself and motivating me at every step to pursue my goal. Thank you for believing in me, even at times when I had almost given up. Thank you for helping me recover from every breakdown. Thank you for bearing with me when I was at my worst and for helping me deliver my very best. Thank you for all the support, care, motivation and love that you have bestowed upon me throughout. This journey would not have been possible without you! I owe this thesis to you.

1. INTRODUCTION

1.1. Aetiology of Periodontal disease

1.1.1. The disease

Microorganisms tend to live in highly complex biofilm communities. These communities are residents of the human body, including the oral environment. The oral microbiome, oral environment and the host strike a fine balance to promote oral health. Shifts in the oral environment or the composition of the microbiota may trigger oral diseases (Lamont, Koo and Hajishengallis 2018) of which the two most common ones in the oral environment are caries, which affect the teeth, and periodontitis, which affects the periodontium. The comparison of the effect of microbial colonization on the tooth in health and in both diseases is depicted in **Figure 1**. Periodontal diseases can range from a mild gingival infection to severe periodontitis. The focus of this thesis is on key microorganisms that are causative agents of periodontitis.

Periodontitis is a chronic, inflammatory, polymicrobial disease, associated with oral biofilms also called dental plaque. The periodontal disease starts at the level of the gingivo-dental sulcus. The persistence of bacteria and their penetration in the connective tissue, lead to a non-specific local inflammatory response and a specific immune response. Simultaneously, the destruction of the periodontal tissues occurs due to the virulence factors of the pathogenic bacteria (proteolytic enzymes, toxins) and also the cells defences stimulated by these bacteria. The bacteria and their products stimulate the resident and migrating host cells to express and release tissue degradation enzymes. All these together, induce an immune response via the release of cytokine by lymphocytes, macrophages and neutrophils.

Gingiva, bone and ligament loss may adversely affect the periodontium resulting in a periodontal pocket where the pathogens flourish. This acts as a subgingival niche with reduced oxygen tensions which favours anaerobic periodontal pathogens such as Bacteroidaceae spp. and Spirochaetes. We focus on *Porphyromonas gingivalis*, considered a keystone pathogen which can subvert the commensal microbiota into a dysbiotic one by modulating the host innate response (Mysak *et al.* 2014). It is often found in patients suffering from chronic periodontitis, mostly along

with another member of the red complex, *Treponema denticola* (Kigure *et al.* 1995; Meuric *et al.* 2013; Zhu *et al.* 2013; Tan *et al.* 2014a; Kumawat *et al.* 2016).



Figure 1: Comparison of the effect of microbial colonization on the tooth in health and in disease (Figure adapted from Lamont, Koo and Hajishengallis 2018).

Periodontitis does not only cause infection, malodour, bone loss or loss of tooth but is also associated with several other systemic disorders (**Figure 2**). Many studies have associated severe or chronic periodontitis to disorders like rheumatoid arthritis, cardiovascular disease, premature birth, diabetes, hemochromatosis and even oral cancer (Hujoel *et al.* 2003; López 2008; Preshaw *et al.* 2012; Koziel, Mydel and Potempa 2014; Beukers *et al.* 2017; Bui *et al.* 2019; Graves, Corrêa and Silva 2019; Nwizu, Wactawski-Wende and Genco 2020). The detailed mechanism behind these associations is not fully understood. However, the particular role of the key oral pathogen *P. gingivalis*, in systemic diseases via bacterial cell dissemination, or release of lipopolysaccharide, fimbriae, outer membrane vesicles, and gingipains demonstrated by several studies has been reviewed (Mei *et al.* 2020).



Figure 2: Association of chronic periodontitis with several systemic disorders

1.1.2. Oral microbiome of healthy individuals

The oral microbiome of healthy individuals is predominated by facultative anaerobes such as streptococci and Actinomyces species. Many other genera (like Capnocytophaga, Eikenella, Fusobacterium. Haemophilus, Lactobacterium. Eubacteria. *Leptotrichia*, Neisseria. Porphyromonas, Prevotella, Propionibacterium, Peptostreptococcus, Staphylococcus, Veillonella, Treponema, Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes and Fusobacteria) are also prevalent in healthy individuals. These species co-exist in equilibrium with the host immune defence systems and form the commensal oral microbiome. Evolutionary processes of mutual adaptations between the host and its microbiota give rise to a diverse oral microbiota which are also affected by diet, hygiene, health and antimicrobial therapies. Protective mechanisms of the periodontium involve select innate host defence mediators which reduce the microbial load on the epithelial-cell surface. The interdependence between the host and the oral microbiota is conserved by the nutrient-rich environment provided by the host while the commensal microbes maintain a homeostasis by secreting metabolites, evading innate immunity, and stimulating host immune responses for protecting host against pathogens (Avila, Ojcius and Yilmaz 2009; Zarco, Vess and Ginsburg 2012).

1.1.3. Oral microbiome of periodontitis patients

Several studies have emphasized on the fact that the oral microbiome is not only specific to each individual but also to the oral site like tongue, subgingiva etc. (Aas et al. 2005). It is also distinct in health and disease. However, in most general terms, the microbiome of patients with periodontitis is predominated by anaerobic gram negative periodontal pathogens of the red (P. gingivalis, T. denticola, Tannerella forsythia) and orange complex (Fusobacterium nucleatum, Prevotella intermedia, Peptostreptococcus micros, Eubacterium nodatum). The members of the green complex (Capnocytophaga gingivalis, Capnocytophaga sputigena, Capnocytophaga ochracea. Campylobacter concisus. Eikenella corrodens. and Actinobacillus actinomycetemcomitans serotype a) are also associated with periodontal disease while the purple (Veillonella parvula, Actinomyces odontolyticus) and the yellow complex (Streptococcus gordonii, Streptococcus intermedius, Streptococcus mitis, Streptococcus oralis, Streptococcus sanguis) mainly consist of facultative anaerobic bacteria associated with oral health (Socransky et al. 1998; Paster et al. 2001, 2006; Ackermann and Reichenbach 2010; Griffen et al. 2012; Abusleme et al. 2013).

The organization of the periodontal bacteria into various colour-coded complexes in the form of a pyramid is depicted in the **Figure 3**. The base of the pyramid comprises of complexes with species that are thought to thrive under healthy conditions or at a very early stage of periodontitis. The central part represents the species which are more dominant in an advanced stage of periodontitis and are thought to bridge base complexes with the pyramidal roof. Finally, the apex or the pyramidal roof comprises of species in the final stage in disease progression.



Figure 3: Bacterial make-up of the subgingival biofilm and its organization into different microbial complexes (Figure adapted from Ackermann and Reichenbach 2010; Socransky *et al.* 1998).

Interestingly microbiome changes during periodontitis are characterized by an increased diversity while most diseases (inflammatory bowel disease, caries) are associated with decreased diversity (Curtis, Diaz and Van Dyke 2020).

Recently a study was performed to identify microbial signatures for health and chronic periodontitis at the genus level. It was carried out from published sequencing data obtained from several different studies (196 subgingival samples from patients with chronic periodontitis and 422 subgingival samples from healthy subjects). Specific genera (*Veillonella, Neisseria, Rothia, Corynebacterium*, and *Actinomyces*) were found to be prevalent (>95%) in health, while other genera (*Eubacterium, Campylobacter, Treponema,* and *Tannerella*) were significantly associated with chronic periodontitis. When restricted to a subgingival sample set with well-defined clinical data, the method permitted the correlation between a simplified dysbiotic ratio (*Porphyromonas, Treponema,* and *Tannerella* to *Rothia* and *Corynebacterium*) and pocket depth (Meuric *et al.* 2017b). Another study in mice showed that oral dysbiosis is strain-dependent. *P. gingivalis* W83 successfully initiated periodontitis in mice and exhibited a specific distinct

microbiota with decreased proportions of taxa usually found in healthy individuals while *P. gingivalis* TDC60-treated mice only experienced moderate lesions (Boyer *et al.* 2020a).

1.1.4. Oral biofilm formation and dysbiosis

The oral biofilm formation is of exceptional complexity due to the wide range of bacterial species involving more than 700 species, the individual variations and the interactions between host and microbiota. A hierarchical model proposes that oral biofilm formation is made possible mainly by early or primary colonizers of the oral environment. These commensal facultative anaerobic bacteria are often related to the oral well-being. They include the streptococci and Actinomyces species. These bacteria adhere to the surface of the tooth coated with salivary proteins that form a pellicle on the tooth surface. The early colonizers (considered as commensals) may further facilitate the adherence of secondary and late colonizers via their surface receptors (**Figure 4**). These late colonizers may consist of the anaerobic pathogens during dysbiotic events responsible for periodontal disease (Kolenbrander *et al.* 2002; Sbordone and Bortolaia 2003; Hojo *et al.* 2009).

Several models, most of them not mutually exclusive were proposed to explain the dysbiosis inside the sub-gingival biofilm leading to periodontal diseases : (1) the overgrowth of pathogenic species (Loesche 1979),(2) the red complex hypothesis as presented in **Figure 3** (Socransky *et al.* 1998); (3) the keystone hypothesis based on the low abundance of specific disruptive pathogenic species which modulate the host immune system (Hajishengallis *et al.* 2011); (4) the polymicrobial synergy and dysbiosis (PSD) hypothesis which focus on specific species interactions leading to disrupted homeostasis independently of a red complex (Hajishengallis and Lamont 2012); (5) the cytokine-related host immune response (Pan, Wang and Chen 2019) and more recently (6) the Inflammation-Mediated Polymicrobial-Emergence and Dysbiotic-Exacerbation (IMPEDE) model which proposed that inflammation plays a central role and modulates the polymicrobial oral biofilm (Van Dyke, Bartold and Reynolds 2020).

Chapter 1



Figure 4: The steps involved in the oral biofilm formation. (Figure adapted from Bakaletz 2004). See text for details.

We propose an additional model highlighting the importance of external factors, iron and oxygen as drivers for dysbiosis which was built based on the particular properties of three representative oral species *S. gordonii*, *P. gingivalis* and *T. denticola* (Chathoth *et al.* 2020) and

clinical observations on the role of iron in the severity of the disease (Meuric *et al.* 2017a; Boyer *et al.* 2018, 2020b).

Indeed, External factors like pH, exposure to oxygen, temperature (Pöllänen, Paino and Ihalin 2013), concentration of substrate, metal ions (Shumi *et al.* 2010), etc., can have an effect on the behaviour of the oral microflora (**Figure 5**). The exact mechanism behind and responsible for the pathogenic switch of the oral microbiota in response to environmental factors has not been clearly understood. We decided to focus on the effect of iron since studies suggested a correlation between iron-overload diseases and severity of periodontitis (Meuric *et al.* 2017a; Boyer *et al.* 2018, 2020b). We restricted the study to an *in vitro* multi-species biofilm model that is presented in the next chapters.



Figure 5: Interaction between periodontal pathogens and other external factors (Figure adapted from Pöllänen, Paino and Ihalin 2013)

1.2. Iron and the three-species oral biofilm model

1.2.1. Iron and its biological significance

Iron is a trace metal essential for the survival of almost all organisms, including bacteria, plants and humans. It exists in the reduced and highly reactive ferrous form (Fe²⁺) or the oxidized and less soluble ferric form (Fe³⁺). Either of its ionic forms participate in major biological processes including photosynthesis and N₂ fixation, methanogenesis, H₂ production and consumption, respiration, the tricarboxylic acid (TCA) cycle, heme synthesis, oxygen transport, gene regulation,

DNA synthesis and an iron-dependent cell death pathway called ferroptosis (Zhang 2014; Bogdan *et al.* 2016; Gozzelino and Arosio 2016). Almost all of these functions are enabled when iron is complexed with proteins, iron-sulfur (Fe-S) clusters, heme-binding proteins or ribonucleotide reductases. However, free ferrous iron, in the presence of hydrogen peroxide, can participate in Fenton chemistry resulting in the highly reactive oxygen species (ROS) hydroxyl radical. Due to this potential toxicity, iron is strictly regulated especially in the presence of oxygen.

Iron is an essential nutrient for bacterial growth and pathogenesis. Most bacteria need around 0.3-4 µM iron to grow (Weinberg 1971). In healthy individuals, iron is maintained in a range of 4-5 g per individual (Zhang, Ghosh and Rouault 2014). However, iron in the human body is tightly sequestered to limit its availability to bacteria. Iron is bound by haemoglobin and ferritin within cells while outside cells, iron is bound by transferrin and lactoferrin (Ganz and Nemeth 2015) thereby restricting free ionic iron availability to only $10^{-12} \mu M$ (Bullen 1981) which is far less than the bacterial iron requirement. Several innate defences are employed by hosts to restrict iron availability for pathogens in case of an infection when pathogens are detected via the interactions of pathogen associated molecular patterns (LPS, peptidoglycan, hypomethylated DNA) and the Toll-like receptors at host cells surface. One of them is involving an increased efflux of iron from the phagosome in which the pathogens are phagocyted and the inhibition of extracellular iron export by macrophages via the induction of hepcidin. Hepcidin is a peptidic hormone which acts by inactivating the cellular iron exporter, ferroportin, which delivers iron to plasma from intestinal cells, macrophages, and hepatocytes (Stocks et al. 2018). Lipocalin-2 expressed in neutrophils and epithelia is also involved in sequestering iron and catecholate siderophores in response to infection to limit bacterial growth (Golonka, Yeoh and Vijay-Kumar 2019). Bacteria therefore must apply various strategies for the acquisition of bound iron in host.

1.2.2. Iron in diseases

Iron imbalance can have derogatory effect on living systems. Free iron can result in the generation of harmful ROS resulting in cell damage and death (Orrenius, Nicotera and Zhivotovsky 2011). Disruption of iron homeostasis can result in either iron deficiency or overload which are both deleterious to health. Iron deficiency can lead to anemia ranging between mild to severe resulting in a compromised iron supply to organs (Clark, Goheen and Cerami 2014; Sankaran and Weiss 2015). On the other hand, iron overload can lead to adverse consequences, such as those

seen in hereditary hemochromatosis (Vujic 2014) and haemolytic anemias (Fibach and Rachmilewitz 2017). Changes in the systemic iron levels may affect the periodontal health. Correlation between the overexpression of genes of iron transport systems and the severity of periodontitis have been reported by comparative metatranscriptomic analysis between the oral microbiota of healthy individuals and periodontitis suffering patients (Duran-Pinedo *et al.* 2014; Yost *et al.* 2015). Interestingly Chakraborty *et al.*, (2014) indicated that patients with iron deficiency anemia (IDA) may be at an increased risk of periodontal destruction in comparison to patients with chronic periodontitis owing to a ROS induced inhibition of superoxide dismutase (SOD) activity. However, Enhos *et al.*, (2009) did not identify IDA as risk for periodontal disease. Studies have associated severe periodontitis with iron overload linked with HFE-related hereditary haemochromatosis (Meuric *et al.* 2017a) and also with dysbiosis in severe periodontitis (Boyer *et al.* 2018). Here, hepcidin deficiency (mutation) and consequently, ferroportin activation, lead to an increased transferrin saturation (TSAT, iron-binding sites of transferrin being occupied by iron), because it induces iron release from macrophages and enterocytes (Lan *et al.* 2018).

1.2.3. Iron in the oral cavity

The main sources of iron in the oral environment are the saliva, gingival crevicular fluid (GCF) and host diet. Dietary intake of iron varies from individual to individual. It has been reported that the salivary samples of a healthy individuals contained only ~9.74 μ g/l (0.2 μ M) of free iron compared to that of periodontal patients with ~26.66 μ g/l (0.5 μ M) when analysed using inductively coupled plasma mass-spectrometry (ICP-MS) (Romano *et al.* 2020). The subgingival biofilm in the periodontal pockets is surrounded by the GCF from the tissues surrounding the gingival crevice. This microbial community is not only influenced by environmental or host-related factors like pH, oxygen, temperature, concentration of substrate, etc. but also by the presence of iron. The GCF which is a serum exudate, contains transferrin, lactoferrin, haemoglobin (during bleeding), and inorganic iron as potential iron sources for microbial growth (Kim, Kim and Kho 2010; Cicek *et al.* 2014; Smalley and Olczak 2017; Guo, Yang and Feng 2018). The average total iron concentration in a periodontal GCF is ~5 mg/l compared to only ~1 mg/l in a healthy serum (Mukherjee 1985).

Moreover, the relationship between mineral elements in saliva and periodontal status in patients with untreated and treated periodontitis compared to periodontally healthy controls was recently accessed by ICP-Mass Spectrometry. Significant increases in copper, sodium, iron and manganese concentrations were observed in saliva of severe periodontitis subjects compared to periodontally healthy controls (Romano *et al.* 2020).

1.2.4. The three-species model and their relation with iron

1.2.4.1. Selected oral bacteria: *Streptococcus gordonii*, *Porphyromonas gingivalis* and *Treponema denticola*

This study is aimed at understanding the effect of various signals in the pathogenic switch of the oral microbiota. Therefore, to mimic the dental plaque we decided to first develop a multi-species oral biofilm model. This oral biofilm model consisted of two periodontal pathogens namely, *P. gingivalis* and *T. denticola*, and a primary colonizer of the dental plaque, *S. gordonii*.

S. gordonii are among the most common colonizers of the dental plaque (Jakubovics 2019). This commensal and Gram-positive facultative anaerobe is a primary/early colonizer, known to promote the co-aggregation and colonization of anaerobic oral pathogens via metabolic interactions (Stinson, Safulko and Levine 1991; Lamont *et al.* 1993, 2002; Amano *et al.* 1994; Rickard *et al.* 2003; Maeda *et al.* 2004a, 2004b; Kuboniwa *et al.* 2006). Exclusive co-localization of *S. gordonii* with *P. gingivalis* has been previously demonstrated in the mouths of human volunteers where *P. gingivalis* specifically localized in streptococcal-rich plaque areas (Slots and Gibbons 1978).

P. gingivalis is a Gram-negative anaerobe rod, referred to as the keystone pathogen in the development of chronic periodontitis (Orth *et al.* 2011). This late colonizer of the dental plaque which is non-motile, asaccharolytic, anaerobic rod forms black-pigmented colonies on blood agar plates due to heme accumulation on its cell-surface. *P. gingivalis* has an absolute requirement for iron and protoporphyrin IX for its growth and survival. It is known to produce a repertoire of virulence factors such as fimbriae, capsules, lipopolysaccharide (LPS), haemagglutinins, gingipains, outer membrane proteins, and outer membrane vesicles.

The interspecies co-aggregation between *S. gordonii* and *P. gingivalis* are among the best characterized. The long fimbriae (FimA) of *P. gingivalis* bind to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) of *S. gordonii* (Maeda *et al.* 2004b) and the *P. gingivalis* short fimbriae (Mfa) engage the streptococcal SspA/B adhesins (Park *et al.* 2005) (**Figure 6**). Further, the

heterotypic biofilm development is promoted by the LuxS-dependent signaling (McNab *et al.* 2003).



Figure 6: Inter-species association between *P. gingivalis* and *S. gordonii* via FimA–GAPDH and Mfa1– SspA/B adhesion-receptor pairs on the surfaces of the organisms (Figure adapted from Lamont and Hajishengallis 2015).

P. gingivalis also co-localizes in the subgingival plaque with the periodontal pathogen, T. *denticola* and have previously shown to exhibit strong synergy in growth, biofilm formation and virulence (Tan et al. 2014a; Ng et al. 2019). T. denticola is an obligate Gram-negative anaerobic spirochete, abundant in deep periodontal pockets (Byrne et al. 2009; Tan et al. 2014a). This late colonizer of the dental plaque is a slow-growing and fastidious and highly fragile anaerobic pathogen (Salvador, Syed and Loesche 1987; Wardle 1997). In vitro, T. denticola utilizes serine, alanine, cysteine, and glycine and generate fermentation products including acetate, lactate, succinate, formate, pyruvate, ethanol, carbon dioxide, hydrogen sulfide (H_2S) , and ammonia (Hespell and Canale-Parola 1971). The products of short-chain fatty-acid fermentation may play a role in disease progression (Niederman et al. 1997). Among the volatile sulfur compounds, methyl mercaptan reduces protein synthesis by human gingival fibroblasts and inhibits cell migration in periodontal ligament cells. H₂S production via cystalysin, disrupts erythrocyte membranes acting as a hemolysin. Therefore, both methyl mercaptan and H_2S are highly toxic and are thought to play an important role in periodontal disease (Johnson, Yaegaki and Tonzetich 1992; Chu et al. 1995; Lancero, Niu and Johnson 1996; Yoshimura et al. 2000). Other virulence factors of T. denticola are leucine-rich repeat protein, cystalysin, dentilisin (proteases), outer sheath proteins, major sheath protein, toxin-antitoxin systems, transposases, trypsin-like protease activity, lipoproteins, outer sheath vesicles, motility and periplasmic flagella.



Figure 7: Metabolic cooperation between P. gingivalis and T. denticola

Close association/proximity between *P. gingivalis* and *T. denticola* are mediated to some extent by *T. denticola* dentilisin or Msp but was not reduced in *T. denticola* dentilisin- or Msp-deficient mutants (Yao *et al.* 1996; Ishihara *et al.* 1998; Hashimoto *et al.* 2003; Rosen, Genzler and Sela 2008), indicating that this interaction is multimodal, involving different proteins and possibly carbohydrate moieties. It is known that the FimA (long fimbrae) and RgpB (Arg-gingipain B) of *P. gingivalis* along with the functional flagella and cytoplasmic filament of *T. denticola* are essential for biofilm formation (Kuramitsu, Chen and Ikegami 2005; Yamada, Ikegami and Kuramitsu 2005). Likewise, *T. denticola* is known to improve the adhesion of *P. gingivalis* by promoting the expression of genes encoding Arg-gingipain A (RgpA), Lys-gingipain (Kgp), and hemagglutinin A (HagA) (Meuric *et al.* 2013). The two species are known to exhibit metabolic symbioses (Tan *et al.* 2014b). *P. gingivalis* utilises the succinate produced by *T. denticola*, while *T. denticola* consumes isobutyric acid generated by *P. gingivalis* (Grenier 1992). *T. denticola* upregulates expression of genes encoding glycine cleavage and reductase system and putative glycine transporter during co-culture with *P. gingivalis. P. gingivalis* increases the rate of hydrolysis of glycine-containing peptides when exposed to *T. denticola* conditioned media. The

increased release of free glycine by *P. gingivalis* is probably beneficial for *T. denticola* as a carbon and energy source (**Figure 7**) (Tan *et al.* 2014b).

Also, *T. denticola* is known to bind to the commensal, early-colonizing *Streptococcus crista* (Yao *et al.* 1996) which may be important for *T. denticola* colonization and persistence during health. There exists direct experimental evidence regarding the possible gene transfer from *T. denticola* to *S. gordonii* (Wang, Chi and Kuramitsu 2002). However, not much is known about the *in vitro* association and growth of *S. gordonii* and *T. denticola* as biofilms.

1.2.4.2. The iron transport systems of the selected oral bacteria

Bacteria compete amongst themselves and with the host for obtaining iron. Indeed, bacteria employ a separate iron acquisition approach for every iron sequestering mechanism employed by the host. Even in healthy individuals, bacteria that scavenge adequate amount of iron have the potential to cause infection. In fact, the overexpression of functions related to the iron acquisition and transport is one of the transcriptomic signatures of pathogenic microbiota (Duran-Pinedo et al. 2014; Yost et al. 2015). The role of iron in the viability and virulence of bacterial pathogens cannot be disregarded and therefore, detailed understanding of the iron uptake systems in bacteria is important. There exist diverse strategies of bacterial iron acquisition, of which few are highly conserved and commonly applied, while the remaining are highly specific for a given bacterium. The various bacterial iron uptake mechanisms include: (1) heme-iron acquisition from hemoproteins (hemoglobins, hemopexin) via secreted proteins (hemophores) or cell surfaceassociated receptors, (2) specific surface-associated binding proteins-mediated or siderophoremediated acquisition of iron released from haemoglobin, transferrin and lactoferrin and (3) free inorganic iron acquisition via ferric iron reductases and associated ferrous iron permeases (Schröder, Johnson and de Vries 2003; Wandersman and Delepelaire 2004). It is important to note that in anaerobic or hypoxic systems such as the subgingival biofilm, free iron is mainly in reduced ferrous state. Therefore, siderophores that bind mainly ferric iron are not common.

1.2.4.2.1. Iron acquisition strategies employed by P. gingivalis

Among oral bacteria, *P. gingivalis* is considered as the keystone pathogen and hence is the most studied and described. It commonly utilizes iron in the form of heme. This iron not only helps for its growth but also contributes to virulence. Due to the absence of siderophores unlike other gram negative bacteria, *P. gingivalis* employs hemagglutinins, hemolysins, gingipains (Kgp, RgpA, RgpB), TonB-dependent outer membrane receptors (HmuR, HusB, IhtA) and hemophore-like proteins (HmuY, HusA) for iron acquisition (Figure 8) (Olczak *et al.* 2005; Smalley and Olczak 2017).

P. gingivalis cells adheres to the surface of erythrocytes using hemagglutinins (Hag A, Hag B, Hag C, Hag D, Hag E) or adhesins which further aid in the invasion of bacteria into host cells and also participate in iron acquisition (Lépine and Progulske-Fox 1996; Travis *et al.* 1997). Hemolysins and other proteases cause disruption of the erythrocyte membrane, resulting in hemoglobin release. The activity of some hemolysins from *P. gingivalis* cells and extracellular vesicles, is regulated by environmental hemin (Kay, Birss and Smalley 1990; Chu *et al.* 1991). Proteases from *P. gingivalis* are either trypsin-like, collagenolytic, or glycylprolyl peptidase. The best-characterized are the cysteine proteases known as gingipains. Gingipains are mainly of two types namely, the arginine-specific gingipains and the lysine-specific gingipain. Arginine-specific gingipains (RgpA and RgpB) are encoded by the rgpA and rgpB genes, and lysine-specific gingipain (Kgp) by the kgp gene (Okamoto *et al.* 1995, 1996). Gingipains participate in nutrient acquisition, adherence and colonization of host cells, neutralization of host defences, manipulation of inflammatory responses, tissue destruction, invasion and dissemination of *P. gingivalis* to systemic sites.



Figure 8: Schematic representation of the various heme acquisition systems in *P. gingivalis* (Figure adapted from Smalley and Olczak 2017). Bacteria adhere to erythrocytes via hemagglutinins (e.g. HagA), adhesion domains of gingipains (Kgp and HRgpA) and other proteins (e.g. HBP35). Hemolysins and other proteases disrupt the erythrocyte membrane, releasing hemoglobin. Oxyhemoglobin (oxyHb) is then oxidized to methemoglobin (metHb) (mainly by Rgps) and hydrolyzed by proteases (mainly by Kgp, RgpB and HRgpA). Heme in metHb, albumin, hemopexin, human hemoproteins is captured by the outer-membrane receptors (e.g. HmuR, HusB) and further transported across the outer membrane into the cell. Iron may be released from heme by the extracellular activity of a protein similar to cobalt chelatases (IhtB) and transported through the outer membrane by the TonB dependent outer-membrane receptor IhtA. Hemophore-like proteins sequester heme from human hemoproteins (e.g. HmuY) or from the cell-surface heme-containing pigment (HusA), and deliver heme to the outer-membrane receptors for internalization (e.g. HmuR and HusB, respectively). The DNA-protective protein from starved cells (PgDps) functions as an intracellular heme reservoir. Excess heme is gradually accumulated on the cell surface as μ-oxo bisheme, resulting in the typical black pigmentation.

Heme in methemoglobin (metHb), or released from human hemoproteins by proteases or hemolysins, is captured by the outer-membrane receptors and further transported across the outer membrane into the cell. TonB-dependent outer-membrane receptor HmuR and a protein homologous to TonB-dependent outer-membrane receptors (HusB) are involved in heme transport through the outer membrane. *P. gingivalis* also uses other outer-membrane receptors. The *iht* operon encodes a homolog of TonB-dependent outer-membrane receptors (IhtA), the protein similar to cobalt chelatases (IhtB), which might play a role in iron acquisition from bacterial cell surface and two other proteins (IhtC and IhtD) with unknown functions (Stojiljkovic and Hantke 1992; Dashper *et al.* 2000; Krieg *et al.* 2009; Cobessi, Meksem and Brillet 2010).

Also, hemophore-like protein, HusA (heme uptake system protein A) is capable of binding heme from pigment extracts of *P. gingivalis* in the form of μ -oxo dimers under heme-limited conditions. HmuY another hemophore-like protein, with HmuR serves as the first unique example of a heme acquisition system, comprising a heme-binding hemophore-like protein and an outermembrane TonB-dependent receptor (Olczak *et al.* 2005).

Researchers have explored the potential role of *S. gordonii* in heme acquisition by *P. gingivalis*. Hydrogen peroxide from *S. gordonii* is thought to oxidize the oxyhemoglobin to metHb releasing heme from hemoglobin which is extracted by *P. gingivalis* by the action of K-gingipain protease and/or the HmuY hemophore-like protein during the co-aggregation of the two species (Brown *et al.* 2018). Recently, it was reported that the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein of *S. gordonii* binds heme enabling exchange via hemophore-like heme-binding proteins of *P. gingivalis* (HmuY) which sequester heme bound to streptococcal GAPDH (Ślęzak *et al.* 2020).

P. gingivalis may also use other systems such as two FeoB proteins. Only one of these, termed FeoB1, is a ferrous iron transporter, while the other, called FeoB2, is a manganese transporter (Nelson *et al.* 2003; Dashper *et al.* 2005). However, the precise role of these proteins in iron uptake is not yet known.

A transcriptomic analyses of *P. gingivalis* gene which were differentially regulated in iron or heme depletion revealed distinct regulons. 39 genes were significantly upregulated in iron-depleted conditions, and 38 genes had reduced expression. 288 genes were significantly upregulated in heme-depleted condition, and 288 genes had reduced expression. Some genes that were drastically affected by iron levels were also regulated by hemin but interestingly the expression of the majority of the genes differentially regulated by iron was not hemin dependent,

Similar results were obtained when comparing the genes regulated by hemin; only a few hemeregulated genes were also regulated in the same way by iron. As expected, the expression of genes encoding proteins that participate in iron uptake and adhesion/invasion of host cells was increased in iron deficiency, while that of genes encoding proteins involved in iron storage, energy metabolism, and electron transport was decreased. Other biological processes affected by hemin deprivation were iron/hemin uptake and metabolism hemin depleted conditions, the most upregulated genes coded for oxidative stress protection. The regulation pathway is yet unclear since the deletion of the putative Fur protein had no effect on the expression of iron-regulated genes (Anaya-Bergman, Rosato and Lewis 2015).

P. gingivalis grown under hemin-limiting conditions were less virulent on a mouse abscess model (Kesavalu, Holt and Ebersole 2003) while a mutant deficient in ferrous iron uptake, FeoB1, has increased survival with oxidative stress and in the presence of host cells (Anaya-Bergman *et al.* 2010).

1.2.4.2.2. Iron acquisition strategies employed by oral anaerobic spirochetes

In periodontitis, spirochetes can account up to 50% of the polymicrobial species population whereas less than 1 % in health, with the genus *Treponema* being the only one found in the mouth (Chan and McLaughlin 2000). Among the oral anaerobic spirochetes, *T. denticola* is the best characterized species due to its prevalence in pathogenesis of periodontal disease. It is particularly associated with severe forms of periodontitis and is found with *P. gingivalis* in deep periodontal pockets (Riep *et al.* 2009; Deng *et al.* 2018). Oral anaerobic spirochetes, like most bacteria, require iron for survival. Iron not being freely available in the oral environment, *T. denticola* employs several iron acquisition methods.

Like *P. gingivalis*, *T. denticola* does not produce siderophores but has other mechanisms for iron uptake. Several lactoferrin and hemin-binding proteins (HbpA, HbpB) have been characterized in *T. denticola* which aid in iron acquisition (Staggs *et al.* 1994; Scott, Siboo and Chan 1996; Xu, Holt and Kolodrubetz 2001). Hemolysin (cystalysin) of *T. denticola* has been reported to lyse erythrocytes and hemoxidize hemoglobin and also possesses hemagglutinin activities (Grenier 1992; Scott *et al.* 1993; Chu *et al.* 1995). Moreover, TroR, which is an iron and manganese-dependent transcriptional repressor, negatively regulates the *tro* operon which may play a crucial
role in manganese and iron homeostasis in *T. denticola* (Brett *et al.* 2008). The *tro* operon (*troABCDR*), is present in all sequenced *Treponema* spp., and codes for highly conserved family of ATP-binding cassette transporters involved in metal cation transport. Its expression is controlled by TroR, a DtxR-like cation-responsive regulatory protein. Recent study has shown that TroR is required for the transcriptional regulation of *troA* in response to iron or manganese. Furthermore, deletion of *troR* results in differential expression of more than 800 genes (Saraithong *et al.* 2020). It was also reported that transcription of *troA*, *troB*, and *troR* was upregulated in *T. denticola* subjected to oxidative stress (McHardy *et al.* 2010).

T. denticola can reduce iron also from inorganic sources but inorganic sources of iron are not commonly available *in vivo*. *T. denticola* can utilize iron from lactoferrin, via the expression of outer membrane protein (OMP) receptors (Staggs *et al.* 1994).

1.2.4.2.3. Iron acquisition by streptococcal species

The role of iron in promoting the growth of gram negative pathogens is extensively studied (Olczak *et al.* 2005; Lewis 2010) but little is known about the involvement of iron in streptococcal infections. Streptococcal species meet their requirement for heme by following methods similar to the gram negative bacteria but employ different strategies for iron acquisition, probably due to the difference in cellular architecture.

Among streptococci, iron uptake in *S. pyogenes* is the most studied (Hanks *et al.* 2005; Montanez 2005). The only known ferrous iron transporter in two *Streptococcus* species (*S. pyogenes* and *S. agalactiae*) is MtsABC (divalent metal transporter of *Streptococcus*) (Janulczyk, Ricci and Björck 2003; Bray, Sutcliffe and Harrington 2009). MtsA is a lipoprotein bound to the cell membrane and aids in transmembrane iron uptake. Heme uptake is enabled by the ABC transporters where heme bound to heme-binding protein (Hbp) and passes through the cell membrane via the permease. The iron uptake from hemoproteins is mediated by the operon *sia* (*Streptococcus* iron acquisition) or *hts* (heme transport *S. pyogenes*) locus. The *sia* locus encodes a hemoprotein receptor Shr, a heme binding protein Shp and an ABC transporter SiaABC. Shr and Shp are anchored to the cell membrane and contribute to bacterial adherence and virulence. Similar to *sia*, another iron transporter named *siu* (streptococcus iron uptake), is also implicated in heme utilization by *S. pyogenes* (Montanez 2005). Likewise, the Shr/Shp/HtsABC(SiaABC) system was

found to be important for heme acquisition in *S. equi* (Meehan *et al.* 2010). However, the detailed mechanism is not known. Interestingly, the ferrous iron transporters (MtsABC25 and FtsABCD50 transporters) were found to be involved in heme utilization in *S. iniae* suggesting that MtsAs exhibit strain dependent differential roles in the iron uptake (Zou *et al.* 2010).



Figure 9: A schematic overview of the major iron, heme and siderophore acquisition systems in *S. pyogenes.* (Figure adapted from Ge and Sun 2014).

Small organic molecules with variable iron affinities called siderophores also mediate iron acquisition. The steps involved are siderophore synthesis, apo-siderophore secretion and the subsequent transmembrane transportation through holo-siderophore specific transporters or through ferrous transporters such as MtsABC. Based on their structures, siderophores are classified into catecholates (e.g. enterobactin), hydroxamates (e.g. ferrichrome) hydroxycarboxylate (e.g. staphyloferrin A) and phenolates (e.g. pyochelin) (Ge, Sun and He 2009; Ge and Sun 2014). Streptococcal species like *S. pyogenes, S. agalactiae, S. equi, S. pneumoniae* acquire iron via siderophores (Brown, Gilliland and Holden 2001; Hanks *et al.* 2005; Clancy *et al.* 2006; Heather *et al.* 2008) while others like *S. gordonii* are not known to synthesize siderophores.

1.3. Hypothesis: The impact of iron and oxygen in the three-species oral biofilm model

The events that may contribute to subgingival dysbiosis- a focus on the interplay between iron, sulfide and oxygen

Iron uptake regulation and oxidative stress defences are tightly connected in bacteria, especially in oxygen exposed species due to the highly reactive hydroxy radical formed by the reaction of iron with hydrogen peroxide. Since low levels of oxygen are always present even in deep periodontal pockets, and since streptococci produce significant amount of hydrogen peroxide in presence of oxygen, we also considered a hypothesis in which not only iron levels but also oxygen plays a role as drivers for dysbiosis. This hypothesis is presented in a review published in 2020: 'The events that may contribute to subgingival dysbiosis- a focus on the interplay between iron, sulfide and oxygen' which is in the following section.

Based on bibliographic data, a hypothetical model that emphasizes the importance of iron, oxygen and sulphur compounds (glutathione and H_2S), in setting up a vicious circle that would promote dysbiosis is proposed. The effect of oxidative stress on the level of glutathione and H_2S produced within the biofilm, and its probable effect on the prevalence of anaerobic pathogens is explained in detail. Finally, strategies for the therapeutic modulation of the microbiota to prevent periodontitis and promote oral health are discussed.

Graphical abstract



Figure 10: Cascade of events leading to dysbiosis in periodontitis: the possible role of oxygen, iron and H_2S in the multifactorial process. (Graphical abstract of 'The events that may contribute to subgingival dysbiosis- a focus on the interplay between iron, sulfide and oxygen', Chathoth *et al.*, 2020)

Downloaded from https://academic.oup.com/femsle/article-abstract/367/14 fnaa100/5860280 by guest on 06 A ligust 2020



FEMS Microbiology Letters, 367, 2020, fnaa100

doi: 10.1093/femsle/fnaa100 Advance Access Publication Date: 20 June 2020 Minireview

MINIREVIEW

The events that may contribute to subgingival dysbiosis: a focus on the interplay between iron, sulfide and oxygen

Kanchana Chathoth¹, Bénédicte Martin¹, Pierre Cornelis^{2,3}, Stéven Yvenou¹, Martine Bonnaure-Mallet^{1,4} and Christine Baysse^{1,*,†}

¹NuMeCan INSERM U1241, CIMIAD, Université de Rennes 1, F-35043 Rennes, France, ²Department of Bioengineering Sciences, Laboratory of Microbiology, Vrije Universiteit Brussel, B-1050 Brussels, Belgium, ³Laboratoire de Microbiologie Signaux et Microenvironnement, LMSM EA4312, Université de Rouen Normandie, Normandie Université, F-27000 Évreux, France and ⁴CHU Pontchaillou Rennes, 35000 Rennes, France

*Corresponding author: NuMeCan INSERM U1241, CIMIAD, 2 Avenue du Professeur Léon Bernard F-35043 Rennes, France. Tel: +33 (0)2 23 23 68 53; Fax: +33 (0)2 99 54 01 37; E-mail: christine.baysse@univ-rennes1.fr

One sentence summary: This review focuses on a probable series of events detrimental to oral health-associated subgingival microbiota in the presence of iron, sulfide and oxygen, and suggests strategies to overcome them.

Editor: Klaus Hantke

[†]Christine Baysse, http://orcid.org/0000-0003-1318-2192

ABSTRACT

This minireview considers the disruption of the host-microbiota harmless symbiosis in the subgingival niche. The establishment of a chronic infection by subversion of a commensal microbiota results from a complex and multiparametric sequence of events. This review narrows down to the interplay between oxygen, iron and sulfide that can result in a vicious cycle that would favor peroxygenic and glutathione producing streptococci as well as sulfidogenic anaerobic pathogens in the subgingival niche. We propose hypothesis and discuss strategies for the therapeutic modulation of the microbiota to prevent periodontitis and promote oral health.

Keywords: oral biofilm; iron; hydrogen sulfide; hydrogen peroxide; oxygen

INTRODUCTION

Unlike the first billion years on earth, oxygen is now abundant in most environments and it shapes the ecology and microbial behavior. Among several effects, oxygen oxidizes soluble ferrous iron into insoluble ferric hydroxides. Oxygen also decomposes Fe-S clusters, which are the essential cofactors of redox processes and metabolisms, and generates reactive oxygen species (ROS). Most microorganisms rely on Fe-S clusters inherited from ancient proteins that have evolved in anoxic conditions rich in iron and sulfide (Imlay 2006). Because of their Fe-S dependency, most microorganisms are sensitive to not only high levels of oxygen and iron deficiency but also excess in ferrous iron that generates hydroxyl radical, the most harmful ROS, in presence of the oxygen derivative H_2O_2 . The so-called anaerobes have restricted their habitat to hypoxic niches or created local hypoxic niche by producing reductive volatile compounds such as hydrogen sulfide H_2S . However, constant hypoxic conditions are rare and these communities are likely to be exposed to changing oxygen levels, which would modify their behavior. The focus of this review is to describe the potential interplay

1

Received: 1 June 2020; Accepted: 19 June 2020

[©] FEMS 2020. All rights reserved. For permissions, please e-mail: journals.permissions@oup.com

between oxygen, iron and sulfide in the oral ecosystem, especially in the subgingival plaque, which consists of hundreds of bacterial species. This ecosystem exposed to ambient oxygen, but composed of both aerobic and anaerobic bacteria, some of them sulfidogenic, is particularly relevant for the topic.

The oral microbiota

Over 500 species have been identified in the oral ecosystem by metagenomic analyses but a large proportion remains uncharacterized (Ai et al. 2017). The oral bacterial composition varies from a commensal biofilm mainly composed of Gram-positive bacteria to a pathogenic biofilm dominated by Gram-negative anaerobic bacteria that can lead to periodontitis, a chronic infection and inflammation of teeth-supporting tissues. Periodontitis is predominantly caused by the pathogens of the red (Porphyromonas gingivalis, Treponema denticola, Tannerella forsythia) and orange complexes (Fusobacterium nucleatum, Prevotella intermedia, Peptostreptococcus micros, Eubacterium nodatum) as described by Socransky et al. (1998). The green complex (Clostridium gingivalis, C. sputigena, C. ochracea, Campylobacter concisus, Eikenella corrodens and Actinobacillus actinomycetemcomitans serotype a) is also associated with periodontal disease. The purple (Veillonella parvula, Actinomyces odontolyticus) and the yellow (Streptococcus gordonii, Streptococcus intermedius, Streptococcus mitis, Streptococcus oralis, Streptococcus sanguis) complexes mainly consist of facultative anaerobic and Gram-positive bacteria associated with oral health. They are also involved in cooperative metabolisms in the periodontal biofilm. In the subgingival plaque, facultative anaerobic or microaerophilic microorganisms predominate. Porphyromonas gingivalis is a keystone pathogen because of its ability to subvert the immune response (Maekawa et al. 2014) and to activate the virulence of pathobionts (Hajishengallis, Darveau and Curtis 2012). The optimal growth rate of P. gingivalis takes place in anaerobic condition, but it possesses an O₂-consuming cytochrome bd oxidase that contributes to its aerotolerant phenotype (Leclerc et al. 2015) as well as an O₂-generating SOD (superoxide dismutase) instead of a non-O2-generating SOR (superoxide reductase) (Lynch and Kuramitsu 1999). Therefore, like the genotypically related Bacteroides fragilis, P. gingivalis may be considered as a nanaerobe, which means an organism that can grow with nanomolar concentrations of oxygen and benefit from it although it does not require oxygen for growth (Morris and Schmidt 2013). A species-specific co-aggregation of P. gingivalis with oral streptococci such as S. gordonii promotes initial colonization (Lamont, Hersey and Rosan 1992). Porphyromonas gingivalis and Treponema denticola exhibit a symbiotic relationship in nutrient utilization, growth promotion and synergetic biofilm formation (Meuric et al. 2013; Ng et al. 2016). It was also suggested that P. gingivalis may benefit from a mutual colonization with Candida albicans, a fungal species commonly encountered in the oral ecosystem. The growth of the filamentous form of the fungi under oxic conditions may offer a protective environment for the growth of P. gingivalis by depleting oxygen (Bartnicka et al. 2019). As described for other nanaerobes such as B. fragilis, the genomes of the pathogens P. gingivalis, Prevotella intermedia and Tannerella forsythia, all from the family of the bacteroidetes, possess a typical cytochrome bd oxidase with a long Q-loop, an hydrophilic region facing the outside of the cells and connecting transmembrane helices of subunit I (Borisov et al. 2011). Some microaerophilic organisms are also part of the oral microbiota, such as Campylobacter concisus (Tanner et al. 1981). Several studies showed C. concisus to be associated with bleeding in young adults with rapidly progressive periodontitis (Kamma, Nakou and Manti 1994, 1995). The presence of O_2 -dependent or O_2 -tolerant microorganisms in the oral biofilm of periodontal patients suggests that the anaerobic-strict picture of the pathogenic subgingival biofilm is controversial and that changing pO_2 (partial pressure of oxygen) may modulate the behavior of the community.

Oxygen in the oral ecosystem

Oxygen is abundant in the oral cavity. The primary source of oxygen is the free entry of atmospheric oxygen through the mouth. Another oxygen supplier is the gingival crevicular fluid (GCF) from the tissues surrounding the gingival crevice or periodontal pocket where the subgingival biofilm generally develops (Loesche et al. 1983). GCF is formed by the passage of fluid from capillaries into the tissues and then in the gingival sulcus. The pO_2 in the periodontal pockets, which are the pathologically deepened gingival sulci, is low but not negligible. During the development of oral biofilms, the redox potential decreases: electrodes in place for up to 7 days in patients showed a fall in oxidation-reduction potential as plaque bacteria accumulated (Kenney and Ash 1969). For reference, under an atmospheric pressure of 760 mmHg (sea level), the partial pressure of oxygen is 160 mmHg. Deep periodontal pockets (7-10 mm) have a pO₂ of 11.6 mmHg and the moderately deep periodontal pockets (5–6 mm) have a pO_2 of 15 mmHg. The pO_2 of healthy sites is unavailable for comparison due to the limitation posed by length of the sensor used. The mean pO2 of untreated periodontal pockets (13.2 mmHg) marks the upper limit of hypoxia (Mettraux, Gusberti and Graf 1984). Moreover, under inflammatory condition, oxygen levels rise due to elevated blood flow and hemoglobin. The pocket pO₂ showed correlation with probing depth and with oxygen saturation of hemoglobin (Tanaka et al. 1998).

Production of H₂O₂

If not the starting event, oxidative stress contributes to the snowball effect due to the interdependence between oxidative stress and inflammation. Streptococci from the mitis and sanguinis groups dominate the initial colonization of the tooth surface (Rosan and Lamont 2000; Mark Welch *et al.* 2016; Wake *et al.* 2016). They are facultative anaerobes and the main producers of H_2O_2 in response to oxygen. This metabolite was first considered beneficial for oral health by maintaining a symbiotic microbiota and preventing pathogens (Kreth, Zhang and Herzberg 2008; Redanz *et al.* 2018).

The sources of microbial H_2O_2 in the oral biofilm have been reviewed by Redanz et al. (2018). Briefly, the main enzyme involved in hydrogen peroxide production is the streptococcal protein SpxB, a pyruvate oxidase that converts pyruvate to acetyl phosphate and H₂O₂ and consumes oxygen (Fig. 1). Minor synthesis of H₂O₂ may arise from detoxifying enzymes from various species such as SOD (superoxide dismutase) and SOR (superoxide reductase) that reduce superoxide anions into less reactive H₂O₂. Another streptococcal enzyme capable of generating H₂O₂ is the FMN-dependent lactate oxidase (LctO) that performs the aerobic oxidation of lactate into pyruvate (Fig. 1). Finally, under conditions where peptone and amino acid are the only available nutrients, such as in saliva, H₂O₂ together with ammonia and α -ketoacid may be produced by the oxidation of amino acid by the L-amino acid oxidase (LAAO) (Fig. 1) (Tong et al. 2008). The generation of H₂O₂ is also a constant process in host eukaryotic cells, which increases during inflammation in response to



Figure 1. Possible H_2O_2 -producing enzymes in oral microbiota. The FMN-dependent lactate oxidase (LctO) performs the aerobic oxidation of lactate into pyruvate. SpxB is a pyruvate oxidase that converts pyruvate to acetyl phosphate and H_2O_2 and consumes oxygen. When peptone and amino acid are the only available nutrients, the oxidation of amino acid by the L-amino acid oxidase (LAAO) produces H_2O_2 together with ammonia and α -ketoacid.

pathogens. When stimulated by pathogens, host cells release cytokines, recruit polymorphonuclear leukocytes that synthesize proteolytic enzymes and ROS. The primary sources of ROS are the NADPH oxidase (Nox), the mitochondrial respiratory chain and SOD (Sculley and Langley-Evans 2002; Sies 2017).

Streptococcus sanguinis and Streptococcus gordonii are often called 'oral health compatible organisms', due to their H₂O₂-related antimicrobial properties against anaerobic pathogens. However, the effect of H₂O₂ on the microbiota cannot be limited to the inhibition of pathogen viability or a global beneficial input on oral health. H₂O₂ is a non-radical product with low reactivity with organic molecules. Its longer life span (1 ms) compared to free radicals such as O₂^{o-} (1 µs) and OH⁻ (1 ns) makes it a good signaling molecule (Dahiya et al. 2013).

The Fenton chemistry and the sources of iron

The toxicity of H₂O₂ mainly depends on transition metal concentration in the surrounding, especially ferrous iron or copper, because of the Fenton reaction $[H_2O_2 + Fe^{2+} \rightarrow \cdot OH + Fe^{3+} + -OH]$ using both substrates to generate the highly reactive hydroxyl radical (OH-). H₂O₂, via the production of OH-, may be toxic for not only other bacteria including potential pathogens but also host cells. The effect of H₂O₂ on eukaryotic oral host cells is either beneficial or deleterious depending on its concentration. At low levels (28.9 µM maximum concentration for 4 h and reduced up to 13.5 µM for 24 h), it can stimulate the proliferation of human periodontal ligament fibroblasts in vitro, but it also displays cytotoxic effect at higher concentrations (1 mM), including on endothelial cells (Choe et al. 2012). Some results suggest that streptococcal H₂O₂ is not only cytotoxic to epithelial cells but also promotes bacterial evasion of the host defense systems in the oral cavity (Okahashi et al. 2014; Liu et al. 2017; Erttmann and Gekara 2019). Moreover, H₂O₂ produced by S. gordonii can serve as an electron acceptor to increase the persistence of A. actinomycetemcomitans by a shift from an anaerobic to an aerobic mode of growth, an interaction termed 'cross-respiration'. Aggregatibacter actinomycetemcomitans is associated with aggressive periodontitis, presumably due to the production of the leukotoxin A (Stacy et al. 2014, 2016). Therefore, the balance between good and bad for oral health may be, among several factors, a matter of concentration (entry, release and turnover) of free ferrous iron and H_2O_2 inside target cells. Oxygen-derived free radical resulting from Fenton reaction serves to amplify the inflammatory response, by favoring the expression of cytokines in endothelial cells, promoting the recruitment of neutrophils and inflammatory cells. As a result of tissue damage, mitochondria, xanthine oxidase and neutrophils produce more reactive oxygen species (Closa and Folch-Puy 2004; Mittal *et al.* 2014).

In the oral environment, iron may come from the gingival crevicular fluid (GCF), saliva and dietary sources. GCF is an inflammatory exudate derived from the periodontal tissues and is composed of serum, tissue breakdown products, inflammatory mediators and sometimes antibodies. During the development of gingivitis, the flow rate of GCF increases (Goodson 2003). In the host, iron is bound in complexes such as lactoferrin, hemoglobin and transferrin. Lactoferrin is a glycoprotein with iron-binding properties, secreted by exocrine glands and neutrophils. It binds to two atoms of iron and retains iron at a much lower pH (up to pH 3.0) than the main systemic transporter transferrin (up to pH 5.5) (Baker and Baker 2005). Lactoferrin is an important defense factor against Streptococcus mutans and periodontopathic bacteria. It has the ability to not only inhibit bacterial growth and biofilm development but also limit freeiron overload, and therefore reduce reactive oxygen formation and inflammatory processes (Berlutti et al. 2011). A correlation between lactoferrin concentration in the GCF and the number of PMNs in GCF was reported (Adonogianaki, Moughal and Kinane 1993). Although lactoferrin can be used as an iron source for some pathogens such as Neisseria gonorrhoeae (Blanton et al. 1990) or Campylobacter jejuni (Miller et al. 2008), its role as an iron source for periodontal bacteria is not yet proven.

Hemic iron is mainly provided by hemoglobin (Hb). Hemoglobin derived from microbleeding in gingival sulci was detected in GCF samples from periodontal sites (Ito *et al.* 2016). Iron acquisition from Hb by oral/periodontal bacteria may be facilitated by the H_2O_2 produced by the streptococci: the initial oxidation of oxyHb into metHb, a form of hemoglobin that contains the ferric [Fe³⁺] form of iron is carried out by H_2O_2 . This further promoted the activity of the gingipains, proteases of *P. gingival*is, which cleave such iron proteins as the first step of heme uptake. Also, the dissociation of the ferric iron from heme protoporphyrin is easier than in the case of ferrous iron (Brown *et al.* 2018). Transferrin is a glycoprotein with two iron-binding sites (composed of 39 cysteines), found in the periodontal pocket together with hemoglobin and lactoferrin. Transferrin and its iron-saturated form, holotransferrin, support the growth of several oral bacteria such as *Campylobacter rectus* (Grenier and Tanabe 2011) and P. gingivalis (Brochu et al. 2001; Goulet et al. 2004). Increased transferrin saturation is generally due to genetic hemochromatosis that provokes iron egress from macrophages and enterocytes in relation with hepcidin deficiency. Increased transferrin saturation is associated with not only increased mortality in patients with sepsis (Lan et al. 2018) but also dysbiosis and severe periodontitis (Boyer et al. 2018). Moreover, as previously stated, iron saturation and transferrin concentration increase in the GCF during periodontitis (Adonogianaki, Moughal and Kinane 1993).

Along with the external sources of iron in the GCF, oxygen radicals, especially superoxide anions, destroy the ubiquitous 4Fe-4S clusters of the host and proteins of the microbiota, releasing free ferrous iron in the surrounding. The fate of the dissolved ferrous iron depends on the properties of the environment such as oxidative status and pH. As the pH increases, the speed of the oxidation of ferrous iron increases and produces ferric oxides and hydroxides. Altogether free iron overload enhanced by oxygen and combined with oxygen derivatives may be one of the triggers that favor the dysbiosis and inflammatory processes, provided some bacteria (pathogens and H_2O_2 producers) can protect themselves against O_2/H_2O_2 toxicity in the presence of ferrous iron.

The role of reductive compounds, H₂S and glutathione

Peroxygenic streptococci limit their reliance to Fe-S cluster protein and use manganese in place of Fe to avoid H₂O₂ self-toxicity (Jakubovics, Smith and Jenkinson 2002; Tseng et al. 2002). However, the sensitivity of anaerobes to oxidative stress mainly relies on the sensitivity of the Fe-S cluster of their pyruvateferredoxin oxidoreductase (PFOR). PFOR is a major metabolic enzyme involved in energy generation through oxidative decarboxylation of pyruvate, as demonstrated with the model Bacteroides thetaiotaomicron (Pan and Imlay 2001; Lu, Sethu and Imlay 2018). The periodontal pathogens P. gingivalis, P. intermedia and T. forsythia use PFOR, which allowed the development of a new antimicrobial compound, amixicile, specifically targeting the anaerobic pathogen via the inhibition of PFOR (Hutcherson et al. 2017). Anaerobes need reduced environment rather than low oxygen concentration for growth. PFOR help bacteria to thrive in a reduced environment because of low-potential electron transfer pathway, in which PFOR passes low-potential electrons toward hydrogen formation and/or NAD reduction. The redox potential measured in saliva was +158 to 542 mV, while it was lower in periodontal pockets (-300 mV) and gingival crevices (-200 mV). Indeed, the redox potential is reduced by microbial metabolism during the development of oral biofilm (Kenney and Ash 1969). Hydrogen sulfide is a metabolite that is produced in high amount by microorganisms in the periodontal environment (Horowitz and Folke 1973). H₂S production add to oral malodor: halitosis is caused by various volatile sulfur compounds (VSCs), including H₂S but also ethanethiol, S-ethyl thioacetate, diethyl disulfide, dimethyl sulfide and methyl mercaptan. The amount of VSCs has been shown to significantly increase in patients with periodontal disease. H₂S produced in subgingival pocket probably contributes to halitosis even though the predominance of methyl mercaptan produced by the microbiota of the tongue has been shown (Yaegaki and Sanada

Downloaded from https://academic.oup.com/femsle/article-abstract/367/14/fnaa100/5860280 by guest on 06 August 2020

1992). Such metabolites with low redox potential (E0 + 0.17 V) potentially contribute to the reductive and protective environment for anaerobes and nanaerobes in subgingival pockets. The effect of H₂S greatly varies with environmental conditions. H₂S molecules dissolve in water and dissociate into H⁺, HS⁻ and S²⁻ ions. HS⁻ and, to a lower extent, H₂S are chemical reductants capable of scavenging ROS and inducing defense response in eukaryotic cells (Xie et al. 2014). H₂S can be as toxic as cyanide due to its ability to bind with and precipitate metal cations. Likewise, for some bacterial species, exogenous H₂S can suppress H₂O₂-mediated oxidative stress. It was shown that H₂S suppressed the oxidative stress in E. coli by sequestering free iron that reacted with H_2O_2 in Fenton chemistry (Mironov et al. 2017). Another study on Streptococci showed that H₂S reduced intracellular bound ferric iron to form unbound ferrous iron that can act as a substrate for Fenton chemistry (Dunning, Ma and Marquis 1998). Therefore, H₂S may be beneficial to oral anaerobes (Lai and Chu 2008) but exerts a toxic effect on other microorganisms, by inhibition of dismutase, catalase and sequestration of trace metals in the surrounding or increase of free cytosolic ferrous iron. The toxic effect of H₂S when studied on streptococci was iron-dependent. The addition of the iron chelator deferoxamine obliterated the bactericidal effect of H₂S (Ooi and Tan 2016). In Shewanella oneidensis, the coordinated effect of H₂S and H₂O₂ was described as being time-dependent. On simultaneous addition of $H_2\mathsf{O}_2$ and $H_2\mathsf{S}$ to cultures, $H_2\mathsf{S}$ promoted $H_2\mathsf{O}_2$ toxicity via catalase inactivation. However, on later supplementation of H₂O₂, H₂S promoted H₂O₂ resistance, probably by lowering external iron concentrations, activating the influx of iron and OxyR-dependent stress defenses (Wu et al. 2015). For anaerobic bacteria, H₂S production helps to maintain a reduced environment and to decrease dissolved O2. A study of Lai and Chu has revealed that H₂S production by T. denticola resulted in a dosedependent removal of dissolved O₂ in the growth cultures (Lai and Chu 2008).

H₂S also increases intracellular glutathione (GSH) synthesis in streptococci. GSH protects streptococci against both their H₂O₂ (Zheng et al. 2013) and H₂S produced by neighboring bacteria (Ooi and Tan 2016). GSH (γ-L-glutamyl-L-cysteinyl-glycine) is an abundant non-protein thiol present in many bacteria and all eukaryotes (Masip, Veeravalli and Georgiou 2006). Glutathione biosynthesis generally occurs via a conserved two-step mechanism in which a γ -glutamylcysteine ligase (GshA) uses Lglutamate and L-cysteine to form γ -glutamylcysteine and then, a glutathione synthetase (GshB) condenses γ -glutamylcysteine with glycine to give glutathione (Fig. 2). H₂S reduces cystine into cysteine in extracellular space and increases the expression of glutamate-cysteine ligase that converts cysteine into γ -glutamyl cysteine, the direct precursor of GSH. The susceptibility of oral streptococci to H₂S is dependent on intracellular glutathione levels, which increase in the presence of the H₂S donor NaHS in vitro (Ooi and Tan 2016).

GSH synthesis by eukaryotic and prokaryotic cells may promote H₂S production by sulfidogenic bacteria if released in the surrounding. H₂S is produced by several anaerobic bacteria of the oral microbiota mainly from cysteine, which originated from the catabolism of cysteine-containing proteins/peptides and glutathione, via cysteine desulfhydration. The possible pathway for production of H₂S from the degradation of GSH is depicted in Fig. 2. As the initial step, the γ -glutamyltransferase GGT breaks down glutathione into cysteinyl glycine (Cys-Gly) and glutamate. Next, the cysteinyl glycinase CGase degrades Cys-Gly into glycine and L-cysteine. Finally, the β C-S lyases, pyridoxal-5'phosphate (PLP)-dependent enzyme (L-cysteine desulfhydrase



(-)

Figure 2. Common pathway of cysteine degradation to H_2S and GSH synthesis. Examples of known coding genes for three representative species of the oral biofilm [Porphyromonas gingivalis TDC60 (PGTDC), Treponema denticola ATCC 35404 (TDE) and Streptococcus gordonii Challis NCTC7868 (SGO)] are: (1) TDE0444; (2) TDE0300; (3) TDE1669, PGTDC60–1459; (4) SGO-1990.



Figure 3. Hypothetical cascade of events leading to dysbiosis in periodontitis: the possible role of oxygen, iron and H_2S in the multifactorial process. The arrows indicate metabolic, regulatory or inhibitory relationships. The numbers in brackets refer to the corresponding chapter in the text. The question mark indicates a link not yet experimentally verified in oral bacteria but identified in other bacteria. Briefly, oxygen always being present in oral sites may promote H_2O_2 production by peroxygenic bacteria, mainly streptococci. The Fenton chemistry involving ferrous iron and H_2O_2 may generate ROS (hydroxyl radical), which in turn may produce more free iron via Fe-S degradation. The generation of toxic hydroxyl radicals may induce the bacterial production of GSH as stress response, and then indirectly induce the formation of H_2S since GSH can act as a substrate in vitro, like other cysteine-containing peptides from the gingival crevicular fluid. H_2S protects anaerobes by lowering the redox potential in local niches. It may trigger more GSH production by H_2O_2 -producing organisms (as described in streptococci), and can reduce ferric iron or can decrease the Fenton chemistry since H_2S can react with non-heme iron, free or present in iron-sulfur cluster containing proteins, to generate insoluble precipitates (FeS). Altogether, this vicious circle starting from O_2 and ferrous iron may promote anaerobic pathogens and inflammation.

LCD for cysteine) catalyze the α,β -elimination of sulfur amino acids [L-cysteine, L-cystathionine, L-cystine, S-(2-aminoethyl)-Lcysteine and S-methyl-L-cysteine] to the corresponding sulfurcontaining molecules (H₂S in case of cysteine), pyruvate and ammonia. The NH₃ produced by this reaction may contribute to the alkaline pH encountered in periodontal pockets. This pathway is certainly relevant in the oral biofilm in vivo since glutathione was identified as a suitable substrate for H₂S production by the major pathogen T. denticola (Chu et al. 2002, 2003, 2008). Recently, a T. denticola mutant lacking the γ glutamyltransferase enzyme GGT was constructed (Chu et al. 2020). The mutant cannot produce H₂S from glutathione. Interestingly, the mutant has also lost the ability to grow in the presence of oxygen when incubated with glutathione and showed a reduced virulence in vitro against human gingival fibroblasts. The data also pointed out that the levels of cell death correlated with the amounts of H₂S produced. Porphyromonas gingivalis does not possess GGT, but it is capable of producing H₂S from cysteinyl glycine, therefore, possessing CGase activity (Tang-Larsen et al. 1995). Neither of the two pathogens possesses the gshA and gshB genes for GSH synthesis. On the contrary, in silico genome analysis (unpublished analysis) indicates that the primary colonizer S. gordonii possesses a GshF-homolog gene. The product of this gene is a bifunctional enzyme that may carry out glutathione biosynthesis by catalyzing both reactions on its own. Several studies pointed out that other pathogenic and free-living bacteria possess a single enzyme to catalyze the γ -glutamylcysteine ligase reaction and the condensation of γ glutamylcysteine with glycine to give glutathione (Gopal et al. 2005; Janowiak and Griffith 2005; Vergauwen, De Vos and Van Beeumen 2006; Stout et al. 2012). Indeed, the SGO-1990 gene product of S. gordonii showed 100% identity with the bifunctional GshF enzyme of S. sanguinis (Cui et al. 2019). What remains to be investigated is whether reactive oxygen species enhance GSH synthesis in streptococci. Such activation was described in different bacteria and is as per the role of GSH in oxidative stress responses. For example, in Pseudomonas aeruginosa, hydrogen peroxide, cumene hydroperoxide and t-butyl hydroperoxide increase the expression of gshA and gshB involved in glutathione synthesis (Wongsaroj et al. 2018). If ROS promotes GSH synthesis in the oral microbiota, oxidative stress conditions from the presence of H₂O₂ and ferrous iron could indirectly trigger H₂S synthesis by pathogenic oral bacteria via GSH cross-feeding and initiate a vicious circle leading to an H₂S-mediated reductive environment where anaerobic bacteria would flourish. The use of GSH by H₂S-producing bacteria may also explain why in GCF, the amount of GSH is lower in cases of chronic periodontal disease compared to healthier sites (Chapple 2002; Grant et al. 2010; Savita et al. 2015).

The cysteine-rich transferrin may also play a role in the oral dysbiosis by not only providing iron but also favoring the synthesis of volatile sulfur compounds. The transferrin concentration increases in the gingival crevicular fluid during periodontitis (Adonogianaki, Moughal and Kinane 1993). As previously said, some bacteria can use it as both iron and nutrient source in the host (Goulet *et al.* 2004). In vitro, iron-saturated transferrin supported the growth of P. gingivalis via degradation of the protein (Brochu *et al.* 2001). Boyer *et al.* (2018) observed a significant link between the periodontitis severity and increased transferrin saturation in patients with iron-overload disease (hemochromatosis). However, the levels of transferrin in the GCF of hemochromatosis patients compared with healthy patients are not known. The use of transferrin as a suitable substrate for H_2S synthesis by oral bacteria remains to be investigated

as well. Finally, following an initial observation that mutants of S. sanguinis and S. gordonii depleted of the pleiotropic carbon catabolite regulator CcpA lost their ability to inhibit H_2O_2 -sensitive strains of S. mutans, the authors identified pyruvate as the compound, overproduced by these mutants and responsible for the protective effect. This study highlighted a new mechanism for regulating H_2O_2 homeostasis within microbiota, which would depend on pyruvate secretion and H_2O_2 scavenging by some members of the community and which will enrich studies on interactions and dysbioses related to oxidative stress (Redanz et al. 2020).

CONCLUSION

This review combines separate data, mainly obtained in vitro, to propose hypotheses that will need to be tested in vivo or in a relevant context such as multispecies biofilms. Oxygen always being present in oral sites, even at low levels, allows H₂O₂ production by peroxygenic bacteria, mainly streptococci. Combined with H_2O_2 , the levels of free ferrous iron may be one of the signals that will promote dysbiosis. Free iron along with the H_2O_2 in the oral sites can give rise to the Fenton reaction and thus produce ROS, which in turn generates more free iron via Fe-S degradation. The generation of toxic hydroxyl radicals may induce the production of GSH by some bacteria, and then indirectly of H₂S since GSH can be used as a substrate. H₂S protects anaerobes and may trigger more GSH production by H₂O₂-producing organisms. H₂S can also reduce ferric iron to promote the Fenton reaction or chelate ferrous iron and decrease the Fenton chemistry in vitro. The main point raised by this speculation is whether oxidative stress in the first step and then H_2S can both induce a significant production of glutathione by oral bacteria so that it can serve as a substrate for the production of H₂S. The whole picture is depicted in Fig. 3. Ultimately, a treatment containing both an oxygen donor and a non-metabolizable iron chelator could be of interest to treat and prevent periodontal diseases as it may favor the development of commensal aerobic organisms without inducing deleterious oxidative stress. Besides, inhibitors of enzymes involved in the production of volatile sulfur compounds may be worth investigating especially on periodontal diseases in relation with increased levels of cysteine-containing proteins such as transferrin (hemochromatosis-related periodontitis). An in vitro study has identified propargylglycine as a potent inhibitor of volatile sulfur compound synthesis in P. gingivalis. The inhibitor hampers the activity of methionine gamma-lyase deaminase (Kandalam et al. 2018). Further detailed and focused investigations on each of these scenarios will widen the understanding concerning the transition from oral health to disease and perhaps open up precise targets against periodontal pathogens. One should not neglect another theory that is worth considering, which is that inflammation plays a central role and modulates the polymicrobial oral biofilm, a model termed 'Inflammation-Mediated Polymicrobial-Emergence and Dysbiotic-Exacerbation' (IMPEDE) (Van Dyke, Bartold and Reynolds 2020). Although our hypothesis has intentionally focused on microbial interactions, the two models are not mutually exclusive.

Conflict of interest. The authors declare that there are no conflicts of interest.

REFERENCES

- Adonogianaki E, Moughal NA, Kinane DF. Lactoferrin in the gingival crevice as a marker of polymorphonuclear leucocytes in periodontal diseases. *J Clin Periodontol* 1993;**20**:26–31.
- Ai D, Huang R, Wen J et al. Integrated metagenomic data analysis demonstrates that a loss of diversity in oral microbiota is associated with periodontitis. *BMC Genomics* 2017;**18**:1041.
- Baker EN, Baker HM. Molecular structure, binding properties and dynamics of lactoferrin. *Cell Mol Life Sci* 2005;**62**:2531–9.
- Bartnicka D, Karkowska-Kuleta J, Zawrotniak M et al. Adhesive protein-mediated cross-talk between *Candida albicans* and *Porphyromonas gingivalis* in dual species biofilm protects the anaerobic bacterium in unfavorable oxic environment. Sci *Rep* 2019;**9**:4376.
- Berlutti F, Pilloni A, Pietropaoli M et al. Lactoferrin and oral diseases: current status and perspective in periodontitis. Ann Stomatol (Roma) 2011;2:10–8.
- Blanton KJ, Biswas GD, Tsai J et al. Genetic evidence that Neisseria gonorrhoeae produces specific receptors for transferrin and lactoferrin. J Bacteriol 1990;**172**:5225–35.
- Borisov VB, Gennis RB, Hemp J et al. The cytochrome bd respiratory oxygen reductases. Biochim Biophys Acta 2011;**1807**: 1398–413.
- Boyer E, Le Gall-David S, Martin B et al. Increased transferrin saturation is associated with subgingival microbiota dysbiosis and severe periodontitis in genetic haemochromatosis. Sci Rep 2018;8:15532.
- Brochu V, Grenier D, Nakayama K et al. Acquisition of iron from human transferrin by Porphyromonas gingivalis: a role for Arg- and Lys-gingipain activities. Oral Microbiol Immunol 2001;16:79–87.
- Brown JL, Yates EA, Bielecki M et al. Potential role for Streptococcus gordonii-derived hydrogen peroxide in heme acquisition by Porphyromonas gingivalis. Mol Oral Microbiol 2018;33: 322–35.
- Chapple ILC. Glutathione in gingival crevicular fluid and its relation to local antioxidant capacity in periodontal health and disease. Mol Pathol 2002;55:367–73.
- Choe Y, Yu J-Y, Son Y-O et al. Continuously generated H_2O_2 stimulates the proliferation and osteoblastic differentiation of human periodontal ligament fibroblasts. J Cell Biochem 2012;**113**:1426–36.
- Chu L, Dong Z, Xu X et al. Role of glutathione metabolism of *Treponema denticola* in bacterial growth and virulence expression. Infect Immun 2002;**70**:1113–20.
- Chu L, Lai Y, Xu X et al. A 52-kDa leucyl aminopeptidase from *Treponema denticola* is a cysteinylglycinase that mediates the second step of glutathione metabolism. *J Biol Chem* 2008;**283**:19351–8.
- Chu L, Wu Y, Xu X et al. Glutathione catabolism by Treponema denticola impacts its pathogenic potential. Anaerobe 2020;**62**:102170.
- Chu L, Xu X, Dong Z et al. Role for recombinant γglutamyltransferase from Treponema denticola in glutathione metabolism. Infect Immun 2003;71:335–42.
- Closa D, Folch-Puy E. Oxygen free radicals and the systemic inflammatory response. *IUBMB Life* 2004;**56**:185–91.
- Cui X, Wan J, Zhang X et al. Efficient glutathione production in metabolically engineered *Escherichia* coli strains using constitutive promoters. *J Biotechnol* 2019;**289**:39–45.
- Dahiya P, Kamal R, Gupta R et al. Reactive oxygen species in periodontitis. J Indian Soc Periodontol 2013;17:411–6.

- Dunning JC, Ma Y, Marquis RE. Anaerobic killing of oral streptococci by reduced, transition metal cations. *Appl Environ Microbiol* 1998;**64**:27–33.
- Erttmann SF, Gekara NO. Hydrogen peroxide release by bacteria suppresses inflammasome-dependent innate immunity. Nat *Commun* 2019;**10**:3493.
- Goodson JM. Gingival crevice fluid flow. Periodontol 2000 2003;31:43–54.
- Gopal S, Borovok I, Ofer A et al. A multidomain fusion protein in Listeria monocytogenes catalyzes the two primary activities for glutathione biosynthesis. J Bacteriol 2005;**187**:3839–47.
- Goulet V, Britigan B, Nakayama K et al. Cleavage of human transferrin by Porphyromonas gingivalis gingipains promotes growth and formation of hydroxyl radicals. Infect Immun 2004;72:4351–6.
- Grant MM, Brock GR, Matthews JB et al. Crevicular fluid glutathione levels in periodontitis and the effect of non-surgical therapy. J Clin Periodontol 2010;**37**:17–23.
- Grenier D, Tanabe S-I. Transferrin as a source of iron for Campylobacter rectus. J Oral Microbiol 2011;3:1–6.
- Hajishengallis G, Darveau RP, Curtis MA. The keystone pathogen hypothesis. Nat Rev Microbiol 2012;**10**:717–25.
- Horowitz A, Folke LE. Hydrogen sulfide production in the periodontal environment. J Periodontol 1973;44:390–5.
- Hutcherson JA, Sinclair KM, Belvin BR et al. Amixicile, a novel strategy for targeting oral anaerobic pathogens. Sci Rep 2017;7:10474.
- Imlay JA. Iron–sulfur clusters and the problem with oxygen. Mol Microbiol 2006;**59**:1073–82.
- Ito H, Numabe Y, Hasphimoto S et al. Correlation between gingival crevicular fluid hemoglobin content and periodontal clinical parameters. J Periodontol 2016;87:1314–9.
- Jakubovics NS, Smith AW, Jenkinson HF. Oxidative stress tolerance is manganese (Mn(2+)) regulated in Streptococcus gordonii. Microbiol Read Engl 2002;**148**:3255–63.
- Janowiak BE, Griffith OW. Glutathione synthesis in Streptococcus agalactiae. One protein accounts for gammaglutamylcysteine synthetase and glutathione synthetase activities. J Biol Chem 2005;**280**:11829–39.
- Kamma JJ, Nakou M, Manti FA. Microbiota of rapidly progressive periodontitis lesions in association with clinical parameters. J Periodontol 1994;65:1073–8.
- Kamma JJ, Nakou M, Manti FA. Predominant microflora of severe, moderate and minimal periodontal lesions in young adults with rapidly progressive periodontitis. J Periodontal Res 1995;30:66–72.
- Kandalam U, Ledra N, Laubach H et al. Inhibition of methionine gamma lyase deaminase and the growth of Porphyromonas gingivalis: a therapeutic target for halitosis/periodontitis. Arch Oral Biol 2018;**90**:27–32.
- Kenney EB, Ash MM. Oxidation reduction potential of developing plaque, periodontal pockets and gingival sulci. J Periodontol 1969;40:630–3.
- Kreth J, Zhang Y, Herzberg MC. Streptococcal antagonism in oral biofilms: Streptococcus sanguinis and Streptococcus gordonii interference with Streptococcus mutans. J Bacteriol 2008;190:4632–40.
- Lai Y, Chu L. Novel mechanism for conditional aerobic growth of the anaerobic bacterium Treponema denticola. Appl Environ Microbiol 2008;**74**:73–9.
- Lamont RJ, Hersey SG, Rosan B. Characterization of the adherence of Porphyromonas gingivalis to oral streptococci. Oral Microbiol Immunol 1992;7:193–7.

- Lan P, Pan K, Wang S et al. High serum iron level is associated with increased mortality in patients with sepsis. Sci Rep 2018;8:11072.
- Leclerc J, Rosenfeld E, Trainini M et al. The cytochrome bd oxidase of Porphyromonas gingivalis contributes to oxidative stress resistance and dioxygen tolerance. PLoS One 2015;10:e0143808.
- Liu C, Mo L, Niu Y et al. The role of reactive oxygen species and autophagy in periodontitis and their potential linkage. Front Physiol 2017;**8**:439.
- Loesche WJ, Gusberti F, Mettraux G et al. Relationship between oxygen tension and subgingival bacterial flora in untreated human periodontal pockets. Infect Immun 1983;**42**:659–67.
- Lu Z, Sethu R, Imlay JA. Endogenous superoxide is a key effector of the oxygen sensitivity of a model obligate anaerobe. Proc Natl Acad Sci USA 2018;115:E3266–75.
- Lynch MC, Kuramitsu HK. Role of superoxide dismutase activity in the physiology of Porphyromonas gingivalis. Infect Immun 1999;67:3367–75.
- Maekawa T, Krauss JL, Abe T *et al.* Porphyromonas gingivalis manipulates complement and TLR signaling to uncouple bacterial clearance from inflammation and promote dysbiosis. Cell Host Microbe 2014;15:768–78.
- Mark Welch JL, Rossetti BJ, Rieken CW *et al.* Biogeography of a human oral microbiome at the micron scale. Proc Natl Acad Sci USA 2016;**113**:E791–800.
- Masip L, Veeravalli K, Georgiou G. The many faces of glutathione in bacteria. Antioxid Redox Signal 2006;**8**:753–62.
- Mettraux GR, Gusberti FA, Graf H. Oxygen tension (pO₂) in untreated human periodontal pockets. *J Periodontol* 1984;**55**:516–21.
- Meuric V, Martin B, Guyodo H et al. Treponema denticola improves adhesive capacities of Porphyromonas gingivalis. Mol Oral Microbiol 2013;**28**:40–53.
- Miller CE, Rock JD, Ridley KA et al. Utilization of lactoferrinbound and transferrin-bound iron by *Campylobacter jejuni*. J Bacteriol 2008;**190**:1900–11.
- Mironov A, Seregina T, Nagornykh M et al. Mechanism of H_2S mediated protection against oxidative stress in Escherichia coli. Proc Natl Acad Sci USA 2017;**114**:6022–7.
- Mittal M, Siddiqui MR, Tran K et al. Reactive oxygen species in inflammation and tissue injury. Antioxid Redox Signal 2014;20:1126–67.
- Morris RL, Schmidt TM. Shallow breathing: bacterial life at low O₂. Nat Rev Microbiol 2013;**11**:205–12.
- Ng HM, Kin LX, Dashper SG et al. Bacterial interactions in pathogenic subgingival plaque. Microb Pathog 2016;94:60–9.
- Okahashi N, Sumitomo T, Nakata M et al. Hydrogen peroxide contributes to the epithelial cell death induced by the oral mitis group of streptococci. PLoS One 2014;9:e88136.
- Ooi XJ, Tan KS. Reduced glutathione mediates resistance to H₂S toxicity in oral streptococci. *Appl Environ Microbiol* 2016;**82**:2078–85.
- Pan N, Imlay JA. How does oxygen inhibit central metabolism in the obligate anaerobe Bacteroides thetaiotaomicron. Mol Microbiol 2001;**39**:1562–71.
- Redanz S, Cheng X, Giacaman RA *et al*. Live and let die: hydrogen peroxide production by the commensal flora and its role in maintaining a symbiotic microbiome. Mol Oral 2018;**33**: 337–52.
- Redanz S, Treerat P, Mu R et al. Pyruvate secretion by oral streptococci modulates hydrogen peroxide dependent antagonism. ISME J 2020;14:1074–88.

- Rosan B, Lamont RJ. Dental plaque formation. *Microbes Infect* 2000;2:1599–607.
- Savita AM, Sarun E, Arora S et al. Evaluation of glutathione level in gingival crevicular fluid in periodontal health, in chronic periodontitis and after nonsurgical periodontal therapy: a clinicobiochemical study. *Contemp Clin Dent* 2015;6:206–10.
- Sculley DV, Langley-Evans SC. Salivary antioxidants and periodontal disease status. Proc Nutr Soc 2002;61:137–43.
- Sies H. Hydrogen peroxide as a central redox signaling molecule in physiological oxidative stress: oxidative eustress. *Redox Biol* 2017;11:613–9.
- Socransky SS, Haffajee AD, Cugini MA et al. Microbial complexes in subgingival plaque. J Clin Periodontol 1998;25:134–44.
- Stacy A, Everett J, Jorth P et al. Bacterial fight-and-flight responses enhance virulence in a polymicrobial infection. Proc Natl Acad Sci USA 2014;111:7819–24.
- Stacy A, Fleming D, Lamont RJ et al. A commensal bacterium promotes virulence of an opportunistic pathogen via cross-respiration. *mBio* 2016;**7**:e00782–16.
- Stout J, De Vos D, Vergauwen B et al. Glutathione biosynthesis in bacteria by bifunctional GshF is driven by a modular structure featuring a novel hybrid ATP-grasp fold. J Mol Biol 2012;416:486–94.
- Tanaka M, Hanioka T, Takaya K et al. Association of oxygen tension in human periodontal pockets with gingival inflammation. J Periodontol 1998;69:1127–30.
- Tang-Larsen J, Claesson R, Edlund MB et al. Competition for peptides and amino acids among periodontal bacteria. J Periodontal Res 1995;**30**:390–5.
- Tanner ACR, Badger S, Lai C-H et al. Wolinella gen. nov., Wolinella succinogenes (Vibrio succinogenes Wolin et al.) comb. nov., and description of Bacteroides gracilis sp. nov., Wolinella recta sp. nov., Campylobacter concisus sp. nov., and Eikenella corrodens from humans with periodontal disease. Int J Syst Bacteriol 1981;31:432–45.
- Tong H, Chen W, Shi W et al. SO-LAAO, a novel L-amino acid oxidase that enables Streptococcus oligofermentans to outcompete Streptococcus mutans by generating H₂O₂ from peptone. J Bacteriol 2008;**190**:4716–21.
- Tseng H-J, McEwan AG, Paton JC et al. Virulence of Streptococcus pneumoniae: psaA mutants are hypersensitive to oxidative stress. Infect Immun 2002;**70**:1635–9.
- Van Dyke TE, Bartold PM, Reynolds EC. The nexus between periodontal inflammation and dysbiosis. *Front Immunol* 2020;**11**:511.
- Vergauwen B, De Vos D, Van Beeumen JJ. Characterization of the bifunctional gamma-glutamate-cysteine ligase/glutathione synthetase (GshF) of Pasteurella multocida. J Biol Chem 2006;281:4380–94.
- Wake N, Asahi Y, Noiri Y *et al.* Temporal dynamics of bacterial microbiota in the human oral cavity determined using an *in situ* model of dental biofilms. *npj Biofilms Microbiomes* 2016;2:16018.
- Wongsaroj L, Saninjuk K, Romsang A et al. Pseudomonas aeruginosa glutathione biosynthesis genes play multiple roles in stress protection, bacterial virulence and biofilm formation. PLoS One 2018;13:e0205815.
- Wu G, Wan F, Fu H et al. A matter of timing: contrasting effects of hydrogen sulfide on oxidative stress response in Shewanella oneidensis. J Bacteriol 2015;**197**:3563–72.
- Xie Z-Z, Shi M-M, Xie L et al. Sulfhydration of p66Shc at cysteine59 mediates the antioxidant effect of hydrogen sulfide. Antioxid Redox Signal 2014;21:2531–42.

- Yaegaki K, Sanada K. Biochemical and clinical factors influencing oral malodor in periodontal patients. J Periodontol 1992;63:783–9.
- Zheng X, Zhang K, Zhou X et al. Involvement of gshAB in the interspecies competition within oral biofilm. J Dent Res 2013;**92**:819–24.

1.4. Objectives of the thesis

The goal of the unit "Nutrition, Metabolisms and Cancer" (University Rennes 1) is to investigate how food, environmental, behavioural and metabolic determinants have an impact on health and diseases, particularly chronic diseases or disorders in different target organs such as the liver, the digestive system and the brain. The CIMIAD Team explores the relationship between microbiota and iron metabolism in order to determine how the oral and digestive microbiota interacts with this metabolism, and if iron overloads have an impact on the virulence of bacteria, by studying the oral microbiota. Through the study of periodontal diseases, it will allow to study the relationships between iron overload, bacteria and lesions.

The main subject of this thesis is to study the "effect of environmental signals on the development of oral biofilms". The oral microbiome may be affected by variations in the oral environment which can trigger a pathogenic switch. These pathogenic anaerobic bacteria can eventually lead to periodontitis which is a chronic inflammatory disease of the tissues supporting the tooth.

To achieve this goal, three bacteria involved in periodontitis, *S. gordonii*, a commensal along with *P. gingivalis* and *T. denticola* which are periodontal pathogens, were chosen to develop an oral biofilm model to analyse their behaviour as multi-species biofilms. The anaerobic periodontal pathogens are sensitive to high levels of oxygen, and restrict their habitat to hypoxic niches or create their own hypoxic niche by producing reductive volatile compounds such as H₂S. Also, sensitivity of bacteria to deficiency of the essential nutrient, iron and also excess of ferrous iron which generate harmful ROS in presence of the oxygen derivative H₂O₂ is well known. Altogether, these factors led us to study the inter-species interactions of the two oral anaerobic pathogens and peroxygenic *S. gordonii* at different iron concentrations.

To enable the study of the environmental signals on the periodontal pathogens, it was essential to develop an *in vitro* biofilm model consisting of the three oral bacteria in a growth medium with a defined and controlled composition. Therefore, a new medium was developed that will enable the mono- and multi-species growth of *S. gordonii*, *P. gingivalis* and *T. denticola* as planktonic cultures and biofilms. This is explained in detail in **Chapter 2.2.** (Research article published in Journal of Microbiological Methods; doi: 10.1016/j.mimet.2018.08.013). In addition,

this medium was used to characterise the production of H₂S by the three species at different iron concentrations (**Chapter 2.3.**, complementary data).

Next, this model was used to test antimicrobial compounds such as an oxygen carriers as therapeutic agents against the oral biofilm model (**Chapter 3.1.**) and to optimize a method for analysing the effectiveness of two antimicrobial gels, one of them being oxygen-based, on multispecies biofilms to decipher their specific effect on each species (submitted to Journal of Applied Microbiology, **Chapter 3.2.**).

The main aim was to investigate the role of iron in the pathogenic switch of the oral microbiota on the basis of a previous study that associated severe periodontitis to hemochromatosis, a dysregulation of the homeostasis of systemic iron (Meuric *et al.*, 2017). This study will enable the analysis of the biofilm structure (e.g. growth, architecture, species distribution) and ratio of each species at different times of incubation corresponding to different stages of biofilm development (adhesion, growth, maturation, detachment). As the first step, the effect of iron and interspecies associations was assessed during biofilm initiation/ attachment (**Chapter 4.1.**). Next, the same was assessed during different stages of biofilm development (**Chapter 4.2.**). The biofilm growth was studied by mathematical modelling (**Chapter 4.3.**). To enable this, the analysis of the behaviour of the mono- and multi-species biofilms at different iron concentrations for biofilm growth, was performed using experimental data which enabled to compute the simulated data in order to mathematically model the behaviour of each of the species in the biofilm. The purpose was to understand the adapted and coordinated behaviour of the multispecies oral biofilm in response to iron levels as it may throw light on the relation between iron overload and the severity of the disease.

The main objectives are summarised as follows:

- 1. To develop a new growth media for the coculture of *P. gingivalis*, *T. denticola* and *S. gordonii*
- 2. Development of *in vitro* models and protocols for testing antimicrobial agents against oral biofilm
- 3. To test the effect of iron on the development of oral biofilms
 - i. To test the effect of iron and bacterial species on biofilm initiation
 - ii. To test the effect of iron and bacterial species on biofilm growth
 - iii. To develop a new mathematical model as a tool to study the interspecies associations and effect of iron on oral biofilm development

2. GROWTH MEDIA FOR ORAL BACTERIA

2.1. Introduction

To gain insight into the complex behaviour of the oral microbiota, which is composed of hundreds of different species, we focused on three species whose co-operations are known to influence the evolution of periodontal disease, namely *S. gordonii* (primary colonizer), *P. gingivalis* and *T. denticola* (anaerobic pathogens) (Ng *et al.* 2019). *P. gingivalis* and *T. denticola* are keystone pathogens associated with the disease (Orth *et al.* 2011). Several studies explored their metabolic interactions *in vitro* because these two bacteria were frequently found in deep periodontal pockets (Byrne *et al.* 2009; Tan *et al.* 2014). Both bacteria species are known as fastidious organisms and all experiments so far were carried out on complex defined or empiric growth media such as OMIZ-M/*T. denticola* CDK (Wyss 1992), or OBGM (Orth *et al.* 2010). Introducing *S. gordonii* in the *in vitro* model is crucial for biofilm initiation and to give insight into the role of commensal strains in the pathogenic switch of microbiomes. The obligate fermenter *S. gordonii* is one of the pioneer bacteria which first attach to supra gingival pellicle, therefore favouring further complexification of the biofilm. *S. gordonii* produces H₂O₂ via a pyruvate oxidase and the resulting oxidative stress is known to affect the survival of other bacterial species such as demonstrated for *S. sanguinis* (Kreth, Zhang and Herzberg 2008).

This study was intended to design a new growth media, for which the known metabolic requirements of each strain were considered as follows: *T. denticola* and *P. gingivalis* only weakly metabolize glucose while preferentially derive energy from amino acid fermentation (Hespell and Canale-Parola 1971). *T. denticola* however is known to exhibit some chemotactic behaviour toward glucose (Ruby *et al.* 2008). *P. gingivalis* prefers to transport small peptides into the cytoplasm rather than amino acids (Takahashi and Sato 2001) while *T. denticola* can use both peptides and amino acids (Smibert, Johnson and Ranney 1984). Both *P. gingivalis* and *T. denticola* are able to degrade and to use bovine serum albumin (BSA). However, as BSA is rich in cysteine, which naturally binds heme (Grinberg, O'Brien and Hrkal 1999), excess of BSA may be a source of heme contaminant. Moreover, high amounts of BSA, or the use of BSA as the sole carbon and nitrogen source were shown to restrict the growth of *P. gingivalis*, presumably because of excessive H₂S production (Oda *et al.* 2007). Acid hydrolysate of casein (CAA) can be taken as a complementary

source of amino acids because it contains not only low iron levels, but also low levels of cysteine and tyrosine amino-acids and no tryptophan. Therefore, a CAA-based medium has to be enriched with a suitable source of these amino-acids. Oral streptococci are dependent on sugars as an energy source. S. gordonii utilizes glucose and possesses multiple transport systems for this substrate (Vadeboncoeur and Pelletier 1997). Other parameters related to auxotrophy have to be considered such as the dependence of T. denticola for thiamine (Tan et al. 2014), isobutyric acid and 2methylbutyric acid (Wyss 1992), as well as the auxotrophy of some strains of *P. gingivalis* for vitamin K or its precursor 1,4-dihydroxy-2-naphthoic acid (Wyss 1992). In addition, cysteine is required by streptococci (Terleckyi and Shockman 1975), and arginine is needed for the aerobic growth of S. gordonii (Jakubovics et al. 2008). Moreover, manganese (Mn) is essential for the oxidative stress tolerance of S. gordonii because Mn levels regulate expression of superoxide dismutase and thiol peroxidase activities (Jakubovics, Smith and Jenkinson 2002). Manganese is also required for microaerobic growth of *P. gingivalis* and plays a role in its protection from peroxide stress (He et al., 2006). Finally, without added hemin, P. gingivalis relies on its ferrochelatase activity that converts PPIX (protoporphyrin IX) and Fe^{2+} into hemin (Kusaba *et al.* 2002). Therefore, growth media have to contain a minimal amount of PPIX to allow de novo heme synthesis in presence of FeSO₄ by *P. gingivalis* without allowing *P. gingivalis* produce excess of heme since we intended to use these medium to control iron source, and hemin can be use as iron source by *P. gingivalis*.

All these nutritional requirements were considered and tested in order to design three new culture media. The first medium MMBC-1 is the simplest medium for the growth of *P. gingivalis*. This medium was then supplemented to allow the growth of *T. denticola* (MMBC-2) and finally *S. gordonii* (MMBC-3). The compositions of these new media are well-defined and the iron concentration, as well as the iron source (hemin or ferrous iron), can be controlled. The growth capacity of each species in these media was tested in planktonic form or in biofilms. The results were published in 2018 in the Journal of Microbiological Methods (**Chapter 2.2**).

2.2. New growth media for oral bacteria

Journal of Microbiological Methods 153 (2018) 10-13

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Note New growth media for oral bacteria

Bénédicte Martin^a, Kanchana Chathoth^a, Souad Ouali^b, Vincent Meuric^a, Martine Bonnaure-Mallet^a, Christine Baysse^{a,*}

^a CIMIAD, U1241 NUMECAN, Université de Rennes 1, 2 av. du Pr. Léon Bernard, 35043 Rennes, France
^b EA1254 Microbiologie-Risques Infectieux, Université de Rennes 1, 2 av. du Pr. Léon Bernard, 35043 Rennes, France

ARTICLE INFO	A B S T R A C T			
Keywords:	New growth media have been designed for the iron-controlled co-cultures of three oral bacteria. These media			
Porphyromonas gingivalis	share a common core composition enabling the switch from mono- to co-cultures, and efficiently promote both			
Treponema denticola	planktonic and biofilm cultures of Pornhyromonas gingivalis. Trenonema denticola and Strentococcus gordonii			
Streptococcus gordonii				
Periodontitis				
Iron				
Growth media				

Periodontitis is characterized by the damage of periodontal tissues causing periodontal pockets and bone loss, leading to tooth loss. The evolution of the disease is linked to a modification of the oral microbiota (Meuric et al., 2017a). Anaerobic bacteria are closely associated with the transition from healthy to pathological conditions. The virulent switch of the microbiota may be influenced by host-related factors such as systemic iron levels. Indeed, a recent study demonstrated that severe periodontitis was associated with the severity of iron burden in patients with HFE-related hereditary haemochromatosis, an autosomal recessive genetic disease leading to iron overload (Meuric et al., 2017b). To further evaluate the impact of iron concentration on the fitness of the oral microbiota, we developed a simplified experimental model with bacterial species for which interactions are known to influence the evolution of periodontal disease, namely Streptococcus gordonii (primary colonizer), Porphyromonas gingivalis and Treponema denticola (anaerobic pathogens)(Ng et al., 2016). P. gingivalis and T. denticola are keystone pathogens strongly associated with the severity of the disease (Orth et al., 2011). They were frequently found together in deep periodontal pockets (Byrne et al., 2009; Tan et al., 2014). Both species are anaerobic fastidious organisms and all experiments so far were carried out in complex media such as OMIZ-M (Wyss, 2007), OBGM (Orth et al., 2010) and BM (Loo et al., 2000). However, to further study the role of iron, it is important to avoid empirical components such as brain heart infusion and yeast extract, which are usually contaminated with high residual concentrations of iron. In this study, new growth media, with only bovine serum albumin (BSA), casamino acids (CAA) and/or glucose as carbon sources, were designed for the individual- or co-cultures of *P. gingivalis, T. denticola* and *S. gordonii* (Medium for Mixed Bacterial Community, MMBC) (Table 1).

A core composition sufficient for the growth of *P. gingivalis* (MMBC-1) was further supplemented with components necessary for the growth of *T. denticola* (MMBC-2) and *S. gordonii* (MMBC-3). The quantification of iron in the MMBC media was performed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS), on X-Series II from Thermo Scientific[®] equipped with collision cell technology (AEM2 Facility, University of Rennes 1), as previously described (Cavey et al., 2015). All three media contained < 0.56 μ M of iron (Table 1), making them suitable for iron metabolism/transport related studies. These media were first tested for planktonic mono- or cocultures in anaerobic conditions at 37 °C.

Strains of *S. gordonii* Challis DL1 (Chen et al., 2004), *P. gingivalis* TDC60 (Watanabe et al., 2011) and *T. denticola* ATCC35405 (Chan et al., 1993) were used. For monocultures, the growth of each species was measured by optical density at 600 nm over a period of time in MMBC media supplemented with hemin or FeSO₄ as iron sources. For cocultures, quantification of each bacterial species at different time

* Corresponding author. *E-mail address:* christine.baysse@univ-rennes1.fr (C. Baysse).

https://doi.org/10.1016/j.mimet.2018.08.013

Received 19 July 2018; Received in revised form 27 August 2018; Accepted 27 August 2018 Available online 28 August 2018

0167-7012/ ${\ensuremath{\mathbb C}}$ 2018 Elsevier B.V. All rights reserved.

37





Table 1

1.

Chapter 2

Medium Type (Iron concentration in µM) _*			Composition	
		MMBC-1	NaH ₂ PO ₄	5 mM
		(0.45 +/- 1.39E-02)*	KCl	5 mM
			MgCl ₂ . 7H ₂ O	5 mM
			Menadione	0.581 µM
			BSA	$3675 \mathrm{mg} \mathrm{L}^{-1}$
			CAA	2500mg L^{-1}
	MMBC-2		Adenine	$1.35 {\rm mg} {\rm L}^{-1}$
	(0.50 +/- 5.37E-04)*		FAD	1mg L^{-1}
			Folic acid	1 mg L^{-1}
			Pyridoxal phosphate	$5 \mathrm{mg}\mathrm{L}^{-1}$
			Fumarate	$0.5 \mathrm{g L^{-1}}$
			Pyruvate	$0.55 \mathrm{g L^{-1}}$
			TPP	25mg L^{-1}
			Inosine	$2.7 \mathrm{mg}\mathrm{L}^{-1}$
			CoA	1mg L^{-1}
			Volatile fatty acids**	0.001%(v/v) each
IMBC-3			D-Biotin	0.05 µM
			Nicotinic acid	0.04 mM
(0.56 +-/ 4.30E-03)*			D-Glucose	$6 g L^{-1}$
			MnSO ₄	10mg L^{-1}
			L-Arginine-HCl	1 mM
			L-Tryptophan	0.1 mM
			Cysteine- HCl	1.3 mM
			L-Glutamic acid	4 mM
			(NH ₄) ₂ SO ₄	$0.6 \mathrm{g L^{-1}}$

* Data are the average concentrations of two independent freshly prepared media.

** Volatile fatty acids: valeric acid, isovaleric acid and isobutyric acid.

points was carried out by qPCR with species-specific 16S rRNA primers as previously described (Ammann et al., 2013; Martin et al., 2017) (Fig. 1). Monocultures of P. gingivalis grew similarly in all MMBC media containing hemin or ferrous iron with protoporphyrin IX (PPIX) (Fig. 1A for MMBC-3). 8 µM of hemin was found as the optimal concentration for P. gingivalis growth (data not shown). The removal of hemin impaired the growth of P. gingivalis and T. denticola (data not shown). Without hemin, the growth of T. denticola was restored by 8 µM of FeSO₄, whereas P. gingivalis needed both FeSO₄ and protoporphyrin IX (PPIX) (data not shown). The optimal concentration of PPIX that promoted the growth of *P. gingivalis* without impairing the growth of *T*. denticola was defined as 0.08 µM. Moreover, addition of PPIX in FeSO4containing MMBC-3 was not required for the growth of S. gordonii (Fig.1B). The growth of T. denticola was enhanced by co-culture with P. gingivalis in MMBC-2 (data not shown) and in MMBC-3 (Fig.1A) such as it was previously observed in the rich OBGM medium which contained yeast extract and brain heart infusion as carbon sources (Tan et al., 2014). Interestingly, an antagonistic behaviour was observed in cocultures of S. gordonii with either P. gingivalis (Fig. 1C) or T. denticola (Fig. 1D). The presence of S. gordonii impeded the growth and survival

of both pathogens.

To assess bacterial growth in sessile conditions, biofilms were grown in sterile μ -slide 8-chambered coverslip (ibiTreat, Ibidi), that were previously coated with 0.22-µm filtered sterile human saliva (25% ν/ν) collected from at least six healthy volunteers (Martin et al., 2017). Formation of three-species biofilms in anaerobic conditions at 37 °C was evaluated in MMBC-3 medium plus hemin or FeSO₄ and PPIX, as compared with brain-heart infusion broth (BHIe) (Biomérieux, France) supplemented with menadione (10 µg mL⁻¹) and hemin (5 µg mL⁻¹). At 24 h, biofilms were stained with 5 µM of Syto[®]40 nucleic acid dye, a blue fluorescent membrane-permeant stain (Molecular Probes, Lieden, The Netherlands) and 40 µM of Propidium Iodide (Thermo Fisher) diluted in PBS before observation *in situ* with a Leica TCS-SP8 confocal laser scanning microscope (Leica Microsystems, Wezlar, Germany). After imaging, bacteria collected from biofilms were quantified by qPCR as described by Martin et al., (2017).

As demonstrated by image analysis and qPCR measurements, biofilms performed in MMBC-3 media displayed similar biomasses as biofilms in BHIe (Fig. 2A), with identical ratios of each species (Fig. 2B). As expected from previous observations of 2-species biofilms



Fig. 1. Planktonic cultures.

A) Mono- and cocultures of *P. gingivalis* and *T. denticola* were grown in MMBC-3 media with 8 μ M of hemin. Quantification of bacterial species in cocultures was carried out by qPCR. Samples were collected at 0 (white bars), 30 (grey bars) and 100 h (black bars) after anaerobic incubation at 37 °C. Pg: monocultures of *P. gingivalis*. Pg(PgTd): quantification of *P. gingivalis* when cocultured with *T. denticola*. Td: monocultures of *T. denticola*. Td(PgTd): quantification of *T. denticola* when cocultured with *P. gingivalis*. Data are the average of triplicate assays. *p* values were calculated with Student's *t*-test. **p* < .05, ***p* < .01.B) *S. gordonii* Challis DL1 was grown in MMBC-3 plus 8 μ M hemin (grey circle), or 8 μ M FeSO₄ together with 0.08 μ M PPIX (grey triangle) or 8 μ M FeSO₄ (black square) at 37 °C in anaerobic conditions. OD values are the average of 3 experiments. C) Growth and survival of *P. gingivalis* and D) *T. denticola* were monitored at 37 °C, in anaerobic condition, in MMBC-3 with 8 μ M hemin, in the presence (grey symbol) or absence (black symbol) of *S. gordonii*. Quantifications of *T. denticola* and *P. gingivalis* in cocultures were carried out by qPCR. Data are the average of 3 experiments performed in triplicates. *p* values were calculated with Student's *t*-test; **p* < .05, ***p* < .01.

(Martin et al., 2017), the proportion of *S. gordonii* in three-species biofilms was always higher than those of *P. gingivalis* and *T. denticola*, probably due to its higher growth rate and antagonistic behaviour. Conclusion

From the simplest medium composition for *P. gingivalis* (MMBC-1), supplemented media were designed and validated for the growth of *P. gingivalis* together with *T. denticola* (MMBC-2) and the growth of the two

anaerobic pathogens together with *S. gordonii* (MMBC-3), allowing an increment in the complexity of the media as per need. These media are suitable for biofilm mode of growth and are specifically designed to control the levels of iron and the nature of iron sources, unlike rich media which are generally used for growing a mixed biofilm, comprising of these fastidious oral bacteria.



Fig. 2. Three-species biofilms in MMBC-3 with different iron sources as compared with BHLe Biofilms

Journal of Microbiological Methods 153 (2018) 10-13

ferent iron sources as compared with BHIe. Biofilms were grown in MMBC-3 medium plus 8 µM hemin or 8 µM FeSO₄/0.08 µM PPIX or in BHIe. 24-hour biofilms stained with Syto®40 nucleic acid dye (Live cells-green colour) and Propidium Iodide (Dead cells-red colour). A). The figure shows representative images of 3 experiments with the 3D maximum z projection. The biomass (µm3/µm2) was estimated using Comstat 2 plugin of ImageJ software V1.43 m (National Institute of Health). B) After 24 h of incubation in anaerobic conditions at 37 °C, the concentration of bacteria was quantified by gPCR: S. gordonii, black bars; P. gingivalis, grey bars; T. denticola, white bars. The data are the average of triplicate assays. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Declarations of interest

None.

Acknowledgement

This work was supported by the Conseil Régional de Bretagne, France (F3/48 CPER), the Fondation "Les Gueules Cassées/Sourire Quand Même" and the Federative Research Structure Biosit (Rennes). We thank M. Ropert for ICP-MS analysis. We also thank all people of the Microscopy Rennes Imaging Center (Biosit) for technical assistance for microscopy experiments.

References

- Ammann, T.W., Bostanci, N., Belibasakis, G.N., Thurnheer, T., 2013. Validation of a quantitative real-time PCR assay and comparison with fluorescence microscopy and selective agar plate counting for species-specific quantification of an *in vitro* subgingival biofilm model. J. Periodontal Res. 48, 517–526.
- Byrne, S.J., Dashper, S.G., Darby, I.B., Adams, G.G., Hoffmann, B., Reynolds, E.C., 2009. Progression of chronic periodontitis can be predicted by the levels of *Porphyromonas* gingivalis and *Treponema denticola* in subgingival plaque. Oral Microbiol. Immunol. 24, 469–477.
- Cavey, T., Ropert, M., de Tayrac, M., Bardou-Jacquet, E., Island, M.-L., Leroyer, P., Bendavid, C., Brissot, P., Loréal, O., 2015. Mouse genetic background impacts both on iron and non-iron metals parameters and on their relationships. Biometals Int. J. Role Met. Ions Biol. Biochem. Med. 28, 733–743.
- Chan, E.C.S., Siboo, R., Keng, T., Psarra, N., Hurley, R., Cheng, S.-L., Iugovaz, I., 1993. *Treponema denticola* (ex Brumpt 1925) sp. nov., nom. rev., and identification of new spirochete isolates from periodontal pockets. Int. J. Syst. Evol. Microbiol. 43,

196-203.

- Chen, C.C., Teng, L.J., Chang, T.C., 2004. Identification of clinically relevant viridans group streptococci by sequence analysis of the 16S-23S ribosomal DNA spacer region. J. Clin. Microbiol. 42, 2651–2657.
- Loo, C.Y., Corliss, D.A., Ganeshkumar, N., 2000. Streptococcus gordonii biofilm formation: identification of genes that code for biofilm phenotypes. J. Bacteriol. 182, 1374–1382.
- Martin, B., Tamanai-Shacoori, Z., Bronsard, J., Ginguené, F., Meuric, V., Mahé, F., Bonnaure-Mallet, M., 2017. A new mathematical model of bacterial interactions in two-species oral biofilms. PLoS One 12, e0173153.
- Meuric, V., Le Gall-David, S., Boyer, E., Acuña-Amador, L., Martin, B., Fong, S.B., Barloy-Hubler, F., and Bonnaure-Mallet, M. (2017a). Signature of microbial dysbiosis in periodontitis. Appl. Environ. Microbiol. 83:e00462–17.
- Meuric, V., Lainé, F., Boyer, E., Le Gall-David, S., Oger, E., Bourgeois, D., Bouchard, P., Bardou-Jacquet, E., Turmel, V., Bonnaure-Mallet, M., et al., 2017b. Periodontal status and serum biomarker levels in HFE haemochromatosis patients. A case-series study. J. Clin. Periodontol. 44, 892–897.
- Ng, H.M., Kin, L.X., Dashper, S.G., Slakeski, N., Butler, C.A., Reynolds, E.C., 2016. Bacterial interactions in pathogenic subgingival plaque. Microb. Pathog. 94, 60–69.
- Orth, R., O'Brien-Simpson, N., Dashper, S., Walsh, K., Reynolds, E., 2010. An efficient method for enumerating oral spirochetes using flow cytometry. J. Microbiol. Methods 80, 123–128.
- Orth, R.K.-H., O'Brien-Simpson, N.M., Dashper, S.G., Reynolds, E.C., 2011. Synergistic virulence of *Porphyromonas gingivalis* and *Treponema denticola* in a murine periodontitis model. Mol Oral Microbiol 26, 229–240.
- Tan, K.H., Seers, C.A., Dashper, S.G., Mitchell, H.L., Pyke, J.S., Meuric, V., Slakeski, N., Cleal, S.M., Chambers, J.L., McConville, M.J., et al., 2014. Porphyromonas gingivalis and Treponema denticola exhibit metabolic symbioses. PLoS Pathog. 10, e1003955.
- Watanabe, T., Maruyama, F., Nozawa, T., Aoki, A., Okano, S., Shibata, Y., Oshima, K., Kurokawa, K., Hattori, M., Nakagawa, I., et al., 2011. Complete genome sequence of the bacterium *Porphyromonas gingivalis* TDC60, which causes periodontal disease. J. Bacteriol. 193, 4259–4260.
- Wyss, C., 2007. Fatty acids synthesized by oral treponemes in chemically defined media. FEMS Microbiol. Lett. 269, 70–76.

2.3. Complementary data

2.3.1. Determination of optimal PPIX concentrations for P. gingivalis and T. denticola growth

P. gingivalis is able to accumulate cell-surface heme-containing pigment in the form of μ oxo bisheme which has inherent catalase activity that helps to protect the cell from oxidative stress (Dashper *et al.* 2009). In order to use the MMBC medium to test our hypothesis that excess iron combined with H₂O₂ produced by the streptococci promote the implantation of anaerobic pathogenic strains (Chathoth *et al.* 2020), we must ensure that our medium does not allow *P. gingvalis* to store this form of heme on its surface and to determine the optimal number of passages in heme-free media that will totally deplete μ -oxo bisheme as iron supply. For this purpose, we have followed the growth of *P. gingivalis* in MMBC-1 medium containing 8 μ M of FeSO₄ and initially 0.8 or 8 μ M of PPIX, and after several passages in PPIX-free media.

Results and discussion

The optimal concentration of hemin promoting growth of *P. gingivalis* was defined as 8 μ M, by measuring the OD₆₀₀ after 72 hours of growth in anaerobic conditions in MMBC-3 with different hemin concentrations (data not shown). Further experiments were carried out with 8 μ M of hemin, when indicated. The removal of hemin impaired the growth of both *P. gingivalis* and *T. denticola* species in absence of iron (data not shown). The growth of *T. denticola* was restored by 8 μ M of FeSO₄ (data not shown) while *P. gingivalis* was unable to grow up to three passages with only FeSO₄ and in the absence of hemin (**Figure 1**). 8 μ M of PPIX (iron-free hemin precursor) plus an equimolar concentration of FeSO₄ were first tested to mimic the usual hemin concentration, but this level of PPIX impaired the growth of *T. denticola* (data not shown). As depicted in **Figure 1**, 0.08 μ M was the minimal concentration of PPIX that is suitable for optimal growth of *P. gingivalis*, and this concentration did not impair the growth of *T. denticola* in MMBC-2 plus FeSO₄ (**Figure 2**). These data also demonstrated that the MMBC-2 medium, although containing BSA, did not contain porphyrin contaminants at levels sufficient to promote the growth of a porphyrin auxotroph such as *P. gingivalis*.

Therefore, supplementation with PPIX was required for the cultures of *P. gingivalis* in absence of hemin and 3 passages in hemin-free medium were sufficient to discard heme storage at cell surface. Interestingly, from *in silico* analysis, *P. gingivalis* possesses only one hemoprotein, NrfA, which shows similarity with nitrite reductase. However, we have demonstrated that *nrfA*

mutation was viable (B. Martin, not published), therefore the auxotrophy for hemin should be related to another metabolic function. Moreover, this study showed that the growth requirement of hemin by *T. denticola* was alleviated when ferrous iron was available, since *T. denticola* could grow in MMBC-3 plus FeSO₄ and without PPIX.



Figure 1. Minimal concentration of PPIX required for *P. gingivalis* growth in absence of hemin. 5 mlcultures in MMBC-1 plus 8 μ M FeSO₄ of *P. gingivalis* TDC60 were inoculated at OD₆₀₀ 0.1 with the indicated concentration of PPIX in μ M: black triangle 0; grey rhombus, 0.8; black circle, 0.08. The OD₆₀₀ of each culture was measured overtime. For each passage (arrows), the inoculum was taken in previous cultures without PPIX. The data are the average of values obtained from 3 cultures in each condition.



Figure 2. Growth of *T. denticola* in MMBC-2 media with different iron sources and in NOS medium. Cultures of *T. denticola* were inoculated at OD₆₀₀ 0.1 and incubated for 72 hours at 37°C in anaerobic condition. (H): 8 μ M hemin; (F): 8 μ M FeSO₄; (F, PPIX): 8 μ M FeSO₄ and 0.08 μ M PPIX.

2.3.2. H₂S quantification in MMBC medium

H₂S is produced by several anaerobic bacteria of the oral microbiota mainly from cysteine, which originated from the catabolism of cysteine-containing proteins/peptides and glutathione, via cysteine desulfhydration. The β C-S lyases, pyridoxal-5'-phosphate (PLP)-dependent enzymes catalyze the α , β -elimination of sulfur amino acids (*L*-cysteine, *L*-cystathionine, *L*-cystine, *S*-(2-aminoethyl)-*L*-cysteine, and *S*-methyl-*L*-cysteine desulfhydrase LCD) along with pyruvate and ammonia. This pathway is certainly relevant in the oral biofilm *in vivo* since it was identified in the major pathogen *T. denticola* (Chu *et al.* 2002, 2003, 2008; Cellini, Bertoldi and Borri Voltattorni 2003; Goulet *et al.* 2004). *P. gingivalis* is capable of producing H₂S from cysteinyl glycine, therefore, possessing LCD activity (Tang-Larsen *et al.* 1995). Genes encoding LCD were identified in the genomes of both strains: TDE1669 for *T. denticola* and PGTDC60-1459 for *P. gingivalis*.

For anaerobic bacteria, H_2S production may help to maintain a reduced environment and to decrease dissolved oxygen. A study of Lai and Chu, (2008) has revealed that H_2S production by *T. denticola* resulted in a dose-dependent removal of dissolved O_2 in the growth cultures.

It was therefore interesting to know if *P. gingivalis* and *T. denticola* were able to produce H₂S in the MMBC medium.

Results and discussion

The ability of the MMBC-3 medium to provide adequate source(s) of substrate for H₂S production was monitored in mono- or in co-cultures. As shown in Figure 3A and 3B, both P. gingivalis and T. denticola grown in MMBC-3 plus hemin produced H₂S as detected by bismuth sulphide precipitation while no detectable amount was produced by S. gordonii. The amount of H₂S increased over time in all cultures containing P. gingivalis and T. denticola, while the colour remained constant in the standards. T. denticola mono-cultures and T. denticola/P. gingivalis cocultures produced visibly more H_2S (++ at 4 hours and +++ at 24 hours) than P. gingivalis monocultures (+ at 4 hours, ++ at 24 hours) for similar cells numbers (same OD_{600nm} in each well). Interestingly, we noticed in this study that, in mono-cultures and at similar cell density, T. denticola produced higher H_2S levels than *P. gingivalis* (Figure 3C). Moreover, in the conditions used, i.e hemin as iron source, the production of H_2S is lower in co-cultures T. denticola/P. gingivalis and T. denticola/P. gingivalis/S. gordonii than in monocultures of T. denticola. In these hemin-rich conditions, P. gingivalis stores hemin at its surface in form of u-oxo bis-heme (Dashper et al. 2009) which is proposed to have a catalase activity over ROS. Since we made the hypothesis that H_2O_2 produced by S. gordonii may trigger H₂S production in high iron levels, the experiment needs to be carried out in conditions of low hemin concentration and excess ferrous iron. MMBC-3 plus 80 μ M of FeSO₄ and 0.8 μ M of PPIX will meet the requirement. However, we will have to implement another method of H₂S detection because preliminary experiments have shown that in the presence of an excess of ferrous iron (in place of hemin), the reagent used here precipitated independently of the presence of H₂S, which induced an irreproducibility of the data.



С



Figure 3. H₂S production in MMBC-3 medium. The production of H₂S was measured with the modified method of Basic et al. (Basic et al., 2015). An OD₆₀₀ of 1 was obtained from overnight cultures of bacteria. Then an equal volume of newly prepared bismuth solution (0.4 M triethanolamine-HCl, pH 8.0; 10 mM bismuth(III)chloride; 20 uM pyridoxal 5-phosphate monohydrate, 20 mM EDTA and 40 mM Lcysteine) was added. A black bismuth sulphide (BS) precipitate is indicative of H₂S production. 100 µl of cultures at OD₆₀₀=1 of mono-cultures of T. denticola (A7-A12), P. gingivalis (B7-B-12), S. gordonii (C7-C12), or co-cultures of T. denticola + P. gingivalis (C1-C6) and T. denticola + P. gingivalis + S. gordonii (B1-B6) were incubated with 100 µl of MMBC-4 plus hemin (1, 2, 3 and 7, 8, 9) medium or 100 µl of bismuth sulfide reagent (4, 5, 6 and 10, 11, 12). Standards (E2-E12) correspond to NaHS diluted in MMBC-3 plus hemin at the following concentrations: 5, 4.5, 4, 3.5, 3, 2.5, 2, 1.5, 1, 0.5, and 0 mM and incubated with bismuth sulphide reagent (only one well out of triplicate is shown in the picture for each concentration). Plates were incubated for 4 hours (A) or 24 hours (B) at 37°C in anaerobic conditions. The experiment was carried out 4 times with similar results. (+) scores the colour intensity due to bismuth sulphide precipitation with H₂S. (C) OD of cultures were then measured at 415 nm over a period of 4 hours after addition of the reagent, which measured both cell density and BS precipitate.Sg: S. gordonii; Pg: P. gingivalis; Td: T. denticola; PgTd: P. gingivalis-T. denticola; SgPgTd: S. gordonii-P. gingivalis-T. denticola.

2.4. Discussion and perspectives

In this study, three new growth media, named MMBC (Medium for Mixed Bacterial Community), were designed for the individual or co-cultures of *P. gingivalis*, *T. denticola* and/or *S. gordonii*, which allow the control of iron and porphyrin. These media were validated for the growth of each bacterium in planktonic and biofilm cultures. Moreover, the concentration of hemin, an oxygen scavenger, can also be controlled in the MMBC composition.

MMBC media would therefore be suitable to test antibacterial compounds against the oral biofilm model. Moreover, it will allow us to test the behaviour of each of the selected strains in presence of iron, as a potential driver for dysbiosis. The synthetic model of the microbial community should also reflect the virulence of the *in vitro* community. Amongst markers of virulence, H₂S production is of particular interest for oral infections. H₂S is the predominant volatile sulphur compound synthesized by oral bacteria and it is prevalent in periodontal pockets. H₂S is cytotoxic to host cells and leads to inflammation via IL-8 increase (Yaegaki 2008; Chen *et al.* 2010; Greabu *et al.* 2016). Moreover, H₂S, may link iron, oxidative stress and sulphur metabolism with virulence trait in oral bacteria, in response to host signal such as cysteine sources or iron overload diseases (Chathoth *et al.* 2020). Therefore, the ability of *P. gingivalis* and *T. denticola* to produce H₂S in MMBC growth media will permit the measure of this virulence factor in the biofilm model in response to iron levels.

These well-defined media will therefore be a useful tool for the study of oral synthetic communities in order, not only to better understand the physiopathology of periodontal diseases, but also to develop new targeted therapies.

3. DEVELOPMENT OF *IN VITRO* MODELS AND PROTOCOLS FOR TESTING ANTIMICROBIAL AGENTS AGAINST ORAL BIOFILM

3.1. Introduction

This chapter gathers two studies that have enabled to test on the *in vitro* model of oral biofilm the effectiveness of potentially active products to fight periodontal diseases related to a dysbiosis of the microbiota.

A first study (Chapter 3.2.) focused on the effect of a liquid solution, provided by HEMARINA, containing an oxygen carrier from Arenicola marina hemoglobin. The tests were carried out in μ -Slide IBIDI which is a chambered coverslip with 8 individual wells. This cell culture chamber is suitable for most microscopy techniques and to grow static biofilms. The effect of 4 mg ml⁻¹ of HEMO₂life[®] (M101) was assessed on the three-species oral biofilm model presented in chapter 2. The 24-hours biofilm was treated for 1 hour with M101, with an adapted protocol to allow the delivery of oxygenated haemoglobin directly into the wells, in anaerobic conditions. The results showed a significant decrease of 65% in the total number of bacteria in comparison to that of the biofilm subjected to the formulation buffer when quantified by qPCR using 16S rRNA primers specific for each species. The treatment induced a significant increase in the total number of bacteria detached from the biofilm. The results obtained by confocal laser microscopy reveal a reduction of about 71% in the bio-volume and 38% in the thickness of the treated biofilm in comparison to the control. These results were presented as a poster in the BIOREGATE Forum in 2018 (Arenicola marina extracellular hemoglobin: a new promising treatment in periodontal disease, December 2018, Conference: 2018 BIOREGATE Forum, 12-14 December, Nantes, France).

As the treatment of periodontitis requires the maintenance of the antimicrobial agent in the periodontal pockets, a formulation in the form of gel is more relevant. We thus optimized the experimental model to meet this requirement. The mixed biofilm was developed on an MBEC system and the protocol adapted to the use of a gel form (**Chapter 3.3.**). The biofilms were subjected to the action of two commercially available, potentially active gels: blue[®]m oral gel (courtesy of the company blue[®]m Europe B.V., Netherlands) and Hyalugel[®]-ADO (Ricerfarma, Milan, Italy). The main ingredients of blue[®]m oral gel are sodium perborate (1.72 % w/w) and

lactoferrin (0.2 % w/w). Sodium perborate when in contact with liquid/saliva releases active oxygen slowly for a longer time duration. Lactoferrin has antimicrobial, anti-inflammatory and anti-carcinogenic properties and also acts as an iron chelator. Hyalugel[®]-ADO mainly consists of hyaluronic acid (0.2 % w/w), which has shown antibacterial activity against several planktonic bacteria and biofilms including *P. gingivalis*. We tested the effect of both gels, on the three-species 6-hour biofilms previously grown on the surface of the pegs and compared the amount of each species with that of untreated biofilms. Both gels showed significant ability in the reduction of the overall biofilm growth in comparison to untreated biofilm. They were especially effective in reducing S. gordonii and T. denticola in the biofilm. S. gordonii, long described as commensal, has been recently classified under 'signatures of dysbiosis' due to its predominance in patients with periodontitis and edentulism. It plays a role in co-aggregation and metabolic interactions with periodontal pathogens and can promote their installation and persistence. T. denticola, a member of the red complex, is known for its metabolic symbiosis, co-aggregation and synergy with the keystone pathogen P. gingivalis. Hence, the reduction of S. gordonii and T. denticola in the oral biofilm model is indeed a promising effect. These data were submitted for publication in the Journal of Applied Microbiology in December 2020 (Chathoth et al., submitted).

3.2. Detachment of oral bacteria in biofilms is induced by a solution containing the haemoglobin of *Arenicola marina*

Abstract

Periodontal diseases are a chronic polymicrobial inflammatory state of the periodontium, which can range from a mild gingival infection to severe periodontitis and tooth loss. A short exposure to a solution containing M101, a marine oxygen carrier, developed by a French company (Hemarina), induces biofilm detachment of a three-species biofilm composed of two periodontal pathogens, *Porphyromonas gingivalis* and *Treponema denticola* and a primary coloniser of the dental plaque, *Streptococcus gordonii*. It is a promising result in the prospect of developing a medical device to decrease the pathogenic microbiota inside the periodontal pocket.

Introduction

Periodontal diseases are a chronic polymicrobial inflammatory state of the periodontium, that can affect up to 90% of the adult population worldwide (Pihlstrom, Michalowicz and Johnson 2005; Kinane, Stathopoulou and Papapanou 2017; Tonetti et al. 2017). They can range from a mild gingival infection to severe periodontitis, tooth loss and can also result in halitosis. It can affect mastication, self-esteem, social relationships (Ayers and Colquhoun 1998; Lee et al. 2003; Petersen and Ogawa 2012; Slots 2013). Moreover, several studies emphasize the association of periodontal diseases with other systemic disorders like rheumatoid arthritis (Bingham and Moni 2013; Koziel, Mydel and Potempa 2014; Araújo, Melo and Lima 2015; Äyräväinen et al. 2017), atherosclerotic cardiovascular disease (Friedewald et al. 2009; Slavicek et al. 2009; Tonetti, Van Dyke, and working group 1 of the joint EFP/AAP workshop 2013; Beukers et al. 2017), diabetes (Mealey 2006; Chang and Lim 2012; Preshaw et al. 2012; Teeuw et al. 2017), adverse pregnancy outcomes (Sanz and Kornman; López 2008; Pitiphat et al. 2008) and even cancer (Hujoel et al. 2003; Tezal et al. 2007; Pendyala et al. 2013; Rajesh et al. 2013). The current treatment options include debridement, surgery, scaling and root planning, use of adjunctive drugs (minocycline, doxycycline, chlorhexidine) and systemic antibiotics (amoxicillin, metronidazole) (Kinane, Stathopoulou and Papapanou 2017). Conventional mechanical therapy has limited effectiveness whereas antiseptics like chlorhexidine, due to their broad antimicrobial spectrum induce dysbiosis and other side effects (Rath and Singh 2013). Furthermore, antibiotics pose a threat of increased antibiotic resistance. Owing to these concerns, there exists the need to discover new approaches to treat periodontal diseases.

Periodontal diseases are commonly caused by the oral microflora that forms a biofilm or dental plaque on and around the surface of the teeth (Pihlstrom, Michalowicz and Johnson 2005). This biofilm initially consists of early colonizers that recognize receptors in the acquired pellicle coating the enamel of the tooth. These early colonizers comprise of facultative anaerobic grampositive bacteria (*Streptococcus* spp., *Actinomyces* spp. etc). Further colonization and disproportionate increase in the number or pathogenicity of late colonizers, including anaerobic pathogens like *Porphyromonas gingivalis* and *Treponema denticola*, eventually result in periodontal disease (Kolenbrander *et al.* 2002; Sbordone and Bortolaia 2003).

P. gingivalis, a keystone pathogen, capable of inducing the formation of a dysbiotic microbial community with an elevated virulence, is a major etiological agent in severe forms of periodontitis. *P. gingivalis* and *T. denticola* are known to form synergistic polymicrobial biofilms. These members of the "red complex" (Holt and Ebersole 2005) are not only associated with severe forms of periodontitis but also demonstrate synergistic virulence upon co-infection (Kuramitsu, Chen and Ikegami 2005; Yamada, Ikegami and Kuramitsu 2005; Zhu *et al.* 2013; Mysak *et al.* 2014; Tan *et al.* 2014). Certain commensals like *Streptococcus gordonii*, act as accessory pathogens facilitating communication and aiding the transition to pathogenicity (Lamont *et al.* 2002; Hajishengallis 2015; Lamont and Hajishengallis 2015).

On account of the close association of anaerobic pathogens with the disease, we have considered the use of oxygen carriers, against the anaerobic bacterial pathogens by using a three-species biofilm model consisting of *P. gingivalis*, *T. denticola* (anaerobic pathogens) and *S. gordonii* (commensal) that we previously developed (Martin *et al.* 2018). These microorganisms were selected owing to the species-specific co-aggregation of *S. gordonii* with *P. gingivalis* (Lamont and Hajishengallis 2015) and the syntrophy and synergy between *P. gingivalis* and *T. denticola* (Grenier 1992; Yamada, Ikegami and Kuramitsu 2005; Orth *et al.* 2011; Zhu *et al.* 2013; Tan *et al.* 2014).

As Brain Heart Infusion (BHI) medium is capable of consuming oxygen (Leclerc *et al.* 2015), and in order to eliminate the interference of empirical components such as peptone or yeast extract present in commonly used rich media for oral bacteria such as OMIZ-M (Oral Microbiology

and Immunology Zurich medium) (Wyss 1992, 2007), OBGM (Oral Bacterial Growth Medium) (Orth *et al.* 2010), BM (Biofilm Medium) (Loo, Corliss and Ganeshkumar 2000) and enriched BHI (Martin *et al.* 2017), we used the newly developed MMBC-3 (Medium for Mixed Bacterial Community, MMBC) that allows us to control strictly the iron exposure of bacteria (Martin *et al.* 2018).

In order to specifically target anaerobic species of periodontal biofilms, we focused on a solution for oxygen delivery based on the high oxygen affinity carrier (M101) developed by Hemarina, (Morlaix, France). M101 is an extracellular haemoglobin extracted from the invertebrate *Arenicola marina* a common marine polychaete annelid living in the intertidal area on the west Atlantic coast between the Northsea and Biarritz and farmed by the company. It has a molecular mass of approximately 3.6 MDa and is composed of 156 globin chains of 8 different types, each globin chain is able to bind oxygen. Also, it contains 42 linker chains of 2 different types with a superoxide dismutase activity (Zal *et al.* 1997; Rousselot *et al.* 2006; Harnois *et al.* 2009) and sulphide-binding ability (Zal *et al.* 1997).

Many studies have been previously performed using M101 revealing a variety of potential applications. It has been widely studied for its ability to carry and transfer oxygen (Le Gall *et al.* 2014; Pape *et al.* 2015) and as a promising therapeutical oxygen carrier (Rousselot *et al.* 2006; Harnois *et al.* 2009; Tsai *et al.* 2012). Its function in organ preservation for transplantation of heart (Teh *et al.* 2017), lung (Glorion *et al.* 2018) and kidney (Thuillier *et al.* 2011; Mallet *et al.* 2014) is noteworthy. M101 is also known to aid in lowering the severity of traumatic brain injury (Moon-Massat *et al.* 2015).

In the current study, the effect of a formulation containing M101 on the three-species periodontal biofilm was evaluated. In the prospect of developing a medical device to decrease the periodontal microbiota, we have measured the biofilm detachment induced by a time-limited treatment.

Material and Methods

Bacterial strains and culture conditions

Strains of *Streptococcus gordonii* Challis DL1 (Chen, Teng and Chang 2004), *Porphyromonas gingivalis* TDC60 (Watanabe *et al.* 2011) and *Treponema denticola* ATCC35405 (Chan *et al.* 1993) were used for the study. The cultures were grown in MMBC-3 (Medium for Mixed Bacterial Community), formulated by our group with FeSO_{4..}7H₂O₂ (Sigma-Aldrich) at 8 μ M and protoporphyrin IX (PPIX, Sigma-Aldrich) at 0.08 μ M as the iron source (Martin *et al.* 2018). *T. denticola* was initially grown in NOS spirochete medium (Leschine and Canale-Parola 1980) and further sub-cultured into MMBC-3 medium supplemented with FeSO₄ (8 μ M) and protoporphyrin IX (PPIX) (0.08 μ M) while *S. gordonii* and *P. gingivalis* were directly grown in MMBC-3 medium supplemented with the iron source. All three micro-organisms were grown in anaerobic condition at 37°C in an anaerobic chamber (MACS 500, Don Whitley Scientific, United Kingdom) with 10% v:v H₂, 10% v:v CO₂ and 80%v:v N₂.

M101

A natural biopolymer, known as M101 (Hemo₂life) and derived from a lugworm (*Arenicola marina*) has been developed by HEMARINA SA (Morlaix, France). The formulation buffer, used as a negative control, is composed of NaCl 90 mM, Na-gluconate 23 mM, Na-acetate 27 mM, KCl 5 mM, MgCl₂ 1.5 mM and CaCl₂ 2.5 mM. M101 and the formulation buffer were obtained from Hemarina (Morlaix, France). Based on preliminary assays, an initial concentration of 4 mg ml⁻¹ M101 diluted in MMBC-3 medium (Martin *et al.* 2018) with FeSO₄ (8 μ M) and PPIX (0.08 μ M) - in order to strictly control the iron content and biochemical forms in the culture medium - was used for all experiments to assess its biological effect.

Multispecies biofilm

Exponential bacterial cultures of *S. gordonii*, *T. denticola* and *P. gingivalis* grown in MMBC-3 medium with the iron source were harvested. A three-species biofilm was grown in sterile open μ -slide 8 chambered coverslip (ibiTreat, Ibidi). The wells of the μ -slide were initially coated with filtered sterile human saliva (Martin *et al.* 2017) and incubated for 30 min before bacterial inoculation of *P. gingivalis* (OD₆₀₀ = 0.1), *S. gordonii* (OD₆₀₀ = 0.05), and *T. denticola* (OD₆₀₀=0.1). After 2 hours, for each well, the inoculum was removed, replaced by fresh medium and the μ -slides were incubated for 24 hours in anaerobic conditions at 37°C before treatment.

Treatment of the 24-hour three-species biofilm

The 24-hour biofilm was treated for 1 hour with M101 (4 mg ml⁻¹) along with two controls i.e. formulation buffer and 70% ethanol, prepared in aerobic environment. The formulation buffer

was used as the negative control for all experiments while 70% ethanol was used as a control in order to set up the microscope parameters for the membrane impermeant dye, propidium iodide, for confocal imaging. The treatment was added to the biofilm in an anaerobic condition in the anaerobic chamber. The detached cells were collected and quantified by qPCR while the biofilm was washed with phosphate buffered saline (PBS), stained and observed using the confocal scanning microscope followed by quantification by qPCR. The experiments were performed in triplicates and repeated thrice.

Confocal microscopy and imaging

The biofilms were washed with PBS for 15 minutes and after recovering the detached cells, they were removed from the anaerobic chamber and were stained with 5 µM of Syto[®] 40 nucleic acid dye (Molecular Probes, Leiden, Netherlands) and 40 µM of propidium iodide (Thermo Fisher, USA) diluted in PBS. The u-slides were then observed *in situ* with a Leica TCS-SP8 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany). An HC PL Apo 63X, 1.4 NA, oil immersion objective lens was used for image capture and a numerical zoom of 1.5 was applied. The 405-nm UV diode and a 420 to 500-nm band-pass emission filter were used to detect all bacteria stained with Syto® 40 while the 514-nm UV diode and a 620 to 650-nm band-pass emission filter were used to detect all bacteria stained with PI. Biofilm stacks ($123 \times 123 \mu m$) acquired at 0.5 µm intervals were scanned with a line average of 2. Leica software (LAS AF V.2.2.1) was used for microscope piloting and image acquisition. Analysis of images based on Syto® 40 and PI fluorescence intensity levels were performed in Comstat 2 plugin in ImageJ software V1.43m (National Institute of Health, USA) to estimate characteristic parameters of biofilms: the biomass, representing the overall volume of the biofilm (expressed in $\mu m^3/\mu m^2$) and the average thickness (defined as the average of thickness over given locations, ignoring pores and voids inside the biofilm). All these parameters are as described previously (Heydorn et al. 2000).

Quantification of bacteria by qPCR

The bacteria (consisting of *S. gordonii*, *P. gingivalis* and *T. denticola*) in the initial inoculum and the bacterial cells detached after treatment (M101/ formulation buffer / 70% ethanol) were centrifuged (7000xg, 20°C, 10 min) and the pellets were resuspended in PBS. Biofilms were collected in PBS (100 μ l). All these samples were further heated (95°C for 20 min) and quantified by qPCR.

For each qPCR, the total reaction volume was 12.5 μ l consisting of 6.25 μ l SYBR 2X Green Master Mix (Eurogentec, Belgium), 1 μ l each of forward and reverse primers (5 μ M) and 1 μ l of DNA template. DNA templates were amplified using the Applied Biosystems apparatus (StepOne Plus). The conditions for qPCR were: an initial holding stage of 95°C for 10 min followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C; a melt curve stage was performed consisting of 15 sec at 95°C followed by a temperature gradient from 60°C to 95° C in 1°C increment step measuring fluorescence at each temperature for 15 sec.

Defined concentrations of DNA standards were set, ranging from 10 to 0.0001 ng with purified genomic DNA from each species which was obtained with the Wizard[®] Genomic DNA Purification Kit (Promega, USA). With the help of the threshold cycles (Ct) obtained and the standard curves, concentrations of the DNA samples were determined. Primers specific for each species targeting the 16S ribosomal RNA were used for the quantification as described previously (Ammann *et al.* 2013), taking into account specific genome weights (2.58x10⁻⁶ ng for *P. gingivalis*, 3.12x10⁻⁶ ng for *T. denticola* and 2.41x10⁻⁶ ng for *S. gordonii*). The primers used were (5'-> 3'): 16S *S. gordonii* F, AAG-CAA-CGC-GAA-GAA-CCT-TA;16S *S. gordonii* R, GTC-TCG-CTA-GAG-TGC-CCA-AC; 16S *P. gingivalis* F, TGG-GTT-TAA-AGG-GTG-CGT-AG; 16S *P. gingivalis* R, CAA-TCG-GAG-TTC-CTC-GTG-AT; 16S *T. denticola* F, CGC-GTC-CCA-TTA-GCT-AGT-TG and 16S *T. denticola* R, TTC-TTC-ATT-CAC-ACG-GCG-TC.

Statistical analysis

All experiments were performed in triplicates and were repeated three times. Statistical analysis was performed using the unpaired student t-test and a p-value of less than 0.05 was considered statistically significant.

Results

We have previously verified in planktonic cultures that M101 at a concentration of 4 mg ml⁻¹ was able to decrease the viability of the anaerobic pathogens *P. gingivalis* and *T. denticola* (Supplementary figure 1), without affecting the facultative anaerobic *S. gordonii* (data not shown).

Effect of the M101 on biofilm parameters

The effect of an initial concentration of M101 at 4 mg ml⁻¹ was assessed on a three-species oral biofilm model previously developed by our team (Martin *et al.* 2018). The three-species 24-
hours biofilm treated for 1 hour with M101 showed a significant decrease of 65% in the total number of bacteria in comparison to that of the biofilm subjected to the formulation buffer (Figure 1A) when quantified by qPCR using 16S rRNA primers specific for each species. The treatment induced a significant increase in the total number of bacteria detached from the biofilm (Figure 1B).

The results obtained by confocal laser microscopy reveal a reduction of about 71% in the biomass and 38% in the thickness of the treated biofilm in comparison to the control (Figure 2). This reduction in biofilm parameters is in agreement with the results obtained by qPCR. There is no difference between the biomass and thickness of the biofilm treated with either formulation buffer or ethanol which is in agreement with the results obtained by qPCR (data not shown).

On evaluating the effect of the treatment on the potential viability of the biofilm, an increased cell staining by a membrane impermeant dye, propidium iodide, was observed in biofilms treated with M101, similar to the ethanol-treated biofilms (Figure 2A). Only about 2 % of the bacteria were potentially alive after treatment as opposed to the result obtained with the formulation buffer which had 25% of potentially live bacteria. These results pertaining to the effect of the treatment on biofilm parameters obtained by qPCR and confocal microscopy are indicative of the efficiency of M101 in the overall reduction of the biofilm.

Effect of M101 on the bacterial composition of the three-species biofilm

The composition of the biofilm and of detached bacteria was assessed by qPCR. The percentage of each species in the biofilm and detached cells are shown in the Figure 1 (C and D). Among the three species, *S. gordonii* was the most predominant species in biofilm as well as in detached cells, with 90% cells being represented by this species, while *P. gingivalis* and *T. denticola* represented only 4 to 7 % of the biofilm species. Interestingly, the ratios of the species in biofilms treated with the M101 solution or formulation buffer were not modified. Likewise, bacterial proportions in detached cells remained the same regardless of the treatment.

In accordance with the decrease of total bacterial species observed in M101- treated biofilms, a substantial reduction in the number of each species by M101 was measured in comparison with biofilms submitted to formulation buffer (Figure 3). *S. gordonii* was reduced by 64%, while the anaerobic pathogens *P. gingivalis* and *T. denticola* were reduced by 70% and 75% respectively. These observations suggest that M101 solution induced an overall detachment of the

biofilm, which is not species-specific and left the ratio among the species unaffected with the commensal microbe, *S. gordonii* being the predominant among them.

Discussion

Eradication of periodontal pathogens with high efficiency, minimal toxicity and side-effects is a challenge. Most oral bacterial pathogens being anaerobic organisms we demonstrated here that a solution containing M101 characterized by a high-oxygen affinity induces biofilm detachment and thus could have a beneficial effect on periodontitis.

Our data show that M101 has an inhibitory effect on the three-species biofilm, resulting in more than half decrease in the total bacterial load. This strong decrease is correlated to an increased detachment of all species from the biofilm. This effect did not specifically target the anaerobic pathogens but the co-aggregation (Rosen, Genzler and Sela 2008; Lamont and Hajishengallis 2015), closely bound nature and vast-networks of inter-microbial interactions (Tan *et al.* 2014) between the three species of our biofilm model probably explain the overall detachment of mixed biofilm. The dominance of the commensal, *S. gordonii* in the biofilm may be due to its high growth rate in comparison to the anaerobes and/or its antagonistic nature towards them as previously observed (Martin *et al.* 2018).

Sudden exposure of the oral biofilm model to the high oxygen affinity extracellular hemoglobin (M101) coming from *Arenicola marina* may create an unexpected stress, likely related to the generation of reactive oxygen species (Bowden and Hamilton 1998). The release of O_2 inside the anaerobic chamber may be very rapid. M101 carries up to 156 O_2 molecules when saturated ($P_{50} = 7 \text{ mmHg}$ at 37°C, (Rousselot *et al.* 2006). M101 releases O_2 without requiring any allosteric effector, when the PO₂ is below the P_{50} . The oxygen or the production of reactive oxygen species induced by exposing bacteria to oxygen, may be the trigger to induce the detachment of the biofilm. Further work is needed to investigate oxygen as an interspecies signal in the periodontal biofilm. We observed as well an increased cell staining by a membrane impermeant dye, propidium iodide, in biofilms treated with M101, similar to the ethanol-treated biofilms, compared to the control samples (Figure 2A). However, the limitation in the live/dead staining approach for anaerobic cultures is the inevitable exposure to atmospheric oxygen during confocal imaging resulting in an overall decrease in the number of live bacteria across all treatments/samples.

Among the current treatment strategies for periodontitis, chlorhexidine is considered the gold standard antiplaque agent (Balagopal and Arjunkumar 2013). However, it must be noted that it is associated with common side effects like staining of the tooth, increased calculus formation and alteration of taste (Rath and Singh 2013: 1). The different classes of antibiotics are also suggested for the treatment of periodontitis (Kapoor *et al.*; Keestra *et al.* 2015). The systemically administered antibiotics have the advantage of reaching inaccessible pathogens in deep periodontal pockets. However, antibiotics pose the threat of antibiotic resistance (Rams, Degener and van Winkelhoff 2014a, 2014b) and cause various side effects. Owing to these limitations, many studies have identified natural antimicrobial compounds of plant (Zhou *et al.* 2014; Sulistyani *et al.* 2016) and marine (Roberts *et al.* 2013) origin to combat periodontal biofilms. These compounds also face the challenges like potential toxicity, susceptibility to proteases, high production costs and unclear mode of action.

M101 can be considered as an potential treatment against periodontal biofilms since it has no known side effects in connection with its applications in tissue repair (Zal and Rousselot 2009), ischemia or anemia pathologies (Rousselot *et al.* 2006; Harnois *et al.* 2009; Tsai *et al.* 2012), organ transplant (Thuillier *et al.* 2011; Mallet *et al.* 2014; Teh *et al.* 2017; Glorion *et al.* 2018). Interestingly, it was shown previously that M101 is also able to bind sulphide on its free cysteine residues or by the opening of the several disulphide bridges contained on their linker chains by a reaction called persulfide formation (Zal *et al.* 1997). Anaerobic periodontal pathogens are involved in the production of volatile sulphur compounds, like H₂S that result is halitosis. M101 can possibly help reduce periodontitis-associated halitosis by sequestering H₂S *in vitro* (Vitvitsky *et al.* 2015; Bostelaar *et al.* 2016). Further studies will help to confirm this hypothesis and identify the mechanisms inducing biofilm detachment.

Acknowledgments and conflict of interests

This work was supported by the Conseil Régional de Bretagne, France (F3/48 CPER), the Fondation "Les Gueules Cassées/Sourire Quand Même" and the Federative Research Structure Biosit (Rennes). We also thank all people of the Microscopy Rennes Imaging Center (Biosit) for technical assistance for microscopy experiments.

We thank Olivier Loreal and Elisabeth Leize-Zal for the critical reading of the manuscript. We thank Emilie Saucisse, who was paid by HEMARINA and did not possess any share of the company. Franck Zal is the founder of HEMARINA and holds shares of the company.

References

- Ammann TW, Bostanci N, Belibasakis GN *et al.* Validation of a quantitative real-time PCR assay and comparison with fluorescence microscopy and selective agar plate counting for species-specific quantification of an *in vitro* subgingival biofilm model. *J Periodontal Res* 2013;**48**:517–26.
- Araújo VMA, Melo IM, Lima V. Relationship between Periodontitis and Rheumatoid Arthritis: Review of the Literature. *Mediators Inflamm* 2015;**2015**:1–15.
- Ayers KM, Colquhoun AN. Halitosis: causes, diagnosis, and treatment. N Z Dent J 1998;94:156–60.
- Äyräväinen L, Leirisalo-Repo M, Kuuliala A *et al.* Periodontitis in early and chronic rheumatoid arthritis: a prospective follow-up study in Finnish population. *BMJ Open* 2017;7:e011916.
- Balagopal S, Arjunkumar R. Chlorhexidine: The Gold Standard Antiplaque Agent. *J Pharm Sci* 2013:5.
- Beukers NGFM, Heijden GJMG van der, Wijk AJ van *et al.* Periodontitis is an independent risk indicator for atherosclerotic cardiovascular diseases among 60 174 participants in a large dental school in the Netherlands. *J Epidemiol Community Health* 2017;**71**:37–42.
- Bingham CO, Moni M. Periodontal disease and rheumatoid arthritis: the evidence accumulates for complex pathobiologic interactions. *Curr Opin Rheumatol* 2013;**25**:345–53.
- Bostelaar T, Vitvitsky V, Kumutima J et al. Hydrogen Sulfide Oxidation by Myoglobin. J Am Chem Soc 2016;**138**:8476–88.
- Bowden GH, Hamilton IR. Survival of oral bacteria. Crit Rev Oral Biol Med 1998;9:54-85.
- Chan ECS, Siboo R, Keng T *et al. Treponema denticola* (ex Brumpt 1925) sp. nov., nom. rev., and Identification of New Spirochete Isolates from Periodontal Pockets. *Int J Syst Bacteriol* 1993;**43**:196–203.
- Chang P-C, Lim LP. Interrelationships of periodontitis and diabetes: A review of the current literature. *J Dent Sci* 2012;7:272–82.
- Chen CC, Teng LJ, Chang TC. Identification of Clinically Relevant Viridans Group Streptococci by Sequence Analysis of the 16S-23S Ribosomal DNA Spacer Region. J Clin Microbiol 2004;42:2651–7.

- Friedewald VE, Kornman KS, Beck JD *et al.* The American Journal of Cardiology and Journal of Periodontology Editors' Consensus: Periodontitis and Atherosclerotic Cardiovascular Disease. *J Periodontol* 2009;**80**:1021–32.
- Glorion M, Polard V, Favereau F *et al.* Prevention of ischemia-reperfusion lung injury during static cold preservation by supplementation of standard preservation solution with HEMO2life® in pig lung transplantation model. *Artif Cells Nanomed Biotechnol* 2018;**46**:1773–80.
- Grenier D. Nutritional interactions between two suspected periodontopathogens, *Treponema* denticola and Porphyromonas gingivalis. Infect Immun 1992;60:5298–301.
- Hajishengallis G. Periodontitis: from microbial immune subversion to systemic inflammation. *Nat Rev Immunol* 2015;**15**:30–44.
- Harnois T, Rousselot M, Rogniaux H et al. High-level Production of Recombinant Arenicola Marina Globin Chains in Escherichia Coli: A New Generation of Blood Substitute. Artif Cell Blood Subs Biotechnol 2009a;37:106–16.
- Heydorn A, Nielsen AT, Hentzer M *et al.* Quantification of biofilm structures by the novel computer program COMSTAT. *Microbiol* 2000;**146 (Pt 10)**:2395–407.
- Holt SC, Ebersole JL. *Porphyromonas gingivalis, Treponema denticola*, and *Tannerella forsythia*: the 'red complex', a prototype polybacterial pathogenic consortium in periodontitis. *Periodontol 2000* 2005;**38**:72–122.
- Hujoel PP, Drangsholt M, Spiekerman C *et al.* An Exploration of the Periodontitis–Cancer Association. *Annal Epidemiol* 2003;**13**:312–6.
- Kapoor A, Malhotra R, Grover V *et al.* Systemic antibiotic therapy in periodontics. *Dent Res J.* 2012;9(5):505.
- Keestra JAJ, Grosjean I, Coucke W *et al.* Non-surgical periodontal therapy with systemic antibiotics in patients with untreated aggressive periodontitis: a systematic review and meta-analysis. *J Periodontal Res* 2015;**50**:689–706.
- Kinane DF, Stathopoulou PG, Papapanou PN. Periodontal diseases. *Nat Rev Dis Primers* 2017;**3**:17038.
- Kolenbrander PE, Andersen RN, Blehert DS *et al.* Communication among Oral Bacteria. *Microbiol Mol Biol Rev* 2002;**66**:486–505.
- Koziel J, Mydel P, Potempa J. The Link Between Periodontal Disease and Rheumatoid Arthritis: An Updated Review. *Curr Rheumatol Rep* 2014;**16**, DOI: 10.1007/s11926-014-0408-9.
- Kuramitsu HK, Chen W, Ikegami A. Biofilm Formation by the Periodontopathic Bacteria *Treponema denticola* and *Porphyromonas gingivalis*. J Periodontol 2005;**76**:2047–51.

- Lamont RJ, El-Sabaeny A, Park Y *et al.* Role of the Streptococcus gordonii SspB protein in the development of Porphyromonas gingivalis biofilms on streptococcal substrates. *Microbiol* 2002;**148**:1627–36.
- Lamont RJ, Hajishengallis G. Polymicrobial synergy and dysbiosis in inflammatory disease. *Trends Mol Med* 2015;**21**:172–83.
- Le Gall T, Polard V, Rousselot M *et al. In vivo* biodistribution and oxygenation potential of a new generation of oxygen carrier. *J Biotechnol* 2014;**187**:1–9.
- Leclerc J, Rosenfeld E, Trainini M *et al.* The Cytochrome bd Oxidase of *Porphyromonas gingivalis* Contributes to Oxidative Stress Resistance and Dioxygen Tolerance. *PLOS ONE* 2015;**10**:e0143808.
- Lee C-H, Kho H-S, Chung S-C *et al.* The relationship between volatile sulfur compounds and major halitosis-inducing factors. *J Periodontol* 2003;74:32–7.
- Leschine SB, Canale-Parola E. Rifampin as a Selective Agent for Isolation of Oral Spirochetes. J Clin Microbiol. 1980 Dec;12(6):792.
- Loo CY, Corliss DA, Ganeshkumar N. *Streptococcus gordonii* Biofilm Formation: Identification of Genes that Code for Biofilm Phenotypes. *J Bacteriol* 2000;**182**:1374–82.
- López R. Periodontal disease and adverse pregnancy outcomes. Evid Based Dent 2008;9:48.
- Mallet V, Dutheil D, Polard V *et al.* Dose-Ranging Study of the Performance of the Natural Oxygen Transporter HEMO ₂ Life in Organ Preservation: Dose-Ranging Study of the Performance of HEMO ₂ Life. *Artif Organs* 2014;**38**:691–701.
- Martin B, Chathoth K, Ouali S *et al.* New growth media for oral bacteria. *J Microbiol Methods* 2018;**153**:10–3.
- Martin B, Tamanai-Shacoori Z, Bronsard J et al. A new mathematical model of bacterial interactions in two-species oral biofilms. *PLOS ONE* 2017;**12**:e0173153.
- Mealey BL. Periodontal disease and diabetes: A two-way street. *J Am Dent Assoc* 2006;**137**:S26–31.
- Moon-Massat P, Mullah SH-E-R, Abutarboush R *et al.* Cerebral Vasoactivity and Oxygenation with Oxygen Carrier M101 in Rats. *J Neurotrauma* 2015;**34**:2812–22.
- Mysak J, Podzimek S, Sommerova P *et al. Porphyromonas gingivalis*: Major Periodontopathic Pathogen Overview. *J Immunol Res* 2014;**2014**:1–8.
- Orth R, O'Brien-Simpson N, Dashper S *et al.* An efficient method for enumerating oral spirochetes using flow cytometry. *J Microbiol Methods* 2010;**80**:123–8.

- Orth RK-H, O'Brien-Simpson NM, Dashper SG *et al.* Synergistic virulence of *Porphyromonas gingivalis* and *Treponema denticola* in a murine periodontitis model. *Mol Oral Microbiol.* 2011;**26**:229–40.
- Pape FL, Bossard M, Dutheil D *et al.* Advancement in recombinant protein production using a marine oxygen carrier to enhance oxygen transfer in a CHO-S cell line. *Artif Cell Nanomed Biotechnol.* 2015;43:186–95.
- Pendyala G, Joshi S, Chaudhari S *et al.* Links demystified: Periodontitis and cancer. *Dent Res J.* 2013;**10**:704–12.
- Petersen PE, Ogawa H. The global burden of periodontal disease: towards integration with chronic disease prevention and control: Global periodontal health. *Periodontol 2000.* 2012;**60**:15–39.
- Pihlstrom BL, Michalowicz BS, Johnson NW. Periodontal diseases. Lancet 2005;366:1809-20.
- Pitiphat W, Joshipura KJ, Gillman MW *et al.* Maternal periodontitis and adverse pregnancy outcomes. *Community Dent Oral Epidemiol* 2008;**36**:3–11.
- Preshaw PM, Alba AL, Herrera D *et al.* Periodontitis and diabetes: a two-way relationship. *Diabetologia* 2012;55:21–31.
- Rajesh KS, Thomas D, Hegde S *et al.* Poor periodontal health: A cancer risk? *J Indian Soc Periodontol* 2013;**17**:706–10.
- Rams TE, Degener JE, van Winkelhoff AJ. Antibiotic Resistance in Human Chronic Periodontitis Microbiota. *J Periodontol* 2014a;**85**:160–9.
- Rams TE, Degener JE, van Winkelhoff AJ. Antibiotic resistance in human peri-implantitis microbiota. *Clin Oral Implants Res* 2014b;**25**:82–90.
- Rath SK, Singh M. Comparative clinical and microbiological efficacy of mouthwashes containing 0.2% and 0.12% chlorhexidine. *Dent Res J.* 2013;**10**:364–9.
- Roberts JL, Khan S, Emanuel C *et al.* An *in vitro* study of alginate oligomer therapies on oral biofilms. *J Dent.* 2013;**41**:892–9.
- Rosen G, Genzler T, Sela MN. Coaggregation of *Treponema denticola* with *Porphyromonas* gingivalis and *Fusobacterium nucleatum* is mediated by the major outer sheath protein of *Treponema denticola*. *FEMS Microbiol Lett.* 2008;**289**:59–66.
- Rousselot M, Delpy E, Drieu La Rochelle C *et al. Arenicola marina* extracellular hemoglobin: a new promising blood substitute. *Biotechnol J.* 2006;1:333–45.
- Sanz M, Kornman K. Periodontitis and adverse pregnancy outcomes: consensus report of the Joint EFP/AAP Workshop on Periodontitis and Systemic Diseases. *J Periodontol* **84**:S164–9.

- Sbordone L, Bortolaia C. Oral microbial biofilms and plaque-related diseases: microbial communities and their role in the shift from oral health to disease. *Clin Oral Invest* 2003;7:181–8.
- Slavicek G, Gruber H, Siegl P *et al.* Periodontitis and atherosclerotic cardiovascular disease. J Stomat Occ Med 2009;**2**:137.
- Slots J. Periodontology: past, present, perspectives: *The state of periodontology*. *Periodontol* 2000 2013;**62**:7–19.
- Sulistyani H, Fujita M, Miyakawa H *et al.* Effect of roselle calyx extract on in vitro viability and biofilm formation ability of oral pathogenic bacteria. *Asian Pac J Trop Med.* 2016;**9**:119–24.
- Tan KH, Seers CA, Dashper SG *et al.* Porphyromonas gingivalis and Treponema denticola Exhibit Metabolic Symbioses. Schneider DS (ed.). *PLoS Pathogens* 2014;**10**:e1003955.
- Teeuw WJ, Kosho MXF, Poland DCW *et al.* Periodontitis as a possible early sign of diabetes mellitus. *BMJ Open Diabetes Res Care* 2017;**5**:e000326.
- Teh ES, Zal F, Polard V *et al.* HEMO₂life as a protective additive to Celsior solution for static storage of donor hearts prior to transplantation. *Artif Cell Nanomed Biotechnol* 2017;**45**:717–22.
- Tezal M, Sullivan MA, Reid ME *et al.* Chronic Periodontitis and the Risk of Tongue Cancer. *Arch Otolaryngol Head Neck Surg* 2007;**133**:450–4.
- Thuillier R, Dutheil D, Trieu MTN *et al.* Supplementation with a New Therapeutic Oxygen Carrier Reduces Chronic Fibrosis and Organ Dysfunction in Kidney Static Preservation: A New O₂ Therapeutic Molecule Improves Static Kidney Preservation. *Am J Transplant*. 2011;**11**:1845–60.
- Tonetti MS, Jepsen S, Jin L *et al.* Impact of the global burden of periodontal diseases on health, nutrition and wellbeing of mankind: A call for global action. *J Clin Periodontol* 2017;**44**:456–62.
- Tonetti MS, Van Dyke TE, working group 1 of the joint EFP/AAP workshop. Periodontitis and atherosclerotic cardiovascular disease: consensus report of the Joint EFP/AAP Workshop on Periodontitis and Systemic Diseases. *J Periodontol* 2013;**84**:S24-29.
- Tsai AG, Intaglietta M, Sakai H *et al.* Microcirculation and NO-CO Studies of a Natural Extracellular Hemoglobin Developed for an Oxygen Therapeutic Carrier. *Curr Drug Discov Technol* 2012.
- Vitvitsky V, Yadav PK, Kurthen A *et al.* Sulfide Oxidation by a Noncanonical Pathway in Red Blood Cells Generates Thiosulfate and Polysulfides. *J Biol Chem* 2015;**290**:8310–20.

- Watanabe T, Maruyama F, Nozawa T *et al.* Complete Genome Sequence of the Bacterium *Porphyromonas gingivalis* TDC60, Which Causes Periodontal Disease. *J Bacteriol* 2011;**193**:4259–60.
- Wyss C. Growth of *Porphyromonas gingivalis*, *Treponema denticola*, *T. pectinovorum*, *T. socranskii*, and *T. vincentii* in a Chemically Defined Medium. *J Clin Microbiol* 1992;**30**:5.
- Wyss C. Fatty acids synthesized by oral treponemes in chemically defined media. *FEMS Microbiol Lett.* 2007;**269**:70–6.
- Yamada M, Ikegami A, Kuramitsu HK. Synergistic biofilm formation by *Treponema denticola* and *Porphyromonas gingivalis*. *FEMS Microbiol Lett.* 2005;**250**:271–7.
- Zal F, Green BN, Lallier FH et al. Quaternary Structure of the Extracellular Haemoglobin of the Lugworm Arenicola marina. A Multi-Angle-Laser-Light-Scattering and Electrospray-Ionisation-Mass-Spectrometry Analysis. Eur J Biochem 1997;243:85–92.
- Zal F, Rousselot M. Utilisation d'une hemoglobine pour la preparation de pansements, et pansements ainsi prepares. 2009 (Patent No. EP2164532B1) European Patent Office.
- Zhou Y, Guan X, Zhu W *et al.* Capsaicin inhibits *Porphyromonas gingivalis* growth, biofilm formation, gingivomucosal inflammatory cytokine secretion, and *in vitro* osteoclastogenesis. *Eur J Clin Microbiol Infect Dis.* 2014;**33**:211–9.
- Zhu Y, Dashper SG, Chen Y-Y *et al. Porphyromonas gingivalis* and *Treponema denticola* Synergistic Polymicrobial Biofilm Development. Brissette CA (ed.). *PLoS ONE* 2013;**8**:e71727.

FIGURES



Figure 1. The oxygenation solution containing M101 induced detachment of oral bacteria from biofilm. A 24-hour, 3 species biofilms consisting of *S. gordonii* (Sg), *P. gingivalis* (Pg) and *T. denticola* (Td) grown in MMBC-3 was subjected to the oxygenation solution containing M101 (4 mg mL⁻¹) ("M101", black bar) or to the formulation buffer (white bar) for 1 hour. Results were obtained by qPCR using 16S rRNA primers specific to each species. Data are the average of three experiments. p-value were calculated using the student's t-test. * p-value <0.01. Figure shows the total number of the bacteria in the biofilm (A) and the total number of bacteria detached (B) after treatment with M101 in comparison to the treatment with the formulation buffer. The table (C) shows percentage of each species in the biofilms shown in (A), while table (D) shows the percentage of each species in the detached cells shown in (B), after treatment with M101 in comparison to the formulation buffer.



Figure 2. The oxygenation solution containing M101 increased cell staining by membrane impermeant **PI dye.** The 24-hour biofilms grown in MMBC-3 were subjected to the oxygenation solution containing M101 (4 mg mL⁻¹) ("M101") or formulation buffer or 70% ethanol for 1 hour and stained with membrane-permeant Syto®40 nucleic acid dye (green –potentially live cells) and membrane-impermeant Propidium Iodide, PI (red - potentially dead cells). (A) Representative images of the surface of biofilms with the 3D maximum z-projection and their respective xz-projection of their thickness. Below the images are the percentage of potentially live (green) and potentially dead (red) bacteria for the respective treatments. (B) Biomass and the average thickness of the biofilms estimated using Comstat 2 plugin in ImageJ software V1.43m (National Institute of Health). The values are the average of 3 independent experiments with the respective standard deviation.



Figure 3. The oxygenation solution containing M101 induced reduction of each species in the biofilm. A 24-hour, 3 species biofilms consisting of *S. gordonii* (Sg), *P. gingivalis* (Pg) and *T. denticola* (Td) grown in MMBC-3 was subjected to the oxygenation solution containing M101 (4 mg mL⁻¹) ("M101") (black bar) or formulation buffer (white bar) for 1 hour. Results were obtained by qPCR using 16S rRNA primers specific to each species. Data are the average of three experiments. p-value were calculated using the student's t-test. * p-value <0.01. Figure shows the number of each strain in the biofilm after treatment in comparison to the biofilm subjected to the stabilization buffer.



Supplementary figure 1

Supplementary figure 1. Antimicrobial activity of the oxygenation solution containing M101 on planktonic cultures. Cultures of *P. gingivalis* (A) and *T. denticola* (B) at an initial OD₆₀₀ =0.1, in MMBC-2 (40) with FeSO₄ and PPIX were subjected to the oxygenation solution containing M101 (4 mg mL⁻¹) ("MMBC-2 + M101", black bars), formulation buffer ("MMBC-2 + fb", white bar), or MMBC-2 media (grey bars). Samples were collected after 240 min of incubation at 37°C in anaerobic condition and stained with Syto®40 and Propidium Iodide for live/dead fluorescence assays. Fluorescence was recorded by excitation at 380 nm or 480 nm and emission at 440 nm or 650 nm for Syto40 and PI respectively, using the POLARstar Omega microplate reader (BMG Labtech). Viability was calculated from a standard curve established from samples containing various amounts of living and dead cells, obtained by mixing bacteria treated or not with 70% ethanol (100 % of dead cells and 100% of live cells). Data are the average of three experiments. p-values were calculated with the student's t-test. * p-value <0.05.

3.3. Test of antibacterial gels against a multispecies oral biofilm with an adapted *in vitro* assay (Publication under review in the Journal of Applied Microbiology)

Kanchana Chathoth^{1*}, Bénédicte Martin¹, Martine Bonnaure-Mallet^{1,2} and Christine Baysse¹ *Corresponding author: kanchana-nandanan.chathoth@univ-rennes1.fr ¹ University of Rennes 1, NUMECAN INSERM U1241, CIMIAD F-35043 Rennes, France ² CHU Pontchaillou, Rennes

Keywords: oral biofilm, antibacterial gels, *Porphyromonas gingivalis, Treponema denticola*, *Streptococcus gordonii*, hyaluronic acid, oxygen, MBECTM assay.

Running headline: Evaluation of gels on oral biofilm.

ABSTRACT

Aim: This work aims to test the effect of two commercial gels against multispecies oral biofilms using an adapted protocol for the Minimum Biofilm Elimination Concentration (MBECTM) assay system.

Methods and Results: Three-species oral biofilms consisting of *Porphyromonas gingivalis*, *Treponema denticola* and *Streptococcus gordonii* were challenged with two gels (Hyalugel®-ADO and blue®m oral gel) of different composition but similar viscosity. The oral biofilm was realised on the peg-lids by using the Mixed Medium for Bacterial Community (MMBC), specifically designed for these bacterial species. Hyalugel®-ADO and blue®m oral gel demonstrated a biofilm reduction of 96.5 % and 97.6 % respectively compared to the untreated control. Interestingly, both gels showed a species-specific reduction of *S. gordonii* and *T. denticola* in the biofilm and also displayed an antibacterial effect on *S. gordonii* and *P. gingivalis* when tested by agar diffusion method.

Conclusions: The MBECTM method was effectively tailored for establishing oral multispecies biofilms on peg-lids and testing gel formulations against them. Both the gels demonstrated biofilm reduction efficacy with a species-specific effect, with a higher efficacy of the blue®m oral gel for total bacteria and pathogen reduction in the biofilm over Hyalugel®-ADO.

Significance and Impact of the Study: This study shows that single exposure of Hyalugel®-ADO and blue®m oral gel was effective against oral biofilms and therefore both gels could be used for the treatment of periodontitis.

INTRODUCTION

Periodontitis is a polymicrobial chronic inflammatory disease of the periodontium caused by the accumulation of dental plaque (Pihlstrom *et al.*, 2005). It is regarded as the second most common disease worldwide, with severe forms affecting up to 10 to 15% of adults (Petersen and Ogawa, 2012) resulting in tooth loss. Many studies have reported an association of severe periodontitis with several systemic disorders (Hujoel *et al.*, 2003; López, 2008; Preshaw *et al.*, 2012; Koziel *et al.*, 2014; Beukers *et al.*, 2017; Bui *et al.*, 2019; Graves *et al.*, 2019) and oral malodor (Lee *et al.*, 2003; Yaegaki, 2008) making it a major socio-economic concern.

Metagenomics analysis of the microbial community in the human subgingival plaque has revealed the presence of over 500 species (Ai *et al.*, 2017). The oral biofilm formation is initiated by early colonizers that consist mainly of facultative anaerobic Gram-positive bacteria such as *Streptococcus* spp. and *Actinomyces* spp. In the oral health-associated biofilm, these Gram-positive cocci and rods predominate. Change in dominant species with an increase of putative pathogens like *Porphyromonas gingivalis*, *Treponema denticola*, *Tannerella forsythia* and *Prevotella intermedia* results in dysbiosis and periodontal disease (Kolenbrander *et al.*, 2002; Meuric *et al.*, 2017).

The present strategies employed for the treatment of periodontitis are root planing and scaling, debridement, use of systemic antibiotics (amoxicillin, metronidazole) and adjunctive drugs (minocycline, doxycycline, chlorhexidine). Mechanical therapy has limited efficacy against periodontitis whereas antibiotics pose a threat of increased antibiotic resistance. Moreover, the gold standard antiplaque agent, chlorhexidine, is known to cause adverse effects like staining of the tooth, increased calculus formation and alteration of taste (Kinane et al., 2017; Rath and Singh, 2013; Jones, 1997). Local and sustained delivery of antibacterial agents at the site of infection may replace systemic drug administration and avoid related side effects.

Gel formulations of antibacterial agents are a promising delivery system for the treatment of periodontitis *via* topical administration. Their advantages include the ease of use, increased retention time at the site of application and controlled drug release. Several authors have demonstrated the effectiveness of gel formulations in reducing microbial content or plaque index (Sauvêtre *et al.*, 1993; Noyan *et al.*, 1997; Paquette *et al.*, 1997; Gomes *et al.*, 2006) in human, animal-based or *in vitro* studies. Similar improvement in probing depth and/or bleeding was reported (Esposito *et al.*, 1996; Graça *et al.*, 1997) on use of gel formulations alone or in conjunction with other modes of treatment. There is an increasing interest in the use of biodegradable and biocompatible compounds like chitosan (İkinci *et al.*, 2002), Q10-based gel and/or 0.8% hyaluronic acid gel (Sharma et al., 2016; Xu et al., 2004), cranberry juice concentrate (H.R. *et al.*, 2017) in gel formulations. The non-toxic and biocompatible properties of such compounds can address the adverse effects of commonly used antimicrobials. These combined with the advantages of gel as an effective delivery system is continuously explored as a treatment strategy against periodontitis.

In this study, we aim to test the efficacy of two gel-based products especially against polymicrobial in vitro oral biofilms to be able to decipher the behaviour of each species in response to the treatment. Therefore, the Minimum Biofilm Elimination Concentration (MBECTM) Assay System (formerly the Calgary Biofilm Device) (Ceri et al., 1999), which has been previously challenged with gel-based products to assess their bactericidal activity on mono-species biofilms (Pseudomonas aeruginosa, Staphylococcus epidermidis a Staphylococcus aureus) (Martineau and Dosch, 2007; Santos et al., 2016) was adapted for polymicrobial oral biofilms. An oral biofilm consisting of two key periodontal pathogens P. gingivalis, T. denticola and a primary colonizer of the dental plaque, S. gordonii was established on the peg-lids. P. gingivalis and T. denticola, coexist in deep periodontal pockets (Kigure et al., 1995; Kumawat et al., 2016) and are associated with severe forms of periodontitis. S. gordonii produces hydrogen peroxide and glutathione that can influence the pathogenic switch of the oral subgingival biofilm (Kreth et al., 2008; Chathoth et al., 2020). Moreover, the species-specific co-aggregation of S. gordonii with P. gingivalis (Lamont and Hajishengallis, 2015) and the syntrophy and synergy between P. gingivalis and T. denticola (Meuric et al., 2013; Zhu et al., 2013; Tan et al., 2014) are well known. The-three species biofilms were grown on saliva-coated pegs to mimic the dental plaque developing initially at the root of teeth. The Mixed Medium for Bacterial Community (MMBC) (Martin et al., 2018) which specifically allows the growth of S. gordonii, P. gingivalis and T. denticola was used. This biofilm model was challenged with two commercial gels called Hyalugel[®]-ADO (Ricerfarma, Milan, Italy) and blue[®]m oral gel (blue[®]m Europe B.V., Netherlands) with different active agents to evaluate their specific effect. Hyalugel®-ADO mainly consists of hyaluronic acid (0.2 %, w/w), a major component of the extracellular matrix of the skin which plays a vital role in skin repair (Neuman et al., 2015). Hyaluronic acid has antibacterial activity against several planktonic bacteria and

biofilms (Ardizzoni et al., 2011; Pirnazar et al., 1999) including *P. gingivalis* (Binshabaib et al., 2020). The main ingredients of blue[®]m oral gel are sodium perborate (1.72 %, w/w) and lactoferrin (0.2 %, w/w). Sodium perborate when in contact with liquid/saliva releases active oxygen slowly for a longer time duration. Oxygen is a known signal for metabolic switches and is a spatiotemporal regulator of microbial virulence (Marteyn et al., 2011; Sengupta et al., 2014). Exposure of periodontal pathogens to oxygen can give rise to metabolic conversion to reactive oxygen species (ROS) that threaten the cellular integrity and affect the survival of oral microorganisms especially the anaerobic pathogens. Lactoferrin has antimicrobial, anti-inflammatory and anti-carcinogenic properties and also acts as an iron chelator (Wang *et al.*, 2019).

METHODS

Strains and media

Strains of *Streptococcus gordonii* Challis DL1 (Chen *et al.*, 2004), *Porphyromonas gingivalis* TDC60 (Watanabe *et al.*, 2011) and *Treponema denticola* ATCC35405 (Chan *et al.*, 1993) were used for the study. The cultures of *S. gordonii* and *P. gingivalis* were grown in MMBC-3 (Medium for Mixed Bacterial Community), with FeSO₄.7H₂O₂ (Sigma-Aldrich) at 8 μ M and protoporphyrin IX (PPIX, Sigma-Aldrich) at 0.08 μ M as the iron source (Martin *et al.*, 2018). *T. denticola* was initially grown in NOS spirochete medium (Leschine and Canale-Parola, 1980) and further sub-cultured in MMBC-3 supplemented with FeSO₄.7H₂O₂ (8 μ M) and PPIX (0.08 μ M). All three micro-organisms were grown in anaerobic condition at 37°C in an anaerobic chamber (MACS 500, Don Whitley Scientific, United Kingdom) with 10% v:v H₂, 10% v:v CO₂ and 80%v:v N₂.

Agar diffusion method

The agar diffusion method was performed for *Streptococcus gordonii* and *Porphyromonas gingivalis*. *Treponema denticola* cannot be tested by this method since it is unable to form colony or a lawn on MMBC-3 agar plate. The bacterial cultures were prepared in MMBC-3 supplemented with FeSO₄.7H₂O₂ (8 μ M) and PPIX (0.08 μ M) and were grown in anaerobic condition. A bacterial suspension containing approximately 1.5×10^8 CFU ml⁻¹ (equivalent to 0.5 McFarland standard) was prepared in 2 ml of MMBC-3 with FeSO₄.7H₂O₂ (8 μ M) and PPIX (0.08 μ M). This culture was poured onto 90 mm plates containing a previously set layer of MMBC-3 agar (prepared by adding the filtered 2X MMBC-3 medium with autoclaved 2X Bacteriological agar at approximately

 60° C) such that the entire surface of the agar is covered by bacteria. The plates were set aside for 5 min and then excess fluid, if any, was removed. Sterile wells of an inner diameter of 6 mm were made on the agar (2 on each plate) and filled with 50 µl of each of the gels (Hyalugel®-ADO and blue®m oral gel) with the help of a 1 ml sterile syringe. At least nine replicates (three biological and three technical) were made for each microorganism. The plates were maintained for 2 hours at room temperature in the appropriate gaseous conditions to allow the diffusion of the agents through the agar and then incubated at 37° C in anaerobic condition for 48 hours for *S. gordonii* and *P. gingivalis*. Zones of inhibition of microbial growth around the wells containing either of the gels were measured using a scale and recorded after the incubation period.

Growth and treatment of the biofilm

The protocol for the growth and treatment of the biofilm is detailed in Figure 1. As the first step, 200 µl of saliva (Pool Human Donors, MyBioSource), filtered (0.20 µm) and diluted twice in sterile distilled water, was added into the wells of Nunc[™] Nunclon[™] 96-well tissue culture microtiter plate. The lid with pegs (Nunc-TSP, polystyrene) was placed over the microtiter plate and incubated in saliva for 30 minutes. Next, the wells of a new microtiter plate were filled with 200 µl of inoculum consisting of S. gordonii (OD_{600nm}:0.05), P. gingivalis (OD_{600nm}:0.1) and T. denticola (OD_{600nm}:0.1). The saliva-coated pegs were immersed in the inoculum by placing the peg-lids over the microtiter plate. This set-up was incubated in anaerobic conditions for 6 hours at 37°C. Meanwhile, the challenge plate consisting of the two gel treatments and MMBC-3 was prepared in a new microtiter plate in aerobic condition by adding 150 µl of each of the treatments or of the medium in individual wells of the 96-well microtiter plate with the aid of 1 ml syringes. The 6-hour three-species biofilms or adherent cells present on the surface of the pegs were subjected to either MMBC-3 or Hyalugel[®]-ADO or blue[®]m oral gel for 1 hour in anaerobic condition at 37°C. After the 1-hour treatment, the pegs were carefully lifted from the challenge plate and further incubated in the microtiter wells containing 200 µl of MMBC-3 for 24 hours in anaerobic conditions at 37°C. This step was done to determine the effect of the treatments on the ability of the bacteria to grow back as either biofilm or planktonic cells. After the 24-hour incubation, the wells of the 96-well microtiter plate comprised of bacteria that detached and proliferated in planktonic form in the well. The pegs consisted of remaining biofilm after treatment. Both samples (planktonic cells and biofilm) are representative of the effectiveness of the treatment. The detached/planktonic cells were collected from the microtiter plate and the biofilm were collected from the surface of the pegs by sonication (30 min) and were quantified by qPCR. Six pegs (two per condition) were also kept for microscopic visualisation.

Confocal microscopy and imaging

Treated and untreated biofilm-containing pegs after a 24-hour incubation in MMBC-3 were subjected to microscopic imaging after staining with 5 μ M of Syto[®]9 green-fluorescent nucleic acid stain (Invitrogen, ThermoFisher Scientific) diluted in PBS. Briefly, six pegs were broken anaerobically from the lid with the help of sterile pliers and were placed in a microtiter plate containing the stain (200 μ l) for 20 min to enable staining of cells along the length of each peg. They were further anaerobically transferred to Syto[®]9 stain-filled (200 μ l) μ -slides (8 chambered coverslip, ibiTreat, Ibidi) for ease of microscopic visualization. The biofilm on the surface of the stained pegs was then observed *in situ* with a Leica TCS-SP5 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany). An HC PL Apo 10X, 0.4 NA oil immersion objective lens was used for image capture and a numerical zoom of 2 was applied. The 488-nm UV diode and a 485 to 500-nm band-pass emission filter were used to detect all bacteria stained with Syto[®]9. Biofilm stacks (123 × 123 μ m) acquired at 1 μ m intervals were scanned with a line average of 2. Also, zoomed images were captured using HC PL Apo 63X, 1.4 NA oil immersion objective lens with a numerical zoom of 5.05.

Leica software (LAS AF V.2.2.1) was used for microscope piloting and image acquisition. Analysis of images based on Syto[®]9 was performed in ImageJ software V1.43m (National Institute of Health, USA) to obtain the maximum z-projection of the images.

Quantification of bacteria by qPCR

The bacteria, consisting of *S. gordonii*, *P. gingivalis* and *T. denticola*, in the initial inoculum and the planktonic/detached cells collected after treatment were centrifuged (8000xg, 20°C, 10 min) and the pellets were resuspended in 150 μ l of Lysis buffer (20 mg ml⁻¹ lysozyme in 20 mM Tris- HCl, 2 mM EDTA, 1.2 % Triton X, PBS, pH 8). Biofilms were collected in 150 μ l Lysis buffer by sonication for 30 min using a water bath sonicator (Ultrasonic cleaner). The biofilm collected from three pegs from the same condition (treated or untreated) were pooled together to increase the sample volume and to compensate for the low biofilm surface area on the pegs

(approximately 44 mm² per peg) (Harrison *et al.*, 2010; MBECTM assay procedural manual, version 2.0, Innovotech). All samples were subjected to DNA extraction using QIAamp DNA Mini kit (Qiagen) according to the manufacturer's instructions with slight modification i.e. the lysis using proteinase K was performed overnight.

Quantitative PCR was performed in a total reaction volume of 12.5 μ l containing 6 μ l TakyonTM Low Rox SYBR[®] MasterMix dTTP Blue (Eurogentec), 0.5 μ l of each primer (5 μ M), and 1 μ l of the sample. Amplification of the extracted DNA template was performed in QuantStudioTM 7 Flex Real-Time PCR System (Applied Biosystems) by initial incubation of 2 min at 55°C and 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. A melt curve stage was performed consisting of 15 sec at 95°C followed by a temperature gradient from 60°C to 95° C with fluorescence measured in an increment of 1°C every 15 sec.

The concentrations of the DNA samples were determined in comparison with the defined concentrations of DNA standards set in the range of 0.0001 to 10 ng with purified genomic DNA from each of the three species. Primers used were specific to each species targeting the 16S ribosomal RNA taking into account specific genome weights (2.58 x 10⁻⁶ ng for *P. gingivalis*, 3.12 x 10⁻⁶ ng for *T. denticola* and 2.41 x 10⁻⁶ ng for *S. gordonii*) (Ammann *et al.*, 2013; Martin *et al.*, 2017). The primers used in this study are listed in Table 1.

Statistical analysis

All the experiments were done with three biological and three technical replicates. Statistical analysis was performed using the two-tailed unpaired student t-test and a p-value of less than 0.05 was considered statistically significant.

RESULTS

Antibacterial activity of Hyalugel[®]-ADO and blue[®]m oral gel

The agar diffusion method was used to evaluate the specific antibacterial effect of the two commercially available gels, namely, Hyalugel[®]-ADO and blue[®]m oral gel against *S. gordonii* and *P. gingivalis*. The diameter of the clear zone around the gel-filled well is indicative of an inhibitory effect. The results are presented in Table 2. Both the gels displayed an antibacterial effect against both the bacterial species. For *S. gordonii*, after 48 hours of incubation, blue[®]m oral gel showed a larger inhibitory zone (24.9 mm) in comparison to Hyalugel[®]-ADO (13.0 mm) (p<0.001).

Similarly, the inhibitory zone observed against *P. gingivalis* for blue®m oral gel (41.1 mm) was significantly larger than for Hyalugel®-ADO (22.4 mm) (p<0.001). These results indicate that both the gel formulations display an antibacterial activity against *S. gordonii* and *P. gingivalis* with blue®m oral gel having better antibacterial activity than Hyalugel®-ADO.

Establishment of the three-species oral biofilm on the peg-lid using MMBC-3 medium

In order to assess the effect of the two gels on the oral multispecies biofilm, the first challenge was to establish an oral biofilm model on the surface of pegs using the MMBC-3 medium. Quantitative PCR analysis revealed that 31-hour (6 hours of growth prior to treatment + 1 hour of treatment + 24 hours of post-treatment growth, see methods and Figure 1) three-species MMBC-3-treated biofilms on peg surface comprised of 2.08×10^7 CFU ml⁻¹ of bacteria (Figure 3A). On the other hand, the 96-well microtiter plate consisted of 4.16×10^9 CFU ml⁻¹ of planktonic or detached bacteria. The presence of bacteria in the biofilm on the peg surface was also confirmed by confocal fluorescent microscopy (Figure 2A and D). The microscopic images are representative of the total biofilm density on the peg surface and do not differentiate between individual species. However, it enables the visualization of clusters of bacteria in the biofilm (Figure 2D).

At the species level, the biofilm on the peg surface and the corresponding planktonic (unattached or detached cells) cells contained a higher proportion of *S. gordonii* in comparison to *P. gingivalis* and *T. denticola* cells (Figure 3B, 3C). Also, we observe that a majority of bacteria remain in the planktonic form for all three species. Altogether, we established the three-species oral biofilm on the peg surface using MMBC-3 medium, which could be further used for testing oral gels.

Effect of antibacterial gels against a 6-hour three-species oral biofilm model

Next, we tested the effect of both gels, Hyalugel[®]-ADO and blue[®]m oral gel on the threespecies 6-hour biofilms on the surface of the pegs and compared the data with untreated biofilms. The concentration of the total bacteria (biofilm + planktonic) was significantly reduced from 4.11 x 10⁹ CFU ml⁻¹ in MMBC-3 control to 5.15×10^6 CFU ml⁻¹ and 1.5×10^7 CFU ml⁻¹ for Hyalugel[®]-ADO and blue[®]m oral gel respectively after treatment of the biofilms for one hour (Figure 3A). The samples treated with Hyalugel[®]-ADO and blue[®]m oral gel displayed a biofilm reduction of 96.5 % and 97.6 % respectively, in comparison to untreated control (taken as 0 % of reduction). This decrease in biofilm concentration due to Hyalugel[®]-ADO and blue[®]m oral gel is also evident in the microscopic images (Figure 2A, 2B, 2C). Similarly, a decrease of over 99.5 % was observed in the concentration of detached/planktonic cells on treatment with either of the gels in comparison to untreated control (Figure 3A).

At the species level, the concentration of *S. gordonii* and *T. denticola* in the biofilm decreased significantly from MMBC-3-treated samples to Hyalugel[®]-ADO to blue[®]m oral gel-treated samples while the concentration of *P. gingivalis* remained unaffected (Figure 3B). Hyalugel[®]-ADO decreased the concentration of planktonic *S. gordonii* and *P. gingivalis* in comparison to MMBC-3 (Figure 3C) while no effect was observed on *T. denticola*. Post-treatment with blue[®]m oral gel, the concentration of planktonic *S. gordonii* decreased whereas blue[®]m oral gel induced significant planktonic *P. gingivalis* and *T. denticola* cells in comparison to MMBC-3.

DISCUSSION

The prime aim of this study was to test two antibacterial gels against multispecies oral biofilm model using the optimized high throughput MBECTM assay. For this we first established the three-species oral biofilms on the peg-lids. The concentration of the biofilm grown on the peg surface using the MMBC-3 medium is 1.5×10^5 CFU (mm²)⁻¹ (growth area per peg is 44 mm²: three pegs pooled together). This value is 650 times lesser in comparison to biofilm grown on μ -slides which is approximately 1 x 10⁸ CFU (mm²)⁻¹ (growth area of each well in a μ -slides is 100 mm²) (Martin *et al.*, 2018). This difference is as expected since the pegs due to the protruding topology vary in structure and surface area from μ -slides. Moreover, sedimentation of bacteria does not occur on pegs like that of μ -slides. However, the overall composition (or ratio) of individual species using identical inocula and growth conditions remained unchanged between pegs and μ -slides. Moreover, the use of the well-defined and iron-controlled MMBC-3, especially designed for the growth of the three species, makes it an appropriate choice for testing different antibacterial compounds.

Next, we compare the efficiency of two commercially available gels in biofilm reduction. It cannot be disregarded that the inherent shear force of the gel owing to its viscosity will have an effect along with their respective active compounds on the removal of biofilm from the peg surface. The similar viscosities, 35000-60000 cP for Hyalugel[®]-ADO, and 25000-50000 cP for blue[®]m oral gel, as provided by respective manufacturers enabled comparison between their effect. The agar

diffusion assay performed in this study enabled the confirmation of the antibacterial effect of the gels. The agar diffusion assay demonstrated an inhibition zone of bacterial growth for *S. gordonii* and *P. gingivalis* grown on MMBC-3 agar plates. Unfortunately, the antibacterial activity against *T. denticola* could not be assessed as this microorganism is known not to form colonies or lawn on agar plates easily (Wardle, 1997). The results of the agar diffusion assay indicate that the differences in antibiofilm activity displayed by the gels, especially against *S. gordonii*, are more likely due to their respective active compounds.

Previous studies have reported the efficacy of 0.8 % of hyaluronic acid in reducing *P. gingivalis* in *in vitro* biofilms (Binshabaib et al., 2020) and the bacteriostatic effect of hyaluronic acid (0.8 %- single application, next 0.2 %- twice a day for two weeks) as adjunct therapy (Eick et al., 2013) on *P. gingivalis* for individuals with chronic periodontitis. In our study, a onetime treatment of Hyalugel[®]-ADO (containing 0.2 % hyaluronic acid) for one hour significantly reduced the concentrations of *S. gordonii* and *T. denticola* but not the level of *P. gingivalis* in the biofilms (Figure 3B). A reduction of the concentration of planktonic *S. gordonii* and *P. gingivalis* on treatment with Hyalugel[®]-ADO is shown in comparison to untreated control (Figure 3C). An antibacterial effect of Hyalugel[®]-ADO against *P. gingivalis* and *S. gordonii* was confirmed by the agar-diffusion test. This could be attributed to a putative bacteriostatic effect of hyaluronic acid as previously demonstrated against planktonic cultures of *P. gingivalis* (MIC: 0.1 %) (Pirnazar et al., 1999) and *Streptococcus mutans* (MIC: 0.025 % from 24 hours onwards) (Ardizzoni et al., 2011) when grown in rich media.

blue[®]m oral gel with sodium perborate (1.72 %, w/w) and lactoferrin (0.2 %, w/w), reduced the biofilm growth of *S. gordonii* and *T. denticola* in comparison to both untreated control and Hyalugel[®]-ADO. This is in accordance with the findings of Cunha et al., (2019): in a randomized controlled clinical trial, they demonstrated antiplaque and anti-gingivitis efficacy of blue[®]m in the form of a toothpaste (containing sodium perborate and lactoferrin) against triclosan-containing toothpaste. The oxygen donor may have a direct effect on the obligate anaerobe *T. denticola* and/or may boost the peroxygenic activity of *S. gordonii* (Chathoth et al., 2020). Arslan et al., (2009) showed that lactoferrin at 0.01 % (w/v) reduces the initial attachment of *Streptococcus gordonii* by iron sequestration. This may explain the decrease in *S. gordonii* in blue[®]m oral gel-treated biofilms. A recent report demonstrated reduced growth of *P. gingivalis* in the presence of the blue[®]m oral gel containing sodium perborate (1.72 %) and lactoferrin (0.2 %) in planktonic culture (Deliberador et al., 2020).

In the present study, no effect of blue[®]m oral gel or Hyalugel[®]-ADO was observed on levels of *P. gingivalis* in biofilm compared to untreated biofilm while more *P. gingivalis* cells were found in the planktonic part of blue[®]m oral gel. Since both gels displayed an antimicrobial effect on *P. gingivalis* with the agar diffusion assay, it would be of interest to assess the viability of the cells remaining in the biofilm. It is known, that in the three-species biofilms, *P. gingivalis* is benefitted by inter-species associations with the other two species which possibly helps in its sustenance (Lamont and Hajishengallis, 2015; Meuric *et al.*, 2013; Zhu *et al.*, 2013; Tan *et al.*, 2014).

To conclude, both gels showed significant ability in the reduction of the overall biofilm growth in comparison to untreated biofilm. They were especially effective in reducing *S. gordonii* and *T. denticola* in the biofilm. *S. gordonii* along with 16 other genera, has been previously classified under 'signatures of dysbiosis' due to its predominance in patients with periodontitis and edentulism (Hunter et al., 2016). Also, its role in co-aggregation and metabolic interactions with other periodontal pathogens is well-known (Hajishengallis and Lamont, 2016; Sakanaka et al., 2015). On the other hand, *T. denticola*, a member of the red complex, is known for its metabolic symbiosis, co-aggregation and synergy with the keystone pathogen *P. gingivalis* (Ito et al., 2010; Meuric et al., 2013; Ng et al., 2019). Hence, the reduction of *S. gordonii* and *T. denticola* in the oral biofilm model is indeed a promising effect.

The species-specific effect of both the gels against *S. gordonii* and *T. denticola* in the biofilm has the potential of preventing oral dysbiosis, co-aggregation of periodontal pathogens and thus periodontal disease progression. Hence multiple exposures/treatments per day with either of the two gels is likely to show better efficacy in the reduction of the dental plaque.

FUNDING SOURCE

This work was supported by the Conseil Régional de Bretagne, France (F3/48 CPER), the Fondation "Les Gueules Cassées/Sourire Quand Même" and the Federative Research Structure Biosit (Rennes).

DECLARATION OF COMPETING INTEREST

The authors declare no competing interests.

ACKNOWLEDGEMENTS

We thank blue[®]m Europe B.V., Netherlands for sending us their product- blue[®]m oral gel, for this study and for product-related information. We also thank the manufacturer of Hyalugel[®]-ADO, Ricerfarma (Milan, Italy) and the distributer, Laboratoire COOPER, Melun, France, for providing the product-related information. Our sincere gratitude to the Microscopy Rennes Imaging Center (Biosit) for their technical assistance for the microscopy experiments.

REFERENCES

Ai, D., Huang, R., Wen, J., Li, C., Zhu, J., and Xia, L.C. (2017). Integrated metagenomic data analysis demonstrates that a loss of diversity in oral microbiota is associated with periodontitis. *BMC Genomics 18*, 1041. DOI: 10.1186/s12864-016-3254-5.

Ammann, T.W., Bostanci, N., Belibasakis, G.N., and Thurnheer, T. (2013). Validation of a quantitative real-time PCR assay and comparison with fluorescence microscopy and selective agar plate counting for species-specific quantification of an *in vitro* subgingival biofilm model. J *Periodontal Res.* 48, 517–526. DOI: 10.1111/jre.12034.

Ardizzoni, A., Neglia, R.G., Baschieri, M.C., Cermelli, C., Caratozzolo, M., Righi, E., Palmieri, B., and Blasi, E. (2011). Influence of hyaluronic acid on bacterial and fungal species, including clinically relevant opportunistic pathogens. *J Mater Sci Mater Med.* 22, 2329–2338. DOI: 10.1007/s10856-011-4408-2.

Arslan, S.Y., Leung, K.P., and Wu, C.D. (2009). The effect of lactoferrin on oral bacterial attachment. *Oral Microbiol Immunol.* 24, 411–416. DOI: 10.1111/j.1399-302X.2009.00537.x.

Beukers, N.G.F.M., Heijden, G.J.M.G. van der, Wijk, A.J. van, and Loos, B.G. (2017). Periodontitis is an independent risk indicator for atherosclerotic cardiovascular diseases among 60174 participants in a large dental school in the Netherlands. *J Epidemiol Community Health* 71, 37–42. DOI: 10.1136/jech-2015-206745.

Binshabaib, M., Aabed, K., Alotaibi, F., Alwaqid, M., Alfraidy, A., and Alharthi, S. (2020). Antimicrobial efficacy of 0.8% Hyaluronic Acid and 0.2% Chlorhexidine against *Porphyromonas gingivalis* strains: An *in vitro* study. *Pak J Med Sci 36*, 111–114. DOI: 10.12669/pjms.36.2.1456.

Bui, F.Q., Almeida-da-Silva, C.L.C., Huynh, B., Trinh, A., Liu, J., Woodward, J., Asadi, H., and Ojcius, D.M. (2019). Association between periodontal pathogens and systemic disease. *Biomed J*. *42*, 27–35. DOI: 10.1016/j.bj.2018.12.001.

Ceri, H., Olson, M.E., Stremick, C., Read, R.R., Morck, D., and Buret, A. (1999). The Calgary Biofilm Device: New Technology for Rapid Determination of Antibiotic Susceptibilities of Bacterial Biofilms. *J Clin Microbiol. 37*, 1771–1776. DOI: 10.1128/JCM.37.6.1771-1776.1999.

Chan, E.C.S., Siboo, R., Keng, T., Psarra, N., Hurley, R., Cheng, S.-L., and Iugovaz, I. (1993). *Treponema denticola* (ex Brumpt 1925) sp. nov., nom. rev., and Identification of New Spirochete Isolates from Periodontal Pockets. *Int J Syst Bacteriol.* 43, 196–203. DOI: 10.1099/00207713-43-2-196.

Chathoth, K., Martin, B., Cornelis, P., Yvenou, S., Bonnaure-Mallet, M., and Baysse, C. (2020). The events that may contribute to subgingival dysbiosis: a focus on the interplay between iron, sulfide and oxygen. *FEMS Microbiol Lett* 367(14). DOI: 10.1093/femsle/fnaa100.

Chen, C.C., Teng, L.J., and Chang, T.C. (2004). Identification of Clinically Relevant Viridans Group *Streptococci* by Sequence Analysis of the 16S-23S Ribosomal DNA Spacer Region. *J Clin Microbiol* 42, 2651–2657. DOI: 10.1128/JCM.42.6.2651-2657.2004.

Cunha, E.J., Auersvald, C.M., Deliberador, T.M., Gonzaga, C.C., Esteban Florez, F.L., Correr, G.M., and Storrer, C.L.M. (2019). Effects of Active Oxygen Toothpaste in Supragingival Biofilm Reduction: A Randomized Controlled Clinical Trial. *Int J Dent.* 2019:3938214. DOI: 10.1155/2019/3938214.

Deliberador, T.M., Weiss, S.G., Rychuv, F., Cordeiro, G., Cate, M.C.L.T., Leonardi, L., Brancher, J.A., and Scariot, R. (2020). Comparative Analysis *in Vitro* of the Application of blue[®]m Oral Gel versus Chlorhexidine on *Porphyromonas gingivalis*: A Pilot Study. *AiM* 10, 194–201. DOI: 10.4236/aim.2020.104015.

Eick, S., Renatus, A., Heinicke, M., Pfister, W., Stratul, S.-I., and Jentsch, H. (2013). Hyaluronic Acid as an Adjunct After Scaling and Root Planing: A Prospective Randomized Clinical Trial. J *Periodontol. 84*, 941–949. DOI: 10.1902/jop.2012.120269.

Esposito, E., Carotta, V., Scabbia, A., Trombelli, L., D'Antona, P., Menegatti, E., and Nastruzzi, C. (1996). Comparative analysis of tetracycline-containing dental gels: Poloxamer- and monoglyceride-based formulations. *Int. J. Pharm.* 142, 9–23. https://doi.org/10.1016/0378-5173(96)04649-2.

Figueiredo de Almeida Gomes, B.P., Vianna, M.E., Sena, N.T., Zaia, A.A., Ferraz, C.C.R., and de Souza Filho, F.J. (2006). *In vitro* evaluation of the antimicrobial activity of calcium hydroxide combined with chlorhexidine gel used as intracanal medicament. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. *102*, 544–550. DOI: 10.1016/j.tripleo.2006.04.010.

Graça, M.A., Watts, T.L.P., Wilson, R.F., and Palmer, R.M. (1997). A randomized controlled trial of a 2% minocycline gel as an adjunct to non-surgical periodontal treatment, using a design with multiple matching criteria. *J Clin Periodontol.* 24, 249–253. DOI: 10.1111/j.1600-051x.1997.tb01838.x.

Graves, D.T., Corrêa, J.D., and Silva, T.A. (2019). The Oral Microbiota Is Modified by Systemic Diseases. *J Dent Res 98*, 148–156. DOI: 10.1177/0022034518805739.

Hajishengallis, G., and Lamont, R.J. (2016). Dancing with the Stars: how Choreographed Bacterial Interactions Dictate Nososymbiocity and Give Rise to Keystone Pathogens, Accessory Pathogens, and Pathobionts. *Trends Microbiol 24*, 477–489. DOI: 10.1016/j.tim.2016.02.010.

Harrison, J.J., Stremick, C.A., Turner, R.J., Allan, N.D., Olson, M.E., and Ceri, H. (2010). Microtiter susceptibility testing of microbes growing on peg lids: a miniaturized biofilm model for high-throughput screening. *Nat Protoc. 5*, 1236–1254. DOI: 10.1038/nprot.2010.71.

H R, R., Dhamecha, D., Jagwani, S., Patil, D., Hegde, S., Potdar, R., Metgud, R., Jalalpure, S., Roy, S., Jadhav, K., Tiwari, N.K., Koduru, S., Hugar, S., Dodamani, S. (2017). Formulation of thermoreversible gel of cranberry juice concentrate: Evaluation, biocompatibility studies and its antimicrobial activity against periodontal pathogens. *Mater Sci Eng C Mater Biol Appl.* 75, 1506–1514. DOI: 10.1016/j.msec.2017.03.054.

Hujoel, P.P., Drangsholt, M., Spiekerman, C., and Weiss, N.S. (2003). An Exploration of the Periodontitis–Cancer Association. *Annals of Epidemiology 13*, 312–316. DOI: 10.1016/s1047-2797(02)00425-8.

Hunter, M.C., Pozhitkov, A.E., and Noble, P.A. (2016). Microbial signatures of oral dysbiosis, periodontitis and edentulism revealed by Gene Meter methodology. *J Microbiol Methods*. *131*, 85–101. DOI: 10.1016/j.mimet.2016.09.019.

İkinci, G., Şenel, S., Akıncıbay, H., Kaş, S., Erciş, S., Wilson, C.G., and Hıncal, A.A. (2002). Effect of chitosan on a periodontal pathogen *Porphyromonas gingivalis*. *Int J Pharm. 235*, 121–127. DOI: 10.1016/s0378-5173(01)00974-7

Ito, R., Ishihara, K., Shoji, M., Nakayama, K., and Okuda, K. (2010). Hemagglutinin/Adhesin domains of *Porphyromonas gingivalis* play key roles in coaggregation with *Treponema denticola*. *FEMS Immunol Med Microbiol 60*, 251–260. DOI: 10.1111/j.1574-695X.2010.00737.x.

Jones CG. (1997) Chlorhexidine: is it still the gold standard? *Periodontol 2000*. Oct;15:55-62. doi: 10.1111/j.1600-0757.1997.tb00105.x. PMID: 9643233.

Kigure, T., Saito, A., Seida, K., Yamada, S., Ishihara, K., and Okuda, K. (1995). Distribution of *Porphyromonas gingivalis* and *Treponema denticola* in human subgingival plaque at different periodontal pocket depths examined by immunohistochemical methods. *J Periodontal Res. 30*, 332–341. DOI: 10.1111/j.1600-0765.1995.tb01284.x.

Kinane DF, Stathopoulou PG, Papapanou PN. (2017). Periodontal diseases. *Nat. Rev. Dis.* Jun 22;3:17038. https://doi.org/10.1038/nrdp.2017.38

Kolenbrander, P.E., Andersen, R.N., Blehert, D.S., Egland, P.G., Foster, J.S., and Palmer, R.J. (2002). Communication among Oral Bacteria. *Microbiol Mol Biol Rev.* 66, 486–505. DOI: 10.1128/mmbr.66.3.486-505.2002.

Koziel, J., Mydel, P., and Potempa, J. (2014). The Link Between Periodontal Disease and Rheumatoid Arthritis: An Updated Review. *Curr Rheumatol Rep 16*. DOI: 10.1007/s11926-014-0408-9.

Kreth J, Zhang Y, Herzberg MC. (2008). Streptococcal antagonism in oral biofilms: *Streptococcus sanguinis* and *Streptococcus gordonii* interference with Streptococcus mutans. *J Bacteriol*. *190*:4632–40. DOI: 10.1128/JB.00276-08.

82

Kumawat, R.M., Ganvir, S.M., Hazarey, V.K., Qureshi, A., and Purohit, H.J. (2016). Detection of *Porphyromonas gingivalis* and *Treponema denticola* in chronic and aggressive periodontitis patients: A comparative polymerase chain reaction study. *Contemp Clin Dent* 7, 481–486. DOI: 10.4103/0976-237X.194097.

Lamont, R.J., and Hajishengallis, G. (2015). Polymicrobial synergy and dysbiosis in inflammatory disease. *Trends Mol Med.* 21, 172–183. DOI: 10.1016/j.molmed.2014.11.004.

Lee, C.-H., Kho, H.-S., Chung, S.-C., Lee, S.-W., and Kim, Y.-K. (2003). The relationship between volatile sulfur compounds and major halitosis-inducing factors. *J. Periodontol.* 74, 32–37. DOI: 10.1902/jop.2003.74.1.32.

Leschine, S.B., and Canale-Parola, E. (1980). Rifampin as a Selective Agent for Isolation of Oral Spirochetes. *J Clin Microbiol.*, 12(6):792-5. DOI: 10.1128/JCM.12.6.792-795.1980.

López, R. (2008). Periodontal disease and adverse pregnancy outcomes. *Evid Based Dent.* 9, 48. DOI: 10.1038/sj.ebd.6400581.

Martin, B., Tamanai-Shacoori, Z., Bronsard, J., Ginguené, F., Meuric, V., Mahé, F., and Bonnaure-Mallet, M. (2017). A new mathematical model of bacterial interactions in two-species oral biofilms. *PLoS ONE 12*, e0173153. DOI: 10.1371/journal.pone.0173153.

Martin, B., Chathoth, K., Ouali, S., Meuric, V., Bonnaure-Mallet, M., and Baysse, C. (2018). New growth media for oral bacteria. *J Microbiol Methods*. *153*, 10–13. DOI: 10.1016/j.mimet.2018.08.013.

Martineau, L., and Dosch, H.-M. (2007). Biofilm reduction by a new burn gel that targets nociception. *J Appl Microbiol.* 103, 297–304. DOI: 10.1111/j.1365-2672.2006.03249.x.

Meuric, V., Martin, B., Guyodo, H., Rouillon, A., Tamanai-Shacoori, Z., Barloy-Hubler, F., and Bonnaure-Mallet, M. (2013). *Treponema denticola* improves adhesive capacities of *Porphyromonas gingivalis*. *Mol Oral Microbiol 28*, 40–53. DOI: 10.1111/omi.12004.

Meuric, V., Le Gall-David, S., Boyer, E., Acuña-Amador, L., Martin, B., Fong, S.B., Barloy-Hubler, F., and Bonnaure-Mallet, M. (2017). Signature of Microbial Dysbiosis in Periodontitis. *Appl. Environ. Microbiol.* 83. DOI: 10.1128/AEM.00462-17. Neuman, M.G., Nanau, R.M., Oruña-Sanchez, L., and Coto, G. (2015). Hyaluronic Acid and Wound Healing. *J Pharm Pharm Sci.* 18, 53–60. DOI: 10.18433/j3k89d.

Ng, H.M., Slakeski, N., Butler, C.A., Veith, P.D., Chen, Y.-Y., Liu, S.W., Hoffmann, B., Dashper, S.G., and Reynolds, E.C. (2019). The Role of *Treponema denticola* Motility in Synergistic Biofilm Formation with *Porphyromonas gingivalis*. *Front. Cell. Infect. Microbiol.* 9. DOI: 10.3389/fcimb.2019.00432.

Noyan, Ü., Yilmaz, S., Kuru, B., Kadir, T., Acar, O., and Büget, E. (1997). A clinical and microbiological evaluation of systemic and local metronidazole delivery in adult periodontitis patients. *J Clin Periodontol.* 24, 158–165. DOI: 10.1111/j.1600-051x.1997.tb00485.x.

Paquette, D.W., Waters, G.S., Stefanidou, V.L., Lawrence, H.P., Friden, P.M., O'Connor, S.M., Sperati, J.D., Oppenheim, F.G., Hutchens, L.H., and Williams, R.C. (1997). Inhibition of experimental gingivitis in beagle dogs with topical salivary histatins. *J Clin Periodontol.* 24, 216–222. DOI: 10.1111/j.1600-051x.1997.tb01834.x.

Petersen, P.E., and Ogawa, H. (2012). The global burden of periodontal disease: towards integration with chronic disease prevention and control: Global periodontal health. *Periodontol* 2000. *60*, 15–39. DOI: 10.1111/j.1600-0757.2011.00425.x.

Pihlstrom, B.L., Michalowicz, B.S., and Johnson, N.W. (2005). Periodontal diseases. *Lancet 366*, 1809–1820. DOI: 10.1016/S0140-6736(05)67728-8.

Pirnazar, P., Wolinsky, L., Nachnani, S., Haake, S., Pilloni, A., and Bernard, G.W. (1999). Bacteriostatic Effects of Hyaluronic Acid. *J Periodontol.* 70, 370–374. DOI: 10.1902/jop.1999.70.4.370.

Pöllänen, M.T., Paino, A., and Ihalin, R. (2013). Environmental Stimuli Shape Biofilm Formation and the Virulence of Periodontal Pathogens. *Int J Mol Sci 14*, 17221–17237. DOI: 10.3390/ijms140817221.

Preshaw, P.M., Alba, A.L., Herrera, D., Jepsen, S., Konstantinidis, A., Makrilakis, K., and Taylor, R. (2012). Periodontitis and diabetes: a two-way relationship. *Diabetologia* 55, 21–31. DOI: 10.1007/s00125-011-2342-y.

Rath SK, Singh M. (2013). Comparative clinical and microbiological efficacy of mouthwashes containing 0.2% and 0.12% chlorhexidine. *Dent Res J (Isfahan)*.10(3):364–9.

Sakanaka, A., Kuboniwa, M., Takeuchi, H., Hashino, E., and Amano, A. (2015). Arginine-Ornithine Antiporter ArcD Controls Arginine Metabolism and Interspecies Biofilm Development of *Streptococcus gordonii*. *J. Biol. Chem.* 290, 21185–21198. DOI: 10.1074/jbc.M115.644401.

Santos, R., Gomes, D., Macedo, H., Barros, D., Tibério, C., Veiga, A.S., Tavares, L., Castanho, M., and Oliveira, M. (2016). Guar gum as a new antimicrobial peptide delivery system against diabetic foot ulcers *Staphylococcus aureus* isolates. *J Med Microbiol.* 65, 1092–1099. DOI: 10.1099/jmm.0.000329.

Sauvêtre, E., Glupczynsky, Y., Labbé, M., Yourassowsky, E., Sauvêtre, E., and Pourtois, M. (1993). The effect of clindamycin gel insert in periodontal pockets, as observed on smears and cultures. *Infection 21*, 245–247. DOI: 10.1007/BF01728900.

Sharma, V., Gupta, R., Dahiya, P., Kumar, M., (2016). Comparative evaluation of coenzyme Q10based gel and 0.8% hyaluronic acid gel in treatment of chronic periodontitis. *J Indian Soc Periodontol.* 20, 374–380. https://doi.org/10.4103/0972-124X.183097

Tan, K.H., Seers, C.A., Dashper, S.G., Mitchell, H.L., Pyke, J.S., Meuric, V., Slakeski, N., Cleal, S.M., Chambers, J.L., McConville, M.J., Reynolds, E.C. (2014). *Porphyromonas gingivalis* and *Treponema denticola* Exhibit Metabolic Symbioses. *PLoS Pathog.* 10, e1003955. DOI: 10.1371/journal.ppat.1003955.

Wang, B., Timilsena, Y.P., Blanch, E., and Adhikari, B. (2019). Lactoferrin: Structure, function, denaturation and digestion. *Crit Rev Food Sci Nutr.* 59, 580–596. DOI: 10.1080/10408398.2017.1381583.

Wardle H. M. (1997). The challenge of growing oral spirochaetes. J. Med. Microbiol. 46, 104– DOI: 116. 10.1099/00222615-46-2-104

Watanabe, T., Maruyama, F., Nozawa, T., Aoki, A., Okano, S., Shibata, Y., Oshima, K., Kurokawa, K., Hattori, M., Nakagawa, I., and Abiko, Y. (2011). Complete Genome Sequence of the Bacterium *Porphyromonas gingivalis* TDC60, Which Causes Periodontal Disease. *J Bacteriol 193*, 4259–4260. DOI: 10.1128/JB.05269-11.

Xu, Y., Höfling, K., Fimmers, R., Frentzen, M., Jervøe-Storm, P.M., (2004). Clinical and Microbiological Effects of Topical Subgingival Application of Hyaluronic Acid Gel Adjunctive to Scaling and Root Planing in the Treatment of Chronic Periodontitis. *J. Periodontol.* 75, 1114–1118. https://doi.org/10.1902/jop.2004.75.8.1114

Yaegaki, K. (2008). Oral malodorous compounds are periodontally pathogenic and carcinogenic. *Japanese Dental Science Review* 44, 100–108. https://doi.org/10.1016/j.jdsr.2008.06.003.

Zhu, Y., Dashper, S.G., Chen, Y.-Y., Crawford, S., Slakeski, N., and Reynolds, E.C. (2013). *Porphyromonas gingivalis* and *Treponema denticola* Synergistic Polymicrobial Biofilm Development. *PLoS ONE 8*, e71727. DOI: 10.1371/journal.pone.0071727.



Figure 1: The process of testing antimicrobial gels on oral biofilms established on the surface of the pegs of the MBECTM apparatus (see methods).



Figure 2: Representative microscopic images of the three-species oral biofilm on the surface of the pegs. Post 1-hour treatment (with MMBC-3, Hyalugel[®]-ADO or blue[®]m oral gel) of the 6-hour biofilms on the surface of the pegs, the pegs were incubated in MMBC-3 for 24 hours. The pegs were further broken from the lid using pliers and stained using Styo[®]9. The stained pegs were visualized using the Leica TCS-SP5 confocal laser scanning microscope. To compare between the three treatments, maximum z-production of the Z stack were taken using 10X oil immersion objective lens and numerical zoom of 2: (A), (B), (C). Magnified images were captured using the 63X oil immersion objective lens, numerical zoom of 5.05: (D), (E), (F).



Figure 3: qPCR quantification of the number of bacteria (CFU ml⁻¹) in the biofilm (collected from the pegs) and in planktonic form (collected from the 96-well microtiter plate). The 6-hour three-species biofilm on the pegs were subjected to MMBC-3 or Hyalugel[®]-ADO or blue[®]m oral gel for 1 hour and further incubated in MMBC-3 for 24 hours. The planktonic cells were collected from the 96-well plate while the biofilm was collected from the pegs and quantified. (A) Total concentration (CFU ml⁻¹) of bacteria (planktonic/detached + biofilm), bacteria in biofilm and bacteria detached or in planktonic form after each treatment. (B) Concentration of each species in the biofilm after each treatment. (C) Concentration of each species in planktonic/ detached cells for each treatment. White bars = MMBC-3, grey bars = Hyalugel[®]-ADO and black bars = blue[®]m oral gel. p-value < 0.05 = *, p-value < 0.01 = **, p-value < 0.001 = ***

TABLES

Table 1: Species-specific primer sequences used in this study

Organism	Sequence (5'→3')
Streptococcus gordonii	F: AAG-CAA-CGC-GAA-GAA-CCT-TA
	R: GTC-TCG-CTA-GAG-TGC-CCA-AC
Porphyromonas gingivalis	F: TGG-GTT-TAA-AGG-GTG-CGT-AG
	R: CAA-TCG-GAG-TTC-CTC-GTG-AT
Treponema denticola	F: CGC-GTC-CCA-TTA-GCT-AGT-TG
	R: TTC-TTC-ATT-CAC-ACG-GCG-TC
Table 2: In vitro inhibition of Hyalugel®-ADO and blue®m oral gel against S. gordonii andP. gingivalis using an agar diffusion assay

Bacterial species	Inhibition diameter* (Mean <u>+</u> Standard Deviation, mm)					
	Hyalugel [®] -ADO	blue [®] m oral gel	P-value			
S. gordonii $(n = 9)$	13.0 <u>+</u> 1.6	24.9 <u>+</u> 1.2	1.8 x 10 ⁻¹¹			
<i>P. gingivalis</i> $(n = 9)$	22.4 <u>+</u> 2.7	41.1 <u>+</u> 1.7	1.2 x 10 ⁻⁰⁹			

* inhibition diameter was measured after a 48-hours treatment with each gel in anaerobic conditions on MMBC-3 agar plates inoculated with 1.5x 10⁸ CFU ml⁻¹ *S. gordonii* or *P. gingivalis.*

3.4. Discussion and perspectives

The model developed in MBEC microtiter plates is promising both for testing the efficacy of new antimicrobials, but also for rapidly screening working hypotheses on factors inducing dysbiosis. Indeed, the growth conditions and physico-chemical parameters of the medium can be modified (addition of iron, oxygen donor etc) and their effect on the maintenance in the biofilms of the three oral bacteria quickly evaluated with a standardized protocol. It will also make it possible to measure the expression of target genes and the effect of mutations. Indeed, as presented in the introduction (Chathoth *et al.*, 2020), genes involved in hydrogen peroxide and glutathione production of *S. gordonii*, as well as the genes enabling H₂S biosynthesis of *T. denticola* and *P. gingivalis* could have a key role in the establishment of dysbiosis.

However, the protocol needs to be improved in order to better achieve its goals. Our method is designed to determine the effect of the treatments on the ability of the bacteria to grow back as either biofilm or planktonic cells. Therefore, the results depend on the bacteria that are alive after 1 hour of treatment. The limitations we encountered to access directly the ratio between live and dead cells are mainly due to the anaerobic nature of two of the targeted bacterial species and the difficulty of culturing them. The reason for not using a Live/Dead stain is because these anaerobic bacteria (*P. gingivalis* and *T. denticola*) will die when exposed to atmospheric oxygen during the process of image acquisition. Therefore, one will not be able to differentiate the effect of the gels from that of atmospheric oxygen. The same concern applies in the use of viability-PCR (using Propidium Monoazide, PMA) since it is difficult to do all the steps in strictly anaerobic conditions. Nor could we determine cell numbers by growing bacteria on agar plate because it is difficult to have viable cells after sonication (to remove the biofilm from pegs) that can itself kill bacteria. Moreover, as sonication is done in aerobic condition, anaerobic bacteria can die.

An improved 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay for evaluating the viability of bacterial cells was published (Wang *et al.* 2010). However, this method is well adapted for aerobic bacteria. The conversion of MTT into formazan crystals is catalyzed by several cellular dehydrogenases, most of which are located in the respiratory chain. Cellular MTT reduction activities are expected to change with the levels of dissolved oxygen.

In theory, the presence of labile nucleic acid (mRNA) in bacterial cells might be an indicator of viability but studies have shown that mRNA was present in cells killed by heat or ethanol. Indeed mRNA was detected in dead *E. coli* cells by RT-PCR and therefore it cannot be an absolute indicator of viability (Sheridan *et al.* 1998). The length of time that mRNA persists seems to depend on both the method by which cells were killed and the samples holding conditions. Moreover, quantification of 16S mRNA is uneasy due to the lack of an adequate housekeeping reference mRNA. Also, the relationship between rRNA and cell state can vary due to life strategy such as viable but dormant cells. Finally, Processing RNA is rather complicated.

To minimize potential degradation bias, an alternative approach, the molecular viability test (MVT), is being developed. MVT targets precursor rRNA (pre-rRNA) which is a more stable molecule and can account for more than 20% of the total RNA in growing cells. Pre-rRNAs are intermediates in rRNA synthesis whose leader and tail fragments are enzymatically removed to yield mature rRNA. When growth slows, pre-rRNA synthesis stops but maturation continues, resulting in active and substantial drainage of pre-rRNA pools. The nucleotide sequences of pre-rRNA leaders and tails are hypervariable and highly species specific. RT-qPCR tests can be designed to detect pre-rRNA synthesis in specific targeted species (Cangelosi *et al.* 2010; Cangelosi and Meschke 2014). The next step to improve our model will be to implement this MVT on the 3-species biofilms treated with antimicrobials.

4. EFFECT OF IRON ON THE DEVELOPMENT OF ORAL BIOFILMS

4.1. Introduction

The oral bacteria form a complex community that can be influenced by host-related factors such as systemic iron levels which may trigger a pathogenic switch. Several studies have associated iron overload with severe periodontitis (Meuric *et al.* 2017; Boyer *et al.* 2018, 2020). However, the direct role of iron in the progression of periodontitis is not known. We aim to study the effect of iron on the process of biofilm initiation or attachment and next on biofilm growth and development of the three bacterial species (*S. gordonii, P. gingivalis, T. denticola*). This study, for the first time, showed the interactions between *in vitro S. gordonii -T. denticola* biofilms during both attachment (**Chapter 4.2.**, manuscript in preparation) and growth phase (**Chapter 4.3.**, manuscript in preparation). With the help of experimental biological experiments on biofilms grown at varying iron levels, we aim to develop a mathematical model as a tool to evaluate the interspecies associations and its effect on biofilm development and growth.

Mathematical models of biofilm dynamics are valuable tools in exploring and understanding the development of biofilms. Often there are myriad mechanisms that are challenging to distinguish experimentally and in such scenarios theoretical investigations are particularly appealing. Martin et al., (2017) has previously developed a mathematical model of bacterial interactions in two-species oral biofilm consisting of S. gordonii and P. gingivalis in a rich growth media. The close association and synergy between P. gingivalis and T. denticola is known in cases of severe periodontitis. To gain further information regarding the effect of iron, we are developing a mathematical model for these three-species oral biofilm (Chapter 4.4.). Unlike the two-species model (Martin et al., 2017), the three-species model will be performed in an ironcontrolled growth media (Martin *et al.*, 2018) at varying iron concentrations. We propose the following hypothesis of interaction: independence, competition between the three species and induction of toxicity by one species on the other. This mathematical model is based on the requirement for different substrates for growth (glucose and proteins) and also different iron concentrations. The mathematical model of bacterial interactions in two-species oral biofilms developed by Martin et al, (2017) will be used as the basis for the development of the model for the three-species oral biofilms.

4.2. Effect of iron and interspecies associations on biofilm initiation by three oral bacteria

Kanchana Chathoth¹, Martine Bonnaure-Mallet^{1,2}, Bénédicte Martin³ and Christine Baysse^{1*g}

*Corresponding author: christine.baysse@univ-rennes1.fr

^d These authors share senior authorship

¹ University of Rennes 1, NUMECAN INSERM U1241, CIMIAD F-35043 Rennes, France

² CHU Pontchaillou, Rennes*

³ SLS France, F-35136 St Jacques de la Lande, France

Keywords: oral biofilm, *Porphyromonas gingivalis, Treponema denticola, Streptococcus gordonii*, Iron.

Running headline: Effect of iron on oral biofilm initiation

ABSTRACT

Bacterial attachment and early biofilm initiation are the first and crucial steps in biofilm development and periodontal disease progression. The balance between attached commensals and pathogens in subgingival sites may be influenced by interspecies associations and oral environmental factors. Since the systemic iron overload disease hemochromatosis has been previously associated with chronic periodontitis, we investigated the effect of iron levels and interspecies associations between an oral commensal *Streptococcus gordonii*, and two periodontal pathogens *Porphyromonas gingivalis* and *Treponema denticola* in biofilm initiation. The ability of these species to form mono-, dual- and three-species 2-hour biofilms at different iron levels was evaluated using quantitative PCR and confocal microscopy. The most noteworthy results observed were the antagonism between the two species in *S. gordonii-T. denticola* biofilms during iron deficiency and co-operation at higher iron concentrations compared to mono-species biofilms. The three-species model favoured the attachment of both pathogens compared to mono-species biofilms and high iron concentrations especiely favoured the attachment of *P. gingivalis* to the nascent biofilm. The thickness of the three-species biofilm also increased with iron concentration. These

results showed that iron levels and interspecies interactions influenced biofilm initiation and therefore may play a role in dysbiosis and progression of periodontal disease.

INTRODUCTION

Bacteria in most natural environments like the subgingival crevice exist in the form of complex polymicrobial communities called biofilms. In the oral environment, dental plaques are the main etiological factor of most oral diseases including periodontitis, a chronic inflammation of gum that causes the destruction of the tooth supporting structures (Becker *et al.* 2002; Socransky, Smith and Haffajee 2002; Kumar et al. 2005; Brito et al. 2007; Göhler et al. 2014). Owing to the complex and dynamic nature of the biofilms, bacteria in the biofilm express phenotypes diverse from their planktonic counterparts like increased antibiotic resistance (Sedlacek and Walker 2007), acid (Welin-Neilands and Svensäter 2007) or oxygen tolerance (Gursoy et al. 2010). The process of the subgingival biofilm formation is initiated by planktonic bacterial cells, consisting of early colonizers (Actinomyces species, streptococci) that attach to surfaces of the oral cavity via the salivary pellicle that contains ligand for bacterial adhesins. Further, secondary colonizers (pathogenic oral bacteria) attach to these pre-colonized bacterial cells via adhesins. The coaggregated bacteria form mature biofilms that engage in co-operative/competitive interactions, metabolic communication and genetic exchange (Sbordone and Bortolaia 2003; Kuboniwa et al. 2006; Orth et al. 2011; Lamont and Hajishengallis 2015; Ng et al. 2019). These can altogether drive the development of pathogenic subgingival plaque. Therefore, initial bacterial attachment and co-aggregation/co-adhesion which is the initial step in dental plaque formation, can impact the emergence and progression of the disease.

Streptococcus gordonii, a primary colonizer, is known to promote the co-aggregation and colonization of anaerobic oral pathogens via metabolic interactions (Stinson, Safulko and Levine 1991; Lamont *et al.* 1993, 1994, 2002; Amano *et al.* 1997; Rickard *et al.* 2003; Maeda *et al.* 2004a, 2004b; Kuboniwa *et al.* 2006). Slots and Gibbons (1978), have demonstrated the exclusive localization of *Porphyromonas gingivalis* in plaque areas rich in streptococcal species when *P. gingivalis* is introduced in the mouths of human volunteers. Co-aggregation of *Streptococcus oralis* and *Actinomyces naeslundii in vivo* (Palmer *et al.* 2003) and of *S. oralis* or *S. gordonii* and *Veillonella* sp. strain PK1910 *in vitro* (Chalmers *et al.* 2008) have been previously reported. Moreover, among anaerobic oral pathogens the synergistic (Ng *et al.* 2019) and symbiotic (Tan *et al.* 2019)

al. 2014) interactions between *P. gingivalis* and *Treponema denticola* are also well known. To better understand the role of biofilm initiation in periodontal disease, it would be pertinent to identify and comprehend the underlying mechanisms of multiple interspecies interactions that occur at the different stages in polymicrobial biofilm communities. This study focuses on early biofilm formation which initiates the dysbiosis. Environmental factors, like iron, can influence this process. Iron is a vital nutrient required for the growth of most microorganisms. Therefore, bacteria are susceptible to not only an iron deficiency but also to iron excess that promotes the production of harmful hydroxyl radical (ROS) in the presence of H₂O₂ producers such as *S. gordonii* (Zhu and Kreth 2012). We focus on iron because researchers have recently associated chronic periodontitis with hemochromatosis which is characterized by an overload of systemic iron (Meuric *et al.* 2017; Boyer *et al.* 2018). Subsequently, iron levels can influence the composition/ratio of commensal to pathogenic bacteria and thus the evolution of the disease. Therefore, in this article, we decided to study the effect of iron and inter-species association/organization on the initial attachment of oral bacterial species that could eventually lead to progression of the disease.

To test this hypothesis, we used a previously established three-species oral biofilm model (Martin et al. 2018) in a newly designed growth medium (MMBC-3), comprising of S. gordonii, P. gingivalis and T. denticola. S. gordonii can facilitate the attachment of late colonizers or periodontal pathogens (Maeda et al. 2004a; Lima, Shi and Lux 2017) and can have an effect on the overall development of the oral biofilm. P. gingivalis is the keystone pathogen often associated with severe forms of the periodontal disease (Orth et al. 2011). T. denticola, along with P. gingivalis is a member of the red complex, and both are found together in deep periodontal pockets in patients suffering from periodontitis (Byrne et al. 2009; Tan et al. 2014). The sequencing data of the subgingival microbiota of 75 patients with hemochromatosis and chronic periodontitis showed that mean pocket depth was positively associated with the presence of *Porphyromonas* as a signature for dysbiosis (Boyer et al. 2020). P. gingivalis and T. denticola are also well known for their nutritional cross-feeding, synergy and syntrophy (Grenier 1992; Meuric et al. 2013; Zhu et al. 2013; Tan et al. 2014; Ng et al. 2019). It has been earlier confirmed that 8 µM of iron (FeSO₄) along with 0.08 μ M of protoporphyrin IX (PPIX) (to allow heme synthesis via the ferrochelatase) was the optimal iron concentration for the growth of P. gingivalis cultures in the MMBC-3 medium (Martin *et al.* 2018), and that these concentrations allowed also the growth of *T. denticola* and *S.* *gordonii*. We therefore based our study on the iron requirement of the keystone pathogen *P*. *gingivalis* and selected the optimal iron level (8 μ M), along with sub-optimal (0.8 μ M) and excess iron levels (80 μ M), keeping PPIX at a constant concentration (0.08 μ M). These three concentrations were used to assess the effect of iron on the attachment of the three oral bacteria as mono-, dual- and three-species biofilms.

MATERIALS AND METHODS

Bacteria and media

The bacteria used in this study are *Streptococcus gordonii* Challis DL1 (Chen, Teng and Chang 2004), *Porphyromonas gingivalis* TDC60 (Watanabe *et al.* 2011) and *Treponema denticola* ATCC 35405 (Chan *et al.* 1993). The MMBC-3 medium with 8 μ M FeSO₄ and 0.08 μ M protoporphyrin IX (PPIX) (Martin *et al.* 2018) was used for growing *S. gordonii* and *P. gingivalis* while *T. denticola* was first cultured in the NOS spirochete medium (Leschine and Canale-Parola 1980) and further sub-cultured in the MMBC-3 with FeSO₄ and PPIX. The three micro-organisms were grown in anaerobic condition at 37°C in an anaerobic chamber (MACS 500, Don Whitley Scientific, United Kingdom) with 10% v:v H₂, 10% v:v CO₂ and 80% v:v N₂.

Bacterial growth conditions as biofilms (to assess initial attachment)

8-chambered polymer coverslip / μ -slide (ibiTreat, Ibidi) was coated with 200 µl of filtered (0.20 µm) and twice diluted (in sterile water) saliva (Pool Human Donors, MyBioSource) for 30 minutes. The saliva was replaced with 200 µl of the inocula consisting of *S. gordonii* (OD_{600nm}=0.05) and/or *P. gingivalis* (OD_{600nm}=0.1) and/or *T. denticola* (OD_{600nm}=0.1) alone and in different combinations to have mono-species, dual-species and three-species biofilms. These culture-containing μ -slides were incubated in anaerobic condition for 2 hours to enable the process of initial bacterial attachment and biofilm initiation in the wells of the slide. After 2 hours, the planktonic cells were removed and the μ -slides containing attached biofilm cells were washed with 200 µl of PBS. These 2-hour biofilms were used for imaging and for bacterial species quantification, as described below.

Confocal laser microscopy and imaging

The attached cells of the 2-hour biofilms were stained using the Syto[®]9 (5 μ M) green-fluorescent nucleic acid stain (Invitrogen, ThermoFisher Scientific) diluted in PBS and incubated

for 15 minutes. These stained cells were observed *in situ* with a Leica TCS-SP5 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany). Using an HC PL Apo 63X, 1.4 NA, oil immersion objective lens, images were captured. However, mono-species 2-hour biofilms of *T. denticola* alone formed scanty biofilms and a magnification of 63X focused either on only biofilm or only void spaces, giving biased images. Hence a magnification of 40 X was used using an HC PL Apo 40X, 1.25-0.75 NA, oil immersion lens to capture clear images of the spirochete. A numerical zoom of 1.5 was applied to all acquisitions. All Syto[®]9 stained bacteria were detected using the 488-nm UV diode and a 485 to 500-nm band-pass emission filter. Biofilm stacks (123 × 123 µm) acquired at 1 µm intervals were scanned with a line average of 2. Leica software (LAS AF V.2.2.1) was used for microscope piloting and image acquisition. Image analyses were performed using the Comstat2 plugin in the ImageJ software V1.43m (National Institute of Health, USA) to estimate characteristic parameters of biofilms: the bio-volume (expressed in µm³/µm²) and the average thickness (µm), roughness co-efficient (Ra) and surface-to-bio-volume ratio. All the parameters are as described previously (Heydorn *et al.* 2000).

After microscopic observation, the stain was removed from the μ -slides' wells and the attached cells/2-hour biofilms were collected in 100 μ l of PBS, centrifuged (8000xg, 10 minutes, 20°C) and the pellets were stored at -20°C for further characterization by qPCR.

qPCR quantification

The attached cells collected in PBS after 2 hours of incubation, comprising of *S. gordonii*, *P. gingivalis* and/or *T. denticola* previously centrifuged (8000xg, 20°C, 10 min) and stored as pellets at -20°C were resuspended in 100µl of PBS. Two hundred µl of the inocula used for each biofilm was also centrifuged and the stored pellets were resuspended in 100 µL of PBS for comparison. These were further heated for 20 min at 95°C. The concentrations of the DNA in these samples were determined by performing quantitative PCR against defined concentrations of DNA standards set in the range of 0.0001 to 10 ng with purified genomic DNA from each of the three species. A total reaction volume of 12.5 µl contained 6.25 µl SYBR 2X Green Master Mix (Eurogentec, Belgium), 1 µl each of forward and reverse primers (5 µM) and 1 µl of DNA template. DNA templates were amplified using the Applied Biosystems apparatus (StepOne Plus). The conditions for qPCR were: an initial holding stage of 95°C for 10 min followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C; a melt curve stage was performed consisting of 15 sec at 95°C

followed by a temperature gradient from 60°C to 95° C in 1°C increment step measuring fluorescence at each temperature for 15 sec. Primers used were specific to each species targeting the 16S ribosomal RNA taking into account specific genome weights (Ammann *et al.* 2013; Martin *et al.* 2017). The primers used in this study are listed in Table 1.

Statistical analysis

All the experiments were done with a minimum of two biological and two technical replicates ($n \ge 4$). Statistical analysis was performed using the two-tailed unpaired student t-test and a p-value of less than 0.05 was considered statistically significant.

RESULTS

Effect of iron and interspecies association on attachment of each species in mono- and multi-species 2-hour biofilms

The effect of iron on the biofilm initiation ability of individual species in the form of mono, dual- and three-species biofilms were analysed and compared. In pure cultures, 8 μ M of iron favoured *S. gordonii* attachment as compared to 0.8 μ M of iron and to 80 μ M of iron, while no difference was observed for *P. gingivalis* and *T. denticola* (Fig. 1). Attached *S. gordonii* levels were comparable in mono-species and dual-species biofilms with *P. gingivalis*. In contrast, attachment of *S. gordonii* cells was promoted by the presence of *T. denticola* and 8 or 80 μ M of iron and reduced at 0.8 μ M, as compared with mono-species conditions (Fig. 1A). Among all biofilms, the concentration of *S. gordonii* were the lowest in the case of three-species biofilms irrespective of iron level. This may imply a detrimental effect of *P. gingivalis* and *T. denticola* when together, on *S. gordonii* development in the early biofilm.

In the case of *P. gingivalis*, very few cells were attached in mono-species conditions (Fig. 1B). The presence of *S. gordonii* and /or *T. denticola* significantly increased the levels of *P. gingivalis* in the 2-hour biofilm, even if at a lower rate with *T. denticola*, independently of the iron concentration used (Fig. 1B and Table 1S). Finally, *P. gingivalis* attachment in three-species biofilm was lower in comparison to that of both dual-species biofilms of either *S. gordonii-P. gingivalis* or *P. gingivalis-T. denticola*. It seems that the positive individual effect of both *S. gordonii* and *T. denticola* on *P. gingivalis* attachment was reduced when they were together in the

inoculum. Iron displayed no effect on *P. gingivalis* attachment, except in the three-species biofilm, with a positive effect at higher iron levels (8 and 80 μ M) (Fig. 1B).

In the case of *T. denticola* (Fig. 1C and Table 1S), highest concentrations were observed in presence of *P. gingivalis* which suggested a beneficial effect of *P. gingivalis* on *T. denticola* at all iron levels. The least concentration of *T. denticola* was observed in either mono-species biofilms or in dual-species biofilms with *S. gordonii* with no significant difference between them. In the three-species biofilm, *S. gordonii* and *P. gingivalis* together favoured the attachment of *T. denticola* when compared to mono-species. Interestingly, the proportion of *T. denticola* cells (approximately 65%) was the highest in comparison to the other two species (*S. gordonii* - 27%, *P. gingivalis* - 8% approximately) in the three-species biofilm at all iron levels. However, the attachment of *T. denticola* in the three-species biofilm was lower than that of dual-species biofilm *P. gingivalis* on *T. denticola* attachment was reduced when *S. gordinii* was also present. Iron affected the levels of *T. denticola* only in the *S. gordonii*-*T. denticola* biofilm, favouring increased levels at 8 and 80 μ M as compared with 0.8 μ M iron. *S. gordonii* at low iron level displayed a detrimental effect on *T. denticola* in mono-species biofilm.

Effect of iron levels and inter-species associations on initial biofilm structure

Among the mono-species biofilms, *P. gingivalis* showed the lowest values for bio-volume and thicknesses (average and maximum thickness) as evaluated by confocal microscopy (Fig. 2) consistent with its negligible initial attachment as measured by qPCR (Fig. 1B, Fig. S1). This negligible initial attachment by *P. gingivalis* is also evident in the microscopic images (Fig. 3). Moreover, *P. gingivalis* mono-species biofilms were the most heterogenous among 2-hour monospecies biofilms (demonstrated by an elevated roughness co-efficient) with high surface-to- biovolume ratio (Fig. 2B, 2C). Mono-species *T. denticola* biofilm on the other hand showed a biovolume higher than that of mono-species *S. gordonii* biofilm in contrast to the lower concentration of sessile cells measured by qPCR (Fig. 2A, Fig. S1). *T. denticola* (5-20 μ m) is much longer than *S. gordonii* (~ 0.5 μ m) and thus accounts for a higher bio-volume. This higher bio-volume of *T. denticola* biofilms distributed along the surface probably also contributed to a roughness coefficient lower than *S. gordonii* biofilms (Fig. 2B). For the dual-species *S. gordonii-P. gingivalis* biofilms, the values for bio-volume, roughness coefficient and surface to bio-volume ratio showed no change due to iron concentration and were similar to that of mono-species *S. gordonii* (Fig. 2), perhaps due to the higher proportion of *S. gordonii* in the *S. gordonii-P. gingivalis* biofilms (Fig. 1A, 1B). The microscopy results were in concurrence with the results of cell concentrations measured by qPCR which also remained unaffected by iron levels. However, the thickness (average and maximum) significantly increased at high iron concentrations (Fig. 2D, 2E) suggesting an effect on the biofilm architecture.

In the case of dual-species *S. gordonii-T. denticola* biofilms, the bio-volume (Fig. 2A) increased at 8 and 80 μ M of iron compared to 0.8 μ M, in accordance with qPCR data (Fig. S1 and Fig. 1A, 1C). Moreover, a significantly lower roughness co-efficient and surface to bio-volume ratio were observed for dual-species *S. gordonii-T. denticola* sessile cells (Fig. 2B, 2C) which can be attributed to the more homogenous distribution of these biofilms across the surface (Fig. 3). The average thickness of dual-species *S. gordonii-T. denticola* was slightly increased at 0.8 and 80 μ M of iron while its maximum thickness remained unaffected by iron concentration (Fig. 2D, 2E).

For the dual-species *P. gingivalis-T. denticola* biofilms, the bio-volume was significantly higher at 8 and 80 μ M of iron when compared to 0.8 μ M (Fig. 2A). However, the bio-volume of the dual-species was lower than that of *T. denticola* mono-species biofilms which is in contrast to the results obtained by qPCR analyses (Fig. 1B, 1C and Fig. S1). This may be attributed to the arrangement of the bacteria in the form of tightly packed clusters as observed in microscopy images (Fig. 3), thus accounting for a lower bio-volume. The lower thickness (average and maximum) of the *P. gingivalis-T. denticola* biofilms in comparison to that of *T. denticola* mono-species biofilms may also be a result of a clustered architecture.

In the case of three-species biofilms, the average and maximum thickness was higher at 8 and 80 μ M of iron than at 0.8 μ M (Fig. 2D, 2E). The average thickness of the three-species biofilm was higher than all other biofilms at all iron levels except at 0.8 μ M iron, where the average thickness of dual-species *S. gordonii-T. denticola* was higher (p<0.05). The roughness co-efficient of the three-species biofilm was high irrespective of iron levels and increased with the concentration of iron (Fig. 2B). The high roughness coefficient at all iron levels may be due to the huge clustering of bacteria (Fig. 3) observed in the three-species biofilm.

DISCUSSION

The impact of iron on the etiology of periodontal diseases is now known (Meuric *et al.* 2017; Boyer *et al.* 2018) and assumptions concerning the behaviour of the oral microbiota in high iron concentrations were raised (Chathoth *et al.* 2020). The aim of this work was to test the model on the early stage of biofilm formation.

S. gordonii biofilm initiation was optimal at 8 μ M iron compared to 0.8 and 80 μ M while no difference in growth rate was observed in planktonic cultures (data not shown). This could be indicative of an acquired iron-preference by sessile *S. gordonii* cells. In the case of dual-species *S. gordonii-T. denticola*, the highest concentrations (8 and 80 μ M) of iron favoured attachment of both the species. Although excess of iron can promote oxidative stress in presence of the peroxygenic streptococcus, H₂S produced by *T. denticola* can serve as a mean to reduce the oxygen tension. Also, Ooi and Tan (2016) reported inhibition of growth and biofilm formation of some streptococcal species due to H₂S derived from *Fusobacterium nucleatum*. However, the authors showed that *S. gordonii* remained unaffected as the intracellular glutathione (GSH) of *S. gordonii* helps overcome H₂S toxicity. It is possible, amongst other hypotheses that in our study, the H₂S produced by *T. denticola* and cellular GSH of *S. gordonii* enable the survival and growth of these two species in the presence of each other at high iron levels (Chathoth *et al.* 2020). The inhibitory effect at the lowest iron concentration on the levels of both *S. gordonii* and *T. denticola* in dualspecies biofilm is of interest and deserve further investigation.

The attachment of *P. gingivalis* was significantly enhanced in *S. gordonii-P. gingivalis* biofilm irrespective of iron levels compared to the mono-species biofilm. This is consistent with previous data. The interspecies co-adhesion systems of these two species are among the best characterized. For instance, *P. gingivalis* long fimbriae (FimA) bind to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) of *S. gordonii* (Maeda *et al.* 2004a) and the *P. gingivalis* short fimbriae (Mfa) engage the streptococcal SspA/B adhesins (Park *et al.* 2005). Kuboniwa *et al.* (2017) also reported that *S. gordonii* 4-aminobenzoate/para-amino benzoic acid (pABA) increased colonization and survival of *P. gingivalis*. After co-adhesion, LuxS-dependent signalling is known to promote heterotypic biofilm development (McNab *et al.* 2003). The initial adaptation to a heterotypic biofilm entailed the regulation of *P. gingivalis* genes involved in metabolic pathways, transport systems and stress responses (Simionato *et al.* 2006). We demonstrated that iron affected

the thickness of the *S. gordonii-P. gingivalis* biofilms: thickness increased with the iron concentration but without affecting the cells numbers, suggesting an impact on biofilm architecture.

In the case of *P. gingivalis-T. denticola* biofilms, both species displayed synergistic biofilm initiation at all three iron levels. These results are consistent with previous observations: Ng *et al.* (2019) reported a 2-fold increase of the dual-species biofilms than the sum of the mono-species biofilms. In our study, 4-fold higher biomass of the dual-species was observed than the additive amounts as mono-species. It is known that the FimA (long fimbrae) and RgpB (Arg-gingipain B) of *P. gingivalis* along with the functional flagella and cytoplasmic filament of *T. denticola* are essential for biofilm formation (Kuramitsu, Chen and Ikegami 2005a; Yamada, Ikegami and Kuramitsu 2005). Likewise, *T. denticola* is known to improve the adhesion of *P. gingivalis* by promoting the expression of genes encoding gingipains and hemagglutinin A (Meuric *et al.* 2013). The two species are known to exhibit metabolic symbioses (Tan *et al.* 2014, Grenier 1992; Kin *et al.* 2020). Interestingly, iron levels showed an effect only on the bio-volume of the *P. gingivalis-T. denticola* biofilms with higher values at 8 and 80 μ M of iron. However, this effect of iron was not observed in the bacterial cell concentrations measured by qPCR. This could be indicative of a different cell length at different concentrations of iron. The length of *T. denticola* cells can range from 5 to 20 μ m (Zhou and Li 2015).

The interspecies co-operative mechanisms favoured *T. denticola* and *P. gingivalis* attachment with *T. denticola* predominance in the three-species biofilms, probably thanks to the same synergistic mechanisms we described for the two-species biofilms. Conversely, *P. gingivalis* and *T. denticola* diminished the attachment of *S. gordonii* in the three-species biofilms which suggests that they may produce an antagonistic compound or start a nutritional competition. Moreover, in comparison to 0.8μ M of iron, higher iron levels increased the concentration of *P. gingivalis* cells in the three-species biofilms. Since, *P. gingivalis* is a minority among the three-species, the bio-volume of the three-species biofilm is not significantly affected by iron levels. Also 8 and 80 μ M of iron increased the thickness of the three-species biofilms compared to 0.8μ M of iron indicating an impact of iron levels on the biofilm architecture. We have previously built a hypothesis (Chathoth *et al.* 2020) that high levels of iron may in turn enhance H₂S production by *P. gingivalis and T. denticola*, especially in cysteine-rich media such as the BSA containing medium used in this study, and therefore may contribute to the prevalence of these anaerobic

pathogens in mixed biofilms. Overall our work reveals the influence of iron and the interspecies association on the initiation of oral biofilm (Fig. 4).

CONCLUSION

In summary, our results revealed an iron-dependant antagonism or cooperation in biofilm initiation of *S. gordonii-T. denticola* biofilms. Additionally, a prevalence of the pathogens and an inhibition of the commensal strains is observed during dysbiotic biofilm initiation. This is particularly true at high iron levels for the keystone pathogen, *P. gingivalis*. Moreover, microscopic analyses revealed the effect of iron on the inter-species organization/architecture. This knowledge will broaden the understanding of the development of subgingival plaque and may help in the future development of anti-attachment strategies to reduce the risk of periodontal disease.

FUNDING SOURCE

This work was supported by the Conseil Régional de Bretagne, France (F3/48 CPER), the Fondation "Les Gueules Cassées/Sourire Quand Même" and the Federative Research Structure Biosit (Rennes).

DECLARATION OF COMPETING INTEREST

The authors declare no competing interests.

REFERENCES

Amano A, Fujiwara T, Nagata H *et al. Porphyromonas gingivalis* fimbriae mediate coaggregation with *Streptococcus oralis* through specific domains. *J Dent Res* 1997;76:852–7. DOI: 10.1177/00220345970760040601

Ammann TW, Bostanci N, Belibasakis GN *et al.* Validation of a quantitative real-time PCR assay and comparison with fluorescence microscopy and selective agar plate counting for species-specific quantification of an *in vitro* subgingival biofilm model. *Journal of Periodontal Research* 2013;48:517–26. DOI: 10.1111/jre.12034

Becker MR, Paster BJ, Leys EJ *et al.* Molecular analysis of bacterial species associated with childhood caries. *J Clin Microbiol* 2002;40:1001–9. DOI: 10.1128/jcm.40.3.1001-1009.2002

Boyer E, Le Gall-David S, Martin B *et al.* Increased transferrin saturation is associated with subgingival microbiota dysbiosis and severe periodontitis in genetic haemochromatosis. *Sci Rep* 2018;8:15532. DOI: 10.1038/s41598-018-33813-0

Boyer E, Martin B, Gall-David SL *et al.* Periodontal pathogens and clinical parameters in chronic periodontitis. *Molecular Oral Microbiology* 2020;35:19–28. DOI: 10.1111/omi.12274

Brito LCN, Teles FR, Teles RP *et al.* Use of multiple-displacement amplification and checkerboard DNA-DNA hybridization to examine the microbiota of endodontic infections. *J Clin Microbiol* 2007;45:3039–49. DOI: 10.1128/JCM.02618-06

Byrne SJ, Dashper SG, Darby IB *et al.* Progression of chronic periodontitis can be predicted by the levels of *Porphyromonas gingivalis* and *Treponema denticola* in subgingival plaque. *Oral Microbiol Immunol* 2009;24:469–77. DOI: 10.1111/j.1399-302X.2009.00544.x

Chalmers NI, Palmer RJ, Cisar JO *et al.* Characterization of a *Streptococcus* sp.-*Veillonella* sp. community micromanipulated from dental plaque. *J Bacteriol* 2008;190:8145–54. DOI: 10.1128/JB.00983-08

Chan ECS, Siboo R, Keng T *et al. Treponema denticola* (ex Brumpt 1925) sp. nov., nom. rev., and Identification of New Spirochete Isolates from Periodontal Pockets. *International Journal of Systematic Bacteriology* 1993;43:196–203. DOI: 10.1099/00207713-43-2-196

Chathoth K, Martin B, Cornelis P *et al.* The events that may contribute to subgingival dysbiosis: a focus on the interplay between iron, sulfide and oxygen. *FEMS Microbiol Lett* 2020;367, DOI: 10.1093/femsle/fnaa100. DOI: 10.1093/femsle/fnaa100

Chen CC, Teng LJ, Chang TC. Identification of Clinically Relevant Viridans Group Streptococci by Sequence Analysis of the 16S-23S Ribosomal DNA Spacer Region. *J Clin Microbiol* 2004;42:2651–7. DOI: 10.1128/JCM.42.6.2651-2657.2004

Göhler A, Hetzer A, Holtfreter B *et al.* Quantitative Molecular Detection of Putative Periodontal Pathogens in Clinically Healthy and Periodontally Diseased Subjects. *PLOS ONE* 2014;9:e99244. DOI: 10.1371/journal.pone.0099244

Grenier D. Nutritional interactions between two suspected periodontopathogens, *Treponema denticola* and *Porphyromonas gingivalis*. *Infect Immun* 1992;60:5298–301. DOI: 10.1128/IAI.60.12.5298-5301.1992

Gursoy UK, Pöllänen M, Könönen E *et al.* Biofilm formation enhances the oxygen tolerance and invasiveness of *Fusobacterium nucleatum* in an oral mucosa culture model. *J Periodontol* 2010;81:1084–91. DOI: 10.1902/jop.2010.090664

Heydorn A, Nielsen AT, Hentzer M *et al.* Quantification of biofilm structures by the novel computer program COMSTAT. *Microbiology (Reading, Engl)* 2000;146 (Pt 10):2395–407. DOI: 10.1099/00221287-146-10-2395

Kin LX, Butler CA, Slakeski N *et al.* Metabolic cooperativity between *Porphyromonas gingivalis* and *Treponema denticola. J Oral Microbiol* 2020;12:1808750. DOI: 10.1080/20002297.2020.1808750

Kuboniwa M, Houser JR, Hendrickson EL *et al.* Metabolic crosstalk regulates *Porphyromonas gingivalis* colonization and virulence during oral polymicrobial infection. *Nat Microbiol* 2017;2:1493–9. DOI: 10.1038/s41564-017-0021-6

Kuboniwa M, Tribble GD, James CE *et al. Streptococcus gordonii* utilizes several distinct gene functions to recruit *Porphyromonas gingivalis* into a mixed community. *Molecular Microbiology* 2006;60:121–39. DOI: 10.1111/j.1365-2958.2006.05099.x

Kumar PS, Griffen AL, Moeschberger ML *et al.* Identification of Candidate Periodontal Pathogens and Beneficial Species by Quantitative 16S Clonal Analysis. *Journal of Clinical Microbiology* 2005;43:3944–55. DOI: 10.1128/JCM.43.8.3944-3955.2005

Kuramitsu HK, Chen W, Ikegami A. Biofilm Formation by the Periodontopathic Bacteria *Treponema denticola* and *Porphyromonas gingivalis*. *Journal of Periodontology* 2005a;76:2047–51. DOI: 10.1902/jop.2005.76.11-S.2047

Kuramitsu HK, Chen W, Ikegami A. Biofilm Formation by the Periodontopathic Bacteria *Treponema denticola* and *Porphyromonas gingivalis*. *J Periodontol* 2005b;76 Suppl 11S:2047–51. DOI: 10.1902/jop.2005.76.11-S.2047

Lamont RJ, Bevan CA, Gil S *et al.* Involvement of *Porphyromonas gingivalis* fimbriae in adherence to *Streptococcus gordonii*. *Oral Microbiology and Immunology* 1993;8:272–6. DOI: 10.1111/j.1399-302x.1993.tb00573.x

Lamont RJ, El-Sabaeny A, Park Y *et al.* Role of the *Streptococcus gordonii* SspB protein in the development of *Porphyromonas gingivalis* biofilms on streptococcal substrates. *Microbiology* 2002;148:1627–36. DOI: 10.1099/00221287-148-6-1627

Lamont RJ, Gil S, Demuth DR *et al.* Molecules of *Streptococcus gordonii* that bind to *Porphyromonas gingivalis. Microbiology (Reading)* 1994;140 (Pt 4):867–72. DOI: 10.1099/00221287-140-4-867

Lamont RJ, Hajishengallis G. Polymicrobial synergy and dysbiosis in inflammatory disease. *Trends in Molecular Medicine* 2015;21:172–83. DOI: 10.1016/j.molmed.2014.11.004

Leschine SB, Canale-Parola E. Rifampin as a selective agent for isolation of oral spirochetes. *J Clin Microbiol* 1980;12:792–5. DOI: 10.1128/JCM.12.6.792-795.1980

Lima BP, Shi W, Lux R. Identification and characterization of a novel *Fusobacterium nucleatum* adhesin involved in physical interaction and biofilm formation with *Streptococcus gordonii*. *Microbiologyopen* 2017;6, DOI: 10.1002/mbo3.444.

Maeda K, Nagata H, Nonaka A *et al.* Oral streptococcal glyceraldehyde-3-phosphate dehydrogenase mediates interaction with *Porphyromonas gingivalis* fimbriae. *Microbes Infect* 2004a;6:1163–70. DOI: 10.1016/j.micinf.2004.06.005

Maeda K, Nagata H, Yamamoto Y *et al.* Glyceraldehyde-3-phosphate dehydrogenase of *Streptococcus oralis* functions as a coadhesin for *Porphyromonas gingivalis* major fimbriae. *Infect Immun* 2004b;72:1341–8. DOI: 10.1128/iai.72.3.1341-1348.2004

Martin B, Chathoth K, Ouali S *et al.* New growth media for oral bacteria. *Journal of Microbiological Methods* 2018;153:10–3. DOI: 10.1016/j.mimet.2018.08.013

Martin B, Tamanai-Shacoori Z, Bronsard J *et al.* A new mathematical model of bacterial interactions in two-species oral biofilms. Amar S (ed.). *PLOS ONE* 2017;12:e0173153. DOI: 10.1371/journal.pone.0173153

McNab R, Ford SK, El-Sabaeny A *et al.* LuxS-based signaling in *Streptococcus gordonii*: autoinducer 2 controls carbohydrate metabolism and biofilm formation with *Porphyromonas gingivalis*. *J Bacteriol* 2003;185:274–84. DOI: 10.1128/jb.185.1.274-284.2003

Meuric V, Lainé F, Boyer E *et al.* Periodontal status and serum biomarker levels in HFE haemochromatosis patients. A case-series study. *J Clin Periodontol* 2017;44:892–7. DOI: 10.1111/jcpe.12760

Meuric V, Martin B, Guyodo H *et al. Treponema denticola* improves adhesive capacities of *Porphyromonas gingivalis. Molecular Oral Microbiology* 2013;28:40–53. DOI: 10.1111/omi.12004

Ng HM, Slakeski N, Butler CA *et al.* The Role of *Treponema denticola* Motility in Synergistic Biofilm Formation With *Porphyromonas gingivalis. Front Cell Infect Microbiol* 2019;9, DOI: 10.3389/fcimb.2019.00432.

Ooi XJ, Tan KS. Reduced Glutathione Mediates Resistance to H₂S Toxicity in Oral Streptococci. *Appl Environ Microbiol* 2016;82:2078–85. DOI: 10.1128/AEM.03946-15

Orth RK-H, O'Brien-Simpson NM, Dashper SG *et al.* Synergistic virulence of *Porphyromonas gingivalis* and *Treponema denticola* in a murine periodontitis model. *Molecular Oral Microbiology* 2011;26:229–40. DOI: 10.1111/j.2041-1014.2011.00612.x

Palmer RJ, Gordon SM, Cisar JO *et al.* Coaggregation-mediated interactions of streptococci and actinomyces detected in initial human dental plaque. *J Bacteriol* 2003;185:3400–9. DOI: 10.1128/jb.185.11.3400-3409.2003

Park Y, Simionato MR, Sekiya K *et al.* Short fimbriae of *Porphyromonas gingivalis* and their role in coadhesion with *Streptococcus gordonii*. *Infect Immun* 2005;73:3983–9. DOI: 10.1128/IAI.73.7.3983-3989.2005

Rickard AH, Gilbert P, High NJ *et al.* Bacterial coaggregation: an integral process in the development of multi-species biofilms. *Trends Microbiol* 2003;11:94–100. DOI: 10.1016/s0966-842x(02)00034-3

Sbordone L, Bortolaia C. Oral microbial biofilms and plaque-related diseases: microbial communities and their role in the shift from oral health to disease. *Clin Oral Invest* 2003;7:181–8. DOI: 10.1007/s00784-003-0236-1

Sedlacek MJ, Walker C. Antibiotic resistance in an in vitro subgingival biofilm model. *Oral Microbiol Immunol* 2007;22:333–9. DOI: 10.1111/j.1399-302X.2007.00366.x

Simionato MR, Tucker CM, Kuboniwa M *et al. Porphyromonas gingivalis* genes involved in community development with *Streptococcus gordonii*. *Infect Immun* 2006;74:6419–28. DOI: 10.1128/IAI.00639-06

Slots J, Gibbons RJ. Attachment of *Bacteroides melaninogenicus* subsp. asaccharolyticus to oral surfaces and its possible role in colonization of the mouth and of periodontal pockets. *Infect Immun* 1978;19:254–64. DOI: 10.1128/IAI.19.1.254-264.1978

Socransky SS, Smith C, Haffajee AD. Subgingival microbial profiles in refractory periodontal disease: Refractory microbial profiles. *Journal of Clinical Periodontology* 2002;29:260–8. DOI: 10.1034/j.1600-051x.2002.290313.x

Stinson MW, Safulko K, Levine MJ. Adherence of *Porphyromonas (Bacteroides) gingivalis* to *Streptococcus sanguis in vitro. Infect Immun* 1991;59:102–8. DOI: 10.1128/IAI.59.1.102-108.1991

Tan KH, Seers CA, Dashper SG *et al. Porphyromonas gingivalis* and *Treponema denticola* Exhibit Metabolic Symbioses. *PLOS Pathogens* 2014;10:e1003955. DOI: 10.1371/journal.ppat.1003955

Watanabe T, Maruyama F, Nozawa T *et al.* Complete Genome Sequence of the Bacterium *Porphyromonas gingivalis* TDC60, Which Causes Periodontal Disease. *J Bacteriol* 2011;193:4259–60. DOI: 10.1128/JB.05269-11

Welin-Neilands J, Svensäter G. Acid tolerance of biofilm cells of *Streptococcus mutans*. *Appl Environ Microbiol* 2007;73:5633–8. DOI: 10.1128/AEM.01049-07

Yamada M, Ikegami A, Kuramitsu HK. Synergistic biofilm formation by *Treponema denticola* and *Porphyromonas gingivalis. FEMS Microbiology Letters* 2005;250:271–7. DOI: 10.1016/j.femsle.2005.07.019

110

Zhou X, Li Y eds. "Chapter 4—Subgingival microbes." *Atlas of Oral Microbiology: From Healthy Microflora to Disease*; Elsevier: Amsterdam, The Netherlands 2015: 67-93. DOI: 10.1016/B978-0-12-802234-4.00004-5

Zhu L, Kreth J. The role of hydrogen peroxide in environmental adaptation of oral microbial communities. *Oxid Med Cell Longev* 2012;2012:717843. DOI: 10.1155/2012/717843

Zhu Y, Dashper SG, Chen Y-Y *et al. Porphyromonas gingivalis* and *Treponema denticola* Synergistic Polymicrobial Biofilm Development. Brissette CA (ed.). *PLoS ONE* 2013;8:e71727. DOI: 10.1371/journal.pone.0071727

FIGURES



Figure 1: Effect of iron and inter-species association on attachment of each species in either mono- or multi-species sessile cells. The concentration of individual species (CFU/ml) in different biofilms (mono-species biofilms: *S. gordonii*-Sg, *P. gingivalis*- Pg and *T. denticola*-Td; dual-species biofilms: *S. gordonii*-*P. gingivalis*: SgPg, *S. gordonii*-*T. denticola*: SgTd, *P. gingivalis*-*T. denticola*: PgTd; three-species biofilms: *S. gordonii*-*P. gingivalis*-*T. denticola*: SgPgTd) were compared at sub-optimal (0.8 μ M), optimal (8 μ M) and excess iron (80 μ M) levels. The graph shows the concentration of *S. gordonii* (A), concentration of *P. gingivalis* (B) and concentration of *T. denticola* (C) in the different 2-hour biofilms. All initial inoculums contained 2.8 x 10⁸ cells of *S. gordonii* and/or 1.2 x 10⁹ cells of *P. gingivalis* and/or 6.3 x 10⁸ cells of *T. denticola* in the various biofilms. * indicates P-value ≤ 0.05 .



Figure 2: Effect of iron on the microscopic parameters obtained for various mono-, dual- and threespecies biofilms. Effect of sub-optimal (0.8 μ M), optimal (8 μ M) and excess (80 μ M) iron levels on the (A) Bio-volume, (B) Roughness co-efficient, (C) Surface-to-bio-volume ratio, (D) Average thickness and (E) Maximum thickness of mono-species (*S. gordonii*-Sg, *P. gingivalis*- Pg and *T. denticola*-Td), dual-species (*S. gordonii-P. gingivalis*: SgPg, *S. gordonii-T. denticola*: SgTd, *P. gingivalis-T. denticola*: PgTd) and threespecies (*S. gordonii-P. gingivalis*-*T. denticola*: SgPgTd) 2-hour biofilms. The microscopic parameters were calculated on the total biofilm in each condition (comprised of all cells irrespective of individual species). * indicates P-value ≤ 0.05 .



Figure 3: Representative microscopic images of bacteria attached at 2 hours in mono-, two- and threespecies biofilms. The sessile cells of various 2-hour biofilms (mono-species: *S. gordonii-* Sg, *P. gingivalis*-Pg, *T. denticola*- Td; dual-species: *S. gordonii-P. gingivalis*: SgPg, *S. gordonii-T. denticola*: SgTd, *P. gingivalis-T. denticola*: PgTd three-species: *S. gordonii-P. gingivalis-T. denticola*: SgPgTd) were stained using Styo®9 and were visualized using the Leica TCS-SP5 confocal laser scanning microscope. The images are representative of the total biofilm in each condition (comprised of all cells irrespective of individual species). The images of these 2-hour biofilms grown at three different iron levels (sub-optimal-0.8 μ M, optimal- 8 μ M, and excess- 80 μ M) were compared. A maximum z-projection of the Z stack was taken using 40X oil immersion objective lens for *T. denticola* mono-species biofilm while the 63X oil immersion objective lens for the remaining. A numerical zoom of 2 was applied. The scale (10 μ m) is shown on the bottom right corner of each image.



Figure 4: Biofilm initiation in the subgingival niche - effect of iron and interspecies associations. The model depicts the antagonistic (red lines) and co-operative (green arrows) behaviour between the three bacterial species *S. gordonii, P. gingivalis* and *T. denticola* as dual-species biofilms (*S. gordonii-P. gingivalis, S. gordonii-T. denticola, P. gingivalis-T. denticola*) and three-species biofilms (*S. gordonii-P. gingivalis-T. denticola*) at sub-optimal (0.8μ M) and higher (8 and 80μ M) iron levels. Briefly, *S. gordonii* enhanced (green arrows) the attachment of *P. gingivalis* in *S. gordonii-P. gingivalis* biofilms at all iron levels. Similarly, both species in *P. gingivalis-T. denticola* biofilms showed synergistic growth (green arrows) at all iron levels. Both species in *S. gordonii-T. denticola* enhanced (green arrows) the attachment of *S. gordonii-T. denticola* enhanced (green arrows) the attachment of *S. gordonii*. In the three-species biofilms, the attachment of both *P. gingivalis* and *T. denticola* was enhanced (green arrows) while attachment of *S. gordonii* was inhibited (red lines) at all iron levels, and attachment of *P. gingivalis* has further enhanced at higher iron levels (dark green arrow). The dotted box indicates only the biofilms that displayed difference in interspecies interactions based on iron levels.

SUPPLEMENTARY DATA



Figure S1: Comparison between mono-, dual- and three-specie 2-hour biofilms. Initial attachment for each species were assessed for mono-species (*S. gordonii*-Sg, *P. gingivalis-* Pg and *T. denticola-*Td), dual-species (*S. gordonii-P. gingivalis:* SgPg, *S. gordonii-T. denticola:* SgTd, *P. gingivalis-T. denticola:* PgTd) and three-species (*S. gordonii-P. gingivalis-T. denticola:* SgPgTd) 2-hour biofilms at sub-optimal (0.8 μ M), optimal (8 μ M) and excess iron (80 μ M) levels. * indicates P-value \leq 0.05.

TABLES

Table 1: List of primers used for the study.

Organism	Sequence $(5' \rightarrow 3')$
Streptococcus gordonii	F: AAG-CAA-CGC-GAA-GAA-CCT-TA
	R: GTC-TCG-CTA-GAG-TGC-CCA-AC
Porphyromonas gingivalis	F: TGG-GTT-TAA-AGG-GTG-CGT-AG
	R: CAA-TCG-GAG-TTC-CTC-GTG-AT
Treponema denticola	F: CGC-GTC-CCA-TTA-GCT-AGT-TG
	R: TTC-TTC-ATT-CAC-ACG-GCG-TC

Iron		Mono-	Dual-species biofilms			Three-
(µM)		species	S. gordonii-	S. gordonii-	P. gingivalis-	species
		biofilm	P. gingivalis	T. denticola	T. denticola	biofilms
0.8	S. gordonii	1.05E+08 <u>+</u>	1.16E+08 <u>+</u>	4.20E+07 <u>+</u>		1.79E+07 <u>+</u>
μМ		2.82E+07	1.02E+08	1.33E+07		2.44E+06
iron	P. gingivalis	2.21E+05 <u>+</u>	1.47E+07 <u>+</u>		7.66E+06 <u>+</u>	4.77E+06 <u>+</u>
		9.76E+04	1.46E+07		3.99E+06	7.36E+05
	T. denticola	1.74E+07 <u>+</u>		5.02E+06 <u>+</u>	8.07E+07 <u>+</u>	4.46E+07 <u>+</u>
		4.36E+06		2.68E+06	3.43E+07	1.45E+07
8 μΜ	S. gordonii	1.42E+08 <u>+</u>	8.76E+07 <u>+</u>	3.53E+08 <u>+</u>		2.07E+07 <u>+</u>
iron		4.11E+07	7.32E+07	7.21E+07		9.75E+06
	P. gingivalis	2.16E+05 <u>+</u>	1.62E+07 <u>+</u>		7.33E+06 <u>+</u>	6.27E+06 <u>+</u>
		6.79E+04	1.42E+07		3.36E+06	1.35E+06
	T. denticola	2.23E+07 <u>+</u>		2.55E+07 <u>+</u>	9.39E+07 <u>+</u>	5.10E+07 <u>+</u>
		8.29E+06		1.43E+07	5.01E+07	1.06E+07
80	S. gordonii	1.01E+08 <u>+</u>	7.86E+07 <u>+</u>	3.72E+08 <u>+</u>		1.71E+07 <u>+</u>
μМ		2.20E+07	6.64E+07	1.05E+08		6.27E+06
iron	P. gingivalis	2.13E+05 <u>+</u>	1.83E+07 <u>+</u>		8.10E+06 <u>+</u>	6.76E+06 <u>+</u>
		7.77E+04	1.43E+07		3.77E+06	8.74E+05
	T. denticola	2.00E+07 <u>+</u>		2.44E+07 <u>+</u>	9.15E+07 <u>+</u>	4.67E+07 <u>+</u>
		6.65E+06		1.14E+07	5.02E+07	9.37E+06

Table 1S: Concentration of each species (CFU/ml) in mono-species and multispecies biofilms at 0.8, 8 and 80 μM of iron.

4.3. Effect of iron and interspecies associations on biofilm growth and development

Kanchana Chathoth¹, Martine Bonnaure-Mallet^{1,2}, Bénédicte Martin3^g and Christine Baysse^{1*g}

*Corresponding author: christine.baysse@univ-rennes1.fr

^d These authors share senior authorship

¹ University of Rennes 1, NUMECAN INSERM U1241, CIMIAD F-35043 Rennes, France

² CHU Pontchaillou, Rennes

³ SLS France, F-35136 St Jacques de la Lande, France

Keywords: oral biofilm, *Porphyromonas gingivalis, Treponema denticola, Streptococcus gordonii,* Iron.

Running headline: Effect of iron on oral biofilm growth

ABSTRACT

Periodontitis is a chronic inflammatory disease of the periodontium characterized by a progressive destruction of the tooth-supporting tissues. The primary cause of periodontitis is the growth of diverse pathogens that flourish in the subgingival niche as dental plaques or biofilms. The composition of these biofilms may be influenced by interspecies interactions (co-operation, competition, and metabolic and genetic exchange) and oral environmental stimulus. A link between the systemic iron overload disease hemochromatosis and severe periodontitis has been previously indicated. However, not much is known about the direct effect of iron on the periodontal biofilm. Therefore, we investigated the effect of iron levels and interspecies associations in an oral biofilm model consisting of *Streptococcus gordonii*, *Porphyromonas gingivalis* and *Treponema denticola* on biofilm growth and development. The effect of iron was evaluated on the ability of these species to form biofilms over a period of 144 hours using quantitative PCR and confocal microscopy. The most noteworthy result observed was that the three-species model showed a preferential growth of all the three species at high iron concentration. Such preference for high iron levels may lead to oral dysbiosis and progression of periodontitis during iron-overloaded diseases.

INTRODUCTION

Periodontitis is a chronic inflammatory disease of the periodontium associated with oral biofilms also known as dental plaques. Severe forms of periodontitis cause bone and tissue loss and loosen the tooth resulting in a periodontal pocket where the pathogens flourish. Chronic

periodontitis is also associated to systemic disorders like rheumatoid arthritis, cardiovascular disease, premature birth, diabetes, hemochromatosis and even oral cancer (Hujoel *et al.* 2003; López 2008; Preshaw *et al.* 2012; Koziel, Mydel and Potempa 2014; Beukers *et al.* 2017; Bui *et al.* 2019; Graves, Corrêa and Silva 2019; Nwizu, Wactawski-Wende and Genco 2020). However, the detailed mechanism behind these associations is not fully known.

The subgingival niche is known to possess reduced oxygen tensions which favours anaerobic periodontal pathogens such as the members of the red complex (*Porphyromonas gingivalis, Treponema denticola*, and *Tannerella forsythia*. These along with primary colonizers (Actinomyces species, streptococci) form subgingival biofilms. The bacteria in such biofilms are known to express phenotypes like increased antibiotic resistance (Sedlacek and Walker 2007), acid (Welin-Neilands and Svensäter 2007) or oxygen tolerance (Gursoy *et al.* 2010) unlike their planktonic counterparts. Several studies have also demonstrated interspecies associations like co-operation, competition, and metabolic and genetic exchange between the various bacterial species in the oral biofilms (Sbordone and Bortolaia 2003; Kuboniwa *et al.* 2006; Orth *et al.* 2011; Lamont and Hajishengallis 2015; Ng *et al.* 2019).

External stimulus due to factors in the oral environment like pH, exposure to oxygen, temperature (Pöllänen, Paino and Ihalin 2013), concentration of substrate, metal ions (Shumi *et al.* 2010), etc., can trigger a pathogenic switch in the oral microflora. Iron being an essential nutrient, is crucial for bacterial growth and biofilm maturation. Excess iron is known to promote Fenton chemistry by the production of harmful hydroxyl radical (ROS) in the presence of hydrogen peroxide. Presence of peroxygenic bacteria such as *Streptococcus gordonii* (Zhu and Kreth 2012) can thus induce Fenton chemistry during conditions of iron excess. Researchers have previously found a link between the iron overload disease hemochromatosis and the severity of periodontitis (Meuric *et al.* 2017; Boyer *et al.* 2018). Indeed, the subgingival microbiota of hemochromatosis patients and patients of chronic periodontitis showed a positive correlation between the mean pocket depth and the presence of *Porphyromonas* which is a signature for dysbiosis (Boyer *et al.* 2020). However, the direct effect of iron on the interspecies associations between bacteria in oral subgingival biofilms is not known.

In this study the effect of iron levels was assessed on interspecies associations in biofilms by analysing the development and growth of an *in vitro* three-species biofilm model previously established and consisting of S. gordonii, P. gingivalis and T. denticola (Martin et al. 2018). The interspecies interactions and binding facilitated through binding between long fimbriae (FimA) of P. gingivalis and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) of S. gordonii (Maeda et al. 2004), likewise the P. gingivalis short fimbriae (Mfa) and the streptococcal SspA/B adhesins (Park et al. 2005) are well known. Similarly, several studies demonstrate the synergy, syntrophy and nutritional cross-feeding between P. gingivalis and T. denticola (Grenier 1992; Meuric et al. 2013: Zhu et al. 2013: Tan et al. 2014: Ng et al. 2019). These two anaerobic pathogens often coexist in deep periodontal pockets (Byrne et al. 2009; Tan et al. 2014) and are associated with severe forms of the periodontal disease (Orth et al. 2011). Based on the planktonic growth of P. gingivalis cultures, 8 µM of iron (FeSO₄) along with 0.08 µM of protoporphyrin IX (PPIX) (to allow heme synthesis via the ferrochelatase) was established as the optimal iron concentration for P. gingivalis in Medium for Mixed Bacterial Community (MMBC) (Martin et al. 2018). Martin et al. (2018) also demonstrated that 8 µM iron also allowed the planktonic growth of T. denticola and S. gordonii and enabled the biofilm growth of the three-species biofilm in the same MMBC medium. In a previous study using same iron concentrations in MMBC medium, an iron-dependent antagonism or cooperation in biofilm initiation was demonstrated between S. gordonii and T. denticola. High concentrations of iron (8 and 80 µM) also favoured P. gingivalis initial attachment in the 3-species biofilm (Chathoth *et al.*, manuscript in preparation). Therefore, based on the iron requirement of the keystone pathogen P. gingivalis, we selected three iron concentrations: 8 µM), 0.8 µM and 80 µM with constant value for PPIX (0.08 µM) to evaluate effect of iron levels on biofilms. Here, the interspecies interactions at these three iron concentrations were studied to assess the effect of iron on the development and growth of the three oral bacteria in biofilms from 24 to 144 hours. For this, we first analyse interspecies associations at 8 µM iron concentration. Then modifications of 10-fold low and high iron levels were assessed on the interspecies associations in the three-species biofilm model.

MATERIALS AND METHODS

Bacteria and media

Bacterial cultures of *Streptococcus gordonii* Challis DL1 (Chen, Teng and Chang 2004), *Porphyromonas gingivalis* TDC60 (Watanabe *et al.* 2011) and *Treponema denticola* ATCC 35405 (Chan *et al.* 1993) grown in MMBC-3 with 8 μM FeSO₄ and 0.08 μM protoporphyrin IX (PPIX) (Martin *et al.* 2018) were used in this study. However, *T. denticola* was first grown in the NOS spirochete medium (Leschine and Canale-Parola 1980) and then sub-cultured in the MMBC-3 with FeSO₄ and PPIX. The three micro-organisms were grown in anaerobic condition at 37°C in an anaerobic chamber (MACS 500, Don Whitley Scientific, United Kingdom) with 10% v:v H₂, 10% v:v CO₂ and 80% v:v N₂.

Growth conditions for biofilms

8-chambered polymer coverslips / μ -slides (ibiTreat, Ibidi) were coated with 200 μ l of filtered (0.20 μ m) and twice diluted (in sterile water) saliva (Pool Human Donors, MyBioSource) for 30 minutes. The saliva was replaced with 200 μ l of the inocula consisting of *S. gordonii* (OD_{600nm}=0.05) and/or *P. gingivalis* (OD_{600nm}=0.1) and/or *T. denticola* (OD_{600nm}=0.1) alone and in different combinations to have mono-species, dual-species and three-species biofilms. For each biofilm, 5 saliva-coated μ -slides were filled with inoculum to have biofilms at different stages of growth (2, 24, 48, 72 and 144 hours). These culture-containing μ -slides were incubated in anaerobic condition for 2 hours after which the planktonic cells were removed from all μ -slides. In one μ -slide (2-hour biofilm) the attached biofilm fresh MMBC-3 medium was added and incubated to complete 24, 48, 72, 144-hour of biofilm growth. Similar to 2-hour biofilms, the planktonic cells were removed from other biofilms (24, 48, 72, 144-hour) after the respective incubation period and the biofilms were washed with 200 μ l of PBS. All biofilms were used for imaging and for bacterial species quantification, as described below.

Confocal laser microscopy and imaging

All biofilms (2, 24, 48, 72 and 144 hours) were stained using the Syto[®]9 (5 µM) greenfluorescent nucleic acid stain (Invitrogen, ThermoFisher Scientific) diluted in PBS and incubated for 15 minutes. These stained cells were observed *in situ* with a Leica TCS-SP5 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany). Using an HC PL Apo 63X, 1.4 NA, oil immersion objective lens, images were captured. However, all mono-species biofilms of *T. denticola* alone formed scanty biofilms and a magnification of 63X focused either on only biofilm or only void spaces, giving biased images. Hence a magnification of 40 X was used using an HC PL Apo 40X, 1.25-0.75 NA, oil immersion lens to capture clear images of the spirochete. A numerical zoom of 1.5 was applied to all acquisitions. All Syto[®]9-stained bacteria were detected using the 488-nm UV diode and a 485 to 500-nm band-pass emission filter. Biofilm stacks (123 × 123 μ m) acquired at 1 μ m intervals were scanned with a line average of 2. Leica software (LAS AF V.2.2.1) was used for microscope piloting and image acquisition. Image analyses were performed using the Comstat2 plugin in the ImageJ software V1.43m (National Institute of Health, USA) to estimate biofilm parameters: the bio-volume (expressed in μ m³/ μ m²) and the average thickness (μ m), roughness co-efficient (Ra) and surface-to-bio-volume ratio as described previously (Heydorn *et al.* 2000). 3-dimentional images were generated using the Imaris Viewer 9.6 software using the biofilm stack obtained using the Leica TCS-SP5 confocal laser scanning microscope.

After microscopic observation, the stain was removed from the μ -slides' wells and the biofilms (either 2, 24, 48, 72 and 144 hours) were collected in 100 μ l of PBS, centrifuged (8000xg, 10 minutes, 20°C) and the pellets were stored at -20°C for further characterization by qPCR.

qPCR quantification

The biofilms (either 2, 24, 48, 72 and 144 hours) collected in PBS, comprising of S. gordonii, P. gingivalis and/or T. denticola previously centrifuged (8000xg, 20°C, 10 min) and stored as pellets at -20°C were resuspended in 100µl of PBS. The inocula (200 µl) used for each biofilm was also centrifuged and the stored pellets were resuspended in 100 uL of PBS for comparison. These were further heated for 20 min at 95°C. The concentrations of the DNA in these samples were determined by performing quantitative PCR against defined concentrations of DNA standards set in the range of 0.0001 to 10 ng with purified genomic DNA from each of the three species. A total reaction volume of 12.5 µl contained 6.25 µl SYBR 2X Green Master Mix (Eurogentec, Belgium), 1 μ l each of forward and reverse primers (5 μ M) and 1 μ l of DNA template. DNA templates were amplified using the Applied Biosystems apparatus (StepOne Plus). The conditions for qPCR were: an initial holding stage of 95°C for 10 min followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C; a melt curve stage was performed consisting of 15 sec at 95°C followed by a temperature gradient from 60°C to 95° C in 1°C increment step measuring fluorescence at each temperature for 15 sec. Primers used were specific to each species targeting the 16S ribosomal RNA taking into account specific genome weights (Ammann et al. 2013; Martin et al. 2017). The primers used in this study are listed in Table 1.

Chapter 4

Statistical analysis

All the experiments were done with a minimum of two biological and two technical replicates $(n \ge 4)$. Statistical analyses were performed using the two-tailed unpaired student t-test and a p-value of less than 0.05 was considered statistically significant.

RESULTS

Effect of iron on the growth of bacteria in mono-species biofilms

The sessile/biofilm growth (between 24 to 144 hours) of the bacteria was assessed at three iron levels: 8 μ M, 0.8 μ M and 80 μ M. At 8 μ M FeSO₄, the mono-species biofilms of *P. gingivalis* showed a steady increase in the concentration of cells from 24 to 144 hours (**Figure 1A**). 10-fold lower iron levels of 0.8 μ M lowered the concentration of *P. gingivalis* cells when compared to 8 μ M of iron from 72 hours onwards, thereby limiting its growth. Whereas, the concentration of *P. gingivalis* cells increased at 80 μ M of iron sooner (at 48 hours) than at 8 μ M, after which, the cell concentration decreased. These results confirmed that 8 μ M of iron was optimal not only for the planktonic growth but also for the sessile growth of *P. gingivalis* cells. The values obtained for the bio-volume of the *P. gingivalis* biofilms by microscopic evaluation were in agreement with the cell concentrations for the three iron levels, with a stark increase when grown in 8 μ M of iron at 144 hours (**Figure 2A**) compared to other iron levels. The roughness coefficient values of *P. gingivalis* biofilms decreased significantly at 8 μ M iron at specifically 144 hours when compared to other iron levels (**Figure 2B**).

The *T. denticola* biofilms attained their maximum cell concentration at either 24 hours (8 μ M iron) or 48 hours (0.8 and 80 μ M iron) which was further maintained (**Figure 1B**). The cell concentration measures for *T. denticola* biofilms were slightly higher at mostly 8 μ M of iron in comparison to other iron levels during its growth (from 24 to 144 hours). This preference for 8 μ M was more prominent in the bio-volume measurements which gradually increased with time (**Figure 2C**). Bio-volume is a measure of the volume occupied by the cell which also takes into account the cell size. The size of *T. denticola* cells are known to vary from 5 to 20 μ m. The increase in bio-volume from 24 to 144 hours, which is not reflected in the cell concentrations may be because *T. denticola* cells grew longer with time (**Supplementary figure 1**). The roughness coefficient of *T. denticola* biofilms tended to increase with time, indicative of biofilm growth as

patches/microcolonies. Roughness was decreased at 80 μ M of iron, as explained by the lower biofilm growth at this iron concentration (**Figure 2D**).

The concentration of cells in *S. gordonii* biofilms remain unaltered irrespective of the iron concentration used (**Figure 1C**). These biofilms reached their maximum cell concentration at 24 hours which was further maintained till 144 hours. The microscopic analyses of the biofilm biovolume showed a fast growth till 24 hours, followed by a gradual increase from 24 to 144 hours but remained unaffected by iron levels. The bio-volume spread uniformly across the surface starting from 24 hours resulting in a low roughness co-efficient indicative of a homogenous biofilm (**Figure 2E, 2F**).

Effect of interspecies interactions at 8 µM of iron for multi-species biofilms

The concentration of each species in multi-species biofilms, evaluated by qPCR, were compared to mono-species biofilms at 8 μ M of FeSO₄ to evaluate reciprocal effects of species. 8 μ M concentration was chosen as the optimal growth concentration for planktonic and sessile *P. gingivalis*. The highest concentration of *S. gordonii* cells was obtained either in mono-species biofilms or in dual-species with *T. denticola*, but only till 48 hours (p ≤ 0.05) in the latter after which it decreased (**Figure 3A**). *S. gordonii* cell concentration was the lowest when with *P. gingivalis* in dual-species biofilms and/or in three-species biofilms (p ≤ 0.05).

The *P. gingivalis* cell concentrations were lower when alone in the initial stages of biofilm growth (24 and 48 hours) as compared to multispecies biofilms but gradually increased and reached maximum cell concentration at 144 hours. *T. denticola* induced a rapid growth of *P. gingivalis* cells in dual-species biofilms till 24 hours which then remained stable and above mono-species concentrations until 72 hours ($p \le 0.05$) (**Figure 3B**). In contrast, in *S. gordonii*-containing dual or three-species biofilms, *P. gingivalis* growth (between 24 to 144 hours) was poor with cell concentrations remaining close to initially attached cells until 144 hours.

T. denticola growth in mono-species or in three-species biofilms was low. In contrast, the presence of *P. gingivalis* in dual-species *P. gingivalis-T. denticola* biofilms allowed a gradual increase of *T. denticola* concentrations from 24 to 72 hours. Also, the *T. denticola* cell concentration in dual-species with *P. gingivalis* remained higher than mono-species biofilms at all

the time points ($p \le 0.05$). Meanwhile, *S. gordonii* decreased *T. denticola* levels in dual-species biofilms after 24 hours compared to the mono-species biofilms.

S. gordonii was the predominant species in dual-species biofilms with *P. gingivalis* and *T. denticola* with 97% between 24 to 144 hours of growth while *P. gingivalis* and *T. denticola* levels were below 2% (**Supplementary figure 2A, 2B**). *T. denticola* predominated the dual species *P. gingivalis-T. denticola* biofilms (**Supplementary figure 2C**) from approximately 80% (at 24 hours) to 94% (at 144 hours). Consecutively, the percentage of *P. gingivalis* in *P. gingivalis-T. denticola* biofilms decreased with time. In the three-species biofilms, the proportion of *S. gordonii* was the highest (above 80 %) followed by *T. denticola* (5-17%) and the least was *P. gingivalis* (2-6%) (**Supplementary figure 2D**).

Microscopic parameters for biofilm growth between the various biofilms were analysed. The bio-volume of all biofilms increased between 2 to 144 hours (**Figure 4A**), indicative of biofilm growth, as already revealed by increase in cell numbers (**Figure 3**). Among the mono-species and multispecies biofilms, the bio-volumes of *P. gingivalis* biofilms were the lowest ($p \le 0.05$), as also demonstrated by cell numbers (**Figure 3B**), while the bio-volume of all *S. gordonii*-containing biofilms were the most important ($p \le 0.05$), which is partly explained by predominance associated with high cell numbers of *S. gordonii* in these biofilms. Among the latter, either *S. gordonii*-*T. denticola* biofilms or the three-species biofilms exhibited the highest bio-volumes between 24 and 72 hours ($p \le 0.05$) (**Figure 4A**).

Kinetics of biofilm growth varied between the types of biofilms. Except for *S. gordonii*-*T. denticola* biofilms, all other types of biofilms grew from 24 to 144 hours. Dual-species *S. gordonii*-*T. denticola* biofilms grew rapidly till 24 hours after which bio-volume decreased. This kinetical trend for bio-volume of *S. gordonii*-*T. denticola* biofilms is in agreement with qPCR cell concentrations. However, bio-volume of *S. gordonii*-*T. denticola* biofilms was higher than expected compared to mono-species bio-volumes measures. In addition, the total number of bacterial cells in *S. gordonii*-*T. denticola* biofilms in contrast to bio-volume measurements. This may be because qPCR measurements are indicative of the cell number (precisely, the number of bacterial chromosomes) while bio-volume is the volume occupied by the cell which also takes into account the size of the cell. Possibly, as previously said, the size of the bacteria increased,
especially *T. denticola* which can increase in size by up to four times, contributing to an increased bio-volume.

Except for *T. denticola* mono-species biofilms, the values of roughness coefficients for all other biofilms tended to decrease with time (**Figure 4B**), indicating more homogenous biofilms. The roughness coefficient was the highest for *P. gingivalis* mono-species ($p \le 0.05$) except at 144 hours in comparison to other biofilms (**Figure 4B**). The corresponding low values of *P. gingivalis* bio-volume (**Figure 4A**) and cell concentrations (**Figure 3B**) result in an uneven biofilm with void spaces (**Figure 5**) increasing the roughness coefficient. The roughness coefficient values of *P. gingivalis-T. denticola* biofilms were also high (**Figure 4B**) as these biofilms were heterogenous/uneven mainly due to the cluster formation by *T. denticola* cells (**Figure 5**). The values of average thickness for the various biofilms followed the same pattern as that of their respective bio-volumes with lowest values ($p \le 0.05$) for *P. gingivalis* mono-species biofilms while the highest values ($p \le 0.05$) for *S. gordonii-T. denticola* biofilms or the three-species biofilms while the highest values ($p \le 0.05$) for *S. gordonii-T. denticola* biofilms or the three-species biofilms while the highest values ($p \le 0.05$) for *S. gordonii-T. denticola* biofilms or the three-species biofilms while the highest values ($p \le 0.05$) for *S. gordonii-T. denticola* biofilms or the three-species biofilms while the highest values ($p \le 0.05$) for *S. gordonii-T. denticola* biofilms or the three-species biofilms (**Supplementary figure 4**).

Effect of iron on the three-species biofilm

The influence of 10-fold lower ($0.8 \mu M FeSO_4$) or higher ($80 \mu M FeSO_4$) iron levels on the growth and architecture of the three-species biofilms in comparison to $8 \mu M FeSO_4$ concentration was studied. The concentration of each species in the biofilms was assessed to decipher the effect of iron on the species growth. In addition, microscopic parameters of the biofilms allowed the interpretation on the effect of iron on biofilm structure/architecture.

In the three-species biofilms, mostly 80 μ M iron after extended time duration favoured the growth of each species. The concentration of *S. gordonii* and *T. denticola* cells was higher at 80 μ M iron at 144 hours (p \leq 0.05) as compared to other iron levels, while *P. gingivalis* cells grew best at 80 μ M iron at all the time points (p \leq 0.05) (**Figure 6A, 6B, 6C**). These results were in contrast to their respective mono-species biofilms where *S. gordonii* cells remained unaffected by iron while *P. gingivalis* and *T. denticola* cells showed a diminished growth at 80 μ M of iron (**Figure 1A, 1C**). The bio-volume of the three-species biofilm was also higher at 80 μ M at 144 hours as compared to 0.8 and 8 μ M while remained unaffected by iron at other time points (**Figure 7A, Figure 8**). This observation is consistent with the values obtained by qPCR where the cell concentration of all three species increase at 80 μ M at 144 hours (**Figure 6**). The roughness coefficient values were low

indicative of evenly spread homogenous biofilms (Figure 7B). Similar to the bio-volume, the average thickness of the three-species biofilms was higher at 80 μ M of iron at especially 144 hours (p ≤ 0.05) (Supplementary figure 5).

DISCUSSION

The interactions between the three species, *S. gordonii*, *P. gingivalis* and *T. denticola* in various two-species and the three-species biofilms and their role in biofilm initiation with respect to their corresponding mono-species biofilms at 8 μ M FeSO₄ have been previously studied (Chathoth *et al.*, manuscript in preparation). In dual species biofilms, the authors reported an increased attachment of *P. gingivalis* by *S. gordonii*, improved attachment of both species in *P. gingivalis-T. denticola* biofilms and attachment of *S. gordonii* enhanced by *T. denticola* in dual-species biofilms compared to mono-species biofilms. Also, *P. gingivalis* and *T. denticola* in the three-species oral biofilm model attached more compared to mono-species biofilms. The proportions and concentrations of bacterial species attached in each type of biofilm will have an influence on the growth and development of mature biofilms.

In the present study, focused on growth phase of biofilm formation, different types of species interactions were witnessed. In the dual-species *S. gordonii-P. gingivalis* biofilms, presence of *P. gingivalis* cells hindered the growth of *S. gordoni*. This inhibitory effect of *P. gingivalis* on *S. gordonii* when grown in the iron-controlled MMBC-3 medium with 8 μ M FeSO₄ is contradictory to previous report in rich BHIe medium (Martin *et al.*, 2017) where *S. gordonii* hampered the *P. gingivalis* cell concentrations. This *P. gingivalis*-induced inhibition of *S. gordonii* needs to be further studied. Our study also showed higher *P. gingivalis* cell concentrations in *S. gordonii*-*P. gingivalis* biofilms than the mono-species biofilms until 24 hours, which is probably the direct effect of the elevated levels of attached *P. gingivalis* cells against mono-species biofilm levels, as reported previously (Chathoth *et al.*, manuscript in preparation).

Direct experimental evidence regarding the possible gene transfer from *T. denticola* to *S. gordonii* has been previously reported (Wang, Chi and Kuramitsu 2002). However, not much is known about the *in vitro* association and growth of *S. gordonii-T. denticola* dual species biofilms. We observe that both *S. gordonii* and *T. denticola* hampered the growth of the other in dual-species biofilms in comparison to their respective mono-species biofilms at 8 µM FeSO₄. The antagonistic effect of *S. gordonii* on *T. denticola* when grown together have been previously reported in

planktonic cultures (Martin *et al.* 2018). The reduction in the *T. denticola* cell concentration may be attributed to the H₂O₂ produced by *S. gordonii* (Kreth, Zhang and Herzberg 2008). The toxic effect of *T. denticola* on *S. gordonii* in these *S. gordonii-T. denticola* biofilms was observed post 48 hours despite previously reported increased levels of attached *S. gordonii* in *S. gordonii-T. denticola* biofilms compared to mono-species biofilms as (Chathoth *et al.*, manuscript in preparation). This toxic effect of *T. denticola* on *S. gordonii* is to be elucidated.

Interactions between *P. gingivalis* with *T. denticola* were previously studied and the observations corroborated our results where higher concentrations of both the species in *P. gingivalis-T. denticola* biofilm compared to the respective mono-species biofilms were observed. The synergistic, symbiotic and co-adhesion systems of *P. gingivalis* with *T. denticola* (Kuramitsu, Chen and Ikegami 2005; Yamada, Ikegami and Kuramitsu 2005; Tan *et al.* 2014; Ng *et al.* 2019) may be responsible for the observed cooperative behaviour between the two species in biofilms. These associations are also pertinent in the case of initial attachment of the two species with increased levels of both species in the dual-species nascent biofilms in comparison to corresponding mono-species biofilm (Chathoth *et al.*, manuscript in preparation). A clustered growth of the two bacteria typical to *P. gingivalis-T. denticola* biofilms was observed which resulted in an uneven biofilm spread along the surface (**Figure 7**).

The three-species 24-hour biofilms grown at 8 μ M iron in this study were comparable to the biofilms reported previously in similar conditions of growth (Martin *et al.* 2018). The proportion of *S. gordonii* was the most predominant among all species (over 90 % among all three species) despite a diminished growth of *S. gordonii* in the presence of *P. gingivalis* and *T. denticola* in the three-species biofilms compared to *S. gordonii* mono-species biofilms. The increased initial cell concentration of *P. gingivalis* in the three-species biofilm compared to mono-species biofilms may be attributed to the beneficial association with *S. gordonii* (McNab *et al.* 2003; Maeda *et al.* 2004; Park *et al.* 2005; Simionato *et al.* 2006) and *T. denticola* (Kuramitsu, Chen and Ikegami 2005; Yamada, Ikegami and Kuramitsu 2005; Tan *et al.* 2014; Ng *et al.* 2019) in the initial attachment of these bacteria (Chathoth *et al.*, manuscript in preparation). Moreover, in this study, the three-species biofilms displayed a prominent effect of iron on both the growth of each species and the architecture of the biofilms at especially 144 hours with an increased preference for 80 μ M of iron (**Figures: 6, 7, 8**). All three bacterial species showed higher growth at 80 μ M of iron in comparison to other iron levels. A similar increase in the concentration of attached *P. gingivalis* and *T. denticola* cells to the nascent three-species biofilm was previously observed (Chathoth *et al.*, manuscript in preparation). This increased attachment of the two pathogens along with an additional effect of 80 μ M FeSO₄ on biofilm growth, especially during later stages of biofilm growth is probably the reason for the increased cell concentration observed in comparison to monospecies biofilms.

Several studies have emphasized on the possible link between iron and severe periodontitis. Comparative metatranscriptomic analysis of oral biofilm from periodontitis patients and healthy individuals have highlighted the correlation between the overexpressed genes of iron transport system and severe periodontitis (Duran-Pinedo et al. 2014; Yost et al. 2015). More recently, researchers have described the correlation between the systemic iron overload in hemochromatosis patients and chronic periodontitis (Meuric et al. 2017; Boyer et al. 2018, 2020). These results are somewhat contradictory since the overexpression of the genes involved in the acquisition of iron generally takes place in conditions of iron deficiency. This suggests that metabolites capable of sequestering iron could be produced secondarily in the mixed biofilm. However, not much is known about the direct effect of iron on the periodontal biofilm. Therefore, through this study we tried to elucidate the direct influence of iron levels and the interspecies bacterial associations in the development of subgingival biofilms. The increased growth of all the three species in our oral biofilm model at high iron level (80 μ M) is in line with a recent hypothesis on iron, among others as a stimulus for periodontitis. Chathoth et al. (2020), hypothesized that high iron levels along with H₂O₂ (from peroxygenic bacteria such as S. gordonii) through the generation of harmful hydroxyl radicals initiate bacterial stress response resulting in glutathione (GSH) production. This may further lead to hydrogen sulphide (H₂S) production by anaerobes (P. gingivalis and T. denticola) especially in BSA containing media such as the one used in this study. Hydrogen sulphide produced by anaerobes result in a reduced redox potential that may contribute to the prevalence of these anaerobic pathogens in mixed biofilms.

CONCLUSION

In short, our work, for the first time described the direct influence of iron on the development of oral biofilms *in vitro*. In this study, the three-species oral biofilm model displayed an increased preference for 80 μ M of iron especially in mature biofilms of 144 hours with elevated

levels of all three species (compared to 0.8 and 8 μ M of iron) unlike the mono-species biofilms. This increase in the three-species biofilm at especially high iron level may lead to oral dysbiosis and progression of periodontitis. This study will therefore enable better understanding of iron-related pathogenesis and will help in the development of preventive strategies to combat periodontitis.

REFERENCES

Ammann TW, Bostanci N, Belibasakis GN *et al.* Validation of a quantitative real-time PCR assay and comparison with fluorescence microscopy and selective agar plate counting for species-specific quantification of an *in vitro* subgingival biofilm model. *J Periodontal Res.* 2013;48:517–26.

Beukers NGFM, Heijden GJMG van der, Wijk AJ van *et al.* Periodontitis is an independent risk indicator for atherosclerotic cardiovascular diseases among 60 174 participants in a large dental school in the Netherlands. *J Epidemiol Community Health* 2017;71:37–42.

Boyer E, Le Gall-David S, Martin B *et al.* Increased transferrin saturation is associated with subgingival microbiota dysbiosis and severe periodontitis in genetic haemochromatosis. *Sci Rep* 2018;8:15532.

Boyer E, Martin B, Gall-David SL *et al*. Periodontal pathogens and clinical parameters in chronic periodontitis. *Mol Oral Microbiol* 2020;35:19–28.

Bui FQ, Almeida-da-Silva CLC, Huynh B *et al.* Association between periodontal pathogens and systemic disease. *Biomed J* 2019;42:27–35.

Byrne SJ, Dashper SG, Darby IB *et al.* Progression of chronic periodontitis can be predicted by the levels of *Porphyromonas gingivalis* and *Treponema denticola* in subgingival plaque. *Oral Microbiol Immunol* 2009;24:469–77.

Chan ECS, Siboo R, Keng T *et al. Treponema denticola* (ex Brumpt 1925) sp. nov., nom. rev., and Identification of New Spirochete Isolates from Periodontal Pockets. *Int J Syst Bacteriol* 1993;43:196–203.

Chathoth K, Martin B, Cornelis P *et al*. The events that may contribute to subgingival dysbiosis: a focus on the interplay between iron, sulfide and oxygen. *FEMS Microbiol Lett* 2020;367, DOI: 10.1093/femsle/fnaa100.

Chen CC, Teng LJ, Chang TC. Identification of Clinically Relevant Viridans Group Streptococci by Sequence Analysis of the 16S-23S Ribosomal DNA Spacer Region. *J Clin Microbiol* 2004;42:2651–7.

Duran-Pinedo AE, Chen T, Teles R *et al.* Community-wide transcriptome of the oral microbiome in subjects with and without periodontitis. *ISME J* 2014;8:1659–72.

Graves DT, Corrêa JD, Silva TA. The Oral Microbiota Is Modified by Systemic Diseases. *J Dent Res* 2019;98:148–56.

Grenier D. Nutritional interactions between two suspected periodontopathogens, *Treponema* denticola and Porphyromonas gingivalis. Infect Immun 1992;60:5298–301.

Gursoy UK, Pöllänen M, Könönen E *et al.* Biofilm formation enhances the oxygen tolerance and invasiveness of *Fusobacterium nucleatum* in an oral mucosa culture model. *J Periodontol* 2010;81:1084–91.

Heydorn A, Nielsen AT, Hentzer M *et al.* Quantification of biofilm structures by the novel computer program COMSTAT. *Microbiol.* 2000;146 (Pt 10):2395–407.

Hujoel PP, Drangsholt M, Spiekerman C *et al.* An Exploration of the Periodontitis–Cancer Association. *Ann Epidemiol* 2003;13:312–6.

Koziel J, Mydel P, Potempa J. The Link Between Periodontal Disease and Rheumatoid Arthritis: An Updated Review. *Curr Rheumatol Rep* 2014;16.

Kreth J, Zhang Y, Herzberg MC. Streptococcal Antagonism in Oral Biofilms: *Streptococcus sanguinis* and *Streptococcus gordonii* Interference with *Streptococcus mutans*. *J Bacteriol* 2008;190:4632–40.

Kuboniwa M, Tribble GD, James CE *et al. Streptococcus gordonii* utilizes several distinct gene functions to recruit *Porphyromonas gingivalis* into a mixed community. *Mol Microbiol* 2006;60:121–39.

Kuramitsu HK, Chen W, Ikegami A. Biofilm Formation by the Periodontopathic Bacteria *Treponema denticola* and *Porphyromonas gingivalis*. *J Periodontol*. 2005;76:2047–51.

Lamont RJ, Hajishengallis G. Polymicrobial synergy and dysbiosis in inflammatory disease. *Trends Mol Med* 2015;21:172–83.

Leschine SB, Canale-Parola E. Rifampin as a selective agent for isolation of oral spirochetes. *J Clin Microbiol* 1980;12:792–5.

López R. Periodontal disease and adverse pregnancy outcomes. Evid Based Dent. 2008;9:48.

Maeda K, Nagata H, Yamamoto Y *et al.* Glyceraldehyde-3-phosphate dehydrogenase of *Streptococcus oralis* functions as a coadhesin for *Porphyromonas gingivalis* major fimbriae. *Infect Immun* 2004;72:1341–8.

Martin B, Chathoth K, Ouali S *et al.* New growth media for oral bacteria. *J Microbiol Methods* 2018;153:10–3.

Martin B, Tamanai-Shacoori Z, Bronsard J et al. A new mathematical model of bacterial interactions in two-species oral biofilms. Amar S (ed.). *PLoS ONE* 2017;12:e0173153.

McNab R, Ford SK, El-Sabaeny A *et al.* LuxS-based signaling in *Streptococcus gordonii*: autoinducer 2 controls carbohydrate metabolism and biofilm formation with *Porphyromonas gingivalis*. *J Bacteriol* 2003;185:274–84.

Meuric V, Lainé F, Boyer E *et al.* Periodontal status and serum biomarker levels in HFE haemochromatosis patients. A case-series study. *J Clin Periodontol* 2017;44:892–7.

Meuric V, Martin B, Guyodo H *et al. Treponema denticola* improves adhesive capacities of *Porphyromonas gingivalis. Mol Oral Microbiol* 2013;28:40–53.

Ng HM, Slakeski N, Butler CA *et al.* The Role of *Treponema denticola* Motility in Synergistic Biofilm Formation With *Porphyromonas gingivalis. Front Cell Infect Microbiol* 2019;9.

Nwizu N, Wactawski-Wende J, Genco RJ. Periodontal disease and cancer: Epidemiologic studies and possible mechanisms. *Periodontol 2000* 2020;83:213–33.

Ooi XJ, Tan KS. Reduced Glutathione Mediates Resistance to H₂S Toxicity in Oral Streptococci. *Appl Environ Microbiol* 2016;82:2078–85.

Orth RK-H, O'Brien-Simpson NM, Dashper SG *et al.* Synergistic virulence of *Porphyromonas gingivalis* and *Treponema denticola* in a murine periodontitis model. *Mol Oral Microbiol* 2011;26:229–40.

Park Y, Simionato MR, Sekiya K *et al.* Short fimbriae of *Porphyromonas gingivalis* and their role in coadhesion with *Streptococcus gordonii*. *Infect Immun* 2005;73:3983–9.

Pöllänen MT, Paino A, Ihalin R. Environmental Stimuli Shape Biofilm Formation and the Virulence of Periodontal Pathogens. *Int J Mol Sci* 2013;14:17221–37.

Preshaw PM, Alba AL, Herrera D *et al.* Periodontitis and diabetes: a two-way relationship. *Diabetologia* 2012;55:21–31.

Sbordone L, Bortolaia C. Oral microbial biofilms and plaque-related diseases: microbial communities and their role in the shift from oral health to disease. *Clin Oral Invest* 2003;7:181–8.

Sedlacek MJ, Walker C. Antibiotic resistance in an *in vitro* subgingival biofilm model. *Oral Microbiol Immunol* 2007;22:333–9.

Shumi W, Lim J, Nam S-W *et al*. Environmental factors that affect *Streptococcus mutans* biofilm formation in a microfluidic device mimicking teeth. *BioChip J* 2010;4:257–63.

Simionato MR, Tucker CM, Kuboniwa M et al. Porphyromonas gingivalis genes involved in community development with *Streptococcus gordonii*. Infect Immun 2006;74:6419–28.

Tan KH, Seers CA, Dashper SG *et al. Porphyromonas gingivalis* and *Treponema denticola* Exhibit Metabolic Symbioses. *PLOS Pathogens* 2014;10:e1003955.

Wang BY, Chi B, Kuramitsu HK. Genetic exchange between *Treponema denticola* and *Streptococcus gordonii* in biofilms. *Oral Microbiol Immunol* 2002;17:108–12.

Watanabe T, Maruyama F, Nozawa T *et al.* Complete Genome Sequence of the Bacterium *Porphyromonas gingivalis* TDC60, Which Causes Periodontal Disease. *J Bacteriol* 2011;193:4259–60.

Welin-Neilands J, Svensäter G. Acid tolerance of biofilm cells of *Streptococcus mutans*. *Appl Environ Microbiol* 2007;73:5633–8.

Yamada M, Ikegami A, Kuramitsu HK. Synergistic biofilm formation by *Treponema denticola* and *Porphyromonas gingivalis*. *FEMS Microbiol Lett* 2005;250:271–7.

Yost S, Duran-Pinedo AE, Teles R *et al.* Functional signatures of oral dysbiosis during periodontitis progression revealed by microbial metatranscriptome analysis. *Genome Med* 2015;7:27.

Zhu L, Kreth J. The role of hydrogen peroxide in environmental adaptation of oral microbial communities. *Oxid Med Cell Longev* 2012;2012:717843.

Zhu Y, Dashper SG, Chen Y-Y *et al. Porphyromonas gingivalis* and *Treponema denticola* Synergistic Polymicrobial Biofilm Development. *PLoS ONE* 2013;8:e71727.

FIGURES



Figure 1: Growth of mono-species biofilms at various iron concentrations. The graph shows the concentration (CFU/ml) of *P. gingivalis* cells (A), *T. denticola* cells (B) and *S. gordonii* cells (C) grown as biofilms/sessile cells across different time points (2, 24, 48, 72 and 144 hours) at three iron concentrations (0.8, 8 and 80 μ M of iron). a = p value between 0.8 and 8 μ M is \leq 0.05; b = p value between 8 and 80 μ M is \leq 0.05.



Figure 2: Microscopic parameters for mono-species biofilms. The graphs show the values for bio-volume (A, C, E) and roughness coefficient (B, D, E) for *P. gingivalis* (A, B), *T. denticola* (C, D) and *S. gordonii* (E, F) mono-species biofilms across different time points (2, 24, 48, 72 and 144 hours) at three iron concentrations (0.8, 8 and 80 μ M of iron). * = p ≤ 0.05.



Figure 3: Effect of interspecies interactions at optimal iron concentration for multi-species biofilms. The concentration of individual species (CFU/ml) in different biofilms (mono-species biofilms: *S. gordonii*, *P. gingivalis* and *T. denticola*; dual-species biofilms: *S. gordonii-P. gingivalis*, *S. gordonii-T. denticola*, *P. gingivalis-T. denticola*; three-species biofilms: *S. gordonii-P. gingivalis-T. denticola*) grown at optimal (8 μ M) iron levels were compared. The graphs show the concentration of *S. gordonii* (A), concentration of *P. gingivalis* (B) and concentration of *T. denticola* (C) in the different biofilms across various time points (2, 24, 48, 72 and 144 hours).



Figure 4: The microscopic parameters obtained for various mono-, dual- and three-species biofilms grown at optimal iron concentration. The microscopic parameters like bio-volume (A) and roughness coefficient (B) were compared between different biofilms (mono-species biofilms: *S. gordonii, P. gingivalis* and *T. denticola*; dual-species biofilms: *S. gordonii-P. gingivalis, S. gordonii-T. denticola, P. gingivalis-T. denticola*; three-species biofilms: *S. gordonii-P. gingivalis-T. denticola*) grown at optimal (8 μM) iron levels across various time points (2, 24, 48, 72 and 144 hours). The microscopic parameters were calculated on the total biofilm in each condition (comprised of all cells irrespective of individual species).



Figure 5: Representative 3-dimentional images obtained for mono and multi-species 72-hour biofilms at optimal iron concentration. The 3-dimentional images were generated using the Imaris Viewer 9.6 software using the biofilm stack obtained using the Leica TCS-SP5 confocal laser scanning microscope. 3-D images of various 72-hour biofilms (A-G) (mono-species: *S. gordonii, P. gingivalis, T. denticola*; dual-species: *S. gordonii-P. gingivalis, S. gordonii-T. denticola, P. gingivalis-T. denticola*; three-species: *S. gordonii-P. gingivalis-T. denticola*) and their respective X-Z projection or thickness (a-g) are shown. The scale (20 µm) is shown on the bottom left corner of each image.



Figure 6: Growth of individual species in *S. gordonii-P. gingivalis-T. denticola* biofilms at various iron concentrations. The graph shows the concentration (CFU/ml) of *S. gordonii* (A) *P. gingivalis* (B) and *T. denticola* (C) cells grown as biofilms/sessile cells across different time points (2, 24, 48, 72 and 144 hours) at three iron concentrations (0.8, 8 and 80 μ M of iron). a = p value between 0.8 and 80 μ M is \leq 0.05; b = p value between 8 and 80 μ M is \leq 0.05.



Figure 7: Microscopic parameters for *S. gordonii-P. gingivalis-T. denticola* biofilms. The graphs show the values for bio-volume (A) and roughness coefficient (B) for *S. gordonii-P. gingivalis-T. denticola* biofilms across different time points (2, 24, 48, 72 and 144 hours) at three iron concentrations (0.8, 8 and 80 μM of iron).



Figure 8: Representative 3-dimentional images obtained for three-species 144-hour biofilms at various iron concentration. The 3-dimentional images were generated using the Imaris Viewer 9.6 software using the Z stack obtained using the Leica TCS-SP5 confocal laser scanning microscope. 3-D images of the 144-hour *S. gordonii-P. gingivalis-T. denticola* biofilms and their respective X-Z projection or thickness are shown at 0.8, 8, and 80 µM of FeSO₄. The scale (20 µm) is shown on the bottom left corner of each image.

TABLES

Table 1: List of primers used for the study.

Organism	Sequence $(5' \rightarrow 3')$
Streptococcus gordonii	F: AAG-CAA-CGC-GAA-GAA-CCT-TA
	R: GTC-TCG-CTA-GAG-TGC-CCA-AC
Porphyromonas gingivalis	F: TGG-GTT-TAA-AGG-GTG-CGT-AG
	R: CAA-TCG-GAG-TTC-CTC-GTG-AT
Treponema denticola	F: CGC-GTC-CCA-TTA-GCT-AGT-TG
	R: TTC-TTC-ATT-CAC-ACG-GCG-TC

SUPPLEMENTARY FIGURES



 $8 \ \mu M$ of iron

Supplementary figure 1: Representative microscopic images of *T. denticola* biofilms at 24 and 144 hours grown at 8 μ M of iron. The sessile *T. denticola* biofilms were stained using Styo®9 and were visualized using the Leica TCS-SP5 confocal laser scanning microscope. A maximum z-projection of the Z stack was taken using 40X oil immersion objective lens and a numerical zoom of 2 was applied. The scale (10 μ m) is shown on the bottom right corner of both images.



Supplementary figure 2: Percentages of each species in dual- and three-species biofilms. The graph shows the percentages of *S. gordonii* and/or *P. gingivalis* and/or *T. denticola* in *S. gordonii-P. gingivalis* biofilms (A), *S. gordonii-T. denticola* biofilms (B), *P. gingivalis-T. denticola* biofilms (C) and *S. gordonii-P. gingivalis-T. denticola* biofilms (D) across different time points (2, 24, 48, 72 and 144 hours) at 8 μM of iron).



Supplementary figure 3: The total bacterial concentration in mono-, dual- and three-species biofilms grown at optimal iron concentration. The bacterial concentrations (sum of each bacterial species) were compared between different biofilms (mono-species biofilms: *S. gordonii, P. gingivalis* and *T. denticola*; dual-species biofilms: *S. gordonii-P. gingivalis, S. gordonii-T. denticola, P. gingivalis-T. denticola*; three-species biofilms: *S. gordonii-P. gingivalis-T. denticola*) grown at optimal (8 μM) iron levels across various time points (2, 24, 48, 72 and 144 hours).



Supplementary figure 4: The average thickness of various mono-, dual- and three-species biofilms grown at optimal iron concentration. The values for average thickness were compared between different biofilms (mono-species biofilms: *S. gordonii*, *P. gingivalis* and *T. denticola*; dual-species biofilms: *S. gordonii-P. gingivalis*, *S. gordonii-T. denticola*, *P. gingivalis-T. denticola*; three-species biofilms: *S. gordonii-P. gingivalis-T. denticola*) grown at optimal (8 µM) iron levels across various time points (2, 24, 48, 72 and 144 hours). The microscopic parameters were calculated on the total biofilm in each condition (comprised of all cells irrespective of individual species).



Supplementary figure 5: Average thickness of *S. gordonii-P. gingivalis-T. denticola* biofilms. The graphs show the values for average thickness for *S. gordonii-P. gingivalis-T. denticola* biofilms across different time points (2, 24, 48, 72 and 144 hours) at three iron concentrations (0.8, 8 and 80 µM of iron).

4.4. Mathematical modelling as a tool to study the interspecies associations and effect of iron on oral biofilm development

INTRODUCTION

Streptococcus gordonii, Porphyromonas gingivalis and Treponema denticola are part of oral bacteria whose co-operations are known to influence the evolution of periodontal disease. Moreover, as already mentioned, systemic iron levels may trigger a pathogenic switch in periodontal multi-species biofilms. To gain insight about interactions between species, a step-bystep analysis of biofilm formation was performed on biofilms composed of S. gordonii, P. gingivalis and T. denticola. In a first step, we studied species reciprocal effects during biofilm initiation (Chathoth *et al*, submitted, **Chapter 4.1.**), analysing adhesion and initial growth of bacteria during the first 2 hours of biofilm formation, focusing on iron effect. In a second step, the phase of biofilm growth and development from 2 to 144 hours was studied, with different combinations of species in two and three-species biofilms, as compared to their respective monospecies counterparts (Chathoth et al, submitted, Chapter 4.2.). The results obtained by the biological experiments on the interspecies interactions at 8 µM iron showed a mutual beneficial effect of both the species in *P. gingivalis-T. denticola* biofilm as compared to the respective monospecies biofilms. In contrast, it was noted that S. gordonii and T. denticola hampered growth of the other in dual-species biofilms in comparison to their respective mono-species biofilms. Finally, in the three-species biofilms, growth of S. gordonii was decreased by P. gingivalis and T. denticola. To evaluate the effect of iron on biofilm growth, three iron levels were tested (0.8, 8 and 80 μ M of FeSO₄). Mono-species biofilms of *P. gingivalis* and *T. denticola*, grew slightly better at 8 µM of iron when compared to either 0.8 or 80 μ M. In the three-species biofilms, all three bacterial species showed higher growth at high levels (80 µM) of iron in comparison to other iron levels.

To fully explore interactions between three species including the effect of iron, we intended to establish a mathematical model based on experimental data. The main objective of the following work was therefore to understand the interspecies associations in the same dual- and three-species experimental biofilms and to decipher the influence of iron levels, focusing on the growth and development phase of biofilm formation.

Mathematical models for microbiological biofilms exist in various forms (Dzianach *et al.* 2019) ranging from simple models which provide empirical correlations to sophisticated and

computationally intense models that give mechanistic and physics-based information that describe the three-dimensional biofilm structure and its association with the environment. The biofilm growth can be modelled using methods which describe either only the species composition with no spatial information (Shen et al. 2016) or provide detailed information on depth of the biofilm structure (Wanner and Reichert 1996). One-dimensional mathematical model of the dental plaque that considers only the planar dental plaque geometry has been previously proposed (Ilie, van Loosdrecht and Picioreanu 2012). The modelling of multi-dimensional multispecies biofilms is possible by multiple approaches. There are mainly either the continuum or continuous mechanical models that assume that adjacent elements are not perceptibly different from each other (Klapper and Dockery 2010) or discrete element-based models which take into account the behaviour of an individual microorganism (Fujikawa 1994; Wimpenny and Colasanti 1997; Picioreanu, van Loosdrecht and Heijnen 1998; Noguera et al. 1999; Kreft et al. 2001). The continuum models are classified as one-dimensional (1D) and multidimensional continuum models. Whereas the discrete models are divided in Cellular Automaton (CA) models and Individual-based Models (IbMs). The individual-based modelling is used to study various multispecies biofilm problems (Picioreanu, Kreft and van Loosdrecht 2004; Fagerlind et al. 2012; Chihara et al. 2015; Martin, Picioreanu and Nerenberg 2015). IbMs can distinguish between spreading mechanisms adopted by different bacteria and operates at the highest spatial resolution level relevant in a biofilm (Picioreanu, Loosdrecht and Lens 2003). However, IbMs are computationally intensive and assume that individual microorganisms behave as hard spheres, which is contrary to the real scenario (Laspidou, Kungolos and Samaras 2010). The Cellular Automaton are represented in an array of small compartments as opposed to the IbM that uses particles located anywhere in space. CA can be used for modelling dual-species (Noguera et al. 1999) and multispecies biofilms to study syntrophic relationship (Tang and Liu 2017), independence or competition of substrates or toxicity caused by one species to another (Martin et al. 2017). CA are especially suitable to model old and aged biofilms, characterized by the presence of cavities (Laspidou, Kungolos and Samaras 2010).

In this study we used the two-dimensional individual based Cellular automaton to represent the growth and development of a three-species biofilm for studying interspecies interactions and influence of iron levels. This study was focussed on the growth of static three-species biofilms without taking cell death into account mainly because of the experimental limitation in measuring the viability of anaerobic bacteria used in this study (as detailed in **Chapter 3.3.**). Also, inclusion of cell death will add another level of complexity to the model. The major challenge was to model the growth of the spirochete bacterium T. denticola owing to its spiral form and length. Therefore, for simplifying the process, we use the initial microscopic parameters like bio-volume, roughness, mean thickness etc. of the mono-species biofilm of T. denticola for simulating its growth. However, it would be of interest to simulate the growth/division of this bacteria by taking into consideration its length and the number of elements it occupies in the lattice. This bacterium, along with the other two, further increase the complexity of the model. In the cellular automaton model, each bacterial cell is considered as an individual, independent entity with a unique state and behaviour. This feature is particularly useful for studying such complex systems like biofilms, where individual heterogeneity is prevalent. The three different species of bacteria used in the present study is a good example for bacterial heterogeneity as they vary in their size and form (S. gordonii-coccus, P. gingivalis- bacillus and T. denticola- bacillus spirochete). The oral biofilms depend on the availability of nutrients/substrates, oxygen supply, iron, pH and its distribution to different parts of the biofilm via diffusion. Growth characteristics and energy metabolism within biofilms are also crucial for their growth and development. Our model will take into account all these parameters along with an additional parameter for damage. Damage can limit cell growth and even lead to cell dispersal which can, in turn, affect biofilm structure and development, without decreasing viability. These parameters were calibrated by measurements made from biological experiments in monospecies biofilms. This mathematical model will help in understanding the nature of the interactions between the three species in the biofilm along with the effect of iron by testing different hypotheses for either independence or competition for substrate between species, mutual benefit/syntrophy or production of toxic molecules. Finally, the validation of the model will be done by comparing the simulated results with those of the experimental results obtained for dual- and three-species biofilms.

As the first step, the biological results from the mono-species biofilms grown from 2 to 72 hours at various iron concentrations were used to obtain simulated results using mathematical equations. The focus of this model being biofilm growth, the experimental data obtained at 144 hours was excluded as the biofilm growth declined post 72 hours. The data of biofilm attached at 2 hours were used to set the initialization parameters and thus were considered as the 0-hour time point and consequently, the other time points were 22, 46 and 70 hours (instead of 24, 48 and 72 hours) in the model. Using these simulated results for biofilm initialization, the dual-species

biofilms were simulated and tested for various hypotheses for species interactions at one iron concentration (8 μ M FeSO₄). Next, the three-species biofilms will be simulated at 8 μ M iron and tested for different hypotheses for interspecies interactions. Finally, the effect of iron on the interaction between the species will be analysed by testing the hypotheses for species interactions between dual- and three-species biofilms at the different iron levels (0.8 and 80 μ M FeSO₄).

METHODS

Mathematical model

The mathematical model in this study is primarily based on and further adapted from the work of Martin *et al.*,(2017). The authors studied a dual-species (*S. gordonii-P. gingivalis*) oral biofilm system with a Cellular Automaton model where three hypotheses on the relationship between the two species of bacteria, namely independence for substrates, competition for substrate, and inhibition of one to another, were tested. The model is being implemented thanks to Dr. Fabrice Mahé from the IRMAR (Institut de Recherche Mathematique de Rennes) from University Rennes 1.

Simulation domain and cellular automaton

The biofilm domain was made up of a two-dimensional lattice of dimensions $L_x \ge L_z$ which was spatially discretized (**Figure 1**). Each element in the lattice can harbour bacterial cells. The bacterial biomass in this element was defined by biomass density $c_x^{(i)}$. When the biomass density reached a value greater than the maximum biomass concentration $c_{xm}^{(i)}$ there is cell division: half of the biomass remained in the same element in the lattice while the other half either randomly took up a free neighbouring element or replaced one of the neighbouring cells. This process was repeated with each replaced element to have a biofilm. The bottom of the lattice represented the substratum upon which the biofilm was allowed to develop. A reservoir of substrate was considered infinite as it was assumed to be larger than the height of the biofilm. The height L_z of the computing domain was set to be equal to the sum of the height of the biofilm and the thickness of a diffusive boundary layer $l_b = 18 \ \mu m$. The boundary conditions for computing the concentration of the substrate $c_s^{(i)}$ were the following: the concentration of the substrate $c_s^{(i)}(t, x, y)$ on the top of the domain Γ_2 was equal to its constant value $c_{s0}^{(i)}$ in the infinite reservoir, the effect of the substratum was modelled by a zero-flux boundary condition, $\partial c_s^{(i)}/\partial z = 0$ on Γ_4 , and a periodic condition was used on the lateral boundaries Γ_1 and Γ_3 to avoid edge effects.



Figure 1: Schematic of the modelling domain and grid of the two-dimensional cellular automaton. (Adapted from Martin *et al.*, 2017). See text for detail.

According to experimental dimensions, the width of the domain L_x was set at 123 µm. To obtain realistic biofilms with small quantities of biomass the size of the volume element (containing bacterial cells and its extracellular constituents) of the lattice Δx was set at 0.615 µm (instead of 1.23 µm in Martin *et al.*, 2017) and there were 200 elements in each row of the two-dimensional grid.

Definition of substrate, bacteria and parameters

The concentration of the substrate for the bacterium *i* (*i* = 1 for *S. gordonii*, *i* = 2 for *P. gingivalis*, *i* = 3 for *T. denticola*) was given by $c_s^{(i)}(t, x, z)$ at the time *t* and the spatial point of coordinates (*x*, *z*). The other parameters specific to each bacterial cell were the maximum specific growth rate $\mu_m^{(i)}$, the yield coefficient $Y_{xs}^{(i)}$ which measures the efficiency of the transformation of the substrate in bacterium bio-volume, the maintenance coefficient $m_s^{(i)}$ and the maximum biomass concentration $c_{xm}^{(i)}$. The mean values of these parameters were reported in **Table 1**. These mean values along with the random draws with a Gaussian distribution were used to generate values for every new bacterium created with a standard deviation of 5% (Kreft, Booth and Wimpenny 1998). As an initial step, the attachment of bacteria on the substratum was modelled by semi-randomly placing elements with bacteria on the substratum without any damage and a random value of biomass concentration between $c_{xm}^{(i)}$ and $c_{xm}^{(i)}/2$, to fit the initial measurements.

The damage concentration in the bacterium *i* was given by $w^{(i)}$.

Model of substrate diffusion and reaction in mono-species biofilms

The substrate diffusion and reaction in mono-species biofilms were modelled at three varying iron levels (0.8, 8 and 80 μ M FeSO₄). For each bacterium *i*, the dynamics of concentration of the substrate $c_s^{(i)}$ was given by the following reaction-diffusion equation,

$$\frac{\partial c_s^{(i)}}{\partial t} = D_s^{(i)} \triangle c_s^{(i)} - r_s^{(i)}(c_s^{(i)}, c_x^{(i)})$$
(1)

where,

 $c_s^{(i)}$ is the substrate concentration $c_x^{(i)}$ is the Biomass concentration $\triangle = \frac{\partial^2}{\partial x^2} + \frac{\partial^2}{\partial z^2}$ is the Laplace operator $D_s^{(i)}$ is the diffusion coefficient $r_s^{(i)}(c_s^{(i)}, c_x^{(i)})$ is the rate of substrate consumption

The rate of substrate consumption depends on the concentration of both biomass and substrate at a considered point and was given by the following equation,

$$r_{s}^{(i)}(c_{s}^{(i)}, c_{x}^{(i)}) = \left(\frac{\mu_{m}^{(i)}}{Y_{xs}^{(i)}} + m_{s}^{(i)}\right) c_{x}^{(i)} \frac{c_{s}^{(i)}}{K_{s}^{(i)} + c_{s}^{(i)}}$$
(2)

where,

 $m_s^{(i)}$ is the maintenance coefficient $\mu_m^{(i)}$ is the maximum specific growth rate $Y_{xs}^{(i)}$ is the growth yield coefficient $K_s^{(i)}$ is the half-saturation coefficient $r_s^{(i)}(c_s^{(i)}, c_x^{(i)})$ is the rate of substrate consumption

The variations in the damage concentrations were governed by the following equation,

$$\frac{\partial w^{(i)}}{\partial t} = \alpha^{(i)} \left[r_s^{(i)}(c_s^{(i)}, c_x^{(i)}) + m_s^{(i)} c_x^{(i)} \right] - \beta^{(i)} w^{(i)}$$
(3)

where,

 $w^{(i)}$ is the damage concentration $\alpha^{(i)}$ is the damage conversion factor $m_s^{(i)} c_x^{(i)}$ is the endogenous metabolism $\beta^{(i)}$ is the rate of damage removal $r_s^{(i)} (c_s^{(i)}, c_x^{(i)})$ is the rate of substrate consumption

The damages are not measured experimentally but were used in the model as a theoretical tool to take into account different possibilities for the alteration in the growth process. The efficiency of the transformation of the substrate to bacterial biomass was supposed to decrease when the level of damage increases in the bacterium and this interdependence between these different biological processes was given by the following equation,

$$\frac{\partial c_x^{(i)}}{\partial t} = \frac{Y_{xs}^{(i)}}{1 + e^{\gamma^{(i)}(w^{(i)} - \delta^{(i)})}} \left(r_s^{(i)}(c_s^{(i)}, c_x^{(i)}) - m_s^{(i)}c_x^{(i)} \right)$$
(4)

where,

 $\gamma^{(i)}$ damage coefficient 1 $\delta^{(i)}$ damage coefficient 2 $m_s^{(i)} c_x^{(i)}$ is the endogenous metabolism e is the Napier's constant (e ~ 2.71828) $r_s^{(i)}(c_s^{(i)}, c_x^{(i)})$ is the rate of substrate consumption

Values of the damage parameters are given in Table 2.

The Equations (3) and (4) were solved by an explicit method of the fourth order obtained by Taylor development with a time step $\Delta t = 1$ hour. Since the diffusion of the substrate was much faster than the growth of the biomass, we solved the stationary equation corresponding to Equation (1) after each modification of the biomass. We used the classical second order central finite difference method with the step Δx to solve it. At the beginning, the substrate concentration was initialized to $c_{s0}^{(i)}$ in the whole domain.

The Equation (1) to (4) allowed the study of the growth of mono-bacterial biofilms.

Simulation of interaction between bacterial species in two-species biofilms

To study the interaction of the two species in the dual-species biofilms (*S. gordonii- P. gingivalis, S. gordonii-T. denticola, P. gingivalis-T. denticola*), different hypotheses were tested by different models: independence and competition for nutrients. The hypothesis to be tested was decided based on the results of the biological experiments obtained.

The first hypothesis (independence) was tested for *S. gordonii-P. gingivalis* and *S. gordonii-T. denticola* biofilms where the limiting nutrient is not the same for each bacterium in the same biofilm: proteins (BSA) for *P. gingivalis* and *T. denticola* while glucose for *S. gordonii*. In this case, there should be no competition for the nutrients between bacterial species. This hypothesis was tested with Equations (1) to (4) solved together for both the species.

The second hypothesis (competition for nutrients) was tested for *P. gingivalis-T. denticola* biofilms where the limiting nutrient, protein is supposed to be the same for both bacteria. The mathematical model was still defined by the Equations (1) to (4) for i = 2 and i = 3 but with $c_s^{(2)} = c_s^{(3)}$. But the abundance of nutrient implies that there is almost no competition and it is independence that is tested.

Experimental biofilms

Bacteria and media

Bacterial cultures of *Streptococcus gordonii* Challis DL1 (Chen, Teng and Chang 2004), *Porphyromonas gingivalis* TDC60 (Watanabe *et al.* 2011) and *Treponema denticola* ATCC 35405 (Chan *et al.* 1993) grown in MMBC-3 with 8 μ M FeSO₄ and 0.08 μ M protoporphyrin IX (PPIX) (Martin *et al.* 2018) were used in this study. However, *T. denticola* was first grown in the NOS spirochete medium (Leschine and Canale-Parola 1980) and then sub-cultured in the MMBC-3 with FeSO₄ and PPIX. The three micro-organisms were grown in anaerobic condition at 37°C in an anaerobic chamber (MACS 500, Don Whitley Scientific, United Kingdom) with 10% v:v H₂, 10% v:v CO₂ and 80% v:v N₂.

Determination of bacterial growth parameters.

S. gordonii, P. gingivalis and T. denticola cultures were grown in the MMBC-3 with 8 μ M FeSO₄ and 0.08 μ M PPIX at 37°C in anaerobic conditions. These cultures were then diluted in 1 ml of the same medium at optical density at 600 nm of 0.1 and incubated in an anaerobic chamber at 37°C. The optical densities were read every two hours for 8 hours for *S. gordonii* cultures while at 0, 7, 24, 31, 48 and 55 hours for *P. gingivalis* and *T. denticola* cultures. These growth experiments were performed either in pure or diluted MMBC-3 with either 0.8, 8 or 80 μ M FeSO₄ and constant PPIX (0.08 μ M). Growth curves were established from optical density measurements from triplicate biological samples which allowed to measure growth parameters such as the specific growth rate μ . The half-saturation coefficient K_s and the maximal specific growth rate μ_m were estimated from the μ calculated at various dilutions of MMBC-3 and the corresponding substrate concentration in the medium.

Glucose consumption experiments

Glucose concentrations were estimated in the iron- (FeSO₄) and PPIX-supplemented MMBC-3 medium at 0 hours and after 24 hours of growth of *S. gordonii* cells in the medium. All measurements were done at Biochemistry-toxicology laboratory, CHU Rennes using a glucose meter. The initial optical density of *S. gordonii* cells were set 0.05 and grown for 24 hours in anaerobic conditions at three different iron concentrations (0.8 μ M, 8 μ M 80 μ M) in 1 ml of MMBC-3 medium. At both time points (0 and 24 hours), the cultures were centrifuged (8000xg, 15 min) and the culture supernatant was subjected to glucose estimation while the cell pellets were heated (95°C, 20 min) and the cell concentration was determined by qPCR. Using the concentration of glucose estimated at 0 and 24 hours the quantity of glucose consumed by *S. gordonii* was obtained.

Protein consumption experiments

The concentrations of proteins (bovine serum albumin -BSA) were estimated in the ironand PPIX-supplemented MMBC-3 medium at 0 hours and after 24 hours of growth of either *P. gingivalis* or *T. denticola* cells in the medium. All measurements were done using the PierceTM BCA Protein Assay Kit. Bacterial cells were grown with an initial optical density of 0.1 for 24 hours in anaerobic conditions at three different iron concentrations (0.8 μ M, 8 μ M or 80 μ M FeSO₄) in 1 ml of MMBC-3 medium. At both time points (0 and 24 hours), the cultures were centrifuged (8000xg, 15 mins) and the culture supernatant was subjected to protein estimation while the cell pellets were heated (95°C, 20 min) and the cell concentration was determined by qPCR. Using the concentration of protein estimated at 0 and 24 hours the quantity of protein consumed by either *P. gingivalis* or *T. denticola* cells was obtained.

RESULTS

Determination of biological parameters

Experimental determination of bacterial growth parameters

Growth parameters necessary for the mathematical model were determined experimentally for the three bacterial species in MMBC-3 medium. In the MMBC-3, c_{s0} of proteins for *P. gingivalis* and *T. denticola* was 7.35 g/l while c_{s0} of glucose for *S. gordonii* was 6 g/l.

Previous reports (Martin *et al.* 2017) and SEM experiments performed on isolated cells allowed us to estimate diameters of bacteria before division (max) and after division (mean). From these values and with an estimation of bacterial density of $\rho = 230$ g/l, the volume and the dry weight of each species were calculated (**Table 3**). Using the percentage of volume occupied by bacteria in a biofilm, the volume and the dry weight of a bacterium (mean and max), the maximum biomass concentration c_{xm} was calculated: 44 $kg_x m^{-3}$ for *S. gordonii*, 38 $kg_x m^{-3}$ for *P. gingivalis* and 46 $kg_x m^{-3}$ for *T. denticola*. In addition, numbers of bacteria produced within 24 hours were evaluated by qPCR. Protein and glucose consumption in bacterial culture was measured after 24 hours. These data combined to bacterial size parameters of **Table 3** allowed the estimation of the yield coefficients Y_{xx} for the three bacterial species (**Table 4**).

For each species, the maximum specific growth rates $\mu_m^{(i)}$, the half saturation coefficient $K_s^{(i)}$ and the maintenance coefficient $m_s^{(i)}$ were calculated from growth curves established by measures of optical densities at 600 nm in mono-species bacterial cultures at different dilutions of MMBC-3 (**Table 5**). The following growth model was considered,

$$\frac{dc_x^{(i)}}{dt}(t) = \mu^{(i)}c_x^{(i)}(t), \quad \text{with } \mu^{(i)} = (\mu_m^{(i)} + \theta^{(i)})\frac{c_s^{(i)}}{K_s^{(i)} + c_s^{(i)}} - \theta^{(i)}$$

where $\theta^{(i)} = m_s^{(i)} Y_{xs}^{(i)}$. An inverse problem was solved to obtain the best values for $\mu_m^{(i)}$, $K_s^{(i)}$ and $\theta^{(i)}$ which fit data of **Table 5** such that,

$$\frac{1}{\mu^{(i)}} \simeq \frac{\frac{K_s^{(i)}}{c_s^{(i)}} + 1}{\mu_m^{(i)} - \theta^{(i)} \frac{K_s^{(i)}}{c_s^{(i)}}}.$$

Value of $m_s^{(i)}$ was deduced from values of $\theta^{(i)}$ and $Y_{xs}^{(i)}$. Results are reported in **Table 1**.

Estimation of damage parameters using mono-species biofilms.

As damage parameters for each species ($\alpha^{(i)}$, $\beta^{(i)}$, $\gamma^{(i)}$ and $\delta^{(i)}$) could not be obtained experimentally, these values were estimated on the basis of experimental mono-species biofilms data obtained at substrate concentrations of 7.35 g/ml for proteins and 6 g/ml for glucose at the three iron concentrations (0.8, 8, 80 μ M FeSO₄).

It is important to mention that results shown in this part are based on average thickness measured on the entire surface of the substratum, including eventual voids, and not an average thickness only measured in biomass-containing pixels. For the mathematical 2D model developed in this study, the simulated value of the bio-volume is fully correlated to the mean thickness for flat biofilms with homogenously scattered bacteria, such as *S. gordonii* mono-species biofilms (low roughness coefficients). Values of mean thickness and roughness coefficient were therefore considered to adjust damage parameters.

For each species, the damage conversion factor $\alpha^{(i)}$, the rate of damage removal $\beta^{(i)}$ and the first coefficient of damage effect $\gamma^{(i)}$ were arbitrarily fixed, while $\delta^{(i)}$ parameter were fitted for each iron concentration using the data of the mono-species biofilms. Results obtained are reported in **Table 2**. The initial repartition of elements containing bio-volume was adjusted to fit the simulated value of the mean thickness and roughness coefficient (and if possible other characteristics like bio-volume or maximum thickness) at 0 hour to the experimental value (which corresponds to 2 hours) presented in the previous section (**Chapter 4.1**). Results of simulations with the estimated parameters at the three iron concentrations (0.8, 8 and 80 μ M FeSO4) for each mono-species biofilm (*S. gordonii*, *P. gingivalis* and *T. denticola* biofilms) are presented in **Figure 2**, **Figure 3** and **Figure 4** respectively.



Figure 2: Biofilm growth simulation for *S. gordonii* mono-bacterial biofilm. The graphs show the mean of 50 simulations in blue line with orange star for the hours of experimental measurements. The dotted lines are the curves of minimum and maximum values while the yellow diamonds are the experimental data at 0.8 μ M (A), 8 μ M (B) and 80 μ M (C) of FeSO₄ for *S. gordonii* biofilms with glucose.

For *S. gordonii* biofilms, a good adequacy was obtained between experimental data from mono-species biofilms and simulations, both for average thickness and roughness coefficient at the three iron concentrations (**Figure 2**) and it was therefore possible to obtain damage parameters for this type of biofilm. We can deduce from Equation (4) that more the value of δ (coefficient 2 of damage effect) is important, less the effect of damage is important. In *S. gordonii* mono-species biofilms, the effect of estimated damages was similar between the three iron concentrations (**Table**)

2) to fit experimental data for which no difference was observed between biofilms at the three iron concentrations (previous Chapter 4.2).



Figure 3: Biofilm growth simulation for *P. gingivalis* mono-bacterial biofilm. The graphs show the mean of 50 simulations in blue line with orange star for the hours of experimental measurements. The dotted lines are the curves of minimum and maximum values while the yellow diamonds are the experimental data at 0.8 μ M (A), 8 μ M (B) and 80 μ M (C) of FeSO₄ for *P. gingivalis* biofilms with proteins.



Figure 4: Biofilm growth simulation for *T. denticola* **mono-bacterial biofilm.** The graphs show the mean of 50 simulations in blue line with orange star for the hours of experimental measurements. The dotted lines are the curves of minimum and maximum values while the yellow diamonds are the experimental data at 0.8 μ M (A), 8 μ M (B) and 80 μ M (C) of FeSO₄ for *T. denticola* biofilms with proteins.

In contrast to *S. gordonii* biofilms, experimental data showed that iron concentrations induced effects on both *P. gingivalis* and T. *denticola* mono-species biofilms. Therefore, for *P. gingivalis* biofilms, δ (coefficient 2 of damage effect) had to be increased with decreasing iron concentration to fit experimental data, with the most prominent effect of damage at 80 µM iron (**Table 2**). Similarly, in the case of *T. denticola* biofilms, δ was adjusted to be the highest at 8 µM of iron followed by 80 µM while the least was at 0.8 µM of iron. Therefore, the maximum effect

of damage was at 0.8 μ M for *T. denticola*. For *P. gingivalis* biofilms, at 8 μ M FeSO₄, it was possible to adjust damage parameters to fit simulated biofilms with experimental data for average thickness and roughness coefficients (**Figure 3**). However, as the present mathematical model does not allow biofilms thicknesses to decrease with time, simulated biofilms obtained at 0.8 and 80 μ M fitted with roughness coefficients of experimental biofilms but did not fit with experimental data for average thickness parameter. Work is still in progress to improve simulation of *P. gingivalis* at these iron concentrations, by developing other parameters that would allow a decrease of biofilm thickness with time. Up to now, it will hence be possible only to simulate *P. gingivalis*-containing biofilms at 8 μ M.

For T. denticola biofilms, a good adequacy was obtained between simulated biofilms and experimental data at all iron concentrations until 46 hours. At 70 hours however, as experimental average thickness decreased, only roughness coefficients were similar between simulated and biological data. In contrast with P. gingivalis situation, only experimental average thickness (including empty spaces) was decreasing with time, whereas bio-volumes and bacterial numbers were stable or increased (see results obtained in the Chapter 4.2.). It would be possible that T. denticola within biofilms reorganize by detachment and subsequent reattachment to modify occupancy of the space within the biofilm, with a more condensed form, inducing a decrease in average thickness measurement (including voids) without lowering average thickness of the biofilm only and not bio-volume. Indeed, T. denticola is a mobile bacterium. Therefore, damage parameters obtained for T. denticola will be used for next steps of mathematical modelling, but results obtained after 46 hours must be considered with caution. Another concern encountered was that the bio-volume of these biofilms were higher than the mean thickness. The maximum value that the bio-volume can reach is a value equal to the mean thickness. Therefore, this incompatibility between these values poses a concern. It is either a shortcoming of the microscopy software or a limitation of the method for acquiring the images or measurements of these parameters. We are trying to troubleshoot the same to address this concern and improve the simulated values.

Figure 5 shows the final images of computed biofilms for biomass density represented along the thickness of the biofilms for each simulated mono-species biofilms at 8μ M of FeSO₄.



Figure 5: Simulated image for mono-bacterial biofilm. The figure shows final output of computer simulation for biomass density in different mono-species biofilms: *S. gordonii* (A), *P. gingivalis* (B) and *T. denticola* at 8 μ M of iron at 72 hours. The colour code represents the value of biomass density (between 0.5 and 1 for biofilm, and 0 (blue) where there is no biofilm). It corresponds to the rate c_x / c_{xm} .
Simulation of bi-bacterial biofilm growth at 8 µM of iron

In order to explore the nature of interactions between individual species in biofilms at one iron concentration (8 μ M FeSO₄), the growth of two-species biofilms was simulated using the mathematical model defined by equations (1) to (4) and damage parameters obtained with monospecies biofilms at 8 μ M iron (**Table 2**). Biofilms were first simulated with two different interaction hypothesis (independence or competition) and compared with experimental data obtained at substrate concentrations of 7.3 g/l for proteins and 6 g/l for glucose. For each hypothesis, two different criteria were used to compare the simulated results to the experimental data: the mean thickness of the biofilm (including voids) and the proportion of each species in the two species biofilms.

The first model was based on an independence of bacterial species for substrate and used Equations (1) to (4). This was tested for the dual-species biofilms, *S. gordonii-P. gingivalis* biofilms and *S. gordonii-T. denticola* biofilms. In this model, each species in the model is dependent on a different substrate: proteins for *P. gingivalis* and *T. denticola* while glucose for *S. gordonii*.

The second model, based on a competition for substrate was tested using Equations (1) to (4), with $c_s^{(2)} = c_s^{(3)}$, with the same substrate for both species to be tested. This model was tested for *P. gingivalis-T. denticola* biofilms.

S. gordonii-P. gingivalis biofilm

As previously shown in "**Chapter 4.2.**", *S. gordonii* was the predominant species in dualspecies biofilms with *P. gingivalis*, with more than 98% being the streptococcal species in these biofilms. *P. gingivalis* cell concentration reached a maximum of 1.9% of total cells in MMBC-3 medium supplemented with 8 μ M FeSO₄ and 0.08 μ M PPIX.

The independence hypothesis was first tested on this biofilm type and comparison with experimental data are shown in **Figure 6A**. Using this model, thickness of the simulated biofilm was lower than the corresponding experimental two-species biofilms. As shown in "**Chapter 4.2**.", experimental two-species biofilms exhibited on the contrary a higher bio-volume and average thickness than expected from the sum of the two mono-species biofilms. This is in agreement with the conclusion of the mathematical simulation that growth of these two bacterial species cannot be

considered as independent. Previous experimental results (refer to **Chapter 4.1.**) showed that *S. gordonii* was facilitating *P. gingivalis* attachment to the substratum, contributing for a higher *P. gingivalis* concentration in two-species biofilms. However, growth of each species from 22 to 70 hours was not improved in two-species conditions as compared to mono-species conditions. Therefore, a more complex interaction between both species must be further explored using another model, including reciprocal effects on bio-volume of the biofilm, independently of cell numbers.

S. gordonii-T. denticola biofilm

As reported in "**Chapter 4.2.**", *S. gordonii* was also the predominant species in dual-species biofilms with *T. denticola*, with more than 98% being the streptococcal species and a maximum of 1.1% of *T. denticola* in these biofilms. *T. denticola* cell concentrations were lower in the presence of *S. gordonii* than when alone in mono-species. Whereas, the concentration of *S. gordonii* in *S. gordonii-T. denticola* biofilm were similar to that of mono-species biofilms till 46 hours, after which it decreased. However, biofilm thickness and bio-volumes were higher in two-species *S. gordonii-T. denticola* than expected from the sum of mono-species biofilms corresponding to each species. The independence hypothesis was first tested on this biofilm type and comparison with experimental data are shown in **Figure 6B**.

Using this model, thickness of the simulated biofilm did not fit with experimental data, as experimental values for biofilm thicknesses increased till 22 hours and then decreased until 70 hours while simulated values kept increasing with time up to 70 hours. Only values for roughness coefficient showed good correlation between experimental and simulated values. Therefore, growth of the two bacterial species cannot be considered as independent for the substrate. Both the species displayed a rapid growth till 22 hours and then the growth decreased. Here, a possible beneficial effect of one species towards the other could result in a rapid initial growth of the biovolume. This initial rapid growth may in turn cause greater damages (decrease in growth) in these biofilms at the later stages of growth resulting in a delayed mutual antagonism. This needs to be confirmed using a more complex model.



Figure 6: Biofilm growth simulation for dual-species biofilm. The graphs show the mean of 50 simulations in blue line with star for the hours of experimental measurements. The dotted lines are the curves of minimum and maximum values while the diamonds are the experimental data at 8 μ M of iron for the independence model for *S. gordonii-P. gingivalis* biofilms(A), independence model for *S. gordonii-T. denticola* biofilms(B) and competition model for *P. gingivalis-T. denticola* biofilms(C).

P. gingivalis-T. denticola biofilm

As shown in "Chapter 4.2.", *T. denticola* predominated the dual species *P. gingivalis-T. denticola* biofilms from approximately 80% (at 24 hours) to 94.5% (at 144 hours) and *P. gingivalis* accounted from around 20% (at 24 hours) to 5.5% (at 144 hours). Both species exhibited cell concentrations higher in two-species than in mono-species biofilms, but the two-species biofilms were less thick than *T. denticola* mono-species biofilms coming from an attachment less important.

Since both species utilize proteins as substrates, the second model, based on a competition for substrate was tested for the *P. gingivalis-T. denticola* biofilms, using Equations (1) to (4), with $c_s^{(2)} = c_s^{(3)}$. However, due to high concentrations of proteins in the medium, this hypothesis could be assimilated to an independence interaction. Using this model, thickness of the simulated biofilm was lower than in experimental two-species biofilms (**Figure 6C**). Indeed, experimental showed that not only the bio-volume but also bacterial cell numbers of both species were higher in two-species biofilms as compared to mono-species biofilms. Therefore, growth of the two bacterial species cannot be considered as based on competition for the substrate. In contrast, a possible mutual benefit between the two bacteria with abundant protein may exist and needs to be validated.

DISCUSSION

The main purpose of this study is to understand biofilm development and growth at varying iron concentrations. For this, as a first step, we aim to study the interspecies interactions and growth of the three bacteria across 2 to 144 hours (or 0 to 142 hours in the model) at 8 μ M of iron in a defined and iron-controlled MMBC-3 medium. This will enable us to understand the behaviour of each species with respect to the other in a three-species biofilm and will help further in marking the variations in growth based on varying iron levels.

Simulations using mathematical model were first performed in mono-species biofilms to set up the biological parameters. Comparison of these simulated biofilms with experimental biofilms showed that growth and substrate parameters were not sufficient to adequately reproduce mono-bacterial experimental biofilms. Differences in growth kinetics between the three kinds of mono-bacterial biofilms could have been attributed to differences in intrinsic bacterial growth rate, which is faster for *S. gordonii* in comparison to the other two bacterial species. But to adjust the simulated values of biofilm thickness with the results of biological experiments, it was necessary to introduce a damage parameter in the mathematical model. Damage concentrations are supposed

to increase and to accumulate with bacterial growth and to limit biofilm growth. This damageinduced effect stopped the growth of S. gordonii and T. denticola mono-species biofilms before 22 hours whereas for *P. gingivalis* biofilms this effect appeared only after 46 hours. However, the P. gingivalis biofilms being sparse, the biofilm thickness was lower than S. gordonii as opposed to the results of Martin et al., (2017) where P. gingivalis biofilms were thicker than S. gordonii biofilms at 48 hours in BHIe medium. The higher thickness of P. gingivalis biofilms can be attributed to both an increased growth and initial attachment of the bacterium when grown in rich BHIe as opposed to the low initial attachment in MMBC-3 (Chathoth et al., manuscript in preparation, Chapter 4.1.). Quorum sensing between bacteria may be responsible for the damage parameter as the concentration of quorum sensing molecules increase with the cellular bacterial density and act as a signal for bacteria to decrease growth (Shao and Demuth 2010). This could explain, at least in part, the differences of behaviour in mono-bacterial biofilm growth between the three species. Likewise, it is known that the development of mixed S. gordonii-P. gingivalis biofilms requires the presence of autoinducer (AI)-2 produced by LuxS (McNab et al. 2003). Moreover, the formation of the dual S. gordonii-P. gingivalis biofilm also required the cbe gene, that putatively encodes a chorismate binding enzyme (Kuboniwa et al. 2006). This gene is thought to be involved in the production of *Pseudomonas* quinolone signal-like signalling molecules (signalling molecule founded in *Pseudomonas* species, (Gallagher et al. 2002) in S. gordonii, which could mediate interspecies communication with P. gingivalis.

The influence of iron levels on the effect of damage on the mono-species biofilms were evaluated and observations were different for each mono-species biofilms. *S. gordonii* biofilms showed no alterations in damages based on iron levels. In contrast, the effect of damages in *P. gingivalis* biofilms was the highest at the highest iron concentration tested (80 μ M FeSO₄). *T. denticola* biofilms on the other hand showed the highest damages at 0.8 μ M while the least damages at 8 μ M of iron. However, set up of damage parameters still needs to be improved to obtain better fits between experimental and simulated thicknesses. Additional parameters such as detachment, cycling/oscillations in growth, cell death, etc. could be added to the model to fully reproduce the biological situation.

The two-species biofilm simulations were performed for *S. gordonii-P. gingivalis*, *S. gordonii-T. denticola* and *P. gingivalis-T. denticola* biofilms in comparison to experimental

biofilms in MMBC-3 medium. The *S. gordonii*-containing experimental two-species biofilms were characterized by a strong predominance of *S. gordonii* over either *P. gingivalis* or *T. denticola* and the architecture of these biofilms was very close to *S. gordonii* mono-species biofilms.

The first mathematical model tested was based on the hypothesis of an independent growth of each species in the biofilm. The biofilms tested for independent growth were *S. gordonii-P. gingivalis* and *S. gordonii-T. denticola* biofilms as each species consumed its own nutrient either protein or glucose with a non-limiting high abundance of both nutrients in the medium. In this case, both simulated bi-bacterial biofilms did not fit the independence hypothesis. Interaction between the species must therefore be further explored using more complex models. For the *P. gingivalis-T. denticola* biofilms, the hypothesis of competition of both bacterial species for the same nutrient was tested as both bacteria have the same nutritional requirement. However, this hypothesis did not fit and suggested a mutually beneficial effect of the two species which needs to be validated. This is consistent with the knowledge regarding the synergy, syntrophy and nutritional cross-feeding between *P. gingivalis* and *T. denticola* (Grenier 1992; Meuric *et al.* 2013; Zhu *et al.* 2013; Tan *et al.* 2014; Ng *et al.* 2019).

Next, the two-species biofilms need to be further tested with more complex models. In the case of *S. gordonii-P. gingivalis* biofilms, the concentration of *S. gordonii* cells is lower in comparison to the mono-species biofilms especially at 46 and 70 hours. This might be indicative of a toxic effect of *P. gingivalis* on *S. gordonii* and therefore, the hypothesis for toxicity can be tested. In contrast, higher values for biofilm thickness was observed at 46 and 70 hours compared to mono-species biofilms suggesting a beneficial effect of one species to the other. It is also possible that a combination of both hypotheses takes place where one species is toxic to the other while the other is beneficial. In the case of *S. gordonii-T. denticola* biofilms, the rapid initial growth observed on the bio-volume data may be due to a beneficial effect of one species towards the other which may result in further decrease in growth due to damages. Here it would be interesting to test an initial beneficial effect of the species followed by toxicity/ damages (especially on *T. denticola* by *S. gordonii*). Finally, for *P. gingivalis-T. denticola* biofilms, both species displayed a growth higher than the sum of mono-species biofilms suggesting a mutually beneficial effect. Therefore, it would be interesting to test this hypothesis for mutual benefit and to model the degree of the benefit contributed by each species.

Finally, the simulated results for three-species biofilms will be further generated by developing new mathematical equations to simulate the interactions in the three-species biofilms. The biological results obtained for the three-species biofilms showed that mutual beneficial effect of *P. gingivalis* and *T. denticola* were abolished by the presence of *S. gordonii* and that the growth of *S. gordonii* was more diminished by both *P. gingivalis* and *T. denticola* than these bacteria alone. Therefore, the hypotheses of toxicity of one species to another can be tested for the three-species interactions. All these together will give a better understanding regarding the influence of each bacterial species on the other and their overall role in the development of the oral biofilms.

REFERENCES

- Chan EC, Siboo R, Keng T *et al.* Treponema denticola (ex Brumpt 1925) sp. nov., nom. rev., and identification of new spirochete isolates from periodontal pockets. *Int J Syst Bacteriol* 1993;**43**:196–203.
- Chen CC, Teng LJ, Chang TC. Identification of clinically relevant viridans group streptococci by sequence analysis of the 16S-23S ribosomal DNA spacer region. *J Clin Microbiol* 2004;**42**:2651–7.
- Chihara K, Matsumoto S, Kagawa Y *et al.* Mathematical modeling of dormant cell formation in growing biofilm. *Front Microbiol* 2015;**6**:534.
- Dzianach PA, Dykes GA, Strachan NJC *et al.* Challenges of biofilm control and utilization: lessons from mathematical modelling. *J R Soc Interface* 2019;**16**:20190042.
- Fagerlind MG, Webb JS, Barraud N *et al.* Dynamic modelling of cell death during biofilm development. *J Theor Biol* 2012;**295**:23–36.
- Fujikawa H. Diversity of the growth patterns of *Bacillus subtilis* colonies on agar plates. *FEMS Microbiol Ecol* 1994;**13**:159–67.
- Gallagher LA, McKnight SL, Kuznetsova MS *et al.* Functions required for extracellular quinolone signaling by *Pseudomonas aeruginosa*. *J Bacteriol* 2002;**184**:6472–80.
- Grenier D. Nutritional interactions between two suspected periodontopathogens, *Treponema* denticola and Porphyromonas gingivalis. Infect Immun 1992;60:5298–301.
- Ilie O, van Loosdrecht MCM, Picioreanu C. Mathematical modelling of tooth demineralisation and pH profiles in dental plaque. *J Theor Biol* 2012;**309**:159–75.
- Klapper I, Dockery J. Mathematical Description of Microbial Biofilms. *SIAM Rev* 2010;**52**:221–65.

- Kreft J-U, Booth G, Wimpenny JWT. BacSim, a simulator for individual-based modelling of bacterial colony growth. *Microbiol* 1998;144:3275–87.
- Kreft JU, Picioreanu C, Wimpenny JW *et al.* Individual-based modelling of biofilms. *Microbiol* 2001;**147**:2897–912.
- Kreth J, Zhang Y, Herzberg MC. Streptococcal Antagonism in Oral Biofilms: *Streptococcus sanguinis* and *Streptococcus gordonii* Interference with *Streptococcus mutans*. *J Bacteriol* 2008;**190**:4632–40.
- Kuboniwa M, Tribble GD, James CE *et al. Streptococcus gordonii* utilizes several distinct gene functions to recruit *Porphyromonas gingivalis* into a mixed community. *Molecular Microbiology* 2006;**60**:121–39.
- Laspidou CS, Kungolos A, Samaras P. Cellular-automata and individual-based approaches for the modeling of biofilm structures: Pros and cons. *Desalination* 2010;**250**:390–4.
- Leschine SB, Canale-Parola E. Rifampin as a selective agent for isolation of oral spirochetes. *J Clin Microbiol* 1980;**12**:792–5.
- Martin B, Chathoth K, Ouali S *et al.* New growth media for oral bacteria. *J Microbiol Methods* 2018;**153**:10–3.
- Martin B, Tamanai-Shacoori Z, Bronsard J *et al.* A new mathematical model of bacterial interactions in two-species oral biofilms. *PLoS ONE* 2017;**12**:e0173153.
- Martin KJ, Picioreanu C, Nerenberg R. Assessing microbial competition in a hydrogen-based membrane biofilm reactor (MBfR) using multidimensional modeling. *Biotechnol Bioeng* 2015;**112**:1843–53.
- McNab R, Ford SK, El-Sabaeny A *et al.* LuxS-based signaling in *Streptococcus gordonii*: autoinducer 2 controls carbohydrate metabolism and biofilm formation with *Porphyromonas gingivalis*. J Bacteriol 2003;**185**:274–84.
- Meuric V, Martin B, Guyodo H et al. Treponema denticola improves adhesive capacities of Porphyromonas gingivalis. Mol Oral Microbiol 2013;28:40–53.
- Ng HM, Slakeski N, Butler CA *et al.* The Role of *Treponema denticola* Motility in Synergistic Biofilm Formation With *Porphyromonas gingivalis*. *Front Cell Infect Microbiol* 2019;**9**.
- Noguera DR, Pizarro G, Stahl DA *et al.* Simulation of multispecies biofilm development in three dimensions. *Water Sci Technol.* 1999;**39**:123–30.
- Picioreanu C, Kreft J-U, van Loosdrecht MCM. Particle-Based Multidimensional Multispecies Biofilm Model. *Appl Environ Microbiol* 2004;**70**:3024–40.
- Picioreanu C, Loosdrecht M, Lens P. Use of mathematical modelling to study biofilm development and morphology. *Biofilms in medicine, industry and environmental biotechnology:*

characteristics, analysis and control. IWA Publishing: London, 2003. - XVI, 610 p. (Integrated environmental technology series) ISBN 1843390191.

- Picioreanu C, van Loosdrecht MC, Heijnen JJ. A new combined differential-discrete cellular automaton approach for biofilm modeling: application for growth in gel beads. *Biotechnol Bioeng* 1998;**57**:718–31.
- Shao H, Demuth DR. Quorum sensing regulation of biofilm growth and gene expression by oral bacteria and periodontal pathogens. *Periodontol 2000* 2010;**52**:53–67.
- Shen Y, Zhao J, de la Fuente-Núñez C *et al.* Experimental and Theoretical Investigation of Multispecies Oral Biofilm Resistance to Chlorhexidine Treatment. *Sci Rep* 2016;6, DOI: 10.1038/srep27537.
- Tan KH, Seers CA, Dashper SG et al. Porphyromonas gingivalis and Treponema denticola Exhibit Metabolic Symbioses. PLoS Pathog 2014;10:e1003955.
- Tang Y, Liu H. Modeling multidimensional and multispecies biofilms in porous media. *Biotechnol Bioeng* 2017;**114**:1679–87.
- Wanner O, Reichert P. Mathematical modeling of mixed-culture biofilms. *Biotechnol Bioeng* 1996;**49**:172–84.
- Watanabe T, Maruyama F, Nozawa T *et al.* Complete Genome Sequence of the Bacterium *Porphyromonas gingivalis* TDC60, Which Causes Periodontal Disease. *J Bacteriol* 2011;**193**:4259–60.
- Wimpenny JWT, Colasanti R. A unifying hypothesis for the structure of microbial biofilms based on cellular automaton models. *FEMS Microbiol Ecol* 1997;**22**:1–16.
- Zhu Y, Dashper SG, Chen Y-Y et al. Porphyromonas gingivalis and Treponema denticola Synergistic Polymicrobial Biofilm Development. PLoS ONE 2013;8:e71727.

TABLES

Symbol	Description	Value	Unit				
Δt	Time step	3600	S				
Δx	Spatial element size	6.15 x 10 ⁻⁷	т				
L_x	Width of the domain	123 x 10 ⁻⁶	т				
l_b	Thickness of the diffusive boundary layer	18 x 10 ⁻⁶	т				
	Streptococcus gordonii i=1						
$c_s^{(1)}$	Substrate concentration (glucose)		$kg_s m^{-3}$				
$c_{s0}^{(1)}$	Substrate concentration in the reservoir	6	$kg_s m^{-3}$				
$K_{s}^{(1)}$	Half saturation coefficient	2.01	$kg_s m^{-3}$				
$D_{s}^{(1)}$	Diffusion coefficient	57 x 10 ⁻¹¹	m^2s^{-1}				
$c_x^{(1)}$	Biomass concentration		$kg_x m^{-3}$				
$c_{xm}^{(1)}$	Maximum biomass concentration	44	$kg_x m^{-3}$				
$\mu_{m}^{(1)}$	Maximum specific growth rate	3.59 x 10 ⁻⁴	<i>s</i> ⁻¹				
$Y_{xs}^{(1)}$	Growth yield coefficient	0.07	$kg_x kg_s^{-l}$				
$m_{s}^{(1)}$	Maintenance coefficient	4.39 x 10 ⁻⁴	$kg_skg_x^{-1}s^{-1}$				
	Porphyromonas gingivalis i=2						
$c_{s}^{(2)}$	Substrate concentration (proteins-BSA)		$kg_s m^{-3}$				
$c_{s0}^{(2)}$	Substrate concentration in the reservoir	7.35	$kg_s m^{-3}$				
$K_{s}^{(2)}$	Half saturation coefficient	0.63	$kg_s m^{-3}$				
$D_{s}^{(2)}$	Diffusion coefficient	5 x 10 ⁻¹¹	m^2s^{-1}				
$c_{x}^{(2)}$	Biomass concentration		$kg_x m^{-3}$				
$c_{xm}^{(2)}$	Maximum biomass concentration	38	$kg_x m^{-3}$				
$\mu_{m}^{(2)}$	Maximum specific growth rate	1.42 x 10 ⁻⁵	s ⁻¹				
$Y_{xs}^{(2)}$	Growth yield coefficient	0.62	$kg_x kg_s^{-l}$				
$m_{s}^{(2)}$	Maintenance coefficient	7.11 x 10 ⁻⁵	$kg_skg_x^{-1}s^{-1}$				
Treponema denticola i=3							
$c_{s}^{(3)}$	Substrate concentration (proteins-BSA)		$kg_s m^{-3}$				
$c_{s0}^{(3)}$	Substrate concentration in the reservoir	7.35	$kg_s m^{-3}$				
$K_{s}^{(3)}$	Half saturation coefficient	1.59	$kg_s m^{-3}$				
$D_{s}^{(3)}$	Diffusion coefficient	5 x 10 ⁻¹¹	m^2s^{-1}				
$c_{x}^{(3)}$	Biomass concentration		$kg_x m^{-3}$				
$c_{xm}^{(3)}$	Maximum biomass concentration	46	$kg_x m^{-3}$				
$\mu_m^{(3)}$	Maximum specific growth rate	2.02 x 10 ⁻⁵	s ⁻¹				
$Y_{xs}^{(3)}$	Growth yield coefficient	0.398	$kg_x kg_s^{-1}$				
$m_{s}^{(3)}$	Maintenance coefficient	8.27 x 10 ⁻⁵	$kg_skg_x^{-1}s^{-1}$				

Table 1: Model parameters and variables

Symbol	Description	Value at different iron concentration			Unit		
		0.8 µM*	8 μΜ	80 µM*			
Streptococcus gordonii i=1							
$w^{(l)}$	Damage concentration				$kg_w m^{-3}$		
$\alpha^{(l)}$	Damage conversion factor	5 x 10 ⁻⁵	5 x 10 ⁻⁵	5 x 10 ⁻⁵	$kg_w kg_s^{-l}$		
$\beta^{(1)}$	Rate of damage removal	1.5 x 10 ⁻⁵	1.5 x 10 ⁻⁵	1.5 x 10 ⁻⁵	s ⁻¹		
$\gamma^{(1)}$	Coefficient 1 of damage effect	500	500	500	$kg_w m^3$		
$\delta^{(l)}$	Coefficient 2 of damage effect	0.0673	0.0679	0.0674	$kg_w m^{-3}$		
	Porphyror	nonas gingival	<i>is i</i> =2				
$w^{(2)}$	Damage concentration				$kg_w m^{-3}$		
$\alpha^{(2)}$	Damage conversion factor	2.78 x 10 ⁻⁵	2.78 x 10 ⁻⁵	2.78 x 10 ⁻⁵	$kg_w kg_s^{-1}$		
$\beta^{(2)}$	Rate of damage removal	1 x 10 ⁻⁷	1 x 10 ⁻⁷	1 x 10 ⁻⁷	s ⁻¹		
$\gamma^{(2)}$	Coefficient 1 of damage effect	500	500	500	$kg_w m^3$		
$\delta^{(2)}$	Coefficient 2 of damage effect	0.0138	0.0125	0.00895	$kg_w m^{-3}$		
Treponema denticola i=3							
$w^{(3)}$	Damage concentration				$kg_w m^{-3}$		
$\alpha^{(3)}$	Damage conversion factor	2.78 x 10 ⁻⁵	2.78 x 10 ⁻⁵	2.78 x 10 ⁻⁵	$kg_w kg_s^{-l}$		
$\beta^{(3)}$	Rate of damage removal	1 x 10 ⁻⁵	1 x 10 ⁻⁵	1 x 10 ⁻⁵	s ⁻¹		
$\gamma^{(3)}$	Coefficient 1 of damage effect	500	500	500	$kg_w m^3$		
$\delta^{(3)}$	Coefficient 2 of damage effect	0.0032	0.0084	0.0075	$kg_w m^{-3}$		

Table 2: Damage parameters and variables

* The damage parameters for 0.8 and 80 μ M iron are not final and need to be improved especially for *Porphyromonas gingivalis* and *Treponema denticola*

Table 3: Size parameters of S. gordonii, P. gingivalis and T. denticola estimated from SEM
experiments and previous report (Martin <i>et al.</i> , 2017).

	Bacterial Length L (µm)			Bacterial Diameter			Volume		Dry weight
				D (µm)			$V(\mu m^3)$		m (g)
<i>S. gordonii-</i> max	0.80			0.50			0.124		2.86 x 10 ⁻¹⁴
<i>S. gordonii-</i> mean	0.50			0.50			0.065		1.51 x 10 ⁻¹⁴
P. gingivalis- max	1.17			0.40			0.126		2.91 x 10 ⁻¹⁴
<i>P. gingivalis</i> -mean	0.78			0.40		0.082		1.87 x 10 ⁻¹⁴	
	Length		Diameter			Number	Volume	Dry	
	Spiral	Turn length	Bacterial	Bacterial	Outer turn	Turn	of turns		weight
	l (μm)	(µm)	L (µm)	D (µm)	d _{se} (µm)	d _s (µm)	ns	V (µm ³)	m (g)
<i>T. denticola-</i> max	15.48	1.23	19.50	0.2	0.5	0.3	12.58	0.610	1.40 x 10 ⁻¹³
<i>T. denticola</i> - mean	7.74	1.23	9.75	0.2	0.5	0.3	6.29	0.304	6.99 x 10 ⁻¹⁴

V = $\pi/4$ (L-D/3) D²; m = ρ v with ρ =2.3 x 10⁻¹³ g μ m⁻³

For *T. denticola*, $L = \sqrt{l^2 + (\pi nsds)^2}$

ns = spiral length/turn length, ds = dse- D

	Number of bacteria	Dry weight of	Substrate	Yield coefficient
	produced by 24h	bacteria	consumed by 24h	Y _{xs}
	(CFU/ml)	produced by 24 h	(g/ml)	
		(g)		
		(g_x)	(g_s)	$(g_{x}g_{s}^{-1})$
S. gordonii	$1.33 \ge 10^{10}$	2 x 10 ⁻⁴	2.69 x 10 ⁻³	0.074
P. gingivalis	1.22 x 10 ¹⁰	2.27 x 10 ⁻⁴	3.65 x 10 ⁻⁴	0.628
T. denticola	1.67 x 10 ⁹	1.17 x 10 ⁻⁴	3.02 x 10 ⁻⁴	0.398

Table 4: Estimation of yield coefficients of S. gordonii, P. gingivalis and T. denticola.

MMBC-3 dilution	None	1.5	2	2.5	3	5
factor						
$c_s^{(1)}$ glucose (g/l)	6	4	3	2.4	2	1.2
$\mu^{(1)}S.$ gordonii (h ⁻¹)	0.9436	0.8511	0.6702	0.6745	0.5895	0.4090
MMBC-3 dilution	None	1.1	1.2	1.6	2	
factor						
c _s ⁽²⁾ protein (g/l)	7.35	6.68	6.12	4.59	3.67	
$\mu^{(2)} P.$ gingivalis (h ⁻¹)	0.0448	0.0343	0.03071	0.02437	0.02437	
MMBC-3 dilution	None	1.1	1.2	1.3	1.6	2
factor						
c _s ⁽³⁾ protein (g/l)	7.35	6.68	6.12	5.65	4.59	3.67
$\mu^{(3)}$ T. denticola (h ⁻¹)	0.0465	0.0351	0.0348	0.0264	0.0235	0.0150

Table 5: Experimental growth parameters for S. gordonii, P. gingivalis and T. denticola

4.5. Complementary data

Effect of iron on the S. gordonii-T. denticola dual-species biofilms

Among the various biofilms, the *in vitro* interactions between the two species in the *S. gordonii*-*T. denticola* biofilms have been studied for the first time. Previously, a direct experimental evidence regarding the possible gene transfer from *T. denticola* to *S. gordonii* has been previously reported (Wang, Chi and Kuramitsu 2002). However, there aren't many reports about the *in vitro* association and growth of these biofilms. The influence of iron levels on the growth and architecture of these biofilms were also studied. The concentrations of 0.8, 8 and 80 of μ M FeSO₄ were selected as sub-optimal, optimal and excess respectively, based on the iron requirement of *P. gingivalis*. The concentration of each species in the *S. gordonii*-*T. denticola* biofilms was assessed to decipher the effect of iron on the biofilm growth. Whereas, microscopic parameters of the multispecies biofilms allowed the interpretation on the effect of iron on biofilm structure/architecture.

Result and discussion

An interesting observation in the biological experiments was the influence of iron on the S. gordonii-T. denticola biofilms. At 0.8 µM of iron, the initial attachment of S. gordonii cells was lower than at 8 µM in two-species biofilms (Chathoth *et al.*, manuscript in preparation, Chapter 4.1.), which resulted in a delayed growth of this species at later time-points (Figure 1). The same was observed for the species T. denticola. Analyses of microscopic parameters also showed that at 0.8 µM of iron, the bio-volume of S. gordonii-T. denticola biofilms remained low from 2 to 144 hours (Figure 2A, Figure 3). The low bio-volume at 0.8 µM of iron, spread unevenly across the surface, resulted in a high roughness coefficient (Figure 2B). However, it is observed that the cell concentrations of both species (Figure 1) did not show a difference between iron concentrations as prominent and clear as in the case of bio-volumes (Figure 2). The exact reason is unknown but this could be attributed to the architecture of the biofilm. Among others, presence of voids in the biofilm architecture at 0.8 μ M iron (Figure 3), would result in a lesser biofilm. It is known that the size of *T. denticola* can range between 5-20 µm at different stages of growth. Higher iron levels (8) and 80 µM FeSO₄) possibly favour longer T. denticola cells thus accounting for a higher biovolume despite of having not very different cell concentrations compared to growth at $0.8 \mu M$ FeSO₄.



Figure 1: Growth of individual species in *S. gordonii-T. denticola* biofilms at various iron concentrations. The graph shows the concentration (CFU/ml) of *S. gordonii* (A) and *T. denticola* (B) cells grown as biofilms/sessile cells across different time points (2, 24, 48, 72 and 144 hours) at three iron concentrations (0.8, 8 and 80 μ M of iron). a = p value between 0.8 and 8 μ M is \leq 0.05; b = p value between 0.8 and 80 μ M is \leq 0.05.



Figure 2: Microscopic parameters for *S. gordonii-T. denticola* **biofilms.** The graphs show the values for bio-volume (A) and roughness coefficient (B) for *S. gordonii-T. denticola* biofilms across different time points (2, 24, 48, 72 and 144 hours) at three iron concentrations (0.8, 8 and 80 μ M of iron). a = p value between 0.8 and 8 μ M is \leq 0.05; b = p value between 0.8 and 80 μ M is \leq 0.05.



Figure 3: Representative 3-dimentional images obtained for 72-hour *S. gordonii-T. denticola* **biofilms at various iron concentration.** The 3-dimentional images were generated using the Imaris Viewer 9.6 software using the Z stack obtained using the Leica TCS-SP5 confocal laser scanning microscope. 3-D images of the 72-hour *S. gordonii -T. denticola* biofilms and their respective X-Z projection or thickness are shown. The scale (20 µm) is shown on the bottom left corner of each image.

This work is the first steps to describe the direct influence of iron on the development of oral biofilms *in vitro*. This study, for the first time showed the interactions between *in vitro S. gordonii -T. denticola* biofilms and the inhibitory effect of 0.8 μ M iron on the growth and architecture of these biofilms. The inhibitory effect of *S. gordonii* on *T. denticola* may be attributed to the H₂O₂ produced by *S. gordonii* (Kreth, Zhang and Herzberg 2008). However, inhibitory effect of *T. denticola* on *S. gordonii* remains to be elucidated. It will be interesting to model this inhibitory or toxic effect of 0.8 μ M iron on the *S. gordonii-T. denticola* biofilms.

4.6. Discussion and perspectives

The first part of the study (**Chapter 4.2**) showed that an iron concentration selected as suboptimal for the growth of *P. gingivalis* (0.8 μ M FeSO₄) reduced the initiation of biofilm formation by *S. gordonii* and *T. denticola* in dual-species biofilms, based on corresponding single species biofilm growth. The addition of *P. gingivalis* to the biofilm removed the iron dependency and enabled both *S. gordonii* and *T. denticola* to initiate similar biofilms irrespective of iron levels. This seems to indicate that at low iron concentration *S. gordonii* and *T. denticola* exhibit an iron-dependent antagonistic behaviour which is alleviated by the addition of *P. gingivalis*. This first study also confirmed the synergistic growth of *P. gingivalis* and *T. denticola* in the early hours of biofilm formation. A new phenomenon has been highlighted: *P. gingivalis* and *T. denticola* both displayed an inhibitory effect on the attachment and growth of *S. gordonii* in the nascent biofilm (at all iron concentrations tested). Finally, this study showed that high iron concentrations (8 and 80 μ M of FeSO₄ i.e. optimal for *P. gingivalis* growth or excess, respectively) increased the *P. gingivalis* concentration in the three-species mixed biofilms as well as the thickness of these biofilms compared to 0.8 μ M of iron.

The growth of the biofilms when monitored over time (Chapter 4.3) also confirmed the synergy between *P. gingivalis* and *T. denticola* when grown together as dual-species biofilms, suggesting a mutually beneficial effect as reported previously (Grenier 1992; Meuric et al. 2013; Zhu et al. 2013; Tan et al. 2014; Ng et al. 2019) at all iron levels tested. However, the presence of both P. gingivalis and T. denticola in the three-species biofilms, was deleterious to the growth of S. gordonii, independently of iron concentration suggesting either an antagonistic effect of the two periodontal pathogen or a competition for nutrients. The mutual beneficial effect of P. gingivalis and T. denticola were abolished by the presence of S. gordonii. Additionally, S. gordonii impaired only the growth of T. denticola, also without an effect of iron levels on this behaviour. The S. gordonii-induced inhibitory effect may be attributed to the H₂O₂ produced by S. gordonii (Kreth, Zhang and Herzberg 2008). This second study confirmed that the better attachment of the three species under high iron concentrations was followed by a higher bio-volume of the mixed biofilm after more than two days of growth. This study, for the first time showed the interactions between in vitro S. gordonii -T. denticola biofilms (Chapter 4.2 and Chapter 4.3) and the inhibitory effect of 0.8 µM iron on mainly the architecture of these biofilms (Chapter 4.5).

It is interesting to note that CFU/ml data obtained by qPCR and bio-volume data, obtained by fluorescence measurement in confocal microscopy, were not always correlated.

These apparent disparities may indicate a change in the size of bacterial cells as the biofilm matures. Thus an increase in bio-volume for a constant or decreasing genomic DNA concentration (CFU/ml) would indicate an increase in bacterial size. For example, the bio-volume of the three-species biofilm at 8 μ M of iron, was higher than all other biofilms on the third day of growth, but this was not the case with their corresponding cell concentrations (CFU/ml). The use of electronic microscopy might allow us to confirm this hypothesis.

In order to optimally combine all these experimental data (microscopic parameters and molecular data), the establishment of a mathematical model of multi-species biofilms was implemented (Chapter 4.4). All the necessary biological data previously described in Chapters 4.2 and 4.3 were collected from biofilms containing one, two and three species at three different iron concentrations. Additional parameters required to establish the model were also obtained either experimentally for each species (growth rates, substrate consumption, yield coefficients, bacterial size and concentration) or computed from simulated mono-species biofilms (damage parameters). Interactions between each species in bi-bacterial biofilms were then assessed at one iron concentration by a first hypothetic relationship between species, i.e. independence for substrate. To this aim, results of this first scenario deduced by the mathematical model were compared to experimental results. As expected from basic scientific knowledge on these three species, mathematical computation showed that this first hypothesis can be rejected. This part is of course not yet completed and it remains to test other interaction hypotheses, namely production of toxic molecules or mutual benefit/syntrophy between each species in bi-bacterial biofilms. Work is still under progress to draw conclusions on the behaviour of each species in relation to each other, depending on iron concentration and biofilm growth kinetics. These are the insights that we will obtain by finalizing this part of the project.

5. CONCLUSION AND PERSPECTIVES

The possible link between iron and severe periodontitis has been emphasized by several studies. Comparative metatranscriptomic analysis of oral biofilm from periodontitis patients and healthy individuals have highlighted the correlation between the overexpressed genes of iron transport system and severe periodontitis (Duran-Pinedo *et al.* 2014; Yost *et al.* 2015). More recently, researchers have described the correlation between the systemic iron overload in hemochromatosis patients and chronic periodontitis (Meuric *et al.* 2017; Boyer *et al.* 2018, 2020). This thesis further demonstrated that iron can directly affect the inter-bacterial interactions within oral biofilms. In the attempt to decipher the effect of iron on the development of an oral biofilm we discovered that iron levels affect the interspecies associations between bacterial species, the proportions in which each species involve in biofilm initiation or attachment and also their development or growth as mature biofilms. Such effect of iron, especially at high concentrations favouring the growth of all the three species in the oral biofilm model, also substantiated the possible link between iron overload and periodontitis.

The first step of this thesis work was to build a synthetic community containing key bacterial species that can influence the dysbiosis phenomenon. *P. gingivalis* and *T. denticola* were chosen because they co-localized *in vivo*, increased pathogenicity during co-infection in animal models and displayed synergetic growth in biofilm (Kin *et al.* 2020). Moreover, we took into account that the initial colonization of tooth surface in the first hours of the biofilm is dominated by oral streptococci (Wake *et al.* 2016). In addition *S. gordonii* is a producer of hydrogen peroxide which was proven to play an important role in maintaining oral symbiotic homeostasis (Redanz *et al.* 2018) but may also have a role in the virulence switch (Chathoth *et al.*, 2020).

The prime hurdle in testing iron against oral biofilms was that rich empirical growth media such as OMIZ-M, OBGM, BM and BHIe commonly used to grow and study the periodontal pathogens contained high residual iron concentration (Wyss 1992; Orth *et al.* 2010; Loo *et al.*, 2000; Martin *et al.*, 2017) making it unsuitable for this study. Owing to this, we first developed an iron-controlled growth media (Mixed Medium for Bacterial Community), especially suited for the three bacteria (Martin *et al.*, 2018). Our new growth media share a common core composition enabling the switch from mono- to co-cultures and efficiently promote both planktonic and biofilm

cultures of *P. gingivalis*, *T. denticola*, and *S. gordonii*. The three-species oral biofilm model established using the MMBC medium was used for this study.

 H_2S is the predominant volatile sulphur compound synthesized by oral bacteria and it is prevalent in periodontal pockets. H_2S , may link iron, oxidative stress and sulphur metabolism with virulence trait in oral bacteria, in response to host signal such as cysteine sources or iron overload diseases (Chathoth *et al.* 2020). Therefore, the ability of this medium to provide adequate source(s) of substrate for H_2S production by the periodontal pathogens will permit the measure of this virulence factor in the biofilm model. The amount of H_2S increased over time in all cultures containing *P. gingivalis* and *T. denticola*. Interestingly, *T. denticola* produced higher H_2S levels than *P. gingivalis*.

As an applied part, the three-species biofilm model was challenged with new products to evaluate their anti-biofilm activity against 24-hour biofilms. A promising oxygen carrier from Arenicola marina hemoglobin in the form of a liquid solution called HEMO2life® (M101) was tested which decreased 65% of the total number of bacteria in comparison to that of the biofilm subjected to the formulation buffer. Gel formulations containing antibacterial agents are a promising delivery system for the treatment of periodontitis via topical administration due to their advantages in the ease of use, increased retention time at the site of application and controlled drug release. We therefore tested two gels: a hyaluronic acid-based product, Hyalugel®-ADO and an oxygen and lactoferrin-based product, blue®m oral gel. The effect on biofilm formation was tested by exposing 6-hour biofilms to these products. A single exposure of Hyalugel®-ADO and blue®m oral gel was effective against oral biofilms with a biofilm reduction of 96.5 % and 97.6 % respectively compared to the untreated control and therefore both gels may be used for the treatment of periodontitis. In this process, we developed a framework for the testing of gels by adapting and improving the existing method thus making it more suited for oral biofilms. The method will then be optimized to monitor the viability of each bacterial species. A coating of the pegs with hydroxyapatite can also be applied to get even closer to *in vivo* conditions.

As the fundamental part, we tested the behaviour of three-species oral biofilm, the interspecies associations in biofilm initiation/attachment followed by biofilm growth and development in response to iron levels (0.8, 8, 80 μ M FeSO₄). The possible role of iron in the pathogenic switch of the oral microbiota is indicated in this work. Firstly, the preference of 8 μ M

of FeSO₄ demonstrated by both periodontal pathogens as mono-bacterial biofilms signifies the importance of iron in their optimal growth and development. Further, the inhibitory effect of low iron levels on the attachment and growth of particularly, *S. gordonii-T. denticola* biofilms is noteworthy and emphasises on the role of iron in growth. Finally, preferential growth of all species in the three-species biofilms at high iron levels at the later stages of growth is an indication of iron-driven oral dysbiosis which can eventually lead to the progression of periodontitis.

Often there are myriad mechanisms that are challenging to distinguish experimentally and in such scenarios theoretical investigations are particularly appealing. Mathematical models of biofilm dynamics are valuable tools in exploring and understanding the development of biofilms. These models can aid in testing various hypotheses or to predict the influence of specific processes on the structure and function of the biofilm (Shen *et al.* 2016; Wanner and Reichert 1996). Therefore, we aimed to use mathematical modelling as a tool to further decipher the effect of iron on the oral biofilm with initial model based on the work of Martin *et al.*, (2017). The measurements made from biological experiments in mono-species biofilms were used to obtain simulated results for two and three-species biofilms which were and will be further tested against different hypotheses for either independence or competition for substrate between species, mutual benefit/syntrophy or production of toxic molecules for dual- and three-species biofilms. Finally, the validation of the model will be done by comparing the simulated results with those of the experimental results obtained. This work is still in progress and will be beneficial to gain detailed knowledge of the effect of iron on the biofilm structure.

Altogether this study enabled a better understanding of the iron-driven pathogenesis of the oral microbiota. The possible role of iron in the pathogenic switch of the oral microbiota is indicated in this work. Further it would be interesting to complete/develop the mathematical models firstly, for interspecies interactions in the three-species biofilms, followed by the effect of iron levels on these interactions. Next, it is of our interest to include the data on the detachment of bacteria corresponding to each time point to add further information on the damages/losses during biofilm development. Additional parameters like cycling or oscillations during the growth cycle, viability (or cell death) would add valuable knowledge to the existing framework to fully reproduce the biological scenario. Finally, it would be of great interest to test the virulent status of the oral pathogens at high iron levels to confirm their role in disease progression.

A metatranscriptomic analysis of differentially expressed genes at different stages of development of the model biofilm grown in iron deficiency or excess will be the next approach to understand, at the molecular level, the interactions between species that may promote an inflammatory microbiota.

6. ANNEXURE

6.1. Poster communications

6.1.1. New growth media for oral bacteria: 4ème rencontre du Groupement d'Intérêt Scientifique 'Nutrition–Alimentation–Métabolisme–Santé'; 2018 Oct 4; Rennes, France

New growth media for oral bacteria

Kanchana Chathoth^{a,}, Bénédicte Martin^a, Souad Ouali^b, Vincent Meuric^a, Martine Bonnaure-Mallet^{a,} and Christine Baysse^a

> ^{*a}</sup> NUMECAN U1241 INSERM CIMIAD, Université de Rennes 1, France* ^{*b*} EA1254 Microbiologie-Risques Infectieux, Université de Rennes 1, France</sup>

Severe periodontitis is related to hemochromatosis, a dysregulation of the homeostasis of systemic iron (Meuric et al., 2017). Periodontitis is initiated by an overgrowth in the oral biofilm of specific Gram-negative anaerobic bacteria that trigger inflammatory processes, and destruction of periodontal tissues. So far, rich empirical growth media such as OMIZ-M (Wyss, 2007), OBGM (Orth et al., 2010) and BM (Loo et al., 2000) containing high residual iron concentration were used to study these periodontal pathogens. A new growth media has been designed for the iron-controlled growth of three oral bacteria namely *Porphyromonas gingivalis, Treponema denticola* and *Streptococcus gordonii*. Our new growth media, Medium for Mixed Bacterial Community, (MMBC) (Martin et al., 2018) shares a common core composition enabling the switch from mono-to co-cultures and efficiently promotes both planktonic and biofilm cultures of *P. gingivalis* (TDC60), *T. denticola* (ATCC35405), and *S. gordonii* (Challis DL1). We believe that the synthetic community modelled in iron- controlled media that we have implemented and tested in this work will be an important step for further studies of iron as an environmental signal that may trigger the pathogenic shift of the oral microbiota.

A new growth media for oral bacteria

Kanchana Chathoth ¹, Bénédicte Martin ¹, Souad Ouali ², Vincent Meuric ¹, Martine Bonnaure-Mallet ¹ and Christine Baysse ¹

1 CIMIAD, U1241 NUMECAN, Université de Rennes 1, 2 av. du Pr. Léon Bernard 35043 Rennes, France

2 EA1254 Microbiologie-Risques Infectieux, Université de Rennes 1, 2 av. du Pr. Léon Bernard 35043 Rennes, France

Introduction

Severe periodontitis is related to hemochromatosis, a dysregulation of the homeostasis of systemic iron (Meuric et al., 2017). Periodontitis is initiated by an overgrowth in the oral biofilm of specific Gram-negative anaerobic bacteria that trigger inflammatory processes, and destruction of periodontal tissues. So far, rich empirical growth media such as OMIZ-M (Wyss, 2007), OBGM (Orth et al., 2010) and BM (Loo et al., 2000) containing high residual iron concentration were used to study these periodontal pathogens. A new growth media has been designed for the ironcontrolled growth of three oral bacteria namely Porphyromonas gingivalis, Treponema denticola and Streptococcus gordonii. Our new growth media, Medium for Mixed Bacterial Community, (MMBC) (Martin et al., 2018) shares a common core composition enabling the switch from mono- to co-cultures and efficiently promotes both planktonic and biofilm cultures of P. gingivalis (TDC60), T. denticola (ATCC35405), and S. gordonii (Challis DL1). We believe that the synthetic community modelled in iron- controlled media that we have implemented and tested in this work will be an important step for further studies of iron as an environmental signal that may trigger the pathogenic shift of the oral microbiotal



References

- Meuric, V., Lainé, F., Boyer, E., Le Gall-David, S., Oger, E., Bourgeois, D., Bouchard, P., Bardou-Jacquet, E., Turmel, V., Bonnaure-Mallet, M., et al., 2017b. Periodontal status and serum biomarker levels in HFE haemochromatosis patients. A case-series study. J. Clin. Periodontol. 44, 892–897.
- Wyss, C., 2007. Fatty acids synthesized by oral treponemes in chemically defined media.FEMS Microbiol. Lett. 269, 70–76.
 Orth, R., O'Brien-Simpson, N., Dashper, S., Walsh, K., Reynolds, E., 2010. An efficient method for enumerating oral
- spirochetes using flow cytometry. J. Microbiol. Methods 80, 123–128.
 Loo, C.Y., Corliss, D.A., Ganeshkumar, N., 2000. Streptococcus gordonii biofilm formation: identification of genes that
- code for biofilm phenotypes. J. Bacteriol. 182, 1374–1382.
 Martin B, Chathoth K, Ouali S, Meuric V, Bonnaure-Mallet M, Baysse C, 2018. New growth media for oral bacteria. J Microbiol Methods. 153, 10-13.



A) Mono- and cocultures of *P. gingivalis* and *T. denticola* grown in MMBC-3 (8 μ M of hemin). Pg: monocultures of *P. gingivalis*. Pg(PgTd): quantification of *P. gingivalis* when cocultured with *T. denticola*. Td: monocultures of *T. denticola*. Td(PgTd): quantification of *T. denticola* when cocultured with *P. gingivalis*. So. *gordonii* grown in MMBC-3 with different iron sources. C) Growth and survival of *P. gingivalis* and D) *T. denticola* in MMBC-3 (8 μ M hemin) with/without S. *gordonii*. p values were calculated with Student's t-test; p < .05. **p < .01.



A) Representative confocal images of 3 biofilms with Live/Dead staining using Syto40 (green) and Propidium lodide (red), with the 3D maximum z projection. The biomass (μm³/μm²) was estimated using Comstat 2 plugin of ImageJ software V1.43m (National Institute of Health). B) 24-hour three-species biofilms quantified by qpcr.

Conclusion

From the simplest medium composition for *P. gingivalis* (MMBC-1), supplemented media were designed and validated for the growth of *P. gingivalis* together with *T. denticola* (MMBC-2) and the growth of the two anaerobic pathogens together with *S. gordonii* (MMBC-3), allowing an increment in the complexity of the media as per need. These media are suitable for biofilm mode of growth and are specifically designed to control the levels of iron and the nature of iron sources, unlike rich media which are generally used for growing a mixed biofilm, comprising of these fastidious oral bacteria.

Acknowledgements

Our sincere thanks to M. Ropert for ICP-MS analysis in the measurement of the iron concentration.



6.1.2. *Arenicola marina* extracellular hemoglobin: a new promising treatment in periodontal disease: 'BIOREGATE' European regenerative medicine forum, 2018 December 12 and 14; Nantes, France

Arenicola marina extracellular hemoglobin: a new promising treatment in periodontal disease

Kanchana Chathoth^{a,}, Bénédicte Martin^a, Emimie Saucisse^a, Martine Bonnaure-Mallet^{a,}, Franck Zal^b, Elisabeth Leize-Zal^b and Christine Baysse^a

^a NUMECAN U1241 INSERM CIMIAD, Université de Rennes 1, France ^b HEMARINA-Aeropole Centre- 296000 Morlaix, France

Periodontitis is a bacterial disease that affects periodontal tissues of teeth and dental implants. It is the first etiology of tooth loss and represents a health issue because this pathology is an etiological or worsening factor of severe chronic systemic diseases (such as diabetes, cardiovascular disease...). Moreover, it has been shown to increase the risk of some cancer (lung or intestine cancer) (Michaud et al., 2018). The evolution of the disease is linked to the modification of the oral microbiota which originally comprises of commensal facultative anaerobes (early colonizers) and strict anaerobic pathogens (late colonizers). The pathogens can therefore be targeted based on their sensitivity to oxygen. Thus oxygenation of the oral biofilm with a solution containing Arenicola marina extracellular hemoglobin (M101), a well described oxygen carrier, should reduce the number of oxygen-sensitive periodontal pathogens, restoring the balance of the oral microbiota. In this study we therefore assessed the effect of M101 on a previously established 3-species 24-hour biofilm model comprising of two periodontal pathogens (*Porphyromonas gingivalis, Treponema denticola*) and one early colonizer (*Streptococcus gordonii*).

Arenicola marina extracellular hemoglobin : a new promising treatment in periodontal disease

Kanchana Chathoth ¹, Bénédicte Martin ¹, Emilie Saucisse ¹, Martine Bonnaure-Mallet ¹, Franck Zal ²,

Elisabeth Leize-Zal² and Christine Baysse¹

¹ CIMIAD, U1241 NUMECAN, Université de Rennes - 1, 2 av. du Pr. Léon Bernard - 35043 Rennes, France

² HEMARINA - Aéropôle Centre - 29600 Morlaix, France

Introduction

Periodontitis is a bacterial disease that affects periodontal tissues of teeth and dental implants. It is the first etiology of tooth loss and represents a health issue because this pathology is an etiological or worsening factor of severe chronic systemic diseases (such as diabetes, cardiovascular disease...). Moreover it has been shown to increase the risk of some cancer (lung or intestine cancer) (Michaud et al., 2018). The evolution of the disease is linked to a modification of the oral microbiota which originally comprises of commensal facultative anaerobes (early colonizers) and strict anaerobic pathogens (late colonizers). The pathogens can therefore be targeted based on their sensitivity to oxygen. Thus, oxygenation of the oral biofilm with a solution containing *Arenicola marina* extracellular haemoglobin (M101), a well described oxygen carrier, should reduce the number of oxygen-sensitive periodontal pathogens, restoring the balance of the oral microbiota.

In this study, we therefore assessed the effect of M101 on a previously established 3-species 24-hour biofilm model comprising of two periodontal pathogens (Porphyromonas gingivalis, Treponema denticola) and one early colonizer (Streptococcus gordonii).

Materials and Methods

Arenicola marina extracellular hemoglobin, M101

M101 is a high molecular weight, extracellular, hexagonal-bilayer, hemoglobin (3600 kDa), extracted from the marine Invertebrate *Arenicola marina* (annelids, polychaete) (Jouan et al., 2001).

It is a giant oxygen carrier that can carry up to 156 molecules of O_2 . Oxygen is released from the molecule simply according to a gradient, in the absence of allosteric effectors, providing the environment with the right amount of O_2 (Zal et al., 1997 - Rousselot et al., 2006).



The characteristics of M101 are currently evaluated by HEMARINA for the preservation of organs in transplantation cases (HEMO₂life®), as an active dressing promoting the healing and oxygenation of hypoxic wounds (HEMHealing®), as a universal oxygen transporter for ischemic diseases (HEMOXYCarrier®), and as an *in vitro* cell growth promoter (HEMOXCell®/HEMUPStream®).



References

- 1. Michaud DS, Lu J, Peacock-Villada AY, Barber JR et al., JNCI J Natl Cancer Inst. 2018, 110 (8).
- Martin B, Chathoth K, Ouali S, Meuric V, Bonnaure-Mallet M, Baysse C, 2018., J Microbiol Methods. 153, 10-13.
 Zal F, Green BN, Lallier FH, Vinogradov SN and Toulmond A. (1997)., Eur J Biochem. 243: 85-92.
- Zai F, Green BN, Lailler FH, Vinogradov SN and Toulmond A. (1997)., Eur J Biochem. 243: 85-92.
 Jouan L, Taveau JC, Marco S, Lallier FH and Lamy JN (2001)., J Mol Biol 305(4): 757-71
- Bousselot M, Le Guen D, Zal F. (2006)., FEBS J 273(7): 1582-96

A 24-hour, 3 species biofilms consisting of S. gordonii (Sg), P. gingivalis (Pg) and T. denticola (Td) grown in MMBC-3 was subjected to 4 mg/mi of M101 or stabilization buffer. Results were obtained by qPCR using 165 rRNA primers specific to each species. Data are the average of three independent experiments with the respective standard deviation. p-value were calculated using the student's t-test. * p-value <0.01 BIOFILM DETACHED Total number of bacteria

Results

M101 induced detachment of bacteria from 3-species biofilm



Percentage of each species in the biofilm and detached cells when considering the total bacteria as the sum of the biofilm and the detached cells for each treatment.



M101 increased cell staining by membrane impermeant PI dye

The 24-hour biofilms grown in MMBC-3 were subjected to 4 mg/ml of M101 or stabilization buffer or 70% ethanol for 1 hour and stained with membrane-permeant Syto®40 nucleic acid dye (green colour-potentially live cells) and membraneimpermeant Propidium Iodide, PI (red colour- potentially dead cells)



(A) Representative images of the surface of biofilm with the 3D maximum z-projection and their respective xzprojection of their thickness. Below the images are the percentage of potentially live (green colour) and potentially dead (red colour) bacteria for the respective treatments.

(B) Biomass and average thickness of the biofilm estimated using Comstat 2 plugin in ImageJ software V1.43m (National Institute of Health). The values are the average of 3 independent experiments with the respective standard deviation.

Conclusion and Perspective

It is concluded that M101 has the potential to reduce the biofilm responsible for periodontitis. Further work will investigate the viability of each species inside the biofilm and the effect of M101 on biofilm formation. These data consolidate the first results obtained by NUMECAN and serve as a proof of concept. They led to the establishment of HEMDental-Care® project, that associates two industrial partners: HEMARINA and HTL and the NUMECAN academic laboratory, to develop a new medical device for the treatment of periodontal disease.

HemDental-Care® project (2019-2021) is certified by Atlanpole Biotherapies and Pôle Mer Bretagne Atlantique clusters, and followed by ID2Santé, it is funded by the Regional Council of Brittany, with the support of Feder funds as part of « l'innovation collaborative au croisement des filières ».





6.1.3. A simple method derived from the Calgary biofilm device for testing antimicrobial gels against oral biofilms: 5ème Rencontre du Groupement d'intérêt Scientifique 'Nutrition–Alimentation–Métabolisme–Santé', 2019 oct 23; Rennes, France

A simple method derived from the Calgary biofilm device for testing antimicrobial gels against oral biofilms

Kanchana Chathoth^{a,b}, Bénédicte Martin^a, Martine Bonnaure-Mallet^{a,b,c}, Olivier Loreal^{a,b} and Christine Baysse^a

^a NUMECAN U1241 INSERM CIMIAD, Université de Rennes 1, France ^b CHU Pontchaillou, 35033 Rennes, France ^c Faculté d'Odontologie, Université de Rennes 1, France

kanchana-nandanan.chathoth@univ-rennes1.fr

Periodontitis is a chronic polymicrobial inflammatory state of the periodontium, which can range from a mild gingival infection to severe periodontitis and tooth loss. Changes in the oral environment can lead to a pathogenic switch in the oral microbiota resulting in periodontitis.

Conventional methods for growing biofilms (microtitre plates, µ slides etc) can pose difficulties in testing antimicrobial gels against biofilms. Treatment of the conventionally-grown biofilm with the gel followed by recovery of detached and sessile bacteria after treatment can be complicated. Hence a simple method was developed using the principle of the Calgary Biofilm Device (CBD) for testing antimicrobial gels against oral biofilms. A protocol with the CBD which consists of the lid with pegs and the 96-well microtitre plate was optimised to establish three-species oral biofilms consisting of two periodontal pathogens *Porphyromonas gingivalis*, *Treponema denticola* and a primary colonizer of the dental plaque, *Streptococcus gordonii* on the surface of the pegs. A method was developed to analyse biofilm composition, detachment and growth in order to test various gel formulations/ antibacterial gels against oral biofilms for the treatment of periodontitis by topical application into periodontal sites. The optimization of the protocol was done using a commercial gel based on hyaluronic acid called Hyalugel[®]ADO (Expanscience Laboratoires, France) and further tests are ongoing using a commercial product called blue[®]m oral gel (blue[®]m Europe B.V., Netherlands).

The oral biofilm model developed on the CBD can be useful not only to test the efficiency of treatments but also to understand the signals that can trigger the pathogenic switch of the oral microbiota.

A simple method derived from the Calgary Biofilm Device for testing antimicrobial gels against oral biofilms

Kanchana Chathoth, Bénédicte Martin, Martine Bonnaure-Mallet, Olivier Loreal, Christine Baysse

1 CIMIAD, U1241 NUMECAN, Université de Rennes 1, 2 av. du Pr. Léon Bernard 35043 Rennes, France



6.1.4. Effect of iron on the development of oral biofilm: Annual meeting of the doctoral school "Biologie-Santé" Université Bretagne Loire 'JBS-UBL', 2019 December 10 and 11; Angers, France

Effect of iron on the development of oral biofilm

Kanchana Chathoth^{a,b}, Fabrice Mahé^c, Bénédicte Martin^a, Martine Bonnaure-Mallet^{a,b,d}, Olivier Loreal^{a,b}, Stéven Yvenou^a and Christine Baysse^a

^a NUMECAN U1241 INSERM CIMIAD, Université de Rennes 1, France ^b CHU Pontchaillou, 35033 Rennes, France ^c IRMAR-UMR CNRS 6625, Université de Rennes 1, France ^d Faculté d'Odontologie, Université de Rennes 1, France

kanchana-nandanan.chathoth@univ-rennes1.fr

Changes in the oral environment can lead to a pathogenic switch in the oral microbiota resulting in periodontitis, a chronic polymicrobial inflammatory state of the periodontium ranging from a mild gingival infection to severe periodontitis and tooth loss. Severe periodontitis is related to hemochromatosis, a dysregulation of the homeostasis of systemic iron (Meuric et al., 2017). We aimed to study the effect of iron as an environmental signal in the pathogenic switch of the oral microbiota. The first step towards this goal was to construct a 3-species oral biofilm model in iron deficient medium in which both iron concentration and iron source may be controlled as the commonly used rich empirical growth media such as OMIZ-M (Wyss, 2007), OBGM (Orth et al., 2010) and BM (Loo et al., 2000) contain high residual iron concentration. The new growth media, Medium for Mixed Bacterial Community, (MMBC) (Martin et al., 2018) shares a common core composition enabling the switch from mono- to co-cultures and efficiently promotes both planktonic and biofilm cultures of two periodontal pathogens P. gingivalis (TDC60), T. denticola (ATCC35405), and a commensal, S. gordonii (Challis DL1). The effect of iron as an environmental signal is now being analyzed in the form of a new mathematical model. We are modelling the evolution of the biofilm in response to different iron levels to decipher the influence of iron on the behaviour of the oral microbiota.

Effect of iron on the development of oral biofilm

Kanchana Chathoth, Fabrice Mahé, Bénédicte Martin, Martine Bonnaure-Mallet, Olivier Loreal, Stéven Yvenou, Christine Baysse CIMIAD, U1241 NUMECAN, Université de Rennes 1, 2 av. du Pr. Léon Bernard 35043 Rennes, France



2. Wyss, C., 2007. Fatty acids synthesized by oral treponemes in chemically defined media. FEMS Microbiol. Lett. 269, 70–76.

3. Orth, R., O'Brien-Simpson, N., Dashper, S., Walsh, K., Reynolds, E., 2010. An efficient method for enumerating oral spirochetes using flow cytometry. J. Microbiol. Methods 80, 123–128.

Loo, C.Y., Corliss, D.A., Ganeshkumar, N., 2000. Streptococcus gordonii biofilm formation: identification of genes that code for biofilm phenotypes. J. Bacteriol. 182, 1374–1382.
 Martin B, Chathoth K, Ouali S, Meuric V, Bonnaure-Mallet M, Baysse C, 2018. New growth media for oral bacteria. J Microbiol Methods. 153, 10-13.
 Martin, B., Tamanai-Shacoori, Z., Bronsard, J., Ginguené, F., Meuric, V., Mahé, F., Bonnaure-Mallet, M., 2017. A new mathematical model of bacterial interactions in two-species oral biofilms. PLoS One 12, e0173153

6.2. Oral communications

6.2.1. New growth media for oral bacteria : 9ième Journée Recherche, Campus Santé 'Chimie, Biologie, Mathématiques et Physique'; 2019 Jan 16; Rennes, France

New growth media for oral bacteria

Kanchana Chathoth^{a,}, Bénédicte Martin^a, Souad Ouali^b, Vincent Meuric^a, Martine Bonnaure-Mallet^{a,} and Christine Baysse^a

> ^{*a}</sup> NUMECAN U1241 INSERM CIMIAD, Université de Rennes 1, France* ^{*b*} EA1254 Microbiologie-Risques Infectieux, Université de Rennes 1, France</sup>

Severe periodontitis is related to hemochromatosis, a dysregulation of the homeostasis of systemic iron (Meuric et al., 2017). Periodontitis is initiated by an overgrowth in the oral biofilm of specific Gram-negative anaerobic bacteria that trigger inflammatory processes, and destruction of periodontal tissues. So far, rich empirical growth media such as OMIZ-M (Wyss, 2007), OBGM (Orth et al., 2010) and BM (Loo et al., 2000) containing high residual iron concentration were used to study these periodontal pathogens. A new growth media has been designed for the iron-controlled growth of three oral bacteria namely *Porphyromonas gingivalis, Treponema denticola* and *Streptococcus gordonii*. Our new growth media, Medium for Mixed Bacterial Community, (MMBC) (Martin et al., 2018) shares a common core composition enabling the switch from mono-to co-cultures and efficiently promotes both planktonic and biofilm cultures of *P. gingivalis* (TDC60), *T. denticola* (ATCC35405), and *S. gordonii* (Challis DL1). We believe that the synthetic community modelled in iron- controlled media that we have implemented and tested in this work will be an important step for further studies of iron as an environmental signal that may trigger the pathogenic shift of the oral microbiota.

6.2.2. A simple method derived from the Calgary biofilm device for testing antimicrobial gels against oral biofilms: 5ème Rencontre du Groupement d'intérêt Scientifique 'Nutrition–Alimentation–Métabolisme–Santé', 2019 oct 23; Rennes, France

A simple method derived from the Calgary biofilm device for testing antimicrobial gels against oral biofilms

Kanchana Chathoth^{a,b}, Bénédicte Martin^a, Martine Bonnaure-Mallet^{a,b,c}, Olivier Loreal^{a,b} and Christine Baysse^a

^a NUMECAN U1241 INSERM CIMIAD, Université de Rennes 1, France ^b CHU Pontchaillou, 35033 Rennes, France ^c Faculté d'Odontologie, Université de Rennes 1, France

kanchana-nandanan.chathoth@univ-rennes1.fr

Periodontitis is a chronic polymicrobial inflammatory state of the periodontium, which can range from a mild gingival infection to severe periodontitis and tooth loss. Changes in the oral environment can lead to a pathogenic switch in the oral microbiota resulting in periodontitis.

Conventional methods for growing biofilms (microtitre plates, µ slides etc) can pose difficulties in testing antimicrobial gels against biofilms. Treatment of the conventionally-grown biofilm with the gel followed by recovery of detached and sessile bacteria after treatment can be complicated. Hence a simple method was developed using the principle of the Calgary Biofilm Device (CBD) for testing antimicrobial gels against oral biofilms. A protocol with the CBD which consists of the lid with pegs and the 96-well microtitre plate was optimised to establish three-species oral biofilms consisting of two periodontal pathogens *Porphyromonas gingivalis*, *Treponema denticola* and a primary colonizer of the dental plaque, *Streptococcus gordonii* on the surface of the pegs. A method was developed to analyse biofilm composition, detachment and growth in order to test various gel formulations/ antibacterial gels against oral biofilms for the treatment of periodontitis by topical application into periodontal sites. The optimization of the protocol was done using a commercial gel based on hyaluronic acid called Hyalugel[®]ADO (Expanscience Laboratoires, France) and further tests are ongoing using a commercial product called blue[®]m oral gel (blue[®]m Europe B.V., Netherlands).

The oral biofilm model developed on the CBD can be useful not only to test the efficiency of treatments but also to understand the signals that can trigger the pathogenic switch of the oral microbiota.

6.2.3. Effect of iron on the development of oral biofilm: BioMetals 2020 Web-symposium, 6-10 July 2020.

Effect of iron on the development of oral biofilm

Kanchana Chathoth^{a,b}, Fabrice Mahé^c, Bénédicte Martin^a, Vincent Meuric^{a,b,d}, Stéven Yvenou^a, Olivier Loreal^{a,b}, Martine Bonnaure-Mallet^{a,b,d} and Christine Baysse^a

^a NUMECAN U1241 INSERM CIMIAD, Université de Rennes 1, France ^b CHU Pontchaillou, 35033 Rennes, France ^c IRMAR-UMR CNRS 6625, Université de Rennes 1, France ^d Faculté d'Odontologie, Université de Rennes 1, France

kanchana-nandanan.chathoth@univ-rennes1.fr

Changes in the oral environment may lead to periodontal diseases that can range from a mild gingival infection to severe periodontitis. Genetic hemochromatosis, characterized by systemic iron overload, favours the occurrence of severe periodontitis (Meuric et al., 2017 - PMID: 28586532). The mechanisms involved are not known. We aim to study the effect of iron excess in the pathogenic switch of the oral microbiota in the form of a new mathematical model. Strategy: As commonly used rich empirical growth media contain high residual iron concentration, we developed a 3-species oral biofilm model in iron-deficient medium which enables control on the concentration and source of iron. This new 'Medium for Mixed Bacterial Community' (MMBC) (Martin et al., 2018 - PMID: 30170019) shares a common core composition enabling the switch from mono- to co-cultures and efficiently promotes both planktonic and biofilm cultures of two periodontal pathogens Porphyromonas gingivalis (TDC60), Treponema denticola (ATCC35405), and a commensal, Streptococcus gordonii (Challis DL1). To better understand the evolution of these bacteria as three-species oral biofilm, we are developing a mathematical model with respect to iron concentration. This model will help to connect the different processes (mutualistic or antagonist relationship) between partners and weigh their relative contributions. To have a comprehensive view, planktonic cultures, mono-, bi- and tri-species biofilms were grown at varying iron concentrations. The biofilm development was monitored by qPCR and confocal fluorescent microscopy. Results: We choose three iron concentrations (0.8, 8, and 80 μ M) corresponding to suboptimal, optimal and excess levels according to the growth rate of P. gingvalis in planktonic culture. Planktonic conditions show no effect of iron on the growth of T. denticola and S. gordonii. Interestingly, 8 µM iron is optimal for the mono-species biofilms of P. gingivalis (with higher thickness and biomass) and T. denticola (with higher biomass). The analyses of composition and physical parameters of bi- and tri-species biofilms are in progress. Finally, we will model the evolution of in vitro mono- or multispecies biofilms to decipher the influence of iron on the behaviour of the oral microbiota. This knowledge would help determine new treatment strategies.

6.2.4. The events that may contribute to subgingival dysbiosis: a focus on the interplay between iron, sulfide and oxygen: BioMetals 2020 Web-symposium, 6-10 July 2020.

The events that may contribute to subgingival dysbiosis: a focus on the interplay between iron, sulfide and oxygen

Kanchana Chathoth^a, Bénédicte Martin^a, Pierre Cornelis^{b,c}, Stéven Yvenou^a, Martine Bonnaure-Mallet^{a,d} and **Christine Baysse^a**

^a NUMECAN U1241 INSERM CIMIAD, Université de Rennes 1, France ^b Normandie Université, Université de Rouen Normandie, Laboratoire de Microbiologie Signaux et Microenvironnement, LMSM EA4312, Évreux, France. ^c Department of Bioengineering Sciences, Laboratory of Microbiology, Vrije Universiteit Brussel, Brussels, Belgium ^d CHU Pontchaillou Rennes, France

christine.baysse@univ-rennes1.fr

The establishment of a chronic infection by subversion of a commensal microbiota results from a complex and multi-parametric sequence of events. This presentation narrows down to the interplay between oxygen, iron and sulfide that can result in a vicious cycle that would favour peroxigenic and glutathione producing streptococci as well as sulfidogenic anaerobic pathogens in the subgingival niche. Briefly, oxygen always being present in oral sites may promote H₂O₂ production by peroxygenic bacteria, mainly streptococci. The Fenton chemistry involving ferrous iron from the oral environment and H_2O_2 may generate ROS (hydroxyl radical) which in turn may produce more free iron via Fe-S cluster degradation. The generation of toxic hydroxyl radicals may induce the bacterial production of glutathione (GSH) as stress response, and then indirectly induce the formation of H₂S since GSH can act as a substrate in vitro, like other cysteine-containing peptides from the gingival crevicular fluid. H₂S protects anaerobes by lowering the redox potential in local niches. It may trigger more GSH production by H₂O₂ producing organisms (as described in streptococci), and can reduce ferric iron. From another point of view, H_2S can decrease the Fenton chemistry since it can react with non-heme iron, free or present in iron-sulfur cluster containing proteins, to generate insoluble precipitates (FeS). Altogether, this vicious circle starting from O₂ and ferrous iron may promote anaerobic pathogens and inflammation. We propose strategies for the therapeutic modulation of the microbiota to prevent periodontitis and promote oral health

7. REFERENCES

- 1. Aas JA, Paster BJ, Stokes LN *et al.* Defining the Normal Bacterial Flora of the Oral Cavity. *J Clin Microbiol* 2005;**43**:5721–32.
- 2. Abusleme L, Dupuy AK, Dutzan N *et al.* The subgingival microbiome in health and periodontitis and its relationship with community biomass and inflammation. *ISME J* 2013;7:1016–25.
- 3. Ackermann K-L, Reichenbach N. Microbiological and genetical diagnostics for advanced risk profiles. *Zeitschrift für Zahnärztliche Implantol* 2010;256-266.
- 4. Amano A, Sharma A, Sojar HT *et al.* Effects of temperature stress on expression of fimbriae and superoxide dismutase by *Porphyromonas gingivalis. Infect Immun* 1994;**62**:4682–5.
- 5. Anaya-Bergman C, He J, Jones K *et al. Porphyromonas gingivalis* Ferrous Iron Transporter FeoB1 Influences Sensitivity to Oxidative Stress. *IAI* 2010;**78**:688–96.
- 6. Anaya-Bergman C, Rosato A, Lewis JP. Iron- and hemin-dependent gene expression of *Porphyromonas gingivalis. Mol oral Microbiol* 2015;**30**:39–61.
- 7. Avila M, Ojcius DM, Yilmaz Ö. The Oral Microbiota: Living with a Permanent Guest. *DNA Cell Biol* 2009;**28**:405–11.
- 8. Bakaletz LO. Developing animal models for polymicrobial diseases. *Nat Rev Microbiol* 2004;**2**:552–68.
- 9. Beukers NGFM, Heijden GJMG van der, Wijk AJ van *et al.* Periodontitis is an independent risk indicator for atherosclerotic cardiovascular diseases among 60 174 participants in a large dental school in the Netherlands. *J Epidemiol Community Health* 2017;**71**:37–42.
- 10. Bogdan AR, Miyazawa M, Hashimoto K *et al.* Regulators of Iron Homeostasis: New Players in Metabolism, Cell Death, and Disease. *Trends Biocheml Sci* 2016;**41**:274–86.
- 11. Boyer E, Le Gall-David S, Martin B *et al.* Increased transferrin saturation is associated with subgingival microbiota dysbiosis and severe periodontitis in genetic haemochromatosis. *Sci Rep* 2018;**8**:15532.
- 12. Boyer E, Leroyer P, Malherbe L *et al.* Oral dysbiosis induced by *Porphyromonas gingivalis* is strain-dependent in mice. *J Oral Microbiol* 2020a;**12**:1832837.
- 13. Boyer E, Martin B, Gall-David SL *et al.* Periodontal pathogens and clinical parameters in chronic periodontitis. *Mol Oral Microbiol* 2020b;**35**:19–28.
- 14. Bray BA, Sutcliffe IC, Harrington DJ. Expression of the MtsA lipoprotein of *Streptococcus agalactiae* A909 is regulated by manganese and iron. *Antonie van Leeuwenhoek* 2009;**95**:101–9.
- 15. Brett PJ, Burtnick MN, Fenno JC *et al. Treponema denticola* TroR is a manganese- and iron-dependent transcriptional repressor. *Mol Microbiol* 2008;**70**:396–409.
- Brown JL, Yates EA, Bielecki M *et al.* Potential role for *Streptococcus gordonii* -derived hydrogen peroxide in heme acquisition by *Porphyromonas gingivalis*. *Mol Oral Microbiol* 2018;**33**:322–35.
- 17. Brown JS, Gilliland SM, Holden DW. A *Streptococcus pneumoniae* pathogenicity island encoding an ABC transporter involved in iron uptake and virulence: Iron uptake by *Streptococcus pneumoniae*. *Mol Microbiol* 2001;**40**:572–85.
- 18. Bui FQ, Almeida-da-Silva CLC, Huynh B *et al*. Association between periodontal pathogens and systemic disease. *Biomed J* 2019;**42**:27–35.
- 19. Bullen JJ. The Significance of Iron in Infection. Clin Infect Dis 1981;3:1127-38.
- 20. Byrne SJ, Dashper SG, Darby IB *et al.* Progression of chronic periodontitis can be predicted by the levels of *Porphyromonas gingivalis* and *Treponema denticola* in subgingival plaque. *Oral Microbiol Immunol* 2009;**24**:469–77.
- 21. Byrne SJ, Dashper SG, Darby IB *et al.* Progression of chronic periodontitis can be predicted by the levels of *Porphyromonas gingivalis* and *Treponema denticola* in subgingival plaque. *Oral Microbiol Immunol* 2009;**24**:469–77.
- 22. Cangelosi GA, Meschke JS. Dead or Alive: Molecular Assessment of Microbial Viability. *Appl Environ Microbiol* 2014;80:5884–91.
- 23. Cangelosi GA, Weigel KM, Lefthand-Begay C et al. Molecular Detection of Viable Bacterial Pathogens in Water by Ratiometric Pre-rRNA Analysis. *AEM* 2010;76:960–2.
- 24. Cellini B, Bertoldi M, Borri Voltattorni C. *Treponema denticola* cystalysin catalyzes β -desulfination of L -cysteine sulfinic acid and β -decarboxylation of L -aspartate and oxalacetate. *FEBS Letters* 2003;**554**:306–10.
- 25. Chakraborty S, Tewari S, Sharma RK *et al.* Impact of iron deficiency anemia on chronic periodontitis and superoxide dismutase activity: a cross-sectional study. *J Periodontal Implant Sci* 2014;**44**:57.
- 26. Chan ECS, McLaughlin R. Taxonomy and virulence of oral spirochetes: Taxonomy and virulence of oral spirochetes. *Oral Microbiol Immunol* 2000;**15**:1–9.
- 27. Chathoth K, Martin B, Cornelis P *et al.* The events that may contribute to subgingival dysbiosis: a focus on the interplay between iron, sulfide and oxygen. *FEMS Microbiol Lett* 2020;**367**.
- 28. Chen W, Kajiya M, Giro G *et al.* Bacteria-Derived Hydrogen Sulfide Promotes IL-8 Production from Epithelial Cells. *Biochem Biophys Res Commun* 2010;**391**:645–50.

- 29. Chu L, Bramanti TE, Ebersole JL *et al.* Hemolytic activity in the periodontopathogen Porphyromonas gingivalis: kinetics of enzyme release and localization. *Infect Immun* 1991;**59**:1932–40.
- 30. Chu L, Burgum A, Kolodrubetz D *et al.* The 46-Kilodalton-Hemolysin Gene from Treponema denticola Encodes a Novel Hemolysin Homologous to Aminotransferases. *Infect Immun* 1995;**63**:8.
- 31. Chu L, Dong Z, Xu X *et al.* Role of Glutathione Metabolism of *Treponema denticola* in Bacterial Growth and Virulence Expression. *Infect Immun* 2002;**70**:1113–20.
- 32. Chu L, Lai Y, Xu X *et al.* A 52-kDa Leucyl Aminopeptidase from *Treponema denticola* Is a Cysteinylglycinase That Mediates the Second Step of Glutathione Metabolism. *J Biol Chem* 2008;**283**:19351–8.
- 33. Chu L, Xu X, Dong Z *et al.* Role for Recombinant -Glutamyltransferase from *Treponema denticola* in Glutathione Metabolism. *Infection and Immunity* 2003;**71**:335–42.
- 34. Cicek D, Dağlı AF, Aydin S *et al.* Does hepcidin play a role in the pathogenesis of aphthae in Behçet's disease and recurrent aphthous stomatitis? *J Eur Acad Dermatol Venereol* 2014;**28**:1500–6.
- 35. Clancy A, Loar JW, Speziali CD *et al.* Evidence for siderophore-dependent iron acquisition in group B streptococcus: Iron transport in group B streptococcus. *Mol Microbiol* 2006;**59**:707–21.
- 36. Clark MA, Goheen MM, Cerami C. Influence of host iron status on *Plasmodium falciparum* infection. *Front Pharmacol* 2014;**5**.
- Cobessi D, Meksem A, Brillet K. Structure of the heme/hemoglobin outer membrane receptor ShuA from *Shigella dysenteriae*: Heme binding by an induced fit mechanism. *Proteins* 2010;**78**:286–94.
- 38. Curtis MA, Diaz PI, Van Dyke TE. The role of the microbiota in periodontal disease. Mariano RJ (ed.). *Periodontol 2000* 2020;**83**:14–25.
- 39. Dashper SG, Ang C-S, Veith PD *et al.* Response of *Porphyromonas gingivalis* to Heme Limitation in Continuous Culture. *JB* 2009;**191**:1044–55.
- 40. Dashper SG, Butler CA, Lissel JP *et al.* A Novel *Porphyromonas gingivalis* FeoB Plays a Role in Manganese Accumulation. *J Biol Chem* 2005;**280**:28095–102.
- 41. Dashper SG, Hendtlass A, Slakeski N *et al.* Characterization of a Novel Outer Membrane Hemin-Binding Protein of *Porphyromonas gingivalis*. *J Bacteriol* 2000;**182**:6456–62.
- 42. Deng Z-L, Sztajer H, Jarek M *et al.* Worlds Apart Transcriptome Profiles of Key Oral Microbes in the Periodontal Pocket Compared to Single Laboratory Culture Reflect Synergistic Interactions. *Front Microbiol* 2018;9:124.

- 43. Duran-Pinedo AE, Chen T, Teles R *et al.* Community-wide transcriptome of the oral microbiome in subjects with and without periodontitis. *ISME J* 2014;**8**:1659–72.
- 44. Enhos S, Duran I, Erdem S *et al.* Relationship Between Iron-Deficiency Anemia and Periodontal Status in Female Patients. *J Periodontol* 2009;**80**:1750–5.
- 45. Fibach E, Rachmilewitz EA. Iron overload in hematological disorders. *La Presse Médicale* 2017;**46**:e296–305.
- 46. Ganz T, Nemeth E. Iron homeostasis in host defence and inflammation. *Nat Rev Immunol* 2015;**15**:500–10.
- 47. Ge R, Sun X, He Q. Iron acquisition by *Streptococcus* species: An updated review. *Front Biol China* 2009;**4**:392.
- 48. Ge R, Sun X. Iron acquisition and regulation systems in *Streptococcus* species. *Metallomics* 2014;**6**:996.
- 49. Golonka R, Yeoh BS, Vijay-Kumar M. The Iron Tug-of-War between Bacterial Siderophores and Innate Immunity. *J Innate Immun* 2019;**11**:249–62.
- 50. Goulet V, Britigan B, Nakayama K *et al.* Cleavage of Human Transferrin by *Porphyromonas gingivalis* Gingipains Promotes Growth and Formation of Hydroxyl Radicals. *Infect Immunity* 2004;**72**:4351–6.
- 51. Gozzelino R, Arosio P. Iron Homeostasis in Health and Disease. IJMS 2016;17:130.
- 52. Graves DT, Corrêa JD, Silva TA. The Oral Microbiota Is Modified by Systemic Diseases. *J Dent Res* 2019;**98**:148–56.
- 53. Greabu M, Totan A, Miricescu D *et al.* Hydrogen Sulfide, Oxidative Stress and Periodontal Diseases: A Concise Review. *Antioxidants (Basel)* 2016;**5**.
- 54. Grenier D. Nutritional interactions between two suspected periodontopathogens, *Treponema denticola* and *Porphyromonas gingivalis*. *Infect Immun* 1992;60:5298–301.
- 55. Griffen AL, Beall CJ, Campbell JH *et al.* Distinct and complex bacterial profiles in human periodontitis and health revealed by 16S pyrosequencing. *ISME J* 2012;**6**:1176–85.
- 56. Grinberg LN, O'Brien PJ, Hrkal Z. The effects of heme-binding proteins on the peroxidative and catalatic activities of hemin. *Free Radic Biol Med* 1999;27:214–9.
- 57. Guo L-N, Yang Y-Z, Feng Y-Z. Serum and salivary ferritin and Hepcidin levels in patients with chronic periodontitis and type 2 diabetes mellitus. *BMC Oral Health* 2018;**18**:63.
- 58. Hajishengallis G, Lamont RJ. Beyond the red complex and into more complexity: the polymicrobial synergy and dysbiosis (PSD) model of periodontal disease etiology. *Microbiol Oral Immunol* 2012;**27**:409–19.

- 59. Hajishengallis G, Liang S, Payne MA *et al.* Low-Abundance Biofilm Species Orchestrates Inflammatory Periodontal Disease through the Commensal Microbiota and Complement. *Cell Host Microbe* 2011;**10**:497–506.
- 60. Hanks TS, Liu M, McClure MJ *et al.* ABC transporter FtsABCD of *Streptococcus pyogenes* mediates uptake of ferric ferrichrome. *BMC Microbiol* 2005;**5**:62.
- 61. Hashimoto M, Ogawa S, Asai Y *et al.* Binding of *Porphyromonas gingivalis* fimbriae to *Treponema denticola* dentilisin. *FEMS Microbiol Lett* 2003;**226**:267–71.
- 62. He J, Miyazaki H, Anaya C *et al.* Role of *Porphyromonas gingivalis* FeoB2 in Metal Uptake and Oxidative Stress Protection. *IAI* 2006;74:4214–23.
- 63. Heather Z, Holden MTG, Steward KF *et al.* A novel streptococcal integrative conjugative element involved in iron acquisition. *Mol Microbiol* 2008;**70**:1274–92.
- 64. Hespell RB, Canale-Parola E. Amino acid and glucose fermentation by *Treponema denticola*. *Archiv Mikrobiol* 1971;**78**:234–51.
- 65. Hojo K, Nagaoka S, Ohshima T *et al.* Bacterial Interactions in Dental Biofilm Development. *J Dent Res* 2009;**88**:982–90.
- 66. Hujoel PP, Drangsholt M, Spiekerman C *et al.* An Exploration of the Periodontitis–Cancer Association. *Ann Epidemiol* 2003;**13**:312–6.
- 67. Ishihara K, Kuramitsu HK, Miura T *et al.* Dentilisin Activity Affects the Organization of the Outer Sheath of *Treponema denticola*. *J Bacteriol* 1998;**180**:3837–44.
- 68. Jakubovics NS, Gill SR, Iobst SE *et al.* Regulation of Gene Expression in a Mixed-Genus Community: Stabilized Arginine Biosynthesis in *Streptococcus gordonii* by Coaggregation with *Actinomyces naeslundii*. *JB* 2008;**190**:3646–57.
- 69. Jakubovics NS, Smith AW, Jenkinson HF. Oxidative stress tolerance is manganese (Mn2+) regulated in *Streptococcus gordonii*. *Microbiol* 2002;**148**:3255–63.
- 70. Jakubovics NS. An ion for an iron: streptococcal metal homeostasis under oxidative stress. *Biochem J* 2019;**476**:699–703.
- Janulczyk R, Ricci S, Björck L. MtsABC Is Important for Manganese and Iron Transport, Oxidative Stress Resistance, and Virulence of *Streptococcus pyogenes*. *IAI* 2003;71:2656– 64.
- 72. Johnson PW, Yaegaki K, Tonzetich J. Effect of volatile thiol compounds on protein metabolism by human gingival fibroblasts. *J Periodontal Res* 1992;**27**:553–61.
- Kay HM, Birss AJ, Smalley JW. Haemagglutinating and haemolytic activity of the extracellular vesicles of *Bacteroides gingivalis* W50. *Oral Microbiol Immunol* 1990;**5**:269– 74.

- Kesavalu L, Holt SC, Ebersole JL. *In vitro* environmental regulation of *Porphyromonas* gingivalis growth and virulence: P. gingivalis environmental regulation. Oral Microbiol Immunol 2003;18:226–33.
- 75. Kigure T, Saito A, Seida K *et al.* Distribution of *Porphyromonas gingivalis* and *Treponema denticola* in human subgingival plaque at different periodontal pocket depths examined by immunohistochemical methods. *J Periodontal Res*1995;**30**:332–41.
- 76. Kim Y-J, Kim Y-K, Kho H-S. Effects of smoking on trace metal levels in saliva: Smoking on trace metal levels in saliva. *Oral Dis* 2010;**16**:823–30.
- 77. Kin LX, Butler CA, Slakeski N *et al.* Metabolic cooperativity between *Porphyromonas gingivalis* and *Treponema denticola*. J Oral Microbiol 2020;**12**:1808750.
- 78. Kolenbrander PE, Andersen RN, Blehert DS *et al.* Communication among Oral Bacteria. *Microbiol Mol Biol Rev* 2002;**66**:486–505.
- 79. Koziel J, Mydel P, Potempa J. The Link Between Periodontal Disease and Rheumatoid Arthritis: An Updated Review. *Curr Rheumatol Rep* 2014;**16**, DOI: 10.1007/s11926-014-0408-9.
- 80. Kreth J, Zhang Y, Herzberg MC. Streptococcal Antagonism in Oral Biofilms: *Streptococcus sanguinis* and *Streptococcus gordonii* Interference with *Streptococcus mutans*. J Bacteriol 2008;**190**:4632–40.
- 81. Krieg S, Huche F, Diederichs K *et al.* Heme uptake across the outer membrane as revealed by crystal structures of the receptor-hemophore complex. *PNAS* 2009;**106**:1045–50.
- Kuboniwa M, Tribble GD, James CE *et al.* Streptococcus gordonii utilizes several distinct gene functions to recruit Porphyromonas gingivalis into a mixed community. *Mol Microbiol* 2006;60:121–39.
- 83. Kumawat RM, Ganvir SM, Hazarey VK *et al.* Detection of *Porphyromonas gingivalis* and *Treponema denticola* in chronic and aggressive periodontitis patients: A comparative polymerase chain reaction study. *Contemp Clin Dent* 2016;7:481–6.
- 84. Kuramitsu HK, Chen W, Ikegami A. Biofilm Formation by the Periodontopathic Bacteria *Treponema denticola* and *Porphyromonas gingivalis*. *J Periodontol* 2005;**76**:2047–51.
- 85. Kusaba A, Ansai T, Akifusa S *et al.* Cloning and expression of a *Porphyromonas gingivalis* gene for protoporphyrinogen oxidase by complementation of a *hemG* mutant of *Escherichia coli*: A *hemG* gene in *Porphyromonas gingivalis. Oral Microbiol Immunol* 2002;**17**:290-5.
- 86. Lai Y, Chu L. Novel Mechanism for Conditional Aerobic Growth of the Anaerobic Bacterium *Treponema denticola*. *Appl Environ Microbiol* 2008;**74**:73–9.
- 87. Lamont RJ, Bevan CA, Gil S *et al.* Involvement of Porphyromonas gingivalis fimbriae in adherence to *Streptococcus gordonii*. *Oral Microbiol Immunol* 1993;**8**:272–6.

- 88. Lamont RJ, El-Sabaeny A, Park Y *et al.* Role of the *Streptococcus gordonii* SspB protein in the development of *Porphyromonas gingivalis* biofilms on streptococcal substrates. *Microbiol* 2002;**148**:1627–36.
- 89. Lamont RJ, Hajishengallis G. Polymicrobial synergy and dysbiosis in inflammatory disease. *Trends Mol Med* 2015;**21**:172–83.
- 90. Lamont RJ, Koo H, Hajishengallis G. The oral microbiota: dynamic communities and host interactions. *Nat Rev Microbiol* 2018;**16**:745–59.
- 91. Lan P, Pan K, Wang S *et al.* High Serum Iron level is Associated with Increased Mortality in Patients with Sepsis. *Sci Rep* 2018;**8**:11072.
- 92. Lancero H, Niu J, Johnson PW. Exposure of Periodontal Ligament Cells to Methyl Mercaptan Reduces Intracellular pH and Inhibits Cell Migration. *J Dent Res* 1996;**75**:1994–2002.
- 93. Lépine G, Progulske-Fox A. Duplication and differential expression of hemagglutinin genes in *Porphyromonas gingivalis*. Oral Microbiol Immunol 1996;**11**:65–78.
- 94. Lewis JP. Metal uptake in host-pathogen interactions: role of iron in *Porphyromonas* gingivalis interactions with host organisms: Metal uptake in host-pathogen interactions. *Periodontol 2000* 2010;**52**:94–116.
- 95. Loesche WJ. Clinical and Microbiological Aspects of Chemotherapeutic Agents Used According to the Specific Plaque Hypothesis. *J Dent Res* 1979;**58**:2404–12.
- 96. López R. Periodontal disease and adverse pregnancy outcomes. *Evidence-Based Dentistry* 2008;9:48.
- 97. Maeda K, Nagata H, Nonaka A *et al.* Oral streptococcal glyceraldehyde-3-phosphate dehydrogenase mediates interaction with *Porphyromonas gingivalis* fimbriae. *Microbes Infect* 2004a;**6**:1163–70.
- 98. Maeda K, Nagata H, Yamamoto Y *et al.* Glyceraldehyde-3-phosphate dehydrogenase of *Streptococcus oralis* functions as a coadhesin for *Porphyromonas gingivalis* major fimbriae. *Infect Immun* 2004b;72:1341–8.
- 99. McHardy I, Keegan C, Sim J-H *et al.* Transcriptional Profiles of *Treponema denticola* in Response to Environmental Conditions. *PLoS ONE* 2010;**5**:e13655.
- 100. McNab R, Ford SK, El-Sabaeny A *et al.* LuxS-based signaling in *Streptococcus gordonii*: autoinducer 2 controls carbohydrate metabolism and biofilm formation with *Porphyromonas gingivalis. J Bacteriol* 2003;**185**:274–84.
- 101. Meehan M, Burke FM, Macken S *et al.* Characterization of the haem-uptake system of the equine pathogen *Streptococcus equi* subsp. equi. *Microbiol* 2010;**156**:1824–35.

- 102. Mei F, Xie M, Huang X *et al. Porphyromonas gingivalis* and Its Systemic Impact: Current Status. *Pathogens* 2020;**9**:944.
- 103. Meuric V, Lainé F, Boyer E *et al.* Periodontal status and serum biomarker levels in HFE haemochromatosis patients. A case-series study. *J Clin Periodontol* 2017a;44:892–7.
- 104. Meuric V, Le Gall-David S, Boyer E *et al.* Signature of Microbial Dysbiosis in Periodontitis. *Appl Environ Microbiol* 2017b;**83**.
- 105. Meuric V, Martin B, Guyodo H *et al. Treponema denticola* improves adhesive capacities of *Porphyromonas gingivalis*. *Mol Oral Microbiol* 2013;**28**:40–53.
- 106. Montanez GE. The streptococcal iron uptake (Siu) transporter is required for iron uptake and virulence in a zebrafish infection model. *Microbiol* 2005;151:3749–57.
- 107. Mukherjee S. The Role of Crevicular Fluid Iron in Periodontal Disease. *J Periodontol* 1985;**56**:22–7.
- 108. Mysak J, Podzimek S, Sommerova P *et al. Porphyromonas gingivalis*: Major Periodontopathic Pathogen Overview. *J Immunol Res* 2014;**2014**:1–8.
- 109. Nelson KE, Fleischmann RD, DeBoy RT *et al.* Complete Genome Sequence of the Oral Pathogenic Bacterium *Porphyromonas gingivalis* Strain W83. *JB* 2003;**185**:5591–601.
- 110. Ng HM, Slakeski N, Butler CA *et al.* The Role of *Treponema denticola* Motility in Synergistic Biofilm Formation With *Porphyromonas gingivalis. Front Cell Infect Microbiol* 2019;9.
- 111. Niederman R, Buyle-Bodin Y, Lu B-Y *et al.* Short-chain Carboxylic Acid Concentration in Human Gingival Crevicular Fluid. *J Dent Res* 1997;**76**:575–9.
- 112. Nwizu N, Wactawski-Wende J, Genco RJ. Periodontal disease and cancer: Epidemiologic studies and possible mechanisms. *Periodontol 2000* 2020;**83**:213–33.
- 113. Oda H, Saiki K, Numabe Y *et al.* Effect of ?-immunoglobulin on the asaccharolytic growth of *Porphyromonas gingivalis*. *J Periodontal Res* 2007;**42**:438–42.
- 114. Okamoto K, Kadowaki T, Nakayama K et al. Cloning and Sequencing of the Gene Encoding a Novel Lysine-Specific Cysteine Proteinase (Lys-Gingipain) in Porphyromonas gingivalis: Structural Relationship with the Arginine-Specific Cysteine Proteinase (Arg-Gingipain). J Biochem 1996;120:398–406.
- 115. Okamoto K, Misumi Y, Kadowaki T *et al.* Structural Characterization of Argingipain, a Novel Arginine-Specific Cysteine Proteinase as a Major Periodontal Pathogenic Factor from *Porphyromonas gingivalis. Arch Biochem Biophys* 1995;**316**:917–25.
- 116. Olczak T, Simpson W, Liu X *et al.* Iron and heme utilization in *Porphyromonas gingivalis*. *FEMS Microbiol Rev* 2005;**29**:119–44.

- 117. Orrenius S, Nicotera P, Zhivotovsky B. Cell Death Mechanisms and Their Implications in Toxicology. *Toxicol Sci*2011;**119**:3–19.
- 118. Orth R, O'Brien-Simpson N, Dashper S *et al*. An efficient method for enumerating oral spirochetes using flow cytometry. *J Microbiol Methods* 2010;**80**:123–8.
- 119. Orth RK-H, O'Brien-Simpson NM, Dashper SG *et al.* Synergistic virulence of *Porphyromonas gingivalis* and *Treponema denticola* in a murine periodontitis model. *Mol Oral Microbiol* 2011;**26**:229–40.
- 120. Pan W, Wang Q, Chen Q. The cytokine network involved in the host immune response to periodontitis. *Int J Oral Sci* 2019;**11**:30.
- 121. Park Y, Simionato MR, Sekiya K *et al.* Short fimbriae of *Porphyromonas gingivalis* and their role in coadhesion with *Streptococcus gordonii*. *Infect Immun* 2005;**73**:3983–9.
- 122. Paster BJ, Boches SK, Galvin JL *et al.* Bacterial Diversity in Human Subgingival Plaque. *J Bacteriol* 2001;**183**:3770–83.
- 123. Paster BJ, Olsen I, Aas JA *et al*. The breadth of bacterial diversity in the human periodontal pocket and other oral sites. *Periodontol 2000* 2006;**42**:80–7.
- 124. Pöllänen MT, Paino A, Ihalin R. Environmental Stimuli Shape Biofilm Formation and the Virulence of Periodontal Pathogens. *Int J Mol Sci* 2013;**14**:17221–37.
- 125. Preshaw PM, Alba AL, Herrera D *et al*. Periodontitis and diabetes: a two-way relationship. *Diabetologia* 2012;**55**:21–31.
- 126. Redanz S, Cheng X, Giacaman RA *et al.* Live and let die: Hydrogen peroxide production by the commensal flora and its role in maintaining a symbiotic microbiome. *Mol Oral Microbiol* 2018;**33**:337–52.
- 127. Rickard AH, Gilbert P, High NJ *et al.* Bacterial coaggregation: an integral process in the development of multi-species biofilms. *Trends Microbiol* 2003;**11**:94–100.
- 128. Riep B, Edesi-Neuss L, Claessen F *et al.* Are Putative Periodontal Pathogens Reliable Diagnostic Markers? *J Clin Microbiol* 2009;47:1705–11.
- 129. Romano F, Castiblanco A, Spadotto F *et al.* ICP-Mass-Spectrometry Ionic Profile of Whole Saliva in Patients with Untreated and Treated Periodontitis. *Biomedicines* 2020;**8**:354.
- 130. Rosen G, Genzler T, Sela MN. Coaggregation of *Treponema denticola* with *Porphyromonas gingivalis* and *Fusobacterium nucleatum* is mediated by the major outer sheath protein of *Treponema denticola*. *FEMS Microbiol Lett* 2008;**289**:59–66.
- 131. Ruby JD, Lux R, Shi W *et al.* Effect of glucose on *Treponema denticola* cell behavior. *Oral Microbiol Immunol* 2008;**23**:234–8.

- 132. Salvador SL, Syed SA, Loesche WJ. Comparison of three dispersion procedures for quantitative recovery of cultivable species of subgingival spirochetes. *J Clin Microbiol* 1987;**25**:2230–2.
- 133. Sankaran VG, Weiss MJ. Anemia: progress in molecular mechanisms and therapies. *Nat Med* 2015;**21**:221–30.
- 134. Saraithong P, Goetting-Minesky MP, Durbin PM *et al.* Roles of TroA and TroR in Metalloregulated Growth and Gene Expression in *Treponema denticola*. *J Bacteriol* 2020;**202**:e00770-19, /jb/202/7/JB.00770-19.atom.
- 135. Sbordone L, Bortolaia C. Oral microbial biofilms and plaque-related diseases: microbial communities and their role in the shift from oral health to disease. *Clin Oral Invest* 2003;7:181–8.
- 136. Schröder I, Johnson E, de Vries S. Microbial ferric iron reductases. *FEMS Microbiol Rev* 2003;27:427–47.
- 137. Scott D, Siboo IR, Chan ECS *et al.* Binding of hemin and Congo red by oral hemolytic spirochetes. *Oral Microbiol Immunol* 1993;**8**:245–50.
- 138. Scott D, Siboo R, Chan ECS. Iron acquisition by oral hemolytic spirochetes: isolation of a hemin-binding protein and identification of iron reductase activity. *Can J Microbiol* 1996;**42**:1072–9.
- 139. Sheridan GEC, Masters CI, Shallcross JA et al. Detection of mRNA by Reverse Transcription-PCR as an Indicator of Viability in Escherichia coli Cells. *Appl Environ Microbiol* 1998;64:1313–8.
- 140. Shumi W, Lim J, Nam S-W *et al.* Environmental factors that affect *Streptococcus mutans* biofilm formation in a microfluidic device mimicking teeth. *BioChip J* 2010;4:257–63.
- 141. Ślęzak P, Śmiga M, Smalley JW *et al. Porphyromonas gingivalis* HmuY and *Streptococcus gordonii* GAPDH—Novel Heme Acquisition Strategy in the Oral Microbiome. *IJMS* 2020;**21**:4150.
- 142. Slots J, Gibbons RJ. Attachment of *Bacteroides melaninogenicus* subsp. asaccharolyticus to oral surfaces and its possible role in colonization of the mouth and of periodontal pockets. *Infect Immun* 1978;**19**:254–64.
- 143. Smalley JW, Olczak T. Heme acquisition mechanisms of *Porphyromonas gingivalis* strategies used in a polymicrobial community in a heme-limited host environment. *Mol oral Microbiol* 2017;**32**:1–23.
- 144. Smibert RM, Johnson JL, Ranney RR. Treponema socranskii sp. nov., Treponema socranskii subsp. socranskii subsp. nov., Treponema socranskii subsp. buccale subsp. nov., and Treponema socranskii subsp. paredis subsp. nov. Isolated from the Human Periodontia. Int J Syst Evol Microbiol, 1984;34:457–62.

- 145. Socransky SS, Haffajee AD, Cugini MA *et al*. Microbial complexes in subgingival plaque. *J Clin Periodontol* 1998;**25**:134–44.
- 146. Staggs TM, Greer MK, Baseman JB *et al.* Identification of lactoferrin-binding proteins from *Treponema pallidum* subspecies pallidum and *Treponema denticola*. *Mol Microbiol* 1994;**12**:613–9.
- 147. Stinson MW, Safulko K, Levine MJ. Adherence of *Porphyromonas* (Bacteroides) gingivalis to Streptococcus sanguis in vitro. Infect Immun 1991;**59**:102–8.
- 148. Stocks CJ, Schembri MA, Sweet MJ *et al.* For when bacterial infections persist: Toll-like receptor-inducible direct antimicrobial pathways in macrophages. *J Leukoc Biol* 2018;**103**:35–51.
- 149. Stojiljkovic I, Hantke K. Hemin uptake system of *Yersinia enterocolitica*: similarities with other TonB-dependent systems in gram-negative bacteria. *EMBO J* 1992;**11**:4359–67.
- 150. Takahashi N, Sato T. Preferential Utilization of Dipeptides by *Porphyromonas gingivalis*. *J Dent Res* 2001;**80**:1425–9.
- 151. Tan KH, Seers CA, Dashper SG et al. Porphyromonas gingivalis and Treponema denticola Exhibit Metabolic Symbioses. PLoS Pathogens 2014a;10:e1003955.
- 152. Tang-Larsen J, Claesson R, Edlund MB *et al.* Competition for peptides and amino acids among periodontal bacteria. *J Periodont Res* 1995;**30**:390–5.
- 153. Terleckyj B, Shockman GD. Amino acid requirements of *Streptococcus mutans* and other oral streptococci. *Infect Immun* 1975;**11**:656–64.
- 154. Travis J, Pike R, Imamura T *et al. Porphyromonas gingivalis* proteinases as virulence factors in the development of periodontitis. *J Periodontal Res* 1997;**32**:120–5.
- 155. Vadeboncoeur C, Pelletier M. The phosphoenol pyruvate:sugar phosphotransferase system of oral streptococci and its role in the control of sugar metabolism. *FEMS Microbiol Rev* 1997;**19**:187–207.
- 156. Van Dyke TE, Bartold PM, Reynolds EC. The Nexus Between Periodontal Inflammation and Dysbiosis. *Front Immunol* 2020;**11**:511.
- 157. VujiÄ M. Molecular basis of HFE-hemochromatosis. Front Pharmacol 2014;5.
- 158. Wake N, Asahi Y, Noiri Y *et al.* Temporal dynamics of bacterial microbiota in the human oral cavity determined using an in situ model of dental biofilms. *npj Biofilms Microbiomes* 2016;**2**:16018.
- 159. Wandersman C, Delepelaire P. Bacterial Iron Sources: From Siderophores to Hemophores. *Annu Rev Microbiol* 2004;**58**:611–47.

- 160. Wang BY, Chi B, Kuramitsu HK. Genetic exchange between Treponema denticola and Streptococcus gordonii in biofilms. *Oral Microbiol Immunol* 2002;17:108–12.
- 161. Wang H, Cheng H, Wang F et al. An improved 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay for evaluating the viability of Escherichia coli cells. *J Microbiol Methods* 2010;82:330–3.
- 162. Wardle HM. The challenge of growing oral spirochaetes. J Med Microbiol 1997;46:104–16.
- 163. Weinberg ED. Roles of Iron in Host-Parasite Interactions. J Infect Dis 1971;124:401-10.
- 164. Wyss C. Growth of *Porphyromonas gzngivalis, Treponema denticola, T. pectinovorum, T. socranskii,* and *T. vincentii* in a Chemically Defined Medium. *J Clin Microbiol* 1992;**30**:5.
- 165. Xu X, Holt SC, Kolodrubetz D. Cloning and Expression of Two Novel Hemin Binding Protein Genes from *Treponema denticola*. *Infect Immun* 2001;**69**:4465–72.
- 166. Yaegaki K. Oral malodorous compounds are periodontally pathogenic and carcinogenic. *Jpn Dent Sci Rev* 2008;**44**:100–8.
- 167. Yamada M, Ikegami A, Kuramitsu HK. Synergistic biofilm formation by *Treponema* denticola and Porphyromonas gingivalis. FEMS Microbiol Lett 2005;250:271–7.
- 168. Yao ES, Lament RJ, Leu SP *et al.* Interbacterial binding among strains of pathogenic and commensal oral bacterial species. *Oral Microbiol Immunol* 1996;**11**:35–41.
- 169. Yoshimura M, Nakano Y, Yamashita Y *et al.* Formation of Methyl Mercaptan froml-Methionine by *Porphyromonas gingivalis. Infect Immun* 2000;**68**:6912–6.
- 170. Yost S, Duran-Pinedo AE, Teles R *et al.* Functional signatures of oral dysbiosis during periodontitis progression revealed by microbial metatranscriptome analysis. *Genome Med* 2015;7:27.
- 171. Zarco M, Vess T, Ginsburg G. The oral microbiome in health and disease and the potential impact on personalized dental medicine: The oral microbiome. *Oral Dis* 2012;**18**:109–20.
- 172. Zhang C. Essential functions of iron-requiring proteins in DNA replication, repair and cell cycle control. *Protein Cell* 2014;**5**:750–60.
- 173. Zhang D-L, Ghosh MC, Rouault TA. The physiological functions of iron regulatory proteins in iron homeostasis an update. *Front Pharmacol* 2014;**5**.
- 174. Zhu Y, Dashper SG, Chen Y-Y et al. Porphyromonas gingivalis and Treponema denticola Synergistic Polymicrobial Biofilm Development. PLoS ONE 2013;8:e71727.
- 175. Zou LL, Wang J, Huang BF *et al.* A Solute-binding Protein for Iron Transport in *Streptococcus iniae. BMC Microbiol* 2010;**10**:309.

RÉSUMÉ

Titre de la thèse : Effet des signaux environnementaux sur le développement du biofilm buccal

Le microbiome buccal, l'environnement buccal et l'hôte sont en équilibre en condition de santé bucco-dentaire. Des changements dans l'environnement buccal peuvent déclencher des maladies parodontales. La parodontite est une maladie polymicrobienne chronique, inflammatoire, associée à la plaque dentaire (ou biofilms buccaux). Elle est associée, non seulement à une infection, mais également à une halitose (mauvaise odeur), une perte osseuse et/ ou une perte de dents. La parodontite est également associée à plusieurs autres troubles systémiques comme la polyarthrite rhumatoïde, les maladies cardiovasculaires, les naissances prématurées, le diabète, l'hémochromatose et même certains cancers. L'évolution de la maladie est liée à une modification du microbiote buccal qui comprend à l'origine des anaérobies facultatifs commensaux (colonisateurs primaires) et des pathogènes anaérobies stricts (colonisateurs tardifs). Cette modification du microbiote est déclenchée par des facteurs de l'environnement buccal. Un lien possible entre l'un de ces facteurs, la concentration en fer, et la sévérité des parodontites a été souligné par plusieurs études. Une analyse métatranscriptomique comparative du biofilm oral de patients atteints de parodontite et d'individus sains a mis en évidence lune corrélation entre l'expression de gènes du métabolisme du fer et la présence et sévérité des parodontites. Plus récemment, des chercheurs ont mis en évidence une corrélation entre la surcharge systémique en fer chez des patients hémochromatosiques et la présence de parodontite chronique avec modification de la composition du microbiote. Cette thèse démontre que le fer peut affecter directement les interactions inter-bactériennes dans les biofilms buccaux.

Pour évaluer l'effet du fer sur le développement d'un biofilm représenté par une communauté synthétique réduite, nous avons étudié l'effet de modifications des concentrations de fer sur les relations entre les espèces bactériennes choisies pour représenter le biofilm buccal. Plus spécifiquement nous avons évalué comment les différents niveaux de fer affectaient les proportions dans lesquelles chaque espèce était impliquée dans l'initiation ou l'attachement du biofilm mais également leur développement ou croissance en tant que biofilms matures.

Les poches parodontales profondes sont caractérisées par de faibles niveaux d'oxygène. Or, les streptocoques produisent des quantités importantes de peroxyde d'hydrogène (H₂O₂) en présence d'oxygène. Sur cette base, nous avons émis l'hypothèse que non seulement les niveaux de fer, mais aussi l'oxygène pouvaient jouer un rôle en tant que facteurs de dysbiose. A partir de données bibliographiques, un modèle hypothétique mettant l'accent sur l'importance du fer, de l'oxygène et des composés soufrés (glutathion et sulfure d'hydrogène- H_2S), dans la mise en place d'un cercle vicieux favorisant la dysbiose a été proposé, L'oxygène toujours présent dans les sites oraux pourrait favoriser la production de H₂O₂ par les streptocoques peroxygéniques. La réaction de Fenton impliquant le fer ferreux et H₂O₂ pourrait alors générer des espèces réactives de l'oxygène (ROS, radical hydroxyle), qui à leur tour pourraient augmenter le taux de fer libre via la dégradation des clusters Fe-S des protéines, qui sont sensibles à l'oxydation. La génération de radicaux hydroxyles toxiques pourrait également activer la production de glutathion par certaines bactéries du microbiote, en tant que réponse au stress oxydant, puis induire indirectement la formation de H₂S puisque le glutathion a été identifié *in vitro* comme un substrat possible de cette biosynthèse, ainsi d'ailleurs que d'autres peptides contenant de la cystéine présente dans le fluide créviculaire gingival. L'H₂S est le composé soufré volatile prédominant synthétisé par les bactéries de la sphère orale surtout dans les poches parodontales. La production d'H₂S protège les bactéries anaérobies en abaissant localement le potentiel redox. Il peut *in vitro* déclencher une plus grande production de glutathion notamment par les organismes péroxygéniques, les streptocoques. Dans l'ensemble, ce cercle vicieux qui pourrait s'enclencher par en présence d'O₂ à une certaine concentration de fer ferreux pourrait favoriser le développement d'agents pathogènes anaérobies, l'inflammation et modifier la composition et le fonctionnement du microbiote.

La première étape de ce travail de thèse a été de construire une communauté synthétique contenant des espèces bactériennes clés qui peuvent influencer le phénomène de dysbiose. *Porphyromonas gingivalis* et *Treponema denticola* ont été choisis parce qu'ils co-localisés *in vivo*, et leur pathogénicité respective augmente lorsqu'ils sont utilisés ensemble pour une co-infection dans des modèles animaux. Ces deux bactéries présentent également une synergie de croissance en biofilm. De plus, nous avons tenu compte du fait que la colonisation initiale de la surface dentaire dans les premières heures du biofilm est dominée par des streptocoques dont *Streptococcus gordonii* est un représentant majeur. En outre, *S. gordonii* est un producteur de peroxyde d'hydrogène qui joue un rôle important dans le maintien de l'homéostasie symbiotique orale, mais qui pourrait également jouer un rôle dans le changement de fonctionnement du microbiote comme exposé précédemment, notamment en excès de fer.

Le principal obstacle dans l'évaluation de l'effet du fer dans les biofilms oraux était que les milieux de croissance empiriques riches tels que OMIZ-M, OBGM, BM et BHIe couramment utilisés pour cultiver et étudier les pathogènes parodontaux contiennent une concentration résiduelle élevée en fer, ce qui les rend inadaptés à cette étude. Pour cette raison, nous avons d'abord développé un milieu de croissance adapté au contrôle de la concentration en fer (Mixed Medium for Bacterial Community), et permettant la croissance des trois espèces bactériennes choisies, en biofilm ou en mode planctonique. À partir de la composition de milieu le plus simple adapté à *P. gingivalis* (MMBC-1), des milieux supplémentés ont été conçus et validés pour la croissance de *P. gingivalis* et *T. denticola* (MMBC-2) puis la croissance des trois espèces incluant *S. gordonii* (MMBC-3). Ces nouveaux milieux de culture partagent une composition de base commune permettant le passage des monocultures aux co-cultures et favorisent efficacement les cultures planctoniques et biofilms de *P. gingivalis*, *T. denticola* et *S. gordonii*. Le modèle de biofilm buccal à trois espèces a été obtenu dans le milieuMMBC-3. Ce milieu permet à *P. gingivalis* et *T. denticola* la production d'H₂S. Par conséquent, la capacité de ce milieu à fournir une ou plusieurs sources adéquates de substrat pour la production d'H₂S par les pathogènes parodontaux permettra d'évaluer l'importance de ce facteur dans le mécanisme de dysbiose.

D'un point de vue appliqué, le modèle de biofilm à trois espèces a été utilisé pour évaluer l'activité anti-biofilm de nouveaux produits. Dans un premier temps, un transporteur d'oxygène contenant de l'hémoglobine d'un ver marin *l'Arenicola marina*, produit sous forme d'une solution liquide appelée HEMO₂life® (ou M101, Hémarina) a été testé. HEMO₂life induit une diminution de 65% du nombre total de bactéries dans un biofilm de 24h en comparaison avec un biofilm soumis à la solution tampon de formulation du produit. Toutefois, les formulations sous forme de gel contenant des agents actifs sont un système de délivrance plus adaptés pour le traitement de la parodontite en raison de plus grandes facilités d'administration, de temps de rétention accru au site d'application et de libération contrôlée du médicament. Nous avons donc testé deux gels : un produit à base d'acide hyaluronique, Hyalugel®-ADO (Ricerfarma, Milan, Italy) et un produit à base de donneur d'oxygène et de lactoferrine, le gel oral blue®m (blue®m Europe B.V., Netherlands). L'effet sur la formation de biofilm a été testé en exposant des biofilms de 6 heures à ces produits grâce à un protocole spécifiquement mis au point pour des formulations à base de gel. Une seule exposition au gel oral Hyalugel®-ADO ou blue®m s'est avérée efficace contre les biofilms avec une réduction de la biomasse des biofilms de 96,5% et 97,6% respectivement par rapport au biofilm contrôle non traité. La méthode ainsi validée pourra être optimisée pour étudier la viabilité de chaque espèce bactérienne lors du traitement.

D'un point de vue fondamental, nous avons testé le comportement du biofilm en réponse à différentes concentrations en fer (0,8, 8, 80 μ M FeSO₄) préalablement choisies par l'analyse de cultures planctoniques. Nous nous sommes intéressés aux interactions interspécifiques dans l'initiation / l'attachement du biofilm et le développement du biofilm. La préférence de 8 μ M de FeSO₄ montrée par les deux pathogènes parodontaux, *P. gingivalis* et *T. denticola* en biofilms mono-bactériens montre que la concentration en fer peut influencer le développement des biofilms buccaux. De plus, de faibles niveaux de fer se caractérisent par une diminution de l'adhésion des cellules bactériennes au support et par une diminution de la croissance des biofilms en particulier des biofilms mixtes composés de *S. gordonii* et *T. denticola*. Enfin, la croissance optimale de toutes les espèces dans les biofilms mixtes tri-bactériens à forte concentration en fer, observée aux derniers stades de croissance du biofilm, est une indication que la dysbiose buccale pourrait être induite par le fer et donc conduire à la progression d'une parodontite.

Il existe souvent une myriade de mécanismes qu'il est difficile de distinguer expérimentalement et dans de tels scénarios, les modèles théoriques sont particulièrement intéressants. Les modèles mathématiques de la dynamique des biofilms sont des outils précieux pour explorer et comprendre le développement des biofilms. Ces modèles peuvent aider à tester diverses hypothèses ou à prédire l'influence de processus spécifiques sur la structure et la fonction du biofilm. Par conséquent, nous avons cherché à utiliser la modélisation mathématique comme un outil pour explorer l'effet du fer sur le biofilm oral. Dans un premier temps, Les données biologiques obtenues avec les biofilms mono-espèces ont été utilisées pour déterminer des paramètres mathématiques spécifiques à chaque espèce, afin d'obtenir des résultats simulés de croissance en biofilms, avec différents scénarios d'interactions entre les espèces : synergie, compétition, toxicité ou indépendance. Dans un second temps les données biologiques recueillies sur les biofilms mixtes et la comparaison avec les modèles mathématiques vont permettre d'identifier les interactions entre chacune des espèces, à différentes concentrations en fer. Cette partie du projet de modélisation est en cours.

Dans l'ensemble, cette étude a permis de mieux comprendre la pathogenèse du microbiote oral induite par les surcharges en fer. La finalisation du modèle mathématique, mais également la prise en compte dans un modèle plus complexe des données biologiques obtenues sur le détachement des bactéries au cours de l'évolution des biofilms à différentes concentrations en fer permettra de mieux caractériser les interactions inter-espèces. Il serait également intéressant de tester in vitro et in vivo, le niveau de virulence des pathogènes buccaux à différentes concentrations en fer. Enfin, une analyse métatranscriptomique de gènes différentiellement exprimés à différents stades de développement du biofilm modèle cultivé à différentes concentrations en fer sera la prochaine approche pour affiner le rôle du fer dans la progression de la maladie parodontale au niveau moléculaire.



Titre : Effet des signaux environnementaux sur le développement du biofilm oral

Mots clés : Streptococcus gordonii, Porphyromonas gingivalis, Treponema denticola, fer, oxygène, biofilm oral, modèle mathématique.

Le microbiote buccal forme une communauté Résumé : complexe. La dysbiose de ce microbiote est à l'origine des maladies parodontales. Parmi de nombreux facteurs pouvant induire cette dysbiose, les niveaux systémiques de fer ont récemment été identifiés comme possibles signaux. Des études antérieures ont suggéré que l'hémochromatose génétique, caractérisée par une surcharge systémique en fer, favorisait le développement de parodontites sévères, mais les mécanismes impliqués ne sont pas élucidés. Par conséquent, ces travaux de thèse ont eu pour objectif d'analyser le comportement de biofilms buccaux à différentes concentrations en fer. La complexité du biofilm buccal a été réduite à trois espèces clefs afin de pouvoir analyser leurs interactions. Les espèces bactériennes ont été sélectionnées pour leur importance dans le développement des maladies parodontales et leurs coopérations métaboliques : Streptococcus gordonii, Porphyromonas gingivalis et Treponema denticola. Pour réaliser ce biofilm multi-espèces, un milieu de croissance permettant de contrôler la concentration en fer et la croissance des trois espèces bactériennes (Mixed Medium for Bacterial Community) a été développé. Ce modèle de biofilm a servi de base pour mener des travaux de recherche appliquée et fondamentale. Des méthodes pour tester l'efficacité de liquides ou gels antimicrobiens sur le biofilm buccal ont été développées. En parallèle, l'effet de différentes concentrations en fer sur l'attachement, l'initiation du biofilm et son développement a été étudié. Cette étude a montré pour la première fois les interactions

entre S. gordonii et T. denticola dans les biofilms. La carence en fer s'est révélée délétère pour l'attachement et la croissance d'un biofilm composé uniquement de ces deux espèces bactériennes. De plus, la présence des trois espèces bactériennes favorise l'attachement des deux pathogènes P. gingivalis et T. denticola par rapport aux biofilms mono-espèces. Enfin, une forte concentration en fer permet une meilleure croissance du biofilm total et de chacune des trois espèces au sein du complexe. Une telle préférence pour des niveaux élevés de fer pour la maturation du biofilm peut expliquer la dysbiose buccale et les parodontites sévères observées lors des maladies de surcharge en fer. Pour optimiser l'analyse des associations entre les espèces bactériennes et l'effet du fer sur ces interactions, un modèle mathématique est en cours d'élaboration. Les premières étapes de ce projet sont présentées dans cette thèse. Les paramètres mathématiques ont été calibrés par des mesures biologiques obtenues sur des biofilms à une espèce bactérienne. Ces paramètres seront utilisés pour tester différentes hypothèses d'interactions entre les espèces : indépendance ou compétition pour le substrat, bénéfice mutuel/syntrophie et/ou production de molécules toxiques. Enfin. la validation du modèle obtenu mathématiquement se fera en comparant les résultats simulés avec les résultats expérimentaux obtenus pour les biofilms bi et triespèces. Dans l'ensemble, cette étude permettra une meilleure compréhension du comportement du biofilm buccal en fonction de la concentration en fer.

Title Effect of environmental signals on the development of oral biofilm

Keywords : Streptococcus gordonii, Porphyromonas gingivalis, Treponema denticola, iron, oxygen, oral biofilm, mathematical model.

Abstract : The oral bacteria form a complex community and their pathogenic switch can be influenced by hostrelated factors such as systemic iron and oxygen levels. genetic Previous studies have suggested that hemochromatosis, characterized by systemic iron overload, favours the occurrence of severe periodontitis but the mechanisms involved are unclear. Therefore, this thesis aimed to analyse the behaviour of multispecies oral biofilms at different iron levels composed of periodontal bacteria selected for their importance in the development of periodontal gordonii. diseases: Streptococcus Porphyromonas gingivalis and Treponema denticola. To realize this multispecies biofilm, an iron-controlled growth medium (Mixed Medium for Bacterial Community) was developed. This work includes applied research work and a fundamental part. In the framework of the development of a system for evaluating the anti-biofilm activity of new products, methods for testing the effectiveness of antimicrobial liquids or gels were developed and used to challenge the 3-species biofilm. To better understand the effect of iron on the behaviour of three-species oral biofilm. the interspecies associations in biofilm initiation/attachment and followed by biofilm growth and development in response to iron levels (0.8, 8, 80 μ M FeSO₄) were investigated. This study, for the first time, showed the interactions between S. gordonii and T. denticola in biofilms

and the effect of iron on these biofilms. The most striking result was the lower initial attachment and growth of this dual-species biofilm at the lower iron concentrations. Moreover, the presence of the three-species altogether favoured the attachment of both pathogens P. gingivalis and T. denticola compared to mono-species biofilms and displayed a preferential growth of all the three species at 80 µM iron at 144 hours. Such preference for high iron levels for biofilm maturation may lead to oral dysbiosis and the progression of periodontitis during iron-overloaded diseases. To further evaluate the inter-bacterial associations, the effect of iron in the evolution of the oral biofilm is being studied in the form of a new mathematical model with T. denticola in a previously established model for P. gingivalis and S. gordonii. The parameters were calibrated by measurements made from biological experiments in mono-species biofilms which will be used to test different hypotheses for either independence or competition for substrate between species, mutual benefit/syntrophy or production of toxic molecules for dualand three-species biofilms. Finally, the validation of the model will be done by comparing the simulated results with those of the experimental results obtained for dualand three-species biofilms. Altogether this study will enable a better understanding of the iron-driven pathogenesis of the oral microbiota.