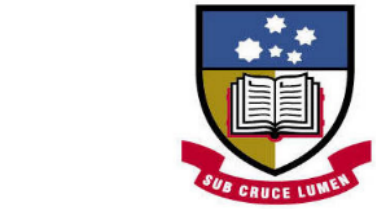
Investigating Microbiome -Targeted Treatments for Chronic Rhinosinusitis:

A novel approach to combat dysbiosis in the nasal microbiome

Martha Alemayehu Menberu



THE UNIVERSITY of ADELAIDE

A thesis submitted for the degree of

Doctor of Philosophy

at the University of Adelaide, Adelaide, Australia

Faculty of Health and Medical Sciences,

School of Medicine, Discipline of Surgery

November 2021

This thesis is dedicated to my loving husband, Mezgebu and kids, Nati and Emi for their patience, encouragement, humor, and making everything possible

Table of Contents

Thesis declaration

This thesis is composed of my original work unless otherwise stated, and contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

I give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

I acknowledge that copyright of all material contained within this thesis resides with the copyright holder(s) of that material. Where appropriate I have obtained copyright permission from the copyright holder to reproduce material in this thesis and have sought permission from co-authors for any jointly authored works included in the thesis.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Martha Alemayehu Menberu

November, 2021

Acknowledgements

First and foremost, I would like to thank God Almighty for giving me the strength, knowledge, ability and opportunity to undertake this research. Without his blessings, this achievement would not have been possible.

I would like to express my sincere gratitude to my principal supervisor, Associate Professor Sarah Vreugde for giving me the opportunity to pursue my PhD in her research team and making me comfortable and confident. I have been extremely lucky to have a supervisor who cared so much about my work, and who took great efforts, starting from initial planning to the careful editing of this thesis. Her excellent guidance, encouragement and invaluable support have ensured the success of this thesis, for which I am very grateful. Thank you Sarah for everything.

I would also like to express my very great appreciation to my co-supervisor, Professor Peter-John Wormald, for his dedication, insightful comments, and inspiring guidance throughout my candidature. His extraordinary experience and extensive helpful discussions are priceless. I thank Professor for his efficient leadership and supervision at all stages of my research. I also thank Professor Alkis Psaltis, for being an excellent guide, with the right advice and instructions throughout my candidature. I greatly appreciated his prompt responses, scientific support and careful attention to detail.

My deepest appreciation goes to Dr. Clare Cooksley for creating a welcoming and productive environment in the laboratory and facilitating my work throughout my candidature. Dr. Clare has been invariably helpful in all aspects and she has always made herself available for support.

I extend my sincere thanks to all the past and present members of the ENT research laboratory, especially Andrew Hayes, Dr. Sha Liu, Dr. Mahnaz Ramezanpour and Catherine Bennett, for

their technical support and kindness throughout my stay in the laboratory. The assistance of George Spyro Bouras in bioinformatics and proteomics analysis warrants special mention. I am blessed to have gotten to know all of you and I am proud to consider you my friends. Furthermore, I would like to thank my colleagues at the ENT department and the Basil Hetzel Institute (BHI), for amazing support in many ways and exceptional team spirit. I appreciate the BHI core facilities and the staffs who have provided this friendly and supportive environment and facilities to do research.

I extremely thank the University of Adelaide for granting me the Adelaide Scholarship International (ASI) award, which made my PhD journey possible.

My sincere thank also goes to Dr. Zelalem Addis for his tremendous support, sharing the ups and downs, and being available whenever needed during a difficult period of my candidature. I am also extremely indebted and thankful to his family (Belaynesh and kids) for a warm welcome in their house and the great hospitality on my first day in Australia. I would also like to thank my friends Dr. Yitayal, Dr. Legesse, Alem, Habtamua and their family who provided a much-needed form of escape from my studies.

Finally, this journey would not have been possible without the support of my family. I would like to thank my father (Alemayehu Menberu), my mother (Asegedech Shigut), my sisters, and my brothers for your continuous love, prayers, encouragement, and support throughout my PhD journey. Special thanks are reserved for my husband, Mezgebu Sahle, who always supports me and wished for my great success. The endless sacrifices you have made for me is beyond measure. To my kids Natanim and Emana, for not complaining my absence and for cheering me up with their laughter and play. I cannot imagine how hard it would have been for me to successfully complete my study without your unconditional love and support.

Publications arising from this thesis

1. **Menberu MA**, Hayes AJ, Liu S, Psaltis AJ, Wormald PJ, Vreugde S. Tween 80 and its derivative oleic acid promote the growth of *Corynebacterium accolens* and inhibit *Staphylococcus aureus* clinical isolates. *Int Forum Allergy Rhinol.* 2021; 11 (4): 810-813.
2. **Menberu MA**, Hayes AJ, Liu S, Wormald PJ, Psaltis AJ, Vreugde S. Fatty acid-induced growth promotion of *Corynebacterium accolens* suppresses pathogenic *Staphylococcus aureus* and *Pseudomonas aeruginosa* isolates in a mixed biofilm model. Prepared in a manuscript format.
3. **Menberu MA**, Liu S, Cooksley C, Hayes AJ, Psaltis AJ, Wormald PJ, Vreugde S. *Corynebacterium accolens* Has Antimicrobial Activity against *Staphylococcus aureus* and Methicillin-Resistant *S. aureus* Pathogens Isolated from the Sinonasal Niche of Chronic Rhinosinusitis Patients. *Pathogens*. 2021; 10 (2):207.
4. **Menberu MA**, Ramezanpour M, Cooksley C, Liu S, Bouras G, Psaltis AJ, Wormald PJ, Vreugde S. Proteomic identification of potential antibacterial products from *Corynebacterium accolens*, a commensal nasal isolate deficient in chronic rhinosinusitis. Prepared in a manuscript format.
5. **Menberu MA**, Cooksley C, Ramezanpour M, Bouras G, Wormald PJ, Psaltis AJ, Vreugde S. *In vitro* and *in vivo* evaluation of probiotic properties of *Corynebacterium accolens* isolated from the human nasal cavity. *Microbiological research*. 2022; 255:126927.
6. Huang S, Liu S, Hon K, Bennett C, Hu H, **Menberu MA**, Wormald PJ, Vreugde S, Psaltis AJ. *Corynebacterium accolens* inhibits *Staphylococcus aureus* induced mucosal barrier disruption. Submitted for publication, November 2021.

Presentations arising from this thesis

The prebiotic potential of free fatty acid compounds

The Queens Elizabeth Hospital Research Expo, Basil Hetzel Institute for Translational Health Research, Adelaide, Australia, October 2019.

Free fatty acids: the potential for prebiotic treatment of a dysbiotic microbiome in chronic rhinosinusitis

The 6th Annual EMBL Australia Postgraduate Symposium (EAPS-2019), Melbourne, Australia, November 2019.

The prebiotic potential of fatty acids (Tween 80 and its derivative Oleic acid) for treatment of nasal microbiota dysbiosis

The 14th Annual Florey Postgraduate Research Conference, Adelaide, Australia, September 2020.

***Corynebacterium accolens* isolated from healthy sinus cavities have antimicrobial activity toward *Staphylococcus aureus* and methicillin-resistant *S. aureus* clinical isolates**

The Queen Elizabeth Hospital Research Expo, Basil Hetzel Institute for Translational Health Research, Adelaide, Australia, October 2020.

***In vitro* and *in vivo* evaluation of potentially probiotic *Corynebacterium accolens* isolated from the human nasal cavity.**

The 15th Annual Florey Postgraduate Research Conference, Adelaide, Australia, September 2021.

Safety and efficacy evaluation of probiotic potential of *Corynebacterium accolens* isolated from the human nasal cavity.

The Queen Elizabeth Hospital Research Expo, Basil Hetzel Institute for Translational Health Research, Adelaide, Australia, October 2021.

List of figures

Chapter 1

Chapter 2

Chapter 4

Chapter 6

List of tables

List of abbreviations

AERD	Aspirin exacerbated respiratory disease
AFS	Allergic fungal sinusitis
ALI	Air-liquid interface
AMPs	Antimicrobial proteins
API	Analytical profile index
BC	Basal cells
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
COPD	Chronic obstructive pulmonary disease
COX	Cyclooxygenase
CRS	Chronic rhinosinusitis
CRSsNP	CRS without nasal polyposis
CRSwNP	CRS with nasal polyposis
CT	Computed tomography
DAMPs	Danger-associated molecular patterns
DNA	Deoxyribonucleic acid
EFRS	Eosinophilic fungal rhinosinusitis
EMT	Epithelial-to-mesenchymal transition
Esp	Extracellular serine protease
FFA	Free fatty acid
FITC	Fluorescein isothiocyanate
FOS	Fructo-oligosaccharide
GERD	Gastroesophgeal reflux disease
GO	Gene ontology

GOS	Galacto-oligosaccardes
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
Ig	Immunoglobulin
IL	Interleukin
LDH	Lactate dehydrogenase
MRSA	Methicillin-resistant Staphylococcus aureus
NF-ƘB	Nuclear factor-kappa B
PAMPs	Pathogen-associated molecular patterns
PCD	Primary ciliary dyskinesia
PGE2	Prostaglandin E2
PRRs	Pattern recognition receptors
QOL	Quality of life
RANTES	Regulated on activation normal T-cell expressed and secreted
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RTF	Rhinosinusitis Task Force
SNOT	Sino-Nasal Outcomes Test
TAGs	Triacylglycerols
TEER	Trans- epithelial electrical resistance,
tGOS	Trans- galacto-oligosaccardes
TLR	Toll-like receptor
Th	T helper type
WGS	Whole genome sequencing
WHO	World Health Organisation
ZO-1	Zonula Occludens-1

Thesis summary

Chronic rhinosinusitis (CRS) is a detrimental inflammatory upper airway disorder with different underlying pathophysiology affecting the mucosa of the nasal cavity and paranasal sinus. Approximately 12.5% of the western populations is affected by this disease with a large socioeconomic impact and compromised quality of life. Microbial dysbiosis that is characterized by alterations in the structure and function of the sino-nasal microbiota composition, with the predominant reduction of *Corynebacteria* is recently linked with the pathogenesis of CRS. As a result, the sino-nasal microbiota has emerged as an attractive therapeutic target. While knowledge of the nasal microbiome is expanding rapidly, the exact microbial dynamics at species or strain level, their interactions and potential therapeutic strategies remain in their infancy. Therefore, understanding the role of both commensal and pathobiont microbes that exist in the sino-nasal tract, with their dynamic interactions between microbes and their hosts, as well as the investigation of innovative therapeutics targeting the microbiota is an active area of current research worldwide. In this thesis, the development of two novel microbiome-targeted treatment strategies were explored including, the fatty acid compounds as prebiotics and commensal *Corynebacteria* as probiotics to restore the normal microbiota in CRS patients. The first part of this thesis reviews the existing literature relating to CRS, the complex role of sino-nasal microbiota both in health and CRS, dysbiosis of the sino-nasal microbiota, and especially focuses on the beneficial function of commensal microbes and several therapeutic strategies for nasal homeostasis. In the second part of the thesis we investigate the prebiotic effect of fatty acid (FA) excipients, Tween 80 and its free fatty acid moiety, Oleic acid on the growth of various nasal commensals including, *C. accolens*, *C. propinquum*, *C. pseudodiphtheriticum* and *S. epidermidis* as well as common nasal pathogens, *S. aureus* and *P. aeruginosa* in planktonic and biofilm forms in an *in vitro* study. As a result, Tween 80 and Oleic acid demonstrated a significant growth promotion effect on

commensal *Corynebacteria*, mainly *C. accolens* and reciprocally an antibacterial and antibiofilm effects against pathogens including, the clinical isolates and reference strains of *S. aureus* at FDA-approved concentration of 0.5% or below. Moreover, an increased growth of *C. accolens* biofilms induced by Tween 80 or Oleic acid showed a significant growth change on *S. aureus* and *P. aeruginosa* biofilms in an *in vitro* mixed commensal-pathogen biofilm model. The findings in this study support the therapeutic potential of FA compounds as prebiotics for the management of dysbiosis-associated CRS.

The third part of the thesis focuses on evaluating the antimicrobial potential of commensal *C. accolens* isolates, which are predominant members of the nasal microbiome against *S. aureus* and methicillin-resistant *S. aureus* (MRSA) isolates from CRS patients. A total of 10 *C. accolens* strains were identified based on microbiological, biochemical and molecular tests among 20 healthy control subjects and used as potential starting strains for exploring the antimicrobial potential toward *S. aureus* pathogens. All *C. accolens* isolates and their secreted proteins exhibited anti-staphylococcal activity in a dose-dependent manner as determined through deferred growth inhibition and micro dilution assays. *C. accolens* strains, in particular C779, C781 and C787 was found to be the best strains with strong antibacterial and antibiofilm effects. Subsequently, we were able to show that the effect is due to production of protein-like substance by *C. accolens*, which was directly involved in the reduction of planktonic growth, biofilm biomass and metabolic activity of *S. aureus* isolates. This finding has led to the exploration of antibacterial protein products from *C. accolens* to realize the development of novel probiotic therapies to promote sinus health. The fourth part of this thesis briefly describes a detailed proteomic analysis of commonly expressed proteins across 6 *C. accolens* strains to identify and characterize antibacterial and other proteins functionally associated with various probiotic properties. As a result, Acetyltransferase, GNAT family protein was found to be the strongest positively correlated abundant protein detected in *C. accolens* associated with strong

antibacterial effect. Besides, commonly expressed *C. accolens* proteins with recognized antimicrobial activity, including the glycosyl hydrolase family 25 and N-acetylmuramoyl-L-alanine amidase as well as many other proteins involved in the survival and adhesion probiotic properties were identified with various abundance level across strains. Thus, all these results hold significant promise to develop more targeted therapy for maintaining nasal homeostasis. For the final part of this thesis, we conducted a combination of *in vitro* and *in vivo* studies, aiming to evaluate the probiotic properties of *C. accolens* nasal isolates. Healthy nasal *C. accolens* strains, C779, C781 and C787 was selected for evaluating potential probiotic features as they demonstrated paramount effect in terms of antimicrobial property. In our *in vitro* experiments, these strains displayed a good adhesion ability to human nasal epithelial cells (HNECs), able to outcompete *S. aureus* for HNEC adhesion, and dampen *S. aureus*-dependent immune activation with no cytotoxic property. Furthermore, Whole genome sequence analysis confirmed them as non-virulent with no detectable antibiotic resistant gene associated with a health risk. In a well-designed *in vivo* experiment in *C. elegans*, the strains were found to be safe and able to protect *C. elegans* from *S. aureus* induced toxicity, giving us valuable insights to launch probiotic *C. accolens* strains and develop novel probiotic therapy in the near future for the management of CRS linked with microbial imbalance. Ultimately, these studies open new paths towards the development of clinically recognized microbiome-targeted treatments, both probiotics and prebiotics for manipulating a stable microbiome ecosystem in CRS. Further work involving a randomised controlled trial is necessary to evaluate changes in nasal microbiota composition and in health outcomes before all treatments can be translated into clinical practice.

CHAPTER 1

Introduction and Literature review

CHAPTER 1: Introduction and Literature review

1.1 Chronic rhinosinusitis - Clinical definition and diagnostic concept

Chronic rhinosinusitis (CRS), formerly known as chronic sinusitis, is one of the most predominant long-lasting inflammatory conditions, affecting people of all age groups world-wide [1]. The definition of CRS is mainly reliant on the basis of various diagnostic symptoms assisting clinicians and researchers for undertaking evidence-based clinical practice and refining discrepancies among studies related to sinus health [2, 3]. In the year 1996, the American Academy of Otolaryngology-Head & Neck Surgery multidisciplinary Rhinosinusitis Task Force (RTF) defined the key symptom-based criteria for the diagnosis of adult rhinosinusitis [4]. The criteria set out by this taskforce included facial pressure or pain, nasal blockage or obstruction, nasal discharge or purulence or discoloured postnasal drainage, anosmia or hyposmia, purulence in the nasal cavity, and fever. Later, in 2003, the RTF's definition was modified to involve radiographic imaging or nasal endoscopic findings along with physical examination and suggestive history [2, 5].

Most of the published guidelines and consensus documents define CRS as a common inflammatory disease process that involves the linings of the nasal cavity and paranasal sinuses persisting for a minimum of 12 weeks duration and, characterized by the presence of two or more of the following diagnostic symptoms. These include facial pain/pressure, nasal blockage, obstruction of the nasal cavity, anterior/posterior mucopurulent rhinorrhoea, and a reduced sense of smell or anosmia. In addition, there should also be a confirmed objective evidence of mucosal changes of the paranasal sinuses and sino-nasal inflammation evidenced with computed tomography (CT) or rhinoscopic findings [6].

1.2 Epidemiology and disease determinants of chronic rhinosinusitis

CRS is thought to be a heterogeneous and highly prevalent chronic disorder across the globe. The estimated prevalence of CRS varies from country to country, as most studies convey on self-reported disease status by a patient instead of recognized diagnostic standards as followed by otolaryngologist experts. Based on the National Health Interview Survey, the burden of CRS among populations in the United States is estimated to be 2-16% [7]. National health statistics data demonstrated that CRS is more prevalent than other frequent chronic respiratory illnesses, such as chronic obstructive pulmonary disease and asthma with the prevalence rate of 3% and 8%, respectively [8]. Moreover, data from a current study reported an estimated prevalence of 4.5 to 12.5% CRS cases in the western population and a lower rate in the developing world [9]. In Australia, more than 8.5% adults are affected by CRS with increased expenditures in usage of both outpatient and other healthcare services [10].

The chronic nature of the disease contributes to significant morbidity and substantial healthcare costs to society. In recent years, the socio-economic burden of CRS has been explored with its direct and indirect costs estimated worldwide. In the United States, the estimated economic burden reported approximately 22 billion US dollar and direct health care costs of 12.5 billion US dollar annually [11, 12]. In addition, the disease has been shown to have significant detrimental effects on the patient's quality of life (QOL) and productivity which have been found to be worse than other chronic syndromes such as chronic heart failure, back pain, angina pectoris and chronic obstructive pulmonary illness [13].

1.3 Clinical manifestations of chronic rhinosinusitis and disease entities

The spectra of clinical manifestations in CRS generally depend on the area of the sino-nasal tract involved. In general, CRS patients present with at least two of the four cardinal signs and

symptoms such as nasal obstruction or congestion, facial pain or pressure, anterior and/or posterior nasal mucopurulent drainage and reduction or loss of sense of smell [14].

In clinical practice, CRS can be categorized into two distinct phenotypes based on the presence or absence of nasal polyps on examination, as CRS with nasal polyposis (CRSwNP) and CRS without nasal polyposis (CRSsNP) [15]. The description of each of these categorizations relies mostly on observable nasal endoscopic findings with the Lund Kennedy scoring system and sino-nasal CT scans with the Lund Mackay scoring system [16]. The distinctive clinical presentation of CRSwNP is characterized by occurrence of bilateral nasal polyps, having a gray-white color and glistening appearance comprised from various gelatinous inflammatory materials and cells. They usually arise from the sinus ethmoid region and are the most distinctive feature of CRSwNP [17]. The existence of sinonasal mucosal inflammation without specific nasal polyposis defines the condition as CRSsNP [17].

Furthermore, the inflammation types of CRS are differentiated based on the activation of the T helper type 1 (Th1) or T helper type 2 (Th2) pathways. In both phenotypes (CRSwNP and CRSsNP), a cellular infiltrate of neutrophils, macrophages, and lymphocytes, along with several pro-inflammatory cytokines associated with Th1 cell involvement is observed. CRSwNP in particular, is distinguished by the presence of an eosinophilic inflammatory infiltrate with a mixed Th1/Th2 cytokine profile that is Th2 biased immune response with a high rate of revision and recalcitrant forms[18, 19].

1.4 Aetiology and pathogenesis of chronic rhinosinusitis

Although there is much evidence elucidating the possible factors contributing to the pathogenesis of CRS, the unifying aetiology and underlying mechanisms that contribute to the disease process and severity are largely unknown. There is a growing body of evidence

supporting an emerging hypothesis that a dysfunctional interaction that occurs at the interface between the host and the environment of the sino-nasal mucosa involving various exogenous agents results in sino-nasal mucosal inflammation [16, 20-22]. CRS is not a single disease entity, but it is increasingly recognized as a complex multifactorial inflammatory disease involving a diverse range of host- associated or intrinsic factors and environmental or extrinsic factors for its long-standing inflammation and pathogenesis [23].

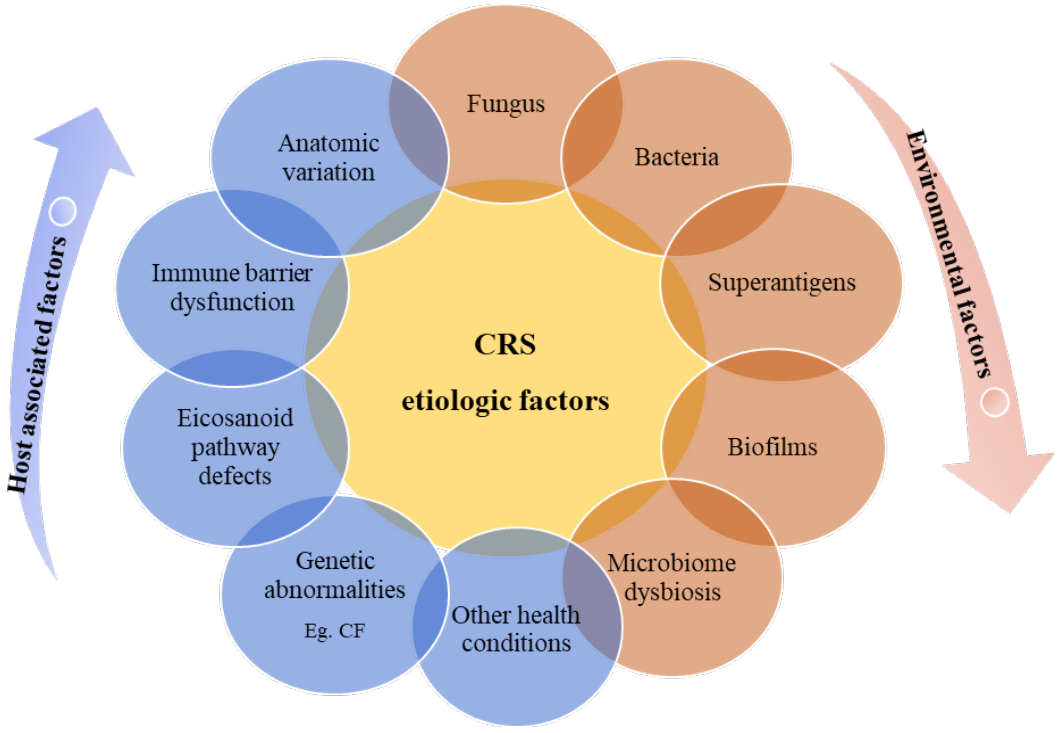


Figure 1.1. A complex Interplay of host and environmental factors contribute to CRS pathophysiology.

1.4.1 Host associated factors in CRS

The vast majority of CRS cases are idiopathic in origin, with only a minority of cases caused by identifiable host factors that predispose patients to developing CRS [24-26]. Some of these host associated factors include anatomic variation, immune barrier dysfunction, diversity in immunological changes, defects in the eicosanoid pathway, genetic factors and other health conditions, and will be discussed in the following paragraphs.

Structural anatomic variation

Anatomic variation in the nose and paranasal sinuses such as septal deviation, enlarged inferior and middle turbinates, variation in ethmoid cell structure, and paradoxical middle turbinates are among the factors causing sino-nasal drainage abnormalities and blockage of sinus ventilation and are thought to be predisposing factors to CRS. These variations can cause obstruction of the ostiomeatal complex affecting the mucociliary transport system which allows CRS to occur and significantly increase the potential for sinus complications [27]. The diagnosis of anatomic variation is well recognized based on clinical symptoms, anterior rhinoscopy, nasal endoscopic findings and computer tomography. Therefore, before surgical procedures, the anatomic variations in the sino-nasal tract should be clearly identified to prevent surgical complications and adequate management to prevent sequelae or recurrence of the disease [28].

Epithelial physical barrier defects

The host sino-nasal epithelium serves as the site of interface with inhaled irritants, commensal organisms and pathogens and, is crucial for the protection of the sino-nasal mucosal interior milieu. Indeed, the mucosal immune system possesses the inherent capability to protect the host from injury induced by environmental agents, and defects in this system could hypothetically contribute to the chronic inflammation characteristic of CRS [24]. Although the exact mechanisms and molecular pathways that lead to these barrier defects are not clearly defined, several studies indicate that epithelial physical barrier defects in patients with CRS can result from inhaled allergens, microbial or virus infections, cytokines, hypoxia, or zinc deficiency [29-31].

Chronic inflammation may lead to a change in the composition of the sinus mucosa in CRS, with a reduction of ciliated cells and an increase in mucus-producing goblet cells. The cilia

beat frequency has been shown to be slow and desynchronized in the setting of CRS [32]. The sinus ostia may also be obstructed by inflamed mucosa, polyps, or inspissated secretions, which can further delay the natural movement of mucus out of the sinus resulting in a chronic mucus stasis in the sinuses, which can serve as a chronic inflammatory stimulus through accumulation of microbes and microbial products [33]. Generally, CRSwNP is characterized by an intense edematous stroma with albumin deposition, formation of pseudocysts, and subepithelial and perivascular inflammatory cell infiltration whereas, CRSsNP is characterized by fibrosis, basement membrane thickening, goblet cell hyperplasia, subepithelial edema, and mononuclear cell infiltration [34].

In the context of CRS, several intercellular adhesion molecules including epithelial cadherin (E-cadherin) and tight junction proteins such as junction adhesion molecule, claudin, occludin, and Zonula Occludens-1 (ZO-1) are involved in modulating the physical barrier structure and function [35, 36]. These molecules/proteins adhere to each other by homotypic binding in the space between adjoining cells, and some have cytoplasmic domains attached to the actin cytoskeleton within the cell.

Innate and adaptive immune response

Immunological changes in the nasal mucosa are also thought to play a prominent role in CRS pathogenesis. Both the innate and adaptive immune responses play critical roles in the host defense and act cooperatively to identify and eliminate infectious threats from the sino-nasal tract [23]. Innate immunity a rapid response and serves as the first line of defense always present in the body and generates a non-specific immune response against the pathogen. Whereas adaptive immunity confers a long-term immunity and generates a specific immune response designed in response to exposure to an external factor/antigen. In theory, defects in the innate immune system may predispose to infection and increased antigenic exposure or

may stimulate inflammation directly via interaction with adaptive immune cells. Once inflammation is present, failure of innate mechanisms to promote resolution and repair may also contribute to disease persistence. Deficiencies in both immune systems and mechanical barrier disruption make the sino-nasal mucosa more susceptible to antigenic exposure and stimulation, leading to either side of the spectrum of chronic inflammation [37].

In patients with CRSwNP, type 2 innate lymphoid cells are thought to be early contributors to the type 2 inflammatory response increased in nasal polyps and are typically driven by the type 2 cytokines interleukin 4 (IL-4), IL-5, and IL-13 [38, 39]. Such inflammation is typically described by infiltration of large numbers of eosinophils, basophils, and mast cells. Epithelium-derived cytokines, such as thymic stromal lymphopoietin and IL-33 show increased levels and enhanced activity in nasal polyps of patients with CRSwNP when compared with healthy sino-nasal tissue [40].

Evidence has demonstrated that innate immune mediators such as Toll-like receptor -2 (TLR2) and IL-22 receptor were expressed differentially in CRS patients compared with controls. In a recent report by Detwiller *et al*., TLR2 is downregulated in patients with both CRSsNP and CRSwNP, whereas IL-22 receptor was overexpressed in patients with CRSsNP, and suggest a potential role of innate immune system dysregulation in the pathophysiology of CRS [41]. In addition, Th17 cells that are a subset of activated CD4+ T cells act as a bridge between adaptive and innate immunity and play crucial roles in the development of autoimmunity, inflammation, and allergic reactions [42]. In a previous study, the Th17 cytokine family (IL-17, IL-22, and IL-26) showed significant disruption of the epithelial barrier, leading to increased paracellular permeability associated with reduced tight junction integrity [43].

The central regulators of the adaptive immune response are T-lymphocytes, and these respond to pathogens once these are presented to them on the surface of a host antigen presenting cells [33]. B-lymphocyte cell lineages, an important part of the adaptive immune response, have also been thought to play a role in the pathogenesis of CRSwNP by secreting mucosal immunoglobulins. For example, CRS tissue, especially nasal polyps, contain large numbers of B-lymphocytes that produce immunoglobulin A (IgA) and IgE. This might reflect increased access of antigenic material to the lamina propria leading to chronic vascular changes in the nasal mucosa, blockage of intracellular fluid transport, and edema of the lamina propria [44].

Innate immune barrier hypothesis

A defective epithelial barrier with a reduced secretion of innate host defense molecules and loss of the physical airway epithelial barrier, along with decreased mucociliary clearance is thought to contribute to frequent colonization with bacteria and enhaced inflammatory response to fungi and other environmental antigens. As a result of a defective mucociliary clearance, there is a local accumulation of pathogen-associated molecular patterns (PAMPs), which are conserved microbial molecular structures and considered crucial for the existence of pathogens, danger-associated molecular patterns (DAMPs) and other antigens and/or allergens, in the sino-nasal cavity that can easily access the underlying mucosal tissues through the defective epithelial barrier [45]. The innate immune system recognizes these damage and danger signals through pattern recognition receptors (PRRs) expressed highly by the nasal epithelium [45]. Altogether, these factors lead to the stimulation of unique signalling pathways that result in recruitment and persistence of adaptive immune responses with development of the clinical symptoms characteristic of the disease. The chronic inflammation associated with CRS that is linked to a defective mechanical and innate immune barrier (that is the immune barrier hypothesis) is presented in Figure 1.2.

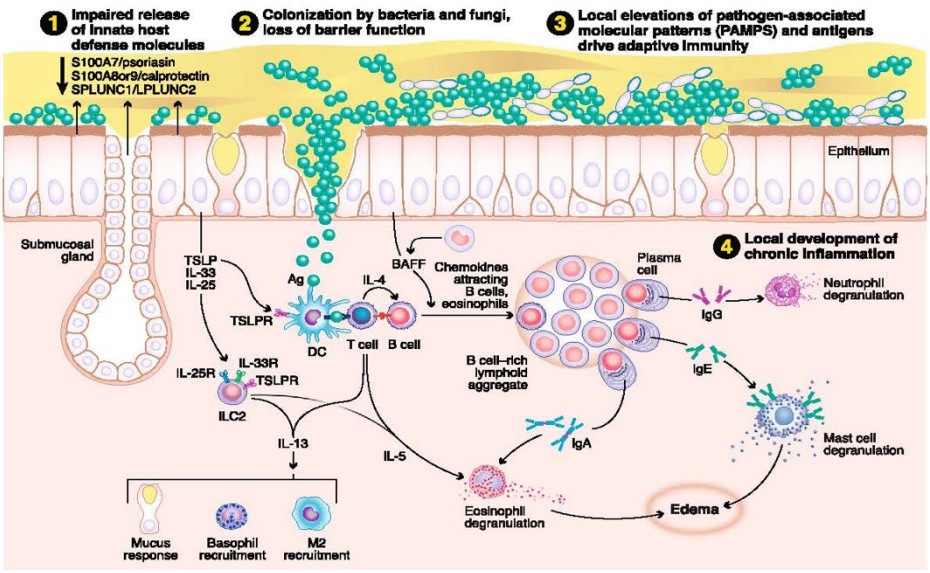


Figure 1.2. Mechanisms of inflammation associated with CRS - the "immune barrier hypothesis" (1) A defective epithelial barrier likely plays a critical role in the initiation and maintenance of chronic inflammation in CRS. These defects include reduced secretion of innate host defense molecules and loss of the airway epithelial barrier, along with decreased mucociliary clearance. (2) This in turn may result in increased colonization by *S. aureus* and, in some cases, fungi. (3) As a result, there is a local accumulation of PAMPS, and other antigens and/or allergens, in the sinonasal cavity that can easily access the underlying mucosal tissues through the defective epithelial barrier. (4) Altogether, this results in the activation of innate effector immune cells (eosinophils, mast cells, ILC2s, etc.) and recruitment and activation of adaptive effector immune cells (T and B cells) to the tissue mucosa [45].

Defects in the eicosanoid pathway

Eicosanoids are important amplifiers and regulators of inflammation in patients with diseases of the airway [21]. Defects in the eicosanoid pathway are characterized by an increased levels of pro-inflammatory cysteinyl leukotrienes and decreased synthesis of anti-inflammatory metabolites such as downregulation of cyclooxygenase-2 (COX-2) accompanied by reduced

levels of prostaglandin E2 (PGE_2), They have recently been implicated as a contributing cause in patients with CRSwNP [46].

Genetic abnormalities

Genetic predisposition is commonly stated as a contributing factor in CRS pathogenesis, although direct evidence is scarce. Cystic fibrosis (CF) is a genetic disorder or an autosomal recessively inherited condition primarily affecting the sino-nasal cavities, lungs and digestive system [47]. This disorder is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, and believed to be involved in the pathogenesis of CRS along with known anatomic, pathologic, and environmental factors [48]. Impaired sino-nasal mucociliary clearance mechanism and altered sino-nasal immune response generally predispose CF patients to develop nasal polyposis and CRS in high incidence and severe disease condition [49, 50]. In addition, an increased prevalence of CRS is observed in patients with primary ciliary dyskinesia (PCD), a rare genetic disorder with structural and functional impaired ciliary motility resulting in chronic disease of the upper and lower airway, which demonstrates the role of ciliary function in maintaining sinus homeostasis [51].

Immune deficiencies

Patients with primary or acquired immune deficiencies like HIV/AIDS, selective IgA deficiency, IgG subclass deficiency, and specific antibody deficiency are at risk of developing a devastating rhinosinusitis condition that can prove difficult to treat. In current clinical practice immunoglobulin deficiencies might not be sufficiently taken into consideration as a cause of CRS [16, 52]. Patients with untreated immunoglobulin deficiency can present with persistent symptomatic CRS despite proper conservative and surgical management. In contrast, patients without evidence of immunoglobulin deficiency have a good prognosis after sinus surgery, with a low rate of recurrence [53].

Other health conditions

The presence of allergy or asthma significantly increases the risk of CRS and appears to correlate with a more significant disease burden and a poorer prognosis [54, 55]. In patients with asthma, poor proliferative epithelial repair responses, improperly formed tight junctions having reduced ZO-1 expression, and anchorage of epithelial cells disruption have been demonstrated. These result in disruption in the formation and/or maintenance of epithelial tight junctions and can cause or exacerbate chronic diseases such as CRS [35].

Previous studies suggest that Aspirin-Exacerbated Respiratory Disease (AERD) is also involved as a potential risk factor for CRS. AERD is a severe upper airway disease characterized by the presence of chronic sino-nasal inflammation and nasal polyps as in CRSwNP. The disease is associated with a severe CRSwNP phenotype and lower health related quality-of-life when compared to CRSwNP patients without aspirin sensitivity [56]. Although there are many notable clinical and pathophysiological differences between CRSwNP and AERD, the development of upper or lower respiratory tract hypersensitivity reaction following the ingestion of a COX-1 inhibitor remains the most prominent clinical feature distinguishing AERD patients from those patients with CRSwNP [57]. On average, AERD patients have worse upper respiratory syndrome with augmented sino-nasal symptoms, mucosal inflammation and more often require revision sinus surgery when compared to CRSwNP patients [58].

Although the exact relationships and underlying mechanisms between gastroesophgeal reflux disease (GERD) and CRS remain controversial, some studies have suggested the causative role of GERD in CRS pathophysiology [59, 60]. GERD is defined as a condition characterized by reflux of stomach contents that causes disturbing symptoms and/or complications [61]. Hanna and Wormald in their review reported that the presence of oesophageal-nasal reflex,

particularly in regard to mucus secretion and symptoms of postnasal drip are implicated as expected contributing factors to worsen nasal symptom scores in CRS [62].

1.4.2 Environmental factors in CRS

CRS is a multifactorial disease and can involve various pathophysiological triggers acting at the interface of the sino-nasal mucosa [63]. Perhaps the most prominent environmental factors that can increase the risk of CRS or worsen clinical symptoms are believed to be fungal infection, bacterial infection, super antigens, biofilms and microbiome dysbiosis [20]. These factors will be discussed in the following paragraphs.

Fungal infection

Although fungi are present in the nares of healthy control subjects and in almost all patients with CRS, various studies have described the role of fungi in the aetiology of fungal-mediated sinus inflammation in CRS. A recent *ex vivo* study showed that fungal antigens such as *Aspergillus*, *Alternaria*, and *Candida* species are less capable of inducing eosinophilia-associated cellular responses in nasal polyps compared with *Staphylococcus aureus* enterotoxin B [64]. The involvement of fungi in rhinosinusitis is frequently reported in two forms; allergic fungal sinusitis (AFS) and eosinophilic fungal rhinosinusitis (EFRS). The AFS form is characterized by an elevated IgE specific to the fungus cultured from mucin [65] Whereas, in the EFRS form , a non-IgE-mediated immune reaction that triggers a humoral and cellular responses (both Th1 andTh2 lymphocytic types) leading to an eosinophilic inflammatory process has been reported [66, 67]. Moreover, Sasama J *et al.* reported the broader role for fungi in CRS pathophysiology, linking the eosinophilic inflammation to the presence of certain molds in the nasal and paranasal cavities [25].

Bacterial infection

Until the advent of next generation sequencing with the potential for comprehensive microbiome profiling, the human sinus cavities were thought to be sterile. The presence of bacteria within the nose and paranasal sinuses (bacterial colonization) of both normal states and patients with CRS has since been shown to be common and has been well documented [5]. However, the role of nasal colonization in the pathophysiology of CRS is far from clear. The most frequently found bacteria in the sino-nasal tract include *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Streptococcus* species (*S. pneumoniae* and *S. viridans*) and *Klebsiella* species [68]. Previous studies have demonstrated an increased colonization rate of *S. aureus* (63.6%) in patients with CRSwNP compared to patients with CRSsNP and control subjects [69]. More importantly, irrespective of an intracellular or extracellular localization in the epithelium, *S. aureus* is able to induce IL-6 synthesis *in vitro*, and might contribute to the Th2 cytokine pattern of inflammation in patients with CRSwNP [70]. As such, *S. aureus* nasal colonization is thought to be a disease modifier by promoting immune dysregulation, disrupting tissue barrier function, promoting impaired mucociliary clearance, driving polyp formation, and causing bacterial dysbiosis leading to recalcitrant disease [71].

The presence of *P. aeruginosa* and other Gram-negative bacilli in the pathogenesis of CRS has increased in recent years, with a higher recovery rate in patients who had sinus surgery than in patients who did not have surgery [72]. Furthermore, *Pseudomonas aeruginosa*, *Enterobacter* spp., methicillin-resistant *Staphylococcus aureus* (MRSA), *Haemophilus influenzae* and *Moraxella catarrhalis* were more frequently recovered in the sinuses of patients who had surgical revision and in those who had been repeatedly treated with antibiotics [73, 74]. This suggests that various bacteria might contribute to or worsen CRS exacerbations.

Superantigens

Although no single bacterial species has been proposed as the primary etiologic agent in CRS, various studies propose that colonizing microbes secrete enterotoxins which can behave as superantigens, capable of generating an immune response with resultant inflammation in the nasal mucosa. [75, 76]. *Staphylococcal* strains are well known to produce many virulence factors and enterotoxins that promote inflammation including the staphylococcal superantigens. These superantigens can activate a subset of T-lymphocytes which bind directly to the T cell receptor in a non-antigen-specific manner [77]

The effect of superantigens on the pathogenesis of CRS has been well recognized. Previous studies have shown a significant association between the presence of *Staphylococcus* species (common producers of superantigenic toxins) and CRSwNP,[78] indicating a potential role for superantigens in the pathogenesis of this phenotype of CRS. Moreover, *Staphylococcal* superantigens have been detected in polyp homogenates and contribute to polyp formation in the nose/sinus, commonly observed in severe CRS conditions as compared with CRSsNP and control subjects [79].

Biofilms

In nature, bacteria exist in two forms; as independent, free-floating planktonic organisms, and as a biofilm matrix. Generally, an estimated 99% of bacteria exist in the form of biofilms and start to proliferate and secrete an extracellular matrix which is highly resistant to antibiotic therapy through preventing antibiotic penetration or limiting antimicrobial efficacy [80, 81]. Biofilm formation and maturation is a multi-step process that comprises both reversible and irreversible stages. As shown in Figure 1.3, the formation of bacterial biofilm begins when free-floating planktonic form (1) of bacteria come in contact with a suitable surface for initial attachment (2), proliferation, permanent attachment with loss of motility (3), biofilm matrix

produced (4), biofilm maturation-different phenotypes, such as persister cells, are present in biofilm (5), and biofilm continues to mature, cells are shed from biofilm and process begins again (6) on a suitable environment.

Bacterial biofilms have been implicated as important features of the endogenous nasal bacteria which play a great role in the disease manifestation of CRS. Biofilms are a complex aggregate of microorganisms encapsulated within a self-produced extracellular matrix composed of polysaccharides, nucleic acids, and proteins which are attached to a moist surface. They provide a mechanism for bacteria to reduce their metabolic rates and serve as a barrier during adverse environmental conditions including host defences and conventional antibiotics [82]. Various bacterial species commonly classified as pathogens including *S. aureus*, *S. pneumoniae*, *P. aeruginosa*, *M. catarrhalis* and *H. influenzae* are able to produce biofilms which are thought to play an important role in the disease manifestation and possibly mediate the adaptive immune response observed in severe cases [83]. Increasing evidence implicate the presence of bacterial biofilms, particularly *staphylococcal* biofilms, in patients with CRS and are linked to more severe and recalcitrant forms of the disease [16, 84].

Biofilm formation is also associated with recurrence of disease and unfavourable outcomes after surgery [85]. Multiple studies have confirmed the presence of bacteria with biofilm-forming capacity in 30-100% of sino-nasal samples from patients with CRS [84, 86, 87]. For example, *S. aureus* is identified in 50% of biofilms from CRS patients and these biofilms are most commonly associated with poor clinical improvement after surgery and recalcitrant outcome [88, 89].

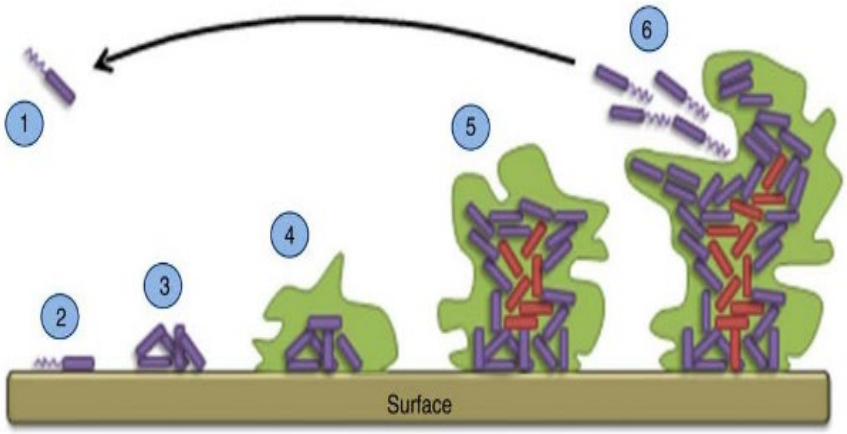


Figure 1.3. Biofilm structure and formation. Initial step includes the introduction of free-floating planktonic bacteria (purple rods) to a surface (grey) with a reversible attachment (1-2), followed by proliferation and irreversible attachment through producing an extracellular matrix (green) (3-4), biofilm maturation, forming characteristic "mushroom" structures due the polysaccharides (5) and, finally, some cells start to detach and the biofilm will disperse (6) [82].

Smoking

Smoking is another factor that contributes to the development and severity of chronic air way inflammation including CRS. Although manifestations of smoking-disordered features in the airways of patients with CRS are complex and diverse, numerous studies have showed convincing evidence that exposure to various inhaled particulates found in tobacco smoke elevates the rate of sinus surgical procedures, delays recovery, and diminishes long-term postoperative outcomes based on the average Sino-Nasal Outcomes Test (SNOT) -16 scores [90-92]. Based on studies in patients with chronic obstructive pulmonary disease (COPD), a lung disease characterized by cough, sputum, and shortness of breath under stress, tobacco smoking is known to induce epithelial-to-mesenchymal transition (EMT) and relevant changes in airway basal cells' (BC) transcriptome, which is critical to the pathogenesis [93]. Tobacco smoking also influences the innate immune function of sino-nasal epithelial cells. The

combination of cigarette smoking with viral infection or exposure to a synthetic viral analogue, poly(I:C) has shown to cause exaggerated expression of the chemotactic cytokine regulated on activation of normal T-cell expressed and secreted (RANTES) as well as the antimicrobial peptide human β-defensin 2 in patients with CRS compared to controls. This has been thought to result in damage of the epithelium, exacerbation of eosinophilic infiltration and contribution to chronic mucosal inflammation [23, 94].

Microbiome dysbiosis

The balance of the local microbiome in the sinuses is increasingly recognized as vital in the initiation or maintenance of CRS [95]. The microbiome is referred to as group or an aggregate of resident microorganisms such as commensal, symbiotic, and pathogenic members in a specific environment, and its presence in the paranasal sinuses is evidently different between CRS patients and healthy individuals [96]. Recent studies have clearly implicated the disruption of nasal microbial communities rather than dominance by specific individual pathogenic bacteria in CRS aetiology. This is characterized as a depletion of several common core microbial groups including *Corynebacterium*, *Propionibacterium*, *Anaerococcus*, *Peptoniphilus* and *Finegoldia*, with reduced microbial diversity and increased overall bacterial load [97, 98]. Moreover, reduced diversity is found in CRS patients following antimicrobial therapy [99]. The focus of this thesis will be to explore the role of sino-nasal microbiome dysbiosis in CRS pathophysiology and investigate novel microbiome-targeted treatments for sinus health and homeostasis.

1.5 The sino-nasal microbiome

The nasal mucosa microbiome has been suggested to play a pivotal role in CRS disease development and the overall nasal health [100]. Even though various studies demonstrated the variation of microbiome composition, there has been no specific beneficial “good’ bacteria or

disease causative “bad” bacteria identified in both CRS and non-CRS patients [101]; however, the balance between them is very important for maintaining health. In the human body of adults, an estimated 10 trillion to 100 trillion microorganisms are reported to reside [102]. It is important to note that, indigenous microorganisms harboured in the nasal cavity are well adapted to the immune system, because of the biological microbe-microbe and host-microbe interaction over time [103].

Analysis of the nasal microbiome has been made using various culture-based methods as well as through advanced high-throughput molecular technology which has allowed the identification of thousands of microbial species that may be present at levels that are too low to detect by direct culture [104]. Although studies report somewhat conflicting results, various studies have described the types and quantities of microorganisms in the complex sino-nasal niche by using advanced molecular- based methods with some of these studies linking traits of the microbiome to CRS disease state [105, 106].

1.5.1 Nasal microbiome in health

The healthy sinus is not sterile, and it appears that not only prevalence, but also abundance of the local microbiota is critical in determining the disease state [107]. Previous studies indicate that bacteria from the genus *Corynebacterium*, *Staphylococcus*, *Streptococcus*, *Dolosigranulum* and *Propionibacterium* were primarily identified as core bacterial groups in the nasal microbiome of healthy humans [108, 109]. The microbiome of the nose in healthy adults has been reported in many microbiome studies with the most dominant families such as *Actinobacteria*, *Firmicutes* , *Proteobacteria*, *Corynebacteriaceae* and *Propionibacteriaceae* [110, 111]. Likewise, a microbiome community variation between healthy and CRS has been previously reported in an advanced molecular sequence analysis

with an overall increment of unique taxa particularly, *Corynebacterium accolens* largely identified in the sinus of healthy controls [112].

1.5.2 Nasal microbiome in CRS

Even though the underlying role of microbial infection in the cause of CRS is yet to be verified, it is postulated that infection due to local microorganisms residing in the sino-nasal cavity elicits an inflammatory response, that results in subsequent chronic alterations and clinical manifestations [113]. Recently, Stephenson *et al.* investigated the sino-nasal microbiome using both conventional culture-based and 16S ribosomal RNA (rRNA) sequencing technologies. In CRS patients, anaerobes were the predominant detected microbes followed by *S. aureus* using molecular methods [108]. In the same study, *S. aureus* and other coagulase negative species of *Staphylococcus* were also detected frequently in 82% of the specimens by culture methods.

The composition of the microbiome and overall quantity of bacteria present in CRS patients versus healthy patients is also reported in other studies. It is evident that there is a marked differences in reported microbial communities between various studies. The causes of such variation are poorly understood, but could include differences among populations of patients (e.g., antibiotic history, ethnicity), methodology, genetics, environmental factors or simply natural variation among different parts of the nasal passages and sinuses. To this end, Cope *et al.* demonstrated the dominant taxa of typical sino-nasal microbiome community members such as *Streptococcaceae*, *Pseudomonadaceae*, *Corynebacteriaceae* or *Staphylococcaceae* and distinct lower abundance bacterial family members exist in the sinus microbiome [114]. With regard to bacterial burden and taxonomic distribution, the most common pathogenic taxa such as *Pseudomonadaceae*, *Lachnospiraceae* and *Mycobacteriaceae* were frequently found in CRS and control subjects with a similar bacterial load, however, *Corynebacterium* species were also identified mainly in CRS patients [115]. Likewise, in the CRS sinus, *Corynebacterium*

tuberculostearicum species and other taxonomic groups such as *Cloacibacterium* and *Alicycliphilus* were reduced significantly compared to healthy subjects, but a possible bacterial group was not identified as pathogens that contribute to exacerbation of inflammation in patients with CRS [112].

1.6 Bacterial dysbiosis in CRS

The importance of understanding the complex microbial communities in the sinuses is emphasized by the concept of dysbiosis, where organisms interact either in a positive or negative way to change the local composition, and interaction with the host [105]. Recent evidence demonstrating an imbalance of the resident microbial community within the paranasal sinuses, termed as nasal microbiota dysbiosis has emerged as a cause in many cases of CRS, as seen by an overabundance of opportunistic pathogens, mainly *S. aureus*, and loss of key commensals belonging to the genus *Corynebacterium*, which leads to a significant loss of epithelial integrity, immune activation and sinus inflammation [116, 117].

The overgrowth of pathogens with increased bacterial load in the context of CRS is often accompanied by dysbiosis with an increased inter- and intra-subject variability and reduced bacterial diversity and this is postulated to contribute to the pathophysiology of CRS [97]. Furthermore, it is thought that disruption of the resident sinus microbiome with a reduction of commensals and dominance of pathobionts or pathogens may mediate a loss of immune homeostasis [118]. For example, Hoggard *et al.* demonstrated an increased variability and bacterial community dysbiosis and showed evidence of their contribution in the pathogenesis of CRS [97]. Interestingly, recent culture-independent studies demonstrated a clear difference in the absolute quantity or density of organisms in CRS and healthy patients [119]. Most notably in CRS patients, a decrement of absolute quantity of known commensal *Corynebacterium* species and an expansion of the pathogenic bacteria including

Staphylococcus, *Haemophilus*, *Moraxella* and *Entrobacteriacea* group have been observed [119]. Figure 1.4 represent the nasal mucosa microbiomes of healthy versus CRS patients.

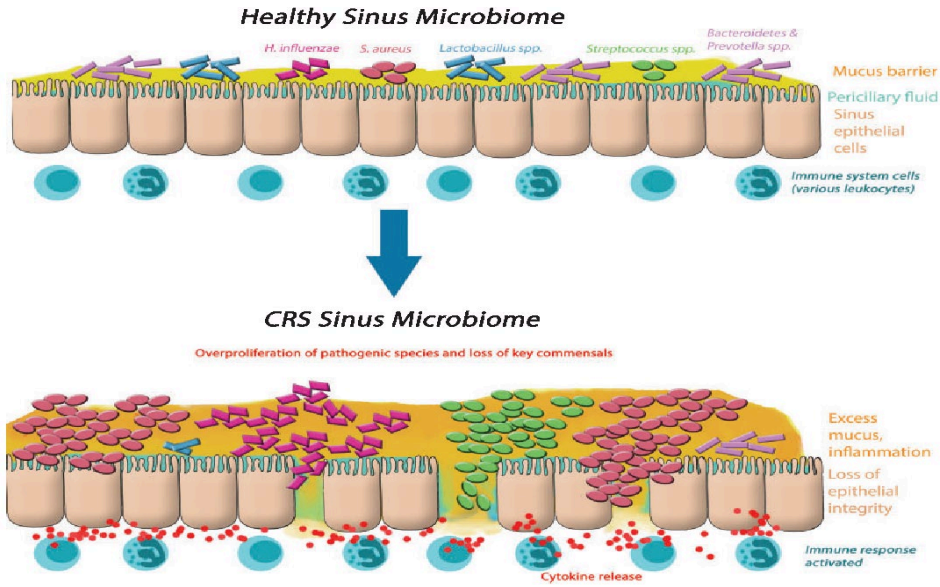


Figure 1.4. The sinus microbiome composition in healthy versus CRS patients. The bacterial taxa implicated here are some of the commensal and pathogenic species that have been involved in CRS disease progression [116].

1.7 Sino-nasal microbial interspecies interaction

Microorganisms in a host interact with each other either positively or negatively to survive the complex community group and maintain the structural composition of the niche. Microbes that normally exist on the linings of nasal cavity can develop a range of either interference competition which occurs by releasing antagonistic substance to inhibit the competitors or exploitative competition to prevent competitors from participating and gaining any nutrient access from the surrounding environment [120, 121]. Several bacterial species isolated from the sinuses demonstrate such interactions. An example of this is the commensal bacterium *S. epidermidis* which can compete with the pathogen *S. aureus*. This occurs via the action of extracellular serine protease, Esp, which is secreted and contributes to a reduction of *S. aureus* biofilm [122]. The enzyme Esp can degrade the *S. aureus* biofilm formation by inhibiting

deoxyribonucleic acid (DNA) release and inactivating autolysins, consequently blocking the important biofilm matrix component [123].

In another study, *C. accolens* showed a positive correlation with *S. aureus* colonization in *in vitro* co-cultivation, suggesting that *S. aureus* can promote the growth of *C. accolens* whereas other *Corynebacterium* species such as *C. pseudodiphtheriticum* showed a negative correlation with *S. aureus*, hindering the development of *S. aureus* colonies [124]. On the contrary, in other studies the pathogen *S. aureus* positively interacts with *C. pseudodiphtheriticum* and negatively interacts with *C. accolens* as implicated in a predominant number of *C. accolens* co-existing with *S. aureus* pathogens [125]. Similarly *Corynebacterium* species, in particular, *C. accolens* interfered with a competitor *S. pneumoniae* in the nasal cavity by using secreted triacylglycerol lipase enzyme (LipS1) [126, 127]. This enzyme can produce oleic acid from triolein (a triglyceride found on human skin and in the sinuses) by hydrolysis. Together with other nutrients, the released oleic acid can suppress the growth of the pathogen *S. pneumoniae* [126, 127]. Characterising such interactions helps to know the role and effect of individual species in maintaining the sinus microbiota.

1.8 Therapeutic strategies in chronic rhinosinusitis

Current strategies for managing CRS are based on medical and surgical treatment with endoscopic sinus surgery, when appropriate medical intervention is unsuccessful. The primarily goals of such management strategies are to minimise inflammation of the mucus, prevent bacterial infection/colonization, restore mucociliary clearance within the sinuses, improve sino-nasal function and maintain quality of life [128]. Yet, there is no complete cure or prevention in CRS management and methodologies are still controversial and lacking.

1.8.1 Medical treatment in CRS

The mainstays of medical treatments for CRS include nasal irrigation with isotonic or hypertonic saline solutions, intranasal corticosteroid sprays, short-term oral corticosteroids and antibiotic use (in both oral and topical preparations) in order to improve sino-nasal symptoms, reduce disease exacerbation and maintain the normal healthy sinus [16, 129]. Although the role and effectiveness of antibiotic therapy for the management of CRS is not fully understood, frequent antibiotic prescription and use in the majority of these patients may contribute to the expansion of antibiotic resistance, which is devastating for both intervention and patient management [130].

Recently, non-antibiotic therapies such as bacteriophages, manuka honey and colloidal silver nanoparticles have become increasingly effective and promising therapeutic options for CRS patients based on their ability to eradicate bacterial biofilms and decrease the recalcitrant condition [131-133]. Bacteriophages (phages) are viruses that infect and replicate in bacteria. They are highly species-specific with regard to their hosts and can thus be used to target pathogenic bacteria without distressing commensal bacteria. Along with their ability to penetrate biofilms, this them an attractive non-antibiotic therapy for treating bacterial infection and biofilms in CRS [134, 135]. Additionally, Fong et al demonstrated in the *in vitro* biofilm model that, a single dose of phage significantly reduced biofilms formed by different *P. aeruginosa* clinical isolates from CRS patients [132]. In addition, manuka honey is largely known for its therapeutic properties in epithelial regeneration and wound-healing. However, based on the *in-vitro* experiment in different studies, manuka honey and its active component, methylglyoxal demonstrated antimicrobial activity against a wide variety of bacteria including *S. aureus*, MRSA and *P. aeruginosa* both the planktonic and biofilms forms [136-138]. Interestingly, intranasal application of colloidal silver has been shown a great clinical benefit in patients with recalcitrant *S. aureus* infected patients. In previous study, topical

colloidal silver application demonstrated a dose-dependent reduction of *S. aureus* biofilms when formed in-vitro [139]. Along with the antimicrobial potential of colloidal silver, its safety has also been reported previously using *in vivo* sino-nasal sheep model. In this study, topical colloidal silver demonstrated continued anti-biofilm effects against *S. aureus* biofilms and appears safe [140].

1.8.2 Surgical treatment in CRS

Endoscopic sinus surgery is an effective and major form of surgical procedure for CRS management, generally reserved for those who have failed to medical therapy. It aims to provide ventilation and drainage of the paranasal sinuses and to enlarge the sinus to access topical medications [141]. Different phenotypes of CRS have specific treatment strategies and approaches, in which CRSwNP is common and a frequent indication to undergo sinus surgical procedures in patient management [15]. There is also evidence that the combination of surgical intervention, careful postoperative care, and medical therapy lead to greater improvement of sino-nasal symptoms and endoscopic findings and promising long-term benefits in CRS [142].

1.8.3 Microbiome-targeted treatments in CRS

Although medical therapy and endoscopic sinus surgery have been shown to improve sino-nasal symptoms and quality of life, prolonged antibiotic administration and previous sinus surgery in patients with CRS is thought to reduce diversity of the sino-nasal microbiome community and lead to emergence of a dominant pathogenic bacteria in the niche [143]. The great difference in microbiome composition as well as absolute quantity of specific organisms in healthy and CRS patients invites the search for - alternative therapeutic options targeting the nasal microbiota.

There has been a recent focus on altering the whole diseased microbiome using microbiome transplantation and this has been successful for treating patients infected with *Clostridium difficile* and promising for inflammatory bowel disease management [144, 145]. However, evidence from a previous study in a mouse model suggests that exogenous antibiotic treatment can reduce the intestinal microbiome species diversity and may facilitate the inflammatory condition through the overgrowth of spore forming bacteria that would be commonly inhibited by the non-pathogenic bacteria [146].

Microbiome-based therapy is currently attracting a lot of attention in CRS management. The possibility of improving sinus health via prebiotics (growth-promoting agents for beneficial bacteria) and probiotics (a live beneficial bacteria) is predicted to be an effective treatment option for manipulating the dysbiotic nasal microbiota [119]. This novel treatment strategy could help not only for disease prevention and treatment but also for reducing the frequent antibiotic prescription and tackling expansion of antimicrobial resistance in the future.

1.9 Prebiotics

1.9.1 Definition and concept of prebiotics

In 1995, Gibson and Roberfroid introduced the concept of prebiotics for the first time as a non-digestible dietary products that can promote or stimulate the growth and activity of one or number commensal bacteria found in the colon, and thus improve the host health [147]. Later on, the definition was modified as fermenting ingredients that can stimulate the growth of entire beneficial bacteria in the gastrointestinal tract and allow specific changes in the composition and activity of gut microbiota to confer the host health and well-being [148]. Non-digestible compounds categorized as potential prebiotics that can provide a health benefit to the intestine should fulfil the following criteria: (a) fights acidity of the gastric environment, hydrolysis through the host enzymes and absorption of gastrointestinal tract; (b) fermented by means of

the intestinal microbiota; and (c) selectively enhances the growth and/or action of intestinal commensal bacteria mainly associated with health and well-being [149]. In addition to those criteria, prebiotics applicable in the food industry must be chemically steady to various treatments necessary for food processing for example low pH, maillard reaction states and heat [148].

In terms of their source, prebiotics occur naturally in numerous dietary food ingredients such as sugar beet, onion, wheat, banana, tomato, soybean, seaweeds, asparagus, etc., and are also synthetically extracted using various raw materials such as sucrose, lactose, starch, and plants [150, 151]. Several oligosaccharides, such as inulin, fructo-oligosaccharide (FOS) and galacto-oligosaccardes (GOS), *trans*-GOS (tGOS), and lactulose are the most extensively studied and well recognized prebiotics to increase *Bifidobacteria* and *Lactobacilli* commensal bacteria [152]. These potential prebiotics are able to stay longer in the stomach and small intestine following intake, and then good bacteria harboured in the large intestine utilise a fermentation process to breakdown the fibres and to use them as fuel for bacterial growth and reproduction, leading to modulation of the gut microbiota [153].

With evidence suggesting that microbiota of the nose may impact on sinus health [106]. There is now increasing attention directed towards prebiotics and their compounds for use in the sino-nasal cavity [119]. Furthermore, the selective nature of prebiotics to stimulate the growth of beneficial bacteria is crucial, as some microorganisms in the nasal tract, mainly *Staphylococci*, are considered as pathobionts that can cause infection under certain conditions [71].

1.9.2 Fatty acid compounds as prebiotics

Fatty acids are long-aliphatic chain hydrocarbons that can be categorized as either saturated, mono-unsaturated, polyunsaturated, or *trans* fats. The differ by their number of carbon atoms, that can range from 12 to 24, and in the presence or absence of double bonds in the hydrocarbon chain and their location [154]. Fatty acids chemically bind to a glycerol moiety to form a complex and structurally essential lipids such as Triglycerides or Triacylglycerols (Figure 1.5), and are the main energy depot of all bacteria within the actinomycetes group [155].

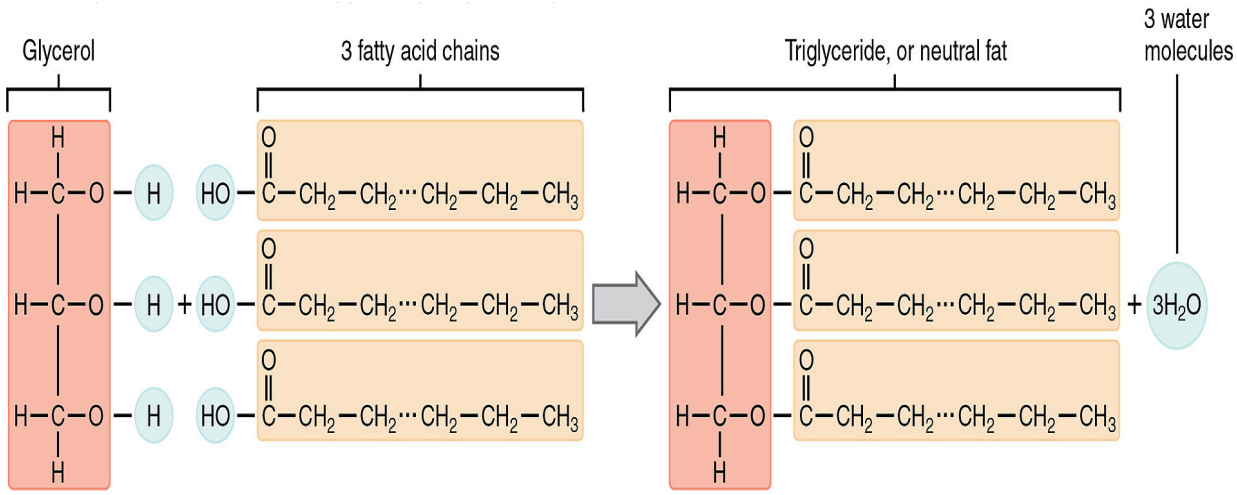


Figure 1.5. Structure of a triacylglycerol molecule. Image source: By OpenStax College, Anatomy & Physiology, Connexions Web site. (http://cnx.org/content/col11496/1.6/), CC-BY-3.0, through Wikimedia Commons.

It is well known that human nostril and skin surface Triacylglycerols (TAGs) enhance the growth of known lipophilic species of Corynebacteria commonly found in the complex nasal microbiota [127]. Complete hydrolysis of triacylglycerols yields a glycerol unit and triester of long-chain fatty acid molecules, which can provide energy for a long time [156].

One potential compound to be used as a prebiotic for rehabilitation and restoring of the sino-nasal microbiome is Polysorbate 80. Polysorbate 80, also known as Tween 80, is a non-ionic surfactant extensively used as an emulsifier, stabilizer or dispersant in pharmaceutical preparations. This compound is commonly used as an excipient in nasal spray formulations to promote the solubilisation and absorption of the active drug [157]. Tween 80 is chemically derived from polyoxyethylene sorbitan and structurally related fatty acid esters, mainly oleic acid [158]. The overall molecular structure of Tween 80 and their derivatives are presented in Figure 1.6.

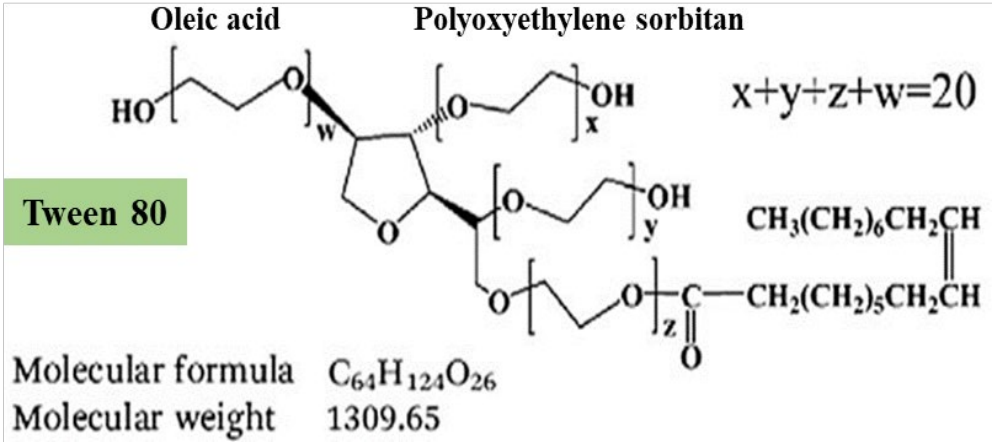


Figure 1.6. Molecular structure of Tween 80, a synthetic surfactant composed of fatty acid esters of polyoxyethylene sorbitan with oleic acid [159].

Few studies have focused on the possible growth-stimulating properties of free fatty acid (FFA) -containing compounds, which contribute to the survival of resident nasal flora. A recent study has shown that a commensal bacterium, *C. accolens*, frequently found in the human nostril requires an exogenous free fatty acid source such as Tween 80, a lipid that contains oleic acid for its growth. This compound has also been shown to exhibit growth inhibition against *S. pneumoniae* and is predicted to be a potential prebiotic in reducing the level of pathogenic bacterial colonization [127]. Various studies have shown the antibacterial and anti-biofilm

activity of Tween 80 against a variety of pathogenic microorganisms, including *S. aureus* and *P. aeruginosa* [160, 161].

Moreover, Tween 80 has been verified to have potential anti-biofilm forming capacity against several nasal pathogens including *P. aeruginosa*. However this appears to be strain dependent with the action of Tween 80 reduced against *P. aeruginosa* PA14 which secretes a lipase to degrade the non- ionic surfactant, showing one mechanism of resistance in this particular bacterium [161]. In another study, Tween 80 showed a growth promotion effect on both planktonic and biofilm forming *S. aureus* species and inhibited *L. monocytogenes* and *P. fluorescens* growth [160]. Tween 80 is also safe, with administration of polysorbate 80 in mice showing a well-tolerated effect on mucosal sites with no evidence of carcinogenic activity [162]. In addition to this potential, Tween 80 has also been approved for nasal spray applications as an ingredient at the recommended dose of 0.5 - 10% [163].

The free fatty acid moiety of Tween 80, oleic acid, has also been reported to have antimicrobial properties against a variety of pathogens including *S. aureus* and MRSA with no toxicity to human cells, and is proposed as a promising therapeutic approach in a dose dependent manner for effective pathogen eradication [164].

Furthermore, oleic acid showed inhibitory activity against *S. pneumoniae* growth by depolarization of the membrane and rupturing of the cell [165]. In general, many compounds containing free fatty acids including Tween 80 and oleic acid have shown an antibacterial effect against a variety of pathogenic microorganisms [160, 164]. However, whether Tween 80 and oleic acid promote or inhibit bacteria in the sino-nasal microbiome (both commensal and pathogenic organisms) is currently unknown. Their effect on the commonly isolated sinus pathogens should therefore be determined to assess whether the nutrient helps or prevents

bacterial growth of different species within the sino-nasal niche and hence whether it promotes a healthy microbiome composition.

1.9.3 Health benefits of prebiotics

Prebiotics have developed as an effective therapeutic approach to restore the balance of microbiota and promote homeostasis. The human nasal microbiota is involved in a range of activities important for sino-nasal health; however, their disruption can lead to substantial metabolic irregularities and complex respiratory disorders [118].

With regard to gut health, prebiotics have been linked with many health benefits such as restoration of the balance of intestinal bacteria, enhancement of the bioavailability and uptake of minerals, reduction in the prevalence and extent of diarrhea, relief of inflammation and other symptoms related with intestinal bowel syndrome, protection against colon cancer, lowering of some risk factors of cardiovascular disease, promotion of weight loss thus prevention of obesity, modulation of the immune system, and regulation of lipid metabolism [166-169]. Bacteria belonging to the genus *Lactobacillus* play a role in assisting in the digestion of lactose for lactose-intolerant individuals, alleviation of constipation, recovery form symptoms of irritable bowel syndrome (IBS), and potentially help protect against traveler's diarrhea [170]. In addition, bifidobacteria exist naturally in the gastero intestinal tract of healthy humans and have a robust attraction to ferment select oligosaccharides, rendering them a common indicator for prebiotic ability. Both lactobacilli and bifidobacteria, are known saccharolytic bacteria, which are frequently found to be beneficial bacteria [171].

In the context of upper respiratory tract infections including CRS, growth-promoting agents such as prebiotics that could potentially have beneficial health effects due to promoting the growth of commensals and reducing pathogenic bacterial growth, modulating the nasal barrier

and regulating the immune system and inflammatory response, is crucial for the regulation of dysbiosis [172].

1.10 Probiotics

1.10.1 Probiotics definition

The term ‘probiotics’ was derived from Greek, meaning ‘for life’. Lilly and Stillwell first introduced this definition to describe secreted products by one bacteria that enhance the growth of another, conferring bacterial growth associated with beneficial properties on its host [173]. In the last two decades, probiotics have been frequently used in foods, especially in fermented dairy products, food supplements as well as drug formulations [174]. According to the World Health Organisation (WHO) the definition of probiotics was refined as ‘live micro-organisms’ that confer a health benefit to the host when consumed in adequate amounts, and are generally acknowledged as safe given their long history of safe use [175]. Various ‘friendly bacteria’ such as *Lactobacillus acidophilus*, *Saccharomyces boulardii*, and *Bifidobacterium infantis* have been discovered for the first time as probiotics with different properties and effects on the human body to treat gastrointestinal complaints [176].

It is believed that a mixture of probiotic products have a wider efficacy with better attainment of conveying health benefits; therefore, many probiotics available on the market often contain numerous bacterial strains from diverse bacterial species, instead of a single strain [177]. Commensal bacteria are often used as probiotic strains for nutritional purposes, disease prevention, health promotion and managing various inflammatory diseases; however, they cannot be termed 'probiotics' until those strains are isolated, well characterized and have evidence of health effects.

1.10.2 Probiotic mechanism of action

Probiotics exert many health effects in a manner beneficial to the host. Although the exact mechanisms by which probiotics achieve their beneficial effects have not been well characterized, they are generally believed to be driven by various mechanisms of action, including promotion of the epithelial barrier integrity, increased adhesion to intestinal mucosa, inhibition of pathogen adhesion, competitive exclusion of pathogenic microorganisms, secretion of anti-microbial substances, and modulation of the immune system [178].

Commensal bacteria can have an enormous role for host epithelial barrier function and protection of the mucosa, and as a result, many probiotic bacteria have been widely studied for their contribution in maintaining the integrity this barrier [179]. Numerous *Lactobacillus* species help maintain the intestinal barrier function as seen in the *in vitro* studies through a higher expression of mucin in human intestinal cell lines (HT29) and *in vivo* by a simultaneous increase in mucin secretion, thus positively controlling epithelial barrier function [180, 181].

The adhesion of certain probiotic strains to the host epithelial cells and mucus are thought to be responsible for the beneficial health effects. The involvement of secreted and surface-associated proteins in bacteria belonging to the genus *Bifidobacterium* and lactic acid bacteria have been reported in facilitating bacterial adhesion to the mucous layer [182]. Moreover, probiotics have been reported to induce the release of mucins and small peptides/proteins from epithelial cells, which in turn are active against pathogenic microorganisms, preventing their adherence and helping the probiotics to occupy the binding sites thereby stabilizing healthy barrier function [183].

Competitive exclusion is one of the mechanisms for certain probiotic bacteria that eliminate or reduce the growth of other bacteria, mainly pathogens, mediated by competition for the existing nutrients and adhesion sites as well as through modification of the host environment to make it unsuitable for the competitors [184]. In this regard, an *in vitro* study by Hirano et al. demonstrated that a strongly adhering strain, *L. rhamnosus* is capable of preventing the attachment of enterohemorrhagic *E. coli* in a human intestinal cell line, likely due to the significant interaction of *L. rhamnosus* with the host cells [185].

Probiotic bacteria also act as a protective barrier against various pathogens through secretion of antimicrobial compounds. It is well known that some probiotic strains produce a diverse array of antibacterial metabolites including bacteriocins and small antimicrobial proteins (AMPs) such as defensins, C-type lectins, cathelicidins and ribonucleases that are responsible for the growth inhibition of bacterial and fungal pathogens [186, 187]. Previous studies have shown that secretion of bioactive substance such as bacteriocin confers probiotic strains with a competitive benefit within the complex microbial niche, as a result of their damage to target cells by formation of pores or cell wall synthesis inhibition, which may directly inhibit the pathogens [188, 189].

It is well known that probiotic bacteria can exert immune-modulatory and anti-inflammatory effects. These effects are due to the ability of probiotic bacteria to interact with epithelial and dendritic cells and with monocytes/macrophages and lymphocytes in the regulation of innate and adaptive immune response [190]. The adaptive immune response depends on B and T lymphocytes, which bind to specific antigens. In contrast, the innate system responds to common assemblies, termed PAMPs, shared by many pathogenic bacteria [191]. It is well documented that probiotics connect with the host by pattern recognition receptors, such as toll-like receptors and nucleotide-binding oligomerization domain-containing protein-like

receptors, which modulate vital signaling pathways, for instance nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinases, to improve or overwhelm activation and impact downstream pathways. This progression is essential for provoking quantified antimicrobial responses with slight inflammatory tissue impairment [192, 193].

It is also important to consider that mechanisms underlying probiotic action engaged against pathogens are varied, diverse and perhaps strain specific. Along the human nasal passages, commensal bacteria that are thought to be probiotics can affect pathobionts or pathogens to maintain nasal homeostasis, through direct inhibition by producing antimicrobials, indirect inhibition by competing for nutrients, environmental pH modification, altering secretion of host-derived metabolites, promotion of host epithelial barrier function or stimulation of the host immune system, and through behaviour modification of pathogens toward commensalism by inactivating the expression of accessory gene regulator (*agr*) quorum sensing system [194]. Figure 1.7 demonstrates the major mechanisms whereby probiotic bacteria might influence the microbiota.

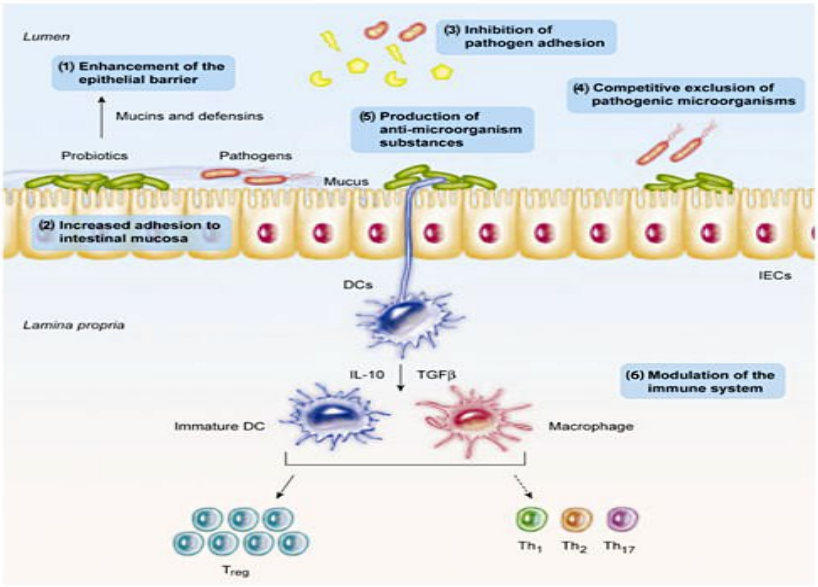


Figure 1.7. Major mechanisms of action of probiotics [178].

1.10.3 Beneficial role of microbes/probiotics in sinus health

Microbes to be used as probiotics for the upper and lower respiratory tract diseases including CRS should be able to modulate nasal epithelial barrier function and interrelate with the innate immune system in order to restore the mucosal barrier integrity and correct the dynamics of the sino-nasal microbiome [195]. Manipulation of the sino-nasal microbiome in CRS patients has potential as an innovative strategy to promote the re-establishment of sino-nasal homeostasis and improve sinus health. Recently, probiotic bacteria have been acknowledged as a potential novel treatment approach in various gut associated diseases such as infectious diarrhoea, antibiotic-associated diarrhoea caused by *Clostridium difficile* and lactose intolerance and thus, when applied in acceptable quantities can provide a host beneficial advantage and interfere with pathogenic organisms [196].

In the gut microbiota, several microorganisms belonging to the genera *Lactobacillus* and *Bifidobacteria* are recognized as the most common probiotic microbes with a diverse potential health effect on the human gut [197]. The potential for probiotic treatment focused on eliminating pathogens while restoring the healthy commensal microbiome in CRS has recently been demonstrated *in vitro* and *in vivo* with several different candidate bacterial strains. In recent times, a number of investigators have proposed the role of beneficial microorganisms or probiotics derived from different genera and species including *Corynebacterium spp*, *Streptococcus spp*, *Lactobacillus spp*, *Bifidobacterium spp* and *Enterococcus spp* in conferring sino-nasal health and homeostasis [115, 198]. Although the potential of probiotics in the treatment of chronic sinus infections has been shown in various *in vitro* and *in vivo* studies, preclinical studies are yet to display efficacy or a clinical benefit in CRS [52, 199]. If preclinical and clinical studies should show promising future results, novel probiotics and prebiotics could provide an inexpensive and safe treatment alternative, reducing antibiotic prescriptions and resistance.

1.10.3.1 Corynebacterium

Corynebacteria play an essential role in nasal health as a result of their capability to interact with common respiratory tract pathogens including *S. aureus* and are thought to have probiotic potential in order to promote the normal nasal microbiome composition [194]. Recent studies have indeed shown that commensal corynebacteria have the potential to maintain a healthy sinus mucosa. For instance, Uehara *et al.* reported the eradication of *S. aureus* and reduction of disease pathology after intranasal administration of *Corynebacterium* species to carriers of *S. aureus* subjects due to competition for survival, suggesting some corynebacteria could be used in probiotic therapy [200]. Another study, using artificially inoculated *C. pseudodiphtheriticum* into the nares of humans showed decreased nasal colonization of potentially pathogenic *S. aureus* bacteria [201]. Moreover, Bomar *et al.* showed that *C. accolens*, the most prominent *Corynebacterium* spp. that colonizes the nasal cavity was able to protect against *S. pneumoniae* nasal colonization and shape the microbiome composition of the nose [127]

1.10.3.2 Staphylococcus

Some organisms of the *Staphylococcus* genus are preventative and therapeutic in managing sino-nasal infections when administered adequately. In particular strains of *S. epidermidis* have been demonstrated to have potentially beneficial interactions against *S. aureus* for manipulation of the microbiome in a mouse model of sinusitis [202]. In this study, nasal co-administration of *S. epidermidis* and the pathogen *S. aureus* exhibited a reduction in goblet cell quantity, an indicator of inflammation in comparison with *S. aureus* inoculated mouse model.

1.10.3.3 Streptococcus

In a molecular-based microbiota study, a mixture of *Streptococcus* probiotic products such as *S. salivarius* 24SMBc and *S. oralis* 89a administered on the nasal microbiota for about 1 week

resulted a significant reduction in *S. aureus* abundance and an increase in the number of entire beneficial microorganisms which could restrict the growth of nasal pathogens [203].

1.10.3.4 Lactobacillus

Strains belonging to the genus *Lactobacillus* are also the most widely recognized strains for nasal probiotic treatments and are generally beneficial in response to reliable and acceptable intake for constructing a healthy nasal micro environment [204]. Interestingly, Abreu *et al.* conducted the first small-scale pilot study to show the potential for topical treatments of *L. sakei* in protecting the sinus epithelial cells. In this study, intranasal administration of *L. sakei* plus *Corynebacterium tuberculostearicum* in the sinusitis experimental mouse model resulted in a reduction in mucin hyper secretion and goblet cells compared to mice administrated with *C. tuberculostearicum* only [115]. Moreover, in another study, orally administered *L. rhamnosus* GG probiotic cocktail led to a reduction in nasal colonization with potentially pathogenic bacteria including *S. aureus*, *S. pneumoniae*, and β-hemolytic streptococci in a group of healthy subjects [205].

In a probiotic screening study, Schwartz *et al.* recently identified a well-tolerated *Lactococcus* probiotic that is involved in an increased secretion of anti-inflammatory cytokine, IL-10 in order to attenuate the disease response in CRS patients [199]. Also, different *Lactobacillus* strains that are isolated from milk and the oral cavity of healthy individuals in other screening study were able to reduce the activity of virulence factors in pathogenic *P. aeruginosa* and also demonstrated the absence of antibiotic resistance [206].

In a randomized, double-blind, placebo-controlled trial by Panigrahi *et al.*, there was a significant reduction in the occurrence of respiratory tract infections with administration of

L. plantarum in 4556 infants for 7 days in the first week of life [207]. Furthermore, the administration of milk with *L. acidophilus* strain L-92 improved patient-reported nasal symptom scores in study subjects with perennial allergic rhinitis compared to the intake of milk with no lactobacillus [208].

Additionally, a randomized controlled trial conducted by Martensson *et al.* demonstrate that 2 weeks' nasal administration of a honeybee LAB microbiome to patients diagnosed with CRSsNP is well tolerated, but neither affects symptom severity nor the microbiological flora/local inflammatory activity [198] .

1.10.3.5 Others

Other potential probiotics belonging to the genus *Bifidobacterium*. The most well-recognized of which is *B. lactis*. This has been shown to prevent upper respiratory tract infection and restore the commensal local microbiota. For example, a clinical double-blind placebo-controlled study in 464 healthy physically-active adults demonstrated that administration of *B. lactis* for 150 days could result in a significant reduction in the risk of upper respiratory tract infections [209]. More recently, nasal application of numerous species of *Bifidobacterium* in combination with *Lactobacillus* for more than 2 weeks has proven safe and well-tolerated but with no significant decrease on CRS disease progression [210]. Furthermore, Habermann *et al.* found a reduced frequency of acute exacerbations of CRS following oral application of *Enterococcus faecalis* Symbioflor-1 over 6 months in a double-blind placebo-controlled multi-center trial [211]. In another clinical trial, 8 weeks of oral treatment of the probiotic *E. faecalis* Symbioflor-1 in 204 children with recurrent rhinosinusitis significantly decreased the frequency and duration of the disease [212].

1.10.3.6 Beneficial Biofilms

Although biofilms are often accepted as potentially destructive for clinical and other industrial fields, many biofilms are beneficial and there are several reports related to the positive use of these biofilms. Beneficial biofilms could be used for wide applications (antibacterial, food fermentation, biofertilizer, biofouling, filtration, prevention of corrosion, antimicrobial agents, wastewater treatment, bioremediation and microbial fuel cells) in food, agricultural, medical, environment and other fields. According to previous reports, certain strains including Bacillus spp., Lactobacillus spp., Enterococcus spp., Pseudomonas spp. (*P. fluorescens*, *P. putida* and *P. chlororaphis*), *Acetobacter aceti*, some fungi and Pseudoalteromonas spp., etc. led to beneficial biofilm formation.

1.11 Probiotic properties required for nasal application

The use of probiotics in the field of upper respiratory tract related diseases including CRS is currently very challenging due to the absence of a well-established monitoring system for probiotic and microbiome-derived treatments [213]. To exert valuable health-promoting effects in a specific host, probiotics must generally possess a variety of helpful properties, as well as properties that allow the microbe to adapt and thrive for the short-term in the target location [214]. Many researchers use various *in vitro* and *in vivo* experimental approaches to screen probiotic strains from the human microbiota. In the *in vitro* experiments, a number of probiotic requirements such as strain identification and characterization, antagonistic activity towards pathogenic microorganisms, adherence to epithelial cell cultures, adhesion competition with pathogenic microorganisms, cytotoxicity, immunomodulatory effects, and presence or absence of antibiotic resistance and virulence factors can be screened by microbial culture, biochemical tests, cell culture, immunological and molecular-based tests [120, 215]. Besides, the safety and efficacy of many probiotic strains can be assessed pre-clinically using *in vivo* experiments in

an animal model, and thus can be imperative for transition of probiotic efficacy to clinical trials in humans [216].

1.11.1 Human origin and strain specificity

On the basis of scientific human microbiome studies, a probiotic bacterium intended for human use should be from human origin and be naturally harboured in the nasal cavity, gastrointestinal tract or elsewhere in the human body [217]. Moreover, correct taxonomic identification of probiotic candidates to the strain and species level is the first step in selection, characterization and safety profile assessment of probiotic strains during the production and commercialization process, as this will help to decipher important information relating to strain/species-associated technical requirements, including bacterial growth conditions, metabolic features and genomic information [218].

For identification and selection of probiotic strains, the use of culture-based phenotypic tests or biochemical identification systems such as analytical profile index (API) are not adequate methods for species or strain level determination, and are considered to be the main cause of mislabelling of certain probiotic organisms reported globally, which likely affects their efficacy and safety assessment [219, 220]. Due to the limitation of phenotypic methods and the progressively significant role displayed by molecular techniques in the field of systematic bacteriology, a variety of DNA techniques based on partial or complete genome sequencing has been largely used for the identification of probiotic bacteria. In recent years, the use of partial or complete sequencing of the ribonucleic acid polymerase beta subunit (*rpoB*)or 16S rRNA gene has become a gold-standard method for identification of closely related bacterial species such as those belonging to the genus *Corynebacterium* [221].

Due to developments in molecular-based technology, whole genome sequencing (WGS) methods have been intensively utilised for the identification of various probiotic candidate strains and investigation of genes involved in antibiotic resistance and virulence [222]. In order to select appropriate patient-specific therapy and achieve a beneficial change, a well-tolerated and effective provision of beneficial microbes is required. Therefore, critical investigation is needed in this area to understand the species or strain level difference of potential probiotic candidates prior to initiation of carefully prepared and rational microbiome manipulation.

1.11.2 Antimicrobial activity against pathogens

Antimicrobial properties are considered an important functional attribute of many probiotics. As the sino-nasal microbiota is a complex environment, composed of diverse genera of microorganisms, introducing probiotics into this vastly competitive niche is very challenging due to species and strain-level variation of interaction between probiotics, commensals and pathogenic microbes [223].

The selection of probiotics is commonly based on the ability to abolish pathogens or competitors. Many probiotics have been reported to exhibit strain specific antimicrobial activity against competing pathogens that may lead to prevention of colonization by pathogens in a specific host [224]. *S. aureus*, *H. influenzae* and *M. catarrhalis* are the main pathobionts identified in the sinuses of patients with CRS and are found dominantly in the sino-nasal niche upon infection [97, 225]. On the other hand, several commensal microbes that can produce bioactive products and compete for nutrients have the potential to inhibit growth of, or kill undesired microorganisms in the sino-nasal niche [222].

In an *in vitro* study using agar spot assays, the potential probiotic strain, *L. casei* AMBR2 inhibited growth of the tested pathogens such as *S. aureus*, *H. influenzae* and *M. catarrhalis*,

possibly by production of bioactive substances and could help in allowing *L. casei* AMBR2 strain to correct dysbiosis of the sino-nasal microbiome [226]. There is also some evidence that commensal *Corynebacterium* and *Dolosigranulum* have an antimicrobial potential significantly involved in specific interactions with upper respiratory tract pathogens including *S. aureus*, *S. pneumoniae* and *M. catarrhalis* and show promise for the development of potential probiotics to treat or prevent a range of respiratory diseases [124, 227].

1.11.3 Adhesion to human nasal epithelial cells

It is generally believed that adherence is an essential feature of most probiotic bacteria to endorse a beneficial action and exert certain beneficial effects on host [228]. The adherence capacity of a probiotic strain to the epithelial and mucosal surfaces of the gastro intestinal tract has also been related to pathogen exclusion in the case of certain bacterial infections [229]. Over the past decades, the Caco-2 cell line that resembles mature enterocytes found in the human intestine has been widely used as an *in vitro* model to determine the adhesion capability of potential probiotic strains isolated from the human gut [230].

The persistence of probiotics in the nasal cavity varies significantly between bacterial genera as well as between strains. For instance, the upper respiratory strain, *Lactobacillus rhamnosus* GG can adhere to the human Calu-3 airway epithelial cells with adhesion percentages of approximately 50% and is able to act as a colonization barrier by inhibiting the common respiratory pathogen *M. catarrhalis* from adhering to the Calu-3 cells [231]. De Boeck *et al.* have also previously investigated the ability of several *Lactobacillus* strains to adhere to Calu-3 cells and found that strains of *L. casei* DSM20178, *L. sakei* AMBR8 and *L. casei* AMBR2 exhibited the greatest adhesion ability to Calu-3 cells in comparison with the other tested strains by the adhesion rate of approximately 12.6%, 11.3%, and 10.4%,

respectively, which enabled them to colonize the human nasopharynx and disturb the binding of pathogens to nasal epithelial cells [226].

1.11.4 Probiotic safety

The safety of probiotics intended for human consumption is of paramount importance in order to avoid any related health threats. As numerous bacteria can have an effect on the balance of microbial composition in the nasal cavity, only those bacterial strains and/or species with a verified positive impact on the host can be designated as potential probiotics [223]. Hence, several organism groups that are well studied and categorized as probiotics such as *Lactobacillus*, *Bifidobacterium*, and yeast are largely considered as safe for gut health and well-being. Moreover, *Enterococcus*, *Streptococcus*, *Bacillus*, and other spore-forming bacteria have been used as probiotic organisms but most are not considered as safe [232]. Theoretically, probiotic strains may be responsible for several adverse effects including systemic infections that cause bacteraemia and septicaemia, harmful metabolic activities, extreme stimulation of the immune system in vulnerable individuals, and transfer of genes conferring antibiotic resistance [223].

Although, there is an increasing interest in the development of novel and safe probiotic strains for the adjunct treatment of dysbiotic microbiota in CRS, the metabolic activity and virulence potential of microbes used as probiotics in a disease processe can vary significantly at the strain level and have been documented in other inflammatory diseases such as atopic dermatitis. Myles *et al*. has shown that, in an atopic dermatitis model, topical microbiome transplantation of commensal *Roseomonas mucosa* isolated from healthy individuals improved the disease outcomes, whereas the same species isolated from atopic dermatitis patients aggravated the disease outcomes [233].

Since numerous types of microbes are explored as probiotics, the potential for pathogenicity or toxicogenicity of a particular microbial species being used must be evaluated to realize safe and effective clinical interventions. For example, in a probiotic *Lactobacillus* species with no identified virulent genes and no decisive evidence of species-associated risk factors, an enormous report of bacterial sepsis particularly, in vulnerable individuals have been documented [234].

An essential parameter such as toxicity of the probiotic bacteria also needs to be tested for safety evaluation of probiotics. An *in vitro* method, lactate dehydrogenase (LDH) enzymatic assay is one that determines the viability of mammalian cells by measuring the secretion of the stable intracellular enzyme LDH upon cell lysis, which is linked with apoptosis or cell death [235]. Baccouri *et al.* demonstrated that *E. faecalis* OB14 and OB15 strains, isolated from traditional Tunisian fermented dairy products did not show any cytotoxic effect *in vitro* following exposure of intestinal cell monolayers compared to a known safe reference strain, *E. faecalis* Symbioflor 1 clone DSM 16431, indicating the strain's potential to avoid the risk of host damage [236].

In response to various concerns about the safety of probiotics, a number of *in vivo* studies in a *Caenorhabditis elegans* (*C. elegans*) worm model have been conducted [236, 237]. *C. elegans* is a small free-living worm that resides in the soil, where it normally feeds on bacteria such as *Escherichia coli* (*E. coli* OP50) and contains a digestive system, nervous system and reproductive system as well as muscle tissue [238]. Because of its morphological simplicity, transparency, appropriateness for genetic exploration, ease of cultivation in the laboratory, and short reproductive cycle and lifespan, *C. elegans* has been established as a potent model organism to study several research questions including toxicity, virulence and mechanism of action of many probiotic bacteria [239]. Results obtained from the *in vivo* assay in *C. elegans*

clearly showed that the *E. faecalis* OB14 and OB15 isolates are not toxic to worms and caused no infection [236]. In another study, feeding a probiotic candidate, *E. faecium* L11 to *C. elegans* worms markedly prolonged their lifespan compared with the control groups worms fed *E. coli* OP50 [237].

It is important to point out that safety is discursively tied to the nature of the particular bacteria being used, and thus expression and transferability of antibiotic resistance determinants from probiotic strains to a harmless member of the microbial community are vital elements of the *in vitro* safety profile assessment in probiotic bacteria. One of the most critical aspects in a statement regarding antibiotic resistance in probiotic bacteria is to distinguish intrinsic or natural resistance from acquired or adaptive resistance. In the acquired classification, it is also important to separate resistance triggered by changes on chromosomal genetic elements from those genes likely to be transmissible such as vancomycin or tetracycline resistance elements that often exist on plasmids or transposons [240]. Moreover, there are a number of intrinsic factors provide evidence of the safety of a potential probiotic, *Lactobacillus GG*, including lack of any plasmids that comprise either transferable or non-transferable chromosomal elements, in particular for vancomycin resistance [241]. The importance of long-lasting colonization or genetic stability as a safety measure for probiotics should not be generalized across all probiotic strains, hosts, or potential locations for colonization. A recent *in vivo* study showed evidence for the transfer of the vancomycin resistance gene, *vanA* from an *Enterococcus* strain to *L. acidophilus*, in an animal model [242].

1.11.5 Probiotic efficacy

Probiotics offer a suitable and biological resolution as part of alternative therapy for various digestive and non-digestive disorders such as inflammatory bowel disease (IBD), IBS, antibiotic-associated diarrhea and *C. difficile* infection, infectious diarrhea, necrotizing

enterocolitis, hepatic encephalopathy, allergic diseases, and cancer prevention [243]. In recent years, the efficacy of using probiotics in the defensive treatment of sino-nasal diseases has gained considerable attention [244].

It has been observed that various probiotics can fight for mucosal surface binding sites in order to exclude pathogens through production of biologically active compounds. For example, Lievin *et al.* demonstrated that resident *Bifidobacterium* strains, CA1 and F9 isolated from the human gastrointestinal tract produce a lipophilic, low molecular weight and anti-bacterial compound that prevents various pathogenic bacteria, including *S. typhimurium* SL1344 and *E. coli* C1845 from adhering to the epithelium and mucosa [245].

Probiotic bacteria can interact with various pathogens in the complex niche through provoking inflammatory responses induced by the respiratory pathogens [246]. *In vitro*, probiotics have been shown to decrease inflammatory cytokines and epithelial permeability. In this regard, many probiotics are able to activate an anti-inflammatory response to produce anti-inflammatory cytokines such as IL-10. Besides, they can elicit a reduction in pro-inflammatory cytokines such as IL-6 during the course of inflammation [247]. Previous studies have recognized different strains of probiotic bacteria, such as *L. amylovorus* DSM 16698 and *L. casei* OLL2768 with the capacity to suppress production of pro-inflammatory cytokines and evade pathogen-induced inflammation [248, 249].

An important approach in which probiotics are able to protect the host against infection induced by pathogenic bacteria is to inhibit pathogen growth and diminish bacterial colonization in the host's intestines, thus keeping overall host-microbial balance [250]. *In vivo* experimental analysis is a critical laboratory tool for assessing the general safety and choice of probiotic bacteria for human health. Li and colleagues have successfully established a model of *C.*

elegans infected by *Vibrio anguillarum* and screened various marine bacteria for potential probiotic properties. In this model it was shown that *Planococcus maritimus* strain ML1206 could protect *C. elegans* against infection and colonization by *V. anguillarum* [251]. In other probiotic based research using the *C. elegans* infection model, isolates of lactic acid bacteria have been verified to protect against tested pathogens such as *E. faecalis*, *S. aureus* and *P. aeruginosa* PA14, resulting in the enhancement of health and lifespan of the nematode [252, 253].

The clinical application of probiotics in ameliorating CRS is very challenging. In general, the efficacy of potential probiotic strains in the treatment of chronic rhinitis and CRS have been investigated in various *in vitro* and *in vivo* assays varies from strain to strain [209, 254]. For example, in a double blind, randomized, placebo-controlled trial, oral intervention of a probiotic strain, *L. rhamnosus* in 77 CRS participants failed to show any significant clinical improvements in the sino-nasal quality of life scores [255]. Therefore, the effectiveness of probiotics and their active constituent's needs to be critically evaluated using a combination of *in vitro* and *in vivo* methods in order to promote health and prevent potential side-effects.

CHAPTER 2

Fatty acids: the potential for prebiotic treatment of a dysbiotic nasal microbiome

CHAPTER 2: Fatty acids: the potential for prebiotic treatment of a dysbiotic nasal microbiome

This chapter includes two papers and addresses the first research aim designed to evaluate the prebiotic potential of Tween 80 and its free fatty acid derivative, oleic acid, to be used as an adjuvant to treatments delivered to the nasal cavity in the context of CRS. The first paper is published as a research note in *International Forum of Allergy & Rhinology* and the second paper is prepared in a manuscript format.

Papers in this Chapter:

I. **Menberu MA**, Hayes AJ, Liu S, Psaltis AJ, Wormald PJ, Vreugde S. Tween 80 and its derivative oleic acid promote the growth of *Corynebacterium accolens* and inhibit *Staphylococcus aureus* clinical isolates. *Int Forum Allergy Rhinol.* 2021; 11 (4): 810-813.

II. **Menberu MA**, Hayes AJ, Liu S, Wormald PJ, Psaltis AJ, Vreugde S. Fatty acid-induced growth promotion of *Corynebacterium accolens* suppresses pathogenic *Staphylococcus aureus* and *Pseudomonas aeruginosa* isolates in a mixed biofilm model. Prepared in a manuscript format.

2.1 Statement of Authorship

Title of Paper	Tween 80 and its derivative oleic acid promote the growth of *Corynebacterium accolens* and inhibit *Staphylococcus aureus* clinical isolates
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Menberu MA, Hayes AJ, Liu S, Psaltis AJ, Wormald PJ, Vreugde S. Tween 80 and its derivative oleic acid promote the growth of *Corynebacterium accolens* and inhibit *Staphylococcus aureus* clinical isolates. International Forum of Allergy & Rhinology. 2021; 11 (4): 810-813.

Principal Author

Name of Principal Author (Candidate)	Martha Alemayehu Menberu		
Contribution to the Paper	Conception and design of the project, acquisition of data, analysis and interpretation of data, drafting the article		
Overall percentage (%)	85		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	21/10/2021

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Andrew James Hayes		
Contribution to the Paper	Interpretation of data, critically revising the article		
Signature		Date	20/10/2021

Name of Co-Author	Sha Liu		
Contribution to the Paper	Acquisition of data, analysis and interpretation of data, manuscript edition		
Signature		Date	18/10/2021

Name of Co-Author	Alkis James Psaltis		
Contribution to the Paper	Conception of the project, critically revising the article		
Signature		Date	18/10/2021

Name of Co-Author	Peter-John Wormald		
Contribution to the Paper	Conception of the project, critically revising the article		
Signature		Date	18/10/2021

Name of Co-Author	Sarah Vreugde		
Contribution to the Paper	Conception and design of the project, critically revising the article		
Signature		Date	18/10/2021

2.2 Paper I: Tween 80 and its derivative Oleic acid promote the growth of *Corynebacterium accolens* and inhibit *Staphylococcus aureus* clinical isolates

Menberu MA, Hayes AJ, Liu S, Psaltis AJ, Wormald PJ, Vreugde S. Tween 80 and its derivative oleic acid promote the growth of *Corynebacterium accolens* and inhibit *Staphylococcus aureus* clinical isolates. *Int Forum Allergy Rhinol.* 2021; 11 (4): 810-813.

Tween 80 and its derivative oleic acid promote the growth of *Corynebacterium accolens and inhibit Staphylococcus aureus* clinicalisolates

Martha Alemayehu Menberu, MSc[1,2], Andrew James Hayes, MPhil[1,3], Sha Liu, PhD[1], Alkis James Psaltis, MBBS (Hons), FRACS, PhD[1], Peter-John Wormald, MD, FRACS[1] and Sarah Vreugde, MD, PhD[1]

Key Words:
therapeutics; chronic rhinosinusitis; bacteriology; nasal cavity; Tween 80; oleic acid

How to Cite this Article:
Menberu MA, Hayes AJ, Liu S, Psaltis AJ, Wormald PJ, Vreugde S. Tween 80 and its derivative oleic acid promotes the growth of *Corynebacterium accolens* and inhibits *Staphylococcus aureus* clinical isolates. *Int Forum Allergy Rhinol*. 2021;11:810–813.

In health, the human nasal cavity is colonized by a wide variety of commensal bacteria and pathobionts and is dominated by Actinobacteria (mainly *Corynebacteriaceae*) and Firmicutes (mainly *Staphylococcaceae*).[1,2] In the context of chronic rhinosinusitis (CRS), however, an imbalance or dysbiosis occurs that is characterized by a decreased relative abundance of Actinobacteria and an overgrowth of pathogens such as *Staphylococcus aureus*.[3]

The fatty acid Tween 80 is commonly used as an excipient in nasal formulations to promote the solubilization of the active drug[4] and is approved by the U.S. Food and Drug Administration (FDA) at a maximum concentration of 0.5%.[5] It is considered to be well tolerated when delivered to mucosal, intradermal, and intravenous sites.[6] Bacteria belonging to the genus Corynebacterium can degrade Tween 80 and use the degradation products (polyoxyethylenic acids and oleic acid) as building blocks to synthesize novel glycolipids that become part of their cell envelope.[7] In contrast, fatty acids can also exhibit concentration-dependent antimicrobial activity against pathogens by interfering with cell membrane permeability.[8]

These unique properties of fatty acid containing formulations and the notion that Tween 80 is already FDA-approved for use in nasal sprays raise the possibility that Tween 80 may have the potential to be used as a prebiotic in topical nasal formulations.

In this study, we aimed to evaluate the dose-dependent activity of Tween 80 and its derivative, oleic acid, on the growth of *Corynebacteriae*, *S. aureus*, and bacteria frequently found in the human nasal cavity using in vitro analysis.

Materials and methods

Twenty-two nasal clinical isolates (CIs) including *C. accolens* (n = 4), *C. propinquum* (n = 3), *C. pseudodiptheriticum* (n = 3), *S. epidermidis* (n = 4), *S. aureus* (n = 4), and *Pseudomonas aeruginosa* (n = 4) and *S. aureus* ATCC25923 (ATCC, Manassas, VA) were used in this study (Human Research Ethics Committee approval number HREC/15/TQEH/132). Tween 80 and oleic acid (Sigma-Aldrich, St. Louis, MO) were diluted at various concentrations in nutrient broth (NB) or brain heart infusion (BHI) (Oxoid, Basingstoke, UK), incubated with bacteria

[1]*Department of Otolaryngology–Head and Neck Surgery, Basil Hetzel Institute for Translational Health Research, University of Adelaide, Woodville, SA, Australia;* [2]*Department of Medical Microbiology, School of Biomedical and Laboratory Sciences, College of Medicine and Health Sciences, University of Gondar, Gondar, Ethiopia;* [3]*Department of Microbiology and Immunology, Peter Doherty Institute for Infection and Immunity, University of Melbourne and The Royal Melbourne Hospital, Melbourne, VIC, Australia*

Correspondence to: Sarah Vreugde, MD, PhD, Department of Otolaryngology–Head and Neck Surgery, Basil Hetzel Institute for Translational Health Research, The Queen Elizabeth Hospital, University of Adelaide, Woodville South, Adelaide, SA 5011, Australia; e-mail: sarah.vreugde@adelaide.edu.au

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Potential conflict of interest: None provided.

Funding sources for the study: The Garnett Passe and Rodney Williams Memorial Foundation (Conjoint Grant to A.J.P and S.V.); The University of Adelaide (International Scholarship to M.A.M.).

Received: 24 August 2020; Revised: 13 October 2020; Accepted: 19 October 2020
DOI: 10.1002/alr.22730
View this article online at wileyonlinelibrary.com.

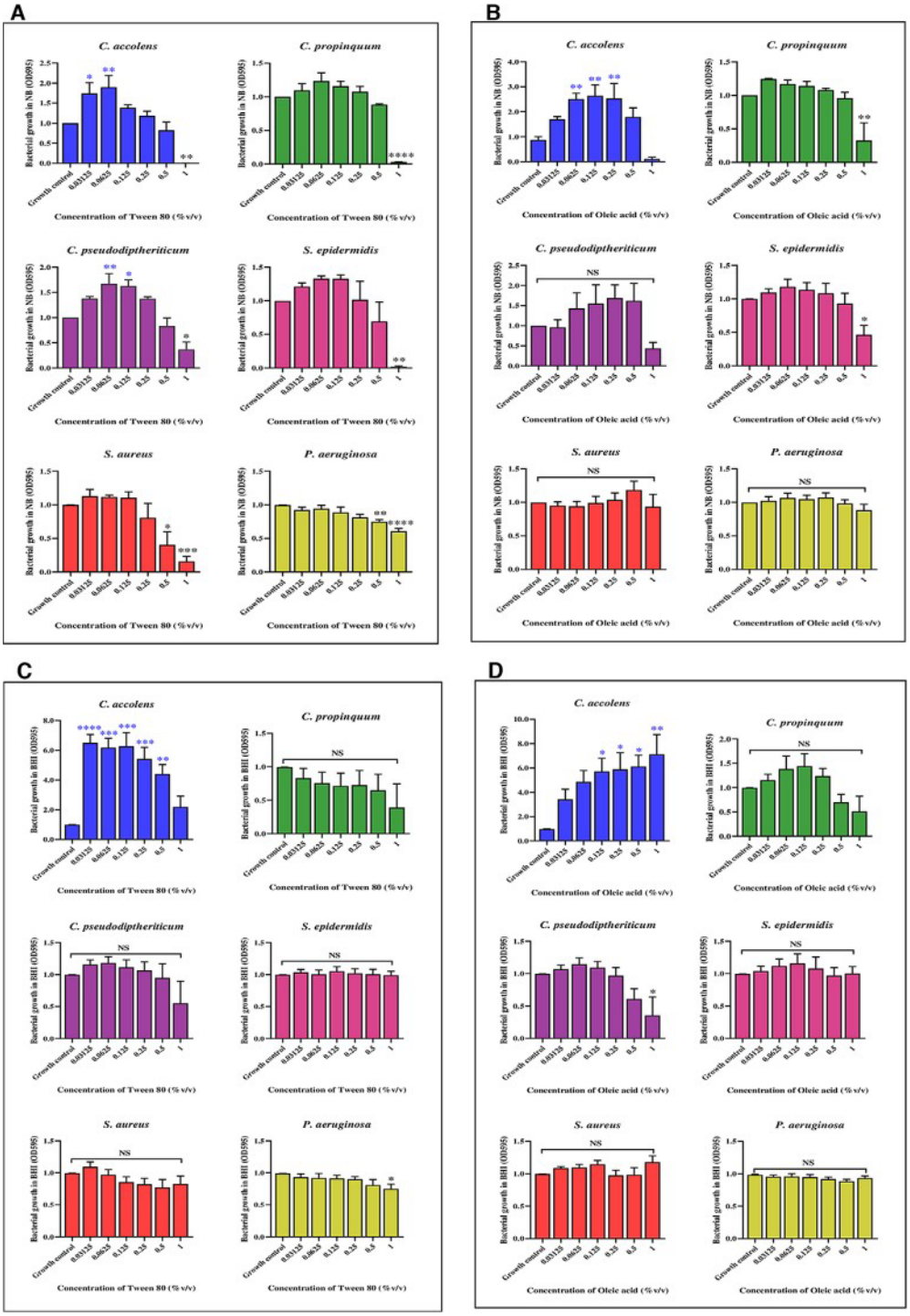


FIGURE 1. (A,B) The effect of Tween 80 (A) and Oleic acid (B) on planktonic growth (OD595) of different bacterial strains after 24 hours in nutrient-poor growth media (NB). Data represent the mean ± SEM of each bacterial strain; *C. accolens* (n = 4), *C. propinquum* (n = 3), *C. pseudodiptheriticum* (n = 3), *S. epidermidis* (n = 4), *S. aureus* (n = 4), and *P. aeruginosa* (n = 4). The experiments were conducted for different treatment concentrations in at least 6 replicates. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, 1-way ANOVA. (C,D). The effect of Tween 80 (A) and oleic acid (B) on planktonic growth (OD595) of different bacterial strains after 24 hours in nutrient-rich growth media (BHI). Data represent the mean ± SEM of each bacterial strain; *C. accolens* (n = 4), *C. propinquum* (n = 3), *C. pseudodiptheriticum* (n = 3), *S. epidermidis* (n = 4), *S. aureus* (n = 4), and *P. aeruginosa* (n = 4). The experiments were conducted for different treatment concentrations in at least 6 replicates. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, 1-way ANOVA. ANOVA = analysis of variance; BHI = brain-heart infusion; NB = nutrient broth; NS = not significant; SEM = standard error of the mean.

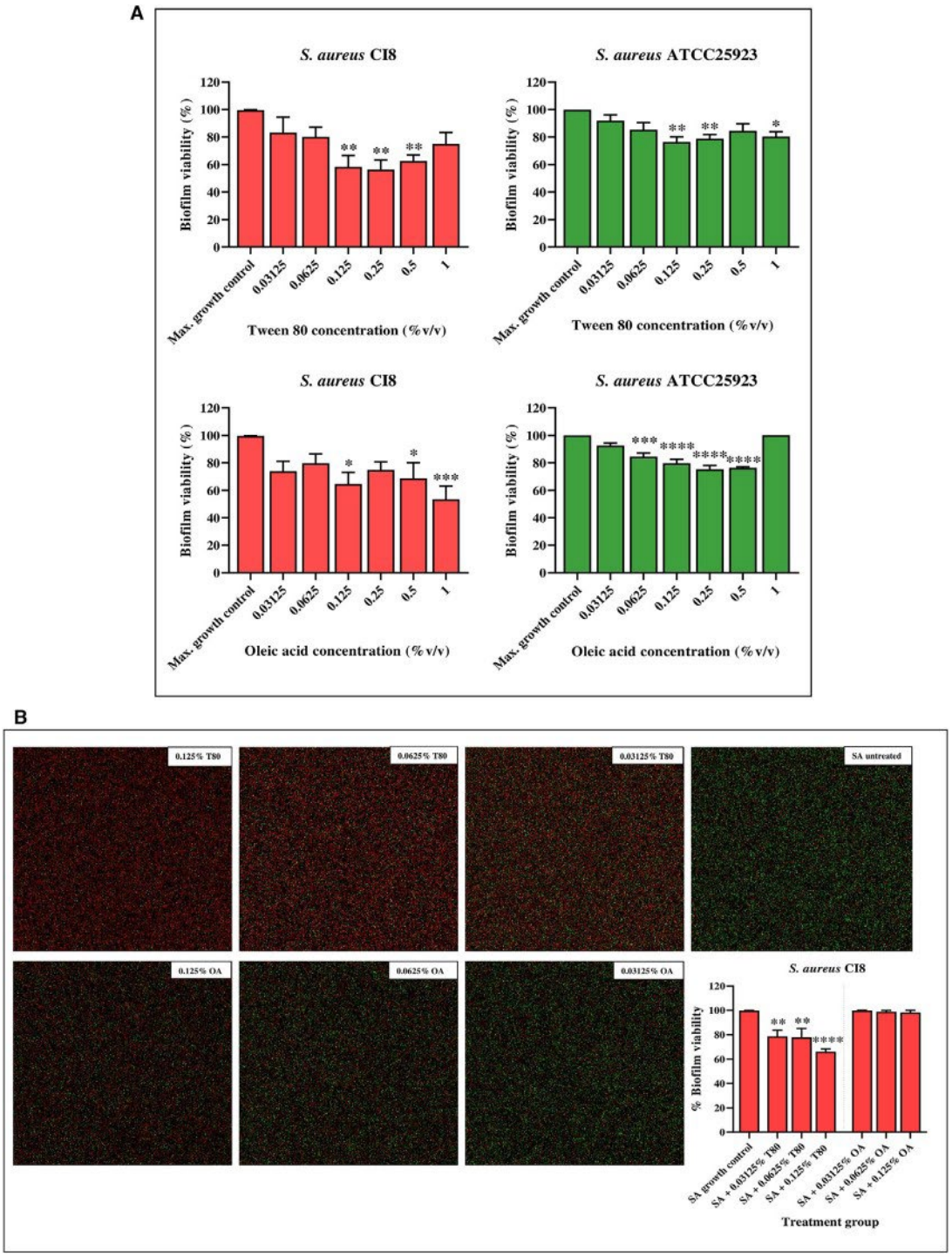


FIGURE 2. (A) Treatment of Tween 80 and oleic acid at concentrations of 0.03125% to 1% (vol/vol) or maximum growth control and its effect on biofilm viability of *S. aureus* clinical isolate (*S. aureus* CI8) and laboratory reference strain (*S. aureus* ATCC25923). The experiments were conducted in each strain at least three times. Data represents the mean $_{+}$ SEM of the three replicates. *$p < 0.05$. **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, 1-way ANOVA. (B). LIVE/DEAD staining and CLSM visualization of *S. aureus* CI8 biofilms treated with Tween 80 or oleic acid in various concentrations. The green color represents live cells whereas red color represents dead cells. Data was presented by calculating the mean + SEM of the fluorescent intensity value of at least 3 microscopic images obtained from 3 replicate experiments. *Statistically significant, *$p < 0.05$, ***$p < 0.001$. ANOVA = analysis of variance; CLSM = confocal laser scanning microscopy; NS = not significant; OA = oleic acid; SA = *S. aureus*; SEM = standard error of the mean; T80 = Tween 80.

for 24 hours followed by determining optical density to determine the minimum inhibitory concentration (MIC). The effects of Tween 80 and oleic acid on the formation of biofilms and on established biofilms were then evaluated by growing *S. aureus* CI8 and ATCC25923 for 48 hours in Tryptone-soya broth (TSB) containing Tween 80 or oleic acid or by adding both compounds to preformed 48-hour biofilms, each time followed by measuring the viability using an Alamar Blue assay on a FLUOstar OPTIMA microplate reader (BMG LABTECH GmbH, Ortenberg, Germany), LIVE/DEAD BacLight staining (Invitrogen Bacterial Viability Kit; Invitrogen, Carlsbad, CA) and confocal laser scanning microscopy (Carl ZEISS 16.0; Carl Zeiss AG, Oberkochen, Germany). One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test using GraphPad Prism version 8.0 (GraphPad Software, Inc., La Jolla, CA) was used for statistical analysis unless specified otherwise. Details of materials and methods are found in this article's online Supporting Information.

Results

In nutrient-poor media (NB, representing the sinonasal environment), the mean growth of *C. accolens* and *C. pseudodiptheriticum* was significantly increased by Tween 80 at low concentrations (up to 1.6-fold in the presence of 0.0625% to 0.125% (vol/vol) for *C. pseudodiptheriticum* and up to 1.9-fold in the presence of 0.03125% to 0.0625% (vol/vol) for *C. accolens*) after 24 hours exposure compared to untreated growth controls ($p < 0.05$). Higher 1% concentrations of Tween 80 (and 0.5% for *S. aureus* and *P. aeruginosa*) significantly reduced the growth of all bacteria tested (Fig. 1A). Furthermore, the mean growth of *C. accolens* (but no other strains) significantly increased following 24 hours oleic acid treatments at 0.0625% to 0.25% concentrations compared to untreated control (up to 3-fold, $p < 0.01$) (Fig. 1B). Similarly, in nutrient-rich media (BHI, used in laboratory conditions), addition of Tween 80 and oleic acid significantly stimulated *C. accolens* growth (at 0.03125% to 0.5% concentration for Tween 80 and 0.125% to 1% (vol/vol) for oleic acid) compared to untreated control (Fig. 1C,D). Adding Tween 80 or oleic acid at the start of biofilm formation, followed by 48 hours incubation, resulted in a significant reduction in biofilm viability for representative isolates *S. aureus* CI8 and ATCC25923 compared to control for various concentrations (between 0.125% and 1% of Tween 80 and oleic acid) ($p < 0.05$) (Fig. 2A). This was also seen using LIVE/DEAD staining where Tween 80 at 0.0315% to 0.125% (but not oleic acid) reduced the formation of 48-hour biofilms compared with untreated control (Fig. 2B). Tween 80 and oleic acid did not reduce the viability of established *S. aureus* biofilms (results not shown).

Discussion

This study showed that Tween 80 at FDA-approved concentrations of 0.5% and below promoted planktonic *C. accolens* growth and reciprocally reduced the viability of *S. aureus* planktonic cells and of newly forming *S. aureus* biofilms. *C. accolens* is considered a benign lipid-requiring commensal species that can degrade human skin triacylglycerols thereby producing free fatty acids that interfere with the growth of pathogens such as *S. pneumoniae*.[9] Such host-microbe-microbe interactions are thought to help shape the human microbiome.[9] A recent international sinonasal microbiome study showed *Corynebacterium* to be the most prevalent genus present in >75% of CRS patients and controls with a significant reduction in its relative abundance in CRS patients compared to controls.[2] The high prevalence of *Corynebacterium* lends itself to the possibility of manipulating that existing microbiome toward homeostasis by promoting its growth. Although further research is needed to validate our findings in the in vivo setting, they support the prebiotic potential of low Tween 80 concentrations to be used in nasal rinse solutions potentially promoting microbiome homeostasis in the context of CRS.

Acknowledgments

We kindly acknowledge Mahnaz Ramezanpour and Clare Cooksley (Department of Otolaryngology–Head and Neck Surgery, Basil Hetzel Institute for Translational Health Research, University of Adelaide, Australia) for their technical support and constructive guidance.

References

1. Bassis C, Tang AL, Young VB, Pynnonen MA. The nasal cavity microbiota of healthy adults. *Microbiome*. 2014;2:1-5.
2. Paramasivan S, Bassiouni A, Shiffer A, et al. The International Sinonasal Microbiome Study (ISMS): a multicentre, multi-national characterization of sinonasal bacterial ecology. *Allergy*. 2020;75:2037-2049.
3. Psaltis AJ, Wormald P-J. Therapy of sinonasal microbiome in CRS: a critical approach. *Curr Allergy Asthma Rep*. 2017;17:59.
4. Trows S, Wuchner K, Spycher R, Steckel H. Analytical challenges and regulatory requirements for nasal drug products in Europe and the US. *Pharmaceutics*. 2014;6:195-219.
5. U.S. Food and Drug Administration (FDA). Inactive Ingredient Search for Approved Drug Products. Silver Spring, MD: FDA; 2020.
6. National Toxicology Program. NTP toxicology and carcinogenesis studies of polysorbate 80 (CAS No. 9005-65-6) in F344/N rats and B6C3F1 mice (feed studies). *Natl Toxicol Program Tech Rep Ser*. 1992;415:1-225.
7. Wang C, Mahrous EA, Lee RE, Vestling MM, Takayama K. Novel polyoxyethylene-containing glycolipids are synthesized in *Corynebacterium matruchotii* and *Mycobacterium smegmatis* cultured in the presence of Tween 80. *J Lipids*. 2011;2011:676535.
8. Kaur G, Mehta S. Developments of polysorbate (Tween) based microemulsions: preclinical drug delivery, toxicity and antimicrobial applications. *Int J Pharm*. 2017;529:134-160.
9. Bomar L, Brugger SD, Yost BH, Davies SS, Lemon KP. *Corynebacterium accolens* releases antipneumococcal free fatty acids from human nostril and skin surface triacylglycerols. *mBio*. 2016;7:e01725-15.

2.3 Statement of Authorship

Title of Paper	Fatty acid-induced growth promotion of *C. accolens* suppresses pathogenic *S. aureus* and *P. aeruginosa* isolates in a mixed biofilm model
Publication Status	☐ Published ☐ Accepted for Publication ☑ Unpublished and Unsubmitted work written in ☐ Submitted for Publication manuscript style
Publication Details	Menberu MA, Hayes AJ, Liu S, Wormald PJ, Psaltis AJ, Vreugde S.

Principal Author

Name of Principal Author (Candidate)	Martha Alemayehu Menberu		
Contribution to the Paper	Conception and design of the project, acquisition of data, analysis and interpretation of data, manuscript preparation		
Overall percentage (%)	85		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	21/10/2021

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

Name of Co-Author	Andrew James Hayes		
Contribution to the Paper	Interpretation of data, critically revising the article		
Signature		Date	20/10/2021

Name of Co-Author	Sha Liu		
Contribution to the Paper	Acquisition of data, analysis and interpretation of data, manuscript edition		
Signature		Date	18/10/2021

Name of Co-Author	Peter-John Wormald		
Contribution to the Paper	Conception of the project, critically revising the article		
Signature		Date	18/10/2021

Name of Co-Author	Alkis James Psaltis		
Contribution to the Paper	Conception of the project, critically revising the article		
Signature		Date	18/10/2021

Name of Co-Author	Sarah Vreugde		
Contribution to the Paper	Conception and design of the project, critically revising the article		
Signature		Date	18/10/2021

2.4 Paper II. Fatty acid-induced growth promotion of *Corynebacterium accolens* suppresses pathogenic *Staphylococcus aureus* and *Pseudomonas aeruginosa* isolates in a mixed biofilm model

Martha A. Menberu[1], Andrew J. Hayes[2], Sha Liu[1], Peter-John Wormald[1], Alkis J. Psaltis[1], Sarah Vreugde[1,*]

[1]*Department of Otolaryngology, Head and Neck Surgery, Basil Hetzel Institute for Translational Health Research, The University of Adelaide, Woodville, South Australia, Australia*

[2]*Department of Microbiology and Immunology, Peter Doherty Institute for Infection and Immunity, The University of Melbourne and The Royal Melbourne Hospital, Melbourne, Victoria, Australia*

[*]Correspondence: sarah.vreugde@adelaide.edu.au

2.4.1 Abstract

Nasal microbiome imbalance or dysbiosis with a reduction in relative abundance of commensal *Corynebacteria* has recently been implicated in the pathophysiology of chronic rhinosinusitis (CRS). Here, we investigated the prebiotic potential of fatty acids such as a common excipient Tween 80 and its derivative oleic acid using *in vitro* approaches. Bacterial isolates retrieved from the nasal cavity, including a nasal commensal *C. accolens* CI09 and pathogens, *S. aureus* CI8 and *P. aeruginosa* CIAus1 were used. The formation and eradication of *P. aeruginosa* biofilms was assessed following treatment with various concentrations of Tween 80 and Oleic acid compounds using AlamarBlue assay and Live/Dead staining techniques. Moreover, the treatment effect on *S. aureus* and *P. aeruginosa* biofilm formation in a mixed biofilm model with commensal *C. accolens* was evaluated using fluorescent in situ hybridisation (FISH) method followed by Confocal Laser Scanning Microscopy (CLSM) image analysis. Tween 80 and Oleic acid, particularly below 0.5% (v/v) concentrations didn't show any effect on *P. aeruginosa* biofilms, when compared to untreated controls. However, both treatments at 1% (v/v) concentration significantly reduced the formation of *P. aeruginosa* biofilms ($p<0.001$) but no impact on already established biofilms. Moreover, in a mixed species biofilm competition, Tween 80 and Oleic acid at or below 0.125% concentration significantly increased *C. accolens* biofilm growth ($P<0.01$) with or without any change on *S. aureus* and *P. aeroginosa* biofilm growth. These results support the potential of low Tween 80 and oleic acid concentrations to be used as growth-stimulating agents or prebiotics for commensal *Corynebacteria* to protect sinus health.

Key words: fatty acids; prebiotics; nasal microbiota; dysbiosis; chronic rhinosinusitis

2.4.2 Introduction

Chronic rhinosinusitis (CRS) is a heterogeneous disorder and highly prevalent global health problem, affecting the mucosal lining of the nasal cavity and paranasal sinuses. This chronic disorder has a complex multifactorial aetiology with various environmental and host factors implicated in the disease process [20]. The healthy human nasal cavity is normally colonized by a diverse group resident microorganism both pathogenic and harmless bacteria and is mainly dominated by the family, *Corynebacteriaceae* and *Staphylococcaceae* [111, 256]. Recently, a nasal microbiota dysbiosis with a reduction in relative abundance of commensal Corynebacteria and an overgrowth of different pathogens, including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Haemophilus influenzae* have been implicated in CRS pathophysiology [119, 256, 257]. The overgrowth of pathogens with increased bacterial load in the context of CRS is often accompanied by dysbiosis with an increased inter- and intra-subject variability and reduced bacterial diversity and this is postulated to contribute to the pathophysiology of CRS [97].

Furthermore, it is thought that disruption of the resident sinus microbiome with a reduction of commensals and dominance of pathobionts or pathogens may mediate a loss of immune homeostasis [118]. In particular, *S. aureus* and *P. aeruginosa*, the most common pathogens in CRS, are able to produce biofilms which are thought to play a great role in the disease manifestation and possibly mediate the adaptive immune response observed in severe cases [83]. In health, the most common bacteria that colonize the nasal cavity are *Corynebacteriaceae* with a prominent presence of *Corynebacterium accolens*, which is thought to have probiotic properties and promotes a normal nasal microbiome composition [194]. Recent studies have indeed shown that commensal bacteria have the potential to shape the microbiome composition and maintain a healthy sinus mucosa [115, 202]. It is well known that

human nostril and Skin Surface Triacylglycerols (TAGs) enhance the growth of known lipophilic members of *Corynebacteria* commonly found in the complex nasal microbiota. Interestingly *C. accolens*, a fatty acid requiring species, secretes the extracellular lipase LipS1 and hydrolyzes surface TAGs, releasing the antibacterial free fatty acid (FFA), oleic acid. These FFAs in turn inhibit nasal pathobionts, particularly pneumococcus, and contribute a beneficial role in shaping the nasal microbiome [127].

Polysorbate 80, also known as Tween 80, is a non-ionic surfactant extensively used as an emulsifier, stabilizer or dispersant in pharmaceutical preparations including nasal spray formulations with an accepted concentration of 0.5% by the US Food and Drug Administration (FDA) [258, 259]. Tween 80 is chemically derived from polyoxyethylene sorbitan and fatty acid esters, mainly oleic acid [158]. Few studies have focused on the possible growth-stimulating properties of FFA-containing compounds, which contribute to the survival of resident nasal flora including *Corynebacteria* genus possibly due to modifying the cell wall permeability [260]. On the other hand, this compound can also display an anti-bacterial effect against pathogens in concentration-dependent manner that led to disrupt the bacterial metabolic activity [261]. For instance, various studies have shown the antibacterial and anti-biofilm activity of Tween 80 against a variety of pathogenic microorganisms, including *S. aureus* and *P. aeruginosa* [160, 161]. The free fatty acid moiety of Tween 80, oleic acid, has also been reported to have antimicrobial properties against a variety of pathogens including Methicillin Resistant *S. aureus* (MRSA) with no toxicity to human cells [164]. The growth stimulation potential of any compounds having a FFA moiety, mainly in promoting the growth of health-associated *Corynebacteria* and reducing the growth of common nasal pathogens, could allow the selection of new prebiotics potentially modify the sino-nasal microbial composition in CRS patients.

In this study, we aimed to evaluate the prebiotic potential of fatty acid (FA) containing excipients that directly stimulate the commensal nasal bacteria in terms of biofilm growth, while reciprocally suppressing the growth of pathogenic nasal bacteria, in order to be used as an adjuvant to treatments delivered to the nasal cavity in the context of CRS. To this end, we have determined the effect of Tween 80 and its free fatty acid moiety, oleic acid on the growth of *C. accolens* nasal bacteria and its effect against most predominant nasal pathogens such as *S. aureus* and *P. aeruginosa* in a mixed commensal-pathogen biofilm model.

2.4.3 Materials and Methods

Bacterial isolates and growth conditions

Ethics clearance for the collection, storage and use of clinical isolates was obtained from The Queen Elizabeth Hospital (TQEH) Human Research Ethics Committee in Adelaide, South Australia (HREC/15/TQEH/132). In this study, different clinical isolates previously identified by our research group from the human nasal swabs, including *C. accolens* CI09, *S. aureus* CI8 and *P. aeruginosa* CIAus1 and a laboratory reference strains, *S. aureus* ATCC25923 and *P. aeruginosa* PA01 were tested. The identification of clinical isolates was performed by the automated Kiestra Total Laboratory Automation (TLA) system (BD Kiestra B.V., The Netherlands) followed by strain verification using matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry (Bruker Daltonik, Bremen, Germany) and stored at - 80°C in glycerol stocks. ATCC25923 and PA01 were purchased from the American Type Culture Collection (ATCC, Manassas, Virginia, USA). Prior to starting experiments, isolates were grown in tryptic soya agar (TSA) and incubated at 37^{o}C under aerobic conditions for 24 hours, with the exception of *C. accolens* which was incubated for 48 hours.

Effect of Tween 80 and Oleic acid on biofilm formation

A clinical isolate, *P. aeruginosa* CIAus1 and a laboratory reference strain, *P. aeruginosa* PA01 were used to determine the effect of Tween 80 and Oleic acid on the formation of biofilms. In brief, 75µl of tryptone-soya broth (TSB) containing either Tween 80 or Oleic acid in a wide concentration range (1% - 0.03125%) was added to clear bottom black 96-well plates. Single colonies of *P. aeruginosa* were mixed in 0.9% saline and standardized to McFarland units of 1.0 (approximately 3×10^8 CFU/ml) followed by a dilution of 1 in 15 using TSB. Then, 75µl of diluted bacterial suspension was inoculated in each well containing treatments. Plates were kept in the dark and incubated at 37°C on a rotating plate for 48 hours. Wells containing no bacteria and untreated wells were used as a negative and untreated growth control, respectively. After incubation, wells were washed twice with 180µl of 1x phosphate buffer saline (PBS) and 180µl of TSB was added for a further 24 hours incubation on a rotating plate. Wells were then washed two times with 200µl of 1xPBS and AlamarBlue assay was performed as described previously [262]. The bacterial viability was determined by measuring the fluorescence intensity every 60 minutes using a plate reader (FLUOstar OPTIMA, Germany) at a wavelength excitation 530nm and emission 590nm until maximum florescence was reached. The anti-biofilm activity of Tween 80 or Oleic acid was calculated as: biofilm killing % = (100 - treated well-background)/ (maximum growth - background) *100; where (background = no bacteria wells, max growth = untreated wells). For each treatment and different concentrations, a minimum of six replicate experiments was conducted.

Effect of Tween 80 and Oleic acid on preformed biofilms

The effect of Tween 80 and oleic acid on established *P. aeruginosa* biofilms was assessed using clinical isolate (*P. aeruginosa* CIAus1) and laboratory reference strain (*P. aeruginosa* PA01). For this investigation, single colonies of *P. aeruginosa* isolates were mixed in saline to

standardize a McFarland unit of 1.0 and then 1 in 15 dilution was made using TSB. Then, 150µl of diluted bacterial suspension was inoculated using clear bottom black 96-well plates and incubated at 37°C on a rotating plate for 48 hours. The established biofilms were washed using 180µl of 1xPBS twice before addition of treatments. 180µl of Tween 80 or oleic acid treatment concentrations from 1% - 0.03125% were added in respective wells and plates were further incubated at 37°C on a rotating plate. After 24 hours incubation, wells were washed two times with 200µl of 1xPBS and AlamarBlue assay performed. Six replicate experiments were performed for each treatment and each bacterial strain.

LIVE/DEAD staining and Confocal Laser Scanning Microscopy (CLSM) examination of biofilms

To verify the anti-biofilm activity of Tween 80 and Oleic acid treatments against *P. aeruginosa* pathogens, both a clinical isolate and a laboratory reference strains of *P. aeruginosa* biofilms were first grown using 8 well chamber slide (cell imaging slide). In brief, single colonies of *P. aeruginosa* were mixed in saline to standardize a McFarland unit of 1.0 and then 1 in 15 dilution was made using TSB. To assess the effect of FAs on inhibition of biofilm formation, 150µl of diluted bacterial suspension was inoculated in each well of the chamber slide containing 150µl of three different concentrations (0.125%, 0.0625% and 0.03125%) of Tween 80 and Oleic acid. Next, chamber slides were incubated at 37°C on a rotator for 48 hours. Wells containing each of the bacteria without treatment were used as positive growth control. Following this, all biofilms in each group were washed twice using 300µl of 1xPBS followed by fixation with 5% glutaraldehyde (Sigma Aldrich, USA) for 45 minutes at room temperature. LIVE/DEAD^{TM} BacLight ^{TM} staining (Invitrogen Bacterial Viability Kit, USA) containing dyes SYTO9 and propidium iodide (PI) was then carried out following the manufacturer's instructions. Briefly, 1.5µl of each dye in 1ml of MilliQ water was incubated on biofilms for 15 minutes in the dark

at room temperature to determine the viability of bacteria within the biofilm. Stained slides were washed twice, mounted and dried prior to examination by CLSM (Carl ZEISS 16.0, Germany). Cells emitting red and green fluorescence were considered as dead and viable cells, respectively. The viability of bacteria was calculated in both treated and untreated biofilm groups as follows: viability % = fluorescent intensity value of live cells/total fluorescent intensity value of live and dead cells * 100. Three replicate experiments were performed in each group.

Biofilm formation assessment and development of mixed-species biofilms

The biofilm forming capability of *C. accolens* CI09 and pathogens, *S. aureus* CI8 and *P. aeruginosa* CIAus1 were evaluated individually using 96-well plates and measuring absorbance at OD_{595} after staining with crystal violet following a static biofilm formation assay as described previously [263, 264] with few modifications. In summary, pure isolated young colonies of *S. aureus* and *P. aeruginosa* were grown from TSA and 48-hour growth of *C. accolens* from TSA was transferred into a sterile glass tube of 0.9% saline and adjusted to 1.0 McFarland turbidity standard. The saline suspension was diluted into TSB at 1:15 ratio and 150 µl of the final suspension was transferred to a sterile flat bottom 96-well CELLSTAR plates. Plates were incubated for 48 hours on a gyratory mixer of 70 rpm at 37ºC. Following incubation, the fluid portion of the culture was aspirated and the biofilm on the plates was washed twice using 180 µl 1x PBS. Then, the biofilm was fixed with 180µl of 95% methanol for 30 minutes. Methanol was aspirated and plates were washed once with 1x PBS and air-dried. Biofilms were then stained with 180 µl per well of 0.2% crystal violet for 60 minutes. Excess stain was removed by gentle washing twice using 1x PBS and left in a dark room for overnight to dry. The crystal violet then was eluted using 180µl of 30% acetic acid and left at room temperature for 30 minutes. The suspension was transferred into a new microplate and

OD of the suspension was measured at 595_{nm} after including 30% acetic acid as negative control. The iMark™, BIO-RAD microplate reader (BMG LABTECH Pty. Ltd. Victoria, Australia) was employed to read the OD. The OD was analysed taking the blank corrected mean off the 3 replicates.

In this study we then developed a dual-species biofilm model of *S. aureus/C. accolens* and *P. aeruginosa/C. accolens* to examine the impact of Tween 80 and oleic acid treatments on biofilm formation in a mixed biofilm. In the first place, the co-existence of *S. aureus* (SA CI8)/*C. accolens* (CA CI09) and *P. aeruginosa* (PA CIAus1)/*C. accolens* (CA CI09) in a biofilm form was optimized using various ratios from the two species cell suspensions following earlier protocols [265, 266] with minor modifications. After optimization, 2-3 colonies of 24 hours culture of SA CI8 and PA CIAus1 and 48 hours culture of CA CI09 were suspended independently in sterile 0.9% saline and adjusted to 1.0 McFarland turbidity standard followed by a dilution of 1:15 in TSB. These suspensions were pooled together at 30:70 ratio of *S. aureus* to *C. accolens* and *P. aeruginosa* to *C. accolens*. Next, using chamber slides mixed biofilms were formed from the final culture suspension, following the same conditions as stated previously in LIVE/DEAD staining experiment.

Treatment of mixed biofilms and Fluorescent In Situ Hybridization (FISH) assay

At the beginning of mixed biofilm formation and after 48-hours mixed biofilm establishment, various concentrations (0.125%, 0.0625% and 0.03125%) of Tween 80 and Oleic acid were added in chamber slides. About 300µl of a mixed culture suspension with no treatment from each group was used as positive growth control. Next, fluorescent in situ hybridisation (FISH) was conducted using a published method with minor modifications [267]. In summary, the probe sequences used were designed to specifically target the 16S rRNA of *S. aureus* (Staaur-

FITC: 5′-GAAGCAAGCTTCTCGTCCG-3′), *P. aeruginosa* (Pseaer-Tred: 5'-GGTAACCGTCCCCCTTGC-3') and *Corynebacteria* (Coryn-PaBl: 16SCorJ557: 5′-GCGACAAACCACCTACGAGCT-3′). The probes targeting *S. aureus* were labelled with fluorescein isothiocyanate (FITC488) and *P. aeruginosa* probes labelled with Texas red (Tred555), both from AdvanDx, Woburn, MA, USA. Whereas, *Corynebacteria* targeting probes labelled with Pacific Blue (PaBl405) were purchased from (PNA Bio Inc, Thousand Oaks, CA, USA). In summary, 48-hr dual species biofilms prepared in 8 well chamber slides in each group were washed twice with 300µl 1xPBS. Then one drop of fixation solution (phosphate-buffered saline with detergent) provided with the kit was added in each well and incubated for 20 minutes at 55ºC. After a fixation step, one drop of specific target probe was added for *S. aureus*/*C. accolens* and *P. aeruginosa*/*C. accolens* mixed biofilms in each specified well and then incubated for 30min at 55ºC. After incubation, the top chamber well was detached and all slides were immersed in 100ml of pre-heated washing buffer at 55ºC for 30 minutes. Then, all slides were allowed to air dry, mounted with mounting medium before microscopic examination. Images were visualized using CLSM (LSM700, Carl ZEISS, Germany) and the mean fluorescent intensity of specific bacteria from treated and untreated group was analysed.

Statistical analysis

The biofilm viability obtained by AlamarBlue assay are presented as mean ± standard error mean (SEM). The effect of each treatment on pathogenic bacterial biofilms as well as the mean intensity value of CLSM results from LIVE/DEAD staining and FISH assay were analyzed statistically by means of one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test using GraphPad Prism version 8.0 (GraphPad Software California, U.S.A). A p value of ≤ 0.05 was considered statistically significant.

2.4.4 Results

Activity of Tween 80 and Oleic acid on established *P. aeruginosa* biofilms

The activity of Tween 80 and oleic acid on established *P. aeruginosa* biofilms was shown in Figure 3. Addition of Tween 80 did not show any effect on *P. aeruginosa* biofilm viability except for a reduction of biofilm viability of *P. aeruginosa* PA01 at the concentrations of 1% ($P<0.001$) and 0.25% ($P<0.01$) (Figure 2.1A). Similarly, compared to untreated maximum growth control, oleic acid treatment led to a modest reduction in viability of *P. aeruginosa* biofilms only at the high 1% concentration for *P. aeruginosa* CIAus1 and PA01 ($P<0.01$) (Figure 2.1B).

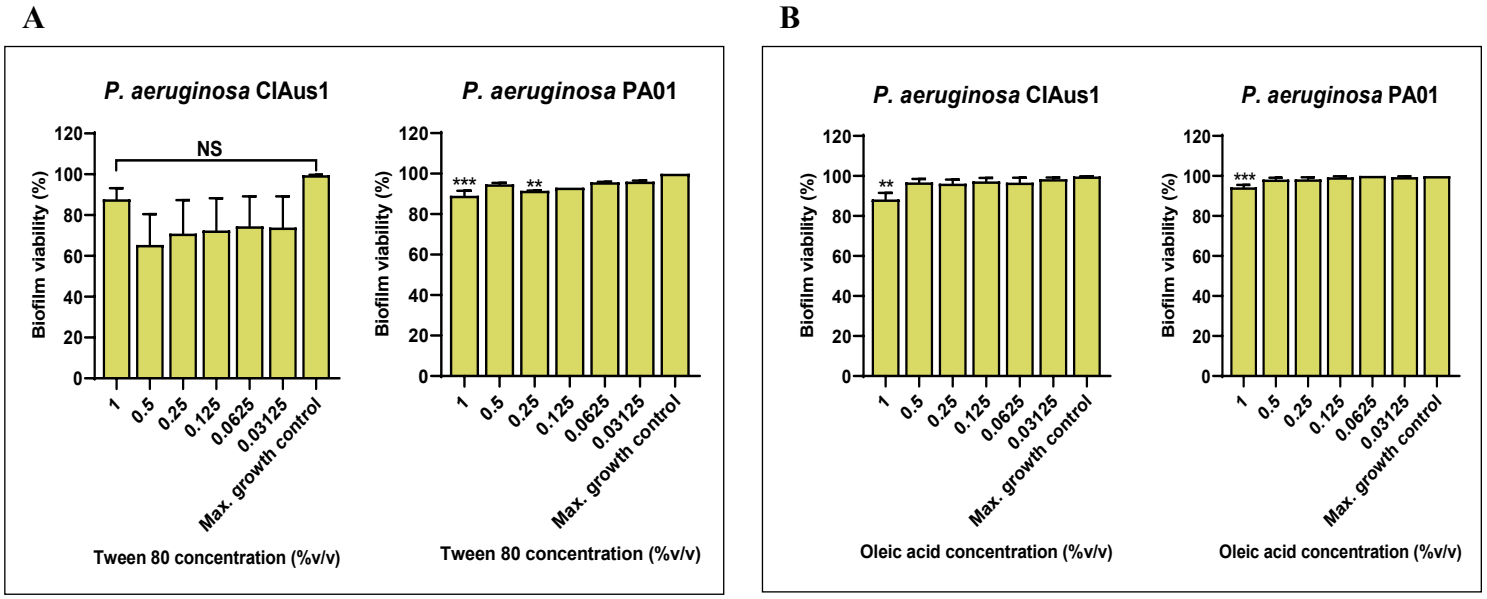


Figure 2.1. Treatment of Tween 80 (A) and oleic acid (B) at various concentrations effect on established *P. aeruginosa* biofilms as a % of positive maximum growth control; (a clinical isolate, *P. aeruginosa* CIAus1; and a laboratory reference strain, *P. aeruginosa* PA01). The experiments were conducted in each strain at least three times. Data represent the mean ± SEM of the three replicates. ** $P<0.01$, *** $P<0.001$, One-way ANOVA; NS, not significant; SEM, standard error of the mean.

Activity of Tween 80 and Oleic acid on biofilm formation by *P. aeruginosa*

In our previous report, we have proved that Tween 80 or oleic acid significantly reduce the formation of biofilms by *S. aureus* CI8 and *S. aureus* ATCC25923 isolates at a concentration of 1% to 0.125% (v/v) (for Tween 80) and 1% to 0.06225% (v/v) (for Oleic acid). Moreover, low Tween 80 concentrations (0.125%, 0.0625% and 0.03125% (v/v)) caused a significant reduction of live cells in *S. aureus* biofilms examined in a Live/Dead analysis [268]. However, compared to the untreated maximum growth control, Tween 80 or oleic acid did not show any significant reduction of biofilm viability for *P. aeruginosa* CIAus1 and PA01 except for a high 1% concentration of Tween 80 where the viability of *P. aeruginosa* PA01 biofilms reduced significantly (P<0001) (Figure 2.2).

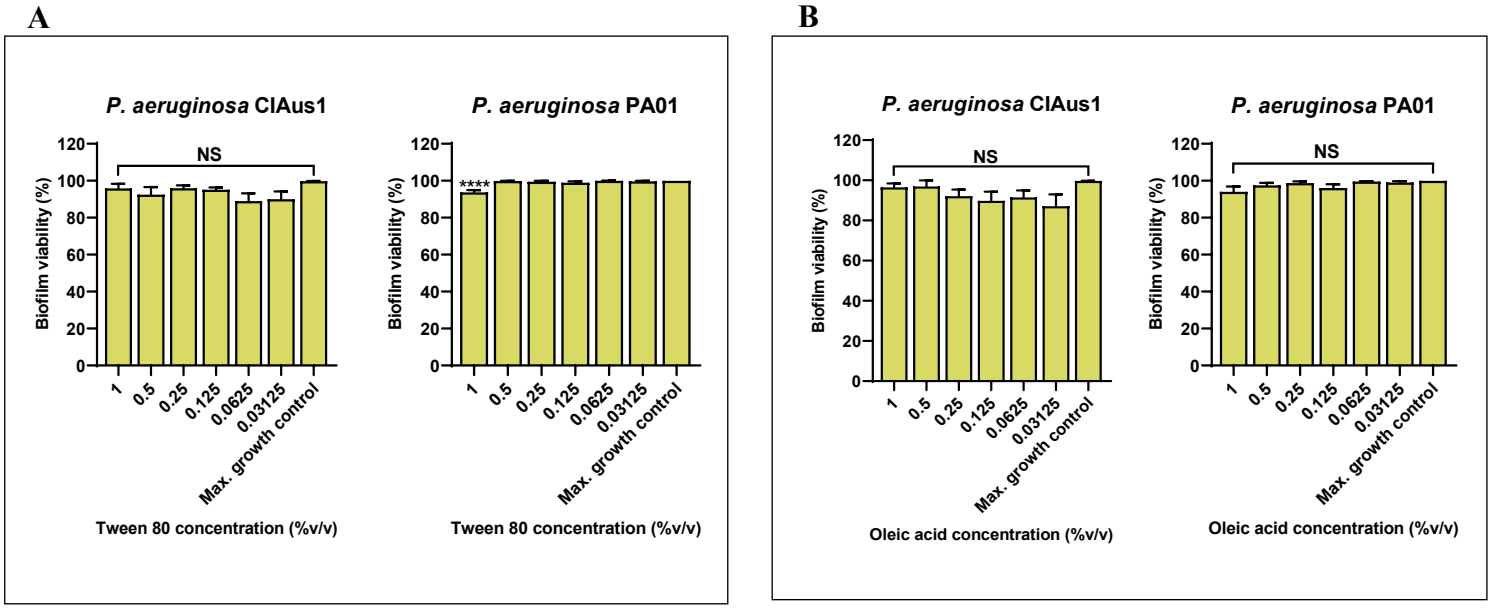


Figure 2.2. Treatment of Tween 80 (A) and Oleic acid (B) at concentrations of 0.03125% - 1% (v/v) and its effect on biofilm formation of a clinical isolate, *P. aeruginosa* CIAus1 and a laboratory reference strain, *P. aeruginosa* PA01). The experiments were conducted in each strain at least three times. Data represents the mean ± SEM of the three replicates. ****P<0.0001, One-way ANOVA; NS, not significant; SEM, standard error of the mean.

We then performed LIVE/DEAD staining to assess the activity of Tween 80 and oleic acid at low concentrations (0.125%, 0.0625% and 0.03125%) on biofilm establishment by *P. aeruginosa*. The treatment concentrations were chosen based on our previous results showing significant reduction of *S. aureus* biofilm viability at some of those concentrations [268]. In *P. aeruginosa* biofilms, we did not observe any apparent change in the mean fluorescent intensity of live and dead cells following Tween 80 and oleic acid treatments at all tested concentrations (Figure 2.3).

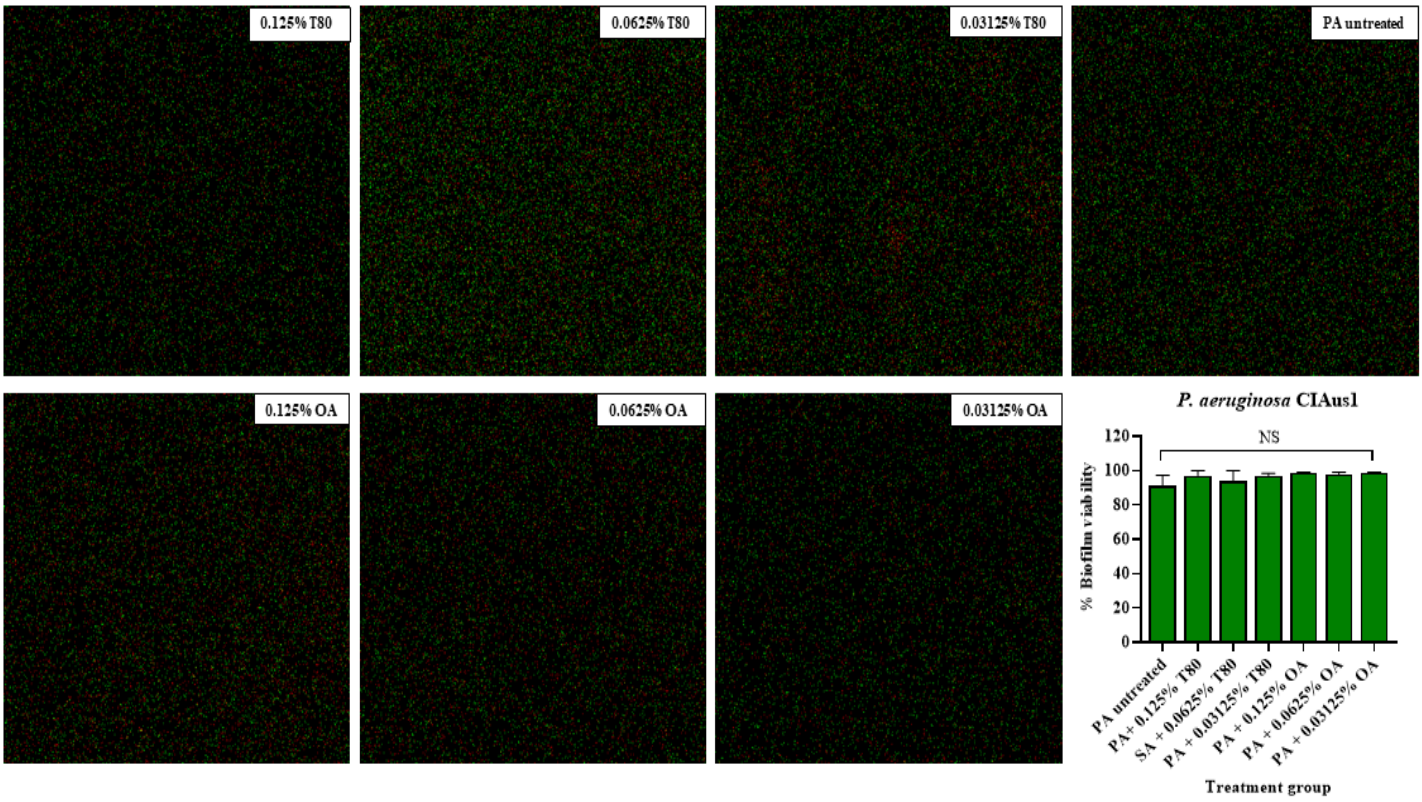


Figure 2.3. CLSM visualization of *P. aeruginosa* CIAus1 biofilms treated with Tween 80 or Oleic acid in various concentrations. The green colour represents live cells whereas red colour represents dead cells. Data was presented by calculating the mean ± SEM of the fluorescent intensity value of at least three microscopic images obtained from three replicate experiments. (PA, *P. aeruginosa*; T80, Tween 80; OA, Oleic acid; NS, not significant; SEM, standard error of the mean).

Impact of Tween 80 and Oleic acid on mixed biofilm formation

The treatments impact on the formation of dual *S. aureus/C. accolens* and *P. aeruginosa/C. accolens* biofilms was examined using FISH and CLSM image analysis. The biofilm forming capability of all tested clinical isolates, *C. accolens* CI09, *S. aureus* CI8, and *P. aeruginosa* CIAus1is shown in Figure 2.4.

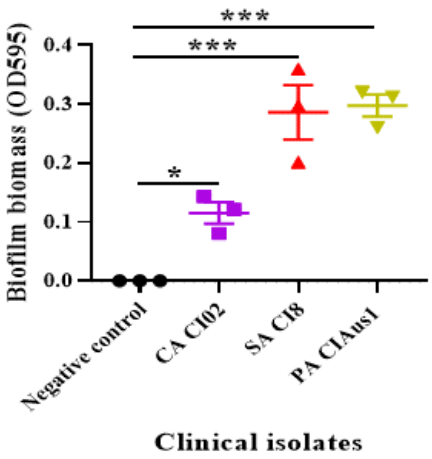


Figure 2.4. Biofilm forming capability of *C. accolens* CI09, *S. aureus* CI8 and *P. aeruginosa* CIAus1 clinical isolates using crystal violate assay. Data represent mean + SEM of three independent replicates. (CA, *C. accolens*; SA, *S. aureus*; PA, *P. aeruginosa*; *statistically significant; ** P<0.01, *** P<0.001).

Addition of Tween 80 or oleic acid at 0.125%, 0.0625% and 0.03125% concentrations for 24 hours did not affect the balance of biofilm establishment between *C. accolens* and *S. aureus* and between *C. accolens* and *P. aeruginosa* clinical isolates (Figure 2.5)

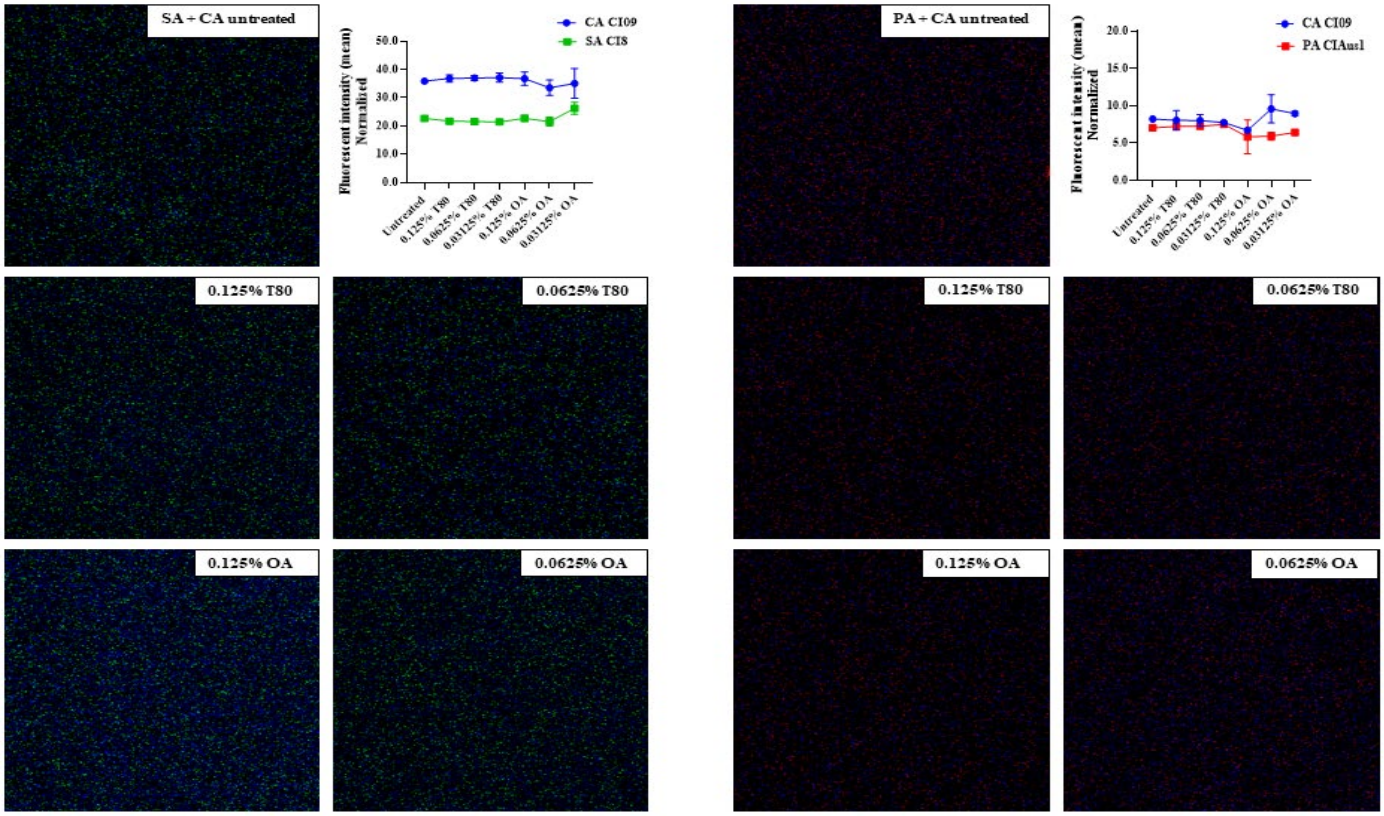


Figure 2.5. FISH followed by CLSM visualization of mixed biofilms of *S. aureus/C. accolens* (left two columns) and *P. aeruginosa/C. accolens* (right two columns) treated with various concentrations of Tween 80 and oleic acid at the time of biofilm formation. Mixed bacterial biofilms labelled using species-specific probes for *S. aureus*, Staph-FITC (Green fluorescing cells), *P. aeruginosa*, Pseu-Tred (Red fluorescing cells) and Corynebacterium species, Coryn-PaBl (Blue fluorescing cells). Data was presented by calculating the mean $\pm$ SEM of the fluorescent intensity value of microscopic images obtained from three replicate experiments. (SA, *S. aureus*; PA, *P. aeruginosa*; CA, *C. accolens*; T80, Tween 80; OA, Oleic acid; SEM, Standard error of the mean).

Impact of Tween 80 and Oleic acid on preformed mixed biofilms

Our FISH and CLSM experiment revealed that, addition of 0.125% Tween 80 on already established mixed biofilms of *S. aureus/C. accolens* significantly reduced *S. aureus* biofilms compared with untreated controls ($P<0.05$) and no significant effect observed on *C. accolens* biofilms. In *P. aeruginosa/C. accolens* mixed biofilms however, both Tween 80 and oleic acid at or below 0.125% concentration significantly increased *C. accolens* biofilms ($P<0.01$) without any change in *P. aeruginosa* biofilms (Figure 2.6).

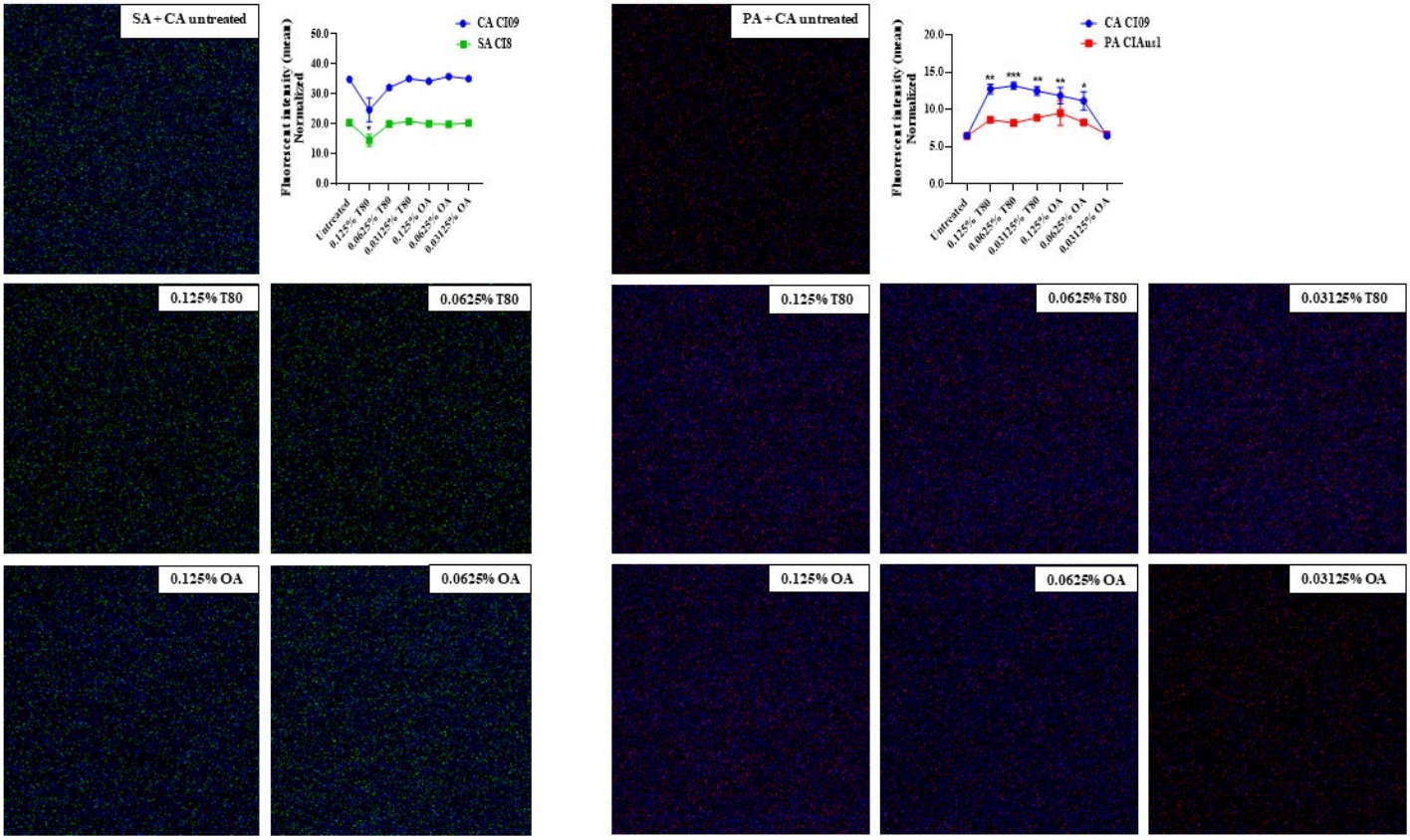


Figure 2.6. FISH and CLSM visualization of preformed mixed biofilms of *S. aureus/C. accolens* (left two columns) and *P. aeruginosa/C. accolens* (right three columns) treated with various concentrations of Tween 80 and oleic acid. Mixed bacterial biofilms labelled using species-specific probes for *S. aureus*, Staph-FITC (Green fluorescing cells), *P. aeruginosa*, Pseu-Tred (Red fluorescing cells) and Corynebacterium species, Coryn-PaBl (Blue fluorescing

cells). Data was presented by calculating the mean ± SEM of the fluorescent intensity value of microscopic images obtained from three replicate experiments. (SA, *S. aureus*; PA, *P. aeruginosa*; CA, *C. accolens*; T80, Tween 80; OA, Oleic acid; *statistically significant, *P<0.05, **P<0.01; ***P<0.001 SEM, Standard error of the mean).

2.4.5 Discussion

This study assessed the activity of a common excipient, Tween 80 and its free fatty acid moiety, oleic acid, on the growth of common nasal bacterial clinical isolates in biofilm form. *P. aeruginosa* biofilm growth were reduced in the presence of higher Tween 80 concentrations of 0.25% and 1%, however the reduction was to a lesser extent (up to 11%). On the contrary, Oleic acid, a Tween 80 derivative and the most common monounsaturated fatty acid in nature, didn't show any effect on the reduction of *P. aeroginosa* biofilm establishment. Furthermore, the growth of *Corynebacterium*, in particular *C. accolens*, was found to be significantly enhanced by Tween 80 and oleic acid at concentrations as low as 0.125 % (v/v) in an established mixed *C. accolens* and *P. aeroginosa* biofilm model when compared to untreated controls.

Bacterial biofilms are thought to play a role in chronic relapsing infections. In particular, *S. aureus* and *P. aeruginosa* dominant biofilms play a role in CRS disease severity and recalcitrance [83]. We found that addition of high (1%) concentration of Tween 80 at the start of biofilm formation significantly lowered the biofilm viability of *P. aeruginosa* biofilms. However, Tween 80 did not show a statistically significant effect on the biofilm viability of established *P. aeruginosa* biofilms. Together, these results indicate that high Tween 80 concentrations might exert their effects on *P. aeruginosa* biofilms at least in part by interfering with the initial stages of biofilm establishment such as attachment to solid surfaces. Tween 80

is indeed a non-ionic surfactant and it is well known that surfactants can degrade and solubilize adhesive components in the matrix of biofilm [269].

Previous studies have also reported variable anti-biofilm activity of Tween 80 against several nasal pathogens including *P. aeruginosa* [161]. Whilst the biofilm metabolic activity of newly forming *P. aeruginosa* biofilms was similarly reduced by high Tween 80 and oleic acid in our study, there were marked differences in how those treatments affected bacterial cell death in *P. aeruginosa* observed in the presence of both Tween 80 and Oleic acid treatments. Interestingly, the action of Tween 80 reduced against *P. aeruginosa* PA14 biofilms appeared to be strain dependent through secretion of lipase enzyme which degrades Tween 80 and release the anti-biofilm oleic acid [161]. This raises the possibility that effects observed on bacterial viability and/or biofilm establishment by Tween 80 might partly be mediated by oleic acid, at least in those microbes including *P. aeruginosa* that secrete lipases. Together these results indicate that oleic acid might contribute to the Tween 80-dependent effects on *P. aeruginosa* biofilm viability but different mechanisms might also play a role in Tween 80-dependent effects on *P. aeruginosa* growth, biofilm formation and in particular cell death. The relationship between lipase secretion by *P. aeruginosa* and antimicrobial and anti-biofilm activity of Tween 80 and oleic acid of various clinical isolates warrants further investigation.

Interestingly, Tween 80 and oleic acid at various low concentrations promoted the growth of *C. accolens* in the presence of *P. aeruginosa* in an established mixed *C. accolens/P. aeruginosa* biofilm model but not in a newly forming *C. accolens/P. aeruginosa* biofilm model nor in a *C. accolens/S. aureus* mixed biofilm model. These findings indicate a positive influence on the growth of established *C. accolens* biofilms potentially by a molecule that might be secreted by

mature *P. aeruginosa* biofilms in the presence of Tween 80 or oleic acid. Further research is required to validate these findings in more clinical isolates and to unravel the mechanisms of this observation.

Emerging evidence indicates differences of the sino-nasal microbiome composition between CRS patients and healthy controls. Dysbiosis in CRS patients is accompanied by changes in community membership and structure, reduced diversity, and increased bacterial load. Bacterial communities in CRS are variably dominated by members of different genera, mainly *Staphylococcus*, *Streptococcus*, *Haemophilus* and *Pseudomonas* whilst in health, the niche is dominated by members of the genera *Corynebacterium* and *Staphylococcus* [97, 98, 256]. Interestingly, a study by Uehara *et al.* showed that *S. epidermidis* and various *Corynebacterium* species were the major bacterial inhabitants in the nares of 156 healthy volunteers and there existed an inverse relationship between *S. aureus* and *Corynebacterium* colonization [200]. Implantation of *Corynebacterium* into the nares of volunteers could eradicate *S. aureus* in more than 70% of cases. Similar bacterial interference occurring between the commensal *S. epidermidis* and *S. aureus* with reduction in *S. aureus* induced inflammation has been shown in a mouse sinusitis model [202]. Together, these studies support the potential of alleviating dysbiosis and associated inflammation by replacement or manipulation of a diseased microbiome into a healthy one. Faecal Microbiota Transplant (FMT) therapy has been used successfully for the treatment of colitis in the context of intestinal dysbiosis and *Clostridium difficile* infections [270] and has inspired recent investigations into the potential of mucus transplant therapy for CRS. Unlike FMT however, where infusion of a mean of 93 grams of healthy human donor faeces (around 10^{13} bacteria) is delivered via colonoscopy [271], healthy mucus inherently has a low volume and microbial biomass. This might limit the success of mucus transplant approaches

and to date, no reports have shown significant benefit with such therapeutic strategies. An alternative or complementary approach to mucus transplant or probiotic therapy could be to manipulate an existing dysbiotic microbiome towards homeostasis by promoting the growth of commensals and suppressing the growth of pathogens using prebiotics. Our previous result indicate that, at least *in vitro*, low concentrations of Tween 80 might indeed have beneficial prebiotic properties by specifically promoting *Corynebacterium* growth while at the same time reducing *S. aureus* growth [268]. Increasing the relative abundance of *Corynebacterium* in CRS might not only reduce *S. aureus* abundance, it might also affect *S. aureus* virulence. Previous research has indeed indicated a reduction in virulence of *S. aureus* when co-cultured with *Corynebacterium* species [272]. Our *in vitro* dual *Corynebacterium/S. aureus* biofilm model failed to show major shifts in growth patterns of both bacteria in the presence of Tween 80 or oleic acid supplementation. Our model used a seeding proportion of *C. accolens/S. aureus* of 70:30 as any reductions in this ratio resulted in an overgrowth of *S. aureus* (Supplementary Figure 2.1). Further research is required to evaluate the potential for promoting the growth of *Corynebacterium* by Tween 80 and oleic acid in the presence of higher *S. aureus* load. In addition, the effect of attenuating *S. aureus* virulence in the presence of *Corynebacterium* at various proportions must be further investigated.

Whereas our results hold promise for Tween 80 at low concentrations to be used as prebiotics for CRS patients, further *in vivo* validation studies are required prior to progressing towards human clinical trials. In particular, it will be interesting to define how the Tween 80-dependent promotion of *Corynebacterium* growth might affect the interaction with *S. aureus* and *P. aeruginosa* and their virulence and pro-inflammatory properties in the *in vitro* and *in vivo* setting.

2.4.6 Conclusions

The FDA approved excipient for nasal spray preparations such as Tween 80 and its derivative oleic acid at low concentrations promoted the growth of *C. accolens* in an established mixed *C. accolens/P. aeruginosa* biofilm model while higher Tween 80 concentrations reduced *P. aeruginosa* biofilm growth. Also, lower Tween 80 and oleic acid concentrations reduced the viability of *S. aureus* biofilms in *C. accolens/ S. aureus* mixed biofilms. Together these findings reflect a good indication for fatty acid compounds, to be exploited as a promising source of prebiotics, particularly Tween 80 and oleic acid in order to correct the health-associated homeostasis of the nasal microbiota.

Author Contributions: Project conceptualization, M.A.M., A.J.H. and S.V.; Experimental design and laboratory investigation, M.A.M. and S.L.; Data management and statistical analysis, M.A.M. and S.L.; Project administration and supervision, S.V., P-J. W. and A.J.P.; Funding acquisition, A.J.P. and S.V.; Manuscript draft preparation and revision, M.A.M.; Manuscript review and editing, A.J.H., S.L., A.J.P., P-J. W. and S.V. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported from Garnett Passe and Rodney Williams Memorial Foundation Conjoint Grant awarded to A.J.P. and S.V.

Acknowledgements: The authors acknowledge to Dr. Clare Cooksley from the Department of Otolaryngology-Head and Neck Surgery, Basil Hetzel Institute for Translational Health Research, University of Adelaide, Australia for her technical support and constructive guidance.

Conflicts of Interest: The authors declare no conflict of interest.

CHAPTER 3

Corynebacterium accolens has antimicrobial activity against *Staphylococcus aureus* and Methicillin-resistant *S. aureus* pathogens isolated from the sinonasal niche of chronic rhinosinusitis patients

CHAPTER 3: *Corynebacterium accolens* has antimicrobial activity against *Staphylococcus aureus* and Methicillin-resistant *S. aureus* pathogens isolated from the sinonasal niche of chronic rhinosinusitis patients

This chapter is a published research article that addresses the second research aim designed to isolate and characterize *Corynebacterium accolens* strains from healthy sinonasal cavities and evaluate their antimicrobial potential against *Staphylococcus aureus* and MRSA clinical isolates from CRS patients.

Menberu MA, Liu S, Cooksley C, Hayes AJ, Psaltis AJ, Wormald PJ, Vreugde S. *Corynebacterium accolens* Has Antimicrobial Activity against *Staphylococcus aureus* and Methicillin-Resistant *S. aureus* Pathogens Isolated from the Sinonasal Niche of Chronic Rhinosinusitis Patients. *Pathogens*. 2021; 10 (2):207.

3.1 Statement of Authorship

Title of Paper	*Corynebacterium accolens* Has Antimicrobial Activity against *Staphylococcus aureus* and Methicillin-Resistant *S. aureus* Pathogens Isolated from the Sinonasal Niche of Chronic Rhinosinusitis Patients
Publication Status	☑ Published ☐ Accepted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style ☐ Submitted for Publication
Publication Details	Menberu MA, Liu S, Cooksley C, Hayes AJ, Psaltis AJ, Wormald PJ, Vreugde S. *Corynebacterium accolens* Has Antimicrobial Activity against *Staphylococcus aureus* and Methicillin-Resistant *S. aureus* Pathogens Isolated from the Sinonasal Niche of Chronic Rhinosinusitis Patients. Pathogens. 2021; 10(2):207.

Principal Author

Name of Principal Author (Candidate)	Martha Alemayehu Menberu		
Contribution to the Paper	Conception and design of the project, acquisition of data, analysis and interpretation of data, drafting the article		
Overall percentage (%)	85		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	21/10/2021

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Sha Liu		
Contribution to the Paper	Acquisition of data, analysis and interpretation of data, manuscript edition		
Signature		Date	18/10/2021

Name of Co-Author	Clare Cooksley		
Contribution to the Paper	Acquisition of data, interpretation of data, critically revising the article		
Signature		Date	19/10/2021

Name of Co-Author	Andrew James Hayes		
Contribution to the Paper	Interpretation of data, critically revising the article		
Signature		Date	20/10/2021

Name of Co-Author	Alkis James Psaltis		
Contribution to the Paper	Conception of the project, critically revising the article		
Signature		Date	18/10/2021

Name of Co-Author	Peter-John Wormald		
Contribution to the Paper	Conception of the project, critically revising the article		
Signature		Date	18/10/2021

Name of Co-Author	Sarah Vreugde		
Contribution to the Paper	Conception and design of the project, critically revising the article		
Signature		Date	18/10/2021

3.2 Publication: *Corynebacterium accolens* has antimicrobial activity against *Staphylococcus aureus* and Methicillin-resistant *S. aureus* pathogens isolated from the sinonasal niche of chronic rhinosinusitis patients

pathogens

MDPI

Article

Corynebacterium accolens Has Antimicrobial Activity against *Staphylococcus aureus* and Methicillin-Resistant *S. aureus* Pathogens Isolated from the Sinonasal Niche of Chronic Rhinosinusitis Patients

Martha Alemayehu Menberu [1,2], Sha Liu [1], Clare Cooksley [1], Andrew James Hayes [3], Alkis James Psaltis [1], Peter-John Wormald [1] and Sarah Vreugde [1,*]

[1] Department of Surgery-Otolaryngology, Head and Neck Surgery, The University of Adelaide, Basil Hetzel Institute for Translational Health Research, Central Adelaide Local Health Network, Woodville 5011, Australia; martha.menberu@adelaide.edu.au (M.A.M.); sha.liu@adelaide.edu.au (S.L.); clare.cooksley@adelaide.edu.au (C.C.); alkis.psaltis@adelaide.edu.au (A.J.P.); peterj.wormald@adelaide.edu.au (P.-J.W.)

[2] Department of Medical Microbiology, School of Biomedical and Laboratory Sciences, College of Medicine and Health Sciences, University of Gondar, Gondar 196, Ethiopia

[3] Department of Microbiology and Immunology, Peter Doherty Institute for Infection and Immunity, University of Melbourne and The Royal Melbourne Hospital, Melbourne 3000, Australia; andrewhayes.bio@gmail.com

* Correspondence: sarah.vreugde@adelaide.edu.au; Tel.: +61-(0)8-8222-6928

Citation: Menberu, M.A.; Liu, S.; Cooksley, C.; Hayes, A.J.; Psaltis, A.J.; Wormald, P.-J.; Vreugde, S. *Corynebacterium accolens* Has Antimicrobial Activity against *Staphylococcus aureus* and Methicillin-Resistant *S. aureus* Pathogens Isolated from the Sinonasal Niche of Chronic Rhinosinusitis Patients. *Pathogens* **2021**, *10*, 207. https://doi.org/10.3390/pathogens10020207

Academic Editor: Bryan Heit

Received: 14 January 2021
Accepted: 11 February 2021
Published: 14 February 2021

Abstract: *Corynebacterium accolens* is the predominant species of the healthy human nasal microbiota, and its relative abundance is decreased in the context of chronic rhinosinusitis (CRS). This study aimed to evaluate the antimicrobial potential of *C. accolens* isolated from a healthy human nasal cavity against planktonic and biofilm growth of *Staphylococcus aureus* (*S. aureus*) and methicillin-resistant *S. aureus* (MRSA) clinical isolates (CIs) from CRS patients. Nasal swabs from twenty non-CRS control subjects were screened for the presence of *C. accolens* using microbiological and molecular techniques. *C. accolens* CIs and their culture supernatants were tested for their antimicrobial activity against eight *S. aureus* and eight MRSA 4CIs and *S. aureus* ATCC25923. The anti-biofilm potential of *C. accolens* cell-free culture supernatants (CFCSs) on *S. aureus* biofilms was also assessed. Of the 20 nasal swabs, 10 *C. accolens* CIs were identified and confirmed with *rpoB* gene sequencing. All isolates showed variable antimicrobial activity against eight out of 8 *S. aureus* and seven out of eight MRSA CIs. Culture supernatants from all *C. accolens* CIs exhibited a significant dose-dependent antibacterial activity ($p < 0.05$) against five out of five representative *S. aureus* and MRSA CIs. This inhibition was abolished after proteinase K treatment. *C. accolens* supernatants induced a significant reduction in metabolic activity and biofilm biomass of *S. aureus* and MRSA CIs compared to untreated growth control ($p < 0.05$). *C. accolens* exhibited antimicrobial activity against *S. aureus* and MRSA CIs in both planktonic and biofilm forms and holds promise for the development of innovative probiotic therapies to promote sinus health.

Keywords: chronic rhinosinusitis; *Corynebacterium accolens*; microbiota; sinus health

1. Introduction

Disruption of the human nasal microbiome homeostasis is found in patients with chronic rhinosinusitis (CRS). CRS is an inflammatory disorder of the mucosa of the nasal cavity and paranasal sinuses, characterized by various clinical manifestations including sinus/facial pain, nasal congestion, rhinorrhoea, post-nasal discharge, and a reduced sense of smell for a minimum of 12 weeks duration [1]. Whilst the aetiology of CRS is thought to be multifactorial, disruption of the microbial community residing in the sinuses, termed dysbiosis, has recently been implicated in CRS pathophysiology, in particular in more severe patients [2]. Dysbiosis is generally described as an imbalance of pathologic and commensal

bacteria, which are involved in the protection against overgrowth of pathobionts or potentially disease-causing organisms [3,4]. In CRS patients, the microbiome is characterised by a decrease in the relative abundance of *Corynebacterium* and an expansion of pathogenic bacteria including *Staphylococcus*, *Haemophilus*, *Moraxella* and *Enterobacteriacea* [5]. Among pathogenic species, *Staphylococcus aureus* is the most frequently isolated species in patients with CRS [6]. Furthermore, exacerbations of CRS due to *S. aureus* and methicillin-resistant *S. aureus* (MRSA) have been reported in severe recalcitrant disease, leading to immune dysregulation, barrier dysfunction, biofilm formation, and worse clinical outcomes [7–9].

Several studies have shown that microbes that typically exist within the mucosa of the nasal cavity compete amongst themselves and inhibit the growth of competitors either by releasing antagonistic substances or by limiting access to nutrients from the surrounding environment [10,11]. For example, *Corynebacterium accolens*, a common commensal nasal species secretes the enzyme triacylglycerol lipase (LipS1) that degrades triacylglycerol to produce free fatty acids that interfere with the growth of *Streptococcus pneumoniae* in the nasal cavity [12,13].

Similarly, probiotic bacteria have been acknowledged as a potential novel treatment in various diseases of the gut linked to dysbiosis, as they can interfere with the growth of pathogenic organisms and provide a host-beneficial advantage [14]. In the context of CRS, manipulation of the sinonasal microbiome has been recognized as an innovative strategy to promote the re-establishment of sinonasal microbiome homeostasis and improve sinus health. As such, the potential of probiotic treatment in CRS has been demonstrated in in vivo models with several candidate bacterial species such as *S. epidermidis* and *Lactobacillus sakei* [15,16]. Similarly, intranasal administration of *Corynebacterium* species to carriers of *S. aureus* resulted in *the* eradication of *S. aureus* in >70% of carriers [17].

To design a probiotic therapy to combat dysbiosis and help shape the microbiome in the context of CRS, a well-tolerated, safe and effective cocktail of beneficial microbes with good antimicrobial activity against pathobionts is required. A recent international sinonasal microbiome study compared the sinonasal microbiome of 410 controls and CRS patients, in which *Corynebacterium* was the most prevalent genus present in >75% of CRS patients and controls with a significant reduction in its relative abundance in CRS patients compared to controls [5]. Together with the notion that *Corynebacterium* species can interfere with the growth of pathogens [12,13,17], these findings support the probiotic potential of *Corynebacterium* species. However, *Corynebacterium* species have also been reported to mediate sinusitis [16]. Therefore, it is important to define the commensal status of *Corynebacteria* at species and strain-level along with their interaction against the most prevalent CRS pathogens, particularly *S. aureus* and MRSA.

This study was designed to isolate and characterize *Corynebacterium accolens* strains from healthy sinonasal cavities and evaluate their antimicrobial and antibiofilm potential against *S. aureus* and MRSA clinical isolates from CRS patients.

2. Results

A total of 36 study subjects, 20 non-CRS controls (8 males and 12 females, aged between 20–70 years old) and 16 CRS patients (8 males and 8 females, aged between 36–90 years old) were included to collect nasal swab samples and identify the clinical isolates. The demographic characteristics and clinical data of each study subject are summarized in Table 1.

Table 1. Demographic and clinical characteristics of the study subjects.

Characteristic	Non-CRS Controls, No. (%)	Patients with CRS, No. (%)
Number of subjects	20	16
Mean age (years)	45.7	64.0
Gender (M/F)	8/12	8/8
Active smoker	0 (0)	1 (6.3)
Asthma	6 (30)	8 (50)
Diabetes mellitus	1 (5)	0 (0)
Cystic fibrosis	0 (0)	0 (0)
GERD	6 (30)	3 (18.8)
Aspirin sensitivity	0 (0)	3 (18.8)
Tonsillitis in the past 6 months	0 (0)	0 (0)
Ear infection in the past 6months	0 (0)	0 (0)
Nasal polyposis	0 (0)	7 (43.8)

Abbreviations: CRS, chronic rhinosinusitis; F, female; GERD, gastroesophageal reflux; M, male.

Based on the phenotypic API 20 Staph and chromogenic MRSA selective agar screening methods, 16 *S. aureus* isolates (8 methicillin-sensitive *S. aureus* (MSSA) and 8 MRSA) were identified from 16 CRS patients. Characteristics of *S. aureus* clinical isolates used in this study are shown in Table S2.

2.1. Identification of C. accolens Isolates

From 20 non-CRS controls, 10 *C. accolens* isolates were identified by the API Coryne test kit with ≥90.0% similarity to known strains of *C. accolens* from the database. The molecular identification of all *C. accolens* isolates with the PCR amplification followed by gel electrophoresis resulted in a DNA fragment of approximately 446 bp in size (Figure S1). In order to confirm the strain level identification, partial *rpoB* gene sequencing was done for all *C. accolens* isolates. As shown in Table 2, the *rpoB* nucleotide sequence BLAST of 10 *C. accolens* isolates showed 96% to 100% similarity and 99% to 100% query coverage with the known culture collection strain, *C. accolens* CIP 104783 (from the Pasteur Institute Collection, Biological Resource Center of Pasteur Institute (CRBIP), Paris, France), GenBank accession number AY492242 identified previously [18].

Table 2. Identification of *Corynebacterium accolens* using API Coryne 20 test system and *rpoB* gene sequencing.

Isolate Code	API Coryne 20 Identification † (% Similarity)	*rpoB* Gene Sequence Identification			
		Strains	% Similarity	% Query Coverage	Accession Number
C778	*C. accolens* (90.0)	*C. accolens*	98.3	100	MT856944
C779	*C. accolens* (95.6)	*C. accolens*	96.0	100	MT856945
C780	*C. accolens* (90.0)	*C. accolens*	97.6	100	MT856946
C781	*C. accolens* (99.4)	*C. accolens*	98.7	100	MT856947
C782	*C. accolens* (95.6)	*C. accolens*	99.5	100	MT856948
C783	*C. accolens* (90.0)	*C. accolens*	98.2	99	MT856949
C784	*C.* accolens (91.4)	*C. accolens*	98.3	100	MT856950
C785	*C. accolens* (90.0)	*C. accolens*	96.6	100	MT856951
C786	*C. accolens* (90.0)	*C. accolens*	97.3	100	MT856952
C787	*C. accolens* (90.0)	*C. accolens*	96.4	100	MT856953

Note: † Results were interpreted based on various biochemical reactions on the API Coryne test strip and % similarity of the isolates were identified by comparing with *C. accolens* isolates deposited from the database (V4.0) using the apiweb™ software.

2.2. Phylogenetic Relationship of the Strains

Comparison of the *rpoB* gene sequences with the corresponding *C. accolens* sequences from the GenBank database showed that *C. accolens* strains were placed in the evolutionary clade of *Corynebacterium* origin. Strains of *C. accolens*, C778 and C779 were clustered together with strains C784 and C785, respectively, with a bootstrap value of 100%. Furthermore, *C. accolens* strain C782 was clustered with a culture collection strain, *C. accolens* ATCC 49726

with a bootstrap value of 99%. The phylogenetic analysis based on the *rpoB* genes of all *C. accolens* clinical isolates and their closest related *Corynebacteria* species are indicated in Figure 1.

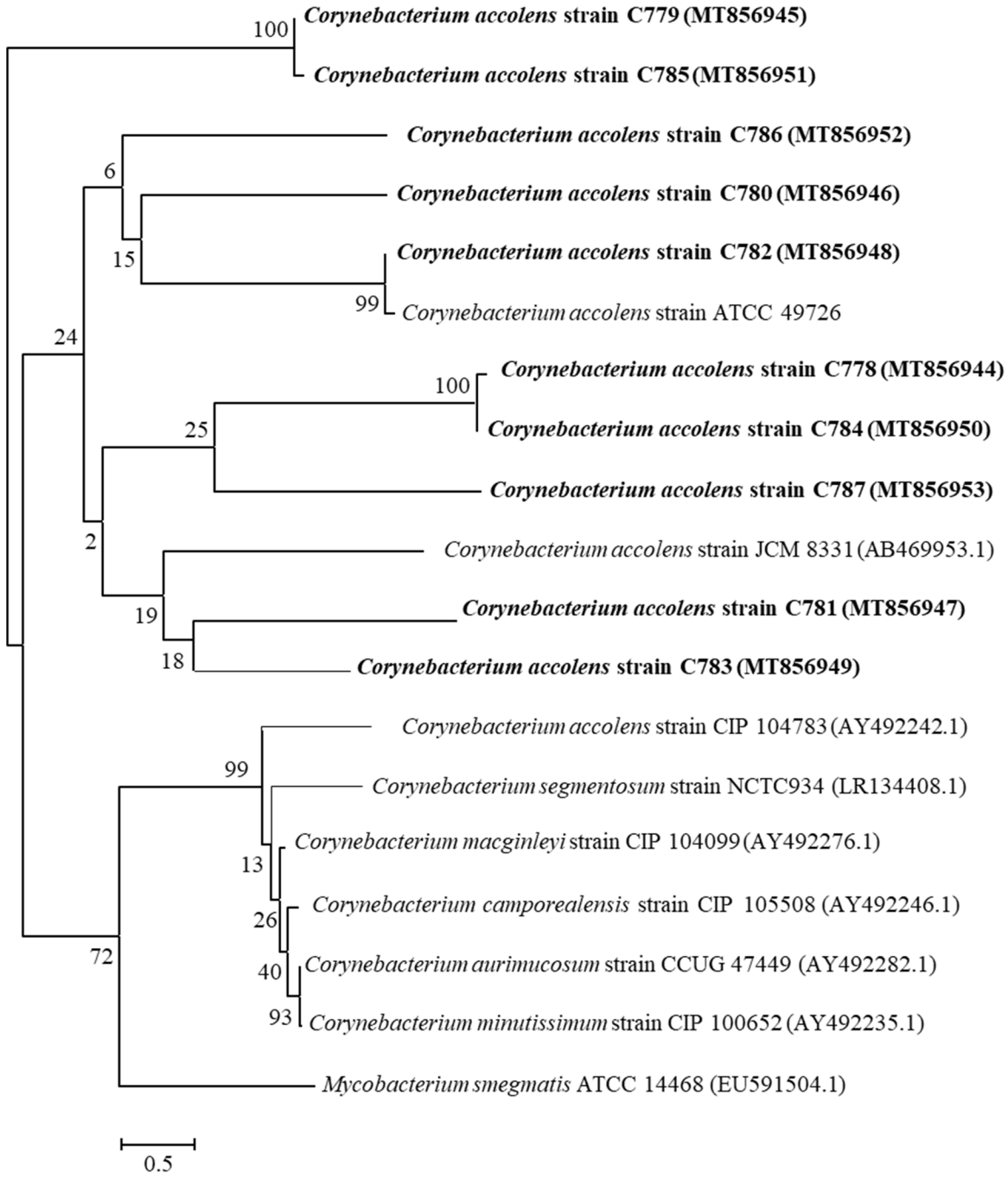


Figure 1. Phylogenetic tree showing the evolutionary relationships between 10 *C. accolens* nasal isolates, a reference strain *C. accolens* ATCC49726 and the type strains of related species (*C. accolens* CIP 104783, *C. accolens* JCM 8331 and other *Corynebacteria* species) based on *rpoB* gene sequences analysed using the neighbour-joining method. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Numbers in parentheses represent the sequence accession number in GenBank. *Mycobacterium smegmatis* ATCC 14468 was used as an outgroup. The scale bar represents 0.5-nucleotide substitutes per position.

2.3. Spectrum of Antimicrobial Activity

Eight methicillin-sensitive *S. aureus* (MSSA) and eight methicillin-resistant *S. aureus* (MRSA) were retrieved from the nasal cavity of CRS patients.

In the deferred growth inhibition assay all, of the *C. accolens* strains showed antagonistic effects against most MSSA and MRSA clinical isolates, but the degree of antagonism varied among the *C. accolens* strains. From all *S. aureus* clinical isolates, only one MRSA strain could not be inhibited by any of the *C. accolens* strains. Most of the *C. accolens* strains showed low inhibitory activities against various strains of MSSA, MRSA and reference strain *S. aureus* ATCC 25923 (inhibition zones of less than 5 mm). Interestingly, three of the isolated strains (*C. accolens* C779, *C. accolens* C781 and *C. accolens* C787), exhibited strong inhibition on the growth of MSSA C26 and MRSA C261 (inhibition zones of more than 8 mm) (Figure 2). *C. accolens* C781 was the most effective strain in inhibiting the growth of eight of eight (100%) MSSA and six of eight (75.0%) MRSA CIs tested. In contrast, *C. accolens* C782 was the least effective strain, showing inhibitory activities against only four of eight (50.0%) MSSA and two of eight (25.0%) MRSA CIs tested. Results are summarized in Table 3.

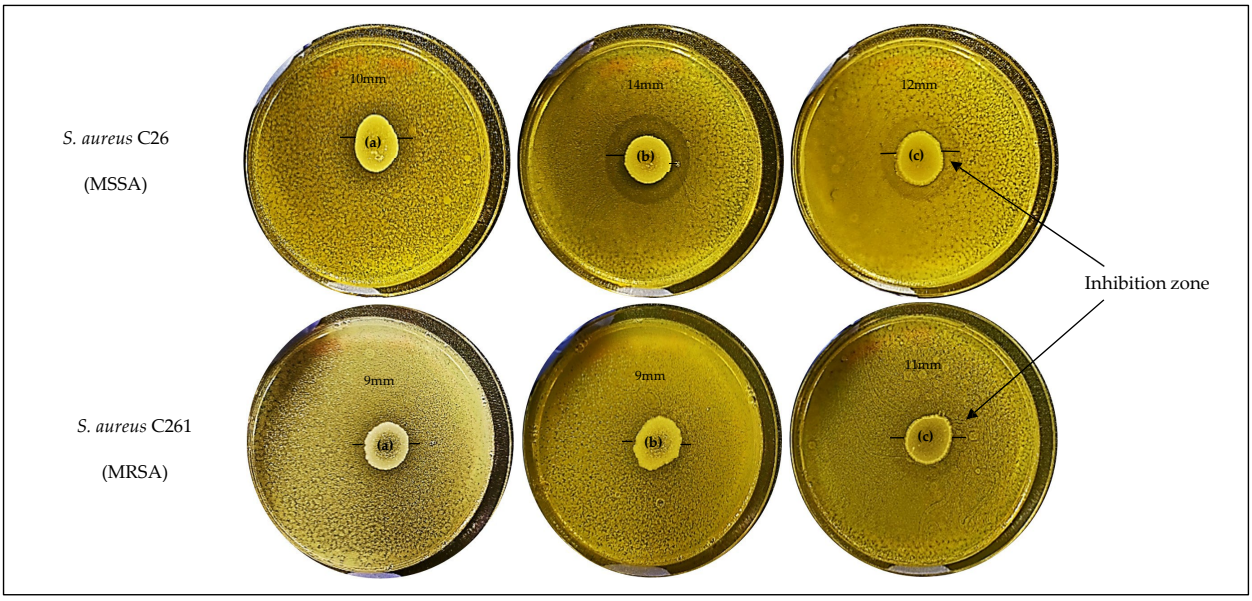


Figure 2. Antagonistic activity of selected *C. accolens* nasal isolates (**a**) *C. accolens* C779, (**b**) *C. accolens* C781 and (**c**) *C. accolens* C787 spotted on a lawn of *S. aureus* clinical isolates, MSSA C26 (top image) and MRSA C261 (bottom image) on tryptone soya agar (TSA) medium. The inhibition zone diameter was measured in at least three replicate experiments and the mean values were taken to score the extent of inhibition. The single line represents the growth inhibition zone. CI, Clinical isolate; MSSA, methicillin-sensitive *S. aureus*; MRSA, methicillin-resistant *S. aureus*.

Table 3. Antagonistic activity of *C. accolens* against *S. aureus* clinical isolates in deferred growth inhibition assay.

Tested Pathogens	Diameter of Growth Inhibition Zone (mm) †										
	Inhibitor Strains										
	C. accolens C778	*C. accolens* C779	*C. accolens* C780	*C. accolens* C781	*C. accolens* C782	*C. accolens* C783	*C. accolens* C784	*C. accolens* C785	*C. accolens* C786	*C. accolens* C787	*C. accolens* ATCC49726
MSSA											
S. aureus C329	−	+	++	+	−	++	++	−	+	+++	+
S. aureus C262	−	++	−	+	+	−	−	−	−	−	−
S. aureus C314	−	−	++	++	−	+	−	−	++	−	++
S. aureus C124	+	+++	+	+	+	−	++	+	−	++	+
S. aureus C5	+	+++	+	+	−	−	+	++	++	++	+
S. aureus C26	++	+++	++	++++	+	+++	++	+	+	++++	+
S. aureus C319	−	+	−	++	+	−	+	++	−	+	−
S. aureus C71	−	−	++	+	−	+	++	−	+	+	+
MSSA (% inhibition)	**3/8 (37.5%)**	**6/8 (75.0%)**	**6/8 (75.0%)**	**8/8 (100%)**	**4/8 (50.0%)**	**4/8 (50.0%)**	**6/8 (75.0%)**	**4/8 (50.0%)**	**5/8 (62.5%)**	**6/8 (75.0%)**	**6/8 (75.0%)**
MRSA											
S. aureus C300	++	+++	++	+++	++	++	−	+	+	+++	++
S. aureus C310	+	−	+	+	−	++	++	+	+	++	+
S. aureus C292	++	+	+	++	−	−	+	−	+	+	+
S. aureus C295	−	−	−	−	−	−	−	−	−	−	−
S. aureus C261	+	+++	++	+++	+	++	+	++	++	++++	++
S. aureus C24	−	+	−	+	−	+	−	−	−	+	−
S. aureus C54	+	−	−	+	−	−	−	−	−	−	−
S. aureus C38	−	+	+	−	−	++	−	−	−	+ +	

Table 3. *Cont.*

Tested Pathogens	Diameter of Growth Inhibition Zone (mm) †										
	Inhibitory Strains										
	C. accolens C778	*C. accolens* C779	*C. accolens* C780	*C. accolens* C781	*C. accolens* C782	*C. accolens* C783	*C. accolens* C784	*C. accolens* C785	*C. accolens* C786	*C. accolens* C787	*C. accolens* ATCC49726
MRSA (% inhibition)	**5/8 (62.5%)**	**5/8 (62.5%)**	**5/8 (62.5%)**	**6/8 (75.0%)**	**2/8 (25.0%)**	**5/8 (62.5%)**	**3/8 (37.5%)**	**3/8 (37.5%)**	**4/8 (50.0%)**	**6/8 (75.0%)**	**5/8 (75.0%)**
S. aureus ATCC25923	+	+	−	+	+	−	+	+	+	+	+
Total (% inhibition)	**9/17 (52.9%)**	**12/17 (70.6%)**	**11/17 (64.7%)**	**15/17 (88.2%)**	**7/17 (41.2%)**	**9/17 (52.9%)**	**10/17 (58.8%)**	**8/17 (47.1%)**	**10/17 (58.8%)**	**13/17 (76.5%)**	**12/17 (70.5%)**

Note: † The extent of inhibition was scored based on the inhibition zone diameter result as: − (0 mm), + (<5 mm), ++ (5–7 mm), +++ (8–10 mm) and ++++ (>10 mm).

2.4. Inhibitory Activity of *C. accolens* Concentrated Cell-Free Culture Supernatants (CFCSs)

All of the *C. accolens* cell-free culture supernatants in the present study exhibited a significant dose-dependent antibacterial activity against all of the *S. aureus* isolates tested (MSSA C5, MSSA C26, MRSA C300, MRSA C261 and ATCC 25923) compared to the control group consisting of *S. aureus* in TSB without CFCS ($p < 0.05$). The highest concentration of 90% CFCS in TSB from all *C. accolens* strains significantly inhibited the planktonic growth of all *S. aureus* isolates ($p < 0.0001$) compared to untreated controls. However, lower concentrations of 30% CFCS in TSB (102 µg/mL) did not show antibacterial activity against any of the *S. aureus* isolates tested ($p > 0.05$) (Figure 3).

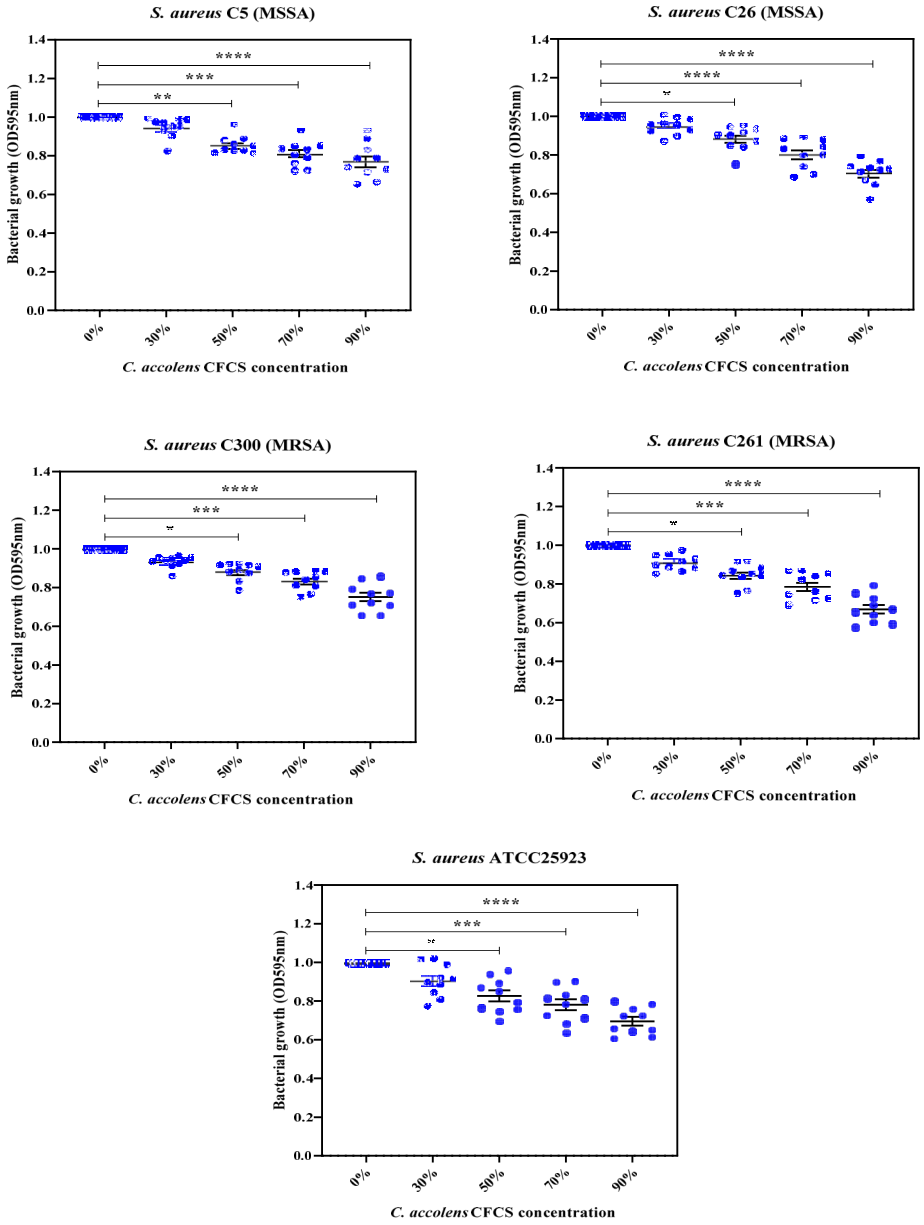


Figure 3. Growth inhibitory effect of *C. accolens* CFCSs (n = 10, represented by blue dots) against *S. aureus* (2 MSSA (C5, C26), 2 MRSA (C300, C261) and *S. aureus* ATCC25923) at various concentrations, 30%, 50%, 70% and 90% (CFCSs diluted in TSB), compared to controls (corresponding volume of TSB + SA, normalized to 1.0 OD value) following 24 h treatment. The results are expressed as means ± SEM of three independent experiments. SA, *S. aureus*; CI, clinical isolate; One-way ANOVA * $p < 0.05$; ** $p < 0.01$; ***$p < 0.001$; **** $p < 0.0001$; SEM, standard error of the means.

C. accolens C779, C781 and C787 showed the highest antagonistic activity in the deferred growth inhibition assay, and their CFCSs demonstrated a strong anti-staphylococcal activity for the three *S. aureus* test strains (MSSA C26, MRSA C261 and ATCC 25923) and were selected for investigation of the dose-dependent growth inhibition of their CFCSs. The highest antimicrobial activity was observed for *C. accolens* C781 CFCS at all tested concentrations (30% to 90%) compared to controls ($p < 0.05$). *C. accolens* C779 and *C. accolens* C787 CFCSs showed significant growth inhibition at concentration of CFCS in TSB higher than 50% against MRSA C261 ($p < 0.05$) and 70% CFCS in TSB against MSSA C26 and *S. aureus* ATCC 25923 strains (Figure 4A–C).

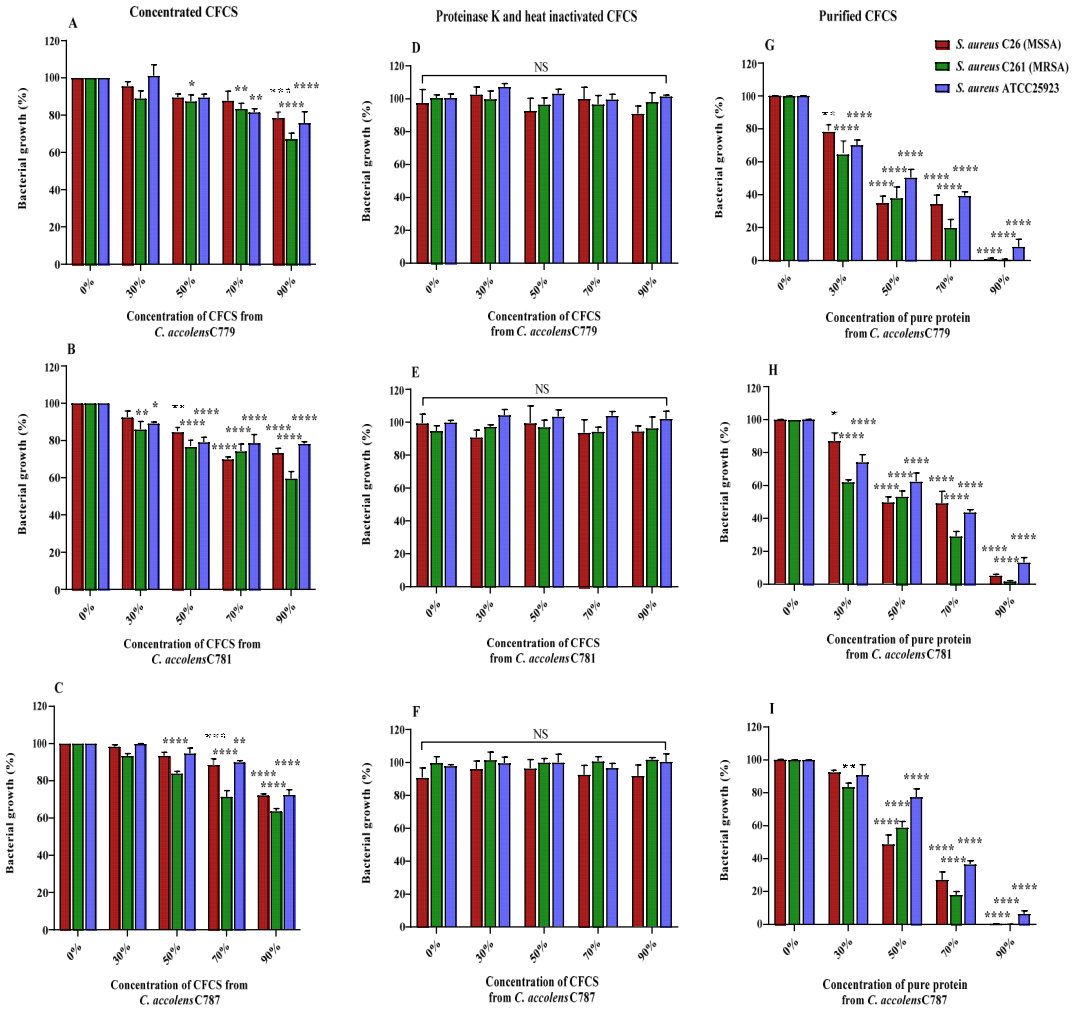


Figure 4. Antibacterial potential of *C. accolens* purified CFCSs (extracted from strains C779, C781 and C787) against *S. aureus* planktonic cells [MSSA C26 (red), MRSA C261 (green) and ATCC25923 (blue)]. (**A–C**) represents treatment of *S. aureus* strains with concentrated CFCSs from *C. accolens* strains C779 (**A**), C781 (**B**) and C787 (**C**) at concentrations of 30–90% or a positive control (corresponding bacterial inoculum in TSB); (**D–F**) represents treatment of *S. aureus* strains with proteinase K and heat inactivated CFCSs from *C. accolens* strains C779 (**D**), C781 (**E**) and C787 (**F**) at concentrations of 30–90% or a positive control (corresponding bacterial inoculum in concentrated TSB treated with proteinase K and heat); (**G–I**) represents treatment of *S. aureus* strains with purified CFCSs from *C. accolens* strains C779 (**G**), C781 (**H**) and C787 (**I**) at concentrations of 30–90% or a positive control (corresponding bacterial inoculum in TSB and MilliQ water, normalized to the water-diluted TSB). Bacterial growth (%) was determined as follows: [(Abs growth control − Abs CFCS treated)/Abs growth control] × 100; where, Abs = mean absorbance. Data presented as means ± SEM of three independent experiments. PK, proteinase K; CTSB, concentrated tryptone soya broth; SA, *S. aureus*; CI, clinical isolate; One-way ANOVA * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; NS, not significant; SEM, standard error of the mean.

2.5. Characterization of the Inhibitory Effect of CFCSs Produced by C. accolens Strains

2.5.1. Effect of Proteinase K and Heat Inactivation

The nature of the inhibitory substance produced by selected *C. accolens* strains was studied by treating their CFCSs with proteinase K and heat. CFCSs from the 3 selected *C. accolens* strains, C779, C781 and C787, at different concentrations completely lost their antimicrobial activity against the selected *S. aureus* strains after treatment with proteinaseK (1 mg/mL) followed by heat (55 °C, 30 min) (Figure 4D–F). This indicated that inhibitory effects of the *C. accolens* strains were due to the proteinaceous nature of active substances.

2.5.2. Effect of Purified Protein Treatment

Purified protein extracts from the selected *C. accolens* CFCSs showed concentration-dependent inhibitory activity against the tested *S. aureus* strains as indicated in Figure 4G–I ($p < 0.05$). Purified protein extracts from *C. accolens* strains C779 and C781 at 30% inhibited the growth of both MSSA C26 ($p < 0.05$), MRSA C261 and ATCC 25923 compared to control ($p < 0.0001$). Higher or 90% concentration of purified protein extracts from all tested *C. accolens* strains, C779, C781 and C787, exhibited a stronger antimicrobial effect ($p < 0.0001$) against all *S. aureus* strains compared to control.

2.6. C. accolens CFCS Inhibits S. aureus and MRSA Biofilm Metabolic Activity

The activity of *C. accolens* CFCS on the metabolic activity of 48-h biofilms formed by 3 representative *S. aureus* strains (MSSA C26, MRSA C261 and ATCC 25923) was evaluated using alamarBlue assays. As shown in Figure 5A–C, CFCSs obtained from *C. accolens* C779, C781 and C787 had a concentration-dependent reduction in metabolic activity of both MSSA C26 and MRSA C261 CIs in established biofilms with values reduced by 23% to 42% compared to respective positive control. Biofilm of *S. aureus* ATCC25923 could be inhibited by about 26% to 29% compared to control by *C. accolens* C781and C787 CFCSat concentrations ranging between 70% and 90%. However, only high concentrations of CFCS of 90% exhibited a significant inhibitory effect on biofilms formed by MSSA C26, MRSA C261 and *S. aureus* ATCC25923. The *C. accolens* CFCS exhibited different anti-biofilm activity against the 3 *S. aureus* strains tested.

2.7. C. accolens CFCS Reduces S. aureus and MRSA Biofilm Biomass

Figure 5D–F show the effects of CFCSs extracted from *C. accolens* strains, C779, C781 and C787 on the *S. aureus* biofilm biomass established by clinical isolates MSSA C26 and MRSA C261 and reference strain ATCC 25923. Although the biofilm of MRSA C261 was less affected than MSSA C26, all tested *C. accolens* CFCSs at the highest concentration (90%) reduced the biofilm biomass of both *S. aureus* clinical isolates (between 28% and 40%). However, the *S. aureus* ATCC 25923 biofilm biomass was not affected by CFCS at any of the concentrations tested.

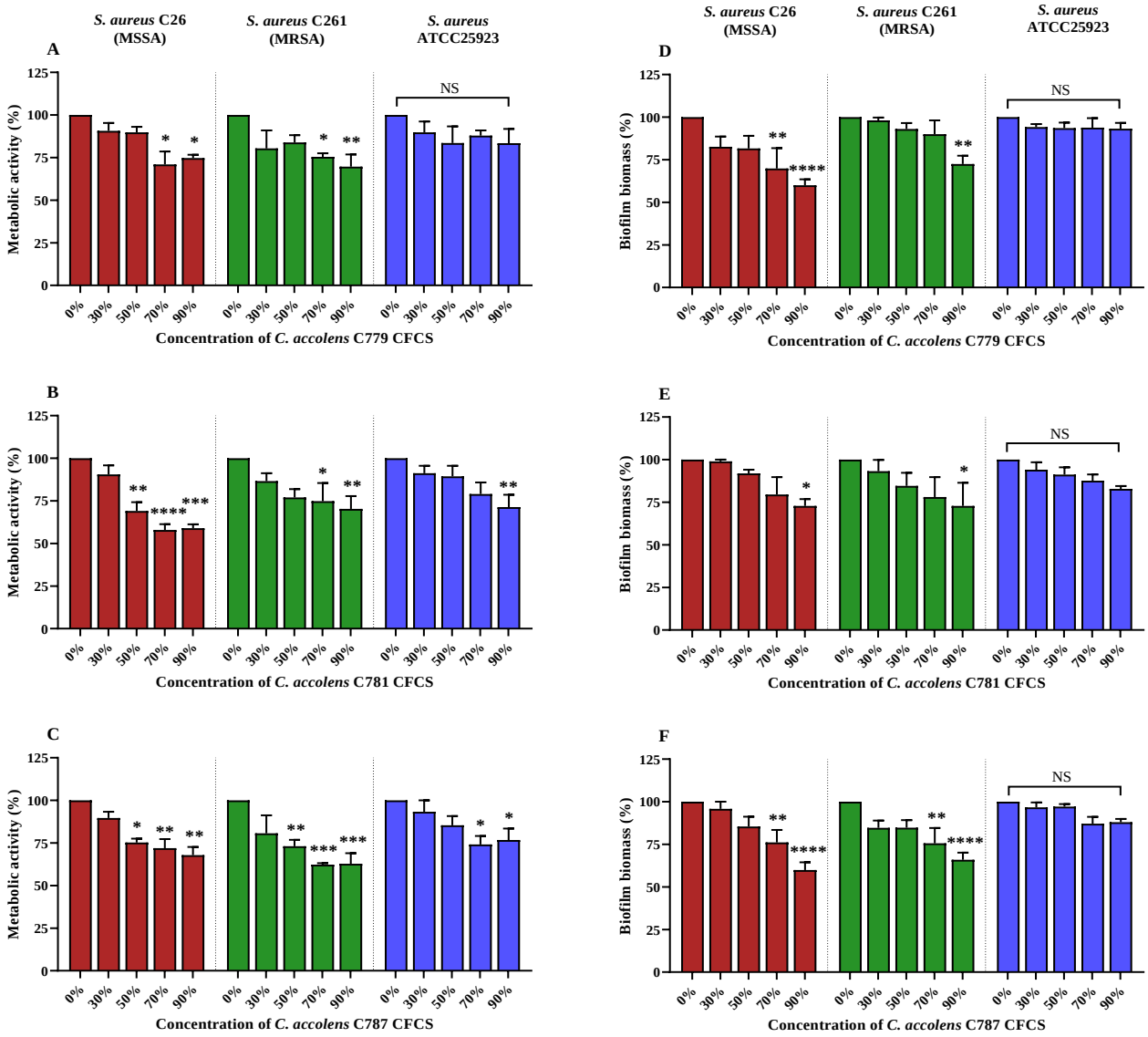


Figure 5. Anti-biofilm potential of *C. accolens* CFCS (extracted from strains C779, C781 and C787) on *S. aureus* biofilms established by clinical isolates (MSSA C26 (red), MRSA C261 (green) and ATCC25923 (blue)). (**A**–**C**) represent reduction of metabolic activity of biofilms formed by *S. aureus* strains (C26, C261 and ATCC25923) normalised to positive control (TSB + corresponding bacterial inoculum) in the presence and absence of CFCS from *C. accolens* strains, C779 (**A**), C781 (**B**) and C787 (**C**) diluted with TSB at different concentrations (30%, 50%, 70% and 90%). (**D**–**F**) represent biofilm biomass reduction of *S. aureus* strains (C26, C261 and ATCC25923) normalised to positive control (TSB + corresponding bacterial inoculum) after 24 hrs incubation with CFCS at different concentrations (30%, 50%, 70% and 90%) extracted from 3 *C. accolens* strains, C779 (**D**), C781 (**E**) and C787 (**F**). Values represent the means ± SEM of at least three independent experiments. Metabolic activity (% inhibition) = ((FI growth control − FI CFCS treated)/FI growth control) × 100, where FI = average fluorescence intensity, and Biofilm biomass (% reduction) = ((Abs growth control—Abs CFCS treated)/Abs growth control) × 100; where, Abs = mean absorbance. SA, *S. aureus*; CI, clinical isolate; One-way ANOVA * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; NS, not significant; SEM, standard error of the mean.

3. Discussion

This study indicates the probiotic potential of *C. accolens* with the potential of this species to help shape a dysbiotic microbiome in the context of CRS by interfering with the growth of MSSA and MRSA in planktonic and biofilm form. Some beneficial nasal bacteria have been evidenced in providing beneficial functions to restore the sinonasal microbiome

composition and improving immune health in patients with CRS through direct pathogen inhibition, secretion of a bioactive molecule or nutrient competition [19]. Our results show that *C. accolens* strains isolated from the sinonasal cavities of non-CRS control patients have antimicrobial activity against MSSA and MRSA strains isolated from the sinonasal cavities of CRS patients. Both MSSA and MRSA planktonic cells and biofilms were sensitive to *C. accolens* and our results indicate a secreted protein to likely be responsible for this activity. Although all *C. accolens* strains had anti-staphylococcal activity, there was a strain-dependent variability in the host range and strength of anti-microbial action.

The human nasal cavity forms a complex microbial ecosystem colonized by several resident microorganisms comprising both commensals and pathobionts [20]. Emerging evidence indicates that *Corynebacteria* are the predominant genus in the sinonasal niche present in >75% of CRS patients and controls; however, the relative abundance of *Corynebacteria* is reduced in patients with CRS [5]. The reduction of *Corynebacteria*, and increased relative abundance of pathobionts such as *S. aureus* in these patients, reflect a potentially disturbed host–microbe–microbe balance that might contribute to the pathophysiology of this disease [2,5,21]. From those studies, it appears that *Corynebacteria* can be in general regarded as a commensal in the sinonasal cavities. This is also in line with our study where *C. accolens* was isolated from the sinonasal cavities of at least 50% of healthy controls. However, an outgrowth of *Corynebacterium* species has also been implicated in CRS [16]. Therefore, it is important to define the commensal status of *Corynebacteria* at the species level. Sequencing of *rpoB* and 16S rRNA genes are the most widely used molecular methods for reliable identification of *Corynebacterium* species, and the *rpoB* gene is considerably more polymorphic than the 16S rRNA gene for members of the genus *Corynebacterium* [18,22]. In this study, *rpoB* gene sequencing confirmed that all isolated *Corynebacterium* strains had a pairwise sequence similarity of 96% to 100% with a culture collection strain *C. accolens* CIP 104783, classifying them as *C. accolens*. Our phylogenetic analysis based on the *rpoB* gene sequences also revealed that some strains such as (C779 and C785), (C778 and C784) and (C781 and C783) were closely related and shared the same clade. Potentially due to their close phylogenetic relationship, these strains tended to have similar antimicrobial properties against *S. aureus* and MRSA.

In this study, all ten *C. accolens* strains were active against a variety of *S. aureus* and MRSA strains. Notably, three of the strains designed as C779, C781 and C787 showed strong inhibition against at least 6/8 (75%) MSSA and 5/8 (62.5%) MRSA CIs tested. In particular, *C. accolens* strain C781 had the widest host range and exhibited inhibitory activity against eight out of eight (100%) *S. aureus* and six out of eight (75%) MRSA CIs. Given that the antimicrobial properties appear similar in MSSA and MRSA, we speculate that the molecular mechanism is likely unrelated to known mechanisms of antibiotic resistance. An increasing amount of research has shown an inverse correlation between *Corynebacterium* and *S. aureus* nasal colonization [23–25]. For example, in a cohort of forty healthy adults, *C. accolens* negatively correlated with *S. aureus* colonization and positively correlated with *C. pseudodiphtheriticum* [23]. Moreover, a previous study by Uehara Y et al. described that frequently implanting *Corynebacterium* species eradicated *S. aureus* colonization in 12 of 17 healthy adult carriers, suggesting the beneficial role of *Corynebacterium* in the abolition of *S. aureus* nasal colonization [17]. Despite the complexity of *Corynebacterium*–*S. aureus* interactions and strain-level variations, those studies are in line with the present study and support the possibility of commensal *C. accolens* strains to be used as probiotic therapy in the context of CRS.

Some studies have also focused on the activity of antibacterial products in commensal *Corynebacterium* CFCSs toward pathogens. For example, a secreted factor by *C. pseudodiphtheriticum*, a closely related *Corynebacterium* species to *C. accolens*, has revealed bactericidal activity against various *S. aureus* strains including MRSA [24]. In our study, the antimicrobial effects by *C. accolens* against *S. aureus* are at least in part due to a secreted antimicrobial substance. Moreover, given the abrogation of this effect by treatment of the CFCS with Proteinase K and heat, the bioactive product is likely a protein or peptide. The

commensal-derived products in a complex sinonasal niche can directly act on challenging the pathogenic bacteria to maintain a well-balanced microbiome. Recently, a novel peptide antibiotic termed lugdunin produced by the nasal and skin commensal *Staphylococcus lugdunensis* has demonstrated strong bactericidal activity against *S. aureus* nasal and skin colonization as well as the immunomodulatory potential to protect the host [26].

The inhibitory activity of *C. accolens*, has been previously reported against *S. pneumoniae* and was mainly due to the production of primary triacylglycerol lipase and release of anti-pneumococcal free fatty acids from representative human nostril and skin surface triacylglycerols [13]. Furthermore, a previous study done by Ramsey MM et al. demonstrated another possibility of interaction between commensal *Corynebacterium* species and *S. aureus* pathobionts with a view to managing *S. aureus* nasal colonization. In this study, the virulence of *S. aureus* was heavily affected by commensal *C. amycolatum*, *C. accolens*, and *C. pseudodiphtheriticum* through altered expression of the *S. aureus* quorum sensing-controlled accessory gene regulator (*agr*) genetic locus involved in colonization and virulence, and shifting bacterial behavior from virulence to a commensal lifestyle [25]. It is particularly interesting to note that the inhibitory activity of commensal *C. accolens* strains in our study is more likely due to proteins affecting the growth of several MSSA and MRSA isolates that are pathogenic in CRS. However, more in-depth studies are needed to identify and characterize the *C. accolens* secreted protein that is responsible for the observed antimicrobial effect and to investigate the unexplored mechanism of action.

It is well known that nasal colonization with *S. aureus* along with MRSA, particularly in biofilm form, is associated with CRS disease recalcitrance and poor outcomes after sinus surgery [27–30]. Biofilms are thought to be the main mediators for disease persistence and treatment failure in various chronic disorders including CRS [30]. To our knowledge, no studies have investigated the anti-biofilm properties of commensal *Corynebacteria*, including *C. accolens*, against *S. aureus* and MRSA. However, Iwase and colleagues have previously shown the activity of another commensal nasal bacterium, *S. epidermidis*, in disrupting biofilm formation and previously established biofilms of *S. aureus* through the production of bioactive extracellular serine protease (Esp) [31]. In our study, CFCSs from selected *C. accolens* strains, C779, C781 and C787, showed a concentration-dependent inhibition of biofilms formed by *S. aureus* and MRSA CIs. Therefore, our findings support the potential use of *C. accolens* or bioactive compounds derived from those strains as antimicrobials against *S. aureus* biofilms.

4. Materials and Methods

4.1. Collection of Clinical Isolates

Ethics clearance for the collection, storage and use of clinical isolates was obtained from TQEH Human Research Ethics Committee (HREC/15/TQEH/132). All study subjects provided their written consent to participate in this study. Nasal swabs were collected at the time of surgery from 16 CRS patients (*S. aureus* clinical isolates) and from 20 non-CRS control patients (*C. accolens* clinical isolates) in a sterile Amies transport medium (Sigma Transwab, MWE Medical Wire, Corsham, UK), placed on ice and immediately transported to our research laboratory for processing.

S. aureus clinical isolates were identified from nasal swabs of CRS patients by culturing on mannitol salt agar (Oxoid, Basingstoke, UK) at 37 °C overnight followed by species-level identification using API 20 Staph test system (bioMerieux, Australia) according to the manufacturer's instructions. All isolates were then screened for MRSA using a super sensitive and specific chromogenic MRSA selective agar (CHROMID® MRSA SMART, bioMerieux, Australia) as described previously [32].

Non-CRS controls were patients undergoing septoplasty with no prior history of CRS, acute sinusitis, tonsillitis and ear infections in the 6 months prior to surgery. Nasal swabs in bacterial transport medium were first vortexed for 60 s and then diluted with phosphate buffer saline (PBS) 1:10. One-hundred-microliter aliquots of diluted samples were overlaid on Columbia agar plates with 5% sheep blood (Thermo Scientific, Oxoid,

Australia) and incubated at 37 °C for 48–72 h. Cultures were inspected daily before colony identification, and visible bacterial colonies were subcultured onto tryptone soya agar (TSA) (Oxoid, Basingstoke, UK) supplemented with 0.8% Tween 80 and incubated for 48 h at 37 °C with 5% CO_2 and screened phenotypically based on colony size and culture morphology. Biochemical characterization of the isolates was performed using the API Coryne test system (BioMérieux NSW, Australia) following the manufacturer's instructions. The isolates were stored at -80 °C in tryptone soya broth (TSB) (Oxoid, Basingstoke, UK) plus 20% (*v*/*v*) sterile glycerol for further analysis.

4.2. C. accolens Genomic DNA (gDNA) Extraction and DNA Quality Control

Bacterial gDNA was extracted from a 48hr culture suspension of the isolates using a DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany), following the manufacturer's recommendations. The concentration of DNA was determined by recording the absorbance at 260 nm (A_{260}) using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The DNA purity was determined from the optical density absorbance value; A_{260}/A_{280} nm ratio. Moreover, the DNA integrity was evaluated through gel electrophoresis. Briefly, 5 µL of each DNA extract was run on 1.8% agarose gel (Sigma-Aldrich, USA) in 1× Tris-Acetate-EDTA (TAE) buffer at 100 Volts for approximately 60 min and stained with 10,000×concentrate SYBR Safe (Invitrogen, Thermo Scientific, city, Canberra, Australia). DNA bands were visualized using the ChemiDoc™ Touch imaging system (Bio-Rad, NSW, Australia).

4.3. Polymerase Chain Reaction (PCR) Amplification of Partial rpoB Gene

PCR was carried out in a T100™ Thermal cycler (Bio-Rad, NSW, Australia) using oligonucleotide primers, C2700F and C3130R (Table S1) according to a previously described protocol with little modification [18]. Briefly, amplification reactions were performed in a final volume of 50 µL containing 5 µL of 10×standard Taq Mg-free buffer, 6 µL of 25 mM $MgCl_2$ solution, 1 µL of 10 mM dNTP mixture (dATP, dTTP, dGTP and dCTP), 0.25 µL of 5.000 U/mL *Taq* DNA polymerase (all from BioLabs inc., Rowley, MA, USA), 1 µL of 10 µM concentration of each forward and reverse primer (Integrated DNA Technologies, SA, Australia), 25.75 µL nuclease-free water and approximately 200 ng/µL of DNA adjusted to 10 µL with nuclease-free water per reaction. Thereafter, PCR mixtures were subjected to 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 50.6 °C for 30 s, and extension at 72 °C for 2 min. A negative control (RNAse free water) and positive control (*C. accolens* ATCC49726, from American Type Culture Collection, Manassas, VA, USA) reaction were set up for every PCR experiment.

Amplified PCR products were separated on a 1.8% agarose gel (Sigma-Aldrich, St. Louis, MO, USA) with 10 µL of 10,000×concentrate SYBR Safe DNA gel stain (Invitrogen, Thermo Scientific, Canberra, Australia) in 1xTris-Acetate-EDTA (TAE) buffer at 100 Volts for 60 min. The gels were visualized using ChemiDoc™ Touch imaging system (Bio-Rad). The size of PCR products was estimated by comparison with a 1kb plus DNA ladder (BioLabs Ltd., Rowley, MA, USA). The primer sequences and amplicon size used for the detection of *Corynebacteria* are described in Supplemental Table S1.

4.4. rpoB Gene Sequencing and Strain Identification of C. accolens

The amplified PCR products were purified from agarose gel using QIAquick Gel extraction kits (Qiagen GmbH, Hilden, Germany) following the manufacturer's extraction protocol. The concentration, purity and integrity of the recovered DNA samples were assessed using the NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and through agarose gel electrophoresis as specified. The purified DNA was then sent to the Australian Genome Research Facility Ltd. (AGRF) for sequencing. All samples were prepared for sequencing following the guide to AGRF sequencing service for Purified DNA (PD) as follows: 10 pmol of a primer (Forward or Reverse) + 12–18 ng of purified DNA + sterile MilliQ water (in a total volume of 12 µL). All sequencing results were

analysed by comparing with NCBI GenBank database using the Blast program (http://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 9 February 2021) for strain identification.

4.5. Phylogenetic Analysis

Based on the *rpoB* gene sequence data, a phylogenetic tree elucidating the relationships between the identified strains was constructed. The nucleotide sequences were aligned through ClustalW program using MEGA (version 7.0) software, and evolutionary analysis was conducted using the neighbour-joining method keeping 1000 bootstrap replications [33]. The analysis involved 19 nucleotide sequences.

4.6. Nucleotide Sequence Accession Numbers

The *rpoB* sequences of 10 *C. accolens* strains, C778, C779, C780, C781, C782, C783, C784, C785, C786 and C787 have been deposited in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank, accessed on 30 December 2020), under the accession numbers MT856944, MT856945, MT856946, MT856947, MT856948, MT856949, MT856950, MT856951, MT856952 and MT856953, respectively.

4.7. Deferred Growth Inhibition Assay

The antagonistic activity of all *C. accolens* clinical strains and a culture collection strain, *C. accolens* ATCC 49726 was evaluated against *S. aureus* clinical isolates and *S. aureus* ATCC 25923 (from ATCC, Manassas, VA, USA) using deferred growth inhibition assays as described previously [34] with modifications. Briefly, a 48 h *C. accolens* culture (20 µL, approximately 10^8 cells) in TSB (test inhibitor strains) was pipetted onto the centre of a TSA plate supplemented with 0.8% Tween 80 and incubated for 48 h at 37 °C with 5% CO_2. Single colonies of a 24 h culture of *S. aureus* (competitor strains) were suspended in sterile 0.9% saline and standardized to McFarland units of 1.0 (approximately $\approx 10^8$ CFU/mL) followed by a dilution of 1:10 in TSB. Next, approximately 250 µL of diluted culture were sprayed over the entire agar surface previously spotted with *C. accolens* and then incubated for a further 18–24 h. After incubation, a photograph was taken and the extent of the growth inhibition zone around the *C. accolens* spot was calculated quantitatively by measuring the diameter of the inhibition zone in millimetres minus the diameter of the central spot of the inhibitor strain. The test was done in triplicate, and the average of the diameters of the inhibition zones was obtained. The extent of inhibition was scored based on the inhibition zone diameter result as – (0 mm), + (<5 mm), ++ (5–7 mm), +++ (8–10 mm) and ++++ (>10 mm).

4.8. Preparation of Concentrated Cell-Free Culture Supernatants (CFCSs) from C. accolens Strains

C. accolens strains were individually grown in 10 mL TSB in a shaking incubator at 37 °C for 48 hrs. The CFCSs were obtained from 48 hr cultures of *C. accolens* in TSB by centrifugation (4000 ×*g*, 4 °C for 10 min) followed by filtration through 0.2 µm sterile syringe filter (Pall Life Sciences, UK). Next, supernatants were passed through 3-kDa filter concentrator (Pierce Protein Concentrator, Thermo Fisher Scientific, Rockford, IL, USA) using centrifugation at 4000×*g*, 4 °C for 1–2 h to collect secreted proteins as described previously [35]. The protein concentration was then determined using Quick Start Bradford Protein Assay Kit (Bio-Rad Laboratories, USA) according to the manufacturer's protocol. All reactions were carried out in duplicate. Concentrated CFCS was then stored as single-use aliquots at −80 °C until use.

4.9. Assessment of Anti-Bacterial Activity Using Concentrated CFCS and Minimum Inhibitory Treatment

The inhibitory activity of CFCS from *C. accolens* isolates was tested against representative *S. aureus* and MRSA isolates following a broth micro-dilution assay protocol as described earlier [36] with minor modifications. The concentrated CFCS were first diluted in various concentration ranges (30%, 50%, 70% and 90%) using TSB. Next, 198 µL of

the diluted mixture was dispensed in 96-well microtiter plates (Life Sciences, Boca Raton, FL, USA) to make CFCS with final concentrations of 102, 170, 238 and 306 µg/mL. The inoculum was then prepared from all tested *S. aureus* isolates by suspending 18–24 h young colonies pre-cultured on TSA in 3 mL of sterile saline (NaCl 0.9% *w*/*v*) and adjusted to 0.5 McFarland turbidity standard (1.0–2.0×10^8 CFU/mL). Following this, 2 µL of bacterial suspension was inoculated to each well, and plates were incubated at 37 °C for 24 h. Wells containing bacteria without supernatant that was grown with the corresponding volume of TSB and sterile TSB-containing wells were used as positive and negative growth controls, respectively. After incubation, bacterial growth was determined by measuring the optical density (OD) at 595 nm using a microplate absorbance reader (iMark™, Bio-Rad, Australia). The inhibitory activity of the supernatant was calculated by comparing OD values between treated and untreated wells. The minimum inhibitory treatment was determined for 3 selected *C. accolens* strains' concentrated CFCSs (*C. accolens* C779, *C. accolens* C781 and *C. accolens* C787). The assays were performed in three replicates, and the antimicrobial activity results are expressed as mean (± standard error of the means).

4.10. Proteinase K and Heat Inactivation of CFCSs

Inactivation experiments of CFCS were carried out using Proteinase K and heat treatment as previously described [37] with minor amendments. An aliquot of CFCS from selected *C. accolens* strains were treated with proteinase K (Sigma-Aldrich, St. Louis, MO, USA), at a final concentration of 1 mg/mL at 37 °C for 5 h. After incubation, the samples were subjected to heat treatment at 55 °C for 30 min to inactivate protease enzymes. Next, the samples were allowed to cool to room temperature for 15 min before application. The antimicrobial activity of samples was then tested against representative *S. aureus* and MRSA clinical isolates and the reference strain *S. aureus* ATCC 25923 using a micro-dilution method in 96-well microtiter plates as specified. Proteinase K was used alone in the corresponding dilution broth (TSB) as a positive control, and wells containing TSB alone were used as a negative control. Three experimental replicates were performed for each protein sample, and data are presented as mean ± SEM of the three experiments.

4.11. Protein Clean-Up from CFCS and Detection of Anti-Bacterial Activity

To remove salts and ionic contaminants such as detergents, lipids and phenolic compounds from CFCS, we used a 2-D Clean-Up Kit (GE-Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions. At completion of the washing steps, 50 µL of sterile MilliQ water was added to resuspend the protein pellet. Following this, the anti-bacterial activity of purified protein samples (cleaned CFCS in sterile MilliQ water and TSB at a ratio of 30%, 50%, 70% and 90% (*v*/*v*)) was tested against representative *S. aureus* and MRSA clinical isolates and *S. aureus* ATCC 25923 as specified. Controls were bacterial inoculum in TSB and sterile MilliQ water at identical volume ratios along with positive growth controls (bacterial inoculum in TSB) and a negative control (sterile TSB). Results are presented as the mean values ± SEM of three independent experiments for each sample.

4.12. Assessment of Anti-Biofilm Activity Using C. accolens CFCSs

4.12.1. Determination of Biofilm Metabolic Activity

To assess the ability of *C. accolens* CFCS to inhibit the metabolic activity of *S. aureus* biofilms, alamarBlue biofilm assay was carried out using clear-bottom black 96-well plates as described previously [38]. Briefly, overnight cultures of *S. aureus* isolates grown in TSA were transferred into a sterile glass tube of 0.9% saline and adjusted to 1.0 McFarland turbidity standard (approximately 3×10^8 CFU/mL). Next, the suspension was diluted into TSB at 1:15 ratio, and 150 µL of the final suspension was transferred to flat-bottom black 96-well microtiter plates and incubated at 37 °C for 48 h in the dark on a rotating shaker to form biofilms. The wells were washed twice with 200 µL 1× PBS to remove planktonic cells and air-dried for 5–10 min. Subsequently, wells were filled with 180 µL of different concentrations of *C. accolens* CFCS diluted in TSB (30%, 50%, 70% and 90%) and incubated

at 37 °C on a rotating shaker for 24 h in the dark. Wells were then washed twice with 200 µL 1 ×PBS and air-dried for 5–10 min. Next, plates were stained with 200 µL alamarBlue (Invitrogen, Thermo Fisher Scientific, Hillsboro, OR, USA) and incubated for 3–5 h at 37 °C on a rotating shaker. Wells containing bacterial culture without CFCS treatment and wells containing TSB without bacterial culture were included as a positive growth control and a sterility control, respectively. The fluorescence intensity of each well was then read every hour by a microplate reader FLUOstar OPTIMA (BMG LABTECH, Ortenberg, Germany) at a wavelength excitation 530 nm and emission 590 nm until maximum fluorescence was reached. Comparing the average fluorescence intensity (FI) of the growth control wells with that of the CFCS treated wells, the inhibition percentages (% inhibition) of metabolic activity was calculated by the following formula: [(FI growth control − FI CFCS treated)/FI growth control] × 100. This assay was performed in triplicate for each treatment.

4.12.2. Determination of Biofilm Biomass

Forty-eight-hour *S. aureus* biofilms treated with CFCS were washed twice with 1xPBS to remove the planktonic cells. The plates were then air-dried for 10 min, and the surface-attached biofilms were stained with 180 µL of 0.1% (v/v) crystal violet per well and incubated at room temperature for 15 min. Subsequently, the crystal violet was removed, and the plates were washed three times with 200 µL per well sterile MilliQ water to remove the unabsorbed stain. Next, 180 µL per well 30% acetic acid was added and incubated on a plate shaker until the crystal violet solubilised. Stained biofilm biomass was determined by measuring absorbance at 595 nm using the microplate reader (iMark™, Bio-Rad, NSW, Australia). All experiments included a sterility control well containing TSB without bacterial culture and a growth control well (as 100% cell mass) containing bacterial culture without CFCS treatment. The mean absorbance (Abs 595 nm) of the samples was determined, and the percentage of biofilm biomass reduction by the CFCS was calculated by the following formula: [(Abs growth control − Abs CFCS treated)/Abs growth control] ×100. All experiments were performed in triplicate and the mean value was calculated with the standard error.

4.13. Statistical Analysis

All the measurements were performed in triplicate, and the values were expressed as mean ± standard error of the mean (SEM). The mean differences in absorbance value between CFCS treated and growth control wells were compared and analysed by One-way analysis of variance (ANOVA) using Dunnett's multiple comparisons test for anti-bacterial and anti-biofilm assays. All experimental data analyses were performed in GraphPad Prism software version 8.0 (GraphPad Software, San Diego, CA, USA). Statistical significance was determined at p-value < 0.05.

5. Conclusions

Taken together, the antimicrobial activity of *C. accolens* strains and their secreted proteins against *S. aureus* and MRSA clinical isolates in planktonic and biofilm form could be useful in the prevention of *S. aureus* outgrowth in the nasal microbiota and opens the possibility for a protective use of *Corynebacteria* against antibiotic-resistant *S. aureus* nasal colonization in a complex niche. Our findings have potential clinical implications towards the development of personalized probiotic therapy and might contribute to shaping the disrupted nasal microbiota in CRS.

Supplementary Materials: The following are available online at https://www.mdpi.com/2076-0817/10/2/207/s1, Table S1: Description of oligonucleotides used for PCR amplification of *rpoB* gene in *C. accolens* isolates, Table S2: Pathogenic *S. aureus* strains (8MSSA and 8MRSA) isolated from the sinonasal cavity of CRS patients used in this study, Figure S1: Identification of *Corynebacterium accolens* isolates by PCR amplification of partial *rpoB* gene (446-bp fragment).

Author Contributions: Project conceptualization, M.A.M., A.J.H. and S.V.; experimental design and laboratory investigation, M.A.M., S.L. and C.C.; data management and statistical analysis, M.A.M. and S.L.; project administration and supervision, S.V., P.-J.W. and A.J.P.; funding acquisition, A.J.P. and S.V.; manuscript draft preparation and revision, M.A.M.; manuscript review and editing, A.J.H., S.L., C.C., A.J.P., P.-J.W. and S.V. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported from Garnett Passe and Rodney Williams Memorial Foundation Conjoint Grant awarded to A.J.P. and S.V; and the University of Adelaide (International Scholarship) to M.A.M.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Human Research Ethics Committee of TQEH (HREC/15/TQEH/132), approved on 18 August 2015.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: We are grateful to Mahnaz Ramezanpour (Department of Otolaryngology-Head and Neck Surgery, Basil Hetzel Institute for Translational Health Research, University of Adelaide, Australia) for her technical support and constructive guidance.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Riechelmann, H. Europäischen Akademie für Allergie und Klinische Immunologie (EAACI) un der European Rhinologic Society (ERS)—Chronic Rhinosinusitis. EPOS 2012 Part I]. *Laryngorhinootologie* **2013**, *92*, 193–201. [PubMed]
2. Hoggard, M.; Biswas, K.; Zoing, M.; Mackenzie, B.W.; Taylor, M.W.; Douglas, R.G. Evidence of microbiota dysbiosis in chronic rhinosinusitis. *Int. Forum Allergy Rhinol.* **2017**, *7*, 230–239. [CrossRef] [PubMed]
3. Copeland, E.; Leonard, K.; Carney, R.; Kong, J.; Forer, M.; Naidoo, Y.; Oliver, B.G.G.; Seymour, J.R.; Woodcock, S.; Burke, C.M.; et al. Chronic rhinosinusitis: Potential role of microbial dysbiosis and recommendations for sampling sites. *Front. Cell. Infect. Microbiol.* **2018**, *8*, 57. [CrossRef] [PubMed]
4. Petersen, C.; Round, J.L. Defining dysbiosis and its influence on host immunity and disease. *Cell. Microbiol.* **2014**, *16*, 1024–1033. [CrossRef] [PubMed]
5. Paramasivan, S.; Bassiouni, A.; Shiffer, A.; Dillon, M.R.; Cope, E.K.; Cooksley, C.; Ramezanpour, M.; Moraitis, S.; Ali, M.J.; Bleier, B.; et al. The international sinonasal microbiome study: A multicentre, multinational characterization of sinonasal bacterial ecology. *Allergy* **2020**, *75*, 2037–2049. [CrossRef]
6. Cleland, E.J.; Bassiouni, A.; Wormald, P.J. The bacteriology of chronic rhinosinusitis and the pre-eminence of Staphylococcus aureus in revision patients. *Int. Forum Allergy Rhinol.* **2013**, *3*, 862–866. [CrossRef]
7. Vickery, T.W.; Ramakrishnan, V.R.; Suh, J.D. The role of Staphylococcus aureus in patients with chronic sinusitis and nasal polyposis. *Curr. Allergy Asthma Rep.* **2019**, *19*, 21. [CrossRef]
8. Fokkens, W.J.; Lund, V.J.; Mullol, J.; Bachert, C.; Alobid, I.; Baroody, F.; Cohen, N.; Cervin, A.; Douglas, R.; Gevaert, P.; et al. European position paper on rhinosinusitis and nasal polyps 2012. *Rhinology* **2012**, *23*, 1–12.
9. Foreman, A.; Wormald, P.J. Different biofilms, different disease? A clinical outcomes study. *Laryngoscope* **2010**, *120*, 1701–1706. [CrossRef]
10. Stubbendieck, R.M.; Straight, P.D. Multifaceted interfaces of bacterial competition. *J. Bacteriol.* **2016**, *198*, 2145–2155. [CrossRef]
11. Hibbing, M.E.; Fuqua, C.; Parsek, M.R.; Peterson, S.B. Bacterial competition: Surviving and thriving in the microbial jungle. *Nat. Rev. Microbiol.* **2010**, *8*, 15. [CrossRef]
12. Speert, D.P.; Wannamaker, L.W.; Gray, E.D.; Clawson, C.C. Bactericidal effect of oleic acid on group A streptococci: Mechanism of action. *Infect. Immun.* **1979**, *26*, 1202–1210. [CrossRef] [PubMed]
13. Bomar, L.; Brugger, S.D.; Yost, B.H.; Davies, S.S.; Lemon, K.P. Corynebacterium accolens releases antipneumococcal free fatty acids from human nostril and skin surface triacylglycerols. *MBio* **2016**, *7*, e01725-15. [CrossRef] [PubMed]
14. Kechagia, M.; Basoulis, D.; Konstantopoulou, S.; Dimitriadi, D.; Gyftopoulou, K.; Skarmoutsou, N.; Fakiri, E.M. Health benefits of probiotics: A review. *ISRN Nutr.* **2013**, *2013*, 481651. [CrossRef]
15. Cleland, E.J.; Drilling, A.; Bassiouni, A.; James, C.; Vreugde, S.; Wormald, P.-J. Probiotic manipulation of the chronic rhinosinusitis microbiome. *Int. Forum Allergy Rhinol.* **2014**, 309–314. [CrossRef]
16. Abreu, N.A.; Nagalingam, N.A.; Song, Y.; Roediger, F.C.; Pletcher, S.D.; Goldberg, A.N.; Lynch, S.V. Sinus microbiome diversity depletion and Corynebacterium tuberculostearicum enrichment mediates rhinosinusitis. *Sci. Transl. Med.* **2012**, *4*, 151ra124. [CrossRef] [PubMed]

17. Uehara, Y.; Nakama, H.; Agematsu, K.; Uchida, M.; Kawakami, Y.; Fattah, A.A.; Maruchi, N. Bacterial interference among nasal inhabitants: Eradication of Staphylococcus aureus from nasal cavities by artificial implantation of *Corynebacterium* sp. *J. Hosp. Infect.* **2000**, *44*, 127–133. [CrossRef]
18. Khamis, A.D.; Raoult, D.; La Scola, B. rpoB gene sequencing for identification of Corynebacterium species. *J. Clin. Microbiol.* **2004**, *42*, 3925–3931. [CrossRef]
19. Desrosiers, M.; Valera, F.C.P. Brave New (Microbial) World: Implications for nasal and sinus disorders. *Braz. J. Otorhinolaryngol.* **2019**, *85*, 675–677. [CrossRef]
20. De Boeck, I.; Wittouck, S.; Martens, K.; Claes, J.; Jorissen, M.; Steelant, B.; van den Broek, M.F.L.; Seys, S.F.; Hellings, P.W.; Vanderveken, O.M.; et al. Anterior nares diversity and pathobionts represent sinus microbiome in chronic rhinosinusitis. *MSphere* **2019**, *4*. [CrossRef]
21. Psaltis, A.J.; Wormald, P.-J. Therapy of sinonasal microbiome in CRS: A critical approach. *Curr. Allergy Asthma Rep.* **2017**, *17*, 59. [CrossRef]
22. Khamis, A.D.; Raoult, D.; La Scola, B. Comparison between rpoB and 16S rRNA gene sequencing for molecular identification of 168 clinical isolates of Corynebacterium. *J. Clin. Microbiol.* **2005**, *43*, 1934–1936. [CrossRef]
23. Wos-Oxley, M.L.; Plumeier, I.; Von Eiff, C.; Taudien, S.; Platzer, M.; Vilchez-Vargas, R.; Becker, K.; Pieper, D.H. A poke into the diversity and associations within human anterior nare microbial communities. *ISME J.* **2010**, *4*, 839–851. [CrossRef]
24. Hardy, B.L.; Dickey, S.W.; Plaut, R.D.; Riggins, D.P.; Stibitz, S.; Otto, M.; Merrell, D.S. Corynebacterium pseudodiphtheriticum exploits Staphylococcus aureus virulence components in a novel polymicrobial defense strategy. *MBio* **2019**, *10*, e02491-18. [CrossRef]
25. Ramsey, M.M.; Freire, M.O.; Gabrilska, R.A.; Rumbaugh, K.P.; Lemon, K.P. Staphylococcus aureus shifts toward commensalism in response to Corynebacterium species. *Front. Microbiol.* **2016**, *7*, 1230. [CrossRef]
26. Bitschar, K.; Sauer, B.; Focken, J.; Dehmer, H.; Moos, S.; Konnerth, M.; Schilling, N.A.; Grond, S.; Kalbacher, H.; Kurschus, F.C.; et al. Lugdunin amplifies innate immune responses in the skin in synergy with host-and microbiota-derived factors. *Nat. Commun.* **2019**, *10*, 1–14. [CrossRef]
27. Thirumazhisi Sachithanandam, S. Rising methicillin-resistant Staphylococcus aureus infections in ear, nose, and throat diseases. *Case Rep. Otolaryngol.* **2014**, *2014*. [CrossRef]
28. Psaltis, A.J.; Weitzel, E.K.; Ha, K.R.; Wormald, P.-J. The effect of bacterial biofilms on post-sinus surgical outcomes. *Am. J. Rhinol.* **2008**, *22*, 1–6. [CrossRef] [PubMed]
29. Singh, P.; Mehta, R.; Agarwal, S.; Mishra, P. Bacterial biofilm on the sinus mucosa of healthy subjects and patients with chronic rhinosinusitis (with or without nasal polyposis). *J. Laryngol. Otol.* **2015**, *129*, 46–49. [CrossRef] [PubMed]
30. Richter, K. Tackling superbugs in their slime castles: Innovative approaches against antimicrobial-resistant biofilm infections. *Microbiol. Aust.* **2019**, *40*, 165–168. [CrossRef]
31. Iwase, T.; Uehara, Y.; Shinji, H.; Tajima, A.; Seo, H.; Takada, K.; Agata, T.; Mizunoe, Y. Staphylococcus epidermidis Esp inhibits Staphylococcus aureus biofilm formation and nasal colonization. *Nature* **2010**, *465*, 346–349. [CrossRef]
32. Dodémont, M.; Verhulst, C.; Nonhoff, C.; Nagant, C.; Denis, O.; Kluytmans, J. Prospective two-center comparison of three chromogenic agars for methicillin-resistant Staphylococcus aureus screening in hospitalized patients. *J. Clin. Microbiol.* **2015**, *53*, 3014–3016. [CrossRef]
33. Saitou, N.; Nei, M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **1987**, *4*, 406–425. [PubMed]
34. Moran, J.C.; Crank, E.L.; Ghabban, H.A.; Horsburgh, M.J. Deferred growth inhibition assay to quantify the effect of bacteria-derived antimicrobials on competition. *J. Vis. Exp.* **2016**, e54437. [CrossRef]
35. Panchatcharam, B.S.; Cooksley, C.M.; Ramezanpour, M.; Vediappan, R.S.; Bassiouni, A.; Wormald, P.J.; Psaltis, A.J.; Vreugde, S. Staphylococcus aureus biofilm exoproteins are cytotoxic to human nasal epithelial barrier in chronic rhinosinusitis. *Int. Forum Allergy Rhinol.* **2020**. [CrossRef] [PubMed]
36. Inturri, R.; Trovato, L.; Volti, G.L.; Oliveri, S.; Blandino, G.; Trovato, L. In vitro inhibitory activity of Bifidobacterium longum BB536 and Lactobacillus rhamnosus HN001 alone or in combination against bacterial and Candida reference strains and clinical isolates. *Heliyon* **2019**, *5*, e02891. [CrossRef] [PubMed]
37. Al Akeel, R.; Mateen, A.; Alharbi, K.K.; Alyousef, A.A.; Al-Mandeel, H.M.; Syed, R. Purification and MIC analysis of antimicrobial proteins from Cucumis sativus L. seeds. *BMC Complement. Altern. Med.* **2018**, *18*, 1–6. [CrossRef]
38. Richter, K.; Ramezanpour, M.; Thomas, N.; Prestidge, C.A.; Wprmald, P.-J.; Vreugde, S. Mind "De GaPP": In vitro efficacy of deferiprone and gallium-protoporphyrin against Staphylococcus aureus biofilms. *Int. Forum Allergy Rhinol.* **2016**, *6*, 737–743. [CrossRef] [PubMed]

CHAPTER 4

Proteomic identification of potential antibacterial products from *Corynebacterium accolens*

CHAPTER 4: Proteomic identification of potential antibacterial products from *Corynebacterium accolens*

This chapter is prepared in a manuscript format and addresses the third research aim designed to identify, characterize and quantify potential antibacterial and other proteins in *Corynebacterium accolens* potentially related to probiotic action.

Menberu MA, Ramezanpour M, Cooksley C, Liu S, Bouras G, Psaltis AJ, Wormald PJ, Vreugde S. Proteomic identification of potential antibacterial products from *Corynebacterium accolens*, a commensal nasal isolate deficient in chronic rhinosinusitis. Prepared in a manuscript format.

4.1 Statement of Authorship

Title of Paper	Proteomic identification of potential antibacterial products from *Corynebacterium accolens*, a commensal nasal isolate deficient in chronic rhinosinusitis
Publication Status	☐ Published ☐ Accepted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style ☐ Submitted for Publication
Publication Details	Menberu MA, Ramezanpour M, Cooksley C, Liu S, Bouras G, Psaltis AJ, Wormald PJ, Vreugde S.

Principal Author

Name of Principal Author (Candidate)	Martha Alemayehu Menberu		
Contribution to the Paper	Conception and design of the project, acquisition of data, analysis and interpretation of data, drafting the article		
Overall percentage (%)	85		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	21/10/2021

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Mahnaz Ramezanpour		
Contribution to the Paper	Acquisition of data, analysis and interpretation of data, manuscript edition		
Signature		Date	19/10/2021

Name of Co-Author	Clare Cooksley		
Contribution to the Paper	Acquisition of data, interpretation of data, critically revising the article		
Signature		Date	19/10/2021

Name of Co-Author	Sha Liu		
Contribution to the Paper	Acquisition of data, analysis and interpretation of data, manuscript edition		
Signature		Date	18/10/2021

Name of Co-Author	George Bouras		
Contribution to the Paper	Analysis and interpretation of data, manuscript edition		
Signature		Date	19/10/2021

Name of Co-Author	Alkis James Psaltis		
Contribution to the Paper	Conception of the project, critically revising the article		
Signature		Date	18/10/2021

Name of Co-Author	Peter-John Wormald		
Contribution to the Paper	Conception of the project, critically revising the article		
Signature		Date	18/10/2021

Name of Co-Author	Sha Liu		
Contribution to the Paper	Acquisition of data, analysis and interpretation of data, manuscript edition		
Signature		Date	18/10/2021

4. Proteomic identification of potential antibacterial products from *Corynebacterium accolens*, a commensal nasal isolate deficient in chronic rhinosinusitis

Martha Alemayehu Menberu[1, 2], Mahnaz Ramezanpour[1], Clare Cooksley[1], Sha Liu1, George Bouras[1], Alkis James Psaltis[1], Peter-John Wormald[1], Sarah Vreugde[1]

[1]Department of Surgery-Otolaryngology, Head and Neck Surgery, The University of Adelaide, Basil Hetzel Institute for Translational Health Research, Central Adelaide Local Health Network, Woodville, South Australia, Australia

[2]Department of Medical Microbiology, School of Biomedical and Laboratory Sciences, College of Medicine and Health Sciences, University of Gondar, Ethiopia

Correspondence

Associate Professor Sarah Vreugde, Department of Otolaryngology, Head and Neck Surgery, Basil Hetzel Institute for Translational Health Research, The Queen Elizabeth Hospital, 28 Woodville Road, Woodville South, SA 5011, Australia.

E-mail: sarah.vreugde@adelaide.edu.au

4.2 Abstract

Chronic rhinosinusitis (CRS) associated dysbiosis is characterized by a significant reduction of Corynebacteria including *C. accolens*. Commensal *C. accolens* nasal isolates, and their secreted proteins, exert antibacterial effects toward common pathogens in CRS including *S. aureus*, and are, as such, considered as potential probiotics. However, the type of antibacterial product and other probiotic traits have not yet been determined. The current study evaluated, the antibacterial effect of secreted proteins from 6 *C. accolens* nasal isolates against clinical isolates (CI) of methicillin-sensitive *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) as well as a *S. aureus* reference strain using a micro dilution assay. A proteomic approach, using one dimensional gel electrophoresis and Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) was used to identify potential antibacterial proteins commonly secreted by *C. accolens* strains, and functional characterization of the most abundant proteins associated with other probiotic properties was performed. Overall, the activity of 300 µg/ml cleaned protein extracts from 6 *C. accolens* strains showed strong inhibitory effects toward MSSA C26 and MRSA C261 CIs. Proteomic analysis of the cleaned protein extracts from 6 *C. accolens* strains identified a total of 1455 distinct proteins. Of these, 595 were common to all strains including the most abundant protein, a putative esterase. In addition, Acetyltransferase, GNAT family protein was the strongest positively correlated protein (Pearson's r = 0.80, p=0.004) detected in *C. accolens* strains with strong antibacterial effect in higher abundance than strains with moderate antibacterial effect against *S. aureus*. Most of the identified proteins in the biological process category belonged to the metabolic processes, cell organization and cellular homeostasis. Moreover, in most *C. accolens* strains, proteins involved in the survival and adhesion probiotic properties such as putative esterase, chaperone protein DnaK, 60 kDa chaperonin and elongation factor thermal unstable (EF-Tu) were identified with strain-specific differences in abundance level. Among the most common abundant proteins, some proteins with recognized antimicrobial activity, such as the

glycosyl hydrolase family 25 and N-acetylmuramoyl-L-alanine amidase were identified. These proteins may be potential anti-microbial products effective against MSSA and MRSA CIs, and hold great promise for the development of a novel probiotic therapy to combat microbiome dysbiosis in CRS.

4.3 Introduction

Chronic rhinosinusitis (CRS) is a complex and clinically challenging inflammatory condition of the sinonasal mucosa associated with significant morbidity to the patient [273]. Molecular and immunological studies have shown an imbalance of resident nasal microbiota, characterized by a reduction in the relative abundance of *Corynebacterium* and an increase in pathobionts, mainly *Staphylococcus aureus* in the context of CRS [98, 274, 275]. Commensal Corynebacteria have gained attention in recent years due to their potential protective role in sinus health and significant antimicrobial properties against pathogens [276, 277]. From the *Corynebacterium* genus, *Corynebacterium accolens* has been widely reported to colonise the healthy sinonasal tract and has been proposed for potential use as a probiotic to promote sinus health [268, 278].

Nasal commensals have been found to use a variety of mechanisms to impact pathogen colonization, including the production of antimicrobials that directly kill or inhibit competitors in a manner beneficial to the host [194]. Studies have demonstrated that various species within the *Corynebacterium* genus have been found to directly antagonize *S. aureus* pathobionts [279, 280]. An example of this is , *C. pseudodiphtheriticum*, a common member of the normal nasal microbiota that has been shown to eliminate both methicillin-sensitive and methicillin-resistant *S. aureus* (MRSA) from the human nose through production of toxic compounds that directly kill incoming competitors [275]. Moreover, colonization by *C. amycolatum*, *C. accolens*, and *C. pseudodiphtheriticum* is negatively correlated with *S. aureus* colonization

[125] and can inhibit the virulence of *S. aureus* through inactivating the accessory gene regulator (*agr*) quorum sensing expression [272].

Although there are many unresolved questions regarding the role of Corynebacteria in promoting nasal health, the secretion of antimicrobial proteins or peptides could be responsible for conferring crucial probiotic traits such as pathogen inhibition, promotion of host epithelial barrier function, and stimulation of the host immune system, making it paramount to explore and identify functional proteins for therapeutic applications [194, 200, 281]. In a previous study, an extracellular triacylglycerol lipase (LipS1) produced by *C. accolens*, demonstrated bactericidal properties against *Streptococcus pneumoniae* potentially due to its hydrolysing ability to release antibacterial free fatty acids from representative human nostril and skin surface triacylglycerols, which in turn protect against Streptococcal nasal colonization [127]. Bacterial lipases used as modulators have been reported to play an important role in pharmaceutical and medicinal applications [282].

We recently demonstrated that *C. accolens* strains have strong antibacterial activity against *S. aureus* and MRSA strains isolated from CRS patients and that their secreted proteins confer a concentration- dependent inhibitory action against those strains in planktonic and biofilm form [278]. Identification and characterization of the secreted bioactive molecules potentially involved in the antimicrobial action is of particular interest and may provide the basis for further investigation into novel therapeutic compounds against *S. aureus* and MRSA.

In the present work, we used a Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)-based proteomics analysis [283, 284] of the *C. accolens* strains' secreted products to identify, characterize and quantify potential antibacterial and other proteins potentially related to probiotic action.

4.4 Materials and methods

Bacterial strains and growth conditions

Ethics approval for the collection, storage and use of clinical isolates was obtained from The Queen Elizabeth Hospital (TQEH) Human Research Ethics Committee in Adelaide, South Australia (HREC/15/TQEH/132). Six different *C. accolens* strains designated as C778, C779, C781, C782, C785, and C787 isolated from the nasal cavity of non-CRS controls (no clinical or radiologic evidence of sinus disease) with demonstrated antimicrobial activity against *S. aureus* and MRSA clinical isolates were used in this study [278]. The strains were cultured on tryptone soya agar (TSA) (Oxoid, Basingstoke, UK) supplemented with 0.8% Tween 80 for 48 hrs at 37ºC with 5% CO_2 and kept in tryptone soya broth (TSB) (Oxoid, Basingstoke, UK) supplemented with 20% (v/v) glycerol at -80 °C until use. MSSA C26 and MRSA C261 were isolated from the sinonasal cavities of CRS patients [278] and identified by an independent diagnostic laboratory (Adelaide Pathology Partners, Adelaide, Australia). *S. aureus* ATCC 25923 was from the American Type Culture Collection (ATCC, Manassas, USA).

Extraction of exoproteins from *C. accolens*

Each strain of *C. accolens* was first grown in 10 ml TSB at 180 rpm in a shaking incubator at 37°C for 48 hrs. Supernatants were obtained by centrifugation (4000× *g*, 4ºC for 10 min) followed by filtration through a 0.2 µm sterile syringe filter (Pall Life Sciences, UK). After which, supernatants were passed through a 3-kDa filter concentrator (Pierce Protein Concentrator, Thermo Fisher Scientific, Rockford, IL, USA) using centrifugation at 4000× *g*, 4ºC for 1-2 hr to collect exoproteins as described previously [285]. The extracted proteins were stored as single-use aliquots at -80ºC until use.

Protein clean-up and Determination of Protein Concentration

Protein purification was performed from the extracted samples using a 2-D Clean-Up Kit (GE-Biosciences, USA) according to the manufacturer's instructions. Protein quantification was performed on the extracted protein samples using Quick Start Bradford Protein Assay Kit (Bio-Rad Laboratories, USA) according to manufacturer's instructions. All reactions were carried out in duplicate.

Anti-bacterial activity of protein extracts

In order to independently confirm and compare the antibacterial potential of secreted protein extracts from *C. accolens* against MSSA C26, MRSA C261 and *S. aureus* ATCC 25923 isolates, a broth micro-dilution assay was performed as previously described with modifications [286]. *C. accolens* exoprotein concentration, clean-up and quantification were performed according to a previously detailed protocol [278]. 198 µl of cleaned exoproteins diluted in TSB (to 300 µg/ml) were dispensed separately in 96-well microtiter plates (Life Sciences, USA). Bacterial suspension was prepared from 24 hr MSSA C26, MRSA C261 and *S. aureus* ATCC 25923, by mixing with 3 ml sterile saline (NaCl 0.9% w/v) and adjusted to 0.5 McFarland turbidity standard (approximately 1.0 - 2.0x10^8 CFU/ml). Following this, 2 µl of bacterial suspension was added to the wells and plates were incubated at 37°C for 24 hrs. After incubation, bacterial growth was determined by measuring the optical density (OD) at 595 nm using a microplate absorbance reader (iMark™, Bio-Rad, Australia). In control tests, bacteria without exoproteins grown in TSB and MilliQ water (to account for the water-diluted TSB) and sterile TSB were used as positive and negative growth controls, respectively. *The antibacterial activity of protein extracts was calculated by* comparing OD values between treated and untreated wells. The percentage of growth inhibition was calculated with the following formula: $[(OD_{growth\ control} - OD_{protein\ treated}) / OD_{growth\ control}] \times 100$. The assays were performed in triplicates for each sample and results are presented as mean (± standard error).

One dimensional (1D) gel electrophoresis

The protein profiling and separation was carried out on all six *C. accolens* strains using 1-D polyacrylamide gel electrophoresis at neutral PH (NuPAGE) test kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instruction. In brief, 15 µl of protein samples were mixed with 5 µl *NuPAGE* lithium dodecyl sulfate (LDS) *sample buffer* (4X) and then heated on dry block heater at 95ºC for 15 min. Next, 20 µl of the protein mixture and 6 µl of full-range (12kDa-225kDa) rainbow protein marker (Bio-Sciences, USA) were loaded into the wells of a NuPAGE 4 to 12%, Bis -Tris Gel. Then, protein gels were run by using 1x running buffer [MOPS Sodium Dodecyl Sulfate (SDS)] at a constant voltage of 100V for 90 mins until the tracking dyes had migrated to the bottom of the gel. Gels were immersed in fixing solution (50 ml methanol, 10 ml Acetic acid and 100 ml sterile MilliQ water) and kept on a rotating shaker at room temperature for 10 min. Following fixation, the resulting gels were stained using Colloidal Blue Staining Kit (Invitrogen, Thermo Fisher Scientific, USA) with continuous shaking for 16-24 hrs. The gels were then washed with sterile MilliQ water and the protein bands were visualized with Coomassie Blue using ChemiDocTM MP Imaging System (Bio-Rad, Australia). Images were analysed by Image Lab software (version 6.0.1). Duplicate experiments were performed for each protein sample.

In gel trypsin digestion of proteins

Each lane of the NuPAGE gels, was cut into 12 pieces using sterile scalpels and transferred into 1.5 ml maximum recovery tubes (Axygen, CA, USA). Dissected gel pieces were washed with 200 µl of sterile distilled water by vortex mixing for 30 seconds. Following this, the gel pieces were de-stained with 100 µl of acetonitrile (ACN) (BDH Laboratory Supplies, Poole, UK) and 50 mM Ammonium Bicarbonate (ABC) (Sigma-Aldrich, MO, USA) in 1:1v/v ratio, reduced with 100 µl freshly prepared 50 mM DTT in 50 mM ABC for 20 min at 56ºC, and alkylated with 100 µl of 100 mM iodoacetamide (both from Sigma-Aldrich, MO, USA)

prepared in 50 mM ABC for 20 mins in the dark at room temperature. The gel pieces were then washed twice with 400 µl sterile water, dehydrated in 100 µl of ACN: 50 mM ABC (1:1v/v) ratio for 5 min with intermittent vortex mixing and this was followed by 30 sec incubation at room temperature in 100 µl of 100% ACN to ensure that all noticeable colloidal stain was removed from the gel pieces. Gel pieces were then dried in a 37ºC oven for approximately 5 min. After reduction and alkylation, gel pieces were digested and rehydrated with trypsin working solution (12.5 ng/µl) containing trypsin (Promega, WI, USA), 50 mM ABC and 50 mM calcium chloride (BDH Chemicals, Vic, Australia) for 18 hrs at 37ºC. The digestion reactions with extracted peptides were transferred into a new tube and centrifuged at 20,000 x *g* for 10 min. The supernatants were collected and transferred into mass spectrometry vials (Thermo Scientific, TN, USA) and then acidified by adding Trifluoroacetic acid (TFA) (Fisher Scientific, Belgium) to a final concentration of 0.5% prior to Liquid chromatography- Tandem Mass Spectrometry (LC-MS/MS) analysis.

Proteomics analysis by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

Tryptic digests were separated by nanoliquid chromatography and analyzed with a compact hybrid quadrupole time-of-flight mass spectrometer (Orbitrap Exploris™ 480, Thermo Scientific, Waltham, MA USA). The mass spectral analysis was performed at the Proteomics Facility, Flinders University, Adelaide, South Australia. The SEQUEST database searching program was used to interpret the MS/MS spectra obtained from the LC-MS/MS runs. The SEQUEST database search algorithm correlates the experimental data of tandem mass spectra of peptides with theoretically generated peptide sequences from a known protein sequence in a database [287]. The obtained mass spectral data were matched to a Uniprot database (https://www.uniprot.org) created using all web-available sequenced *C. accolens* protein databases, to identify the proteins. The online Uniprot database was used to obtain Gene

Ontology (GO) of genes corresponding to the common identified proteins in *C. accolens* (top 25 abundant) and to characterize the functional categories as well as specific protein function.

Statistical analysis

Statistical analyses of the *in vitro* antimicrobial activity were conducted with GraphPad prism 9.0.0 software. Experiments were performed at least in triplicate. Data are presented as the mean ± standard error of the mean (SEM) for each *C. accolens* strain activity. One-way ANOVA followed by the Tukey's multiple comparisons test was employed to investigate statistical differences. The R Core Team statistical software was used to determine the pair-wise protein differences and adjusted multiple comparisons between strains. The common and unique proteins identified across the six strains of *C. accolens* were listed based on their abundance. The relative protein abundance was estimated based on the number of spectra generated from LC-MS/MS measurements matching to a given protein identified through SEQUEST search. Pearson's correlation analysis was performed to determine the correlation between *C. accolens* common protein abundance and *S. aureus* growth inhibition. This analysis gives information about the magnitude of the association, or correlation, as well as the direction of the relationship. Correlations between abundance and bacterial growth inhibition were tested for significance for each expressed protein against the null hypothesis that the correlation was 0 (using the cor. test function in R). P-values were adjusted for multiple comparisons using the Benjamani-Hochberg method, with the false discovery rate set to 5%. The relative abundance of an individual protein was calculated through dividing the total number of spectra matching to a given protein by the total number of spectra matching to top 25 abundant proteins detected in each strain sample. A *P*-value of <0.05 was considered statistically significant.

4.5 Results

Antibacterial activity of *C. accolens* extracellular proteins

The total cleaned exoprotein concentration from the 6 *C. accolens* strains was similar and ranged from 373 to 377 µg/ml. Cleaned exoprotein extracts (containing 300 µg/ml) from all 6 *C. accolens* strains demonstrated significant antibacterial action against representative *S. aureus* clinical isolates (MSSA C26 and MRSA C261) as well as a reference *S. aureus* strain, ATCC 25923 ($p<0.0001$) with a range of 58% to 99% growth reduction (Figure 4.1). *C. accolens* strains C779, C781 and C787 had the strongest antibacterial effect (87% to 99% inhibition) whilst *C. accolens* strains C778, C782 and C785 had a moderate antibacterial effect (58% to 73% inhibition).

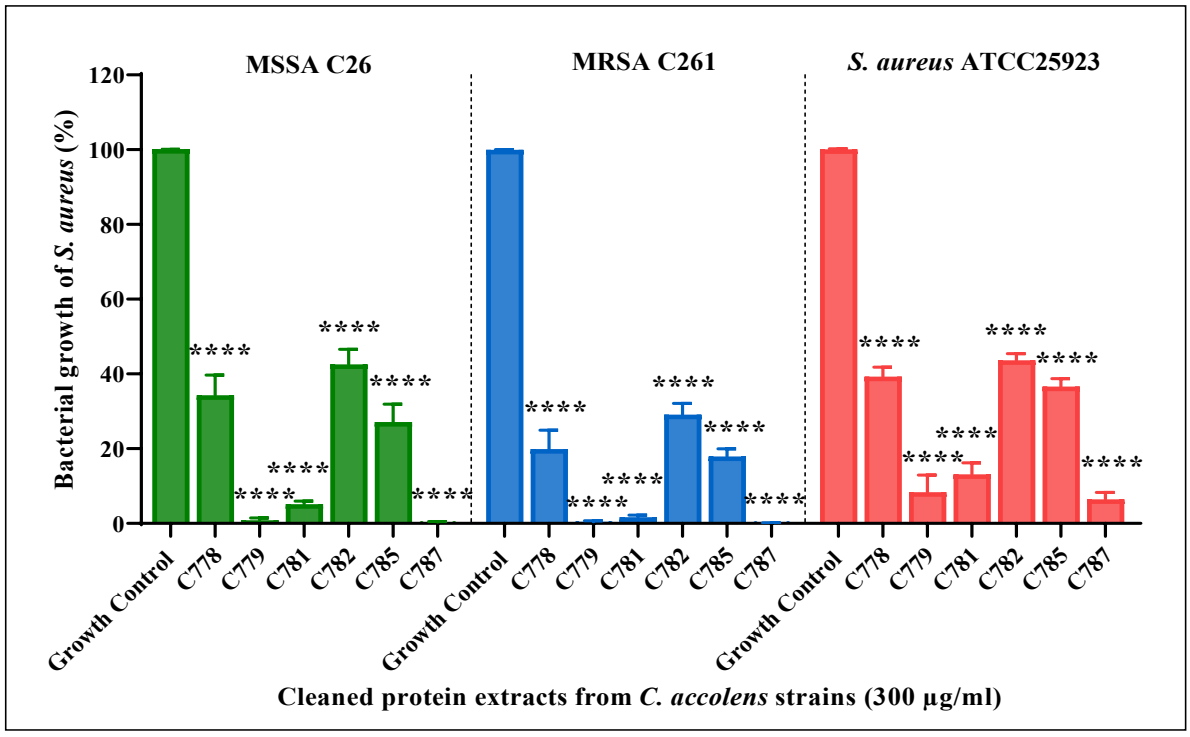


Figure 4.1. Antibacterial potential of *C. accolens* exoproteins against *S. aureus* planktonic cells. Cleaned protein extract from *C. accolens* strains (C778, C779, C781, C782, C785 and C787) at 300 µg/ml concentration was added to MSSA C26 (green), MRSA C261 (blue) and ATCC25923 (red) cultures and the Optical Density value was measured after 24 hrs. Results

were expressed as percentage of bacterial growth compared to the growth control [*S. aureus* in TSB and MilliQ water (to account for the water-diluted TSB)]. Data presented as means ± SEM of three replicate experiments. MSSA, methicillin-sensitive *S. aureus*; MRSA, methicillin-resistant *S. aureus*; TSB, tryptone soya broth; One-way ANOVA ***p<0.001; ****p<0.0001; SEM, standard error of the mean.

Protein profiling of *C. accolens* strains using 1D Nu-PAGE

To compare the secreted protein expression profiles among strains, exoproteins from 6 *C. accolens* strains were separated using one dimensional gel electrophoresis. We identified a diverse pattern of proteins, distributed over a wide range of molecular weights (mainly between 17-76 kDa) in all *C. accolens* strains. The electrophoretic patterns displayed no clear distinct protein profiles or variations of individual bands in both intensity and distribution associated with a strong and moderate antibacterial effect of the *C. accolens* strains. Two representative gel images of exoprotein profiles with stain free and coomassie blue are shown in Figure 4.2A and 4.2B.

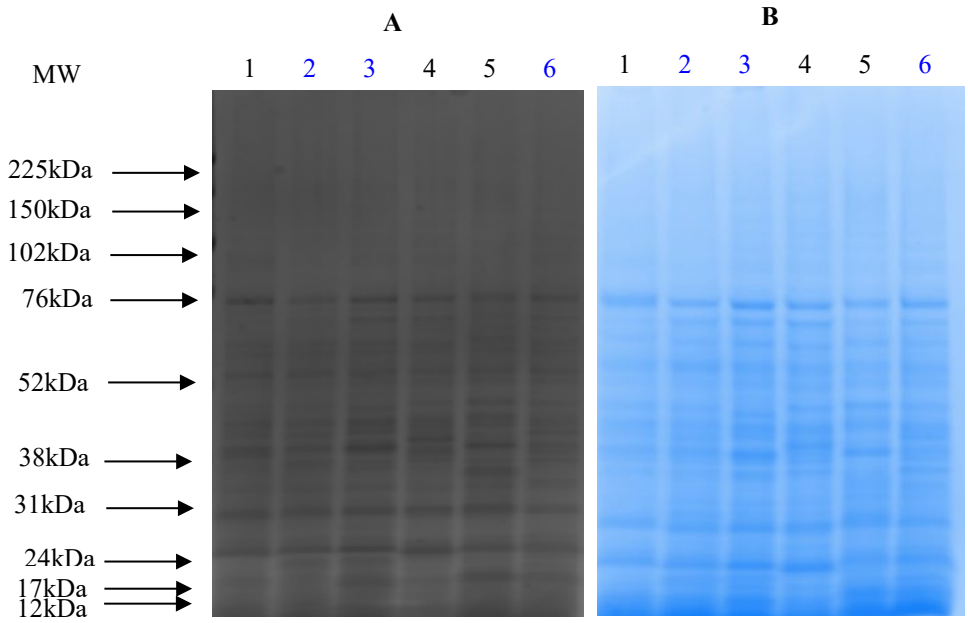


Figure 4.2. Overview of the exoprotein patterns from *C. accolens* strains separated by 1D Nu-PAGE assay. Lanes 1-6 represent the exoprotein bands from *C. accolens* strains C778 (1), C779 (2), C781 (3), C782 (4), C785 (5), and C787 (6) gel images in a stain-free gel (A) and coomassie blue stained gel (B). Numbers in blue colour indicate *C. accolens* strains with strong antimicrobial activity. MW; molecular weight marker (225-12kDa), kDa; kilodalton.

Identification of proteins among *C. accolens* strains

A total of 1455 distinct proteins were identified among the exoproteins from the six *C. accolens* strains, of which 595 proteins were common to all strains **(**Supplementary Table 4.1). A list of the top 25 most abundant proteins (according to abundance) commonly expressed in all *C. accolens* strains is presented in Table 4.1. From those common proteins the most abundant protein was Putative esterase (cmtC) with a molecular weight of 71.3 kDa followed by NlpC/P60 family protein that has a molecular weight of 21.5 kDa and is classified as a hypothetical protein of unknown function. We also identified 254 unique proteins across the six strains, of which 32, 23, 52, 45, 45, and 57 proteins were unique to strains C778, C779, C781, C782, C785, and C787, respectively (Supplementary Table 4.2). A boxplot of the average in abundance of unique proteins identified in each strain is shown in Figure 4.3.

Table 4.1. Proteins commonly expressed in six *C. accolens* strains (top 25 most abundant) identified by SEQUEST search

Accession	Protein description	Gene name	Number of AAs	Protein abundance	MW (kDa)	Average coverage (%)	Average number of matched peptides	Average sequest score
E0N0K3	Putative esterase	cmtC	649	6.36E+09	71.3	61.25	61	1432.65
E0MZ10	NlpC/P60 family protein	HP↓	209	3.94E+09	21.5	49.5	20	450.72
E0MXB4	Glycosyl hydrolase family 25	HP↓	394	2.73E+09	41.6	40.75	17.5	371.09
E0MXC7	META domain-containing protein	HP↓	177	1.52E+09	18.5	65.75	16.25	347.93
E0MVI7	Periplasmic binding protein	fecS	316	1.04E+09	34.2	43.75	14.75	265.1
E0MVM0	ErfK/YbiS/YcfS/YnhG	HP↓	263	9.63E+08	29	20.75	6.25	112.75
E0MYQ9	Putative monovalent cation/H+ antiporter subunit C	mnhC	158	9.23E+08	17.1	8	1	1.79
E0N0D0	Chaperone protein DnaK	dnaK	620	9.13E+08	66.8	48.5	27.75	272.01
E0MW06	Uncharacterized protein	HP↓	190	8.31E+08	20.4	48	7.75	155.79
E0N068	Hydrolase, alpha/beta domain protein	HP↓	384	7.47E+08	41.5	33.75	17	319.36
E0MV38	Dihydrolipoyl dehydrogenase	lpdA	470	7.32E+08	50.4	33.75	13	272.78
E0MWT6	Enolase	eno	425	6.89E+08	45.1	41.75	12.75	149.59
E0MXY2	NlpC/P60 family protein	HP↓	498	6.62E+08	52.1	23.25	7.5	159.74
E0MVG2	Elongation factor Tu	tuf	396	6.51E+08	44	39.25	12.75	223.7
E0MXJ7	Putative ribosomal protein S1	rpsA	487	6.21E+08	53.8	38.25	16.75	196.42
E0MWL3	Trypsin	HP↓	500	6.20E+08	51	21.5	7.5	154.35
E0MZU3	Cysteine synthase	cysK	311	5.80E+08	32.3	45	13.25	194.24
E0N0M2	N-acetylmuramoyl-L-alanine amidase	csp	632	5.47E+08	66.7	26.75	18	235.88
E0N0C6	Aldehyde dehydrogenase (NAD) family protein	aldA2	506	5.19E+08	55.3	33	13.75	234.91

E0MZ09	NlpC/P60 family protein	HP$^{\downarrow}$	347	4.82E+08	37	27	10.5	151.28
E0MUU3	Trypsin	HP$^{\downarrow}$	401	4.81E+08	40.6	21.75	6	53.23
E0MW05	Uncharacterized protein	HP$^{\downarrow}$	422	4.80E+08	46.7	45	15.75	236.79
E0MZN7	Periplasmic binding protein	hmuT2	391	4.71E+08	42.6	39	11	153.59
E0N072	60 kDa chaperonin	groL	547	4.69E+08	57.5	29.5	14.5	214.36
E0MWN3	Transglycosylase-like domain protein	HP$^{\downarrow}$	391	4.47E+08	41.2	18.75	7.5	206.53

HP$^{\downarrow}$: Hypothetical Protein

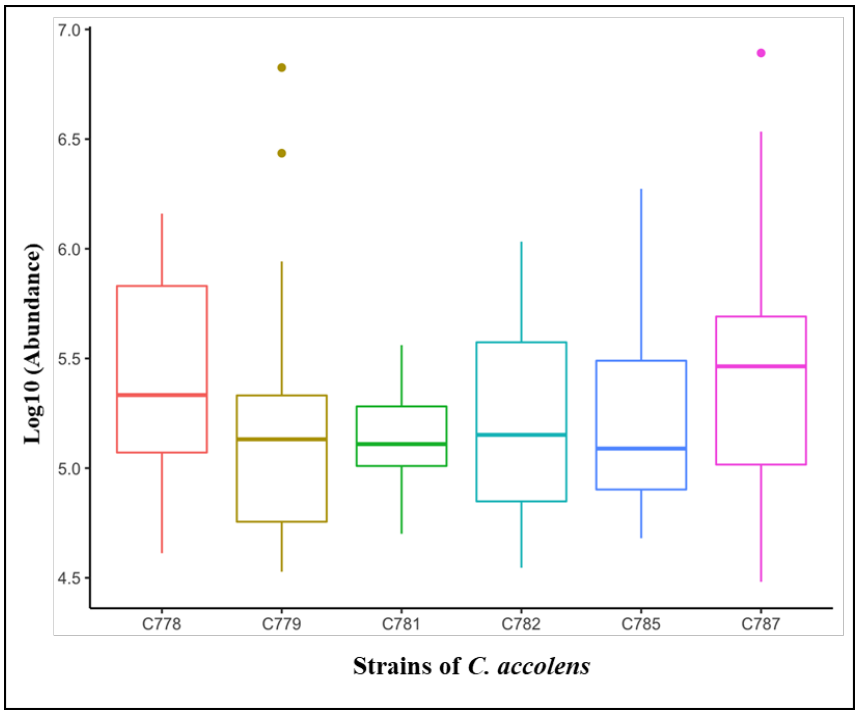


Figure 4.3. The distribution of unique protein abundances amongst the 6 *C. accolens* strains (C778, C779, C781, C782, C785, and C787). Data shows the average in abundance of the unique proteins expressed by each strain and is presented as log10 abundance.

Association between abundance of common proteins in *C. accolens* and *S. aureus* growth inhibition

The 3 *C. accolens* strains C779, C781 and C787 with the strongest antibacterial effect against *S. aureus* isolates (87% to 99% inhibition) and the 3 *C. accolens* strains C778, C782 and C785 with a moderate antibacterial effect (between 58% to 73% inhibition) were selected for further proteomic correlation analysis. Pearson correlation measures the strength and direction of a linear relationship between two variables. From 595 commonly expressed *C. accolens* proteins, the abundance of a total of 14 proteins was positively correlated with *S. aureus* growth inhibition (Pearson's $r > 0.5$, $p<0.05$). The strongest significant positive correlation was detected for an Acetyltransferase, GNAT family protein (Pearson's $r = 0.80$, $p=0.004$) followed by Cell envelope-like function transcriptional attenuator common domain protein (Pearson's r

= 0.73, p=0.01), Septum-form domain-containing protein (Pearson's r = 0.67, p=0.02), Glycosyl hydrolase family 3 N-terminal domain protein (Pearson's r = 0.66, p=0.03), and Putative monovalent cation/H+ antiporter subunit C (Pearson's r = 0.65, p=0.04) (Figure 4.4). 103 common *C. accolens* proteins were negatively correlated (Pearson's r < -0.5) with the inhibition of *S. aureus* growth. A list of all common abundant proteins positively and negatively correlated with the *S. aureus* growth inhibition is shown in Supplementary Table 4.3.

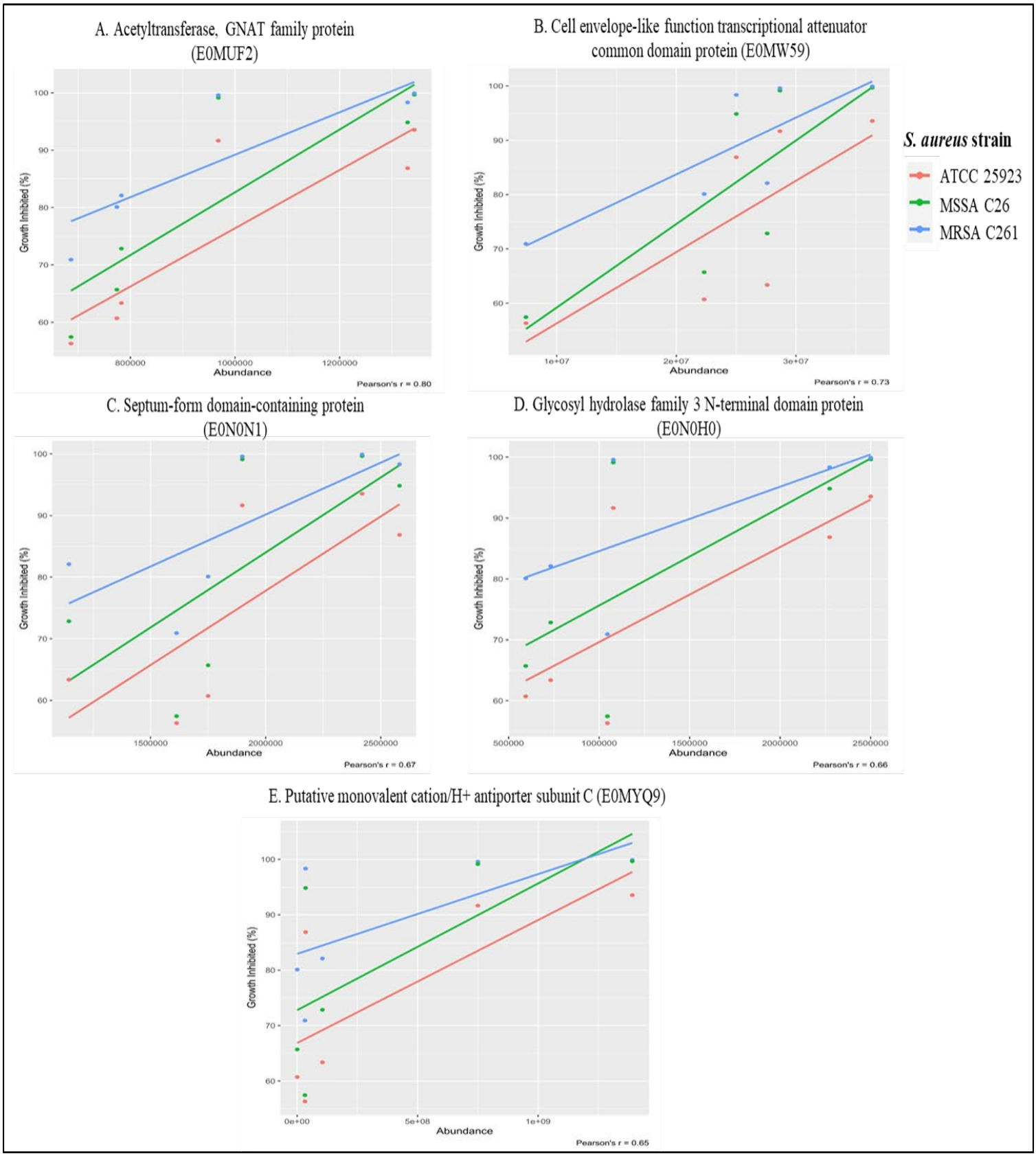


Figure 4.4. The plot showing positive correlation between abundance versus *S. aureus* growth inhibition (%) for Acetyltransferase, GNAT family protein (A), Cell envelope-like function

transcriptional attenuator common domain protein (B), Septum-form domain-containing protein (C), Glycosyl hydrolase family 3 N-terminal domain protein (D), and Putative monovalent cation/H+ antiporter subunit C (E) commonly identified from *C. accolens* strains [3 with strong inhibition effect (87% to 99%) and 3 with moderate inhibition effect (58% to 73%) against MSSA C26 (green), MRSA C261 (blue) and *S. aureus* ATCC 25923 (red)]. Number in parenthesis indicate the gene accession number for the specified protein.

Functional classification of common proteins in *C. accolens*

To further investigate the functional categories of common proteins identified in *C. accolens* strains, we analysed the top 25 most abundant proteins using the database Universal Protein Resource (UniProt) [288]. Abundance-based Gene Ontology (GO) enrichment was also applied to describe each protein function [289]. According to the molecular function category, most of the common abundant proteins identified were associated with catalytic activity (61%). Beside this, 22% of common abundant proteins in this category identified were related with nucleotide and protein binding, 9% were related with metal ion binding, and 8% were related with RNA binding and structural molecule activity (Figure 4.5A).

In the biological process classifications, common abundant proteins related with metabolic processes occupied the largest part (90%) (Figure 4.5B). In addition, cellular component of the common abundant proteins identified was mainly occupied by integral component of membrane (57%), followed by cytoplasm (29%) and ribosome (14%), which described the localization processes of a gene product at the molecular level (Figure 4.5C).

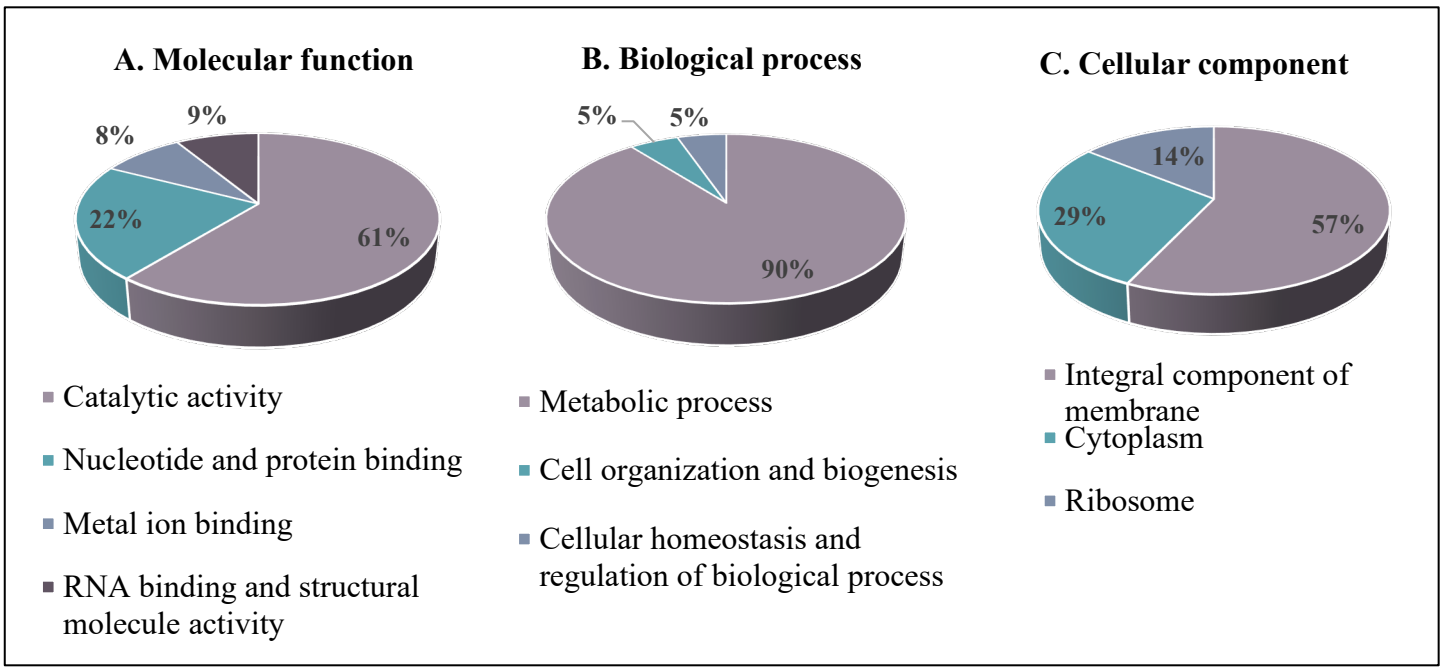


Figure 4.5. Functional classification of abundant proteins in all *C. accolens* strains (n=6) using GO classification from the UniProtKB database. Descriptive information on the functional enrichment and number of commonly expressed proteins involved in (A) Molecular function, (B) Biological process, and (C) Cellular component are indicated. Data shows the percentage of proteins commonly identified in *C. accolens* (top 25 most abundant) involved in each functional category.

Table 4.2. List of GO categories of commonly expressed *C. accolens* proteins and their functional classification.

Functional classification	Gene ontology category
Molecular function	GO: 0009253 - peptidoglycan catabolic process GO: 0016998 - cell wall macromolecule catabolic process GO: 0009252 - peptidoglycan biosynthetic process GO: 0006096 - glycolytic process GO: 0006535 - cysteine biosynthetic process from serine GO: 0016620 - oxidoreductase activity GO: 0006457 - protein folding GO: 0045454 - cell redox homeostasis GO: 0042026 - protein refolding GO: 0006412 - translation
Biological process	GO:0050348 - trehalose O-mycolyltransferase activity GO:0003796 - lysozyme activity GO:0016740 - transferase activity GO:0016491 - oxidoreductase activity GO:0005524 - ATP binding GO:0051082 - unfolded protein binding GO:0016787 - hydrolase activity GO:0004148 - dihydrolipoyl dehydrogenase activity GO:0050660 - flavin adenine dinucleotide binding GO:0000287 - magnesium ion binding GO:0004634 - phosphopyruvate hydratase activity GO:0003924 - GTPase activity GO:0005525 - GTP binding GO:0003746 - translation elongation factor activity GO:0003676 - nucleic acid binding GO:0004252 - serine-type endopeptidase activity GO:0004124 - cysteine synthase activity GO:0008745 - N-acetylmuramoyl-L-alanine amidase activity GO:0008270 - zinc ion binding
Cellular component	GO: 0016021 - integral component of membrane GO: 0005737 - cytoplasm GO: 0005840 - ribosome

Comparative and functional analysis of the common abundant proteins in *C. accolens* strains

The top 25 most abundant commonly expressed *C. accolens* proteins were analysed to assess a strain level difference in individual protein abundance and characterize their functions. Several proteins involved in the catalytic activity and biological processes were identified in the *C. accolens* proteome with notable strain level variation in abundance (Figure 4.6). For example, a putative esterase involved in the trehalose O-mycolyltransferase activity (GO: 0050348) was found in strains C781, C785 and C779 at a higher relative abundance of 48.2%, 34.5% and 31.2%, respectively when compared to other strains. In addition, glycosyl hydrolase family 25 proteins involved in the peptidoglycan catabolic process (GO: 0009253) was expressed by strain C778, C779, C781, C782 and C785 at high relative abundance (from 10.5% to 19.0%) but also found at lower relative abundance (3.0%) in strain C787. Another protein involved in the peptidoglycan catabolic process such as N-acetylmuramoyl-L-alanine amidase was also identified in strain C781 with a higher relative abundance (>4.0%) compared to other strains C778, C779, C781 and C782 with a lower relative abundance (< 4.0%). Putative monovalent cation involved in oxidoreductase activity (GO: 0016491) was identified at higher relative abundance (25.5%) only in C779 and C787 strains compared to other strains (<1.8% relative abundance). Furthermore, the relative abundance of ErfK/YbiS/YcfS/YnhG protein involved in peptidoglycan biosynthetic process (GO: 0009252) was found to be higher in strain C782 (11.4%) and C787 (14.5%) compared to the other strains. However, there was no significant difference in relative abundance of most common proteins including chaperone protein DnaK, dihydrolipoyl dehydrogenase, EF-Tu, enolase, putative ribosomal protein S1 and cysteine synthase identified among the six *C. accolens* strains. In fact, members of each of these protein families have been found to be 'moonlighting proteins'. This proteins comprise a subset of multifunctional proteins in which one polypeptide chain exhibits more than one physiologically relevant biochemical or biophysical function [290].

While moonlighting functions vary among microbial species there is a common theme for roles in adherence and in immune regulation. In many instances, DnaK were identified as cell wall associated or surface located in different bacterial species with and without pathogenic properties. Furthermore, moonlighting functions for EF-Tu are associated with a role in adherence to a range of host molecules and host cells [291].

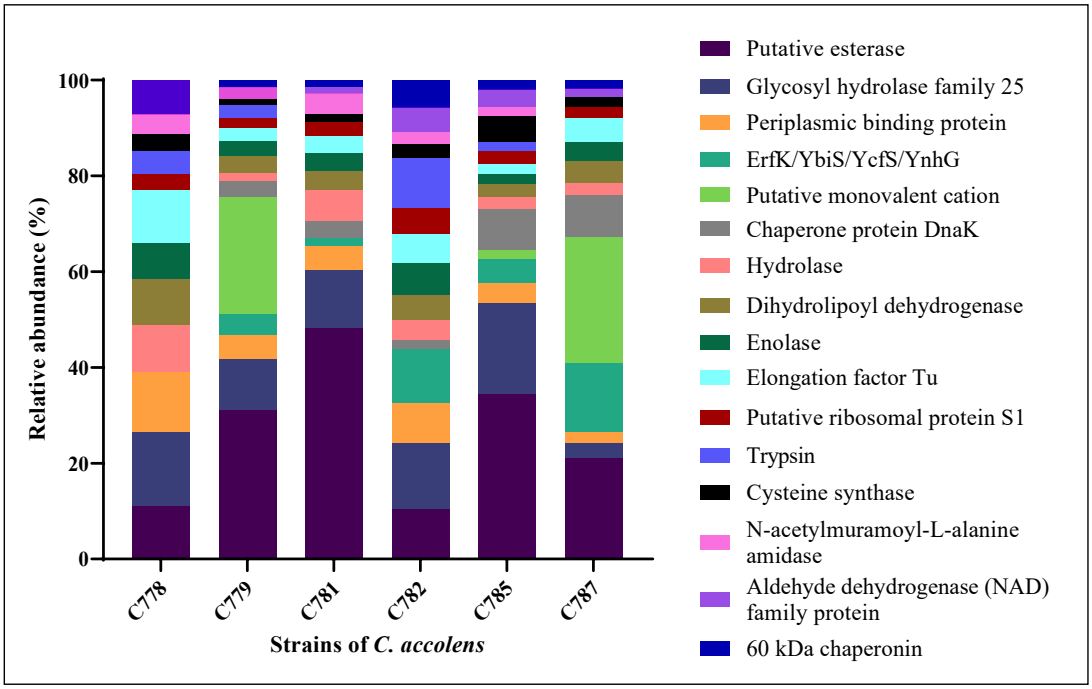


Figure 4.6. Comparative analysis of proteins commonly identified in six *C. accolens* strains (C778, C779, C781, C782, C785, and C787), top 25 most abundant proteins that are involved in molecular function and biological processes. Coloured bars represent the relative abundance of an individual protein as a percentage of the total proteins commonly present in each strain.

4.6 Discussion

This paper reports the application of a proteomic approach to identify the extracellular bioactive products or key proteins involved in the antimicrobial activity of *C. accolens* clinical isolates against *S. aureus*. *Corynebacteria* have been the focus of much recent attention due to their ability to exhibit an antimicrobial action against common nasal pathogens including *S.*

aureus [200]. In this study, the secreted protein extracts of 6 *C. accolens* strains isolated from healthy nasal cavities were used for antimicrobial protein identification and functional characterization, as the presence of proteinaceous antimicrobial compounds in these strains has been previously demonstrated in an earlier study published from our department [278]. Our findings confirm strong antimicrobial properties of *C. accolens* exoproteins from all 6 tested strains against representative *S. aureus* and MRSA strains. From 595 commonly expressed *C. accolens* proteins, the abundance of a total of 14 proteins was positively correlated with *S. aureus* growth inhibition and the strongest significant positive correlation was detected for an Acetyltransferase, GNAT family protein followed by Cell envelope-like function transcriptional attenuator common domain protein, Septum-form domain-containing protein, Glycosyl hydrolase family 3 N-terminal domain protein, and Putative monovalent cation/H+ antiporter subunit C.

Probiotic bacteria exert their functions in different ways, among which antimicrobial activity is suggested to be one of the most important requirements in reducing colonization and infection by pathogens [194, 292]. Here we confirmed that protein extracts from *C. accolens* strains C779, C781, and C787 demonstrated a strong antibacterial effect (up to 87% growth inhibition) and strains C778, C782, and C785 showed a moderate antibacterial effect (up to 58% growth inhibition) against *S. aureus* and MRSA clinical isolates, which were likely due to secreted proteins or peptides. There is an increasing number of reports on the identification and characterization of some other probiotic isolates involved in the production of natural antibiotic-like molecules that directly kill or inhibit the growth of pathogenic microorganisms [293, 294]. However, this is the first comprehensive data set to our knowledge of common proteins expressed by *C. accolens* strains in relation to their antibacterial activity and their functional classification using proteomics analysis.

The analysis of the *C. accolens* proteome in this study identified 1455 distinct proteins across the six strains, of which 595 were common to all strains. Pearson correlation analysis between the *C. accolens* commonly expressed abundant proteins and the growth inhibition of *S. aureus* identified Acetyltransferase, GNAT family protein as a candidate protein involved in the anti-staphylococcal effects. This protein belongs to an important family of proteins and plays a variety of anabolic and catabolic roles in both prokaryotes and eukaryotes. The protein promotes acetylation affecting a large number of substrates, from small molecules such as aminoglycoside antibiotics to macromolecules and produces an essential metabolite, UDP-*N*-acetylglucosamine (UDP-GlcNAc) [295, 296]. However, as for the majority of the identifiable bacterial GNATs, the present *C. accolens* GNAT protein's biochemical function is not known and in particular, a potential role in effecting antistaphylococcal activity requires further study.

Furthermore, a greater proportion (90%) of common proteins from the top 25 most abundant identified in *C. accolens* were involved in the biological process's category. This included proteins involved in metabolic processes (seventeen proteins), cell organization and biogenesis (one protein), cellular homeostasis and regulation of biological processes (one protein) as well as six proteins of unknown function. In fact, biological processes are vital for the life of an organism and contribute to cellular maintenance [297]. This report provided detailed functional proteomic data regarding the most common abundant proteins identified in *C. accolens*.

Several studies suggest that proteomics analysis is a valuable tool to identify distinctive traits of beneficial bacterial strains and their function, to select potential probiotic strains for clinical application [298]. In our study, a putative esterase was identified as the most common abundant protein in *C. accolens*. Putative esterase is known to be a crucial enzyme found in humans and bacteria with a primary role for cleaving ester bonds [299]. In addition, this enzyme helps in the hydrolysis of triacylglycerol and xenobiotic detoxification, and is utilized in a variety of

applications including drug therapy [300]. Wall et al. reported that putative esterase expressed from a commensal lactic acid bacterium *Lactobacillus reuteri*, was involved in altering the cell wall and thus increasing the cells tolerance towards harsh environmental conditions [301]. This protein is therefore likely to be an important player in managing the survival of the bacteria in the sinonasal tract.

Other proteins such as chaperone protein DnaK and 60 kDa chaperonin, involved in protein folding (GO: 0006457) and ATP binding (GO: 0005524) were found in the five *C. accolens* strains (C779, C781, C782, C785 and C787), with a relative abundance of 1.5% to 9.0%. The expression of these proteins is generally species and strain dependent [291], and plays numerous roles in probiotic bacteria including proper structural folding, synthesis, and stabilization [302]. In addition, they have been associated with the adhesion process of probiotic bacteria such as *Lactobacillus helveticus* T159 [303]. Another essential protein identified is elongation factor Tu which functions in translation elongation (GO: 0003746) during polypeptide synthesis. Evidence has revealed that elongation factor Tu expressed from different probiotic *Lactobacillus* species plays a role in the competitive exclusion of pathogenic bacteria such as *Enterococcus faecalis and* mediates the attachment of bacteria to mucins, Caco-2 cells and human intestinal cells [290, 304]. Thus the presence of chaperone protein DnaK, 60 kDa chaperonin and elongation factor Tu proteins in most of the *C. accolens* strains in our study indicates their importance for proper protein biosynthesis and may also be involved in the attachment process to the human nasal epithelial cells.

The bacterial cell surface is the first physical barrier to defend cells from the external environmental. In the present study, ErfK/YbiS/YcfS/YnhG protein involved in the peptidoglycan biosynthetic process was found in most *C. accolens* strains tested except in strain C778. Changes in the expression of peptidoglycan synthesis enzymes were seen when

strains of *Bifidobacterium animalis* ssp. *lactis* were exposed to stress conditions and thus resulted in alteration of the cell surface compositions and probiotic properties [305]. We have also identified a putative monovalent cation involved in oxidoreductase activity (GO: 0016491) that showed more than 25% relative abundance in two *C. accolens* strains, C779 and C787. The main function of this protein is to catalyze the oxidation of one compound with the reduction of another in a catalytic role, and conformational change during catalytic turnover [306]

Although several proteins were commonly identified in the *C. accolens* proteomes, some proteins such as glycosyl hydrolase family 25 and N-acetylmuramoyl-L-alanine amidase were functionally linked to bacterial cell wall degradation (GO:0009253) and defence response to gram-positive bacterium (GO:0050830). Glycosyl hydrolase family 25, also called O-Glycosyl hydrolases, comprises enzymes with known lysozyme activity that hydrolyse the β-1, 4-glycosidic bond between *N*-acetylmuramic acid (NAM) and *N*-acetylglucosamine (NAG) residues in the peptidoglycan found in the bacterial cell wall [307]. Previously, the lytic properties of this enzyme as an antimicrobial agent were studied *in vitro* and *in vivo*, and resulted in the effective prevention and elimination of *S. pneumoniae* upper respiratory tract colonization, demonstrating a remarkable specificity against medically important drug resistant pathogens [308, 309]. Therefore, glycosyl hydrolase family 25 identified in *C. accolens* strains may be one of the candidates as an antibacterial agent as all strains demonstrated antibacterial activity toward *S. aureus* and MRSA pathogens.

The other protein, which is known to be involved in the degradation of bacterial peptidoglycan, N-acetylmuramoyl-L-alanine amidase, was also identified in all *C. accolens* strains. This protein has been investigated as a potential antimicrobial agent that resulted in the breakdown of the peptidoglycan layer found in both gram positive and gram negative bacterial cell walls,

reducing the occurrence of undesirable microorganisms [307, 310]. It is generally accepted that several probiotic bacteria including commensal *C. accolens* isolates are able to release bioactive compounds to inhibit pathogens and maintain the healthy homeostasis [127]. Therefore, the antimicrobial effects elicited by all *C. accolens* strains in this study, might be due to the common expression of glycosyl hydrolase family 25 and N-acetylmuramoyl-L-alanine amidase antimicrobial proteins.

Taken together, these findings provide novel insights into the identification of proteins that play a crucial role in the antimicrobial potential and other probiotic properties of *C. accolens* isolates, and hold great promise for the selection of an ideal probiotic candidate for clinical application in CRS-associated microbiome dysbiosis.

4.7 Conclusion

This study opens the way for the use of a proteomics approach for preliminary selection and development of *C. accolens* probiotic therapy through a focused approach. Expression of Acetyltransferase, GNAT family protein in *C. accolens* strains with strong antibacterial effects was higher than in strains with moderate antibacterial effect and its abundance was correlated with the observed antistaphylococcal effects. Proteins such as glycosyl hydrolase family 25 and N-acetylmuramoyl-L-alanine amidase could clearly be linked to the observed antibacterial activity in nasal commensal *C. accolens* strains. Furthermore, putative esterase, the most common abundant protein that likely contributes to bacterial survival, and chaperone protein DnaK, 60 kDa chaperonin and EF-Tu proteins, that potentially act as adhesion promoting factors were identified. In the future, intensive in vivo studies will be carried out to explore the novel health promoting role of *C. accolens* isolates for the successful treatment of CRS.

CHAPTER 5

In vitro and *in vivo* evaluation of probiotic properties of *Corynebacterium accolens* isolated from the human nasal cavity

CHAPTER 5: *In vitro* and *in vivo* evaluation of probiotic properties of *Corynebacterium accolens* isolated from the human nasal cavity

This chapter is a published research article that addresses the fourth research aim designed to evaluate the probiotic potential of *Corynebacterium accolens* strains' safety and efficacy using a combination of *in vitro* and *in vivo* approaches.

Menberu MA, Cooksley C, Ramezanpour M, Bouras G, Wormald PJ, Psaltis AJ, Vreugde S. *In vitro* and *in vivo* evaluation of probiotic properties of *Corynebacterium accolens* isolated from the human nasal cavity. *Microbiological research*. 2022; 255:126927.

5.1 Statement of Authorship

Title of Paper	*In vitro* and *in vivo* evaluation of probiotic properties of *Corynebacterium accolens* isolated from the human nasal cavity
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Menberu MA, Cooksley C, Ramezanpour M, Bouras G, Wormald PJ, Psaltis AJ, Vreugde S. *In vitro* and *in vivo* evaluation of probiotic properties of *Corynebacterium accolens* isolated from the human nasal cavity. Microbiological Research. 2022; 255:126927.

Principal Author

Name of Principal Author (Candidate)	Martha Alemayehu Menberu		
Contribution to the Paper	Conception and design of the project, acquisition of data, analysis and interpretation of data, drafting the article		
Overall percentage (%)	85		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	21/10/2021

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Clare Cooksley		
Contribution to the Paper	Acquisition of data, interpretation of data, critically revising the article		
Signature		Date	19/10/2021

Name of Co-Author	Mahnaz Ramezanpour		
Contribution to the Paper	Acquisition of data, analysis and interpretation of data, manuscript edition		
Signature		Date	19/10/2021

Name of Co-Author	George Bouras		
Contribution to the Paper	Analysis and interpretation of data, manuscript edition		
Signature		Date	19/10/2021

Name of Co-Author	Peter-John Wormald		
Contribution to the Paper	Conception of the project, critically revising the article		
Signature		Date	18/10/2021

Name of Co-Author	Alkis James Psaltis		
Contribution to the Paper	Conception of the project, critically revising the article		
Signature		Date	18/10/2021

Name of Co-Author	Sarah Vreugde		
Contribution to the Paper	Conception and design of the project, critically revising the article		
Signature		Date	18/10/2021

5.2 Publication: *In vitro* and *in vivo* evaluation of probiotic properties of *Corynebacterium accolens* isolated from the human nasal cavity

Microbiological Research 255 (2022) 126927

Contents lists available at ScienceDirect

Microbiological Research

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

In vitro and *in vivo* evaluation of probiotic properties of *Corynebacterium accolens* isolated from the human nasal cavity

Martha Alemayehu Menberu [a,b], Clare Cooksley [a], Mahnaz Ramezanpour [a], George Bouras [a], Peter-John Wormald [a], Alkis James Psaltis [a], Sarah Vreugde [a,*]

[a] Department of Surgery-Otolaryngology, Head and Neck Surgery, The University of Adelaide, Basil Hetzel Institute for Translational Health Research, Central Adelaide Local Health Network, Woodville, SA, Australia

[b] Department of Medical Microbiology, School of Biomedical and Laboratory Sciences, College of Medicine and Health Sciences, University of Gondar, Ethiopia

ARTICLE INFO

Keywords:
Probiotic
Corynebacteriumaccolens
Human nasal cavity
Dysbiosis
Chronic rhinosinusitis

ABSTRACT

Corynebacterium accolens strains are increasingly recognized as beneficial bacteria that can confer a health benefit on the host. In the current study, the probiotic potential of three *C. accolens* strains, C779, C781 and C787 derived from a healthy human nasal cavity were investigated. These strains were examined for their adhesion to HNECs, competition with *Staphylococcus aureus* for adhesion, toxicity, induction of IL-6, antibiotic susceptibility and the presence of antibiotic resistance and virulence genes. Furthermore, the safety and efficacy of strains were evaluated *in vivo* using *Caenorhabditis elegans*. The adhesion capacity of *C. accolens* to HNECs was strain-dependent. Highest adhesion was observed for strain C781. None of the *C. accolens* strains tested caused cell lysis. All strains were able to outcompete *S. aureus* for cell adhesion and caused a significant decrease of IL-6 production by HNECs co-exposed to *S. aureus* when compared to the control groups. All strains were sensitive or showed intermediate sensitivity to 10 different antibiotics. Whole Genome Sequence analysis showed *C. accolens* C781 and C787 did not possess antibiotic resistance genes whereas strain C779 harboured 5 genes associated with resistance to Aminoglycoside, Chloramphenicol and Erythromycin. In addition, no virulence genes were detected in any of the 3 strains. Moreover, the tested strains had no detrimental effect on worm survival and induced protection from *S. aureus*-mediated infection. Taken all together, *C. accolens* strains, C781 and C787 displayed probiotic potential and hold promise for use in clinical applications for combating dysbiosis in chronic rhinosinusitis.

1. Introduction

In recent years, imbalance of nasal microbiota or dysbiosis has been linked to chronic mucosal inflammatory diseases such as Chronic Rhinosinusitis (CRS) (Bordin et al., 2016; Hoggard et al., 2017). This notion has increased a search for the identification and selection of potentially probiotic bacteria that exert a beneficial health effect in maintaining nasal homeostasis and immune modulation (Rutten et al., 2011; Schwartz et al., 2016). According to the World Health Organization (WHO), probiotics are defined as viable, non-pathogenic micro-organisms which when administered in certain numbers confer a health benefit on the host (Hotel and Cordoba, 2001).

The beneficial effects of probiotics in a variety of inflammatory diseases including CRS have been previously reported (Perrin et al., 2014; Roos et al., 2011; Marchisio et al., 2015; Mårtensson et al., 2017). Probiotic application may improve the healthy microbial composition within the sinonasal microbiota and enhance the immune responses through competitive exclusion as well as antagonistic action against pathogens (Brugger et al., 2016). Commensal nasal bacteria from the genus *Corynebacterium* are thought to be the most dominant members of the human nasal microbiota and known to have beneficial effects in the host. It has been demonstrated that *Corynebacterium accolens* can reduce the growth of *Staphylococcus aureus,* Methicillin-Resistant *S. aureus* (MRSA) and *Streptococcus pneumoniae* nasal pathogens *in- vitro* (Menberu et al., 2021; Bomar et al., 2016). This reduction may be due to the production of toxic compounds (Hardy et al., 2019), inactivation of expression of the accessory gene regulator (*agr*) quorum sensing system of pathogens (Ramsey et al., 2016), ability to hydrolyse and release

* Corresponding author at: Department of Otolaryngology, Head and Neck Surgery, Basil Hetzel Institute for Translational Health Research, The Queen Elizabeth Hospital, 28 Woodville Road, Woodville South, SA, 5011, Australia.
E-mail address: sarah.vreugde@adelaide.edu.au (S. Vreugde).

https://doi.org/10.1016/j.micres.2021.126927
Received 23 July 2021; Received in revised form 26 October 2021; Accepted 22 November 2021
Available online 23 November 2021

antibacterial free fatty acids (Bomar et al., 2016), and secretion of proteinaceous antimicrobial substances (Menberu et al., 2021). *S. aureus* is the most common pathogenic component of the CRS microbiome and is associated with an increased disease severity, higher rate of revision surgery and poorer postoperative outcomes (Feazel et al., 2012; Cleland et al., 2013). Moreover, biofilm-mediated diseases due to *S. aureus* are also linked to a more severe and recalcitrant outcome in patients with CRS (Foreman et al., 2009).

In recent times, beneficial bacteria or probiotics have been recommended as preventive and/or adjunctive treatment alternatives to antibiotics for patients with a dysbiotic microbiota in CRS (Psaltis and Wormald, 2017; Mukerji et al., 2009). The mechanisms underlying the health-promoting effects of probiotic bacteria to restore sinus health are not elucidated in detail, and consideration has been given to their ability to counter pathogens by producing antibacterial peptides and other antibacterial metabolites, competing for cell surface receptors to inhibit adherence of pathogens, and starve the pathogens for nutrients (Stubbendieck and Straight, 2016). In the case of the gut microbiota, there is clear evidence that the commensal probiotic bacteria have direct action on epithelial cells, maintaining the strength of tight junctions, decreasing pro-inflammatory cytokine production, preventing programmed cell death and interacting with lymphocytes with an increased release of anti-inflammatory cytokines (Wells et al., 2017; Wieërs et al., 2020).

The probiotic functional capacity greatly varies among strains belonging to the same genera (Myles et al., 2018). In order to consider certain bacteria for use as probiotics, candidate strains must be able to demonstrate antagonistic activity against pathogens. These properties could prove very useful in screening for probiotic products (Cleland et al., 2014; De Boeck et al., 2021). Apart from this, successful probiotic bacteria for the treatment of CRS should be able to colonize the nasal cavity by adhering to the nasal epithelium, prevent the attachment of pathogens and stimulate their removal from the infected sinonasal tract (Cervin, 2018). Another important trait to be proven, in order to address safety concerns, is the absence of antibiotic resistance determinants (Imperial and Ibana, 2016). Furthermore, an *in vivo Caenorhabditis elegans* (*C. elegans*) model represents a suitable and inexpensive screening method to study the health-promoting traits of probiotic strains. An increasing number of studies have used this model and have shown that consumption of potential probiotic bacteria can extend the lifespan of nematodes and alter host defence mechanisms (Guantario et al., 2018; Sim et al., 2018).

The antimicrobial ability of *C. accolens* strains isolated from the human nasal cavity have been reported to have beneficial health effects, particularly related to the growth inhibition of *S. aureus* pathogens obtained from CRS patients (Menberu et al., 2021). However, other probiotic characteristics, functional properties and safety aspects in those strains have not yet been described. Therefore, this study aims to evaluate the probiotic potential of *C. accolens* strains' safety and efficacy using a combination of *in vitro* and *in vivo* approaches.

2. Methods

2.1. Bacterial strains and collection of human nasal epithelial cells

Ethics approval for the collection, storage and use of clinical isolates and primary human nasal epithelial cells (HNECs) from both CRS and non-CRS patients was granted by The Queen Elizabeth Hospital (TQEH) Human Research Ethics Committee in Adelaide, South Australia (HREC/15/TQEH/132). Three *C. accolens* clinical isolates (C779, C781 and C787), previously isolated from the nasal cavity of non-CRS subjects, were used in this study (Menberu et al., 2021). The identification of *C. accolens* was based on culture morphology and colony size determination, Analytical Profile Index (API) Coryne test system (BioMèrieux NSW, Australia) and a molecular method using Polymerase Chain Reaction (PCR) amplification and sequencing of the *rpoB* gene (Menberu et al., 2021). *S. aureus* clinical strains, Methicillin- sensitive *S. aureus* (MSSA) C26 and MRSA C261, isolated from the nasal swabs of CRS patients and a laboratory reference strain, *S. aureus* ATCC 25923 (American Type Culture Collection, Manassas, USA) were used as test pathogens.

2.2. Primary human nasal epithelial cell harvesting and culture

HNECs were collected from the inferior turbinate of patients with CRS during surgery using cytology brushes (Medico, Melbourne, Australia) as described previously (Ramezanpour et al., 2019, 2016) The nasal brushings were transported using basal medium (PneumaCult Ex Plus; STEMCELL Technologies, Cambridge, UK) and then processing of the cells from the brushes was performed after centrifugation at 525 x *g* for 7 min. at 4 °C and suspending in 2 mL of complete PneumaCult-Ex Plus medium supplemented with 2% PneumaCult-Ex Plus 50x supplement, 50 units/mL penicillin, 50 µg/mL streptomycin, and 2.5 µg/mL amphotericin B (Invitrogen, Gaithersburg, MD, USA). The pellet was resuspended using a 2-ml syringe to create a single-cell suspension in a 100-mm diameter petri dish coated with anti-CD68 antibody (Dako, Glostrup, Denmark) for 20 min. to deplete the cell suspension of macrophages. Following this, HNECs were seeded on type 1 collagen-coated T25 flasks (Thermo Fisher Scientific, Waltham, MA, USA) and incubated at 37 °C in a 5% CO_2 atmosphere in a humidified incubator for approximately 5–7 days until confluent.

2.3. Adhesion capacity of C. accolens to primary HNECs

The adhesion of *C. accolens* strains, C779, C781 and C787 to primary HNECs was assessed based on the method according to Rajoka et al. (Rajoka et al., 2018) with modifications. To determine the adhesive ability of the *C. accolens* strains, HNECs (0.2×10^6 cells/mL) were seeded on a 12-well culture plate (Greiner bio-one Cell Star, Germany) and grown at 37 °C in a 5% CO_2 atmosphere until the cells were fully confluent. The overnight cultures of *C. accolens* strains were harvested by centrifugation and re-suspended in antibiotic-free Ex-plus cell culture medium to an appropriate dilution [approximately 1×10^6 colony forming units (CFU)/mL]. After that, the HNECs were challenged with each *C. accolens* strain for 2 h at 37 °C, 5% CO_2 atmosphere. After incubation, the cells were washed twice with 1x phosphate-buffer saline (PBS) to remove non-adherent bacteria and lysed with 1% Triton X-100 solution for 15 min. incubation with gentle agitation. The cell lysates were then serially diluted and plated onto Tryptone Soya Agar (TSA) plates supplemented with 0.8 % Tween 80 to determine the number of adherent bacteria. The plates were incubated at 37 °C for 48 h. The percentage of bacterial adhesion was calculated as follows: Adhesion % = (adhered bacteria/total added bacteria) × 100.

2.4. Inhibitory effect of C. accolens on pathogenic S. aureus adhesion to primary HNECs

The inhibitory effect of the *C. accolens* strains (C779, C781 and C787) on pathogenic *S. aureus* adhesion was evaluated using a competition assay, as previously described (Guantario et al., 2018) with a few modifications. In brief, HNECs were co-treated with each *C. accolens* strain (1×10^6 CFU/mL) and an equal number of MSSA C26, MRSA C261, and *S. aureus* ATCC 25923 (1×10^6 CFU/mL) in antibiotic-free PneumaCult-Ex Plus medium for 2 h at 37 °C with 5% CO_2. After incubation, the HNECs were washed twice with 1x PBS and lysed by the addition of 1% Triton X-100 for 15 min. with gentle agitation. Adhering viable cells of each *S. aureus* and *C. accolens* strain were quantified by plating serial dilutions of HNEC lysates onto 0.8 % Tween 80 supplemented TSA plates for *C. accolens* and *S. aureus* selective Mannitol Salt Agar plates (Oxoid, Basingstoke, UK) followed by incubating for 24 –48 hrs at 37°c to count viable cells.

2.5. Cytotoxicity studies

HNEC-monolayers grown on 12-well tissue culture collagen-coated plates (Greiner bio-one Cell Star, Germany) were first treated with different strains of *C. accolens*, *S. aureus* and a combination of *C. accolens* and *S. aureus* at approximately 1 $\times 10^6$ CFU/mL concentration each, followed by incubation at 37 °C for 2 h. Lactate dehydrogenase (LDH) release was measured as an index of cytotoxicity from the supernatants, using a Cytotoxicity Detection Kit (Promega, Madison, USA) to determine cell viability. In brief, 50 µL of the supernatant from each sample was transferred to a 96 well polystyrene plate (Sigma-Aldrich, USA), and 50 µL of LDH reagent was added to the supernatant and incubated for 30 min. in the dark at room temperature. The maximal LDH release of cells was determined by addition of lysis solution (10 % Triton X-100). The background level (0% LDH release) was determined with antibiotic free Ex-plus culture medium. The absorbance values of prepared samples were recorded at 490 nm on a FLUOstar Optima plate reader (BMG Labtech, Ortenberg, Germany), and relative viability was calculated relative to the total LDH levels of negative controls (untreated cells).

2.6. Quantification of IL-6 protein level

Interleukin-6 (IL-6) protein level was measured from the supernatant of HNEC monolayers cultures after 2 h exposure to 1 $\times 10^6$ CFU/mL *C. accolens*, *S. aureus* and a combination of *C. accolens* and *S. aureus* isolates. Enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences, Franklin Lakes, NJ, USA) for IL-6 was used according to the manufacturer's instructions. The absorbance was read at 450 nm using a FLUOstar OPTIMA plate reader (BMG Labtech, Ortenberg, Germany). All measurements were carried out in duplicate and results were expressed in pg/mL. A standard curve was used to calculate for protein concentration (detection range from 800 pg/ml to 12.5 pg/mL).

2.7. Antibiotic susceptibility assay

The antibiotic susceptibility of *C. accolens* isolates was assessed using the disk diffusion method as described previously (Pennekamp et al., 1996), with some modifications. The antibiotics tested were Ciprofloxacin (CIP, 5 µg/disk), Gentamicin (CN, 10 µg/disk), Erythromycin (E, 15 µg/disk), Tetracycline (TE, 30 µg/disk), Rifampicin (RD, 5 µg/disk), Vancomycin (VA, 5 µg/disk), Imipenem (IPM, 10 µg/disk), Cefuroxime Sodium (CXM, 30 µg/disk), Sulphamethoxazole/Trimethoprim (SXT, 25 µg/disk) and Penicillin G (P, 5 Units/disk). Antibiotic discs were obtained from Oxoid (Basingstoke, UK). The broth cultures of *C. accolens* isolates were first prepared in TSB for 48 h and adjusted to 0.5 McFarland standards (equivalent to 1×10^8 CFU ml^{-1}). Then, 100 µl of freshly prepared bacterial suspensions were spread onto Muller Hinton Agar (MHA) supplemented with 0.8 % Tween 80. The antibiotic discs were placed on the surface of the agar and the plates were incubated aerobically at 37 °C with 5% CO_2 for 24 –48 hrs. The diameter of inhibition zone was measured from the centre of the disk and, the susceptibility of isolates was scored as resistant, intermediary susceptible, or susceptible, according to the EUCAST clinical breakpoints for *Corynebacterium* spp. (EUCAST, 2015), and the disk diffusion cut-off values suggested by Barberis et al. (Barberis et al., 2018). Each experiment was performed in triplicate.

2.8. Whole genome sequencing (WGS), assembly and analysis

Pure cultures from the three *C. accolens* strains, C779, C781 and C787 were subjected to WGS as described previously (Hasman et al., 2014). WGS was performed *via* a commercial partner (SAPathology Partners, Adelaide, Australia). The isolates were grown on TSA (Sigma-Aldrich) plates supplemented with 0.8 % Tween 80 at 37 °C for 48 h. Thereafter, genomic DNA was extracted using the MN NucleoSpin®Microbial DNA (Machery-Nagel GmbH and Co.KG, Duren, Germany). Sequencing libraries were prepared using a modified protocol for the Nextera XT DNA library preparation kit with 150 bp paired end reads (Illumina Inc., San Diego, Ca, USA). Briefly, genomic DNA was fragmented, followed by the amplification of Nextera XT indices (Illumina Inc., San Diego, CA, USA) to the DNA fragments using a low-cycle PCR reaction. Subsequently, the amplicon library was manually purified and normalised. Sequencing was performed on the Illumina NextSeq 550 platform with NextSeq 500/550 Mid-Output kit v2.5 (Illumina Inc., San Diego, Ca, USA). Quality checks were conducted on the raw FASTQ reads using FASTQC (Andrews, 2010). Following this, the genomes were assembled using Unicycler v 0.4.8 (Wick et al., 2017). The assembled genomes were then run through TYGS, the Type Strain Genome Server, in order to confirm that the sequenced reads were *C. accolens* (Meier--Kolthoff and Göker, 2019).

Following this, the presence of antibiotic resistance genes and virulence factors in the 3 *C. accolens* genomes was identified by searching the comprehensive antibiotic resistance database (CARD) (http://arpcard.mcmaster.ca/) and virulence factors database (VFDB) (http://www.mgc.ac.cn/VFs/main.htm), respectively using abricate (https://github.com/tseemann/abricate) (Seemann, 2019). BLAST analysis against the nucleotide (NT) database was used to investigate potential extrachromosomal components of the assembled genomes (Altschul et al., 1990; Information 1988).

Furthermore, comparative genomic analysis was undertaken between all 3 *C. accolens* strains. Roary v 3.13.0 was used to construct the pangenome (Page et al., 2015), while Snippy v 4.6.0 was used to identify variants compared to the known reference strain, ATCC 49725 (Seemann, 2015). Snippy was also used to identify pairwise variants between the 3 isolates.

2.9. Genome accession numbers

The whole genome sequences for 3 *C. accolens* isolates have been deposited in GenBank under the following accession numbers: JAHWRA000000000 (*C. accolens* C779), JAHWQZ000000000 (*C. accolens* C781) and JAHWQY000000000 (*C. accolens* C787).

2.10. In vivo safety studies in C. elegans

To assess the safety of *C. accolens* nasal isolates, an *in vivo* toxicity assessment in *C. elegans* AU37 (glp-4; sek-1) was carried out as previously described (Sharma et al., 2019) with minor modifications. Eggs of *C. elegans* were first isolated from mature adults using a hypochlorite solution (5% Sodium hypochlorite and 4 M NaOH) and allowed to hatch on fresh Nematode Growth Medium (NGM) agar plates seeded with *Escherichia coli* strain OP50 as a food source (Hunt, 2017) in order to obtain a synchronized *C. elegans* population. The synchronized nematodes at fourth larval (L4) stage were then collected in OGM medium containing 95 % M9 buffer, 5% brain heart infusion broth (Oxoid, Basingstoke, UK), 10 µg/mL cholesterol (Sigma-Aldrich). Next, 20 nematodes were exposed to 25 µl of a 48 -h bacterial culture from *C. accolens* strains (C779, C781 and C787) adjusted to 1 $\times 10^6$ CFU/mL in OGM medium using a sterile 96-well microtiter plate (Life Sciences, USA), and incubated at 25 °C for 24, 48, and 72 h time intervals. Uninfected nematodes in OGM medium were used as a control group. The number of viable and dead nematodes was counted daily using a light microscope at 40x magnification, and the percentage of surviving worms was calculated by the following formula: survival (%) (live worms/total worms used)* 100. A worm was considered dead when it failed to move or respond to touch. The experiment was performed in triplicate.

2.11. In vivo efficacy in a C. elegans infection model

To examine the efficacy of *C. accolens*, synchronized nematodes, *C. elegans* AU37 (*glp-4*; *sek-1*), were first grown to L4 stage, suspended in

OGM medium and added into 96-well plates with at least 20 worms per well (Richter et al., 2017). Nematodes were then infected with 25 µl of an overnight culture of *S. aureus* isolates (MSSA C26, MRSA C261 and ATCC 25,923) adjusted to 2×10^8CFU/mL in OGM medium and exposed to 25 µl of treatment with *C. accolens* strains (C779, C781 and C787), each with 1×10^6 CFU/mL concentration. Uninfected nematodes in OGM medium as well as infected but untreated nematodes were used as controls. The number of viable and dead nematodes was assessed every 24 h over 3 days incubation at 25 °C. Subsequently, the bacterial load per worm was determined at day 3. Briefly, worms were thoroughly washed 3 times with M9 buffer containing 1 mM sodium azide and in 1xPBS for the removal of excess of the bacteria attached to the surface of the nematodes. Next, nematodes were mechanically disrupted by vortexing the worms in microtubes with 1.0 mm silicon carbide beads for 10 min (Daintree Scientific, TAS, Australia). Whole worm lysates were serially diluted and plated onto TSA supplemented with 7% NaCl and incubated overnight at 37 °C aerobically for counting CFUs of respective isolates. Each experiment was performed in triplicate.

2.12. Statistical analysis

The statistical analyses were performed with GraphPad prism 9.0.0 software. All data are presented as mean±standard error mean (SEM) of three experiments with duplicate or triplicate measurements. The results from *in vitro* experiments were analysed using one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test for adhesion assay and with Dunnett's multiple comparisons test for adhesion competition, cytotoxicity and IL-6 quantification assays. The results from *in vivo* experiments in *C. elegans* were evaluated using one-way ANOVA with Tukey's multiple comparisons test. In each assay, results were compared with an appropriate control group. Differences with *p*-values < 0.05 were considered significant.

3. Results

3.1. Adhesion of C. accolens to HNECs

To confirm the adhesion capacity of the 3 *C. accolens* strains to nasal epithelium, we used an *in vitro* model of HNECs from CRS patients. *C. accolens* strains were able to adhere to HNECs with a variable adhesion ability after 2 h incubation (Fig. 1). Among the tested strains, *C. accolens* C779 and C781 possessed a strong adhesion capacity (63 %) and (70 %), respectively to HNECs. Strain C787 displayed a lower (50 %) adhesive capacity in comparison with strains C779 ($p < 0.01$) and C781 ($p < 0.0001$).

3.2. Reduction of S. aureus adhesion to HNECs in the presence of C. accolens nasal isolates

We next determined the capability of the *C. accolens* strains to inhibit the adhesion of *S. aureus* isolates derived from CRS patients. All *C. accolens* and *S. aureus* isolates diluted in antibiotic-free Ex-plus media were able to grow on their respective selective agar (data not shown). In the absence of *C. accolens*, all 3 *S. aureus* strains tested had strong adherence to HNECs of >80 %. Co-incubation with all of the *C. accolens* strains tested significantly inhibited the adhesion of *S. aureus* strains, with a significant reduction in adhesion of MSSA C26 (from 34 to 44%) **(**Fig. 2A**)**, MRSA C261 (from 24 to 31%) **(**Fig. 2B**)** and ATCC 25923 (from 41 to 50%) **(**Fig. 2C**)** to HNECs when compared to controls (*S. aureus* alone) ($p < 0.0001$).

3.3. C. accolens strains are not toxic to HNEC monolayers after 2 h of exposure

Cell viability was assessed by measuring LDH release from HNEC-monolayers derived from CRS patients. A 2 -h exposure to *C. accolens*

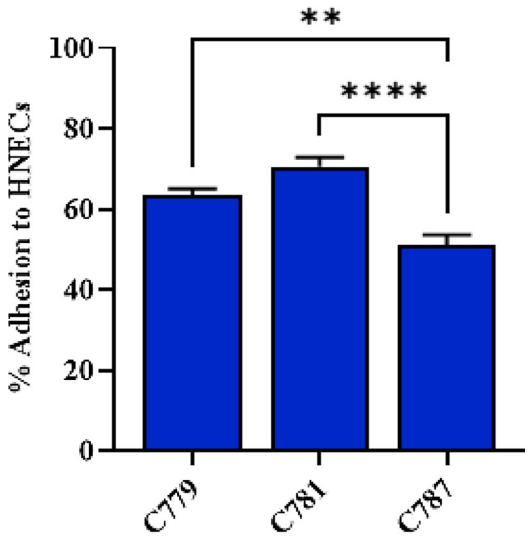


Fig. 1. *C. accolens* adhesion to primary HNECs. The percentage of viable *C. accolens* strains C779, C781, and C787 adhering to HNECs from an initial inoculum of 1×10^6 CFU/mL. Columns represent the mean ± SEM of two independent experiments, each performed in triplicate. **$p < 0.01$; **** $p < 0.0001$; ns, no significant difference, ANOVA, followed by Tukey's multiple comparisons test. CFU, colony forming units; SEM, standard error of the means.

C779, C781 and C787 at 1×10^6 CFU/mL showed no significant increase in LDH release in HNECs from patients with CRS **(**Fig. 3**A)** ($p > 0.05$). In contrast, exposure with all 3 *S. aureus,* MSSA C26, MRSA C261 and ATCC 25,923, at 1×10^6 CFU/mL for 2 h significantly reduced the viability of HNEC monolayer cultures compared with the negative control (media only) ($p < 0.0001$). The presence of C. accolens strain, C779 significantly reduced MSSA C26 induced cytotoxicity. *C. accolens* C781 had the strongest effect, reducing *S. aureus* induced cytotoxicity to background levels for all 3 *S. aureus* strains. The cytotoxicity effects upon exposure to different *C. accolens* and *S. aureus* strains, are shown in Fig. 3**B**.

3.4. No significant effect on IL-6 production following exposure of HNEC monolayers from CRS patients to C. accolens alone and in combination with S. aureus

We then evaluated the capability of *C. accolens* to modulate immune activation (Azad et al., 2018). Exposure of HNECs to *C. accolens* C779, C781 and C787 for 2 h at a concentration of approximately 1×10^6 CFU/mL resulted in no significant difference in IL-6 secretion compared to the negative control ($p > 0.05$). In contrast, exposure to all 3 *S. aureus,* MSSA C26, MRSA C261 and ATCC 25923, at 1×10^6 CFU/mL for 2 h significantly induced the release of IL-6 compared with the negative control (media only) ($p < 0.0001$). Co-culture with all 3 *C. accolens* strains significantly reduced *S. aureus*-dependent IL-6 production for all 3 *S. aureus* strains. *C. accolens* C787 had the strongest effect reducing *S. aureus*-dependent IL-6 production for all 3 *S. aureus* strains. The effects of *C. accolens* alone, *S. aureus* alone and their combination on IL-6 release from HNECs are presented in Fig. 4.

3.5. Antibiotic susceptibility

We then tested the antibiotic susceptibility for *C. accolens* for a panel of 10 antibiotics. The results for *C. accolens* C779, C781 and C787 are illustrated in Table 1, in comparison with the laboratory reference strain, *C. accolens* ATCC 49726. Overall, all tested strains were sensitive to all antibiotics tested except for C779 that showed intermediate sensitivity to erythromycin.

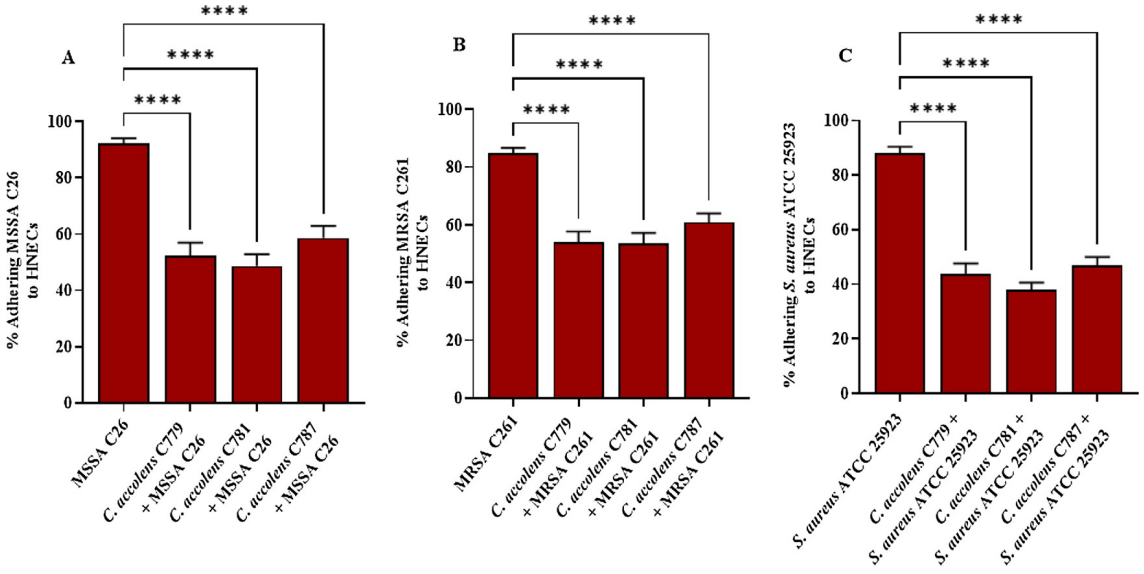


Fig. 2. Reduction of *S. aureus* adhesion to HNECs by *C. accolens*. Cell counts of viable *S. aureus* strains, MSSA C26 (A), MRSA C261 (B) and ATCC 25925 (C) adhering to HNECs treated with: *S. aureus* alone (control) and *S. aureus* in combination with *C. accolens* strains, C779, C781 and C787. Columns represent the mean ± SEM of two independent experiments, each performed in triplicate. Data are reported as percentage of bacteria recovered after plating compared to inoculum. **** $p < 0.0001$, one-way ANOVA, followed by Dunnett's multiple comparisons test. SEM, standard error of the means.

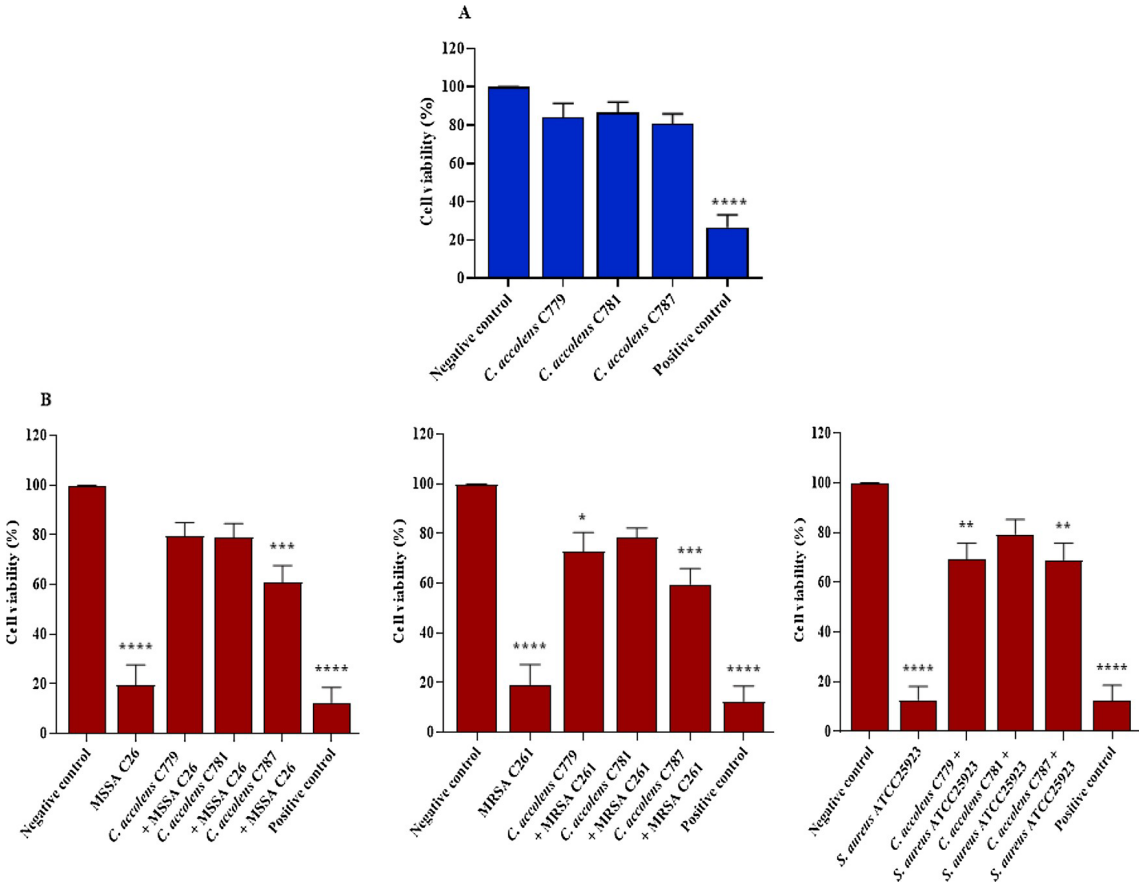


Fig. 3. Cell viability of HNEC monolayers derived from CRS patients after 2 h exposure to *C. accolens* strains alone (C779, C781 and C787) (A), combination of *C. accolens* with *S. aureus* strains (MSSA C26, MRSA C261 and ATCC 25923) and *S. aureus* alone (B), at 1×10^6 CFU/mL cell concentrations for each strain. Negative control (medium) and positive control (10 % Triton X-100). Cell viability was calculated relative to the negative control. The values are shown as means ± SEM, n = 3. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns, not significant, One-way ANOVA, followed by Dunnett's multiple comparisons test. SEM, standard error of the mean; HNEC, human nasal epithelial cell; CFU, colony forming units; CRS, chronic rhinosinusitis.

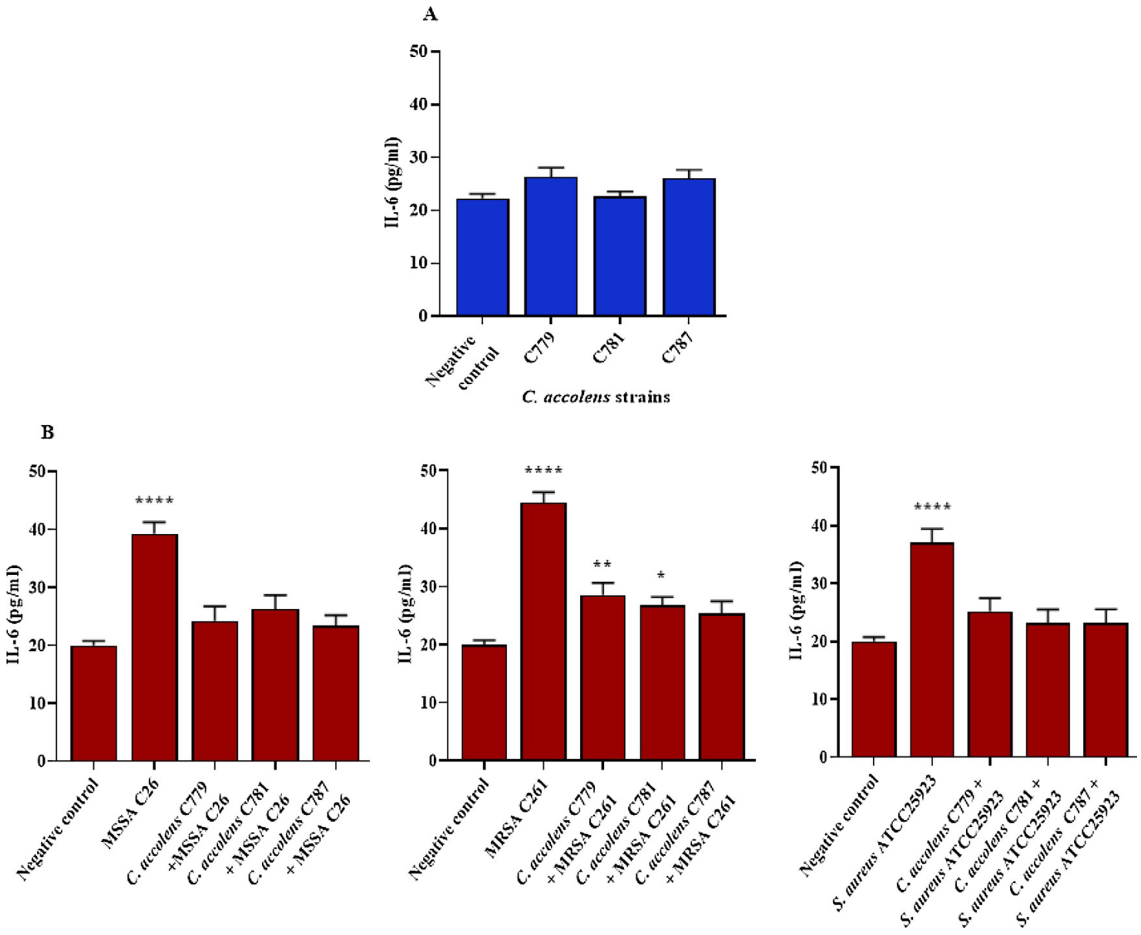


Fig. 4. Interleukin-6 secretion by HNEC monolayers derived from CRS patients after 2 h exposure to *C. accolens* strains alone (C779, C781 and C787) (A), combination of *C. accolens* with *S. aureus* strains (MSSA C26, MRSA C261 and ATCC 25,923) and *S. aureus* alone (B), at 1×10^6 CFU/mL concentrations for each strain. Negative control is untreated cell (medium). Data shown as a mean ± SEM, n = 3. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$, ns, not significant. One-way ANOVA, followed by Dunnett's multiple comparisons test. SEM, standard error of the mean; HNEC, human nasal epithelial cell; CFU, colony forming units; CRS, chronic rhinosinusitis.

Table 1
Antibiotic susceptibility profile of *C. accolens* strains isolated from the healthy human nasal cavity.

Antibiotic agents	Disk potency	Zone diameter breakpoint (mm)*			*C. accolens* strains			
		S ≥	I	R ≤	C779	C781	C787	ATCC 49726
Ciprofloxacin (CIP)	5μg	25	–	24	S	S	S	S
Gentamicin (CN)	10μg	23	–	22	S	S	S	S
Erythromycin (E)	15μg	23	21–22	20	I	S	S	S
Tetracycline (TE)	30μg	24	–	23	S	S	S	S
Rifampicin (RD)	5μg	30	25–29	24	S	S	S	S
Vancomycin (VA)	5μg	17	–	16	S	S	S	S
Imipenem (IPM)	10μg	21	16–20	15	S	S	S	S
Cefuroxime Sodium (CXM)	30μg	25	–	24	S	S	S	S
Sulphamethoxazole/ Trimethoprim (SXT)	25μg	16	13–15	12	S	S	S	S
Penicillin G	5U	28	22–27	21	S	S	S	S

* Inhibition zone diameter interpreted according to EUCAST, 2015 and CLSI 2016 guidelines. S indicates susceptible to antibiotics; I indicates intermediary susceptible to antibiotics.

3.6. Analysis of antibiotic resistance and virulence genes

The summary assembly statistics for each *C. accolens* strain are presented in Table 2. Analysis using abricate showed that no antibiotic resistance genes were detected in the genome of *C. accolens* C781 and C787 isolates. However, the C779 genome was found to contain 5 antibiotic-resistance genes in the CARD database, associated with resistance to Aminoglycoside (*APH (3′′)-Ib*, *APH (6)-Id* and *APH (3′)-Ia*), Chloramphenicol (*Cmx*) and Erythromycin (*ErmX*), a representative of the macrolide antibiotics. In our phenotypic study, Chloramphenicol was not determined for susceptibility, due to its controversy for clinical use and its association with aplastic anaemia and bone marrow suppression (Shukla et al., 2011). These resistance genes were harboured on a section of the C779 genome. During assembly, this genome was identified as a putative extra-chromosomal component, with length 70.472 kbp. BLAST analysis against the NT database revealed that this

Table 2
WGS assembly summary statistics.

C. accolens strains	Total Length (bp)	GC %	Number of Contigs	N50 (bp)	Plasmid Present
C779	2,491,477	58	41	253,945	Yes, Length 70,472
C781	2,437,515	58	44	240,416	No
C787	2,513,312	58	79	138,931	No

Note: Contig, overlapping DNA sequences; N50, nucleotide sequence length.

component was a 94.45 % match to an unnamed *C. kefirresidentii* plasmid (Genbank Accession CP067011.1) along 56 % of its genome. This indicates that the antibiotic resistance genes were likely harboured on *C. accolens* C779 plasmids. Furthermore, results of sequence data showed no recognized virulence factor genes identified in any of the *C. accolens* genome.

Besides, comparative genomic analysis showed that there is a large amount of diversity between the 3 *C. accolens* strains. The pangenome of the 3 isolates identified by Roary consisted of 2803 genes, with 1913 being part of the core genome shared by all 3 isolates. There were 641 genes unique to only one of the three isolates - C779 had 262 unique genes, C781 had 175 unique genes and C787 had 204 unique genes. Further, each isolate had tens of thousands of variants compared to the reference strain ATCC 49725 (61753, 61763 and 57593 respectively for C779, C781, C787), with a significant amount of the ATCC 49275 being unaligned for each isolate (138446, 180,398 and 144,062 bases respectively, out of a total ATCC genome length of 2,437,186). There were also tens of thousands of pairwise variants between the 3 isolates (48,046 for C779 *vs* C781, 47,709 for C779 *vs* C787 and 47,658 for C787 *vs* C781). This suggests that each isolate and the reference strain are not closely related.

3.7. Safety evaluation of *C. accolens* strains in *C. elegans*

To investigate *in vivo* toxicity, the effect of *C. accolens* on the survival of *C. elegans* was studied. *C. accolens* C779, C781 and C787 at a concentration of 1 × 10^6 CFU/mL did not affect the viability of nematodes compared to control uninfected worms ($p > 0.05$), with an average survival rate of 96 %, 88 % and 79 % at days 1, 2 and 3, respectively **(**Fig. 5**)**. This indicates that the presence of *C. accolens* had no adverse effects on the survival of *C. elegans*.

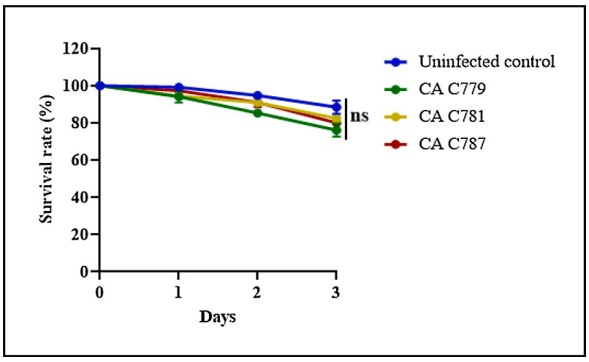


Fig. 5. Effect of the *C. accolens* strains, C779 (Green), C781 (Yellow) and C787 (Red) on *C. elegans* survival over 3 days at a concentration of 1 × 10^6 CFU/mL compared to uninfected control treated with OGM media (Blue). Data represent the mean ± SEM of three independent experiments. Statistical analysis was performed by one-way ANOVA, followed by Tukey's multiple comparisons test. ANOVA = analysis of variance; ns, not significant; SEM, standard error of the mean; CFU, colony forming units.

3.8. Assessment of *C. accolens* ability to protect *C. elegans* against *S. aureus* infection

C. accolens C779, C781 and C787 were assessed for their ability to protect *C. elegans* from *S. aureus* induced toxicity *in vivo*. All 3 *S. aureus* strains gradually reduced the viability of the worms over a 3-day period with a survival rate of 29 %, 22 % and 44 % for the MSSA C26, MRSA C261 and ATCC 25923 respectively compared to an average survival rate of uninfected worms of 88 % at day 3. Exposure to all 3 *C. accolens* at 1 × 10^6 CFU/mL consistently improved the survival of *C. elegans* infected with any of the 3 *S. aureus* isolates at different time points up to day 3. However, the *C. accolens* strains varied in their ability to protect live worms from MSSA C26 infection (50%–64% survival rates) **(**Fig. 6A**)**, MRSA C261 infection (30%–46% survival rates) **(**Fig. 6B**)** and *S. aureus* ATCC 25923 infection (56%–61% survival rates) **(**Fig. 6C**)** at day 3. *C. accolens* strain C781 demonstrated the highest protection against MSSA C26 infection (64 % survival rate) and against *S. aureus* ATCC 25923 infection (61 % survival rate), respectively and was as effective as C779 against ATCC25923 (62 % survival rate) at day 3 when compared to uninfected worms.

In both MSSA C26 and *S. aureus* ATCC 25923 infected worms, a reduced bacterial load was observed after exposure to *C. accolens* strains, C781 and C787 ($p < 0.01$) compared to the respective infected worms in the absence of *C. accolens* **(**Fig. 7**A and C)**. In addition, *C. accolens* C779 and C781 induced a significant reduction of the *S. aureus* bacterial load

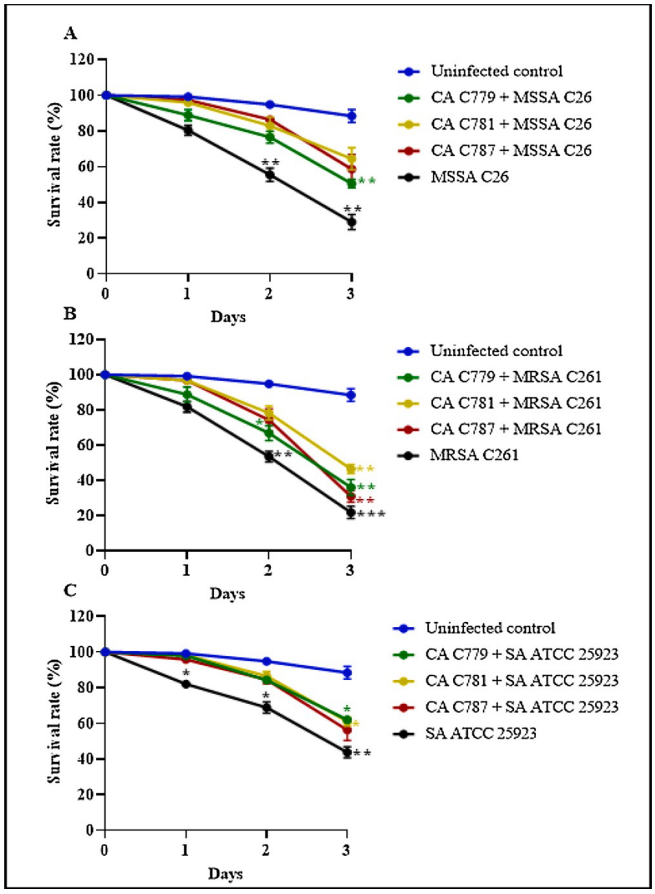


Fig. 6. *C. elegans* survival rate (%) over 3 days in MSSA C26 (A), MRSA C261 (B) and *S. aureus* ATCC 25923 (C) infection under potential protection by 3 different *C. accolens* strains, C779 (Green), C781 (Yellow) and C787 (Red) at 1 × 10^6 CFU/mL concentration compared to uninfected controls (Blue). Data represent the mean ± SEM of three independent experiments. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$, One-way ANOVA, followed by Tukey's multiple comparisons test. SEM, standard error of the mean; CFU, colony forming units.

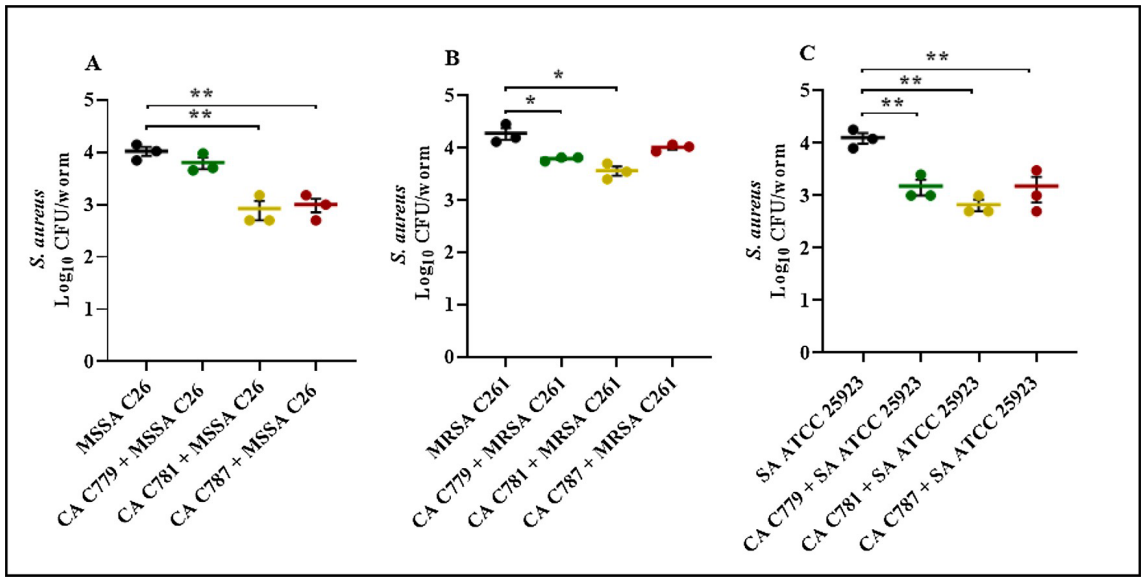


Fig. 7. Reduction of the *S. aureus* bacterial load in *C. elegans*. Plate counting of *S. aureus* bacterial colonisation (Log10 CFU/nematode) after 3 days infection with MSSA C26 (A), MRSA C261 (B) and *S. aureus* ATCC 25923 (C) and treatment with *C. accolens* strains, C779 (Green), C781 (Yellow) and C787 (Red) at 1×10^6 CFU/mL concentration compared to untreated infection controls (Blue). Data represent the mean ± SEM of three independent experiments. One-way ANOVA, followed by Tukey's multiple comparisons test. *$p < 0.05$, **$p < 0.01$. ANOVA = analysis of variance; SEM, standard error of the mean; CFU, colony forming units.

[log10 of 3.8 CFU/worm for C779 (68 % reduction) and 3.6 CFU/worm for C781 (81 % reduction)] in MRSA C261 infected worms compared to MRSA C261 infection control: log10 of 4.3 CFU/worm ($p < 0.05$) **(**Fig. 7B**)**. C781 had the strongest effect across all 3 *S. aureus* strains resulting in an average log10 of 3.1 CFU/worm (97 % reduction) against ATCC25923 infection control: log 10 of 4.1 CFU/mL ($p < 0.01$).

4. Discussion

Nasal commensals belonging to the genus *Corynebacterium*, including *C. accolens*, are increasingly recognized as potential probiotic bacteria that could be used as an adjunct treatment for CRS patients affected by dysbiotic microbiota (Hardy et al., 2019; Uehara et al., 2000). In a previous study, we found *C. accolens* have strong antimicrobial activity against pathogenic *S. aureus* (Menberu et al., 2021). Antimicrobial activity is considered one of the important features of probiotics as it promotes competition with pathogenic bacteria (Brugger et al., 2016; Bomar et al., 2016). In the present study, we further demonstrated the probiotic potential of 3 *C. accolens* strains, sourced from healthy human nasal cavities. Our data indicates that *C. accolens* reduces *S. aureus* adhesion to HNECs and reduces *S. aureus*-dependent IL-6 secretion and cytotoxicity. *C. accolens* did not have cytotoxic properties *in vitro* and *in vivo* and could protect *C. elegans* from *S. aureus* induced toxicity.

Bacterial adhesion to epithelial cells and mucosal layers is one of the selection criteria of probiotic strains for topical application in the context of CRS (Cervin, 2018). Some species of non-pathogenic *Corynebacterium* are known to have a good adherence capacity to human respiratory epithelial cell lines (Moura-Costa et al., 2008) and mucus (Souza et al., 2012), and may attain protective effects against bacterial pathogens by directly blocking adhesion locations (Kanmani et al., 2017). In our *in vitro* study, the three *C. accolens* strains, C779, C781 and C787, showed a strong adherence ability to HNECs, ranging from 63 % to 70 % after 2 h exposure, which is in line with earlier reports on adhesion capability of other commensal *Corynebacterium* species (Alibi et al., 2021; Hayashi et al., 1985). Whilst further testing is required, our results suggest that *C. accolens* strains have the ability to adhere to epithelial cells of the sinonasal cavity and given the prominence of *Corynebacterium* within the sinonasal niche (Paramasivan et al., 2020), it is postulated that this binding also occurs *in vivo*. Overall, the phenotypic differences seen between the 3 *C. accolens* isolates could be related to their genetic differences, with a large amount of variation between the strains. A far broader analysis of the *C. accolens* pangenome by sequencing a larger cohort of isolates and conducting a genome-wide association study (GWAS) is required to identity genetic differences associated with phenotypic differences.

The probiotic potential of *C. accolens* is furthermore supported by our findings that it significantly inhibited the adhesion of MSSA C26, MRSA C261 and *S. aureus* ATCC 25923 to HNECs, indicating that *C. accolens* could compete with *S. aureus* for nasal epithelial cell adhesion. Similarly, *C. accolens* isolated from the healthy sinonasal cavity significantly reduced the adhesion of *S. aureus* to epithelial cells in an *in vitro* bacterial community model (Ménard et al., 2020). Together, these findings suggest that *C. accolens* can regulate and reduce the adhesion of *S. aureus* to epithelial cells including HNECs, possibly due to their strong affinity to HNECs and competition for space. In *Corynebacterium*, the adhesive pili subunits and Sortase A proteins are known to play a role in adherence to host tissue (López-Medrano et al., 2008; Mandlik et al., 2007). Similarly, *S. aureus* also uses Sortase isoforms to bind to host fibronectin and blocking Sortase-dependent fibronectin binding not only reduces *S. aureus* adhesion, it also reduces its virulence (Oh et al., 2006). Previous studies have furthermore shown that *Corynebacterium* species may compete at similar sites of *S. aureus* adhesion to epithelial cells (Lina et al., 2003). Therefore, *C. accolens*-dependent reduction of *S. aureus* adhesion and cytotoxicity might be the result of competition for binding to identical host proteins where presumably *C. accolens* has stronger affinity and binding capacity and outcompetes *S. aureus*. Further assays are required to test this hypothesis.

Some *Corynebacterium* species are generally recognized as safe along the human nasal passages (Ramsey et al., 2016; Lappan and Peacock, 2019). Here, the three *C. accolens* strains at a concentration of 1×10^6 CFU/mL did not display cytotoxic effects when applied to HNECs compared with their negative controls. Moreover, all 3 *C. accolens* strains exhibited a significant increase in HNEC viability when co-incubated with *S. aureus* compared to cells incubated with *S. aureus* only. Moreover, no antibiotic resistance genes and virulence factors were found in *C. accolens* strains C781 and C787, suggesting these two strains are considered potential probiotics for nasal application. On the other hand, resistance to Aminoglycoside, Chloramphenicol and

Erythromycin encoded by plasmids have been detected in strain C779. Plasmids represent one of the mobile elements used as vehicle for the transfer of antibiotic resistant genes in bacteria (Álvarez-Cisneros and Ponce-Alquicira, 2018). As a result, the antibiotic resistance genes against Aminoglycosides, Chloramphenicol and Erythromycin in C779 strain could represent extrinsic or acquired by a horizontal gene transfer (HGT) between bacterial genera in a complex niche, and this might be associated with a health risk. A previous report by Kanmani et al. indicated the protective effect of respiratory commensals in mice challenged with bacterial and viral pathogens, with a significant reduction of cytotoxicity in mice treated with commensal *C. pseudodiphtheriticum* 090104 compared to non-treated mice (Kanmani et al., 2017). In another study, nasal administration of the same strain 090104 improved the resistance of mice to respiratory pathogens with a reduction of tissue damage (Ortiz Moyano et al., 2020). Also, non-pathogenic bacteria and their metabolites have been shown to prolong *C. elegans* longevity, and are suggested as beneficial bacteria or probiotics (Khan et al., 2018). Kim et al. also reported that an oral commensal, *C. durum* extended the lifespan of *C. elegans* by releasing Phenethylamine and N-acetylphenethylamine metabolites, which has been associated with the regulation of *C. elegans* lifespan (Kim et al., 2020).

Several probiotic strains were furthermore reported to protect *C. elegans* against infection mediated by several pathogens (Oliveira et al., 2017; Ikeda et al., 2007). In line with those studies, our result also showed *C. accolens* did not reduce the viability of *C. elegans* and a reduction of *S. aureus* dependent *in vivo* toxicity was observed when *C. accolens* was co-administered with *S. aureus*. The higher worm survival in nematodes infected with both *C. accolens* and *S. aureus* is likely at least in part due to the competition of the two bacteria for space.

Commensal bacteria can regulate homeostasis also indirectly by modulating host immune responses (Brugger et al., 2016; Ortiz Moyano et al., 2020). IL-6 is an important pro-inflammatory cytokine, and its elevated level plays a major role in the development of numerous inflammatory diseases including CRS (Turner et al., 2018; Rincon and Irvin, 2012). In our study, 2 h exposure of *C. accolens* strains on HNEC monolayers at a concentration of 1×10^6 CFU/mL did not increase IL-6 cytokine production in comparison to negative control. Similarly, a reduction of IL-6 was also found after 2 h exposure of intestinal epithelial cells with different probiotic bacteria (Čitar et al., 2015). Our results also indicate that *C. accolens* suppressed *S. aureus*-dependent IL-6 production in cultured HNECs monolayers *in vitro* indicating *C. accolens* strains have the capacity to dampen *S. aureus*-dependent immune activation.

Together, our results indicate the commensal nature of *C. accolens* with the potential for this strain to be used as a probiotic to combat *S. aureus* colonisation and virulence. Further studies are required to evaluate the molecular mechanism of *C. accolens* action against *S. aureus* adhesive capacity and virulence. Furthermore, *in vivo* probiotic efficacy in relevant mammalian models are required to support the potential for clinical translation.

5. Conclusion

Nasal *C. accolens* strains, C779, C781 and C787 displayed probiotic features, both *in vitro* and *in vivo*. All strains showed significant adhesion to HNECs and could reduce *S. aureus* adhesion to HNECs with no apparent cytotoxic effects. Importantly, strains C781 and C787 revealed notable immunomodulatory potential by reducing the secretion of IL-6 in HNECs when co-treated with *S. aureus* and absence of antibiotic resistance and virulence genes. By virtue of having these beneficial features with their recognized antimicrobial ability, the *C. accolens* strains, C781 and C787 are considered safe and can be explored as novel probiotics to combat dysbiosis in CRS patients. Further *in vivo* trials are required to fully understand the probiotic potential for these strains to promote sinonasal health.

Author statement

Martha A. Menberu and Sarah Vreugde: Project conceptualization. Martha A. Menberu, Clare Cooksley and Mahnaz Ramezanpour: Experimental design and laboratory investigation. Martha A. Menberu and George Bouras: Data management and statistical analysis. Sarah Vreugde and Peter-John Wormald: Project administration and supervision. Alkis J. Psaltis and Sarah Vreugde: Funding acquisition. Martha A. Menberu: Manuscript draft preparation and revision. Clare Cooksley, Mahnaz Ramezanpour, Peter-John Wormald, Alkis J. Psaltis and Sarah Vreugde: Manuscript review and editing. All authors have read and agreed to the manuscript.

Declaration of Competing Interest

The authors declare no conflicts of interest regarding this manuscript.

Acknowledgments

This work was supported by the Garnett Passe and Rodney Williams Memorial Foundation Conjoint Grant to Alkis J. Psaltis and Sarah Vreugde and the University of Adelaide International Scholarship to Martha A. Menberu.

References

Alibi, S., Ramos-Vivas, J., Selma, W.B., Mansour, H.B., Boukadida, J., Navas, J., 2021. Virulence of clinically relevant multidrug resistant *Corynebacterium striatum* strains and their ability to adhere to human epithelial cells and inert surfaces. Microb. Pathog. 155, 104887.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215 (3), 403–410.

Álvarez-Cisneros, Y.M., Ponce-Alquicira, E., 2018. Antibiotic Resistance in Lactic Acid Bacteria. In Antimicrobial Resistance-A Global Threat. IntechOpen.

Andrews, S., 2010. FastQC: A Quality Control Tool for High Throughput Sequence Data. Version 0.11. 2. http://www.

Azad, M., Kalam, A., Sarker, M., Wan, D., 2018. Immunomodulatory effects of probiotics on cytokine profiles. Biomed Res. Int. 2018.

Barberis, C.M., Sandoval, E., Rodriguez, C.H., Ramírez, M.S., Famiglietti, A., Almuzara, M., Vay, C., 2018. Comparison between disk diffusion and agar dilution methods to determine in vitro susceptibility of Corynebacterium spp. Clinical isolates and update of their susceptibility. J. Glob. Antimicrob. Resist. 14, 246–252.

Bomar, L., Brugger, S.D., Yost, B.H., Davies, S.S., Lemon, K.P., 2016. *Corynebacterium accolens* releases antipneumococcal free fatty acids from human nostril and skin surface triacylglycerols. MBio. 7 (1), e01725–01715.

Bordin, A., Sidjabat, H.E., Cottrell, K., Cervin, A., 2016. Chronic rhinosinusitis: a microbiome in dysbiosis and the search for alternative treatment options. Microbiol. Aust. 37 (3), 149–152.

Brugger, S.D., Bomar, L., Lemon, K.P., 2016. Commensal–pathogen interactions along the human nasal passages. PLoS Pathog. 12 (7).

Cervin, A.U., 2018. The potential for topical probiotic treatment of chronic rhinosinusitis, a personal perspective. Front. Cell. Infect. Microbiol. 7, 530.

Čitar, M., Hacin, B., Tompa, G., Štempelj, M., Rogelj, I., Dolinšek, J., Narat, M., Matijašić, B.B., 2015. Human intestinal mucosa-associated Lactobacillus and Bifidobacterium strains with probiotic properties modulate IL-10, IL-6 and IL-12 gene expression in THP-1 cells. Benef. Microbes 6 (3), 325–336.

Cleland, E.J., Bassiouni, A., Wormald, P.J., 2013. The bacteriology of chronic rhinosinusitis and the pre-eminence of *Staphylococcus aureus* in revision patients. Int. Forum Allergy Rhinol. 3 (8), 642–646.

Cleland, E.J., Drilling, A., Bassiouni, A., James, C., Vreugde, S., Wormald, P.J., 2014. Probiotic manipulation of the chronic rhinosinusitis microbiome. Int. Forum Allergy Rhinol. 4 (4), 309–314.

De Boeck, I., Spacova, I., Vanderveken, O.M., Lebeer, S., 2021. Lactic acid bacteria as probiotics for the nose? Microb. Biotechnol. 14 (3), 859–869.

EUCAST, T., 2015. European committee on antimicrobial susceptibility testing. Breakpoint tables for interpretation of MICs and zone diameters 5, 2015.

Feazel, L.M., Robertson, C.E., Ramakrishnan, V.R., Frank, D.N., 2012. Microbiome complexity and *Staphylococcus aureus* in chronic rhinosinusitis. Laryngoscope 122 (2), 467–472.

Foreman, A., Psaltis, A.J., Tan, L.W., Wormald, P.-J., 2009. Characterization of bacterial and fungal biofilms in chronic rhinosinusitis. Am. J. Rhinol. Allergy 23 (6), 556–561.

Guantario, B., Zinno, P., Schifano, E., Roselli, M., Perozzi, G., Palleschi, C., Uccelletti, D., Devirgiliis, C., 2018. In vitro and in vivo selection of potentially probiotic lactobacilli from Nocellara del Belice table olives. Front. Microbiol. 9, 595.

Hardy, B.L., Dickey, S.W., Plaut, R.D., Riggins, D.P., Stibitz, S., Otto, M., Merrell, D.S., 2019. *Corynebacterium pseudodiphtheriticum* exploits *Staphylococcus aureus* virulence components in a novel polymicrobial defense strategy. MBio. 10 (1).

Hasman, H., Saputra, D., Sicheritz-Ponten, T., Lund, O., Svendsen, C.A., Frimodt-Møller, N., Aarestrup, F.M., 2014. Rapid whole-genome sequencing for detection and characterization of microorganisms directly from clinical samples. J. Clin. Microbiol. 52 (1), 139–146.
Hayashi, A., Yanagawa, R., Kida, H., 1985. Adhesion of *Corynebacterium renale* and *Corynebacterium pilosum* to the epithelial cells of various parts of the bovine urinary tract from the renal pelvis to vulva. Vet. Microbiol. 10 (3), 287–292.
Hoggard, M., Biswas, K., Zoing, M., Wagner Mackenzie, B., Taylor, M.W., Douglas, R.G., 2017. Evidence of microbiota dysbiosis in chronic rhinosinusitis. Int. Forum Allergy Rhinol. 7 (3), 230–239.
Hotel, A.C.P., Cordoba, A., 2001. Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. Prevention. 5 (1), 1–10.
Hunt, P.R., 2017. The *C. Elegans* model in toxicity testing. J. Appl. Toxicol. 37 (1), 50–59.
Ikeda, T., Yasui, C., Hoshino, K., Arikawa, K., Nishikawa, Y., 2007. Influence of lactic acid bacteria on longevity of *Caenorhabditis elegans* and host defense against *Salmonella enterica* serovar enteritidis. Appl. Environ. Microbiol. 73 (20), 6404.
Imperial, I.C., Ibana, J.A., 2016. Addressing the antibiotic resistance problem with probiotics: reducing the risk of its double-edged sword effect. Front. Microbiol. 7, 1983.
Kanmani, P., Clua, P., Vizoso-Pinto, M.G., Rodriguez, C., Alvarez, S., Melnikov, V., Takahashi, H., Kitazawa, H., Villena, J., 2017. Respiratory commensal bacteria *Corynebacterium pseudodiphtheriticum* improves resistance of infant mice to respiratory syncytial virus and *Streptococcus pneumoniae* superinfection. Front. Microbiol. 8, 1613.
Khan, F., Jain, S., Oloketuyi, S.F., 2018. Bacteria and bacterial products: foe and friends to *Caenorhabditis elegans*. Microbiol. Res. 215, 102–113.
Kim, J.H., Bang, I.H., Noh, Y.J., Kim, D.K., Bae, E.J., Hwang, I.H., 2020. Metabolites produced by the oral commensal bacterium *Corynebacterium durum* extend the lifespan of *Caenorhabditis elegans* via SIR-2.1 overexpression. Int. J. Mol. Sci. 21 (6), 2212.
Lappan, R., Peacock, C.S., 2019. Corynebacterium and Dolosigranulum: future probiotic candidates for upper respiratory tract infections. Microbiol. Aust. 40 (4), 172–177.
Lina, G., Boutite, F., Tristan, A., Bes, M., Etienne, J., Vandenesch, F., 2003. Bacterial competition for human nasal cavity colonization: role of staphylococcal agr alleles. Appl. Environ. Microbiol. 69 (1), 18–23.
López-Medrano, F., García-Bravo, M., Morales, J., András, A., San Juan, R., Lizasoain, M., Aguado, J., 2008. Urinary tract infection due to *Corynebacterium urealyticum* in kidney transplant recipients: an underdiagnosed etiology for obstructive uropathy and graft dysfunction-results of a prospective cohort study. Clin. Infect. Dis. 46 (6), 825–830.
Mandlik, A., Swierczynski, A., Das, A., Ton-That, H., 2007. *Corynebacterium diphtheriae* employs specific minor pilins to target human pharyngeal epithelial cells. Mol. Microbiol. 64 (1), 111–124.
Marchisio, P., Santagati, M., Scillato, M., Baggi, E., Fattizzo, M., Rosazza, C., Stefani, S., Esposito, S., Principi, N., 2015. *Streptococcus salivarius* 24SMB administered by nasal spray for the prevention of acute otitis media in otitis-prone children. Eur. J. Clin. Microbiol. Infect. Dis. 34 (12), 2377–2383.
Mårtensson, A., Abolhalaj, M., Lindstedt, M., Mårtensson, A., Olofsson, T.C., Vásquez, A., Greiff, L., Cervin, A., 2017. Clinical efficacy of a topical lactic acid bacterial microbiome in chronic rhinosinusitis: a randomized controlled trial. Laryngoscope Investig. Otolaryngol. 2 (6), 410–416.
Meier-Kolthoff, J.P., Göker, M., 2019. TYGS is an automated high-throughput platform for state-of-the-art genome-based taxonomy. Nat. Commun. 10 (1), 1–10.
Ménard, G., Bonnaure-Mallet, M., Donnio, P.-Y., 2020. Adhesion of *Staphylococcus aureus* to epithelial cells: an in vitro approach to study interactions within the nasal microbiota. J. Med. Microbiol. 69 (10), 1253–1261.
Menberu, M.A., Liu, S., Cooksley, C., Hayes, A.J., Psaltis, A.J., Wormald, P.-J., Vreugde, S., 2021. *Corynebacterium accolens* has antimicrobial activity against *Staphylococcus aureus* and methicillin-resistant *S. Aureus* pathogens isolated from the sinonasal niche of chronic rhinosinusitis patients. Pathogens. 10 (2), 207.
Moura-Costa, L., Bahia, R., Carminati, R., Vale, V., Paule, B., Portela, R., Freire, S.M., Nascimento, I., Schaer, R., Barreto, L., 2008. Evaluation of the humoral and cellular immune response to different antigens of *Corynebacterium pseudotuberculosis* in Canindé goats and their potential protection against caseous lymphadenitis. Vet. Immunol. Immunopathol. 126 (1–2), 131–141.
Mukerji, S.S., Pynnonen, M.A., Kim, H.M., Singer, A., Tabor, M., Terrell, J.E., 2009. Probiotics as adjunctive treatment for chronic rhinosinusitis: a randomized controlled trial. Otolaryngol. Head. Neck Surg. 140 (2), 202–208.
Myles, I.A., Earland, N.J., Anderson, E.D., Moore, I.N., Kieh, M.D., Williams, K.W., Saleem, A., Fontecilla, N.M., Welch, P.A., Darnell, D.A., 2018. First-in-human topical microbiome transplantation with *Roseomonas mucosa* for atopic dermatitis. JCI Insight 3 (9).
Oh, K.B., Oh, M.N., Kim, J.G., Shin, D.S., Shin, J., 2006. Inhibition of sortase-mediated *Staphylococcus aureus* adhesion to fibronectin via fibronectin-binding protein by sortase inhibitors. Appl. Microbiol. Biotechnol. 70 (1), 102–106. https://doi.org/10.1007/s00253-005-0040-8.
Oliveira, A., Oliveira, L.C., Aburjaile, F., Benevides, L., Tiwari, S., Jamal, S.B., Silva, A., Figueiredo, H.C., Ghosh, P., Portela, R.W., 2017. Insight of genus Corynebacterium: ascertaining the role of pathogenic and non-pathogenic species. Front. Microbiol. 8, 1937.
Ortiz Moyano, R., Raya Tonetti, F., Tomokiyo, M., Kanmani, P., Vizoso-Pinto, M.G., Kim, H., Quilodrán-Vega, S., Melnikov, V., Alvarez, S., Takahashi, H., 2020. The ability of respiratory commensal bacteria to beneficially modulate the lung innate immune response is a strain dependent characteristic. Microorganisms. 8 (5), 727.
Page, A.J., Carla, A.C., Martin, H., Vanessa, K.W., Sandra, R., Matthew, T.H., Maria, F., Daniel, F., Jacqueline, A.K., Julian, P., 2015. 'Roary: rapid large-scale prokaryote pan genome analysis'. Bioinformatics. 31, 3691–3693.
Paramasivan, S., Bassiouni, A., Shiffer, A., Dillon, M.R., Cope, E.K., Cooksley, C., Ramezanpour, M., Moraitis, S., Ali, M.J., Bleier, B., 2020. The international sinonasal microbiome study: a multicentre, multinational characterization of sinonasal bacterial ecology. Allergy. 75 (8), 2037–2049. https://doi.org/10.1111/all.14276.
Pennekamp, A., Pünter, V., Zbinden, R., 1996. Disk diffusion, agar dilution and the E-test for susceptibility testing of *Corynebacterium jeikeium*. Clin. Microbiol. Infect. 2 (3), 209–213.
Perrin, Y., Nutten, S., Audran, R., Berger, B., Bibiloni, R., Wassenberg, J., Barbier, N., Aubert, V., Moulin, J., Singh, A., 2014. Comparison of two oral probiotic preparations in a randomized crossover trial highlights a potentially beneficial effect of *Lactobacillus paracasei* NCC2461 in patients with allergic rhinitis. Clin. Transl. Allergy 4 (1), 1–12.
Psaltis, A.J., Wormald, P.-J., 2017. Therapy of sinonasal microbiome in CRS: a critical approach. Curr. Allergy Asthma Rep. 17 (9), 59.
Rajoka, M.S.R., Hayat, H., Sarwar, S., Mehwish, H., Ahmad, F., Hussain, N., Shah, S., Khurshid, M., Siddiqu, M., Shi, J., 2018. Isolation and evaluation of probiotic potential of lactic acid bacteria isolated from poultry intestine. Microbiology. 87 (1), 116–126.
Ramezanpour, M., Moraitis, S., Smith, J.L., Wormald, P., Vreugde, S., 2016. Th17 cytokines disrupt the airway mucosal barrier in chronic rhinosinusitis. Mediators Inflamm. 2016.
Ramezanpour, M., Bolt, H., Psaltis, A., Wormald, P.J., Vreugde, S., 2019. Inducing a mucosal barrier-sparing inflammatory response in laboratory-grown primary human nasal epithelial cells. Curr. Protoc. Toxicol. 80 (1), e69.
Ramsey, M.M., Freire, M.O., Gabrilska, R.A., Rumbaugh, K.P., Lemon, K.P., 2016. *Staphylococcus aureus* shifts toward commensalism in response to Corynebacterium species. Front. Microbiol. 7, 1230.
Richter, K., Thomas, N., Zhang, G., Prestidge, C.A., Coenye, T., Wormald, P.-J., Vreugde, S., 2017. Deferiprone and gallium-protoporphyrin have the capacity to potentiate the activity of antibiotics in *Staphylococcus aureus* small colony variants. Front. Cell. Infect. Microbiol. 7, 280.
Rincon, M., Irvin, C.G., 2012. Role of IL-6 in asthma and other inflammatory pulmonary diseases. Int. J. Biol. Sci. 8 (9), 1281.
Roos, K., Simark-Mattsson, C., Håkansson, E.G., Larsson, L., Sandberg, T., Åhrén, C., 2011. Can probiotic lactobacilli eradicate persistent carriage of meticillin-resistant *Staphylococcus aureus*? J. Hosp. Infect. 78 (1), 77–78.
Rutten, N., Besseling-Van der Vaart, I., Klein, M., De Roock, S., Vlieger, A., Rijkers, G., 2011. In vitro assessment of the immunomodulatory effects of multispecies probiotic formulations for management of allergic diseases. Benef. Microbes 2 (3), 183–192.
Schwartz, J.S., Peres, A.G., Endam, L.M., Cousineau, B., Madrenas, J., Desrosiers, M., 2016. Topical probiotics as a therapeutic alternative for chronic rhinosinusitis: a preclinical proof of concept. Am. J. Rhinol. Allergy 30 (6), e202–e205.
Seemann, T., 2015. Snippy: Fast Bacterial Variant Calling From NGS Reads (v4. 6.0).
Seemann, T., 2019. abricate. Github.
Sharma, K., Pooranachithra, M., Balamurugan, K., Goel, G., 2019. Multivariate analysis of increase in life span of *Caenorhabditis elegans* through intestinal colonization by indigenous probiotic strains. Probiotics Antimicrob. Proteins 11 (3), 865–873.
Shukla, P., Bansode, F.W., Singh, R.K., 2011. 'Chloramphenicol toxicity: a review'. J. Med. Med. Sci. 2, 1313–1316.
Sim, I., Keun-Tae, P., Gayeung, K., Jong-Ho, K., Young-Hee, L., 2018. 'Probiotic potential of *Enterococcus faecium* isolated from chicken cecum with immunomodulating activity and promoting longevity in *Caenorhabditis elegans*'. J. Microbiol. Biotechnol. 28, 883–892.
Souza, M.Cd., Santos, L.Sd., Gomes, D.L.R., Sabbadini, P.S., Santos, C.Sd., Camello, T.C. F., Asad, L.M., Rosa, A.C., Nagao, P.E., Hirata Júnior, R., 2012. Aggregative adherent strains of *Corynebacterium pseudodiphtheriticum* enter and survive within HEp-2 epithelial cells. Memãşrias Do Inst. Oswaldo Cruz 107 (4), 486–493.
Stubbendieck, R.M., Straight, P.D., 2016. Multifaceted interfaces of bacterial competition. J. Bacteriol. 198 (16), 2145–2155.
Turner, J.H., Chandra, R.K., Li, P., Bonnet, K., Schlundt, D.G., 2018. Identification of clinically relevant chronic rhinosinusitis endotypes using cluster analysis of mucus cytokines. J. Allergy Clin. Immunol. 141 (5), 1895-1897. e1897.
Uehara, Y., Nakama, H., Agematsu, K., Uchida, M., Kawakami, Y., Fattah, A.A., Maruchi, N., 2000. Bacterial interference among nasal inhabitants: eradication of *Staphylococcus aureus* from nasal cavities by artificial implantation of Corynebacterium sp. J. Hosp. Infect. 44 (2), 127–133.
Wells, J.M., Brummer, R.J., Derrien, M., MacDonald, T.T., Troost, F., Cani, P.D., Theodorou, V., Dekker, J., Méheust, A., De Vos, W.M., 2017. Homeostasis of the gut barrier and potential biomarkers. American Journal of Physiology-Gastrointestinal and Liver Physiology. 312 (3), G171–G193.
Wick, R.R., Judd, L.M., Gorrie, C.L., Holt, K.E., 2017. Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. PLoS Comput. Biol. 13 (6), e1005595.
Wieërs, G., Belkhir, L., Enaud, R., Leclercq, S., Philippart de Foy, J.-M., Dequenne, I., de Timary, P., Cani, P.D., 2020. How probiotics affect the microbiota. Front. Cell. Infect. Microbiol. 9, 454.

CHAPTER 6

Corynebacterium accolens inhibits *Staphylococcus aureus* induced mucosal barrier disruption

CHAPTER 6: *Corynebacterium accolens* inhibits *Staphylococcus aureus* induced mucosal barrier disruption

This chapter is prepared in a manuscript format and addresses the fifth research aim designed to evaluate the interactions between clinical isolates of *Corynebacterium accolens* and *Staphylococcus aureus*, cultured from the sinonasal mucosa, and their effect on the mucosal barrier.

Huang S, Liu S, Hon K, Bennett C, Hu H, **Menberu MA**, Wormald PJ, Vreugde S, Psaltis AJ. *Corynebacterium accolens* inhibits *Staphylococcus aureus* induced mucosal barrier disruption. Submitted for publication, November 2021.

6.1 Statement of Authorship

Title of Paper	*Corynebacterium accolens* inhibits Staphylococcus aureus induced mucosal barrier disruption
Publication Status	☐ Published ☐ Accepted for Publication ☑ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Huang S, Liu S, Hon K, Bennett C, Hu H, Menberu MA, Wormald PJ, Vreugde S, Psaltis AJ.

Principal Author

Name of Principal Author	Shuman Huang		
Contribution to the Paper	Conception and design of the project, acquisition of data, analysis and interpretation of data, manuscript preparation		
Overall percentage (%)	85		
Certification:	This paper reports on original research I conducted during the period of my Research Fellowship and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	28/10/2021

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Sha Liu		
Contribution to the Paper	Acquisition of data, analysis and interpretation of data, manuscript edition		
Signature		Date	18/10/2021

Name of Co-Author	Karen Hon		
Contribution to the Paper	Acquisition of data, analysis and interpretation of data, manuscript edition		
Signature		Date	28/10/2021

Name of Co-Author	Catherine Bennett		
Contribution to the Paper	Acquisition of data, analysis and interpretation of data, manuscript edition		
Signature		Date	28/10/2021

Name of Co-Author	Hua Hu		
Contribution to the Paper	Acquisition of data, analysis and interpretation of data, manuscript edition		
Signature		Date	28/10/2021

Name of Co-Author	Martha Alemayehu Menberu		
Contribution to the Paper	Acquisition of data, analysis and interpretation of data, manuscript edition		
Signature		Date	28/10/2021

Name of Co-Author	Peter-John Wormald		
Contribution to the Paper	Conception of the project, critically revising the article		
Signature		Date	18/10/2021

Name of Co-Author	Sarah Vreugde		
Contribution to the Paper	Conception and design of the project, critically revising the article		
Signature		Date	18/10/2021

Name of Co-Author	Alkis James Psaltis		
Contribution to the Paper	Conception of the project, critically revising the article		
Signature		Date	18/10/2021

6. *Corynebacterium accolens* inhibits *Staphylococcus aureus* induced mucosal barrier disruption

Shuman Huang[1,2,3], Sha Liu[1,2], Karen Hon[1,2], Catherine Bennett[1,2], Hua Hu[1,2,4],Martha Menberu [1,2], Peter-John Wormald[1,2], Sarah Vreugde[1,2], Alkis J Psaltis[1,2]*

[1]Department of Surgery-Otolaryngology Head and Neck Surgery, Basil Hetzel Institute for Translational Health Research, Central Adelaide Local Health Network, Woodville South, Australia.

[2]Adelaide Medical School, The University of Adelaide, Adelaide, Australia.

[3]Department of Rhinology, The ENT Hospital, The First Affiliated Hospital ofZhengzhou University, Zhengzhou 450052, China.

[4]Department of Otolaryngology, Head and Neck Surgery, Shanghai GeneralHospital, Shanghai Jiaotong University, Shanghai 200080, China.

*Correspondence: alkis.psaltis@adelaide.edu.au; Tel.: +618-822-27158

Running title: *C. accolens* inhibits *S. aureus* induced mucosal barrier disruption

Keywords: *Corynebacterium accolens*, *Staphylococcus aureus*, supernatant, planktonic, biofilm, TER

6.2 Abstract

Previous sinonasal microbiota research has identified *Corynebacterium* and *Staphylococcus* genera to be among the most prevalent core micro-organismsin both health and disease. *Corynebacterium accolens* (*C. accolens*) is a common nasal colonizer, while *Staphylococcus aureus* (*S. aureus*) is typically considered a pathogenic organism and has been associated with more severesinus disease and treatment resistance. This study aims to evaluate the interaction between *C. accolens* and *S. aureus in vitro*. Clinical isolates of *C. accolens* and *S. aureus* from sinonasal swabs of healthyand chronic rhinosinusitis (CRS) patients, and primary human nasal epithelialcells (HNECs) cultured at air-liquid interface (ALI) from cellular brushings of healthy and CRS patients were used for this study. A total of three *C. accolens*clinical isolates, one *S. aureus* clinical isolate, and one *S. aureus* reference strain (ATCC51650) were used. Supernatants of all isolates grown alone and in co-cultures were tested for their effects on trans-epithelial electrical resistance (TER), FITC-Dextran permeability, Lactate Dehydrogenase (LDH), and IL6 and IL8 secretion of HNECs. Confocal scanning laser microscopy and immunofluorescence were used to visualize apical junctional complexes. The antimicrobial activity of *C. accolens* supernatants on planktonic and biofilm *S. aureus* growth was also assessed. The *C. accolens* supernatants of three clinical strains (at 60% and 30% concentration) were shown to significantly inhibit the growth of both the planktonic *S. aureus* reference and clinical strain, whilst no inhibition of the *S. aureus* biofilm growth was observed. The *C. accolens* supernatant caused no change in the TER or FITC-Dextran permeability of the HNEC-ALI cultures whilst *S. aureus* strains had a detrimental effect. Conditioned media of *C. accolens* co-cultured with both strains of *S. aureus* delayed the *S. aureus*- dependent mucosal barrier damage in a dose-dependent manner. This study found that *C. accolens* supernatants can inhibit the growth of the *S. aureus* planktonic bacteria, but not *S. aureus* biofilms. Additionally, *C. accolens* could reduce *S. aureus*-induced damage to the mucosal barrier.

6.3 Introduction

Microbiota can be defined as ecological communities of commensal and pathogenic microorganisms found in and on all multicellular organisms. Microbial communities encode millions of genes and associated functions, which act alongside those of human cells to maintain homeostasis [311]. A wealthof studies have now established microbiota as being an important contributor to essential mammalian functions including metabolism [312]; biosynthesis [313]; neurotransmission [314, 315]; and immunomodulation [316, 317]. The host-microbiota interface is particularly important with evidence now suggesting that many chronic inflammatory diseases are associated with significant shifts in the localmicrobiota towards inflammatory configurations [318]. A better understanding of the microbiota associated with such conditions may therefore be the key to unraveling their underlying pathogenesis and ultimately facilitate the development of new treatments.

Chronic Rhinosinusitis (CRS) is a chronic inflammatory condition of the paranasal sinus mucosa with unclear pathogenesis, although microbial disturbances and dysregulated inflammation are both thought to play an important role. Sinonasal microbiota studies have demonstrated that healthy individuals and CRS patients have a similar overall bacterial burden and sharemany common phyla [119], although patients with CRS tend to have reduced bacterial diversity with an expansion of pathogenic micro-organisms. Similar toother chronic inflammatory conditions it is possible that pathogen colonization and microbiota imbalance may be the initial causes of the chronic immune response and inflammation seen in this condition [318].

Previous research from our department assessed a large international patient cohort to characterize the sinonasal microbiota and its global geographical variations in both health and sinus disease. Using 16S rRNA gene sequencing, we found *Corynebacterium* and

Staphylococcus to be amongst the most dominant genera in the majority of sampled patients, irrespective of the disease state [225]. Unfortunately, due to current well documented limitations of the short-read16S rRNA gene sequencing, it was not possible to accurately characterize the bacteria genus to the species level. It is known that several different *Corynebacterium* species reside within the nose, the majority of which are believed to exist as commensals with important protective functions. *C. accolens*, for example, is a common nasal colonizer and can inhibit Streptococcal growth via the release of oleic acids from the hydrolysis of host triacylglycerols [127]. Meanwhile, *Staphylococcus*, and in particular *S. aureus* has typically been viewed as a pathogenic organism within the nose, and its presence is associated with recalcitrant CRS and worse postoperative outcomes [202, 319, 320]. Our previous research also showed *C. accolens* isolated from a healthy human nasal cavity exhibited antimicrobial activity against planktonic and biofilm growth of S. *aureus* and methicillin- resistant *S. aureus* isolated from CRS patients [278]. This study aims to evaluate the interactions between clinical isolates of *C. accolens* and *S. aureus,* cultured from the sinonasal mucosa, and their effect on the mucosal barrier in an *in vitro* setting.

6.4 Materials and Methods

The study was approved by the Central Adelaide Local Health Network Human Research Ethics Committee (HREC/15/TQEH/132) and written informed consent was obtained from participants before collection of microbial swabs and primary human nasal epithelial cells (HNECs).

Sample collection

Bacterial swabs were used to sample the middle meatus of CRS patients and non-CRS controls. Cytobrushes (EndoScan Brush, Medico, Melbourne) were used intraoperatively to harvest primary human nasal epithelial cells (HNECs) from the inferior turbinate mucosa. Control

patients were patients undergoing endoscopic skull base procedures without clinical or radiological evidence of sinus disease. CRS patients fulfilled the diagnostic criteria set out in the positionpapers by the American Academy of Otolaryngology and Head and Neck Surgery and the European Position Statement (EPOS) on CRS [16, 321].

Bacterial assay

Bacterial culture

C. accolens clinical isolates (C1, C2, and C3) were isolated and identified using molecular and microbiological techniques detailed by Menberu et al [268, 278]; *S.aureus* clinical isolates (SC) were identified by SA Pathology and *S. aureus* reference strain (ATCC51650, SA) was purchased from ATCC (Manassas, USA). All bacterial strains were frozen in 20% glycerol at -80°C. Isolates (*S. aureus* and *C. accolens)* were thawed and cultured at 37°C for 24 hours on 1.5% trypticase soy agar (TSA) plates with 0.5% Tween 80 (Sigma-Aldrich, St. Louis,USA). One single colony of *C. accolens* and *S. aureus* was resuspended in 2ml 0.9% saline to adjust to McFarland (McF)=0.5. 50µl of the resuspended bacterial solution was added to a 50ml centrifuge tube containing 5ml of trypticase soy broth (TSB) and 0.5% Tween 80 and incubated at 37°C on an orbital shaking incubator at 180rpm for 24 hours. The overnight bacterial culture was then diluted with TSB containing 0.5% Tween 80 to an absorbance of 0.05 at a wavelength of 600nm (OD600) (SmartSpec 3000, Biorad, CA, USA). 10ml of the diluted bacterial suspension was transferred into a 100ml centrifuge tubeand incubated at 37°C on an orbital shaking incubator at 180rpm. The OD600 was measured hourly to prepare a standard growth curve of the bacteria.

Conditioned media harvest and exoprotein quantification

For planktonic conditioned media, 0.5McF *C. accolens* and *S. aureus* suspension in 0.9% saline were obtained using a single colony from a plate grown on 1.5% TSA with 0.5% Tween 80 at 37℃ for 24 hours. The bacterial suspension was diluted at 1:100 in TSB with 0.5% tween 80 in a 50ml falcon tube, then, the suspension of *C. accolens* or *S. aureus* was incubated at 180rpm in a 37℃ incubator in air for 24 hours. For *C. accolens* and *S. aureus*co-cultures, a fixed number of *S. aureus* (5 x 10^5 CFU) with *C. accolens* in different ratios (50%, 70%, and 90%) was incubated at 180 rpm in a 37℃ incubator in air for 24 hours. The supernatant from single cultures and co-cultures was harvested after 24 hours after spinning down and filtered through a 0.22µm syringe filter (Pall Corporation, San Diego, USA).

For biofilm conditioned media, 1McF *C. accolens* and *S. aureus* suspension was diluted 1:15 in TSB with 0.5% Tween 80 to form biofilm in 6-well plates [322].The suspension of *C. accolens*, *S. aureus,* and S. *aureus* co-cultured with *C.accolens* in different ratios (50%,70%, and 90%) were incubated for 48 hours at 37ºC on a gyratory shaker at 70 rpm in air. The supernatant was harvested as above. Then the protein concentration was determined using Nano Orange protein quantitation kit (Invitrogen, Carlsbad, CA, USA). The experiment was repeated three times.

Antibacterial assay

Planktonic bacteria assay

100µl of *S. aureus* suspension (0.5McF) was grown in TSB with 0.5% Tween 80 in a 96-well plate (Corning Incorporated, Corning NY, USA) containing different concentrations (20%-90% v/v) of *C. accolens* (clinical isolates 1 to 3) conditioned media and incubated for 24 hours at 180rpm in 37℃ in air. *S. aureus* treated with TSB with 0.5% Tween 80 used as positive control and TSB with 0.5% Tween 80 as a negative control. OD600 was measured to determine the growth of bacteria. All treatments were carried out in six replicates and the entire

experimental procedure was repeated three times.

Confocal laser scanning microscopy

The planktonic inhibition was confirmed with confocal laser scanning microscopy [323]. Briefly, one drop of cells from above was spotted on the glassslide and left to air dry. Cells were then stained with a LIVE/DEAD BacLight Bacterial Viability Kit (Life Technologies Australia, Mulgrave, Victoria, Australia) according to the manufacturer's instructions. The stained cells were examined at 20x magnification using a confocal laser scanning microscope (Zeiss LSM700, Carl Zeiss AG, Oberkochen, Germany). The experiment was repeated three times.

Biofilm assay

Black 96-well microplates (Costar; Corning Incorporated, Corning, NY, USA) were used to form biofilms. The microplates containing 150µl of *S. aureus* (1McF of bacterial suspension in 0.9% saline diluted 1:15 with TSB) were incubated for 48 hours at 37ºC on a gyratory shaker at 70rpm to allow for biofilm formation. TSB was used as a negative control. After 48 hours, biofilms were rinsed three times with 1x phosphate-buffered saline (PBS) to remove planktonic bacteria. S. *aureus* biofilm was then treated with *C. accolens* planktonic and biofilm conditioned supernatants (in 20% - 100% v/v) for 24 hours.

S. aureus biofilms treated with TSB only were used as a positive control. After treatment, the viability of the biofilms was determined by alamarBlue (Sigma- Aldrich, St. Louis, MO, USA) [278]. Briefly, the microplates were incubated with 200µl diluted (1:10 ratio) of alamarBlue at 37ºC for three hours, and the fluorescence intensity of the samples was measured using a FLUOstar Optima 96-well fluorescence microplate reader (BMG Labtech, Ortenberg, Germany). All assays were carried out in six replicates and the experiment was repeated three times.

Cell culture assays with primary human nasal epithelial cells (HNECs)

Primary human nasal epithelial cells

HNECs were cultured as previously described [31, 324]. Briefly, extracted HNECs were treated with anti-CD68 (Dako, Glostrup, Denmark) at 37ºC for 20 min to remove macrophages. Then HNECs were seeded in collagen-coated T75 cell culture flasks (Corning Incorporated, NY, USA) and grown in Ex- medium consisting of PneumaCult™-Ex Plus Basal Medium (STEMCELL Technologies, Tullamarine, VIC, Australia), PneumaCult™-Ex Plus 50x Supplement (STEMCELL Technologies, Tullamarine, VIC, Australia), and penicillin-streptomycin (Thermo Scientific, Walthman, MA, USA). The seeded HNECs were incubated at 37℃ with a 95% humidity incubator supplied with 5% CO_2 and inspected daily under light microscopy.

Air liquid interface culture

Once HNECs achieved 80-100% confluence, cells were detached by treating with 0.05% trypsin (Thermo Scientific, Waltham, MA, USA) and were neutralized with 10% fetal bovine serum in PBS. Cells were resuspended in Ex-medium after centrifugation. Cell suspensions were then seeded onto collagen IV-coated apical chambers of Transwells, onto polyester membranes with a pore size of 0.4µm (BD Biosciences, San Jose, California, USA). 500µl Ex-medium was added in the basolateral chamber. Cells were given two days to settle and the medium from the apical chamber was removed completely and the basolateral chamber medium was changed to PneumaCult™-ALI Basal Medium (STEMCELL Technologies, Tullamarine, VIC, Australia); PneumaCult™-ALI 10X Supplement; penicillin-streptomycin/amphotericin B(Thermo Scientific, Walthman, MA, USA); PneumaCult™-ALI Maintenance Supplement (STEMCELL, Vancouver, Canada). The basolateral chamber medium was changed every three days. The cells were cultured for 17 up to 21days.

Trans-epithelial electrical resistance (TER)

TER was measured by using an EVOM volt-ohmmeter (World Precision Instruments, Sarasota, FL, USA). Briefly, 100µl of B-ALI medium was added tothe apical chamber of ALI cultures to form an electrical circuit across the cell monolayer and into the basal chamber. Cultures were maintained at 37°C during the measurement period using a heating platform (LEC Instrument, Australia). Only wells displaying baseline resistance readings greater than 700Ω/cm2 were used for the experiments. *S. aureus* and *C. accolens* co-cultured conditioned media in different ratios were added to the apical chambers of each Transwell and TER measurements were obtained at times 0, 0.5, 1, and 2h. TSB and 2% Triton X-100 (Promega, Madison, WI, USA) were tested alongsidethe negative control and positive control respectively. The experiment was repeated three times.

FITC-Dextran Permeability Assay

Paracellular permeability was assessed by measuring the apical-to-basolateralflux of FITC-Dextran 4kDa (Sigma, Saint Louis, USA). Briefly, after treating thecells for two hours, the apical chambers were filled with 3 mg/ml of FITC - Dextran and incubated for two hours at 37°C. Samples were then taken from the basolateral compartment and transferred to a clear bottom black 96-well plate (Corning-Costar Corp., Cambridge, United Kingdom), and the fluorescence was measured with a FLUOstar Optima 96-well fluorescence microplate reader (BMG Labtech, Ortenberg, Germany) at excitation and emission wavelengths of 485nm and 520nm. The experiment was repeated three times.

Immunofluorescence staining

Cells were fixed with 2.5% formalin in PBS for 10min on the ice. Fixed samples were permeabilized with 1 % sodium dodecyl sulfate (SDS) for 10 min on ice. The permeabilized cells were then blocked for one hour with a serum-free blocker (Dako, Glostrup, Denmark) at

room temperature (RT). Cells were incubated with primary antibody (Rabbit anti-claudin-1(1:50; Invitrogen, Carlsbad, CA, USA); Mouse anti-zonula occludens (ZO)-1(1:100; Invitrogen, Carlsbad, CA, USA)) overnight at 4ºC. After washing, cells were then incubated with secondary antibody (Donkey anti-rabbit Cy3 (1:200; Jackson ImmunoResearch Labs Inc., West Grove, PA, USA); Donkey anti-mouse IgG Alexa Fluor 488 (1:200; Jackson ImmunoResearch Labs Inc., West Grove, PA,USA)) for one hour at RT. 200ng/ml of 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, MO, USA) for 10min at RT. Cells were then mountedwith a fluorescence anti-fade mounting medium (Dako, Glostrup, Denmark) and were covered with coverslips. Images were examined with a confocal laser- scanning microscope LSM700 (Zeiss Microscopy, Jena, Germany) and imageswere processed with ZEN Imaging Software (Carl Zeiss AG, Oberkochen, Germany). The experiment was performed in triplicates.

Cell Cytotoxicity Assay

The medium was collected from the basal chambers of each sample following the last TER measurements (2 hours) and cytotoxicity was determined using the lactate dehydrogenase (LDH) release kit (Promega, Madison, WI, USA) according to the manufacturer's instructions [22]. Briefly, 50µl of the medium from each condition was transferred to a new plate, and 50µl of LDH reagent was added to the supernatant and incubated for 30min in the dark at room Tº. Absorbance was read using a microplate reader at 490nm (BMG Labtech, Ortenberg, Germany). The relative viability was calculated relative to the LDH levels of negative controls and positive controls. The experiment was performedin triplicates.

Enzyme-linked immunosorbent assay (ELISA)

The medium was collected from the basolateral compartment of treated HNEC - ALI cultures after exposure with bacteria-conditioned media. Interleukin-6 (IL-6)and Interleukin-8 (IL-8)

levels were estimated with an ELISA kit (BD Biosciences,New Jersey, USA), according to the manufacturer's instructions. All measurements were performed in triplicates using a FLUOstar OPTIMA plate reader (BMG Labtech, Ortenberg, Germany). The IL-6 and IL-8 concentration was calculated from a standard curve and corrected for protein concentration.

Statistical analysis

GraphPad Prism 9.0 (San Diego, CA, USA) was used for statistical analysis. One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test or Tukey's multiple comparisons test was used to compare the differences between multiple groups. A *P-value* of < 0.05 was considered as statistically significant.

6.5 Results

Antibacterial effects of *C. accolens* conditioned media against planktonic and biofilm *S. aureus*

To understand if *C. accolens* conditioned media can inhibit *S. aureus* planktonic growth, three *C. accolens* strains (C1 to C3) were selected and their planktonicexoproteins were harvested. The *S. aureus* clinical isolate and ATCC51650 were treated with different concentrations of *C. accolens* exoproteins (20% to 90% v/v). The supernatants of all 3 tested *C. accolens* strains were shown to significantly inhibit the growth of planktonic *S. aureus* in a dose-dependent way. There was significant growth inhibition of the reference strain at > 50% v/v andof the *S. aureus* clinical strain at 30% v/v (Figure 6.1a). This was further confirmed using LIVE/DEAD BacLight Bacterial Viability staining (Figure 6.1b). Biofilm growth of either the reference or clinical strain was not inhibited by the same concentrations of *C. accolens* conditioned media (Supplementary Figure 6.1).

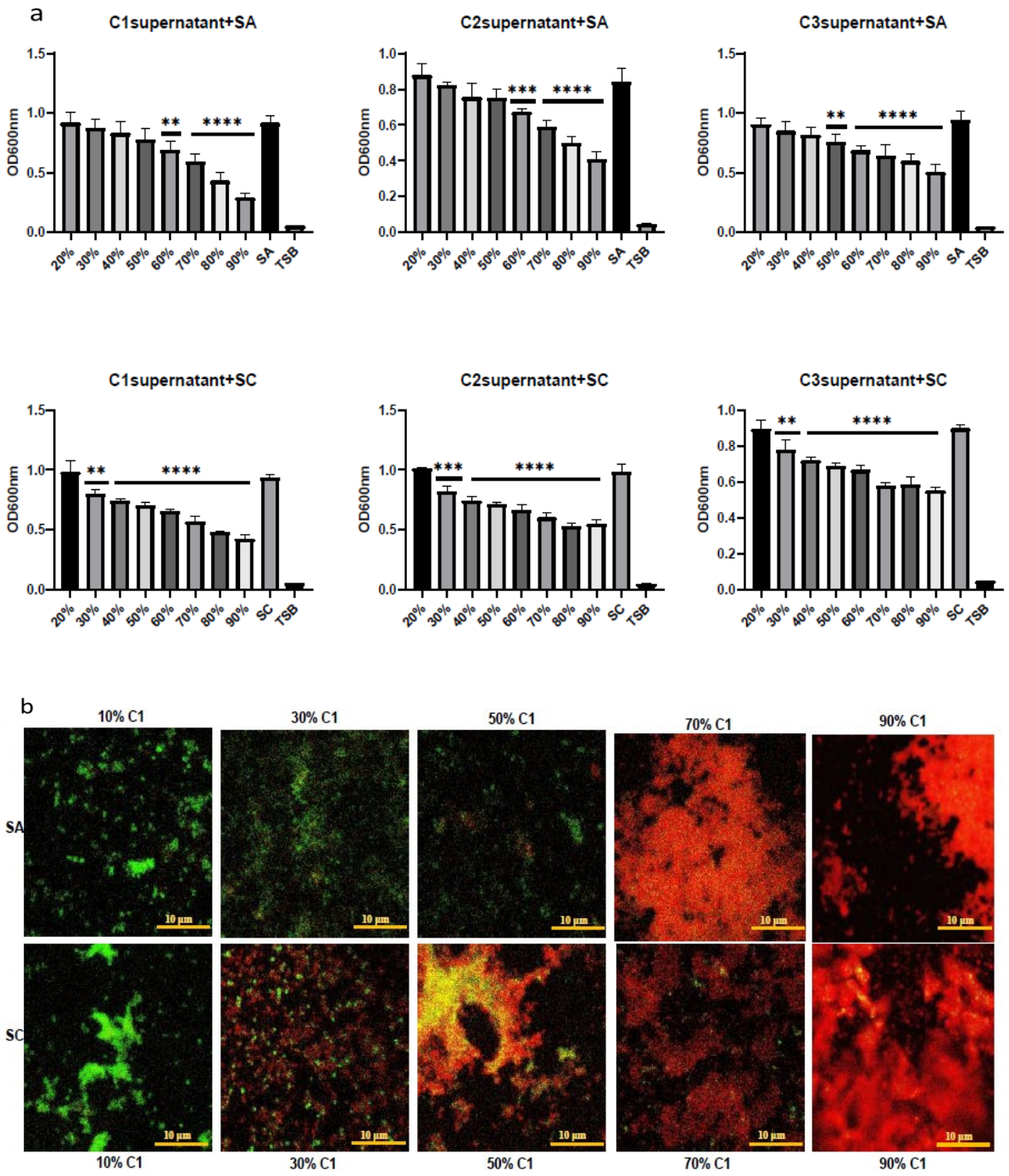


Figure 6.1. Antibacterial effects of *C. accolens* supernatants against planktonic *S. aureus*. Different concentrations of supernatants from 3 clinical isolates of *C. accolens* were added to treat planktonic SA and SC. OD600 was measured to determine the bacterial growth (a). LIVE/DEAD BacLight Bacterial Viability stainingof *S. aureus* planktonic bacteria was used to show dead (PI, red) and live (SYTO® 9, green) bacteria in samples representative of each treatment group (b). C1= *C. accolens* supernatant exoprotein from *C. accolens* clinical isolate

1, C2= *C. accolens* supernatantexoprotein from *C. accolens* clinical isolate 2, C3= *C. accolens* supernatant exoproteinfrom *C. accolens* clinical isolate 3, SA= *S. aureus* ATCC 51650, SC= *S. aureus* clinicalstrain. **: $P < 0.01$, ***: $P < 0.001$, ****: $P < 0.0001$. Experiments were performed three times.

Effect of *C. accolens* on *S. aureus* exoprotein secretion

To assess if *C. accolens* had any effect on *S. aureus* total exoprotein secretion, one *S. aureus* clinical isolate and *S. aureus* ATCC51650 were co-cultured with different numbers of *C. accolens* bacteria. Both planktonic and biofilm conditioned media were harvested and proteins quantified with no significant difference detected suggesting that *C. accolens* did not affect the overall *S. aureus* exoprotein secretion (Supplementary Figure 6.2).

Effect of *C. accolens* on *S. aureus* induced barrier disruption

Trans-epithelial electrical resistance (TEER)

HNECs from 3 control patients and 3 CRSsNP were used to examine the effectof bacterial conditioned media on the integrity and transcellular permeability of HNEC-ALI cultures. *C. accolens* conditioned media did not affect the TEER, while the reference and clinical strains of *S. aureus* conditioned media reducedthe TEER significantly within 15 minutes. Conditioned media from the mixed *C.accolens* - S. *aureus* cultures induced a time and dose-dependent reduction in TEER. With a higher number of the *C. accolens* (70% and 90%), a statistically significant reduction in TEER took one hour to occur, while at lower numbers of *C. accolens* (50%), a significant reduction in TEER occurred within 30 minutes (Figure 6.2).

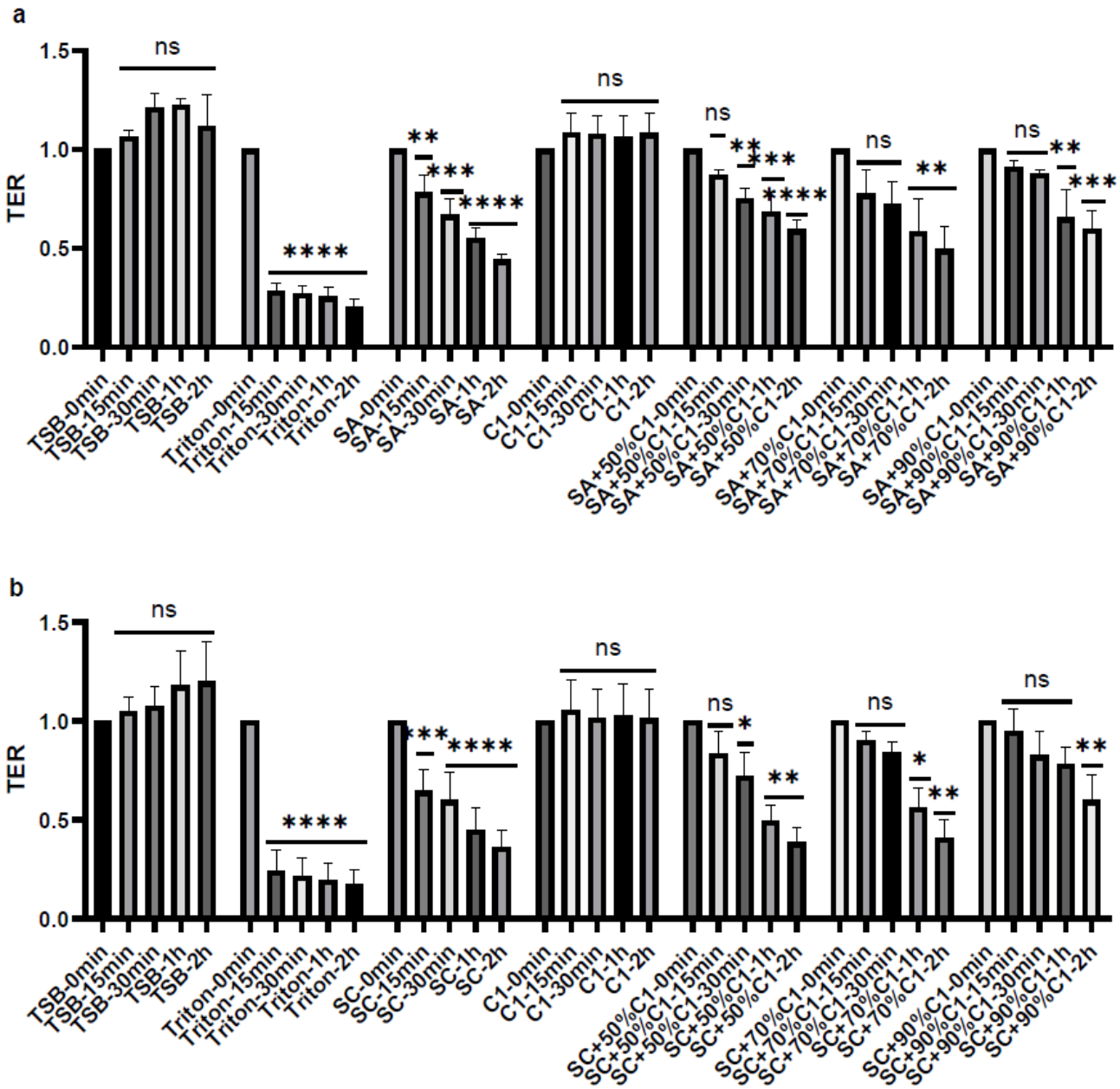


Figure 6.2. TER Assay. The exoprotein of SA (a) or SC (b) co-cultured with *C. accolens* in different ratio were added to HNECs, and TER measurements were obtained. C1= *C. accolens* clinical isolate 1, SA= *S. aureus* ATCC51650, SC= *S. aureus* clinical strain. ns: $P > 0.05$, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, ****: $P < 0.0001$. Experiments were performed three times.

FITC-Dextran Permeability Assay

To examine the effect of bacterial conditioned media on the paracellular permeability of the epithelial cell layer, a FITC-Dextran Permeability Assay was performed after exposure of HNEC-ALI cultures to the supernatants of three clinical isolates of *C. accolens* and the reference and clinical strains of *S. aureus* co-cultured conditioned media after two hours. The supernatant of *C. accolens* appeared to have no significant effect on the paracellular permeability of the HNEC-ALI cultures when applied alone. Both the reference and clinical strains of *S. aureus* significantly increased the permeability of the epithelial celllayer. When the co-cultured *S. aureus* and *C. accolens* conditioned media were applied, a higher ratio of *C. accolens* (90%) resulted in a higher reduction of *S.aureus*-dependent effects on paracellular permeability than when a lower ratio (50%) of *C. accolens* was applied (Figure 6.3).

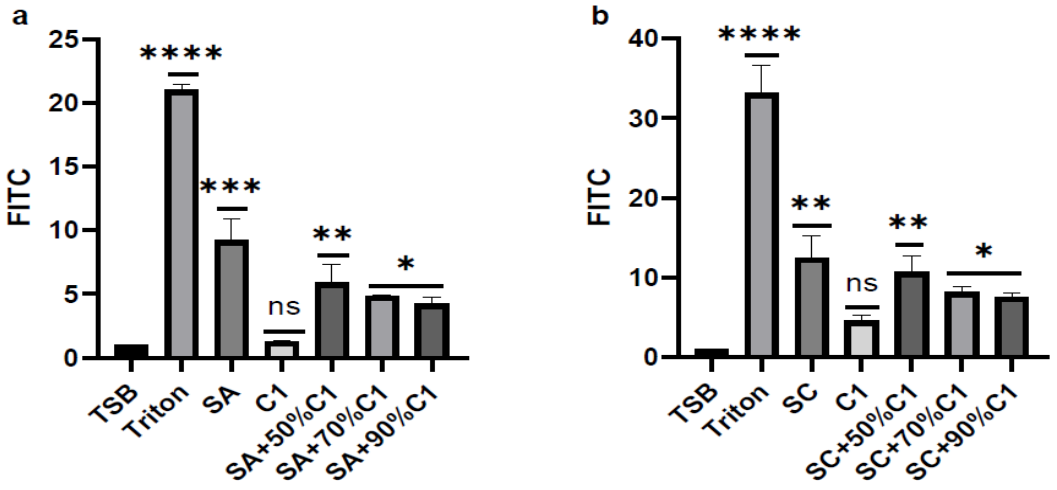


Figure 6.3. FITC Assay. The FITC-Dextran were added to HNECs after two hours treatment with the supernatants in different ratio's, fluorescence was measured. C1= *C.accolens* clinical isolate 1, SA= *S. aureus* ATCC51650, SC= *S. aureus* clinical strain. ns: $P > 0.05$, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, ****: $P < 0.0001$. Experiments were performed three time.

6.1.1.1 Immunofluorescence staining

Zonula Occludens-1 (ZO-1) and claudin-1 immunofluorescence were evaluated after the application of the various bacterial co-cultured supernatants to test their effect on the tight junction protein expression and immune localization. *C.accolens* conditioned media had no significant effect on either tight junction protein (Figure 6.4). The exoproteins from both the reference and clinical strains of *S. aureus* significantly reduced the ZO-1 and claudin-1 expression. Furthermore, as the number of *C. accolens* increased during the co-culture, the effect of the *S. aureus* and *C. accolens* co-cultured conditioned media on boththight junction proteins decreased.

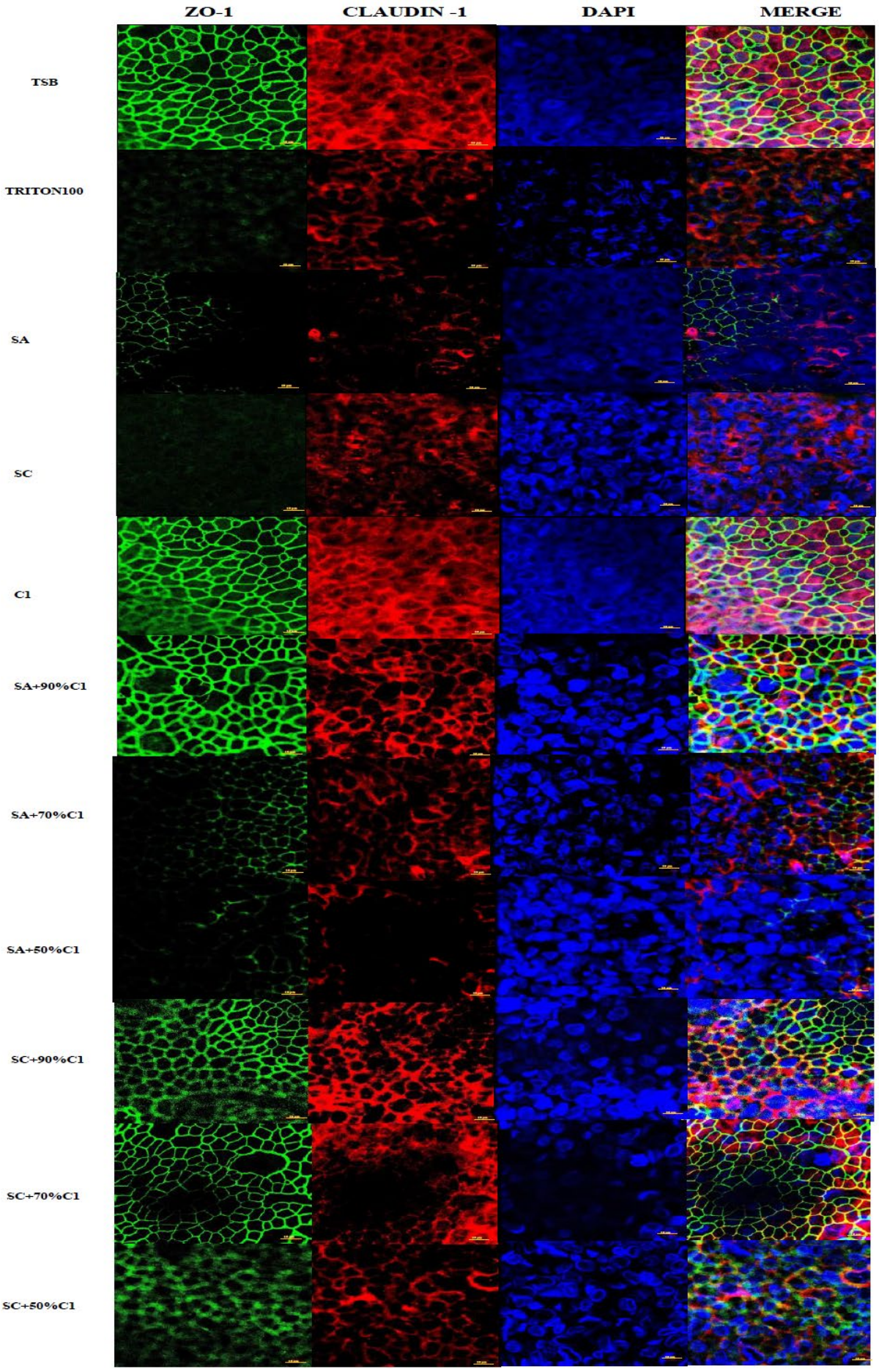

ZO-1
CLAUDIN -1
DAPI
MERGE
TSB
TRITON100
SA
SC
C1
SA+90%C1
SA+70%C1
SA+50%C1
SC+90%C1
SC+70%C1
SC+50%C1

Figure 6.4. Immunofluorescence staining of HNECs after treatment. The effects of exoprotein of SA and SC co-cultured with *C. accolens* in different ratio's on ZO-1 and claudin-1 expression. ALI cultured cells were stained with antibodies to ZO-1 (green), claudin-1 (red) and DAPI to resolve nuclei (blue). TSB was used as the negative control.Triton-100 was used as the positive control. Images were examined with confocal laser- scanning microscope (Scale bar=10 μm). C= *C. accolens*, SA= *S. aureus* ATCC51650, SC= *S. aureus* clinical strain.

Cell Cytotoxicity Assay

Using LDH as a surrogate marker for cytotoxicity, no significant cytotoxicity was observed for the HNEC-ALI cultures following exposure to the conditioned media of either *C. accolens or S. aureus* when applied alone or together (Supplementary Figure 6.3).

Inflammatory Mediator production

Using ELISA to quantitate IL-6 and IL-8 release from HNECs exposed to conditioned media, we observed no significant increase in either IL-6 or IL-8 secretion with *C. accolens*. A significant increase was observed with exposureto both the reference and clinical strains of *S. aureus;* lower rates of both IL-6 and IL-8 secretion were observed when *S. aureus* was co-cultured with various concentrations of *C. accolens* (Figure 6.5a and b).

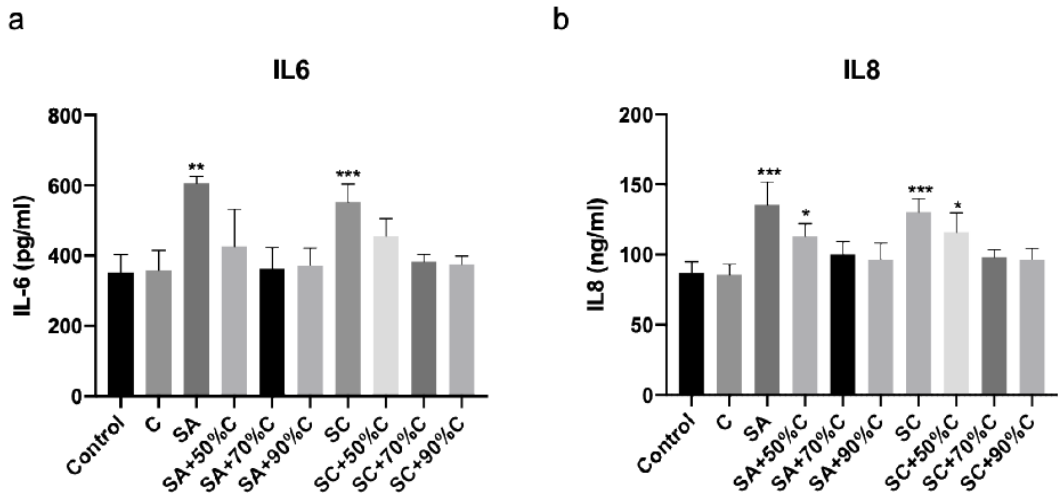


Figure 6.5. IL-6 and IL-8 ELISA Assay. The IL-6 (a) and IL-8 (b) of HNECs after treatment with the exoprotein of SA and SC co-cultured with *C. accolens* in different ratio were determined. C= *C. accolens*, SA= *S. aureus* ATCC51650, SC= *S. aureus* clinical strain. Experiments were performed with three replicates. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

6.6 Discussion

C. accolens is typically considered a commensal bacterium in the sinuses of healthy patients. It is hypothesized to have a "gate-keeping" function against pathogenic bacteria including *S. aureus* although a mechanistic link has not yet been established [124]. Previous microbiota research in our department has shown that while *Corynebacterium* and *Staphylococcus* species are among the most common organisms isolated from the sinuses of both healthy and CRS patients, CRS tend to exhibit a relative reduction in *Corynebacterium* load and expansion of *Staphylococcus* species in the diseased state [225]. This study provides the direct impact that *C. accolens* exoprotein has on *S. aureus* growth and exoprotein activity *in vitro*.

C. accolens is a lipid-requiring species, with Bomar et al. [127] finding *C. accolens* to grow more robustly within Brain Heart Infusion (BHI) broth supplemented with 100ug/ml of either triolein or trilinolein, but poorly, if at all, within BHI broth supplemented with solvent alone.

Consistent with Bomars' study, we also found *C. accolens* to grow better in TSB containing 0.5% Tween 80 rather than TSB alone [268]. This is most likely due to the lipid supplementation provided by tween 80. Interestingly, we also showed *S. aureus* to grow well in TSB (containing 0.5% Tween 80), suggesting that both organisms can exist in similar *in vitro* conditions. This may be supported by earlier work by Yan et al [124] who also found that *C. accolens* growth could be co-cultured with *S. aureus.*

In this study, *C. accolens* conditioned media could directly affect the planktonic growth of both the reference and clinical strains of S. *aureus.* This is consistent with the findings of Menberu et al which showed that *C. accolens* exhibited antimicrobial activity against *S. aureus* and MRSA CIs in both planktonic and biofilm forms [278]. This supports the findings of other studies that demonstrate a negative correlation between *S. aureus* abundance and *Corynebacterium* abundance [200, 325, 326] and may explain the typical lack of *S. aureus* expansion inthe non-diseased state. It also supports the hypothesis of the "gate-keeping" function of this commensal bacteria. Interestingly, we did not observe the sameeffect of the *C. accolens* supernatant on *S. aureus* biofilm growth, suggesting that the biofilm structure confers some protection against the *C. accolens* supernatant. The resistance of biofilms against naturally occurring and synthetic therapeutic agents has been well documented and explains their association with the chronic disease state [117, 327].

Consistent with the reported commensal role of *C. accolens* [328, 329], we did not observe any detrimental effects of its supernatant on epithelial integrity, membrane permeability, or cellular viability. This was in contrast with the supernatant of *S. aureus* which resulted in marked reductions in TEER and increases in FITC-Dextran permeability when applied to HNECs in our study. This is in concordance with previous studies which demonstrate a detrimental effect of *S. aureus* exotoxins on sinonasal epithelium [330, 331]. Intracellular localization of S. *aureus*

in patients with CRS has been previously reported and has been shown to be associated with poor prognostic features and treatment resistance [332-334]. We hypothesize that it is through their exoprotein secretion that *S. aureus* bacteria not only exert their pathogenic effect but also disrupt thenormal barrier function and gain access to their intracellular location to facilitate disease persistence.

A novel and important finding of this study also supports the proposed protective action of *C. accolens*, which was the ratio-dependent increase in time to epithelial disruption and membrane permeability when *S. aureus* was co- cultured with the *C. accolens.* It appeared that the more of the *C. accolens* present, the longer it took for the *S. aureus* supernatant to cause significant damage. This suggests that protective factors secreted by *C. accolens* can dampen the inflammation caused by *S. aureus*. This is further supported by the reduction in the release of IL-6 and IL-8, potent inflammatory cytokines, observed when the supernatant of *S. aureus* was co-cultured with the *C. accolens.* Further proteomic and molecular work is now needed to identify what exactly these factors are.

Although this study is limited by its *in vitro* study design, a small sample of clinical isolates tested, and lack of functional testing, these findings do support the consistent observation of both clinical and microbiota sinus studies; the association of *S. aureus* with more severe disease and the collapse of the diverse healthy microbiota in patients with CRS [113, 277, 335]. Furthermore, we report for the first time a mechanistic link by which commensal bacteria like *C. accolens* keep pathogenic bacteria in check, through the secretions of their exoproteins. Reduction in the number of these gate-keeping bacteria may therefore be implicated as an inciting event in the pathogenesis of CRS.

6.7 Conclusion

In conclusion, this study demonstrated that *C. accolens* supernatant can inhibitthe growth of *S. aureus* planktonic bacteria, and also the deleterious effects of *S. aureus* exoproteins on the sinonasal epithelium. Further research is needed to better characterize the proteins that mediate their effect and their precise mode of action.

Funding

The author(s) disclosed receipt of the financial support from Conjoint grant from The Garnett Passe and Rodney Williams Memorial Foundation to AJP.

Competing interests

The author(s) declared no potential conflicts of interests.

CHAPTER 7

General discussion, conclusion and future research perspectives

CHAPTER 7: General discussion, conclusion and future research perspectives

The significant amount of research on the microbiome in recent years has led to a more-robust understanding of the microbiome and its role in human health. The complex microbiota-microbiome and microbiome-host interactions are dynamic, involving a variety of mechanisms and makes vital contributions to energy homeostasis, metabolism, epithelial health, immunologic activity, and neurodevelopment [102].

Microbiome research in the field of otolaryngology is still in its infancy but rapidly growing. In recent times, nasal microbiota alterations or dysbiosis in the sinus mucosa and paranasal sinuses have been evidenced extensively in patients with CRS [119]. Despite many available therapeutics, CRS due to microbial ecosystem imbalance remains a great public health problem worldwide [336]. Given increasing evidence on the persistent reduction in relative abundance of Actinobacteria, mainly *Corynebacterium* and an overgrowth of Firmicutes, mainly *Staphylococcus* in CRS, manipulation of the nasal microbiota could be a novel strategy for the reestablishment of nasal homeostasis.

The pivotal role of nasal microbiota in the function and integrity of sino-nasal mucosa, protection against pathogens, maintenance of host immunity and homeostasis [337], emphasizing the importance of targeting beneficial microbes, particularly a lipophilic *Corynebacterium* and associated metabolites for therapeutic development. This aspect coupled with the potential impact of nasal microbiota manipulation on clinical medicine opens up the opportunity for developing alternative microbiome-based therapeutics such as prebiotics and probiotics to improve microbe - microbe and host - microbe balance in CRS. The main purpose of the research presented in this thesis was to investigate the prebiotic activity of a free fatty

acid containing compound, Tween 80 and the probiotic properties of healthy *Corynebacterium* nasal isolate, specifically *C. accolens* using *in vitro* and *in vivo* approaches.

In chapter 2 of this thesis, the growth stimulatory activity of a common excipient Tween 80 and its free fatty acid Oleic acid on nasal commensals and their anti-bacterial and ant-biofilm activity against nasal pathogens was investigated. Tween 80 is a mono-unsaturated fatty acid (Oleic acid) derivative commonly used as an excipient in nasal formulations and growth promoter in laboratory media particularly for lipophilic microorganisms [157, 338]. The results in this study demonstrate that both Tween 80 and oleic acid at the FDA approved concentrations of 0.5% and below stimulated the planktonic growth of nasal commensal, *C. accolens* and reduced growth of *S. aureus* planktonic cells and newly forming biofilms. More than 50% of bacterial colonization in CRS is demonstrated in form of biofilms largely by *S. aureus* and are associated with the recalcitrant condition and disease severity [85, 339, 340]. High concentration of fatty acid containing compounds, including those tested in this study, have been shown to possess anti-bacterial and anti-biofilm property against pathogens [160], but this current work reported no significant impact on already established *S. aureus* and *P. aeruginosa* biofilms. I also show the growth stimulatory activity of low Tween 80 and Oleic acid concentrations on commensal *C. accolens* in an established mixed *C. accolens* and *P. aeroginosa* biofilms. Overall, these data suggest the potential of low Tween 80 and oleic acid concentrations to be used as prebiotic by stimulating the commensal *C. accolens* growth.

Tween 80 can enhance growth of various beneficial microorganisms including the lactobacilli genus and protect cells against adverse environmental circumstances. These effects are actually related to the fact that the oleic acid moiety of Tween 80 can be integrated into the cell membrane, which disturbs cell membrane properties [158]. Besides, it was displayed that exogenous fatty acids can be directly incorporated into the cells, and that supplementation of

oleic acid limits fatty acid production in beneficial Lactobacilli, where low production of responsible enzymes may possibly play a role [149]. The underlying mechanism by which these fatty acid compounds influence a specific microbial growth is still unclear and should therefore be explored in future before clinical application.

The work presented in chapter 3 of this thesis demonstrates the anti-bacterial and anti-biofilm potential of *C. accolens* nasal isolates against pathogens. Antimicrobial activity is one of the primary requirements for the selection of potentially beneficial bacteria as probiotics [227]. Interestingly, cell free culture supernatants from selected *C. accolens* strains, C779, C781 and C787 also showed a concentration-dependent inhibition on planktonic as well as biofilm growth *S. aureus* clinical isolates tested. Biofilms are in fact, the main mediators for disease persistence and treatment failure in various chronic disorders including CRS [340]. The virulence of *S. aureus* can be heavily affected in response to commensal *Corynebacterium* including, *C. accolens*, *C. amycolatum* and *C. pseudodiphtheriticum* by shifting its virulence behaviour toward a commensal lifestyle [272]. However, it is particularly interesting to note that the antimicrobial activity of all tested *C. accolens* strains are more likely through the production of bioactive proteins, leading to identify and functionally characterize these molecules, the objective of the experiments in chapter 4 of this thesis.

In chapter 4, I have described several expressed proteins from *C. accolens* functionally linked to the antimicrobial activity and other probiotic properties. Indeed, Acetyltransferase, GNAT family protein, glycosyl hydrolase family 25 and N-acetylmuramoyl-L-alanine amidase showed a strong positive correlation with the observed anti-staphylococcal activity described in this chapter. In addition, higher relative abundance of putative esterase was identified across all *C. accolens* strains tested. This protein is well recognized in altering the cell wall & increasing cells tolerance towards harsh environments [301], which likely contributes to

survival and adhesion process of *C. accolens*. Overall, these proteins could hold the key to novel therapeutic development and commercialization.

It is essential to remark that the use of potential probiotic Corynebacteria cell cultures and culture supernatants was proposed as an approach to bypass the contrary effects of antibiotic therapy and to avoid the impact of antibiotic resistance established by the microorganism. Also it is noteworthy that the virulence and pathogenicity of *S. aureus* infections includes a biofilm-forming ability that allows serious recalcitrant CRS disorder. Using cultures bacterial cells and cell-free culture supernatants, we have shown that *C. accolens* nasal strains exhibit anti-microbial and anti-biofilm activities against *S. aureus* pathogens. Thus, nasal commensal strains of *C. accolens* could be utilized to combat microbiome dysbiosis and *S. aureus* associated CRS cases. However, future research is needed to identify and characterize more targeted antibacterial proteins across several *C. accolens* isolates. In this case, the focus would be on the possible synergistic effects of combining probiotic *C. accolens* supernatants with anti-Staphylococcal agents.The mechanism behind its antimicrobial activity also remains to be elucidated.

As described in chapter 5 of this thesis, *C. accolens* strains, C779, C781 and C787 are considered safe with good adhesion ability to HNECs and preventive effect on *S. aureus* mediated cytotoxicity and inflammation. It has been demonstrated previously that *Corynebacterium* species may compete at the same sites of *S. aureus* adhesion to epithelial cells [325]. The safety results in these *in vitro* assessments are consistent with previous reports for different isolates of *Corynebacterium* [341, 342]. Notably, *C. accolens* strain C781 displayed a higher worm survival rate with a reduction of *S. aureus* load in infected nematodes when compared to the other strains tested. A report from a previous study showed the ability

of some non-pathogenic bacteria and their metabolites to prolong *C. elegans* longevity, and are suggested as beneficial bacteria or probiotics [343].

The study described here is the first on the inhibitory effects of *C. accolens* on nasal pathogenic bacteria, mainly *S. aureus*. All tested *C. accolens* isolates fulfilled various criteria to be used as a probiotic microorganisms. Furthermore, those strains inhibited *S. aureus* planktonic growth and biofilm formation. Overall, these data support the probiotic indication of our tested *C. accolens* strains for safe use in nasal application. Hence, we suggest that those *C. accolens* strains may be appropriate probiotic isolates for the management of dysbiotic microbiota in CRS without adverse effects, but more in-depth pre-clinical and clinical studies are yet to be conducted before translation to the clinical practice.

Chapter 6 aimed to evaluate the interactions between *C. accolens* and *S. aureus* as well as their effect on the mucosal barrier resulted a beneficial property of *C. accolens* on host health with a remarkable anti-staphylococcal activity. Various beneficial bacteria can interact with pathogens in the complex microenvironment with the capacity to decrease inflammatory cytokines, epithelial permeability and pathogen growth [115, 198]. The finding indicates that exoproteins extracted from *C. accolens* was responsible for the protection of epithelial cell disruption, membrane permeability and inflammation caused by *S. aureus*. However, the specific mechanism of action by which these secreted metabolites mediate protection of the mucosal barrier remains to be investigated further.

In conclusion, the data generated in this thesis has offered the prebiotic potential of a free- fatty acid containing compound, Tween 80 in promoting growth of nasal commensal *C. accolens* and inhibiting pathobionts. Further, healthy *C. accolens* strains, in particular, C779, C781 and C787 and their metabolites showed a wide range of antimicrobial activity against various *S.*

aureus pathobionts and proved to be safe with robust protection against *S. aureus* induced infection and inflammation. This finding provides probiotic potential of *C. accolens* with biotherapeutic evidence that could help to manipulate a dysbiotic microbial ecosystem in CRS. With several sino-nasal microbiome studies underway supporting the benefits of prebiotics and probiotics, a comprehensive clinical evidence that can be translated into clinical practice is needed to ensure implementation. It is clearly point out that, nasal microbiota plays an imperative role in the health of the host, and contributes actively in the expansion of a wide-range of diseases. Therefore, the critical roles of the nasal microbiota should be explored at a much deeper level, and microbiome-based diagnosis and treatment approaches will be used for the development of future personalized medicine. Ultimately, the findings from this PhD together with future preclinical and clinical studies will provide novel insights not only for developing individualized prebiotic and probiotic therapy but also to pave the way for clinical translation and shape the sino-nasal microbial ecosystem in the face of dysbiosis.

APPENDICES

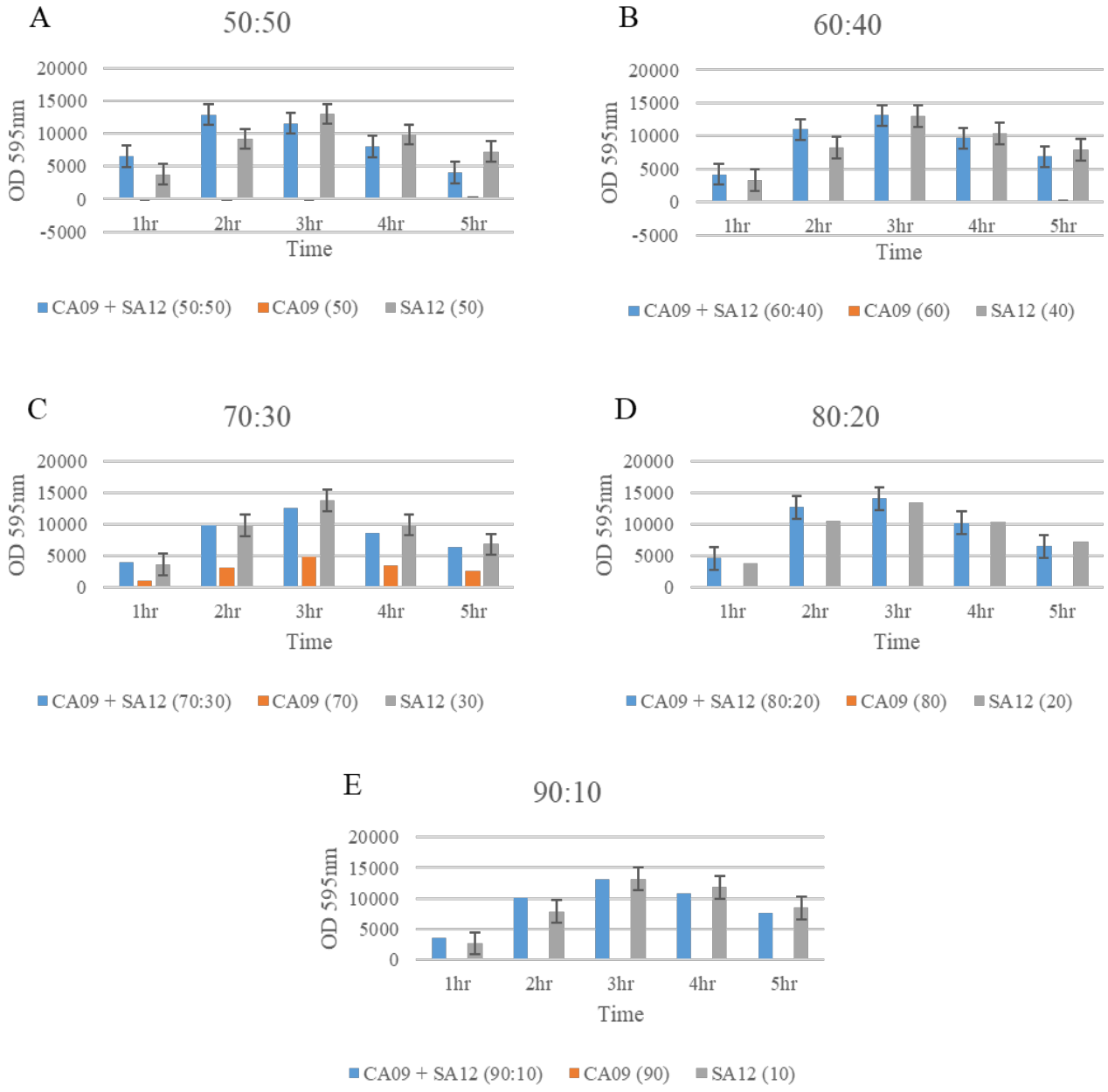
APPENDICES

Appendix I. Additional publications produced during my PhD candidature

1. Getachew A, Tadie A, G Hiwot M, Guadu T, Haile D, G Cherkos T, Gizaw Z, **Menberu MA**. Environmental factors of diarrhea prevalence among under five children in rural area of North Gondar zone, Ethiopia. *Italian journal of pediatrics*. 2018; 44(1):1-7.
2. Ali S, **Menberu MA**, Dagnew M, Gebrecherkos T. Vancomycin-resistant enterococci and its associated risk factors among HIV-positive and-negative clients attending Dessie referral hospital, Northeast Ethiopia. *International journal of microbiology*. 2018; 18; 2018.
3. Belyhun Y, Moges F, Endris M, Asmare B, Amare B, Bekele D, Tesfaye S, **Menberu MA**, Biadgelegne F, Mulu A, Assefa Y. Ocular bacterial infections and antibiotic resistance patterns in patients attending Gondar Teaching Hospital, Northwest Ethiopia. *BMC research notes*. 2018; 11(1):1-7.
4. Tsige Y, Tadesse S, Tefera MM, Amsalu A, **Menberu MA**, Gelaw B. Prevalence of Methicillin-Resistant *Staphylococcus aureus* and Associated Risk Factors among Patients with Wound Infection at Referral Hospital, Northeast Ethiopia. *Journal of pathogens*. 2020; 24; 2020.
5. Getachew A, Tadie A, Haile D, Guadu T, **Menberu MA**, Gizaw Z, G Hiwot M, G Cherkos T. Bacteriological Quality of Household Drinking water in North Gondar Zone, Ethiopia; a Community Based Cross-Sectional Study. *Applied Water Science*. 2021; 11:189.

Appendix II. Supporting information for Chapter 2

Additional material provided by the authors to supplement paper II written in manuscript format in Chapter 2.



Supplementary Figure 2.1: Co-existance assessment for *C. accolens* (CA09) and *S. aureus* (SA12) biofilms in a different ratios of *C.accolens*:*S. aureus* (A) 50:50; (B) 60:40; (C) 70:30; (D) 80:20 and (E) 90:10. Biofilm viability was determined by measuring the fluorescent intensity at OD 595nm. The experiments were conducted in at least three times. Data represents the average of the three replicates.

Appendix III. Supporting information for Chapter 3

Additional material provided by the authors to supplement a published research article in Chapter 3.

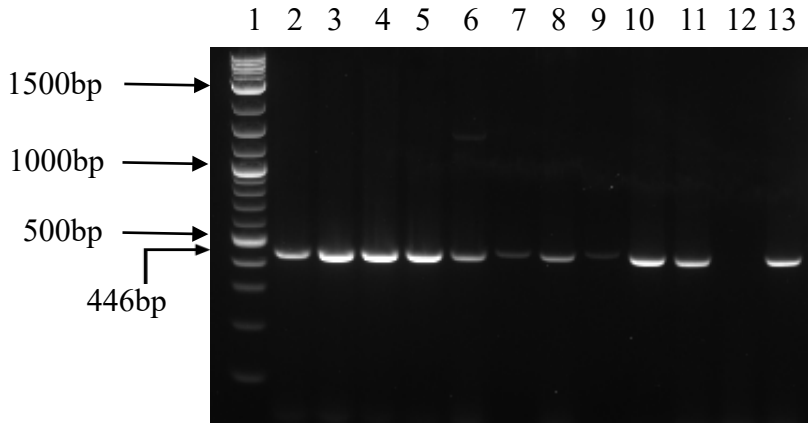
Supplementary Table 3.1. Description of oligonucleotides used for PCR amplification of *rpoB* gene in *C. accolens* isolates

Primer name	Primer sequence (5'-3')	Amplicon size (bp)
C2700F	5'-CGWATGAACATYGGBCAGGT-3'	446bp
C3130R	5'-TCCATYTCRCCRAARCGCTG-3'	

Supplementary Table 3.2. Pathogenic *S. aureus* strains (8MSSA and 8MRSA) isolated from the sinonasal cavity of CRS patients used in this study

Strain code number	Source	Strain property
S. aureus C329	CRS nasal swab	
S. aureus C262	CRS nasal swab	
S. aureus C314	CRS nasal swab	
S. aureus C124	CRS nasal swab	MSSA
S. aureus C5	CRS nasal swab	
S. aureus C26	CRS nasal swab	
S. aureus C319	CRS nasal swab	
S. aureus C71	CRS nasal swab	
S. aureus C300	CRS nasal swab	
S. aureus C310	CRS nasal swab	
S. aureus C292	CRS nasal swab	
S. aureus C295	CRS nasal swab	MRSA
S. aureus C261	CRS nasal swab	
S. aureus C24	CRS nasal swab	
S. aureus C54	CRS nasal swab	
S. aureus C38	CRS nasal swab	
S. aureus ATCC25923	Bacterial culture collection	

Abbreviations: CRS, Chronic rhinosinusitis; MSSA, methicillin sensitive *S. aureus*; MRAS, methicillin resistant *S. aureus*



Supplementary Figure 3.1: Identification of *Corynebacterium accolens* isolates by PCR amplification of partial *rpoB* gene (446-bp fragment). Lane 1: 1 kb plus DNA ladder. Lane 2-11: *rpoB* gene amplicon from *C. accolens* strains (C778 to C787). Lane 12: Negative control (5 µl of RNAse free water). Lane 13: Positive control (*C. accolens* ATCC49726).

Appendix IV. Supporting information for Chapter 4

Additional material provided by the authors to supplement paper written in manuscript format in Chapter 4.

Supplementary Table 4.1. Commonly expressed proteins (n=595) in six *C. accolens* strains

Accession Number	Protein description	Gene name	Average protein abundance
E0N0K3	Putative esterase	cmtC	1.29E+09
E0MZ10	NlpC/P60 family protein	HP↓	1.11E+09
E0MXB4	Glycosyl hydrolase family 25	HP↓	5.42E+08
E0MW06	Uncharacterized protein	HP↓	4.49E+08
E0MYQ9	Putative monovalent cation/H+ antiporter subunit C	mnhC	3.86E+08
E0MXC7	META domain-containing protein	HP↓	3.01E+08
E0MVM0	ErfK/YbiS/YcfS/YnhG	HP↓	2.90E+08
E0MVI7	Periplasmic binding protein	fecS	2.41E+08
E0N0D0	Chaperone protein DnaK	dnaK	2.32E+08
E0MV38	Dihydrolipoyl dehydrogenase	lpdA	1.99E+08
E0MXY2	NlpC/P60 family protein	HP↓	1.95E+08
E0MVG2	Elongation factor Tu	tuf	1.94E+08
E0N068	Hydrolase, alpha/beta domain protein	HP↓	1.83E+08
E0MWT6	Enolase	eno	1.79E+08
E0N017	Uncharacterized protein	HP↓	1.66E+08
E0MXJ7	Putative ribosomal protein S1	rpsA	1.37E+08
E0N0C6	Aldehyde dehydrogenase (NAD) family protein	aldA2	1.31E+08
E0MZU3	Cysteine synthase	cysK	1.29E+08
E0MWL3	Trypsin	HP↓	1.27E+08
E0MZN7	Periplasmic binding protein	hmuT2	1.27E+08
E0MW05	Uncharacterized protein	HP↓	1.26E+08
E0MWE4	Transglycosylase-like domain protein	rpfA	1.26E+08
E0MXA0	ATP synthase subunit beta	atpD	1.15E+08
E0MZ09	NlpC/P60 family protein	HP↓	1.12E+08
E0N0M2	N-acetylmuramoyl-L-alanine amidase	csp	1.09E+08

E0N072	60 kDa chaperonin	groL	9.51E+07
E0MWJ8	Peptidase, M23 family	HP↓	9.20E+07
E0MV37	Putative esterase	cmt	9.19E+07
E0MUU3	Trypsin	HP↓	8.95E+07
E0MY12	Transaldolase	tal	8.48E+07
E0MWN3	Transglycosylase-like domain protein	HP↓	8.34E+07
E0MZ62	Oxidoreductase, FAD/FMN-binding protein	nemA	7.85E+07
E0MYM9	Elongation factor Ts	tsf	7.69E+07
E0MUK2	Ferritin	ftn	7.68E+07
E0MX98	ATP synthase subunit alpha	atpA	7.64E+07
E0MW07	Uncharacterized protein	HP↓	7.63E+07
E0MYU3	Uncharacterized protein	HP↓	7.24E+07
E0MW12	Uncharacterized protein (Fragment)	HP↓	6.79E+07
E0MY17	Triosephosphate isomerase	tpiA	6.66E+07
E0MZR6	Ribonucleoside-diphosphate reductase subunit beta	nrdF2	6.64E+07
E0MZU7	Succinate CoA transferase	actA	6.63E+07
E0MY68	Endolytic murein transglycosylase	mltG	6.56E+07
E0MW65	Phosphoglucomutase/phosphomannomutase, alpha/beta/alpha domain II	manB2	6.51E+07
E0MWD3	Periplasmic binding protein	feuS	6.50E+07
E0MZ17	Cytochrome aa3 subunit 2	ctaC	6.46E+07
E0MV56	Uncharacterized protein	HP↓	6.19E+07
E0MYA8	Uncharacterized protein	HP↓	6.12E+07
E0MYB4	Chlorite O(2)-lyase	HP↓	5.95E+07
E0MYM7	Ribosome-recycling factor	frr	5.88E+07
E0MYE3	Alkyl hydroperoxide reductase AhpD	ahpD	5.54E+07
E0MZ23	Probable cytosol aminopeptidase	pepA	5.53E+07
E0MY19	Glyceraldehyde-3-phosphate dehydrogenase	gap	5.43E+07
E0MY18	Phosphoglycerate kinase	pgk	5.24E+07
E0N0K4	Putative esterase	HP↓	5.14E+07
E0MZK0	Malate dehydrogenase	mdh2	4.94E+07
E0MUN5	Peptidase family M13	pepO	4.93E+07
E0N0P4	Superoxide dismutase	sodA2	4.75E+07
E0MVT3	NLPA lipoprotein (Fragment)	metQ	4.74E+07
E0MUH2	Penicillin-binding protein, transpeptidase domain protein	HP↓	4.68E+07
E0MVM5	DNA-directed RNA polymerase subunit alpha	rpoA	4.60E+07
E0MWC1	Gram-positive signal peptide protein, YSIRK family	HP↓	4.59E+07

E0MW04	Carboxyl transferase domain protein	accD	4.53E+07
E0N0J0	Secretory lipase	HP↓	4.49E+07
E0MUV6	HtaA domain protein	HP↓	4.37E+07
E0MZM3	Alanine dehydrogenase	ald	4.29E+07
E0MZZ7	Response regulator receiver domain protein	HP↓	4.24E+07
E0MYS5	Pyruvate kinase	pyk	4.00E+07
E0MYW1	Uncharacterized protein	HP↓	3.98E+07
E0N0I3	Phosphoenolpyruvate carboxykinase [GTP]	pckA	3.96E+07
E0MVR5	60 kDa chaperonin	groL	3.90E+07
E0MYS1	Glutamate dehydrogenase	gdhA	3.86E+07
E0MVD4	Periplasmic binding protein	HP↓	3.75E+07
E0MWS7	Uncharacterized protein	HP↓	3.66E+07
E0MVH4	Uncharacterized protein	HP↓	3.63E+07
E0MZL0	Trigger factor	tig	3.56E+07
E0MVX2	DJ-1/PfpI family protein	HP↓	3.47E+07
E0MY61	Creatinase	yqhT	3.33E+07
E0MY40	Uncharacterized protein	HP↓	3.32E+07
E0MYX5	Antigen 84	wag	3.26E+07
E0MWS0	Ribose-phosphate pyrophosphokinase	prs	3.24E+07
E0MWF4	Citrate (Si)-synthase (Fragment)	gltA3	3.23E+07
E0MWF7	Oxidoreductase, aldo/keto reductase family protein	dkgB	3.10E+07
E0MYW2	Uncharacterized protein	HP↓	3.03E+07
E0N019	Uncharacterized protein	HP↓	2.94E+07
E0N0J9	AMP-binding enzyme (Fragment)	fadD4	2.92E+07
E0MVS2	GMP synthase [glutamine-hydrolyzing]	guaA	2.91E+07
E0MYL7	Mycothione reductase	mtr	2.89E+07
E0MU99	Succinate-semialdehyde dehydrogenase	gabD	2.80E+07
E0MZ74	CRISPR system CASCADE complex protein CasC	casC	2.79E+07
E0MVT2	Uncharacterized protein (Fragment)	HP↓	2.75E+07
E0MV29	SGNH_hydro domain-containing protein	HP↓	2.74E+07
E0MVD5	Phosphoglucomutase/phosphomannomutase, alpha/beta/alpha domain II	manB	2.73E+07
E0MYE4	Antioxidant, AhpC/TSA family	dirA	2.71E+07
E0MUY9	Catalase	cat	2.71E+07
E0MV30	Mycothiol-dependent formaldehyde dehydrogenase	HP↓	2.68E+07
E0N0B1	Fructose-bisphosphate aldolase	fbaA	2.64E+07
E0MYW8	Uncharacterized protein	HP↓	2.60E+07

E0MVH8	50S ribosomal protein L23	rplW	2.58E+07
E0N0M1	UDP-galactopyranose mutase	glf	2.56E+07
E0MWA8	Putative hydrolase	HP↓	2.53E+07
E0MU65	50S ribosomal protein L9	rplI	2.47E+07
E0MW59	Cell envelope-like function transcriptional attenuator common domain protein	HP↓	2.46E+07
E0MZ29	Glycine cleavage system H protein	gcvH	2.37E+07
E0MZZ8	Thiamine pyrophosphate enzyme, C-terminal TPP binding domain protein	poxB	2.37E+07
E0MVS0	Inosine-5'-monophosphate dehydrogenase	guaB	2.33E+07
E0MU72	Inositol 1-phosphate synthase	HP↓	2.29E+07
E0MX71	Molybdate ABC transporter, periplasmic molybdate-binding protein	modA	2.21E+07
E0MV03	Cyclic nucleotide-binding domain protein	ntcA	2.14E+07
E0MVK0	50S ribosomal protein L24	rplX	2.14E+07
E0MYX0	Uncharacterized protein	HP↓	2.13E+07
E0MXW7	ATP phosphoribosyltransferase	hisG	2.12E+07
E0MX60	Histidine ammonia-lyase	hutH	2.11E+07
E0N0Q8	Universal stress family protein	uspA2	2.11E+07
E0MWW0	D-fructose-1,6-bisphosphate 1-phosphohydrolase class 2	glpX	2.07E+07
E0MZH4	ABC transporter, substrate-binding protein	HP↓	1.98E+07
E0MWB7	Periplasmic binding protein	HP↓	1.97E+07
E0MXK3	Universal stress family protein	uspA	1.96E+07
E0N0P1	L-lactate dehydrogenase	ldh	1.95E+07
E0MVL8	Adenylate kinase	adk	1.94E+07
E0MZ28	Aminomethyltransferase	gcvT	1.87E+07
E0MUC4	Thioredoxin reductase	trxB	1.86E+07
E0MX06	2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase	dapD	1.84E+07
E0MWX4	Nuclease-like protein	nucH	1.81E+07
E0MU90	Ribonucleoside-diphosphate reductase	nrdF	1.73E+07
E0MZL3	Oxidoreductase, aldo/keto reductase family protein	morA	1.72E+07
E0N035	Periplasmic binding protein	fetS	1.71E+07
E0MZ14	Cytochrome bc1 complex cytochrome c subunit	qcrC	1.70E+07
E0MUF8	Peptidyl-prolyl cis-trans isomerase	ppiA	1.69E+07
E0MWV9	Fumarate hydratase class II	fum	1.68E+07
E0MYL5	Probable malate:quinone oxidoreductase	mqo	1.67E+07
E0MW15	Biotin--[acetyl-CoA-carboxylase] ligase	birA	1.65E+07

E0MYN0	30S ribosomal protein S2	rpsB	1.63E+07
E0MVZ2	Thymidine phosphorylase	deoA	1.59E+07
E0MVN5	ESAT-6-like protein	HP↓	1.56E+07
E0MVK9	50S ribosomal protein L6 (Fragment)	rplF	1.54E+07
E0MXT6	Pyridine nucleotide-disulfide oxidoreductase	ndh	1.51E+07
E0N088	Exodeoxyribonuclease III	xth	1.51E+07
E0MXA9	Electron transfer flavoprotein domain protein	etfB	1.50E+07
E0MUC0	YceI-like domain protein	HP↓	1.48E+07
E0MWB6	Periplasmic binding protein	HP↓	1.47E+07
E0N0M4	Glycerol kinase	glpK	1.46E+07
E0MVE7	50S ribosomal protein L7/L12	rplL	1.46E+07
E0MWJ3	Glucose-6-phosphate isomerase	pgi	1.46E+07
E0MXR9	Oxoglutarate dehydrogenase inhibitor	odhI	1.44E+07
E0MVI2	30S ribosomal protein S3	rpsC	1.44E+07
E0MXM6	Ornithine cyclodeaminase	arcB	1.42E+07
E0MWP0	Uncharacterized protein	HP↓	1.41E+07
E0MW13	Uncharacterized protein (Fragment)	HP↓	1.36E+07
E0MZ34	Glutamine synthetase	glnA	1.34E+07
E0MV88	Glutamate-1-semialdehyde 2,1-aminomutase	hemL	1.31E+07
E0MZI7	50S ribosomal protein L21	rplU	1.30E+07
E0MX65	Arginine--tRNA ligase	argS	1.30E+07
E0MVE1	Transcription termination/antitermination protein NusG	nusG	1.29E+07
E0MWG1	Uncharacterized protein (Fragment)	HP↓	1.26E+07
E0MYF2	Phosphoenolpyruvate-protein phosphotransferase	ptsP	1.26E+07
E0MZN4	ErfK/YbiS/YcfS/YnhG	lppS	1.24E+07
E0MZ87	Aldehyde dehydrogenase (NAD) family protein	gabD2	1.22E+07
E0MXS8	6-phosphogluconate dehydrogenase, decarboxylating	gnd	1.21E+07
E0MUQ4	LGFP repeat protein	HP↓	1.20E+07
E0MWS6	Uncharacterized protein	HP↓	1.19E+07
E0MVP8	Glutamine--fructose-6-phosphate aminotransferase [isomerizing]	glmS	1.16E+07
E0N0M9	Serine--tRNA ligase	serS	1.15E+07
E0MY98	Probable transcriptional regulatory protein HMPREF0277_1482	yebC	1.15E+07
E0MXF0	D-3-phosphoglycerate dehydrogenase	serA	1.14E+07
E0MYK2	Transcription termination/antitermination protein NusA	nusA	1.13E+07
E0MZD8	Aminotransferase, class I/II	aecD	1.11E+07
E0MZ02	Phospho-2-dehydro-3-deoxyheptonate aldolase	aroF	1.11E+07

E0MVY1	MmgE/PrpD family protein	prpD	1.09E+07
E0MWX0	Intracellular protease, PfpI family	yhbO	1.09E+07
E0MWV0	Serine hydroxymethyltransferase	glyA	1.09E+07
E0MXU8	Peptidase, M24 family (Fragment)	pepE	1.08E+07
E0MVI5	30S ribosomal protein S17	rpsQ	1.06E+07
E0N096	Acetate kinase	ackA	1.04E+07
E0MY02	FeS assembly protein SufD	sufD	1.03E+07
E0MVD6	Deoxyribose-phosphate aldolase	deoC	1.03E+07
E0MXG4	Glutamate--tRNA ligase	gltX	1.03E+07
E0MZH3	Gamma-glutamyl phosphate reductase	proA	1.02E+07
E0N0K0	AMP-binding domain-containing protein (Fragment)	HP↓	1.01E+07
E0MYP2	YhgE/Pip domain protein	HP↓	1.01E+07
E0MXC6	Glutamyl-tRNA(Gln) amidotransferase subunit A	gatA	9.90E+06
E0MZ78	Peroxiredoxin, Ohr subfamily	ohr	9.80E+06
E0MZV3	Phosphate-binding protein PstS	pstS	9.70E+06
E0N0B4	Orotate phosphoribosyltransferase	pyrE	9.62E+06
E0MVZ9	Sulfurtransferase	rhdA	9.56E+06
E0N055	D-alanyl-D-alanine carboxypeptidase/D-alanyl-D-alanine-endopeptidase	dacB	9.51E+06
E0MXB0	Electron transfer flavoprotein domain protein (Fragment)	etfA	9.50E+06
E0N074	Peptidase dimerization domain protein	HP↓	9.48E+06
E0MUU9	Putative phage head-tail adaptor	HP↓	9.37E+06
E0N028	Lysine--tRNA ligase	lysS	9.27E+06
E0MY03	FeS assembly protein SufB	sufB	9.04E+06
E0N056	Inorganic pyrophosphatase	ppa	9.04E+06
E0MW98	ZnMc domain-containing protein	HP↓	8.97E+06
E0MUL6	Aldehyde dehydrogenase (NAD) family protein	betB	8.89E+06
E0MXW6	Aspartate ammonia-lyase	aspA	8.88E+06
E0MV31	Metallo-beta-lactamase domain protein	HP↓	8.83E+06
E0N0G9	VanW-like protein	HP↓	8.70E+06
E0MXD3	Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit B	gatB	8.70E+06
E0MYG4	Uncharacterized protein	HP↓	8.62E+06
E0MUZ7	YqeY-like protein	HP↓	8.57E+06
E0MXP3	Uncharacterized protein	HP↓	8.54E+06
E0MXK5	Uncharacterized protein	HP↓	8.34E+06
E0MUC5	Thioredoxin	trxA	8.30E+06
E0MZB2	Glycine--tRNA ligase	glyS	8.22E+06

E0MUJ9	AMP nucleosidase	amn	8.22E+06
E0MX67	Homoserine dehydrogenase	thrA	8.19E+06
E0N090	Uncharacterized protein	HP↓	8.14E+06
E0MVE2	50S ribosomal protein L11	rplK	8.12E+06
E0MV61	AMP-binding enzyme	fadD2	8.07E+06
E0MZL6	Uncharacterized protein	HP↓	8.06E+06
E0MUL4	Flavin reductase	HP↓	8.05E+06
E0MYH4	PspA/IM30 family protein	HP↓	8.00E+06
E0MW61	Nucleotidyl transferase	mpg	7.97E+06
E0MX26	Urocanate hydratase	hutU	7.88E+06
E0MXW3	Formate--tetrahydrofolate ligase	fhs	7.85E+06
E0MW81	Ribosome hibernation promoting factor	raiA	7.83E+06
E0MU66	Single-stranded DNA-binding protein	ssb	7.83E+06
E0MVA0	Uncharacterized protein	HP↓	7.79E+06
E0MZS6	Thioredoxin-like_fold domain-containing protein	HP↓	7.73E+06
E0MV65	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase	gpm	7.56E+06
E0MVY3	Citrate synthase (unknown stereospecificity) (Fragment)	prpC	7.55E+06
E0MW73	Mannose-6-phosphate isomerase	manA	7.54E+06
E0MX21	Uncharacterized protein	HP↓	7.54E+06
E0MUL5	Oxidoreductase, short chain dehydrogenase/reductase family protein	ydfG	7.27E+06
E0MVE3	50S ribosomal protein L1	rplA	7.23E+06
E0MYV3	Uncharacterized protein	HP↓	7.13E+06
E0MVK1	50S ribosomal protein L5	rplE	7.11E+06
E0MWU4	Transcription elongation factor GreA	greA	7.06E+06
E0MWB0	Uncharacterized protein	HP↓	7.05E+06
E0MY00	Cysteine desulfurase	sufS	6.99E+06
E0MXW1	M18 family aminopeptidase	pepC	6.97E+06
E0MY11	Transketolase	tkt	6.95E+06
E0MZP0	Antioxidant, AhpC/TSA family	bcp	6.65E+06
E0MXK2	Universal stress family protein	HP↓	6.62E+06
E0MYQ0	30S ribosomal protein S16	rpsP	6.51E+06
E0MZ19	Iron-sulfur cluster assembly accessory protein	iscA	6.48E+06
E0N098	Pyridine nucleotide-disulfide oxidoreductase	fprA2	6.47E+06
E0MUW7	Uncharacterized protein	HP↓	6.44E+06
E0MVN9	30S ribosomal protein S9	rpsI	6.42E+06
E0MVG0	30S ribosomal protein S7	rpsG	6.37E+06

E0N097	Phosphate acetyltransferase	pta	6.27E+06
E0MWN6	Uncharacterized protein	HP↓	6.24E+06
E0MX61	Uncharacterized protein (Fragment)	HP↓	6.09E+06
E0MXH4	3-isopropylmalate dehydratase small subunit	leuD	6.07E+06
E0MX89	Peptide chain release factor 1	prfA	6.02E+06
E0MYM8	Uridylate kinase	pyrH	6.00E+06
E0MVG1	Elongation factor G	fusA	5.90E+06
E0MVX3	Uncharacterized protein	HP↓	5.79E+06
E0MWM9	Methionine--tRNA ligase	metG	5.76E+06
E0MZP5	Hydrolase, alpha/beta domain protein	pip	5.71E+06
E0MWU5	Uncharacterized protein	HP↓	5.67E+06
E0N0A5	Adenylosuccinate synthetase	purA	5.62E+06
E0MV77	Hydroxymethylbilane synthase (Fragment)	hemC	5.49E+06
E0MYC0	Uncharacterized protein	HP↓	5.45E+06
E0MVG3	ABC transporter, substrate-binding protein, family 5	HP↓	5.41E+06
E0MZU1	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	murA	5.38E+06
E0MV90	Antioxidant, AhpC/TSA family	HP↓	5.36E+06
E0MVS8	Uncharacterized protein	HP↓	5.34E+06
E0MX08	Succinyl-diaminopimelate desuccinylase	dapE	5.33E+06
E0N006	ABC transporter, substrate-binding protein	HP↓	5.25E+06
E0MWF5	Citrate synthase (unknown stereospecificity) (Fragment)	HP↓	5.23E+06
E0MXX1	L-cysteine:1D-myo-inositol 2-amino-2-deoxy-alpha-D-glucopyranoside ligase	mshC	5.22E+06
E0MVL1	30S ribosomal protein S5	rpsE	5.14E+06
E0MWY9	Uncharacterized protein	HP↓	5.12E+06
E0MYG9	Protein RecA	recA	5.11E+06
E0MV78	Hydroxymethylbilane synthase (Fragment)	hemC	5.07E+06
E0MV84	Coproporphyrinogen III oxidase	hemG	5.07E+06
E0MVI1	50S ribosomal protein L22	rplV	5.02E+06
E0MY80	Thiol peroxidase	tpx	5.01E+06
E0MUD7	Beta sliding clamp	dnaN	4.98E+06
E0N0A4	Uncharacterized protein	HP↓	4.98E+06
E0MZI0	Glutamate 5-kinase	proB	4.95E+06
E0MYY0	Cell division protein FtsZ	ftsZ	4.88E+06
E0MXH9	Uncharacterized protein	HP↓	4.85E+06
E0MUK0	Phosphoribosyl transferase domain protein	gpt	4.82E+06

E0MWT9	Ppx/GppA phosphatase family protein	HP↓	4.79E+06
E0MYQ1	Acyltransferase	wbpC	4.74E+06
E0MX99	ATP synthase gamma chain	atpG	4.74E+06
E0MY60	Elongation factor P	efp	4.69E+06
E0N0B8	Chaperone protein ClpB (Fragment)	clpB2	4.62E+06
E0MU59	Thioredoxin	trxA	4.58E+06
E0MVE6	50S ribosomal protein L10	rplJ	4.53E+06
E0N0A3	Uncharacterized protein	HP↓	4.53E+06
E0MW95	Uncharacterized protein	HP↓	4.49E+06
E0MVW1	Isocitrate dehydrogenase [NADP]	icd	4.49E+06
E0MVF9	30S ribosomal protein S12	rpsL	4.43E+06
E0MZZ3	Adenylosuccinase (Fragment)	purB2	4.36E+06
E0MZZ4	Phosphoribosylamine--glycine ligase	purD	4.24E+06
E0MWB9	Uncharacterized protein	HP↓	4.21E+06
E0MX97	ATP synthase subunit delta	atpH	4.14E+06
E0MVV7	Pyridoxal 5'-phosphate synthase subunit PdxS	pdxS	4.04E+06
E0MU58	PspA/IM30 family protein	HP↓	4.03E+06
E0MXX3	Uncharacterized protein	HP↓	4.00E+06
E0MWJ7	Peptidase, M23 family	HP↓	3.98E+06
E0MZ53	Putative zinc ribbon domain protein	HP↓	3.96E+06
E0MYP1	50S ribosomal protein L19	rplS	3.92E+06
E0MX63	Putative iron-sulfur cluster-binding protein	ykgF	3.88E+06
E0MY73	Aspartate--tRNA(Asp/Asn) ligase	aspS	3.83E+06
E0MXU9	Creatinase_N domain-containing protein (Fragment)	HP↓	3.83E+06
E0MWB2	UPF0182 protein HMPREF0277_0796	HP↓	3.79E+06
E0MU70	Penicillin-insensitive transglycosylase	ponA	3.79E+06
E0MY78	Histidine--tRNA ligase	hisS	3.77E+06
E0MYI3	4-hydroxy-tetrahydrodipicolinate synthase	dapA	3.71E+06
E0N069	Polyphosphate kinase 2	ppk2	3.66E+06
E0N099	Putative phosphoribosylglycinamide formyltransferase 2	purT	3.58E+06
E0MV02	Metallo-beta-lactamase domain protein	HP↓	3.55E+06
E0N0B6	Rhodanese-like protein	sseA	3.51E+06
E0MXT0	Magnesium transport protein CorA	corA	3.50E+06
E0MVX1	Uncharacterized protein	HP↓	3.50E+06
E0MZL4	Ribose 5-phosphate isomerase	rpiB	3.42E+06
E0MWU6	Mycothiol S-conjugate amidase	mca	3.38E+06

E0MZ76	CRISPR system CASCADE complex protein CasA	casA	3.29E+06
E0MYR7	Ribonuclease 3	rnc	3.27E+06
E0MXK9	Translation initiation factor IF-3	infC	3.26E+06
E0MYD8	UDP-glucose 4-epimerase	galE	3.26E+06
E0MYY9	UDP-N-acetylmuramoyl-tripeptide--D-alanyl-D-alanine ligase	murF	3.25E+06
E0N0J6	Carboxyl transferase domain protein	pccB	3.24E+06
E0MWP1	Antibiotic biosynthesis monooxygenase	HP↓	3.23E+06
E0MZ39	Threonine synthase	thrC	3.21E+06
E0MUX6	Lipid II isoglutaminyl synthase (glutamine-hydrolyzing) subunit MurT	murE	3.19E+06
E0MU73	Transcriptional regulator, MarR family	HP↓	3.18E+06
E0N0P3	Peptide methionine sulfoxide reductase MsrA	msrA	3.16E+06
E0MZV8	UPF0678 fatty acid-binding protein-like protein HMPREF0277_2042	HP↓	3.15E+06
E0MU77	Uncharacterized protein	HP↓	3.13E+06
E0MUB6	tRNA adenylyltransferase	HP↓	3.12E+06
E0MWY0	Uncharacterized protein	HP↓	3.10E+06
E0MXX6	Raf-like protein	ybcL	3.10E+06
E0MXF2	FAH family protein	fahA2	3.05E+06
E0MVJ9	50S ribosomal protein L14	rplN	3.05E+06
E0MVM4	30S ribosomal protein S4	rpsD	3.01E+06
E0MU64	Replicative DNA helicase	dnaB	3.00E+06
E0MVH9	50S ribosomal protein L2	rplB	2.98E+06
E0MWV6	Acetyltransferase, GNAT family	HP↓	2.93E+06
E0MYZ1	Penicillin-binding protein dimerization domain protein (Fragment)	ftsI	2.93E+06
E0MWK0	Bifunctional purine biosynthesis protein PurH	purH	2.91E+06
E0MY44	Dihydroorotase	pyrC	2.90E+06
E0MU89	Flavin reductase	nqr	2.90E+06
E0MUZ8	Penicillin-insensitive transglycosylase	pon	2.89E+06
E0MVX9	Pyridine nucleotide-disulfide oxidoreductase	lpdA	2.87E+06
E0MXT8	Dyp-type peroxidase family protein	tyrA2	2.87E+06
E0N041	Aminopeptidase	pepC2	2.84E+06
E0MX15	Glycosyl hydrolase family 32	HP↓	2.83E+06
E0MZL7	Alanine aminopeptidase	pepN	2.82E+06
E0MZA8	TPM_phosphatase domain-containing protein	HP↓	2.80E+06
E0MUP0	Oxidoreductase, short chain dehydrogenase/reductase family protein	HP↓	2.77E+06
E0MXH8	D-alanine--D-alanine ligase	ddlA	2.75E+06
E0MXL1	50S ribosomal protein L20	rplT	2.72E+06

E0MUY4	Aspartokinase	lysC	2.70E+06
E0MVN8	50S ribosomal protein L13	rplM	2.67E+06
E0MXL4	Phenylalanine--tRNA ligase alpha subunit	pheS	2.67E+06
E0MVJ8	NLPA lipoprotein	HP↓	2.64E+06
E0N0F8	dCTP deaminase, dUMP-forming	dcd	2.62E+06
E0MVL0	Ribosomal protein L18	rplR	2.58E+06
E0MWG3	OsmC-like protein	HP↓	2.58E+06
E0MXM4	Argininosuccinate lyase (Fragment)	argH	2.55E+06
E0MVK8	30S ribosomal protein S8	rpsH	2.55E+06
E0MYA7	Putative Tat-translocated enzyme	HP↓	2.55E+06
E0MVM2	30S ribosomal protein S13	rpsM	2.55E+06
E0MVY2	Methylisocitrate lyase	prpB	2.53E+06
E0MY59	Transcription antitermination protein NusB	nusB	2.52E+06
E0MXE7	Ketol-acid reductoisomerase (NADP(+))	ilvC	2.50E+06
E0MYS0	Uncharacterized protein	HP↓	2.48E+06
E0MVZ8	Carbamoyl-phosphate synthase L chain, ATP binding domain protein	bccA	2.43E+06
E0MWL4	Molybdopterin binding domain protein	pcd	2.38E+06
E0MXU0	YceI-like domain protein	HP↓	2.38E+06
E0MZM6	Energy-dependent translational throttle protein EttA	ettA	2.35E+06
E0MVH7	50S ribosomal protein L4	rplD	2.34E+06
E0MZV5	Mycothiol acetyltransferase	mshD	2.32E+06
E0MYJ8	Ribosome-binding factor A	rbfA	2.30E+06
E0MUK8	Uncharacterized protein	HP↓	2.29E+06
E0MVU1	Bifunctional protein FolD	folD	2.26E+06
E0MZR1	Nicotinate phosphoribosyltransferase	pncB	2.21E+06
E0MZS5	SGNH_hydro domain-containing protein	HP↓	2.21E+06
E0MV33	dTDP-4-dehydrorhamnose reductase	rfbD	2.20E+06
E0MU34	2-oxoglutarate dehydrogenase, E2 component, dihydrolipoamide succinyltransferase (Fragment)	sucB	2.20E+06
E0MXY5	(2R,3S)-2-methylisocitrate dehydratase	acnA	2.20E+06
E0MXM2	Argininosuccinate synthase	argG	2.13E+06
E0MYZ0	Uncharacterized protein (Fragment)	HP↓	2.13E+06
E0MXF5	Uncharacterized protein	HP↓	2.13E+06
E0MUH0	Non-specific serine/threonine protein kinase	pknB	2.12E+06
E0MZ56	Pyruvate dehydrogenase (acetyl-transferring) (Fragment)	aceE	2.11E+06
E0MVY5	Pyruvate carboxylase	pyc	2.10E+06

E0MX32	Sodium/proline symporter	putP	2.10E+06
E0MYM0	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase (flavodoxin)	ispG	2.09E+06
E0N0E4	Putative 5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase	HP↓	2.04E+06
E0MWW9	Ribosome-binding ATPase YchF	ychF	2.02E+06
E0MX66	Diaminopimelate decarboxylase	lysA	1.99E+06
E0MYC5	Inositol-1-monophosphatase	suhB2	1.97E+06
E0MV99	1,4-dihydroxy-2-naphthoyl-CoA synthase	menB	1.96E+06
E0MYD6	RNA polymerase sigma factor	sigB	1.96E+06
E0N091	ABC transporter, ATP-binding protein	HP↓	1.95E+06
E0MVQ3	Uncharacterized protein	HP↓	1.95E+06
E0MYI9	Putative polyribonucleotide nucleotidyltransferase (Fragment)	pnp	1.95E+06
E0MY36	S-adenosylmethionine synthase	metK	1.93E+06
E0N0N1	Septum_form domain-containing protein	HP↓	1.90E+06
E0MVC5	Polyprenyl synthetase	ispB	1.90E+06
E0MUN8	Mycobacterial cell wall arabinan synthesis protein	HP↓	1.90E+06
E0N016	CarD-like protein	carD	1.88E+06
E0MV83	Uroporphyrinogen decarboxylase	hemE	1.88E+06
E0MZ46	Glutamine synthetase	glnA2	1.88E+06
E0MXJ0	ABC transporter, substrate-binding protein, family 3	HP↓	1.87E+06
E0MYK7	Proline--tRNA ligase	proS	1.87E+06
E0MVW7	Tryptophan--tRNA ligase	trpS	1.84E+06
E0MZI6	50S ribosomal protein L27	rpmA	1.82E+06
E0MYH6	Competence/damage-inducible domain protein CinA	cinA	1.78E+06
E0MUF7	DAK1 domain protein	dhaK	1.77E+06
E0MZZ1	Phosphoribosylaminoimidazole-succinocarboxamide synthase	purC	1.77E+06
E0MY56	Uncharacterized protein	HP↓	1.75E+06
E0MY58	Polyphosphate:nucleotide phosphotransferase, PPK2 family	ppk2	1.75E+06
E0MYT3	1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamino] imidazole-4-carboxamide isomerase	hisA	1.73E+06
E0MV40	Succinate dehydrogenase cytochrome B subunit, b558 family	sdhC	1.70E+06
E0MY27	6,7-dimethyl-8-ribityllumazine synthase	ribH	1.69E+06
E0MXY1	Coproporphyrin III ferrochelatase	hemH	1.69E+06
E0MZ18	Asparagine synthase (Glutamine-hydrolyzing)	asnB	1.65E+06
E0MZ95	Conserved carboxylase domain protein	oadA	1.64E+06
E0MYP0	Signal peptidase I	lepB	1.63E+06
E0MW82	Protein translocase subunit SecA	secA	1.62E+06

E0MUF4	Uncharacterized protein	HP↓	1.62E+06
E0MW77	Response regulator receiver domain protein	mtrA	1.61E+06
E0MZW2	Amidophosphoribosyltransferase	purF	1.60E+06
E0MX78	Nitrate reductase, beta subunit	narH	1.60E+06
E0MZJ6	Bifunctional protein FolC	folC	1.59E+06
E0MXH3	3-isopropylmalate dehydratase large subunit	leuC	1.59E+06
E0N0C9	Protein GrpE	grpE	1.54E+06
E0MUH6	FHA domain protein	HP↓	1.54E+06
E0MYY3	UDP-N-acetylmuramate--L-alanine ligase	murC	1.54E+06
E0N0N7	Cell envelope-like function transcriptional attenuator common domain protein	HP↓	1.52E+06
E0MYB3	Peptide-methionine (R)-S-oxide reductase	msrB	1.49E+06
E0MY75	Luciferase-like monooxygenase, FMN-dependent, CE1758 family	HP↓	1.49E+06
E0MZ72	CRISPR system CASCADE complex protein CasE	casE	1.49E+06
E0MUF1	Polysaccharide deacetylase	pgdA	1.47E+06
E0MV87	Phosphogluconate dehydrogenase (Decarboxylating), NAD binding domain protein	HP↓	1.47E+06
E0MYL9	Penicillin-binding protein, transpeptidase domain protein	HP↓	1.46E+06
E0MXE8	DUF262 domain-containing protein	HP↓	1.46E+06
E0MYV1	L-threonine dehydratase	ilvA	1.44E+06
E0MZS3	NH(3)-dependent NAD(+) synthetase	nadE	1.43E+06
E0MXD4	GroES-like protein	adh	1.42E+06
E0MXD0	ATP-dependent 6-phosphofructokinase	pfkA	1.42E+06
E0MV92	ResB-like protein	resB	1.41E+06
E0MVP1	Phosphoglucosamine mutase	glmM	1.40E+06
E0MYJ2	Riboflavin biosynthesis protein	ribF	1.40E+06
E0MZP4	SPFH/Band 7/PHB domain protein	HP↓	1.40E+06
E0MV54	Uncharacterized protein	HP↓	1.40E+06
E0MUM9	Glyoxalase family protein	HP↓	1.38E+06
E0MZH0	Ribosomal silencing factor RsfS	rsfS	1.37E+06
E0N0H0	Glycosyl hydrolase family 3 N-terminal domain protein	nagA2	1.37E+06
E0MUY5	Aspartate-semialdehyde dehydrogenase	asd	1.37E+06
E0MW49	Transporter, gluconate:H+ symporter family	gntP	1.36E+06
E0MY93	Protein translocase subunit SecD	secD	1.36E+06
E0MZZ2	ADSL_C domain-containing protein	HP↓	1.34E+06
E0MW18	N5-carboxyaminoimidazole ribonucleotide synthase	purK	1.33E+06

E0N013	Cysteine--tRNA ligase (Fragment)	cysS	1.33E+06
E0MWT5	Uncharacterized protein	HP↓	1.31E+06
E0MXK6	Metallo-beta-lactamase domain protein	HP↓	1.30E+06
E0MWW5	4-hydroxy-3-methylbut-2-enyl diphosphate reductase	ispH	1.30E+06
E0MVX8	Amidohydrolase	amiA	1.30E+06
E0MX28	ATP-dependent RNA helicase DeaD	deaD2	1.29E+06
E0MVF2	Uncharacterized protein (Fragment)	HP↓	1.28E+06
E0MY57	Uncharacterized protein	HP↓	1.27E+06
E0MWR7	Peptidyl-tRNA hydrolase	pth	1.27E+06
E0MUQ1	NAD(P)H quinone oxidoreductase, PIG3 family	cryZ	1.26E+06
E0MWQ3	Peptide chain release factor 3	prfC	1.26E+06
E0MU85	GroES-like protein (Fragment)	HP↓	1.25E+06
E0MVA5	o-succinylbenzoate synthase	menC	1.24E+06
E0MY15	6-phosphogluconolactonase	pgl	1.23E+06
E0MV10	Uncharacterized protein	HP↓	1.23E+06
E0MYG1	Diaminopimelate epimerase	dapF	1.23E+06
E0MX30	Uncharacterized protein	HP↓	1.22E+06
E0MW53	ABC transporter, substrate-binding protein, QAT family	HP↓	1.19E+06
E0MZ73	CRISPR system CASCADE complex protein CasD	casD	1.19E+06
E0MUJ2	M20_dimer domain-containing protein (Fragment)	HP↓	1.19E+06
E0MYC4	Uncharacterized protein	HP↓	1.18E+06
E0MUN6	Abhydrolase_3 domain-containing protein	HP↓	1.18E+06
E0MWC5	Histidinol-phosphatase	hisN	1.18E+06
E0MV08	Serine protease	cvpA	1.16E+06
E0MUP1	FAD binding domain protein	HP↓	1.14E+06
E0MWZ6	Ferredoxin	fdxA	1.14E+06
E0MYM2	1-deoxy-D-xylulose 5-phosphate reductoisomerase	dxr	1.13E+06
E0MV80	Delta-aminolevulinic acid dehydratase	hemB	1.11E+06
E0MX35	Ser/Thr phosphatase family protein	HP↓	1.09E+06
E0MY01	FeS assembly ATPase SufC	sufC	1.08E+06
E0MWA9	Endopeptidase La	HP↓	1.06E+06
E0MXF1	3-isopropylmalate dehydrogenase	leuB	1.05E+06
E0MWK3	Uncharacterized protein	HP↓	1.03E+06
E0MYI8	Putative polyribonucleotide nucleotidyltransferase (Fragment)	HP↓	1.02E+06
E0MY82	Peptidyl-prolyl cis-trans isomerase, cyclophilin-type (Fragment)	ppi	1.01E+06
E0MZD1	AMP-binding enzyme	HP↓	1.00E+06

E0MZQ9	DUF2017 domain-containing protein	HP↓	9.93E+05
E0MUF2	Acetyltransferase, GNAT family	HP↓	9.81E+05
E0MZ13	Cytochrome bc1 complex Rieske iron-sulfur subunit	petA	9.76E+05
E0MWS9	Transcriptional regulator, TetR family	HP↓	9.72E+05
E0MW01	Uncharacterized protein	HP↓	9.70E+05
E0MXH7	Glycerol-3-phosphate dehydrogenase [NAD(P)+]	gpsA	9.64E+05
E0MV58	AI-2 synthesis protein	luxS	9.62E+05
E0N0K1	Cutinase	HP↓	9.56E+05
E0MXJ9	Dephospho-CoA kinase	coaE	9.55E+05
E0MUU5	Putative tRNA adenosine deaminase-associated protein	HP↓	9.39E+05
E0MZ93	Biotin carboxylase	accC	9.35E+05
E0MV43	Uncharacterized protein	HP↓	9.31E+05
E0MXE3	Dihydroxy-acid dehydratase	ilvD	9.24E+05
E0MXZ9	SUF system FeS assembly protein, NifU family	HP↓	9.21E+05
E0MVB9	Putative 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylic-acid synthase (Fragment)	menD	9.16E+05
E0MYE0	Uncharacterized protein	HP↓	8.84E+05
E0MVT1	Methionine import ATP-binding protein MetN	metN2	8.59E+05
E0N0A0	Putative phosphoribosylglycinamide formyltransferase 2	purT2	8.46E+05
E0MXP6	CobQ/CobB/MinD/ParA nucleotide binding domain protein	soj2	8.43E+05
E0MV60	AMP-binding enzyme	fadD	8.38E+05
E0MXU3	Glycosyltransferase, group 2 family protein	ppmC	8.36E+05
E0MY91	ABC transporter, substrate-binding protein, family 5	dciAE	8.35E+05
E0MY24	Nucleotide-binding protein HMPREF0277_1408	HP↓	8.28E+05
E0MZM0	Cys/Met metabolism PLP-dependent enzyme	metC	8.09E+05
E0N0Q2	DUF2020 domain-containing protein	HP↓	7.94E+05
E0MVM3	30S ribosomal protein S11	rpsK	7.89E+05
E0MVF8	Uncharacterized protein	HP↓	7.88E+05
E0MWF2	Uncharacterized protein	HP↓	7.84E+05
E0MZR8	Ribonucleoside-diphosphate reductase	nrdE	7.82E+05
E0N0I5	tRNA (guanine-N(7)-)-methyltransferase	trmB	7.61E+05
E0N0Q6	Transcriptional regulator, TetR family	HP↓	7.35E+05
E0MYT8	Histidinol-phosphate aminotransferase	hisC	7.31E+05
E0MU93	Leucine--tRNA ligase	leuS	7.17E+05
E0MYI2	Ribonuclease J	rnj	7.12E+05
E0MYM3	Uncharacterized protein	HP↓	7.05E+05

E0MZV6	Uncharacterized protein	HP↓	7.04E+05
E0MXB2	Aminotransferase, class V	iscS	6.98E+05
E0MXI6	Phosphopantetheine adenylyltransferase	coaD	6.85E+05
E0MV70	Uncharacterized protein	HP↓	6.84E+05
E0MZ50	GalKase_gal_bdg domain-containing protein	HP↓	6.82E+05
E0MWD0	Cell division protein FtsX	ftsX	6.82E+05
E0MXE9	Uncharacterized protein	HP↓	6.62E+05
E0N0J7	Thioesterase domain protein (Fragment)	pks	6.57E+05
E0MVL7	Protein translocase subunit SecY	secY	6.42E+05
E0MVG9	Uncharacterized protein	HP↓	6.33E+05
E0N0F5	Putative aminotransferase AlaT	aspC	6.31E+05
E0MUE2	Uncharacterized protein	HP↓	6.19E+05
E0MXH0	YhgE/Pip domain protein	HP↓	6.19E+05
E0N060	Hypoxanthine phosphoribosyltransferase	hpt	6.05E+05
E0MUH7	FHA domain protein	HP↓	6.03E+05
E0MVZ0	Nucleoside transporter, NupC family	yutK	5.90E+05
E0N022	ATPase family associated with various cellular activities (AAA)	clpC	5.89E+05
E0MZK2	ATP-dependent Clp protease ATP-binding subunit ClpX	clpX	5.79E+05
E0MW79	Lipoprotein LpqB	lpqB	5.74E+05
E0MYS7	Prolipoprotein diacylglyceryl transferase (Fragment)	lgt	5.67E+05
E0MZK8	ATP-dependent Clp protease proteolytic subunit	clpP	5.58E+05
E0MZP8	Formate C-acetyltransferase	pflB	5.53E+05
E0MUC2	Alanine aminopeptidase	pepN	5.49E+05
E0MX22	2-oxo acid dehydrogenase acyltransferase (Catalytic domain) (Fragment)	odhA	5.40E+05
E0MZT2	Efflux ABC transporter, permease protein (Fragment)	HP↓	5.39E+05
E0MU51	Penicillin-binding protein, transpeptidase domain protein (Fragment)	pbpB	5.36E+05
E0MY20	L-lactate permease	lctP	5.16E+05
E0MVF3	DNA-directed RNA polymerase subunit beta	rpoB	5.13E+05
E0MYH7	CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatidyltransferase	pgsA	5.11E+05
E0MYP7	Ribosome maturation factor RimM	rimM	5.05E+05
E0MY63	3-dehydroquinate synthase	aroB	5.00E+05
E0MW14	ABC transporter, ATP-binding protein	HP↓	4.96E+05
E0MV42	Succinate dehydrogenase/fumarate reductase iron-sulfur subunit	frdB	4.92E+05
E0MZA0	Acetyl-CoA C-acetyltransferase	pcaF	4.92E+05
E0MXL9	Acetylornithine aminotransferase	argD	4.84E+05
E0MWT8	Uncharacterized protein	HP↓	4.68E+05

E0MV06	Redoxin family protein	HP↓	4.64E+05
E0MZT3	ABC transporter, ATP-binding protein	HP↓	4.54E+05
E0MV95	Uncharacterized protein	HP↓	4.53E+05
E0MZL1	Uncharacterized protein	HP↓	4.46E+05
E0MZ57	PDH_E1_M domain-containing protein (Fragment)	HP↓	4.44E+05
E0MXZ8	FeS_assembly_P domain-containing protein	HP↓	4.40E+05
E0N0J8	Beta-ketoacyl synthase, N-terminal domain protein (Fragment)	pks2	4.36E+05
E0MWK5	DUF294_C domain-containing protein	HP↓	4.32E+05
E0MX25	Tat pathway signal sequence domain protein	HP↓	4.30E+05
E0MVF4	DNA-directed RNA polymerase subunit beta'	rpoC	4.29E+05
E0MVL3	50S ribosomal protein L15	rplO	4.27E+05
E0MUL8	Choline dehydrogenase	betA	4.15E+05
E0MWZ4	Uncharacterized protein	HP↓	4.14E+05
E0MZ12	Cytochrome bc1 complex cytochrome b subunit	HP↓	4.11E+05
E0MY92	Protein-export membrane protein SecF	secF	3.87E+05
E0MY88	5'-nucleotidase, C-terminal domain protein	nuc	3.73E+05
E0MYE6	Uncharacterized protein	HP↓	3.66E+05
E0MUC6	N-acetylmuramoyl-L-alanine amidase	amiD	3.64E+05
E0MU57	NYN domain-containing protein	HP↓	3.43E+05
E0MVC0	TPP_enzyme_C domain-containing protein (Fragment)	HP↓	3.38E+05
E0N052	GTP cyclohydrolase 1	folE	3.36E+05
E0MYY6	Mur ligase middle domain protein	murD2	3.31E+05
E0MZM1	Transporter, small conductance mechanosensitive ion channel MscS family protein	HP↓	3.30E+05
E0MUE5	DNA gyrase subunit A	gyrA	3.29E+05
E0MX37	Transcriptional regulator, MarR family	HP↓	3.25E+05
E0MX27	Imidazolonepropionase	hutI	3.24E+05
E0N0K2	Uncharacterized protein	HP↓	3.17E+05
E0N094	ABC transporter, substrate-binding protein, family 3	HP↓	3.08E+05
E0MUP5	GtrA-like protein	HP↓	3.05E+05
E0MX75	Copper-containing nitrite reductase	HP↓	3.05E+05
E0MXT7	Cyclopropane-fatty-acyl-phospholipid synthase	cfa2	2.91E+05
E0MXM1	Arginine repressor	argR	2.80E+05
E0MYX7	Uncharacterized protein	HP↓	2.25E+05
E0MVW3	Exodeoxyribonuclease III	HP↓	2.17E+05
E0MY22	WhiA_N domain-containing protein (Fragment)	HP↓	2.14E+05

E0MYX2	Isoleucine--tRNA ligase	ileS	1.87E+05
E0MUN2	Transmembrane amino acid transporter protein	sdaC	1.58E+05
E0MX16	O-methyltransferase	HP⸸	1.58E+05

HP⸸: Hypothetical Protein

Supplementary Table 4.2. Unique proteins in *C. accolens* strains

***C. accolens* strain C778**		
Accession Number	**Protein description**	**Gene name**
E0MU53	Uncharacterized protein	HP⸸
E0MY49	Helicase C-terminal domain protein	HP⸸
E0N0K5	Uncharacterized protein	HP⸸
E0N012	Uncharacterized protein (Fragment)	HP⸸
E0MXM3	Putative argininosuccinate lyase (Fragment)	argH
E0MZ91	2Fe-2S iron-sulfur cluster binding domain protein	pht
E0MUI4	Uncharacterized protein	HP⸸
E0N059	FtsH, extracellular (Fragment)	hflB
E0MY51	N6_N4_Mtase domain-containing protein	HP⸸
E0MZT9	Transposase	tnp3520a2
E0MZF7	Uncharacterized protein (Fragment)	HP⸸
E0MZD5	Isopentenyl-diphosphate Delta-isomerase	idi
E0N0E0	LPXTG-motif cell wall anchor domain protein	HP⸸
E0MZW7	Death-on-curing family protein	doc
E0MYA3	Lipid A biosynthesis (KDO)2-(Lauroyl)-lipid IVA acyltransferase	htrB
E0MXG9	YhgE/Pip domain protein	HP⸸
E0N048	Uncharacterized protein	HP⸸
E0MYD4	L-serine dehydratase	sdaA
E0MU42	Molybdopterin domain-containing protein (Fragment)	HP⸸
E0MUA1	Anthranilate synthase component 1	trpE
E0MV74	HAD hydrolase, family IB	HP⸸
E0MVN4	Type VII secretion-associated protein, Rv3446c family	HP⸸
E0MVP7	CCG domain-containing protein	HP⸸
E0MWP8	Uncharacterized protein	HP⸸
E0MX36	AAA_23 domain-containing protein	HP⸸
E0MZ77	CRISPR-associated helicase Cas3	cas3

E0MZD2	Uncharacterized protein	HP$^{\downarrow}$
E0MZG7	ComEA protein	HP$^{\downarrow}$
E0MZP9	Xanthine permease	pbuX
E0MZT6	Uncharacterized protein	HP$^{\downarrow}$
E0N0D5	LPXTG-motif cell wall anchor domain protein	HP$^{\downarrow}$
E0N0F2	Cysteine-rich domain protein	HP$^{\downarrow}$

HP$^{\downarrow}$: Hypothetical Protein

***C. accolens* strain C779**		
Accession Number	**Protein description**	**Gene name**
E0MZI5	Uncharacterized protein	HP$^{\downarrow}$
E0MZR0	ATP-dependent Clp protease adapter protein ClpS	clpS
E0MWJ5	Chorismate mutase	HP$^{\downarrow}$
E0MZI3	Uncharacterized protein	HP$^{\downarrow}$
E0N0P9	Uncharacterized protein	HP$^{\downarrow}$
E0MVG4	Putative phosphonate C-P lyase system protein PhnK	HP$^{\downarrow}$
E0MZY1	Uncharacterized protein	HP$^{\downarrow}$
E0MW25	Sugar-binding domain protein	HP$^{\downarrow}$
E0MUS5	Magnesium transporter MgtE	mgtE
E0MZ45	Bifunctional glutamine synthetase adenylyltransferase/adenylyl-removing enzyme	glnE
E0MUA7	Transporter, dicarboxylate/amino acid:cation Na+/H+ symporter family protein	HP$^{\downarrow}$
E0MUQ9	Uncharacterized protein	HP$^{\downarrow}$
E0MWL1	50S ribosomal protein L32	rpmF
E0MU32	Low molecular weight phosphotyrosine protein phosphatase	ptpA
E0MVI0	30S ribosomal protein S19	rpsS
E0MVB4	Uncharacterized protein	HP$^{\downarrow}$
E0MVA4	HNH endonuclease domain protein	HP$^{\downarrow}$
E0MU95	Uncharacterized protein	HP$^{\downarrow}$
E0MYP3	Tex-like protein N-terminal domain protein	yhgF
E0MV48	Uncharacterized protein	HP$^{\downarrow}$
E0MVQ6	tRNA N6-adenosine threonylcarbamoyltransferase	tsaD
E0N023	Uncharacterized protein	HP$^{\downarrow}$
E0N0G4	Histidine kinase	HP$^{\downarrow}$

HP$^{\downarrow}$: Hypothetical Protein

C. accolens strain C781		
Accession Number	**Protein description**	**Gene name**
E0N0J2	Uncharacterized protein	HP↓
E0MYF6	HNH endonuclease domain protein	HP↓
E0MZ88	DUF2236 domain-containing protein	HP↓
E0MXP2	TPPK_C domain-containing protein	HP↓
E0N0G6	Ribbon-helix-helix protein, CopG family	HP↓
E0MYV8	Uncharacterized protein	HP↓
E0N0E2	LPXTG-motif cell wall anchor domain protein	HP↓
E0MVM8	Type VII secretion protein EccB	eccB
E0N084	LytR_C domain-containing protein	HP↓
E0MXV8	Proteasome accessory factor PafA2	HP↓
E0MYN6	Uncharacterized protein	HP↓
E0MUF6	5-methyltetrahydropteroyltriglutamate–homocysteine methyltransferase	metE
E0MZF3	Deferrochelatase/peroxidase	HP↓
E0MWG5	Ammonium transporter	amt
E0MY06	ABC transporter, ATP-binding protein	HP↓
E0MY47	TIGR01777 family protein	HP↓
E0MUT0	Antibiotic biosynthesis monooxygenase	HP↓
E0MXN9	Putative ribosomal RNA large subunit methyltransferase J	tlyA
E0MWI6	Glutaredoxin	HP↓
E0MXN0	Glycine oxidase ThiO	thiO
E0MYP6	tRNA (guanine-N (1)-)-methyltransferase	trmD
E0N054	tRNA (Ile)-lysidine synthase	tilS
E0MYB8	TRAM domain protein	HP↓
E0N0M8	Acyltransferase	plsC2
E0MW67	Assimilatory sulfite reductase (ferredoxin)	nirA
E0MV97	AMP-binding enzyme	menE
E0MUD8	DNA replication and repair protein RecF	recF
E0MZC9	Uncharacterized protein	HP↓
E0MZK4	Uncharacterized protein	HP↓
E0MWT2	Quinolinate synthase A	nadA
E0MYH8	YCII domain-containing protein	HP↓
E0MV25	Adenylate/guanylate cyclase catalytic domain protein	HP↓
E0MX33	SNF2 family N-terminal domain protein	HP↓

E0MX76	Respiratory nitrate reductase, gamma subunit	narI
E0MZG1	Uncharacterized protein	HP↓
E0MU31	SURF1-like protein	HP↓
E0MV28	AMP-binding enzyme	HP↓
E0MVB6	NAD_binding_9 domain-containing protein	HP↓
E0MVD9	Deoxyribonucleoside regulator	deoR
E0MVX5	Uncharacterized protein	HP↓
E0MW70	Probable membrane transporter protein	HP↓
E0MWP7	MMPL family protein	HP↓
E0MXC9	Drug resistance MFS transporter, drug:H+ antiporter-2 family	HP↓
E0MXU4	Apolipoprotein N-acyltransferase	lnt
E0MXZ7	ABC transporter, ATP-binding protein	HP↓
E0MYG2	tRNA dimethylallyltransferase	miaA
E0MYL4	Protein adenylyltransferase SelO	selO
E0MYR0	NADH-ubiquinone/plastoquinone (Complex I) family protein	nuoL
E0MZN6	Iron chelate uptake ABC transporter, FeCT family, permease protein	hemU
E0MZW0	Uncharacterized protein	HP↓
E0N0A8	Uncharacterized protein	HP↓
E0N0F0	ABC transporter, ATP-binding protein	HP↓

HP↓: Hypothetical Protein

***C. accolens* strain C782**		
Accession Number	**Protein description**	**Gene name**
E0MYB6	3'-5' exonuclease	HP↓
E0MW83	Uncharacterized protein	HP↓
E0MYP4	Uncharacterized protein	HP↓
E0MW76	Thymidylate kinase	tmk
E0MWW7	RmuC domain protein	rpsI2
E0MZX6	Transporter, gluconate:H+ symporter family	gntT
E0MVZ1	Putative cytidine deaminase	cdd
E0MWV8	Transcriptional regulator, TetR family	HP↓
E0MWM7	Ribosomal RNA small subunit methyltransferase I	rsmI
E0N0F4	Uncharacterized protein	HP↓
E0MV39	DNA-binding helix-turn-helix protein	ramB

E0N009	Abhydrolase_2 domain-containing protein	HP$^{\downarrow}$
E0N092	Hydrolase, NUDIX family	HP$^{\downarrow}$
E0MVG7	ABC transporter, permease protein	HP$^{\downarrow}$
E0MU96	M	NA
E0N0H6	Uncharacterized protein	HP$^{\downarrow}$
E0MXA3	Endonuclease NucS	nucS
E0MXF3	Methyltransferase domain protein	HP$^{\downarrow}$
E0MWI7	Dihydrofolate reductase	folA
E0MWX5	5-oxoprolyl-peptidase	pcp
E0N0S0	Uncharacterized protein	HP$^{\downarrow}$
E0N0G5	Response regulator receiver domain protein	cgtR
E0MZ89	DUF2236 domain-containing protein	HP$^{\downarrow}$
E0MW63	Uncharacterized protein	HP$^{\downarrow}$
E0N043	Acetyltransferase, GNAT family	HP$^{\downarrow}$
E0MWR3	Response regulator receiver domain protein	HP$^{\downarrow}$
E0MXT2	Hydroxymethylpyrimidine kinase	thiD
E0MY89	RelA/SpoT family protein	relA
E0MVJ5	PTS system sucrose-specific IIBC component	scrA
E0MZU4	Serine acetyltransferase	cysE
E0MU61	Copper-exporting ATPase	actP
E0MVS3	HNH endonuclease domain protein	HP$^{\downarrow}$
E0MVY0	DNA-binding helix-turn-helix protein	HP$^{\downarrow}$
E0MW64	Uncharacterized protein	HP$^{\downarrow}$
E0MWD4	Iron chelate uptake ABC transporter, FeCT family, permease protein	feuB
E0MWJ6	ATP-dependent DNA helicase	pcrA
E0MWY5	Transporter, betaine/carnitine/choline family	opuD2
E0MX74	Uncharacterized protein	HP$^{\downarrow}$
E0MXE5	Acetolactate synthase	ilvB
E0MXM5	Amino acid permease	ansP
E0MXS4	Uncharacterized protein	HP$^{\downarrow}$
E0MZE8	BCCT family transporter	opuD3
E0MZH1	Probable nicotinate-nucleotide adenylyltransferase	nadD
E0MZH8	Diphtheria toxin repressor	HP$^{\downarrow}$
E0MZM9	LysR substrate binding domain protein	HP$^{\downarrow}$

HP$^{\downarrow}$: Hypothetical Protein

C. accolens strain C785		
Accession Number	**Protein description**	**Gene name**
E0MZK3	Putative 3-oxoacyl-[acyl-carrier-protein] reductase	fabG
E0MW19	N5-carboxyaminoimidazole ribonucleotide mutase	purE
E0MVJ7	Methionine import ATP-binding protein MetN	metN
E0MWA5	DNA helicase	uvrD
E0MZT4	Oxidoreductase, zinc-binding dehydrogenase family protein	HP↓
E0MUS9	Methyltransferase domain protein	HP↓
E0MVT4	Error-prone DNA polymerase (Fragment)	dnaE2
E0MYB5	Uncharacterized protein	HP↓
E0MY76	Putative FMN reductase	HP↓
E0MW45	Amidase	pncA
E0MYA5	Histidine triad domain protein	HP↓
E0MWE6	Uncharacterized protein	HP↓
E0MWQ8	Peptidyl-tRNA hydrolase	pth
E0MWY3	Selenocysteine-specific translation elongation factor (Fragment)	selB
E0MW21	Ribokinase	rbsK
E0N0L4	Transcriptional regulator, PadR family	HP↓
E0N0L3	Uncharacterized protein	HP↓
E0MWP6	Transcriptional regulator, TetR family	HP↓
E0N0N3	Phosphoglycerate mutase family protein	HP↓
E0MUS2	Hydrolase, NUDIX family	utp
E0MUY3	Uncharacterized protein	HP↓
E0MWW6	Uncharacterized protein	HP↓
E0MZ41	AAA domain-containing protein	HP↓
E0MZ64	Uncharacterized protein	HP↓
E0MYH2	ABC transporter, ATP-binding protein	HP↓
E0MUB5	Putative ACR, COG1678	HP↓
E0MZP1	Transcriptional regulator, TetR family	HP↓
E0MVV6	Uncharacterized protein	HP↓
E0MUF0	Putative 3-methyladenine DNA glycosylase	HP↓
E0MUJ6	Uncharacterized protein	HP↓
E0MUK3	Uncharacterized protein	HP↓
E0MW27	Aldose 1-epimerase	HP↓
E0MWF9	Transporter, SSS family	HP↓

E0MWP3	Cof-like hydrolase	supH
E0MXG5	Uncharacterized protein	HP$^{\downarrow}$
E0MYJ4	4'-phosphopantetheinyl transferase family protein	HP$^{\downarrow}$
E0MYL2	Histidine kinase	cstS
E0MYS9	Trp region conserved hypothetical membrane protein	HP$^{\downarrow}$
E0MZ08	Glycosyltransferase, group 1 family protein	HP$^{\downarrow}$
E0MZ33	RDD family protein	HP$^{\downarrow}$
E0MZB3	Transcriptional regulator, ArsR family	HP$^{\downarrow}$
E0MZN5	ABC transporter, ATP-binding protein	HP$^{\downarrow}$
E0N0B5	Uncharacterized protein	HP$^{\downarrow}$
E0N0D4	Response regulator receiver domain protein	HP$^{\downarrow}$
E0N0N2	Uncharacterized protein	HP$^{\downarrow}$

HP$^{\downarrow}$: Hypothetical Protein

***C. accolens* strain C787**		
Accession Number	**Protein description**	**Gene name**
E0MVD3	Iron chelate uptake ABC transporter, FeCT family, permease protein	fepD
E0MU20	DNA-(apurinic or apyrimidinic site) lyase	nei
E0MXK1	UvrABC system protein B	uvrB
E0MVN6	ESAT-6-like protein	HP$^{\downarrow}$
E0MUV1	Iron chelate uptake ABC transporter, FeCT family, permease protein	HP$^{\downarrow}$
E0MZC8	AAA_16 domain-containing protein	HP$^{\downarrow}$
E0MX59	Transcriptional regulator, IclR family, C-terminal domain protein	HP$^{\downarrow}$
E0MZK7	Amino acid/peptide transporter	dtpT
E0MY25	UvrABC system protein C	uvrC
E0MU54	Uncharacterized protein	HP$^{\downarrow}$
E0MZB0	Uncharacterized protein (Fragment)	HP$^{\downarrow}$
E0MUP3	Uncharacterized protein	HP$^{\downarrow}$
E0MYC6	ROK family protein	ppgK
E0MWP2	Acyltransferase	HP$^{\downarrow}$
E0MWU8	Isoprenyl transferase	uppS
E0MU25	Integrase catalytic domain-containing protein (Fragment)	HP$^{\downarrow}$
E0MZG2	Translocator protein, LysE family	HP$^{\downarrow}$
E0MWD1	SsrA-binding protein	smpB

E0MU74	Universal stress family protein	HP‡
E0MZA3	DNA primase	dnaG
E0MX53	Uncharacterized protein	HP‡
E0MY04	Transcriptional regulator, ArsR family	HP‡
E0MWR9	50S ribosomal protein L25	rplY
E0N0F1	DUF2236 domain-containing protein	HP‡
E0MVI9	Iron chelate uptake ABC transporter, FeCT family, permease protein	HP‡
E0MW41	Phosphoenolpyruvate-dependent sugar phosphotransferase system, EIIA 2	fruA
E0MYT0	Phosphoribosyl-AMP cyclohydrolase	hisI
E0MUY7	Sigma-70, region 4 (Fragment)	sigC
E0MUE9	Transcriptional regulator, MarR family	HP‡
E0MYC9	Uncharacterized protein	HP‡
E0MYA4	CDP-alcohol phosphatidyltransferase	HP‡
E0MVT8	bPH_2 domain-containing protein	HP‡
E0MUP4	Uncharacterized protein	HP‡
E0MXP0	NAD kinase	nadK
E0MXU1	Uncharacterized protein	HP‡
E0MYM1	S2P endopeptidase	HP‡
E0MWG4	Peptidase_S9 domain-containing protein	HP‡
E0MXV0	DEAD/DEAH box helicase	HP‡
E0MU41	Uncharacterized protein (Fragment)	HP‡
E0MYC8	Type III restriction enzyme, res subunit	HP‡
E0MUC7	Transporter, major facilitator family protein	HP‡
E0MYV9	Pseudouridine synthase	rluD
E0MXX5	Uncharacterized protein	HP‡
E0MXL8	Acetylglutamate kinase	argB
E0MUB7	Hydrolase, NUDIX family	HP‡
E0MYN2	Tyrosine recombinase XerC	xerD2
E0MVR3	Uncharacterized protein	HP‡
E0MUM6	Translocator protein, LysE family	HP‡
E0MUZ1	Uncharacterized protein	HP‡
E0MV68	Uncharacterized protein	HP‡
E0MW62	Transcriptional regulator WhiB	whiB
E0MWU1	Uncharacterized protein	HP‡
E0MWY6	Zinc transporter ZupT	zipped
E0MYG6	Uncharacterized protein	HP‡

E0MZE2	ABC transporter, permease protein	HP↓
E0N0R4	Uncharacterized protein	HP↓
E0N0S4	7,8-diamino-pelargonic acid aminotransferase	bioA

HP↓: Hypothetical Protein

Supplementary Table 4.3.

A. Commonly expressed *C. accolens* proteins positively correlated with *S. aureus* growth inhibition

Accession Number	Protein description	Gene name	Average protein abundance	Correlation	Adjusted p-value
E0MUF2	Acetyltransferase, GNAT family	HP↓	9.81E+05	0.80	0.004
E0MW59	Cell envelope-like function transcriptional attenuator common domain protein	HP↓	2.46E+07	0.73	0.014
E0N0N1	Septum_form domain-containing protein	HP↓	1.90E+06	0.67	0.028
E0N0H0	Glycosyl hydrolase family 3 N-terminal domain protein	nagA2	1.37E+06	0.66	0.031
E0MYQ9	Putative monovalent cation/H+ antiporter subunit C	mnhC	3.86E+08	0.65	0.037
E0MUF4	Uncharacterized protein	HP↓	1.62E+06	0.56	0.096
E0MX30	Uncharacterized protein	HP↓	1.22E+06	0.53	0.130
E0MVH8	50S ribosomal protein L23	rplW	2.58E+07	0.53	0.130
E0MZ78	Peroxiredoxin, Ohr subfamily	ohr	9.80E+06	0.52	0.136
E0MYA7	Putative Tat-translocated enzyme	HP↓	2.55E+06	0.52	0.139
E0MXY1	Coproporphyrin III ferrochelatase	hemH	1.69E+06	0.52	0.143
E0MW98	ZnMc domain-containing protein	HP↓	8.97E+06	0.51	0.144
E0MZN4	ErfK/YbiS/YcfS/YnhG	lppS	1.24E+07	0.51	0.145
E0MUN2	Transmembrane amino acid transporter protein	sdaC	1.58E+05	0.51	0.153

HP↓: Hypothetical Protein

B. Commonly expressed *C. accolens* proteins negatively correlated with *S. aureus* growth inhibition

Accession Number	Protein description	Gene name	Average protein abundance	Correlation	Adjusted p-value
E0MU77	Uncharacterized protein	HP↓	3.13E+06	-0.87	0.001
E0N0A0	Putative phosphoribosylglycinamide formyltransferase 2	purT2	8.46E+05	-0.84	0.003
E0MVX9	Pyridine nucleotide-disulfide oxidoreductase	lpdA	2.87E+06	-0.83	0.003
E0MU85	GroES-like protein (Fragment)	HP↓	1.25E+06	-0.83	0.003
E0MZS6	Thioredoxin-like_fold domain-containing protein	HP↓	7.73E+06	-0.81	0.004
E0MU99	Succinate-semialdehyde dehydrogenase	gabD	2.80E+07	-0.81	0.004
E0N0F8	dCTP deaminase, dUMP-forming	dcd	2.62E+06	-0.80	0.004
E0MXY2	NlpC/P60 family protein	HP↓	1.95E+08	-0.80	0.004
E0N0C6	Aldehyde dehydrogenase (NAD) family protein	aldA2	1.31E+08	-0.79	0.006
E0MYZ0	Uncharacterized protein (Fragment)	HP↓	2.13E+06	-0.79	0.006
E0MV84	Coproporphyrinogen III oxidase	hemG	5.07E+06	-0.78	0.006
E0MXC6	Glutamyl-tRNA(Gln) amidotransferase subunit A	gatA	9.90E+06	-0.78	0.006
E0MUY9	Catalase	cat	2.71E+07	-0.78	0.006
E0N074	Peptidase dimerization domain protein	HP↓	9.48E+06	-0.76	0.009
E0MZU7	Succinate CoA transferase	actA	6.63E+07	-0.76	0.009
E0MWS7	Uncharacterized protein	HP↓	3.66E+07	-0.76	0.009
E0N0J8	Beta-ketoacyl synthase, N-terminal domain protein (Fragment)	pks2	4.36E+05	-0.75	0.011
E0MYL5	Probable malate:quinone oxidoreductase	mqo	1.67E+07	-0.75	0.011
E0N0M4	Glycerol kinase	glpK	1.46E+07	-0.74	0.012
E0N097	Phosphate acetyltransferase	pta	6.27E+06	-0.74	0.012
E0MV06	Redoing family protein	HP↓	4.64E+05	-0.73	0.014
E0MZS3	NH(3)-dependent NAD(+) synthetase	nadE	1.43E+06	-0.73	0.014
E0N0M9	Serine--tRNA ligase	serS	1.15E+07	-0.73	0.014
E0MWL3	Trypsin	HP↓	1.27E+08	-0.72	0.016
E0MVY1	MmgE/PrpD family protein	prpD	1.09E+07	-0.72	0.017
E0MXT8	Dyp-type peroxidase family protein	tyrA2	2.87E+06	-0.72	0.017
E0MVS2	GMP synthase [glutamine-hydrolyzing]	guaA	2.91E+07	-0.71	0.017
E0MVS0	Inosine-5'-monophosphate dehydrogenase	guaB	2.33E+07	-0.71	0.017
E0MXG4	Glutamate--tRNA ligase	gltX	1.03E+07	-0.71	0.017
E0MXM4	Argininosuccinate lyase (Fragment)	argH	2.55E+06	-0.71	0.017

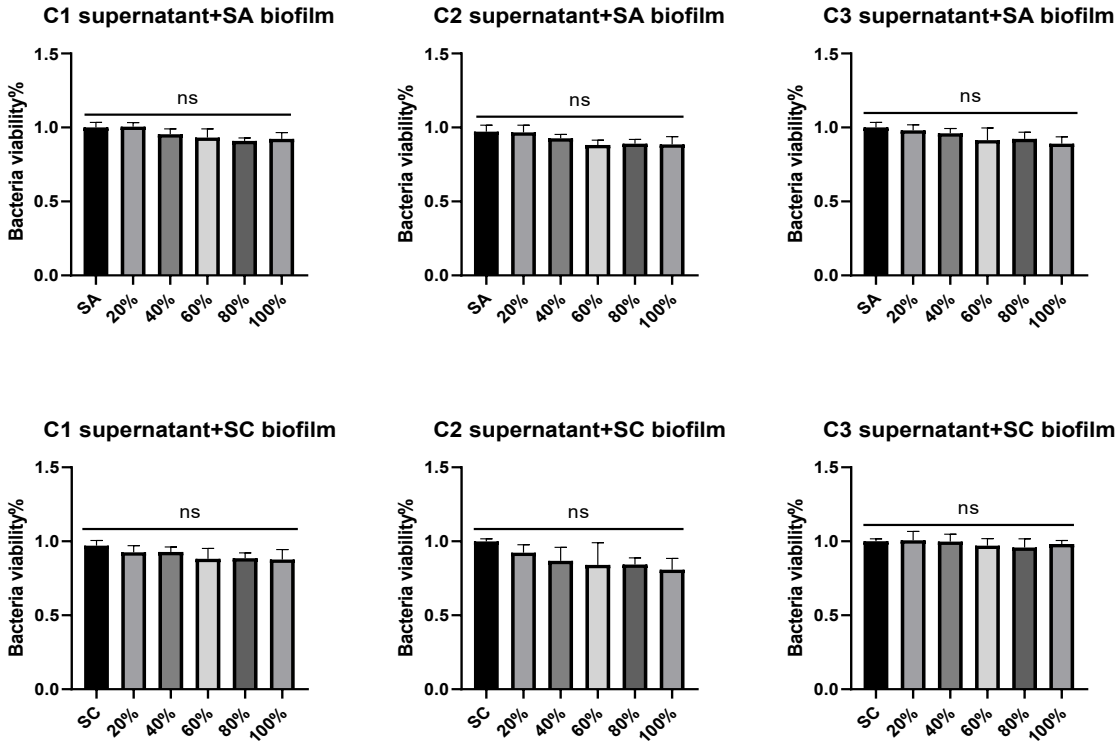
E0MVB9	Putative 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylic-acid synthase (Fragment)	menD	9.16E+05	-0.71	0.017
E0N0B6	Rhodanese-like protein	sseA	3.51E+06	-0.71	0.018
E0MXD3	Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit B	gatB	8.70E+06	-0.70	0.018
E0N0Q8	Universal stress family protein	uspA2	2.11E+07	-0.70	0.018
E0MZT3	ABC transporter, ATP-binding protein	HP↓	4.54E+05	-0.70	0.019
E0MX06	2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase	dapD	1.84E+07	-0.69	0.020
E0MWK0	Bifunctional purine biosynthesis protein PurH	purH	2.91E+06	-0.69	0.020
E0MXC7	META domain-containing protein	HP↓	3.01E+08	-0.69	0.022
E0MY57	Uncharacterized protein	HP↓	1.27E+06	-0.68	0.025
E0MVI7	Periplasmic binding protein	fecS	2.41E+08	-0.68	0.026
E0MY80	Thiol peroxidase	tpx	5.01E+06	-0.68	0.026
E0MV33	dTDP-4-dehydrorhamnose reductase	rfbD	2.20E+06	-0.67	0.028
E0MYY9	UDP-N-acetylmuramoyl-tripeptide--D-alanyl-D-alanine ligase	murF	3.25E+06	-0.67	0.028
E0MZN7	Periplasmic binding protein	hmuT2	1.27E+08	-0.67	0.030
E0MX66	Diaminopimelate decarboxylase	lysA	1.99E+06	-0.65	0.037
E0MY03	FeS assembly protein SufB	sufB	9.04E+06	-0.65	0.039
E0N060	Hypoxanthine phosphoribosyltransferase	hpt	6.05E+05	-0.65	0.039
E0N013	Cysteine--tRNA ligase (Fragment)	cysS	1.33E+06	-0.65	0.039
E0MX65	Arginine--tRNA ligase	argS	1.30E+07	-0.64	0.045
E0MVY3	Citrate synthase (unknown stereospecificity) (Fragment)	prpC	7.55E+06	-0.64	0.045
E0MXB0	Electron transfer flavoprotein domain protein (Fragment)	etfA	9.50E+06	-0.64	0.046
E0MZR8	Ribonucleoside-diphosphate reductase	nrdE	7.82E+05	-0.63	0.046
E0MYL7	Mycothione reductase	mtr	2.89E+07	-0.63	0.046
E0MZR1	Nicotinate phosphoribosyltransferase	pncB	2.21E+06	-0.63	0.046
E0MVV7	Pyridoxal 5'-phosphate synthase subunit PdxS	pdxS	4.04E+06	-0.63	0.046
E0MWF2	Uncharacterized protein	HP↓	7.84E+05	-0.63	0.046
E0MUL6	Aldehyde dehydrogenase (NAD) family protein	betB	8.89E+06	-0.63	0.047
E0MZ56	Pyruvate dehydrogenase (acetyl-transferring) (Fragment)	aceE	2.11E+06	-0.63	0.048
E0MVZ2	Thymidine phosphorylase	deoA	1.59E+07	-0.63	0.048
E0MYG1	Diaminopimelate epimerase	dapF	1.23E+06	-0.62	0.049
E0MWJ3	Glucose-6-phosphate isomerase	pgi	1.46E+07	-0.62	0.055
E0N0P1	L-lactate dehydrogenase	ldh	1.95E+07	-0.61	0.057

E0MX25	Tat pathway signal sequence domain protein	HP↓	4.30E+05	-0.61	0.057
E0MZH3	Gamma-glutamyl phosphate reductase	proA	1.02E+07	-0.61	0.061
E0MVZ0	Nucleoside transporter, NupC family	yutK	5.90E+05	-0.61	0.061
E0MW18	N5-carboxyaminoimidazole ribonucleotide synthase	purK	1.33E+06	-0.60	0.062
E0MY36	S-adenosylmethionine synthase	metK	1.93E+06	-0.60	0.062
E0MWK5	DUF294_C domain-containing protein	HP↓	4.32E+05	-0.60	0.063
E0MYY0	Cell division protein FtsZ	ftsZ	4.88E+06	-0.60	0.064
E0MZI0	Glutamate 5-kinase	proB	4.95E+06	-0.60	0.064
E0MY78	Histidine--tRNA ligase	hisS	3.77E+06	-0.60	0.065
E0MW14	ABC transporter, ATP-binding protein	HP↓	4.96E+05	-0.59	0.074
E0MVP1	Phosphoglucosamine mutase	glmM	1.40E+06	-0.59	0.074
E0MZ46	Glutamine synthetase	glnA2	1.88E+06	-0.58	0.080
E0MZZ1	Phosphoribosylaminoimidazole-succinocarboxamide synthase	purC	1.77E+06	-0.58	0.082
E0N022	ATPase family associated with various cellular activities (AAA)	clpC	5.89E+05	-0.57	0.088
E0MXE7	Ketol-acid reductoisomerase (NADP(+))	ilvC	2.50E+06	-0.57	0.088
E0MZB2	Glycine--tRNA ligase	glyS	8.22E+06	-0.57	0.088
E0MVX1	Uncharacterized protein	HP↓	3.50E+06	-0.57	0.089
E0MUU5	Putative tRNA adenosine deaminase-associated protein	HP↓	9.39E+05	-0.57	0.089
E0MY60	Elongation factor P	efp	4.69E+06	-0.57	0.089
E0MV03	Cyclic nucleotide-binding domain protein	ntcA	2.14E+07	-0.56	0.096
E0N0Q2	DUF2020 domain-containing protein	HP↓	7.94E+05	-0.56	0.096
E0MYS5	Pyruvate kinase	pyk	4.00E+07	-0.56	0.096
E0MVN9	30S ribosomal protein S9	rpsI	6.42E+06	-0.56	0.101
E0MZ73	CRISPR system CASCADE complex protein CasD	casD	1.19E+06	-0.55	0.103
E0MYE4	Antioxidant, AhpC/TSA family	dirA	2.71E+07	-0.55	0.103
E0MX15	Glycosyl hydrolase family 32	HP↓	2.83E+06	-0.55	0.103
E0MZ62	Oxidoreductase, FAD/FMN-binding protein	nemA	7.85E+07	-0.55	0.107
E0MWB6	Periplasmic binding protein	HP↓	1.47E+07	-0.55	0.111
E0N0I5	tRNA (guanine-N(7)-)-methyltransferase	trmB	7.61E+05	-0.54	0.113
E0MWT8	Uncharacterized protein	HP↓	4.68E+05	-0.54	0.113
E0MX63	Putative iron-sulfur cluster-binding protein	ykgF	3.88E+06	-0.54	0.114
E0MXH3	3-isopropylmalate dehydratase large subunit	leuC	1.59E+06	-0.53	0.124
E0MZ34	Glutamine synthetase	glnA	1.34E+07	-0.53	0.124
E0MVX3	Uncharacterized protein	HP↓	5.79E+06	-0.53	0.130

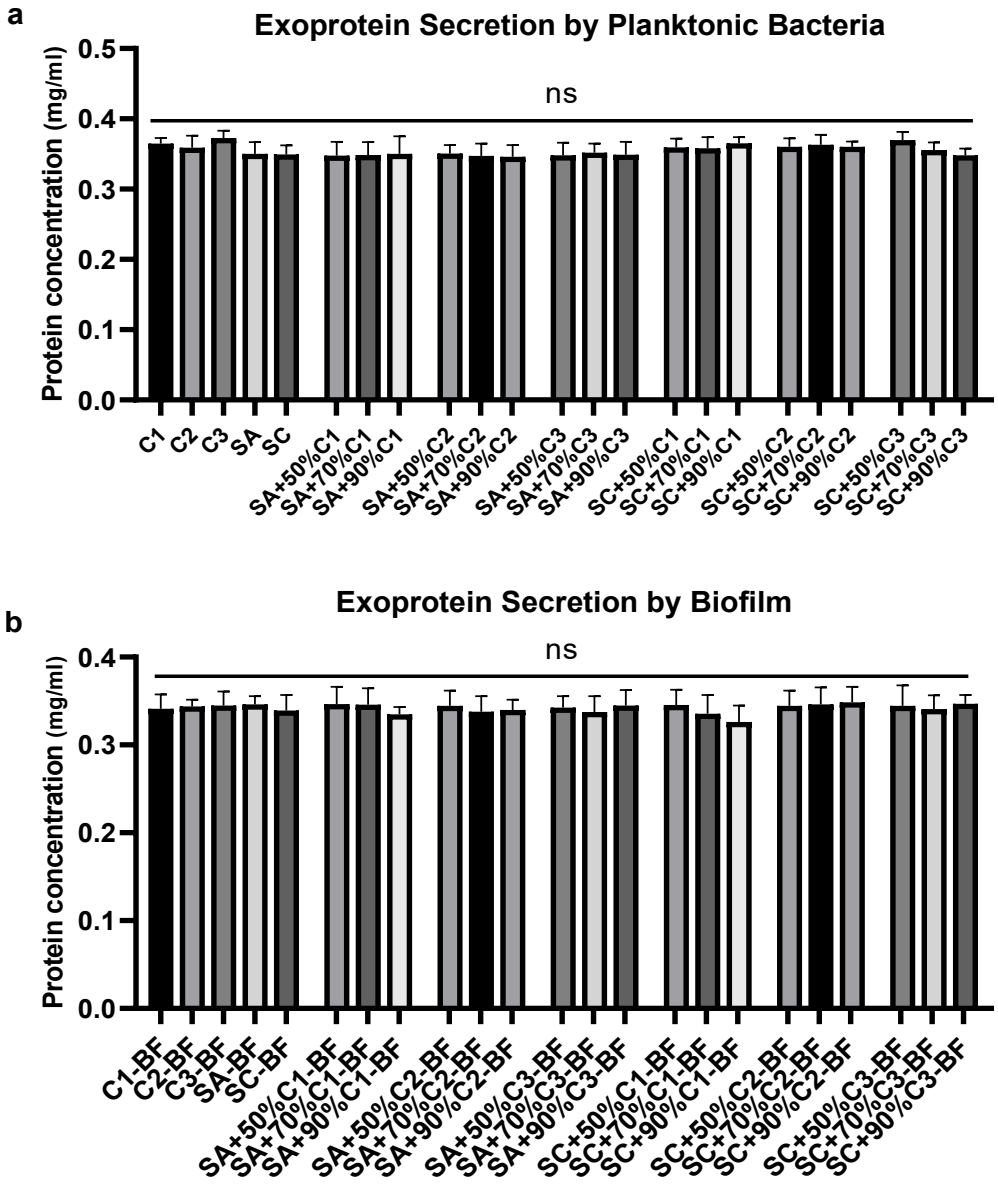
E0MX32	Sodium/proline symporter	putP	2.10E+06	-0.53	0.130
E0MWV6	Acetyltransferase, GNAT family	HP↓	2.93E+06	-0.53	0.130
E0MX16	O-methyltransferase	HP↓	1.58E+05	-0.52	0.133
E0MXA0	ATP synthase subunit beta	atpD	1.15E+08	-0.52	0.134
E0MVM4	30S ribosomal protein S4	rpsD	3.01E+06	-0.52	0.135
E0MYY3	UDP-N-acetylmuramate--L-alanine ligase	murC	1.54E+06	-0.51	0.147
E0MWT9	Ppx/GppA phosphatase family protein	HP↓	4.79E+06	-0.51	0.155

Appendix V. Supporting information for Chapter 6

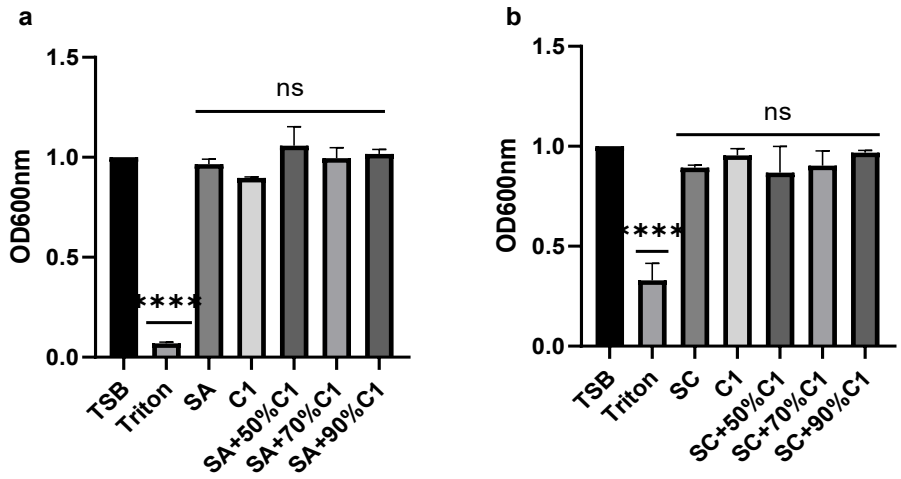
Additional material provided by the authors to supplement paper written in manuscript format in Chapter 6.



Supplementary Figure 6.1. Antibacterial effects against *S. aureus* biofilms. The different concentrations of *C. accolens* 1-3 supernatants were used to treat SA and SC biofilms. Cell viability was calculated. C1= *C. accolens* supernatant exoprotein from *C. accolens* clinical isolate 1, C2= *C. accolens* supernatant exoprotein from *C. accolens* clinical isolate 2, C3= *C. accolens* supernatant exoprotein from *C. accolens* clinical isolate 3, SA= *S. aureus* ATCC51650, SC= *S. aureus* clinical strain. ns: $P > 0.05$. Experiments were performed with six replicates for three times.



Supplementary Figure 6.2. Exoprotein quantification. *S. aureus* and *C. accolens* bacteria in different proportions (50%, 70%, 90%) were co-cultured, the exoprotein in the supernatant of planktonic (a) and biofilm (b) forms were determined. C1= *C. accolens* clinical isolate 1, C2= *C. accolens* clinical isolate 2, C3= *C. accolens* clinical isolate 3, SA= *S. aureus* ATCC51650, SC= *S. aureus* clinical strain, BF= biofilm. ns: $P > 0.05$. Experiments were performed three times.



Supplementary Figure 6.3. LDH Assay. The LDH of HNECs after two hours treatment with the supernatants of SA and SC co-cultured with *C. accolens* in different ratios were determined, absorbance was read using a microplate reader at 490nm after 30min incubation. C= *C. accolens*, SA= *S. aureus* ATCC51650, SC= *S. aureus* clinical strain. Experiments were performed with three replicates. ns: $P > 0.05$, ****: $P < 0.0001$.

REFERENCES

1. Almutairi, A.F.N., et al., *Acute and Chronic Sinusitis Causes and Management.* The Egyptian Journal of Hospital Medicine, 2017. **68**(3): p. 1513-1519.
2. Meltzer, E.O., et al., *Rhinosinusitis: establishing definitions for clinical research and patient care.* Journal of allergy and clinical immunology, 2004. **114**(6): p. 155-212.
3. Scadding, G., et al., *BSACI guidelines for the management of rhinosinusitis and nasal polyposis.* Clinical & Experimental Allergy, 2008. **38**(2): p. 260-275.
4. Committee, R.T.F., *Report of the rhinosinusitis task force committee meeting. Alexandria, Virginia, August 17, 1996.* Otolaryngol Head Neck Surg, 1997. **117**(3 Pt 2): p. S1-68.
5. Benninger, M.S., et al., *Adult chronic rhinosinusitis: definitions, diagnosis, epidemiology, and pathophysiology.* Otolaryngology-Head and Neck Surgery, 2003. **129**(3): p. S1-S32.
6. Riechelmann, H., *Europäischen Akademie für Allergie und Klinische Immunologie (EAACI) un der European Rhinologic Society (ERS).[Chronic Rhinosinusitis.* EPOS, 2012: p. 193-201.
7. Blackwell, D.L. and L. Tonthat, *Summary Health Statistics for US Children: National Health Interview Survey, 1999.* 2003.
8. Blackwell, D.L., J.W. Lucas, and T.C. Clarke, *Summary health statistics for US adults: national health interview survey, 2012.* Vital and health statistics. Series 10, Data from the National Health Survey, 2014(260): p. 1.
9. DeConde, A.S. and Z.M. Soler, *Chronic rhinosinusitis: epidemiology and burden of disease.* American journal of rhinology & allergy, 2016. **30**(2): p. 134-139.
10. Speight, J., et al., *Diabetes MILES-Australia (Management and Impact for Long-term Empowerment and Success): methods and sample characteristics of a national survey*

of the psychological aspects of living with type 1 or type 2 diabetes in Australian adults. BMC Public Health, 2012. **12**(1): p. 120.

11. Smith, K.A., R.R. Orlandi, and L. Rudmik, *Cost of adult chronic rhinosinusitis: a systematic review.* The Laryngoscope, 2015. **125**(7): p. 1547-1556.
12. Caulley, L., et al., *Direct costs of adult chronic rhinosinusitis by using 4 methods of estimation: results of the US Medical Expenditure Panel Survey.* Journal of Allergy and Clinical Immunology, 2015. **136**(6): p. 1517-1522.
13. Gliklich, R.E. and R. Metson, *The health impact of chronic sinusitis in patients seeking otolaryngologic care.* Otolaryngology—Head and Neck Surgery, 1995. **113**(1): p. 104-109.
14. Hamilos, D.L., E.H. Holbrook, and D.G. Deschler, *Chronic rhinosinusitis: clinical manifestations, pathophysiology, and diagnosis.* UpToDate. Waltham: UpToDate Inc. http://www. uptodate. com. Accessed on, 2017. **7**.
15. Hamilos, D.L., *Chronic rhinosinusitis: epidemiology and medical management.* Journal of allergy and clinical immunology, 2011. **128**(4): p. 693-707.
16. Fokkens, W.J., et al., *European position paper on rhinosinusitis and nasal polyps 2012.* Rhinology. Supplement, 2012. **23**: p. 3 p preceding table of contents, 1-298.
17. Orlandi, R.R., et al. *International consensus statement on allergy and rhinology: rhinosinusitis*. in *International forum of allergy & rhinology*. 2016. Wiley Online Library.
18. Van Zele, T., et al., *Differentiation of chronic sinus diseases by measurement of inflammatory mediators.* Allergy, 2006. **61**(11): p. 1280-1289.
19. ten Brinke, A., et al., *Chronic sinusitis in severe asthma is related to sputum eosinophilia.* Journal of Allergy and Clinical Immunology, 2002. **109**(4): p. 621-626.

20. Lam, K., R. Schleimer, and R.C. Kern, *The etiology and pathogenesis of chronic rhinosinusitis: a review of current hypotheses.* Current allergy and asthma reports, 2015. **15**(7): p. 41.

21. Van Crombruggen, K., et al., *Pathogenesis of chronic rhinosinusitis: inflammation.* Journal of Allergy and Clinical Immunology, 2011. **128**(4): p. 728-732.

22. Tan, B.K., R.P. Schleimer, and R.C. Kern, *Perspectives on the etiology of chronic rhinosinusitis.* Current opinion in otolaryngology & head and neck surgery, 2010. **18**(1): p. 21.

23. Lee, S. and A.P. Lane, *Chronic rhinosinusitis as a multifactorial inflammatory disorder.* Current infectious disease reports, 2011. **13**(2): p. 159-168.

24. Stevens, W.W., et al., *Chronic rhinosinusitis pathogenesis.* Journal of Allergy and Clinical Immunology, 2015. **136**(6): p. 1442-1453.

25. Sasama, J., et al., *New paradigm for the roles of fungi and eosinophils in chronic rhinosinusitis.* Current opinion in otolaryngology & head and neck surgery, 2005. **13**(1): p. 2-8.

26. Bachert, C., et al., *Role of staphylococcal superantigens in upper airway disease.* Current opinion in allergy and clinical immunology, 2008. **8**(1): p. 34-38.

27. Ratnawati, L.M. and I.P.Y. Pradiptha, *Anatomic Variation of CT Scan in Chronic Rhinosinusitis Patients in Sanglah Provincial General Hospital.* Biomedical and Pharmacology Journal, 2019. **12**(04): p. 2083-2086.

28. Zinreich, S. and T. Gotwald, *Radiographic anatomy of the sinuses.* Disease of the Sinuses: Diagnosis and Management. Lewiston, Newyork: BC Decker Inc, 2001: p. 13-27.

29. Jiao, J., C. Wang, and L. Zhang, *Epithelial physical barrier defects in chronic rhinosinusitis.* Expert review of clinical immunology, 2019. **15**(6): p. 679-688.

30. Hariri, B.M. and N.A. Cohen, *New insights into upper airway innate immunity.* American Journal of Rhinology & Allergy, 2016. **30**(5): p. 319-323.

31. Murphy, J., et al., *Mucosal zinc deficiency in chronic rhinosinusitis with nasal polyposis contributes to barrier disruption and decreases ZO-1.* Allergy, 2018. **73**(10): p. 2095-2097.

32. Gudis, D., K.-q. Zhao, and N.A. Cohen, *Acquired cilia dysfunction in chronic rhinosinusitis.* American journal of rhinology & allergy, 2012. **26**(1): p. 1-6.

33. Sedaghat, A.R., *Chronic rhinosinusitis*, in *Infections of the Ears, Nose, Throat, and Sinuses*. 2018, Springer. p. 155-168.

34. Bachert, C., et al., *Nasal polyposis: from cytokines to growth.* American journal of rhinology, 2000. **14**(5): p. 279-290.

35. Holgate, S.T., *Epithelium dysfunction in asthma.* Journal of Allergy and Clinical Immunology, 2007. **120**(6): p. 1233-1244.

36. Wise, S.K., et al. *Interleukin-4 and interleukin-13 compromise the sinonasal epithelial barrier and perturb intercellular junction protein expression*. in *International forum of allergy & rhinology*. 2014. Wiley Online Library.

37. Kern, R.C., et al., *Perspectives on the etiology of chronic rhinosinusitis: an immune barrier hypothesis.* American journal of rhinology, 2008. **22**(6): p. 549-559.

38. Mjösberg, J.M., et al., *Human IL-25-and IL-33-responsive type 2 innate lymphoid cells are defined by expression of CRTH2 and CD161.* Nature immunology, 2011. **12**(11): p. 1055-1062.

39. Shaw, J.L., et al., *IL-33–responsive innate lymphoid cells are an important source of IL-13 in chronic rhinosinusitis with nasal polyps.* American journal of respiratory and critical care medicine, 2013. **188**(4): p. 432-439.

40. Nagarkar, D.R., et al., *Thymic stromal lymphopoietin activity is increased in nasal polyps of patients with chronic rhinosinusitis.* Journal of allergy and clinical immunology, 2013. **132**(3): p. 593-600. e12.

41. Detwiller, K.Y., et al., *Differential expression of innate immunity genes in chronic rhinosinusitis.* American journal of rhinology & allergy, 2014. **28**(5): p. 374-377.

42. Shen, Y., et al., *Impaired balance of Th17/Treg in patients with nasal polyposis.* Scandinavian journal of immunology, 2011. **74**(2): p. 176-185.

43. Ramezanpour, M., et al., *Th17 cytokines disrupt the airway mucosal barrier in chronic rhinosinusitis.* Mediators of inflammation, 2016. **2016**.

44. Tieu, D.D., R.C. Kern, and R.P. Schleimer, *Alterations in epithelial barrier function and host defense responses in chronic rhinosinusitis.* Journal of allergy and clinical immunology, 2009. **124**(1): p. 37-42.

45. Hulse, K.E., *Immune mechanisms of chronic rhinosinusitis.* Current allergy and asthma reports, 2016. **16**(1): p. 1.

46. Pérez-Novo, C.A., et al., *Prostaglandin, leukotriene, and lipoxin balance in chronic rhinosinusitis with and without nasal polyposis.* Journal of Allergy and Clinical Immunology, 2005. **115**(6): p. 1189-1196.

47. Nick, J.A. and D.M. Rodman, *Manifestations of cystic fibrosis diagnosed in adulthood.* Current opinion in pulmonary medicine, 2005. **11**(6): p. 513-518.

48. Wang, X., et al., *Increased prevalence of chronic rhinosinusitis in carriers of a cystic fibrosis mutation.* Archives of Otolaryngology–Head & Neck Surgery, 2005. **131**(3): p. 237-240.

49. Johnson, B.J., G.W. Choby, and E.K. O'Brien, *Chronic rhinosinusitis in patients with cystic fibrosis—Current management and new treatments.* Laryngoscope Investigative Otolaryngology, 2020. **5**(3): p. 368-374.

50. Robertson, J.M., E.M. Friedman, and B.K. Rubin, *Nasal and sinus disease in cystic fibrosis.* Paediatric respiratory reviews, 2008. **9**(3): p. 213-219.

51. Laura, C., et al., *Nasal Polyposis in Patients with Primary Ciliary Dyskinesia.* J Otolaryngology ENT Res, 2017. **7**(4): p. 00210.

52. Cervin, A.U., *The potential for topical probiotic treatment of chronic rhinosinusitis, a personal perspective.* Frontiers in cellular and infection microbiology, 2018. **7**: p. 530.

53. Kennedy, D.W., *Pathogenesis of chronic rhinosinusitis.* Annals of Otology, Rhinology & Laryngology, 2004. **113**(5_suppl): p. 6-9.

54. Gao, W.-X., et al., *Occupational and environmental risk factors for chronic rhinosinusitis in China: a multicentre cross-sectional study.* Respiratory research, 2016. **17**(1): p. 1-7.

55. Lötvall, J., L. Ekerljung, and B. Lundbäck, *Multi-symptom asthma is closely related to nasal blockage, rhinorrhea and symptoms of chronic rhinosinusitis-evidence from the West Sweden Asthma Study.* Respiratory research, 2010. **11**(1): p. 163.

56. Jang, D.W., et al., *Aspirin sensitivity does not compromise quality-of-life outcomes in patients with Samter's triad.* The Laryngoscope, 2014. **124**(1): p. 34-37.

57. Chang, J.E., et al. *Aspirin-exacerbated respiratory disease: burden of disease*. in *Allergy and Asthma Proceedings*. 2012. OceanSide Publications.

58. Stevens, W.W. and R.P. Schleimer, *Aspirin-exacerbated respiratory disease as an endotype of chronic rhinosinusitis.* Immunology and Allergy Clinics, 2016. **36**(4): p. 669-680.

59. Kim, S.Y., et al. *Gastroesophageal reflux disease increases the risk of chronic rhinosinusitis: a nested case-control study using a national sample cohort*. in *International forum of allergy & rhinology*. 2019. Wiley Online Library.

60. Leason, S.R., et al., *Association of gastro-oesophageal reflux and chronic rhinosinusitis: systematic review and meta-analysis*. 2017.

61. Vakil, N., et al., *The Montreal definition and classification of gastroesophageal reflux disease: a global evidence-based consensus.* American Journal of Gastroenterology, 2006. **101**(8): p. 1900-1920.

62. Hanna, B.C. and P.J. Wormald, *Gastroesophageal reflux and chronic rhinosinusitis.* Current opinion in otolaryngology & head and neck surgery, 2012. **20**(1): p. 15-18.

63. Min, J.-Y. and B.K. Tan, *Risk factors for chronic rhinosinusitis.* Current opinion in allergy and clinical immunology, 2015. **15**(1): p. 1.

64. Okano, M., et al., *Role of fungal antigens in eosinophilia-associated cellular responses in nasal polyps: a comparison with enterotoxin.* Clinical & Experimental Allergy, 2011. **41**(2): p. 171-178.

65. Schubert, M.S., *Allergic fungal sinusitis: pathophysiology, diagnosis and management.* Medical Mycology, 2009. **47**(Supplement_1): p. S324-S330.

66. Ferguson, B.J., *Eosinophilic mucin rhinosinusitis: a distinct clinicopathological entity.* The Laryngoscope, 2000. **110**(5): p. 799-813.

67. Shin, S.-H., et al., *Chronic rhinosinusitis: an enhanced immune response to ubiquitous airborne fungi.* Journal of Allergy and Clinical Immunology, 2004. **114**(6): p. 1369-1375.

68. Pandak, N., et al., *Bacterial colonization or infection in chronic sinusitis.* Wiener klinische Wochenschrift, 2011. **123**(23-24): p. 710-713.

69. Van Zele, T., et al., *Staphylococcus aureus colonization and IgE antibody formation to enterotoxins is increased in nasal polyposis.* Journal of Allergy and Clinical Immunology, 2004. **114**(4): p. 981-983.

70. Sachse, F., et al., *Staphylococcus aureus invades the epithelium in nasal polyposis and induces IL-6 in nasal epithelial cells in vitro.* Allergy, 2010. **65**(11): p. 1430-1437.

71. Vickery, T.W., V.R. Ramakrishnan, and J.D. Suh, *The role of Staphylococcus aureus in patients with chronic sinusitis and nasal polyposis.* Current allergy and asthma reports, 2019. **19**(4): p. 21.

72. Brook, I. and E.H. Frazier, *Correlation between microbiology and previous sinus surgery in patients with chronic maxillary sinusitis.* Annals of Otology, Rhinology & Laryngology, 2001. **110**(2): p. 148-151.

73. Bolger, W.E., *Gram negative sinusitis: an emerging clinical entity?* American Journal of Rhinology, 1994. **8**(6): p. 279-284.

74. Brook, I., P.A. Foote, and J.N. Hausfeld, *Increase in the frequency of recovery of meticillin-resistant Staphylococcus aureus in acute and chronic maxillary sinusitis.* Journal of medical microbiology, 2008. **57**(8): p. 1015-1017.

75. T PROFT, J., *Bacterial superantigens.* Clinical and experimental immunology, 2003. **133**(3): p. 299.

76. Van Kaer, L., *How superantigens bind MHC.* The Journal of Immunology, 2018. **201**(7): p. 1817-1818.

77. Schubert, M.S., *A superantigen hypothesis for the pathogenesis of chronic hypertrophic rhinosinusitis, allergic fungal sinusitis, and related disorders.* Annals of Allergy, Asthma & Immunology, 2001. **87**(3): p. 181-188.

78. Bachert, C., et al., *Allergic rhinitis, rhinosinusitis, and asthma: one airway disease.* Immunology and Allergy Clinics, 2004. **24**(1): p. 19-43.

79. Seiberling, K.A., et al., *Superantigens and chronic rhinosinusitis: detection of staphylococcal exotoxins in nasal polyps.* The Laryngoscope, 2005. **115**(9): p. 1580-1585.

80. Patel, R., *Biofilms and antimicrobial resistance.* Clinical Orthopaedics and Related Research®, 2005. **437**: p. 41-47.

81. Stewart, P.S. and J.W. Costerton, *Antibiotic resistance of bacteria in biofilms.* The lancet, 2001. **358**(9276): p. 135-138.

82. Ramakrishnan, Y., et al., *Biofilms in chronic rhinosinusitis: what is new and where next?* The Journal of laryngology and otology, 2015. **129**(8): p. 744.

83. Foreman, A., et al., *Adaptive immune responses in Staphylococcus aureus biofilm–associated chronic rhinosinusitis.* Allergy, 2011. **66**(11): p. 1449-1456.

84. Foreman, A. and P.J. Wormald, *Different biofilms, different disease? A clinical outcomes study.* The Laryngoscope, 2010. **120**(8): p. 1701-1706.

85. Psaltis, A.J., et al., *The effect of bacterial biofilms on post-sinus surgical outcomes.* American journal of rhinology, 2008. **22**(1): p. 1-6.

86. Ramadan, H.H., J.A. Sanclement, and J.G. Thomas, *Chronic rhinosinusitis and biofilms.* Otolaryngology—Head and Neck Surgery, 2005. **132**(3): p. 414-417.

87. Psaltis, A.J., et al., *Confocal scanning laser microscopy evidence of biofilms in patients with chronic rhinosinusitis.* The Laryngoscope, 2007. **117**(7): p. 1302-1306.

88. Bendouah, Z., et al., *Biofilm formation by Staphylococcus aureus and Pseudomonas aeruginosa is associated with an unfavorable evolution after surgery for chronic sinusitis and nasal polyposis.* Otolaryngology—Head and Neck Surgery, 2006. **134**(6): p. 991-996.

89. Foreman, A., et al., *Characterization of bacterial and fungal biofilms in chronic rhinosinusitis.* American journal of rhinology & allergy, 2009. **23**(6): p. 556-561.

90. Smith, T.L., et al., *Predictive factors and outcomes in endoscopic sinus surgery for chronic rhinosinusitis.* The Laryngoscope, 2005. **115**(12): p. 2199-2205.

91. Briggs, R.D., et al., *Smoking in chronic rhinosinusitis: a predictor of poor long-term outcome after endoscopic sinus surgery.* The Laryngoscope, 2004. **114**(1): p. 126-128.

92. Ramadan, H.H. and R.A. Hinerman, *Smoke exposure and outcome of endoscopic sinus surgery in children.* Otolaryngology—Head and Neck Surgery, 2002. **127**(6): p. 546-548.

93. Shaykhiev, R. and R.G. Crystal, *Early events in the pathogenesis of chronic obstructive pulmonary disease. Smoking-induced reprogramming of airway epithelial basal progenitor cells.* Annals of the American Thoracic Society, 2014. **11**(Supplement 5): p. S252-S258.

94. Yamin, M., et al., *Cigarette smoke combined with Toll-like receptor 3 signaling triggers exaggerated epithelial regulated upon activation, normal T-cell expressed and secreted/CCL5 expression in chronic rhinosinusitis.* Journal of allergy and clinical immunology, 2008. **122**(6): p. 1145-1153. e3.

95. Miraglia Del Giudice, M., et al., *Nasal microbiome in chronic rhinosinusitis.* Minerva Pediatrica, 2020.

96. Cho, D.-Y., R.C. Hunter, and V.R. Ramakrishnan, *The microbiome and chronic rhinosinusitis.* Immunology and Allergy Clinics, 2020. **40**(2): p. 251-263.

97. Hoggard, M., et al. *Evidence of microbiota dysbiosis in chronic rhinosinusitis*. in *International forum of allergy & rhinology*. 2017. Wiley Online Library.

98. Copeland, E., et al., *Chronic rhinosinusitis: potential role of microbial dysbiosis and recommendations for sampling sites.* Frontiers in cellular and infection microbiology, 2018. **8**: p. 57.

99. Liu, C.M., et al. *Medical therapy reduces microbiota diversity and evenness in surgically recalcitrant chronic rhinosinusitis*. in *International forum of allergy & rhinology*. 2013. Wiley Online Library.

100. Dimitri-Pinheiro, S., R. Soares, and P. Barata, *The Microbiome of the Nose—Friend or Foe?* Allergy & Rhinology, 2020. **11**: p. 2152656720911605.

101. Rawls, M. and A.K. Ellis, *The microbiome of the nose.* Annals of Allergy, Asthma & Immunology, 2019. **122**(1): p. 17-24.

102. Peterson, J., et al., *The NIH human microbiome project.* Genome research, 2009. **19**(12): p. 2317-2323.

103. Lee, J.T., C.M. Kim, and V. Ramakrishnan, *Microbiome and disease in the upper airway.* Current opinion in allergy and clinical immunology, 2019. **19**(1): p. 1.

104. Stearns, J.C., et al., *Culture and molecular-based profiles show shifts in bacterial communities of the upper respiratory tract that occur with age.* The ISME journal, 2015. **9**(5): p. 1246-1259.

105. Boase, S., et al., *The microbiome of chronic rhinosinusitis: culture, molecular diagnostics and biofilm detection.* BMC infectious diseases, 2013. **13**(1): p. 210.

106. Mahdavinia, M., et al., *The nasal microbiome in patients with chronic rhinosinusitis: analyzing the effects of atopy and bacterial functional pathways in 111 patients.* Journal of Allergy and Clinical Immunology, 2018. **142**(1): p. 287-290. e4.

107. Wilson, M.T. and D.L. Hamilos, *The nasal and sinus microbiome in health and disease.* Current allergy and asthma reports, 2014. **14**(12): p. 1-10.

108. Stephenson, M.-F., et al., *Molecular characterization of the polymicrobial flora in chronic rhinosinusitis.* Journal of Otolaryngology--Head & Neck Surgery, 2010. **39**(2).

109. Whelan, F.J., et al., *The loss of topography in the microbial communities of the upper respiratory tract in the elderly.* Annals of the American Thoracic Society, 2014. **11**(4): p. 513-521.

110. Shilts, M.H., et al., *Minimally invasive sampling method identifies differences in taxonomic richness of nasal microbiomes in young infants associated with mode of delivery.* Microbial ecology, 2016. **71**(1): p. 233-242.

111. Bassis, C.M., et al., *The nasal cavity microbiota of healthy adults.* Microbiome, 2014. **2**(1): p. 1-5.

112. Aurora, R., et al., *Contrasting the microbiomes from healthy volunteers and patients with chronic rhinosinusitis.* JAMA otolaryngology–head & neck surgery, 2013. **139**(12): p. 1328-1338.

113. Wagner Mackenzie, B., et al., *Bacterial community collapse: a meta-analysis of the sinonasal microbiota in chronic rhinosinusitis.* Environmental microbiology, 2017. **19**(1): p. 381-392.

114. Cope, E.K., et al., *Compositionally and functionally distinct sinus microbiota in chronic rhinosinusitis patients have immunological and clinically divergent consequences.* Microbiome, 2017. **5**(1): p. 53.

115. Abreu, N.A., et al., *Sinus microbiome diversity depletion and Corynebacterium tuberculostearicum enrichment mediates rhinosinusitis.* Science translational medicine, 2012. **4**(151): p. 151ra124-151ra124.

116. Bordin, A., et al., *Chronic rhinosinusitis: a microbiome in dysbiosis and the search for alternative treatment options.* Microbiology Australia, 2016. **37**(3): p. 149-152.

117. Mahdavinia, M., et al., *A comprehensive review of the nasal microbiome in chronic rhinosinusitis (CRS).* Clinical & Experimental Allergy, 2016. **46**(1): p. 21-41.

118. Cope, E. and S. Lynch, *Novel microbiome-based therapeutics for chronic rhinosinusitis.* Current allergy and asthma reports, 2015. **15**(3): p. 9.

119. Psaltis, A.J. and P.-J. Wormald, *Therapy of sinonasal microbiome in CRS: a critical approach.* Current allergy and asthma reports, 2017. **17**(9): p. 59.

120. Stubbendieck, R.M. and P.D. Straight, *Multifaceted interfaces of bacterial competition.* Journal of bacteriology, 2016. **198**(16): p. 2145-2155.

121. Hibbing, M.E., et al., *Bacterial competition: surviving and thriving in the microbial jungle.* Nature Reviews Microbiology, 2010. **8**(1): p. 15.

122. Iwase, T., et al., *Staphylococcus epidermidis Esp inhibits Staphylococcus aureus biofilm formation and nasal colonization.* Nature, 2010. **465**(7296): p. 346-349.

123. Chen, C., et al., *Secreted proteases control autolysin-mediated biofilm growth of Staphylococcus aureus.* Journal of Biological Chemistry, 2013: p. jbc. M113. 502039.

124. Yan, M., et al., *Nasal microenvironments and interspecific interactions influence nasal microbiota complexity and S. aureus carriage.* Cell host & microbe, 2013. **14**(6): p. 631-640.

125. Wos-Oxley, M.L., et al., *A poke into the diversity and associations within human anterior nare microbial communities.* The ISME journal, 2010. **4**(7): p. 839-851.

126. Speert, D.P., et al., *Bactericidal effect of oleic acid on group A streptococci: mechanism of action.* Infection and immunity, 1979. **26**(3): p. 1202-1210.

127. Bomar, L., et al., *Corynebacterium accolens releases antipneumococcal free fatty acids from human nostril and skin surface triacylglycerols.* MBio, 2016. **7**(1): p. e01725-15.

128. Friedlander, S.L., *Medical Management of Chronic Rhinosinusitis in Children and Adults*, in *Diseases of the Sinuses*. 2014, Springer. p. 373-387.

129. Schlosser, R.J. and Z.M. Soler, *Evidence-based treatment of chronic rhinosinusitis with nasal polyps.* American journal of rhinology & allergy, 2013. **27**(6): p. 461-466.

130. Smith, S.S., et al., *National burden of antibiotic use for adult rhinosinusitis.* Journal of Allergy and Clinical Immunology, 2013. **132**(5): p. 1230-1232.

131. Ooi, M.L., et al., *Topical Colloidal Silver for the Treatment of Recalcitrant Chronic Rhinosinusitis.* Frontiers in microbiology, 2018. **9**: p. 720.

132. Fong, S.A., et al., *Activity of bacteriophages in removing biofilms of Pseudomonas aeruginosa isolates from chronic rhinosinusitis patients.* Frontiers in cellular and infection microbiology, 2017. **7**: p. 418.

133. Lee, V.S., et al. *Manuka honey sinus irrigation for the treatment of chronic rhinosinusitis: a randomized controlled trial*. in *International forum of allergy & rhinology*. 2017. Wiley Online Library.

134. Vilas Boas, D., et al., *Discrimination of bacteriophage infected cells using locked nucleic acid fluorescent in situ hybridization (LNA-FISH).* Biofouling, 2016. **32**(2): p. 179-190.

135. Speck, P.G. and P.-J. Wormald, *Is phage therapy suitable for treating chronic sinusitis Staphylococcus aureus infection?* 2018, Future Medicine.

136. Niaz, K., et al., *Health benefits of manuka honey as an essential constituent for tissue regeneration.* Current Drug Metabolism, 2017. **18**(10): p. 881-892.

137. Alandejani, T., et al., *Effectiveness of honey on Staphylococcus aureus and Pseudomonas aeruginosa biofilms.* Otolaryngology—Head and Neck Surgery, 2009. **141**(1): p. 114-118.

138. Miyake, M.M. and B.S. Bleier. *Future topical medications in chronic rhinosinusitis*. in *International forum of allergy & rhinology*. 2019. Wiley Online Library.

139. Goggin, R., et al. *Colloidal silver: a novel treatment for Staphylococcus aureus biofilms?* in *International forum of allergy & rhinology*. 2014. Wiley Online Library.

140. Rajiv, S., et al. *Topical colloidal silver as an anti-biofilm agent in a Staphylococcus aureus chronic rhinosinusitis sheep model*. in *International Forum of Allergy & Rhinology*. 2015. Wiley Online Library.

141. Orlandi, R.R., T.T. Kingdom, and P.H. Hwang. *International consensus statement on allergy and rhinology: rhinosinusitis executive summary*. in *International forum of allergy & rhinology*. 2016. Wiley Online Library.

142. Smith, T.L., et al. *Medical therapy vs surgery for chronic rhinosinusitis: a prospective, multi-institutional study with 1-year follow-up*. in *International forum of allergy & rhinology*. 2013. Wiley Online Library.

143. Feazel, L.M., et al., *Microbiome complexity and Staphylococcus aureus in chronic rhinosinusitis.* The Laryngoscope, 2012. **122**(2): p. 467-472.

144. Rossen, N.G., et al., *Findings from a randomized controlled trial of fecal transplantation for patients with ulcerative colitis.* Gastroenterology, 2015. **149**(1): p. 110-118. e4.

145. Colman, R.J. and D.T. Rubin, *Fecal microbiota transplantation as therapy for inflammatory bowel disease: a systematic review and meta-analysis.* Journal of Crohn's and Colitis, 2014. **8**(12): p. 1569-1581.

146. Lawley, T.D., et al., *Antibiotic treatment of Clostridium difficile carrier mice triggers a supershedder state, spore-mediated transmission, and severe disease in immunocompromised hosts.* Infection and immunity, 2009. **77**(9): p. 3661-3669.

147. Gibson, G.R. and M.B. Roberfroid, *Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics.* The Journal of nutrition, 1995. **125**(6): p. 1401-1412.

148. Gibson, G.R., et al., *Dietary modulation of the human colonic microbiota: updating the concept of prebiotics.* Nutrition research reviews, 2004. **17**(2): p. 259-275.

149. Wang, Y., *Prebiotics: Present and future in food science and technology.* Food Research International, 2009. **42**(1): p. 8-12.

150. Al-Sheraji, S.H., et al., *Prebiotics as functional foods: A review.* Journal of functional foods, 2013. **5**(4): p. 1542-1553.

151. de Jesus Raposo, M.F., A.M.M.B. De Morais, and R.M.S.C. De Morais, *Emergent sources of prebiotics: seaweeds and microalgae.* Marine drugs, 2016. **14**(2): p. 27.

152. Varzakas, T., et al., *Innovative and fortified food: Probiotics, prebiotics, gmos, and superfood*, in *Preparation and processing of religious and cultural foods*. 2018, Elsevier. p. 67-129.

153. Ouwehand, A.C., et al., *Prebiotics and other microbial substrates for gut functionality.* Current opinion in biotechnology, 2005. **16**(2): p. 212-217.

154. Burdge, G.C. and P.C. Calder, *Introduction to fatty acids and lipids.* Intravenous Lipid Emulsions, 2015. **112**: p. 1-16.

155. Maurya, R.K., S. Bharti, and M.Y. Krishnan, *Triacylglycerols: fuelling the hibernating Mycobacterium tuberculosis.* Frontiers in cellular and infection microbiology, 2019. **8**: p. 450.

156. Shen, P.-L., et al., *Identification of characteristic fatty acids to quantify triacylglycerols in microalgae.* Frontiers in plant science, 2016. **7**: p. 162.

157. Trows, S., et al., *Analytical challenges and regulatory requirements for nasal drug products in Europe and the US.* Pharmaceutics, 2014. **6**(2): p. 195-219.

158. Navari, R.M., *HTX-019: polysorbate 80-and synthetic surfactant-free neurokinin 1 receptor antagonist for chemotherapy-induced nausea and vomiting prophylaxis.* Future Oncology, 2019. **15**(3): p. 241-255.

159. Rabiee, H., et al., *Enhancement in permeation and antifouling properties of PVC ultrafiltration membranes with addition of hydrophilic surfactant additives: Tween-20 and Tween-80.* Journal of Environmental Chemical Engineering, 2016. **4**(4): p. 4050-4061.

160. Nielsen, C.K., et al., *Effects of Tween 80 on growth and biofilm formation in laboratory media.* Frontiers in microbiology, 2016. **7**: p. 1878.

161. Toutain-Kidd, C.M., et al., *Polysorbate 80 inhibition of Pseudomonas aeruginosa biofilm formation and its cleavage by the secreted lipase LipA.* Antimicrobial agents and chemotherapy, 2009. **53**(1): p. 136-145.

162. Program, N.T., *NTP Toxicology and Carcinogenesis Studies of Polysorbate 80 (CAS No. 9005-65-6) in F344/N Rats and B6C3F1 Mice (Feed Studies).* National Toxicology Program technical report series, 1992. **415**: p. 1.

163. Marx, D., G. Williams, and M. Birkhoff, *Intranasal drug administration—An attractive delivery route for some drugs*, in *Drug Discovery and Development-From Molecules to Medicine*. 2015, InTech.

164. Chen, C.-H., et al., *An innate bactericidal oleic acid effective against skin infection of methicillin-resistant Staphylococcus aureus: a therapy concordant with evolutionary medicine.* J. Microbiol. Biotechnol, 2011. **21**(4): p. 391-399.

165. Clementi, E.A., et al., *A complex of equine lysozyme and oleic acid with bactericidal activity against Streptococcus pneumoniae.* PloS one, 2013. **8**(11): p. e80649.

166. Panesar, P.S., S. Kumari, and R. Panesar, *Biotechnological approaches for the production of prebiotics and their potential applications.* Critical reviews in biotechnology, 2013. **33**(4): p. 345-364.

167. Pokusaeva, K., G.F. Fitzgerald, and D. van Sinderen, *Carbohydrate metabolism in Bifidobacteria.* Genes & nutrition, 2011. **6**(3): p. 285-306.

168. Peña, A., *Intestinal flora, probiotics, prebiotics, synbiotics and novel foods.* Revista Española de Enfermedades Digestivas, 2007. **99**(11): p. 653.

169. Lomax, A. and P. Calder, *Probiotics, immune function, infection and inflammation: a review of the evidence from studies conducted in humans.* Current pharmaceutical design, 2009. **15**(13): p. 1428-1518.

170. Salminen, S. and A. Von Wright, *Lactic acid bacteria: microbiological and functional aspects*. Vol. 139. 2004: CRC Press.

171. Salyers, A.A., *Energy sources of major intestinal fermentative anaerobes.* The American Journal of Clinical Nutrition, 1979. **32**(1): p. 158-163.

172. Luoto, R., et al., *Prebiotic and probiotic supplementation prevents rhinovirus infections in preterm infants: a randomized, placebo-controlled trial.* Journal of Allergy and Clinical Immunology, 2014. **133**(2): p. 405-413.

173. Lilly, D.M. and R.H. Stillwell, *Probiotics: growth-promoting factors produced by microorganisms.* Science, 1965. **147**(3659): p. 747-748.

174. Ganguly, N., et al., *ICMR-DBT guidelines for evaluation of probiotics in food.* The Indian journal of medical research, 2011. **134**(1): p. 22.

175. Hotel, A.C.P. and A. Cordoba, *Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria.* Prevention, 2001. **5**(1): p. 1-10.

176. Podolsky, S.H., *Metchnikoff and the microbiome.* The Lancet, 2012. **380**(9856): p. 1810-1811.

177. Ouwehand, A.C., et al., *Effectiveness of multi-strain versus single-strain probiotics: current status and recommendations for the future.* Journal of clinical gastroenterology, 2018. **52**: p. S35-S40.

178. Plaza-Diaz, J., et al., *Probiotic mechanisms of action.* Ann Nutr Metab, 2012. **61**: p. 160-174.

179. Bermudez-Brito, M., et al., *Probiotic mechanisms of action.* Annals of Nutrition and Metabolism, 2012. **61**(2): p. 160-174.

180. Otte, J.-M. and D.K. Podolsky, *Functional modulation of enterocytes by gram-positive and gram-negative microorganisms.* American Journal of Physiology-Gastrointestinal and Liver Physiology, 2004. **286**(4): p. G613-G626.

181. Caballero-Franco, C., et al., *The VSL# 3 probiotic formula induces mucin gene expression and secretion in colonic epithelial cells.* American Journal of Physiology-Gastrointestinal and Liver Physiology, 2007. **292**(1): p. G315-G322.

182. Sanchez, B., M.C. Urdaci, and A. Margolles, *Extracellular proteins secreted by probiotic bacteria as mediators of effects that promote mucosa–bacteria interactions.* Microbiology, 2010. **156**(11): p. 3232-3242.

183. Kim, Y.S. and S.B. Ho, *Intestinal goblet cells and mucins in health and disease: recent insights and progress.* Current gastroenterology reports, 2010. **12**(5): p. 319-330.

184. Servin, A.L., *Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens.* FEMS microbiology reviews, 2004. **28**(4): p. 405-440.

185. Hirano, J., et al., *The effect of Lactobacillus rhamnosus on enterohemorrhagic Escherichia coli infection of human intestinal cells in vitro.* Microbiology and immunology, 2003. **47**(6): p. 405-409.

186. Gallo, R.L. and L.V. Hooper, *Epithelial antimicrobial defence of the skin and intestine.* Nature Reviews Immunology, 2012. **12**(7): p. 503-516.

187. Coloretti, F., et al., *Antifungal activity of lactobacilli isolated from salami.* FEMS microbiology letters, 2007. **271**(2): p. 245-250.

188. O'Shea, E.F., et al., *Production of bioactive substances by intestinal bacteria as a basis for explaining probiotic mechanisms: bacteriocins and conjugated linoleic acid.* International journal of food microbiology, 2012. **152**(3): p. 189-205.

189. Hassan, M., et al., *Natural antimicrobial peptides from bacteria: characteristics and potential applications to fight against antibiotic resistance.* Journal of applied microbiology, 2012. **113**(4): p. 723-736.

190. Gómez-Llorente, C., S. Munoz, and A. Gil, *Role of Toll-like receptors in the development of immunotolerance mediated by probiotics.* Proceedings of the Nutrition Society, 2010. **69**(3): p. 381-389.

191. D'amelio, P. and F. Sassi, *Gut microbiota, immune system, and bone.* Calcified tissue international, 2018. **102**(4): p. 415-425.

192. Van Zyl, W.F., S.M. Deane, and L.M. Dicks, *Molecular insights into probiotic mechanisms of action employed against intestinal pathogenic bacteria.* Gut Microbes, 2020. **12**(1): p. 1831339.

193. Lebeer, S., J. Vanderleyden, and S.C. De Keersmaecker, *Host interactions of probiotic bacterial surface molecules: comparison with commensals and pathogens.* Nature Reviews Microbiology, 2010. **8**(3): p. 171-184.

194. Brugger, S.D., L. Bomar, and K.P. Lemon, *Commensal–pathogen interactions along the human nasal passages.* PLoS pathogens, 2016. **12**(7).

195. Martens, K., et al., *Probiotics for the airways: potential to improve epithelial and immune homeostasis.* Allergy, 2018. **73**(10): p. 1954-1963.

196. Kechagia, M., et al., *Health benefits of probiotics: a review.* ISRN nutrition, 2013. **2013**.

197. Petersen, C. and J.L. Round, *Defining dysbiosis and its influence on host immunity and disease.* Cellular microbiology, 2014. **16**(7): p. 1024-1033.

198. Martensson, A., et al., *Clinical efficacy of a topical lactic acid bacterial microbiome in chronic rhinosinusitis: a randomized controlled trial. Laryngoscope Investig Otolaryngol 2: 410–416*. 2017.

199. Schwartz, J.S., et al., *Topical probiotics as a therapeutic alternative for chronic rhinosinusitis: a preclinical proof of concept.* American Journal of Rhinology & Allergy, 2016. **30**(6): p. e202-e205.

200. Uehara, Y., et al., *Bacterial interference among nasal inhabitants: eradication of Staphylococcus aureus from nasal cavities by artificial implantation of Corynebacterium sp.* Journal of Hospital Infection, 2000. **44**(2): p. 127-133.

201. Kiryukhina, N., et al., *Use of Corynebacterium pseudodiphtheriticum for elimination of Staphylococcus aureus from the nasal cavity in volunteers exposed to abnormal microclimate and altered gaseous environment.* Probiotics and antimicrobial proteins, 2013. **5**(4): p. 233-238.

202. Cleland, E.J., et al. *Probiotic manipulation of the chronic rhinosinusitis microbiome*. in *International forum of allergy & rhinology*. 2014. Wiley Online Library.

203. De Grandi, R., et al., *Putative microbial population shifts attributable to nasal administration of Streptococcus salivarius 24SMBc and Streptococcus oralis 89a.* Probiotics and antimicrobial proteins, 2019. **11**(4): p. 1219-1226.

204. Pellaton, C., et al., *Intragastric and intranasal administration of Lactobacillus paracasei NCC2461 modulates allergic airway inflammation in mice.* International journal of inflammation, 2012. **2012**.

205. Glück, U. and J.-O. Gebbers, *Ingested probiotics reduce nasal colonization with pathogenic bacteria (Staphylococcus aureus, Streptococcus pneumoniae, and β-hemolytic streptococci).* The American journal of clinical nutrition, 2003. **77**(2): p. 517-520.

206. Alexandre, Y., et al., *Screening of Lactobacillus spp. for the prevention of Pseudomonas aeruginosa pulmonary infections.* BMC microbiology, 2014. **14**(1): p. 1-10.

207. Panigrahi, P., et al., *A randomized synbiotic trial to prevent sepsis among infants in rural India.* Nature, 2017. **548**(7668): p. 407-412.

208. Ishida, Y., et al., *Clinical effects of Lactobacillus acidophilus strain L-92 on perennial allergic rhinitis: a double-blind, placebo-controlled study.* Journal of dairy science, 2005. **88**(2): p. 527-533.

209. West, N.P., et al., *Probiotic supplementation for respiratory and gastrointestinal illness symptoms in healthy physically active individuals.* Clinical Nutrition, 2014. **33**(4): p. 581-587.

210. Mårtensson, A., et al., *Clinical efficacy of a topical lactic acid bacterial microbiome in chronic rhinosinusitis: a randomized controlled trial.* Laryngoscope investigative otolaryngology, 2017. **2**(6): p. 410-416.

211. Habermann, W., et al., *Reduction of acute recurrence in patients with chronic recurrent hypertrophic sinusitis by treatment with a bacterial immunostimulant (Enterococcus faecalis Bacteriae of human origin.* Arzneimittel-forschung, 2002. **52**(8): p. 622-627.

212. Kitz, R., et al., *Probiotic E. faecalis—adjuvant therapy in children with recurrent rhinosinusitis.* Open Medicine, 2012. **7**(3): p. 362-365.

213. De Boeck, I., et al., *Lactic acid bacteria as probiotics for the nose?* Microbial Biotechnology, 2021. **14**(3): p. 859-869.

214. Martinelli, M., et al., *Probiotics' efficacy in paediatric diseases: which is the evidence? A critical review on behalf of the Italian Society of Pediatrics.* Italian journal of pediatrics, 2020. **46**(1): p. 1-13.

215. Rosenberg, H.F., J.C. Masterson, and G.T. Furuta, *Eosinophils, probiotics, and the microbiome.* Journal of leukocyte biology, 2016. **100**(5): p. 881-888.

216. Cho, D.-Y., et al., *Sinus microanatomy and microbiota in a rabbit model of rhinosinusitis.* Frontiers in cellular and infection microbiology, 2018. **7**: p. 540.

217. Consortium, H.M.P., *Structure, function and diversity of the healthy human microbiome.* nature, 2012. **486**(7402): p. 207.

218. Sanders, M.E., *Probiotics: definition, sources, selection, and uses.* Clinical infectious diseases, 2008. **46**(Supplement_2): p. S58-S61.

219. Masco, L., et al., *Culture-dependent and culture-independent qualitative analysis of probiotic products claimed to contain bifidobacteria.* International journal of food microbiology, 2005. **102**(2): p. 221-230.

220. Vankerckhoven, V., et al., *Biosafety assessment of probiotics used for human consumption: recommendations from the EU-PROSAFE project.* Trends in Food Science & Technology, 2008. **19**(2): p. 102-114.

221. Khamis, A., D. Raoult, and B. La Scola, *rpoB gene sequencing for identification of Corynebacterium species.* Journal of clinical microbiology, 2004. **42**(9): p. 3925-3931.

222. Stubbendieck, R.M., et al., *Competition among nasal bacteria suggests a role for siderophore-mediated interactions in shaping the human nasal microbiota.* Applied and environmental microbiology, 2019. **85**(10): p. e02406-18.

223. Wu, Y., et al., *Effect of probiotics on nasal and intestinal microbiota in people with high exposure to particulate matter≤ 2.5 μm (PM2. 5): a randomized, double-blind, placebo-controlled clinical study.* Trials, 2020. **21**(1): p. 1-10.

224. Karska-Wysocki, B., M. Bazo, and W. Smoragiewicz, *Antibacterial activity of Lactobacillus acidophilus and Lactobacillus casei against methicillin-resistant Staphylococcus aureus (MRSA).* Microbiological research, 2010. **165**(8): p. 674-686.

225. Paramasivan, S., et al., *The international sinonasal microbiome study: A multicentre, multinational characterization of sinonasal bacterial ecology.* Allergy, 2020.

226. De Boeck, I., et al., *Lactobacilli have a niche in the human nose.* Cell Reports, 2020. **31**(8): p. 107674.

227. Lappan, R. and C.S. Peacock, *Corynebacterium and Dolosigranulum: future probiotic candidates for upper respiratory tract infections.* Microbiology Australia, 2019. **40**(4): p. 172-177.

228. Castagliuolo, I., et al., *Beneficial effect of auto-aggregating Lactobacillus crispatus on experimentally induced colitis in mice.* FEMS Immunology & Medical Microbiology, 2005. **43**(2): p. 197-204.

229. Garcia-Gonzalez, N., et al., *Adhesion properties of food-associated Lactobacillus plantarum strains on human intestinal epithelial cells and modulation of IL-8 release.* Frontiers in microbiology, 2018. **9**: p. 2392.

230. Sambuy, Y., et al., *The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics.* Cell biology and toxicology, 2005. **21**(1): p. 1-26.

231. Van den Broek, M., et al., *Multifactorial inhibition of lactobacilli against the respiratory tract pathogen Moraxella catarrhalis.* Beneficial microbes, 2018. **9**(3): p. 429-439.

232. Doron, S. and D.R. Snydman, *Risk and safety of probiotics.* Clinical Infectious Diseases, 2015. **60**(suppl_2): p. S129-S134.

233. Myles, I.A., et al., *First-in-human topical microbiome transplantation with Roseomonas mucosa for atopic dermatitis.* JCI insight, 2018. **3**(9).

234. Vesterlund, S., et al., *Safety assessment of Lactobacillus strains: presence of putative risk factors in faecal, blood and probiotic isolates.* International journal of food microbiology, 2007. **116**(3): p. 325-331.

235. Ghoneim, M.A. and S.S. Moselhy, *Impact of probiotic-supplemented diet on the expression level of lactate dehydrogenase in the leukocytes of rabbits.* Toxicology and industrial health, 2014. **30**(3): p. 225-232.

236. Baccouri, O., et al., *Probiotic potential and safety evaluation of Enterococcus faecalis OB14 and OB15, isolated from traditional tunisian testouri cheese and rigouta, using physiological and genomic analysis.* Frontiers in microbiology, 2019. **10**: p. 881.

237. Sim, I., et al., *Probiotic potential of Enterococcus faecium isolated from chicken cecum with immunomodulating activity and promoting longevity in Caenorhabditis elegans.* Journal of microbiology and biotechnology, 2018. **28**(6): p. 883-892.

238. Samuel, B.S., et al., *Caenorhabditis elegans responses to bacteria from its natural habitats.* Proceedings of the National Academy of Sciences, 2016. **113**(27): p. E3941-E3949.

239. Komura, T., et al., *Mechanism underlying prolongevity induced by bifidobacteria in Caenorhabditis elegans.* Biogerontology, 2013. **14**(1): p. 73-87.

240. Mathur, S. and R. Singh, *Antibiotic resistance in food lactic acid bacteria—a review.* International journal of food microbiology, 2005. **105**(3): p. 281-295.

241. Salminen, M.K., et al., *The efficacy and safety of probiotic Lactobacillus rhamnosus GG on prolonged, noninfectious diarrhea in HIV patients on antiretroviral therapy: a*

randomized, placebo-controlled, crossover study. HIV clinical trials, 2004. **5**(4): p. 183-191.

242. Mater, D.D., et al., *A probiotic Lactobacillus strain can acquire vancomycin resistance during digestive transit in mice.* Journal of Molecular Microbiology and Biotechnology, 2008. **14**(1-3): p. 123-127.

243. Michail, S., et al., *Clinical efficacy of probiotics: review of the evidence with focus on children.* Journal of pediatric gastroenterology and nutrition, 2006. **43**(4): p. 550-557.

244. Kramer, M.F. and M.D. Heath, *Probiotics in the treatment of chronic rhinoconjunctivitis and chronic rhinosinusitis.* Journal of allergy, 2014. **2014**.

245. Lievin, V., et al., *Bifidobacterium strains from resident infant human gastrointestinal microflora exert antimicrobial activity.* Gut, 2000. **47**(5): p. 646-652.

246. Ortiz Moyano, R., et al., *The ability of respiratory commensal bacteria to beneficially modulate the lung innate immune response is a strain dependent characteristic.* Microorganisms, 2020. **8**(5): p. 727.

247. Čitar, M., et al., *Human intestinal mucosa-associated Lactobacillus and Bifidobacterium strains with probiotic properties modulate IL-10, IL-6 and IL-12 gene expression in THP-1 cells.* Beneficial microbes, 2015. **6**(3): p. 325-336.

248. Finamore, A., et al., *Lactobacillus amylovorus inhibits the TLR4 inflammatory signaling triggered by enterotoxigenic Escherichia coli via modulation of the negative regulators and involvement of TLR2 in intestinal Caco-2 cells and pig explants.* PLoS One, 2014. **9**(4): p. e94891.

249. Takanashi, N., et al., *Advanced application of bovine intestinal epithelial cell line for evaluating regulatory effect of lactobacilli against heat-killed enterotoxigenic Escherichia coli-mediated inflammation.* BMC microbiology, 2013. **13**(1): p. 1-16.

250. McFarland, L.V., C.T. Evans, and E.J. Goldstein, *Strain-specificity and disease-specificity of probiotic efficacy: a systematic review and meta-analysis.* Frontiers in medicine, 2018. **5**: p. 124.

251. Li, Y.-X., et al., *Planococcus maritimus ML1206 Isolated from Wild Oysters Enhances the Survival of Caenorhabditis elegans against Vibrio anguillarum.* Marine drugs, 2021. **19**(3): p. 150.

252. Chelliah, R., et al., *In vitro and in vivo defensive effect of probiotic LAB against Pseudomonas aeruginosa using Caenorhabditis elegans model.* Virulence, 2018. **9**(1): p. 1489-1507.

253. El-Bestawy, E., Y. Gohar, and S. Wefky, *Antimicrobial phenolic compounds from Enterococcus faecium S29 (EU 158188): characterization and production optimization.* International Journal of Environmental Science and Technology, 2017. **14**(3): p. 497-508.

254. Wassenberg, J., et al., *Effect of Lactobacillus paracasei ST11 on a nasal provocation test with grass pollen in allergic rhinitis.* Clinical & Experimental Allergy, 2011. **41**(4): p. 565-573.

255. Mukerji, S.S., et al., *Probiotics as adjunctive treatment for chronic rhinosinusitis: a randomized controlled trial.* Otolaryngology—Head and Neck Surgery, 2009. **140**(2): p. 202-208.

256. Paramasivan, S., et al., *The international sinonasal microbiome study (ISMS): a multi-centre, multi-national characterization of sinonasal bacterial ecology.* Allergy, 2020.

257. Cleland, E.J., et al., *The bacterial microbiome in chronic rhinosinusitis: richness, diversity, postoperative changes, and patient outcomes.* American journal of rhinology & allergy, 2016. **30**(1): p. 37-43.

258. Food, U. and D. Administration, *Inactive ingredient search for approved drug products.* FDA Database: Silver Spring, MD, USA, 2017.

259. Sarfaraz, K.N., *HANDBOOK OF PHARMACEUTICAL MANUFACTURING FORMULATIONS: Volume Five, Over...-the-counter Products*. 2019: CRC PRESS.

260. Wang, C., et al., *Novel polyoxyethylene-containing glycolipids are synthesized in Corynebacterium matruchotii and Mycobacterium smegmatis cultured in the presence of Tween 80.* Journal of lipids, 2011. **2011**.

261. Kaur, G. and S. Mehta, *Developments of Polysorbate (Tween) based microemulsions: Preclinical drug delivery, toxicity and antimicrobial applications.* International journal of pharmaceutics, 2017. **529**(1-2): p. 134-160.

262. Richter, K., et al. *Mind "De GaPP": in vitro efficacy of deferiprone and gallium-protoporphyrin against Staphylococcus aureus biofilms*. in *International forum of allergy & rhinology*. 2016. Wiley Online Library.

263. González, S., et al., *The behavior of Staphylococcus aureus dual-species biofilms treated with bacteriophage phiIPLA-RODI depends on the accompanying microorganism.* Appl. Environ. Microbiol., 2017. **83**(3): p. e02821-16.

264. Ishiwada, N., et al., *Clinical and bacteriological analyses of bacteremia due to Corynebacterium striatum.* Journal of infection and chemotherapy, 2016. **22**(12): p. 790-793.

265. Gutierrez, D., et al., *Two Phages, phiIPLA-RODI and phiIPLA-C1C, Lyse Mono- and Dual-Species Staphylococcal Biofilms.* Appl Environ Microbiol, 2015. **81**(10): p. 3336-48.

266. Sillankorva, S., P. Neubauer, and J. Azeredo, *Phage control of dual species biofilms of Pseudomonas fluorescens and Staphylococcus lentus.* Biofouling, 2010. **26**(5): p. 567-75.

267. Thurnheer, T., R. Gmür, and B. Guggenheim, *Multiplex FISH analysis of a six-species bacterial biofilm.* Journal of microbiological methods, 2004. **56**(1): p. 37-47.

268. Menberu, M.A., et al. *Tween 80 and its derivative oleic acid promote the growth of Corynebacterium accolens and inhibit Staphylococcus aureus clinical isolates*. in *International Forum of Allergy & Rhinology*. 2020. Wiley Online Library.

269. Roy, R., et al., *Strategies for combating bacterial biofilms: A focus on anti-biofilm agents and their mechanisms of action.* Virulence, 2018. **9**(1): p. 522-554.

270. Rubin, T.A., et al., *Fecal microbiome transplantation for recurrent Clostridium difficile infection: report on a case series.* Anaerobe, 2013. **19**: p. 22-26.

271. Cammarota, G., G. Ianiro, and A. Gasbarrini, *Fecal microbiota transplantation for the treatment of Clostridium difficile infection: a systematic review.* Journal of clinical gastroenterology, 2014. **48**(8): p. 693-702.

272. Ramsey, M.M., et al., *Staphylococcus aureus shifts toward commensalism in response to Corynebacterium species.* Frontiers in microbiology, 2016. **7**: p. 1230.

273. Bachert, C., et al., *Adult chronic rhinosinusitis.* Nature Reviews Disease Primers, 2020. **6**(1): p. 1-19.

274. Lee, K., et al., *Heterogeneity of microbiota dysbiosis in chronic rhinosinusitis: potential clinical implications and microbial community mechanisms contributing to sinonasal inflammation.* Frontiers in cellular and infection microbiology, 2018. **8**: p. 168.

275. Hardy, B.L., et al., *Corynebacterium pseudodiphtheriticum exploits Staphylococcus aureus virulence components in a novel polymicrobial defense strategy.* MBio, 2019. **10**(1).

276. Kumpitsch, C., et al., *The microbiome of the upper respiratory tract in health and disease.* BMC biology, 2019. **17**(1): p. 87.

277. Biswas, K., et al., *The nasal microbiota in health and disease: variation within and between subjects.* Frontiers in microbiology, 2015. **6**: p. 134.

278. Menberu, M.A., et al., *Corynebacterium accolens Has Antimicrobial Activity Against Staphylococcus aureus and Methicillin-Resistant S. aureus Pathogens Isolated from the Sinonasal Niche of Chronic Rhinosinusitis Patients.* Pathogens, 2021. **10**(2): p. 207.

279. Lemon, K.P., et al., *Comparative analyses of the bacterial microbiota of the human nostril and oropharynx.* MBio, 2010. **1**(3).

280. McKenney, P.T. and E.G. Pamer, *From hype to hope: the gut microbiota in enteric infectious disease.* Cell, 2015. **163**(6): p. 1326-1332.

281. Donia, M.S., et al., *A systematic analysis of biosynthetic gene clusters in the human microbiome reveals a common family of antibiotics.* Cell, 2014. **158**(6): p. 1402-1414.

282. Chandra, P., R. Singh, and P.K. Arora, *Microbial lipases and their industrial applications: a comprehensive review.* Microbial Cell Factories, 2020. **19**(1): p. 1-42.

283. Aebersold, R. and M. Mann, *Mass spectrometry-based proteomics.* Nature, 2003. **422**(6928): p. 198-207.

284. Nesvizhskii, A.I., *Protein identification by tandem mass spectrometry and sequence database searching*, in *Mass Spectrometry Data Analysis in Proteomics*. 2007, Springer. p. 87-119.

285. Panchatcharam, B.S., et al. *Staphylococcus aureus biofilm exoproteins are cytotoxic to human nasal epithelial barrier in chronic rhinosinusitis*. in *International Forum of Allergy & Rhinology*. 2020. Wiley Online Library.

286. Inturri, R., et al., *In vitro inhibitory activity of Bifidobacterium longum BB536 and Lactobacillus rhamnosus HN001 alone or in combination against bacterial and Candida reference strains and clinical isolates.* Heliyon, 2019. **5**(11): p. e02891.

287. Sadygov, R.G., *Using SEQUEST with theoretically complete sequence databases.* Journal of the American Society for Mass Spectrometry, 2015. **26**(11): p. 1858-1864.

288. Consortium, U., *UniProt: a worldwide hub of protein knowledge.* Nucleic acids research, 2019. **47**(D1): p. D506-D515.

289. Martin, D., et al., *GOToolBox: functional analysis of gene datasets based on Gene Ontology.* Genome biology, 2004. **5**(12): p. R101.

290. Ramiah, K., C.A. van Reenen, and L.M. Dicks, *Surface-bound proteins of Lactobacillus plantarum 423 that contribute to adhesion of Caco-2 cells and their role in competitive exclusion and displacement of Clostridium sporogenes and Enterococcus faecalis.* Research in microbiology, 2008. **159**(6): p. 470-475.

291. De Angelis, M. and M. Gobbetti, *Environmental stress responses in Lactobacillus: a review.* Proteomics, 2004. **4**(1): p. 106-122.

292. Cimermancic, P., et al., *Insights into secondary metabolism from a global analysis of prokaryotic biosynthetic gene clusters.* Cell, 2014. **158**(2): p. 412-421.

293. Lippolis, R., et al., *Comparative secretome analysis of four isogenic Bacillus clausii probiotic strains.* Proteome science, 2013. **11**(1): p. 28.

294. Azkargorta, M., et al., *In-depth proteomics and natural peptidomics analyses reveal antibacterial peptides in human endometrial fluid.* Journal of Proteomics, 2020. **216**: p. 103652.

295. Dyda, F., D.C. Klein, and A.B. Hickman, *GCN5-related N-acetyltransferases: a structural overview.* Annual review of biophysics and biomolecular structure, 2000. **29**(1): p. 81-103.

296. Mio, T., et al., *Saccharomyces cerevisiae GNA1, an essential gene encoding a novel acetyltransferase involved in UDP-N-acetylglucosamine synthesis.* Journal of Biological Chemistry, 1999. **274**(1): p. 424-429.

297. Robles, T.F. and J.E. Carroll, *Restorative biological processes and health.* Social and personality psychology compass, 2011. **5**(8): p. 518-537.

298. Bianchi, L., et al., *A Combined Proteomics, Metabolomics and In Vivo Analysis Approach for the Characterization of Probiotics in Large-Scale Production.* Biomolecules, 2020. **10**(1): p. 157.

299. Holmquist, M., *Alpha beta-hydrolase fold enzymes structures, functions and mechanisms.* Current Protein and Peptide Science, 2000. **1**(2): p. 209-235.

300. Lavis, L.D., T.-Y. Chao, and R.T. Raines, *Synthesis and utility of fluorogenic acetoxymethyl ethers.* Chemical science, 2011. **2**(3): p. 521-530.

301. Wall, T., et al., *The early response to acid shock in Lactobacillus reuteri involves the ClpL chaperone and a putative cell wall-altering esterase.* Applied and environmental microbiology, 2007. **73**(12): p. 3924-3935.

302. Pragya, P., et al., *High-resolution mass spectrometry-based global proteomic analysis of probiotic strains Lactobacillus fermentum NCDC 400 and RS2.* Journal of proteomics, 2017. **152**: p. 121-130.

303. Montoro, B.P., et al., *Proteomic analysis of Lactobacillus pentosus for the identification of potential markers involved in acid resistance and their influence on other probiotic features.* Food microbiology, 2018. **72**: p. 31-38.

304. Granato, D., et al., *Cell surface-associated elongation factor Tu mediates the attachment of Lactobacillus johnsonii NCC533 (La1) to human intestinal cells and mucins.* Infection and immunity, 2004. **72**(4): p. 2160-2169.

305. Ruiz, L., et al., *Cell envelope changes in Bifidobacterium animalis ssp. lactis as a response to bile.* FEMS microbiology letters, 2007. **274**(2): p. 316-322.

306. Stepanova, A., A. Valls, and A. Galkin, *Effect of monovalent cations on the kinetics of hypoxic conformational change of mitochondrial complex I.* Biochimica et Biophysica Acta (BBA)-Bioenergetics, 2015. **1847**(10): p. 1085-1092.

307. Nelson, D.C., et al., *Endolysins as antimicrobials*, in *Advances in virus research*. 2012, Elsevier. p. 299-365.

308. Loeffler, J.M., S. Djurkovic, and V.A. Fischetti, *Phage lytic enzyme Cpl-1 as a novel antimicrobial for pneumococcal bacteremia.* Infection and immunity, 2003. **71**(11): p. 6199-6204.

309. Nelson, D., L. Loomis, and V.A. Fischetti, *Prevention and elimination of upper respiratory colonization of mice by group A streptococci by using a bacteriophage lytic enzyme.* Proceedings of the National Academy of Sciences, 2001. **98**(7): p. 4107-4112.

310. Pang, X.-Y., et al., *Gene knockout and overexpression analysis revealed the role of N-acetylmuramidase in autolysis of Lactobacillus delbrueckii subsp. bulgaricus Ljj-6.* PloS one, 2014. **9**(8): p. e104829.

311. Yatsunenko, T., et al., *Human gut microbiome viewed across age and geography.* nature, 2012. **486**(7402): p. 222-227.

312. Trompette, A., et al., *Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis.* Nature medicine, 2014. **20**(2): p. 159-166.

313. Lynch, S.V. and O. Pedersen, *The human intestinal microbiome in health and disease.* New England Journal of Medicine, 2016. **375**(24): p. 2369-2379.

314. Cryan, J.F. and S.M. O'Mahony, *The microbiome-gut-brain axis: from bowel to behavior.* Neurogastroenterology & Motility, 2011. **23**(3): p. 187-192.

315. Bhattacharjee, S. and W.J. Lukiw, *Alzheimer's disease and the microbiome.* Frontiers in cellular neuroscience, 2013. **7**: p. 153.

316. Fulde, M. and M.W. Hornef, *Maturation of the enteric mucosal innate immune system during the postnatal period.* Immunological Reviews, 2014. **260**(1): p. 21-34.

317. Lloyd, C.M. and B.J. Marsland, *Lung homeostasis: influence of age, microbes, and the immune system.* Immunity, 2017. **46**(4): p. 549-561.

318. Hand, T.W., et al., *Linking the microbiota, chronic disease, and the immune system.* Trends in endocrinology & metabolism, 2016. **27**(12): p. 831-843.

319. Archer, N.K., et al., *Staphylococcus aureus biofilms: properties, regulation, and roles in human disease.* Virulence, 2011. **2**(5): p. 445-459.

320. Singhal, D., et al., *Staphylococcus aureus biofilms: Nemesis of endoscopic sinus surgery.* The Laryngoscope, 2011. **121**(7): p. 1578-1583.

321. Miljkovic, D., et al., *Naive and effector B-cell subtypes are increased in chronic rhinosinusitis with polyps.* American journal of rhinology & allergy, 2018. **32**(1): p. 3-6.

322. Dong, D., et al., *Distribution and inhibition of liposomes on Staphylococcus aureus and Pseudomonas aeruginosa biofilm.* PLoS One, 2015. **10**(6): p. e0131806.

323. Ramezanpour, M., et al., *Primary human nasal epithelial cells: a source of poly (I: C) LMW-induced IL-6 production.* Scientific reports, 2018. **8**(1): p. 1-8.

324. Li, J., et al., *Pseudomonas aeruginosa exoprotein-induced barrier disruption correlates with elastase activity and marks chronic rhinosinusitis severity.* Frontiers in cellular and infection microbiology, 2019. **9**: p. 38.

325. Lina, G., et al., *Bacterial competition for human nasal cavity colonization: role of staphylococcal agr alleles.* Applied and environmental microbiology, 2003. **69**(1): p. 18-23.

326. Zhang, Y., et al., *Effects of isosorbide mononitrate loaded nanoparticles conjugated with anti-Staphylococcus aureus α-toxin on Staphylococcus aureus biofilms.* Experimental and therapeutic medicine, 2020. **19**(2): p. 1267-1274.

327. Dong, D., et al., *Inhibition of Staphylococcus aureus and Pseudomonas aeruginosa biofilms by quatsomes in low concentrations.* Experimental Biology and Medicine, 2020. **245**(1): p. 34-41.

328. Frank, D.N., et al., *The human nasal microbiota and Staphylococcus aureus carriage.* PloS one, 2010. **5**(5): p. e10598.

329. Murphy, J., et al., *Staphylococcus Aureus V8 protease disrupts the integrity of the airway epithelial barrier and impairs IL-6 production in vitro.* The Laryngoscope, 2018. **128**(1): p. E8-E15.

330. Malik, Z., et al. *Staphylococcus aureus impairs the airway epithelial barrier in vitro*. in *International forum of allergy & rhinology*. 2015. Wiley Online Library.

331. Tan, N.C.W., et al. *Intracellular Staphylococcus aureus: the Trojan horse of recalcitrant chronic rhinosinusitis?* in *International forum of allergy & rhinology*. 2013. Wiley Online Library.

332. Ou, J., et al. *Association of intracellular Staphylococcus aureus with prognosis in chronic rhinosinusitis*. in *International forum of allergy & rhinology*. 2016. Wiley Online Library.

333. Ou, J., et al., *The persistence of intracellular Staphylococcus aureus in the sinuses: a longitudinal study.* Rhinology, 2017. **55**(4): p. 305-311.

334. Choi, E.B., et al., *Decreased diversity of nasal microbiota and their secreted extracellular vesicles in patients with chronic rhinosinusitis based on a metagenomic analysis.* Allergy, 2014. **69**(4): p. 517-526.

335. Ramakrishnan, V.R., et al., *The microbiome of the middle meatus in healthy adults.* PloS one, 2013. **8**(12): p. e85507.

336. Wang, J.C., et al. *Association of the sinonasal bacterial microbiome with clinical outcomes in chronic rhinosinusitis: a systematic review*. in *International forum of allergy & rhinology*. 2020. Wiley Online Library.

337. Sivasubramaniam, R. and R. Douglas, *The microbiome and chronic rhinosinusitis.* World journal of otorhinolaryngology-head and neck surgery, 2018. **4**(3): p. 216-221.

338. Johnsson, T., et al., *Cellular fatty acid profiles of Lactobacillus and Lactococcus strains in relation to the oleic acid content of the cultivation medium.* Applied and Environmental Microbiology, 1995. **61**(12): p. 4497-4499.

339. Singh, P., et al., *Bacterial biofilm on the sinus mucosa of healthy subjects and patients with chronic rhinosinusitis (with or without nasal polyposis).* The Journal of Laryngology & Otology, 2015. **129**(1): p. 46-49.

340. Richter, K., *Tackling superbugs in their slime castles: innovative approaches against antimicrobial-resistant biofilm infections.* Microbiology Australia, 2019. **40**(4): p. 165-168.

341. Sustrova, T., et al., *Effect of probiotics on the viability of porcine and human neutrophils in vitro.* Veterinární medicína, 2017. **62**(12): p. 637-646.

342. Janda, W.M., *Corynebacterium species and the coryneform bacteria part I: new and emerging species in the genus Corynebacterium.* Clinical Microbiology Newsletter, 1998. **20**(6): p. 41-52.

343. Khan, F., S. Jain, and S.F. Oloketuyi, *Bacteria and bacterial products: foe and friends to Caenorhabditis elegans.* Microbiological research, 2018. **215**: p. 102-113.