Human Commensal Microbiota That Inhibit the Growth of Respiratory Tract Pathogens

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LAY ABSTRACT

The human respiratory tract harbours commensal and pathogenic bacteria, and the latter cause most of the lower respiratory tract infections. The commensal bacteria help to train the immune system and impede the growth of pathogens through colonization resistance. A previous study by the Surette lab identified bacterial isolates from the respiratory tract that inhibit the growth of select pathogens, among them, a particular strain of *Staphylococcus aureus.* Based on the results of the earlier study, I hypothesized that the respiratory tract bacteria is a good source of commensals that can inhibit the growth of *S. aureus* and other respiratory pathogens, such as *Streptococcus pneumoniae, Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. To find potential therapeutic bacteria, I screened ~5000 respiratory tract isolates from the Surette lab's strain collection for the ability to impair growth of target pathogens. Additionally, I further characterized the activity of the previously identified *S. aureus* strain against various Lactobacillalles strains and used comparative genomics to identify potential biosynthetic genes required for biosynthesis of molecules with antibacterial activity within the genome of *S. aureus*. The research reported in this thesis demonstrates that many commensal bacteria that live within our airways have the ability to inhibit the growth of bacterial pathogens. This work may provide a new source of antibiotics against respiratory infections and new strategies to reduce susceptibility to infections in vulnerable populations.

ABSTRACT

Lower respiratory tract infectious diseases are a world-wide healthcare burden with bacterial pathogens accounting for a large portion of primary and secondary infections. The human respiratory tract is home to hundreds of species of microbes that comprise the human airway microbiome. These commensals play a crucial role in human health in part by providing colonization resistance against pathogens. In a previous study from the Surette lab it was shown that specific bacterial isolates from the respiratory microbiome inhibits the growth of pathogens aerobically. This included an isolate of *Staphylococcus aureus* which inhibited the growth of *Enterococcus faecium*. This activity was further characterized in this thesis and the underlying mechanism was explored through comparative genomics. As well, this observation provided proof-of-concept for a large-scale screen for additional isolates which inhibit pathogen growth. I hypothesized that the respiratory tract microbiota included many other bacteria capable of inhibiting the growth of respiratory tract pathogens in both aerobic and anaerobic environments, and that anaerobic conditions will identify new activities not detected aerobically. To examine and identify potential beneficial bacteria, I have screened ~5000 respiratory tract bacteria from the Surette lab's airway isolate collection against four pathogens: *Streptococcus pneumoniae, Staphylococcus aureus, Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. The respiratory tract commensals were pinned onto the pathogen-lawn and their interaction was expressed as zones of clearing or altered growth phenotypes of the pathogen. The results of the screen showed that anti-pathogen activity was a common feature of respiratory tract commensals. In particular, *S. pneumoniae* was inhibited by taxonomically diverse members of the microbiota representing three phyla

(Proteobacteria, Firmicutes and Actinobacteria). Many of the facultative anaerobes that inhibited *S. pneumoniae* expressed their activity in anerobic conditions.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AIC	**Airway Isolate collection**
BHI	**Brain Heart Infusion**
CFS	**Cell Free Supernatant**
GI	**Gastro-Intestinal**
H	**Halo Phenotype**
H_20_2	**Hydrogen Peroxide**
NRPS	**Nonribosomal Peptide Synthetase Pathway**
LRT	**Lower Respiratory Tract**
M	**Mutual Killing Phenotype**
MALDI-ToF	**Matrix Assisted Laser Desorption Ionization Time-Of-Flight**
M9	**Minimal Media**
MRS	**De Mann Rogosa & Sharpe**
MDR	**Multidrug-resistant**
NRPS	**Nonribosomal Peptide Synthetase Pathway**
NP	**Nasopharynx**
O_2	**Oxygen**
OP	**Oropharynx**
PKS	**Polyketide Synthetase Pathway**
RNI	**Reactive Nitrogen Intermediate**

T	**Short-Range Phenotype**
THY	**Todd Hewitt Yeast**
URT	**Upper Respiratory Tract**
V	**Long-Range Phenotype**
WGS	**Whole Genome Sequencing**
WT	**Wild Type**
ZOI	**Zone of Inhibition**

DECLARATION OF ACADEMIC ACHIEVEMENT

The “R- analysis of the unique hit- isolates and hierarchical clustering tree (UPGMA) in chapter 3 was done by Jake C. Szamosi.

MALDI-ToF of the re-screened hit- isolates and isolation of the pure strains was done with the assistance of Victoria A. Marko and Michael G. Surette.

The WGS and antiSMASH analysis of 18D11 and 14F12 was completed with the assistance of Michael. G. Surette.

The author completed the rest of the work in this study.

CHAPTER 1.

INTRODUCTION

The human microbiome refers to the bacteria, fungi, and viruses that live in and on our body and has co-evolved with us (Dave, Higgins, Middha, & Rioux, 2012). The human microbiome is known to play important roles in almost all aspects of health and disease (Moloney, Desbonnet, Clarke, Dinan, & Cryan, 2014). In addition, the microbiome is known to be a source of products that affect both microbe-microbe and microbe-host interactions (Donia & Fischbach, 2016) Colonization resistance is the ability of the commensal micro-organisms of the human microbiome to limit colonization by pathogens and, therefore, infection (Pickard, Zeng, Caruso, & Núñez, 2017). The prevention of colonization by pathogens is achieved directly through competition for limited resources (nutrients and space) and bacterial-killing, and indirectly via stimulation of the host's immune system (Mousa, Athar, Merwin, & Magarvey, 2017). This microbial warfare, in concert with the immune system, can prevent or limit pathogens from colonizing the host, which is often a prerequisite for infection.

1.1. The Respiratory Tract Microbiome

While much of the research has focused on the gastrointestinal tract, the microbiome of the airways is also important in human health. The microbiome of the respiratory tract plays a crucial role in colonization resistance against respiratory pathogens, thus reducing susceptibility to infection (de Steenhuijsen Piters, Sanders, & Bogaert, 2015). The upper respiratory tract (URT) represents the distal segment of the airways and its main role is to conduct humidified and purified air to the lungs (Sahin-Yilmaz & Naclerio, 2011). The microbiota of the URT resides in the four main

compartments: anterior nares, sinuses, nasopharynx, and oropharynx (de Steenhuijsen Piters et al., 2015). These niches are anatomically close to each other but provide differences in conditions (temperature, humidity, O_2/CO_2 partial pressure, nutrients) that drive the growth of diverse but overlapping communities (Man, De Steenhuijsen Piters, & Bogaert, 2017). These micro-environments in the URT accommodate strict anaerobes and facultative anaerobes in less ventilated areas, such as the sinuses (Brook, 2006), tonsillar crypts (Samant, Sandoe, High, & Makura, 2009) and underneath the mucus layer (Worlitzsch et al., 2002). The anaerobic bacterial isolates that flourish in the URT are present in higher cell densities than obligate aerobes and facultative anaerobes (Brook, 2004).

The main phyla colonizing the upper respiratory tract are Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria (Dethlefsen, McFall-Ngai, & Relman, 2007), and the most abundant bacterial organisms are members of the *Staphylococcaceae*, *Propionbibacteriaceae, Corynebacteriaceae, Prevotellaceae, Streptococcaceae, Veillonellaceae, Fusobacteriaceae* and *Neisseriaceae* families (Charlson et al., 2011). Isolates such as: *Gemella, Granulicatella, Streptococcus*, and *Veillonella* species were found to be common in the healthy oral microbiome (Aas, Paster, Stokes, Olsen, & Dewhirst, 2005). Most of these representatives of the URT microbiome are commensals that live in symbiosis with the host however, pathogenic species of *Streptococcaceae* and *Staphylococcaceae* can be asymptomatic colonizers of the URT that have the potential to cause infection when colonizing the lower respiratory tract (LRT) (Siegel & Weiser, 2015).

1.2. Bacterial interaction in the URT

The competition between bacterial communities for resources, referred to as colonization resistance, is an important mechanism that modulates the microbiome and the immune system (de Steenhuijsen Piters et al., 2015). Colonization resistance occurs amongst commensals as well as between commensals and pathogens (Skaar, 2010). In addition to passive competition, bacteria can employ antimicrobial peptides or other antibacterial substances that inhibit the growth of competitors (Iwase, Uehara, Shinji et al., 2010). For example, *S. pneumoniae* colonization is prevented by some resident viridans group streptococci (Bernstein at al., 2006; Crowe, Sanders, & Longley, 1973). In addition, *Cutibacterium spp*., commensal isolates of the skin and oral cavity (Brook & Frazier, 1991) are recognized for their antimicrobial properties (Corvec, 2018). The propionic acid production by *Cutibacterium* (Moore & Cato, 1963) is found to inhibit the growth of *S. aureus* (Wang et al., 2014). The innate and adaptive immune systems, act to maintain a microbiome in equilibrium and to promote the health of the respiratory tract (Whitsett & Alenghat, 2015). This microbiome-immune system homeostasis is important in health (Wu & Wu, 2012) and for maintaining colonization resistance (Lazar et al., 2018).

The main innate mechanisms of the immune system in the respiratory tract include the mucous layer that traps foreign particles (viruses, bacteria, particulate matter) before air reaching the lungs, the antimicrobial substances secreted by the epithelial cells in the mucus layer which inhibit the growth of pathogens directly or indirectly (by triggering an immune response) and mucociliary clearance. The latter, displaces the foreign particles entrapped in the mucus layer from the conductive airways toward the oral cavity (Lambert & Culley, 2017; Möller, Häußinger, Ziegler-Heitbrock, & Heyder, 2006; Whitsett &

Alenghat, 2015). The adaptive mechanisms of the immune system include the humoral and the cellular responses (Newton, Cardani, & Braciale, 2016).

Until recently it was believed that the lung was a sterile niche as it is an organ with low levels of nutrients and a thin mucus layer that impedes bacterial growth (Dickson, Erb-Downward, Martinez, & Huffnagle, 2016). However, studies have shown that the microbiome of the LRT in healthy subjects is a transient community that is seeded by the microbiota that inhabit the URT (Morris, Beck, Schloss, et al., 2013). The bacterial cells that colonize the LRT originate by inspiration, microaspiration and mucosal dispersal. Meanwhile, their elimination from the respiratory tract is facilitated by the air flow during expiration and innate and adaptive immune mechanisms (Fig. 1).

A healthy lung microbiome is considered a transient community with a low rate of organisms that colonize the niche. The proportion of the bacteria that manage to grow in the niche depends on the balance between the microorganisms that immigrate into the lung and those that are eliminated from it (Dickson et al., 2016). When colonization resistance and the host's immune responses fail to prevent colonization of pathogens in the URT it leads to a higher probability for pathogens to invade and colonize the LRT. Infections of the LRT functionally compromise the respiratory tract and are considered life threatening conditions. LRT infections are associated with increased mucus production, higher presence of inflammatory cells in the lungs, and decreased ventilation: perfusion ratio. As a result, the exchange of gases in the alveoli decreases and may lead to severe clinical conditions (Moldoveanu et al., 2008; Pascoal et al., 2015)

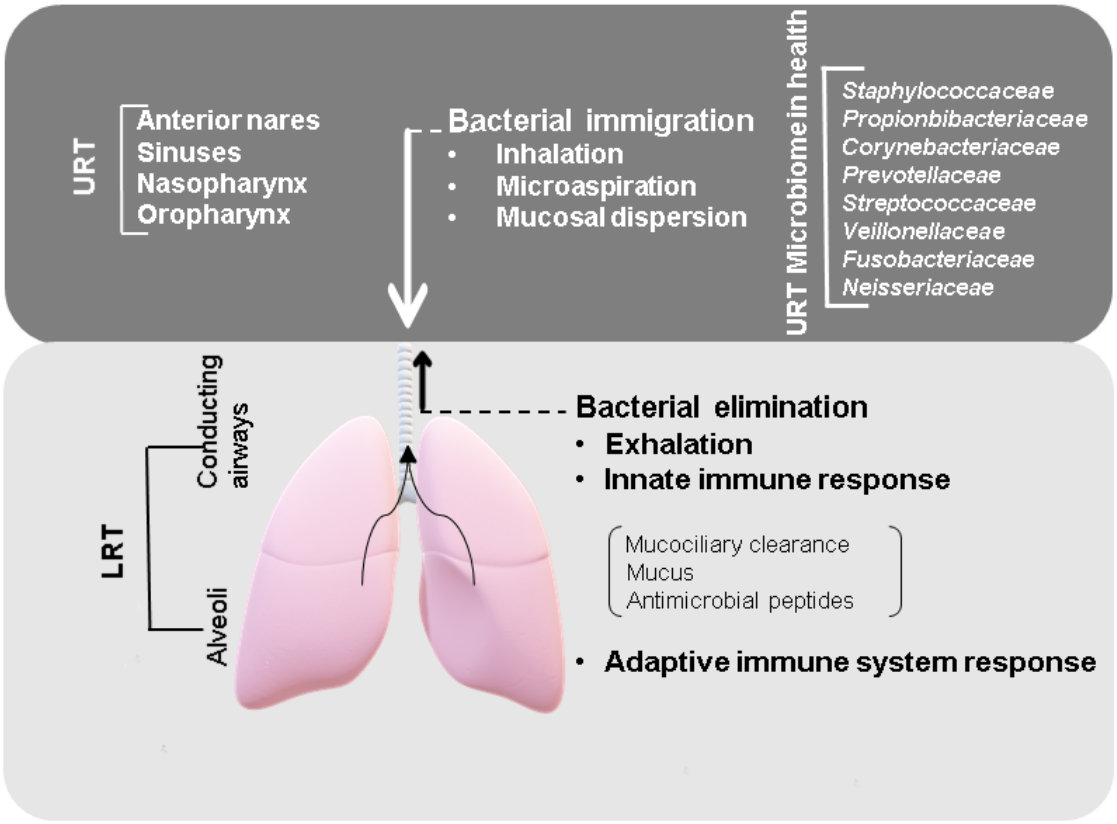

Figure 1. LRT microbial load is driven by the URT microbiome & host's immune responses.

The microbial composition of the LRT is determined by equilibrium between microbial immigration, microbial elimination, and the reproduction of its members. The URT microbiome migrates into the lower respiratory tract through inhalation of air, microaspiration of the saliva and food/water, and mucosal dispersion. The bacterial elimination process from the alveoli towards the URT is accomplished by the exhalation of the air and the immune mechanisms (mucociliary clearance, mucus and antimicrobial peptides secreted from epithelial cells and adaptive immune responses) (modified from Dickson, 2015) (Dickson & Huffnagle, 2015).

1.3. Microbiome Aging and Respiratory Infections

The URT microbiome is colonized at birth and is shaped by multiple factors, including the birth delivery method (Van Nimwegen et al., 2011). The local URT microbiome helps structure the local immune system and matures in tandem with it (Herbst

et al., 2011; Mendez, Banerjee, Bhattacharya, & Banerjee, 2019). The URT tract microbiome matures over the first few years of life (Stearns et al., 2015). Like the gut microbiome, it is thought that the microbiome of the airways is stable throughout most of adulthood but changes later in life. While the anterior nares and oropharyngeal microbiome are distinct throughout most of life, this biogeography is lost in the elderly (Whelan et al., 2014). Additionally, age-related changes in the URT microbiome occur in parallel with immunosenescence. Studies in murine models of aging suggests that this loss of microbiome-immune homeostasis is not driven independently by either age-related microbiome alterations or immunosenescence (Thevaranjan et al., 2017). Lifetime incidence of respiratory disease are highest in the very young and the elderly. These age groups are both characterized by unstable microbiome-immune interactions. A specific motivation for this study is the increased susceptibility to respiratory infections by the elderly as a consequence of the loss of colonization resistance. Respiratory infections in older adults (>55 yr) have costly short-term and long-term consequences and pneumonia is the most economically costly infectious disease in Canada (Kwong et al., 2010), therefore novel intervention methods are urgently needed.

1.4. Bacterial Antagonism

Bacterial communities have seemingly symbiotic relationships with the host (Dethlefsen et al., 2007); however, microorganisms may have antagonistic interaction with each other (Tano, Olofsson, Grahn-Håkansson, & Holm, 1999). The antagonistic relationship between bacteria involves a wide range of mechanisms, starting from basic resource competition like depleting and/or sequestering nutrients from the environment, to

sophisticated ones like contact dependent and contact independent killing mechanisms (Hibbing, Fuqua, Parsek, & Peterson, 2010; Skaar, 2010; Zipperer et al., 2016) (Fig. 2). The contact dependent mechanisms require cell contact to occur. Bacterial cells inject toxins into adjacent cells by using one of four different mechanisms (Klein, Ahmad, & Whitney, 2020). Meanwhile, contact independent antagonism is achieved when bacteria release bioactive molecules into the surrounding environment that can inhibit neighbouring cells.

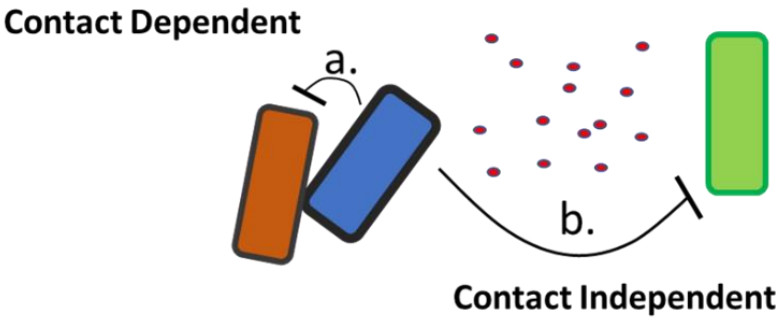

Figure 2. General mechanisms of inter-bacterial killing.

a) Contact dependent interaction (e.g. Type I, IV, VI and VII Secretion Systems) results from direct transfer of toxins from producers to target cell. b) Contact independent inhibition involves natural products/secondary metabolites that are released into the environment and act on nearby cells. (e.g. bacteriocins, lantibiotics, conventional antibiotics).

The variety of bioactive molecules that have the potential to inhibit the growth of related or unrelated bacterial cells is quite extensive. They can be simple chemical structures such as NO_2 (Nathan & Shiloh, 2000) and H_2O_2 (Ramos-Montañez et al., 2008), or more complex compounds such as antibiotic small molecules, peptides or modified peptides. The molecules typically thought of as antibiotics are secondary metabolites, synthesized by bacteria in three pathways: NRPS (nonribosomal peptide synthetase pathway), PKS (polyketide synthetase pathway) and mixed NRPS-PKS enzymatic

pathway (Wase & Wright, 2008; Zacharof & Lovitt, 2012). Environmental organisms are the traditionally studied source of these molecules and these microorganisms do not appear to be abundant in the human microbiome. Human cells also produce antimicrobial peptides that also modulate the immune system (Mookherjee, Anderson, Haagsman, & Davidson, 2020). The ribosomally synthesized peptides (post-translationally modified or not) with antibacterial properties produced by bacteria are referred here as bacteriocins.

1.5. Bacteriocins

Bacteriocins are small ribosomally synthesized peptides produced by bacterial cells that usually target related strains (Hols, Ledesma-García, Gabant, & Mignolet, 2019). They differ from antibiotics in molecular structure and size, synthesis pathway and range of activity. Bacteriocins are generally small, heat-stable peptides, with a narrow-range activity toward bacteria phylogenetically related to the producing strain (Müller-Auffermann, Grijalva, Jacob, & Hutzler, 2015). However, the range of activity of bacteriocin can be broad; for example, both nisin and pediocin target a wide range of Gram-positive bacteria. Bacteriocins produced by facultative anaerobes can inhibit the target strain in both aerobic and anaerobic conditions (Yang, Fan, Jiang, Doucette, & Fillmore, 2012). The classification of bacteriocins based on their chemical structure is summarized in Table 1. The bacteriocins included in class I and II are small proteins divided further in subclasses based on the structure, modification after translation and their mode of action. Members of class I include lantibiotics, sactipeptides, glycocins, and lasso-peptides (Hols, Ledesma-García, Gabant, & Mignolet, 2019). Class II bacteriocins are heat-stable non-modified proteins, that are divided in four subclasses based on structure. This class consist of pediocin-like, two-peptides, circular, and nonpediocin-like linear bacteriocins (Sánchez

et al., 2007). Meanwhile, class III and IV incorporate large heat-liable molecules. The class III includes bacteriolysins (group A) and non-lytic compounds (group B) (Yang, Lin, Sung, & Fang, 2014). The bacteriocins in the class IV are lipid or carbohydrate-conjugated peptides that lyse bacterial cells (Hols et al., 2019).

Table 1. Bacteriocin classification and characteristics.

Class	Group	Heat-liability	Modified[1]	Size	Chemical structure	Citation
I	A B	stable	yes	<4 kDa	protein	(Hols et al., 2019) (Zacharof & Lovitt, 2012)
II	A B C D	stable	no/minor	<10 kDa	protein	(Hols et al., 2019) (Nissen-Meyer, Oppegård, Rogne, Haugen, & Kristiansen, 2010) (Netz et al., 2002)
III	A B	sensitive	no/ minor	>10 kDa	protein	(Güllüce, Karadayı, & Barış, 2013) (Hols et al., 2019)
IV		sensitive	yes	>10 kDa	Lipid/ carbohydrate-proteins	(Stepper et al., 2011)

[1]- in addition to the cleavage of the pre-protein

1.6. Bacterial Pathogens of the Respiratory Tract

Respiratory tract infections are a major cause of morbidity and mortality worldwide and *Streptococcus pneumoniae* is the foremost etiological factor that contributes to mortality in children (<5 yr) and in the elderly (>70 yr) (Troeger et al., 2018). Pathogens of the respiratory tract are predominantly viruses and bacteria that can cause upper and

lower respiratory infections. The URT infections like influenza, acute otitis media, pharyngitis, tonsillitis, and laryngitis are more common in children and they often precipitate to LRT infections (Benediktsdóttir, 1993). Respiratory infections tend to have a seasonal pattern and may convey a range of symptoms, from mild to severe (Dowell & Shang Ho, 2004). The common airway pathogens that asymptomatically colonize the URT are *Streptococcus pneumoniae*, *Hemophilus influenzae*, *Staphylococcus aureus, Moraxella catarrhalis and Neisseria meningitidis* (Bogaert et al., 2011; Kovács et al., 2020; Shak, Vidal, & Klugman, 2013). Two particularly important LRT pathogens that are common nosocomial pathogens and cause disease in immuno-compromised individuals are *Pseudomonas aeruginosa and Klebsiella pneumoniae.* Both pathogens asymptomatically colonize the URT of healthy adults and elderly (Schenck, Surette, & Bowdish, 2016; Whelan et al., 2014) and URT of health care personnel (Hassoun et al., 2015). Although, the colonization of the URT is often a requirement for LRT infections these pathogens cause LRT disease. The research undertaken in this thesis has focused on five pathogens described in more detail here.

1.6.1. Streptococcus pneumoniae

Streptococcus pneumoniae is an important pathogen that asymptomatically colonizes the URT and causes a range of infections such as pneumonia, acute otitis media and meningitis (Bogaert, de Groot, & Hermans, 2004). *S. pneumoniae* produces a number of different virulence factors including neuraminidases, hyaluronidase, pneumococcal surface protein A (PspA), phosphorylcholine, Pav A, and pyruvate oxidase (Kadioglu, Weiser, Paton, & Andrew, 2008). Pyruvate oxidase enables these bacteria produce

hydrogen peroxide (H_2O_2) in the presence of oxygen (O_2). *In vitro*, H_2O_2 kills *S. pneumoniae* and other bacteria growing nearby and also damages the DNA in epithelial alveolar cells, leading to apoptosis and intensifying the detrimental effect of *S. pneumoniae* in the lungs (Rai et al., 2015). Moreover, H_2O_2 suppresses inflammasomes which are components of the innate immune response (Erttmann & Gekara, 2019). Most likely the inactivation of this mechanism of the native immune response promotes the colonization of other bacteria in the URT. The direct impact of H_2O_2 produced by *S. pneumoniae* and oral streptococci is shown to inhibit the growth of the airway pathogens such as *S. aureus* and *P. aeruginosa* (Regev-Yochay, Trzciński, Thompson, Malley, & Lipsitch, 2006; Scoffield & Wu, 2016). Scoffield and colleagues showed that *in vitro* inhibition of *P. aeruginosa* by H_2O_2-producing streptococci depended on the presence of nitrite in the medium. Peroxynitrite, a reactive nitrogen intermediate (RNI), was generated and accumulated intracellularly on *P. aeruginosa,* causing cell death (Scoffield & Wu, 2015). On the other hand, in anaerobic growth conditions streptococci do not generate H_2O_2.

1.6.2. Staphylococcus aureus

Staphylococcus aureus is a Gram-positive, commensal bacteria in the order Bacillales (Becker, Heilmann, & Peters, 2014) that asymptomatically colonises the URT of 20%-30% of the population (Newstead, Varjonen, Nuttall, & Paterson, 2020; Van Belkum et al., 2009; Wos-Oxley et al., 2010). *S. aureus* is the most common pathogen of early onset ventilator-associated pneumonia (VAP) (Gastmeier et al., 2009). Methicillin resistant *S. aureus* (MRSA) are multidrug-resistant (MDR) strains and have high rates of infection throughout the world, with Canada among the countries with the highest incidence rates

(Diekema, Pfaller, Shortridge, Zervos, & Jones, 2019). *S. aureus* produces several haemolysins and heat-stable toxins as well as bacteriocins (O'Sullivan, Rea, O'Connor, Hill, & Ross, 2019). The common bacteriocins are BacCh91 (class Ia), staphylococcin C55 and aureocin A70 (class IIb), aureocin A53 (class IId), and aureocyclicin 4185 (class IV) (Ceotto, Nascimento, Brito, & Bastos, 2009; Newstead et al., 2020). The narrow range of activity of these bacteriocins targets respectively: *Micrococcus luteus, Lactococcus lactis* (Wladyka et al., 2013), *S. aureus*, *Micrococcus luteus* (Navaratna, Sahl, & Tagg, 1998)*, Listeria monocytogenes*, *S. aureus* (Netz, Pohl, et al., 2002)*, E. faecium, L. innocua , M. luteus, S. aureus, S. epidermidis, S. simulans* (Netz, Bastos, & Sahl, 2002), and *M. luteus* (Potter, Ceotto, Coelho, Guimarães, & Bastos, 2014). On the other hand, bacteriocins produced by coagulase-negative, skin-resident staphylococci are found to inhibit MRSA strains (O'Sullivan et al., 2019). The bacteriocins produced by commensals of the human microbiome are gaining attention as potential treatment option against MRSA.

1.6.3. *Enterococcus faecium, Enterococcus faecalis*

Enterococcus faecium and *Enterococcus faecalis* are Gram-positive, facultative anaerobic bacteria in the order Lactobacillales (Yilmaz et al, 2014). They were part of the group *D* streptococci and until 1984 classified as *Streptococcus* (Foulquié Moreno, Sarantinopoulos, Tsakalidou, & De Vuyst, 2006). Enterococci are generally considered residents of intestinal microbiota, but they are also antibiotic-resistant and hospital-acquired pathogens (Lebreton, Willems, & Gilmore, 2014). These pathobionts develop antimicrobial resistance, but also acquire it from other members of the microbiome (Sood, Malhotra, Das, & Kapil, 2008). The most common nosocomial infections they cause are: urinary tract infection, bacteremia and abdominal post-surgery wound (Morrison, &

Wenzel, 1986; Sood et al., 2008). Additionally, enterococci were isolated from the airways in VAP patients and the initial site of colonization for *E. faecalis* was the oropharynx (Lund, Agvald-Öhman, Hultberg, & Edlund, 2002). This might suggest that the LRT infections can also initiate as a URT colonisation by enterococci. They colonize and maintain colonisation in the host through adhesion in the tissues and thrive by overgrowing in the niche. The virulence factors of enterococci include: aggregation substance, lipoteichoic acid, protease, hyaluronidase, cytolysin, enterocins, enterocin AS-48 and enterolysin A (Jett, Huycke, & Gilmore, 1994). Cytolysin is a combination of haemolytic toxin and bacteriocin that lyses both prokaryotic and eukaryotic cells (Coburn & Gilmore, 2003), meanwhile, enterocins, AS-48 and enterolysin A are bacteriocins with a wide range of activity (Cintas, Casaus, Håvarstein, Hernández, & Nes, 1997; Franz, Van Belkum, Holzapfel, Abriouel, & Gálvez, 2007; Galvez, Gimenez-Gallego, Maqueda, 1989). These bioactive compounds contribute further to dysbiosis in the host's microbiota and promote disease. Lactobacilli and *Enterococcus hirae* are known to inhibit *E. faecalis and E. faecium* by bacteriocin activity (Abee, Klaenhammer, & Letellier, 1994; Sánchez et al., 2007).

1.6.4. *Pseudomonas aeruginosa*

P. aeruginosa is a Gram-negative, rod-shaped, facultative anaerobe predominantly environmental organism and is an opportunistic pathogen. It colonizes the LRT of cystic fibrosis and Chronic Obstructive Pulmonary Disease patients (Curran, Bolig, & Torabi-Parizi, 2018). It is the leading cause of morbidity and mortality in cystic fibrosis patients (Moradali, Ghods, & Rehm, 2017). *P. aeruginosa* is persistent in the environment and is an opportunistic pathogen in immuno-deprived patients due to numerous virulence factors and intrinsic resistance against antimicrobials (Veesenmeyer, Hauser, Lisboa, & Rello, 2009). *P. aeruginosa* produces numerous proinflammatory molecules and toxins and

causes tissue impairments in the host (Jaffar-Bandjee et al., 1995; Moradali et al., 2017). The bacterium uses quorum sensing, biofilm formation, toxins and bacteriocins to outcompete other bacteria (Tashiro, Yawata, Toyofuku, Uchiyama, & Nomura, 2013). The common bacteriocins produced by *P. aeruginosa* include R- and F-type tailocins, S-type pyocins and lectin-like (L-type) (Ghequire & Öztürk, 2018). These bacteriocins target mainly *Pseudomonas* strains, although the R-type tailocins inhibit growth of other Gram-negatives as well (Michel-Briand & Baysse, 2002; Parret, Temmerman, & Mot, 2005). Conversely, the bacteriocin activity of *L. acidophilus* (Al-Mathkhury, Ali, & Ghafil, 2011) and the cell free supernatant (CFS) of *Streptococcus salivarius* strain M18 can inhibits biofilm formation of *P. aeruginosa* (Tunçer & Karaçam, 2020). *P. aeruginosa* grows very well *in vitro* in different O_2 concentrations; however, in anaerobic conditions it requires alternative electron acceptors such as nitrate (Wu et al., 2005).

1.6.5. *Klebsiella pneumoniae*

Klebsiella pneumoniae is a Gram-negative facultative anaerobe bacterium in the family *Enterobacteriaceae* that is commonly associated with hospital-acquired infections (Podschun & Ullmann, 1998). *K. pneumoniae* infections including pneumonia, urinary tract infections, bacteremia, and liver abscesses (Paczosa & Mecsas, 2016). As a MDR pathogen with emerging hyper-virulent strains, *Klebsiella*-related infections can be challenging to control. The most studied virulence factor of the bacterium is the capsular polysaccharide which decreases phagocytosis by macrophages (Cortés et al., 2002; Paczosa & Mecsas, 2016). *Klebsiella* produce bacteriocins that include microcin E492 and klebicins. Microcin E492 is produced by *K. pneumoniae* and induces apoptosis in human

cells (Hetz, Bono, Barros, & Lagos, 2002). Klebicins are produced by various plant-derived *Klebsiella* strains and inhibit growth of MDR *K. pneumoniae* (Denkovskienė et al., 2019).

1.7. RATIONALE HYPOTHESIS AND AIMS

The human microbiota is a source of antimicrobial substances and has been under-explored (Shekhar, Schenck, & Petersen, 2018). Colonization of the URT is often a prerequisite for infection by respiratory pathogens and the URT provides a niche for pathogens to colonize and establish populations that can disseminate to other sites and cause disease. Colonization resistance limits the ability of pathogens to colonize the airways. One mechanism of colonization resistance is direct microbe-microbe antagonism whereby bacteria directly inhibit the growth of other bacteria through exploitation of resources and production of bioactive compounds. This project focuses on exploring the microbiota of the upper respiratory tract for bacteria and bacterial products that inhibit respiratory pathogens.

I hypothesize that the respiratory tract microbiota harbours a rich diversity of antimicrobial activities and that these activities includes obligate anaerobes and genes expressed under anaerobic conditions for facultative anaerobes.

This project had two aims:

Aim 1: To further characterize the antibacterial activity of *Staphylococcus aureus* strain 18D11 against *Enterococcus faecium* which was originally identified by Dr. Steve Bernier.

Aim 2: To carry out a screen of aerobes/facultative anaerobes and obligate anaerobes from our human respiratory tract isolate collection (4652 strains) against *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Klebsiella pneumoniae under* aerobic and anaerobic conditions to identify commensal-produced antimicrobials.

CHAPTER 2. Characterization of *Staphylococcus aureus* Strain 18D11 Inhibitory Activity Against Lactobacillales

2.1. INTRODUCTION

Antimicrobial resistance is recognized as a global health crisis and is increasing due to widespread antibiotic misuse and cross transmission of resistant bacteria and dissemination of antimicrobial resistance genes, leading to the necessity to discover new antimicrobial therapeutics (Amann, Neef, & Kohl, 2019; Golkar, Bagasra, & Gene Pace, 2014; Halperin et al., 2016). The World Health Organization has generated a list of pathogens for which new antibiotics are urgently needed. The original ESKAPE pathogen list (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* species) has recently been expanded to 12 species including *Streptococcus pneumoniae* (Tacconelli et al., 2018) . Consequently, there is a recognized need to look for alternatives to conventional antibiotics (Mulani, Kamble, Kumkar, Tawre, & Pardesi, 2019). One group of antimicrobials that merits further consideration are bacteriocins, derived from commensal microbes (Lopetuso et al., 2019). Unlike antibiotics produced by soil bacteria, these natural products have evolved to function in mucosal surfaces (i.e. the site of initial colonization).

Staphylococcus aureus and *Enterococcus faecium* are both members of the ESKAPE pathogen list thus, a burden for the health care system (Rice, 2010). *Enterococcus faecium* belongs to the Lactobacillales order and was classified under the *Streptococcus* genera until 1984 (Schleifer & Kilpper-Balz, 1984).

A previous screen carried out by Dr Steve Bernier in the Surette lab identified commensal bacteria that could inhibit the growth of ESKAPE pathogens. One activity identified in that screen was the inhibition of the *Enterococcus faecium* target strain by one specific

Staphylococcus aureus strain (18D11) from the Surette lab collection. Other *S. aureus* strains in the screen did not inhibit the *E. faecium* strain. The inhibition was displayed as a zone of clearing around a colony of *S. aureus* 18D11 on an *E. faecium* 22H6 lawn. As zones of inhibition indicate contact independent interaction, the *S. aureus* 18D11 was hypothesized to produce a diffusible bioactive molecule that kills *E. faecium* 22H6. Dr. Bernier established the assay conditions for the activity assessment of *S. aureus* 18D11 on *Enterococcus*. He tested *E. faecium* 22H6 with a collection of *S. aureus* from the Surette lab collection and showed that the activity was not expressed by any of them, implying that *S. aureus* strain 18D11 was unique among these *S. aureus* strains. Additionally, Dr. Bernier screened *S. aureus* 18D11 with a collection of MDR enterococci (Wright Clinical Collection) and observed that the activity of strain 18D11 was inhibitory for most of the enterococci in the collection. In this study, I validated the aforementioned findings of Dr. Bernier, evaluated the activity of *S. aureus* 18D11 and *E. faecium* 22H6 in anaerobic conditions, as well as assessed and characterized further the activity of *S. aureus* 18D11. The later, was done by expanding the screen of strain 18D11 to bacterial strains related to *Enterococcus.* In addition, I attempted to isolate the bioactive molecule and analyze it further. Whole genome sequencing (WGS) of *S. aureus* 18D11 was also used to facilitate the identification of potential antimicrobial gene clusters.

2.2. MATERIALS AND METHODS

2.2.1. Bacterial Isolates and Growth Conditions

The bacterial strains included in the screen were all recovered from -80°C 15% glycerol stocks. *Staphylococcus aureus* 18D11, *Staphylococcus aureus* 14F12,

Enterococcus faecium 22H6 and other *Enterococcus* strains were grown on Brain Heart Infusion (BHI) 1.5 % agar (unless otherwise specified) for 24 h at 37°C under 5% CO_2. For growth in broth culture, single colonies of 18D11, 14F12 and 22H6 were inoculated in 5 ml BHI broth and incubated with shaking (200 rpm) at 37°C for 20 h.

Streptococcus strains were grown on Todd Hewitt agar supplemented with 0.5% yeast extract (THY) and 2% catalase (100 µl) at 37ºC under 5% CO_2 incubator for 24 h and broth cultures were inoculated from single colonies and grown without shaking (static) in 5 ml THY at 37ºC under 5% CO_2 (Regev-Yochay, Trzcinski, Thompson, Lipsitch, & Malley, 2007).

Lactobacillus strains were grown in De Mann, Rogosa and Sharpe (MRS) agar media (pH 6) and incubated for 48 h at 37ºC. Single colonies were inoculated in 5 ml MRS and grown (static) at 37ºC for 48 h.

Staphylococcus aureus strains were pinned from 96 well glycerol stocks into 150 ml BHI (broth) 96 well plates and were grown at 37°C for 20 h with shaking (200 rpm).

2.2.2. The Bacterial Interaction Assays Between *S. aureus* 18D11 and *Enterococcus, Streptococcus* and *Lactobacillus* Strains

To assess the activity of *S. aureus* 18D11 with *Enterococcus, Streptococcus* and *Lactobacillus* (target strains are summarized in Table 5), strains were grown in broth culture and each overnight was diluted to OD_{600} 0.6 except, *S. aureus* 18D11 and 14F12 which were used at OD_{600} ≥2.0. Duplicate spots of 100 µl of the target strains (OD_{600} 0.6) were spotted on agar plates (*Enterococcus* was grown on BHI, *Streptococcus* in THY and *Lactobacillus* on MRS) and left to dry. Then, 5 µl of *S. aureus* overnight cultures were inoculated in the centre of each target spot and the assays were grown at conditions

specified above for each target. This allowed up to 4 target strains to be tested per 100 mm petri plate. Zones of clearing were identified by visual inspection, measured, and photographed. The photographs in this study was performed with camera Canon PowerShot G12.

2.2.3. Multiplexed (96 Strain) Inhibition Assay of an *S. aureus* Strain Collection Against *E. faecium* 22H6 on Solid Medium

To scale up the screen of *S. aureus* strains for activity against *E. faecium* 22H6 target strain, I multiplexed the plate assay using 96 pin replicators. *E. faecium* 22H6 was grown as described above and 200 µl (OD_{600} 0.6) was spread on BHI plates (150 mm x 15 mm) with sterile glass beads. After the lawn was dry, the *Staphylococcus* strains' overnights (grown in 96 well plates) were inoculated onto the *E. faecium* lawn with a 96 well pin replicator. The plates were then incubated for 24 h at 37°C under 5% CO_2. Zones of clearing were identified by visual inspection, measured, and photographed.

2.2.4. Well and Disc Diffusion Assays with *S. aureus* 18D11 CFS

The cell free supernatants (CFS) was prepared from 5 ml of *S. aureus* 18D11 cultures grown for 16 h. The diffusion assays were done with the swab lawn of *E. faecium* 22H6 or by spreading 100 µl on BHI agar. The culture was spin down at 4000 x g for 20 min and the CFS was filter sterilized (syringe filter 0.22 µm pore size). Ten µl of CFS was assayed by spotting directly on a blank sterile paper disc placed on the lawn of the indicator strain, *E. faecium* 22H6. The assays were incubated for 24 h at 37°C under 5% CO_2 and the activity was assessed for visible zone of clearing around the disk. The well diffusion assays were infused with 50 µl CFS into wells punched into the lawn seeded with 100 µl

(OD_{600} 0.6) of the indicator strain. After 24 h incubation at 37°C under 5% CO_2, the assays were evaluated for zones of clearing around the wells.

In addition, a methanol precipitate extract (50% final volume) of the 18D11 culture was also assayed in a lawn of *E. faecium* 22H6. 1.5 ml culture of *S. aureus* 18D11 was spin down on 11200 x g for 5 min. The CFS was aliquoted (750 µl) in 1.5 ml microcentrifuge tubes and 750 µl of methanol was added to each CFS volume. The mixture was left in room temperature for 24 h. Immediately after, the mixture was spin down in 21900 x g for 30 min, CFS was discarded, and the pellet was left to air dry for 12 h. The dried pellet was then resuspended in 50 µl of Minimal media (M9) and 10 µl was infused on a blank sterile paper disc placed on the lawn of the indicator strain. The plate was incubated for 24 h and was assessed for zone of clearing of the 22H6 around the disc.

2.2.5. Embedding *S. aureus* in Alginate Beads

S. aureus 18D11 cells were recovered from 1 ml culture by centrifugation for 10 min at 6000 x g and suspended in 1 ml 0.9% NaCl solution and then mixed with 4 ml 2.5% sterile alginate solution. The mixture was then transferred to a 5 ml syringe and the drops were forced through the syringe (needle) and slowly suspended into 100 ml of 100 mM $CaCl_2$ solution. They were left there to harden for 15 min and washed 3 times with distilled H_2O. The beads were transferred into the 15 ml broth media [BHI, MRS and M9 was used in this study] to grow for 24 h. After 24 h, CFS of 2 ml culture was used to test in a lawn of *E. faecium* 22H6 and activity was assessed the same as in the paragraph above.

2.2.6. Whole Genome Sequencing and AntiSMASH Prediction of Biosynthetic Gene Clusters

To facilitate the identification of possible antimicrobial activity produced by *S. aureus* 18D11, a genomic approach was undertaken. The whole genome sequencing (WGS) of *S. aureus* 18D11, and another *S. aureus*, strain 14F12, from our collection that does not produce the activity, was completed. Genomic DNA was purified from the isolates using the Wizard genomic DNA purification kit (Promega, Fitchburg, WI), and DNA concentrations were determined with the Qubit 4.0 Fluorometer double-stranded DNA using the High Sensitivity Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) (Brumfield et al., 2020). Illumina sequencing libraries were prepared with Nextera XT DNA Library kit (Illumina, San Diego, CA) as per manufacturer's instructions. Genomes were assembled from 250 bp paired end Illumina HiSeq reads and assembled using Unicycler (Wick, Judd, Gorrie, & Holt, 2017) and the assembled genomes were annotated with Prokka (Seemann, 2014) using default parameters. To identify potential genes that may be involved in antibacterial activity, each genome was analyzed using antiSMASH 4.0 (Blin et al., 2017) (https://antismash.secondarymetabolites.org/#!/start). This software predicts bacterial secondary metabolites including small ribosomally derived peptides such as bacteriocins.

2.3. RESULTS

2.3.1. Validation of *S. aureus* 18D11 Activity Against *E. faecium* 22H6

In the previous study by Dr. Steve Bernier, *S aureus* strain 18D11 was identified as having antimicrobial activity against *E. faecium strain* 22H6. He established the assay

conditions; I validated the inhibitory activity of *S. aureus* 18D11 on a lawn of *E. faecium* 22H6. *S. aureus* 18D11 generally generated a 4mm clear zone of inhibition (ZOI) on a lawn of *E. faecium* 22H6. The *S. aureus* 14F12 was used as negative control and showed no inhibition. I carried out the inhibition assay under anaerobic conditions and a similar ZOI was observed (Fig. 3). The zone of clearing anaerobically was the same size as aerobically.

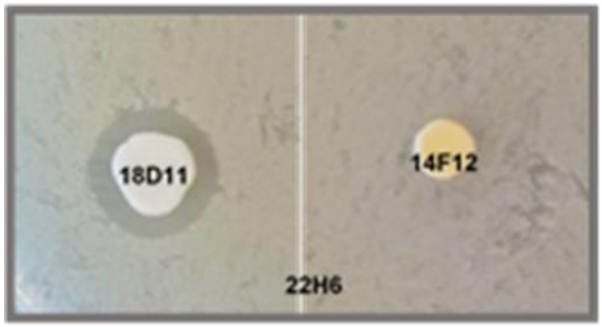

Figure 3. Anaerobic growth inhibition of *E. faecium* 22H6 by *S. aureus* 18D11.

The ZOI is displayed around *S. aureus* 18D11 but not on the negative control strain, *S. aureus* 14F12.

2.3.2. Strain Specificity of *S. aureus* 18D11 anti-Enterococcal Activity

To further evaluate the anti-enterococcal activity of *S. aureus* 18D11, I tested the activity against a collection of 114 isolates of vancomycin-resistant *Enterococcus* (Table 2) under aerobic conditions. This collection of *Enterococcus* included 95 *Enterococcus sp*. (not identified to species in the strain collection), 18 *E. faecalis* and 1 *E. faecium* isolate. *S aureus* 18D11 exhibited a broad range of anti-enterococcal activity with clear zones of inhibition detected against 101 isolates of 114 *Enterococcus* strains. The ZOI ranged from 2 mm to 4 mm diameter.

Table 2. Growth inhibition of *Enterococcus spp.* strains by *S. aureus* 18D11 under aerobic conditions

Enterococcus strains	Inhibition (%)	No inhibition (%)
Enterococcus sp.	93 (98)	2(2)
Enterococcus faecium	1 (100)	0
Enterococcus faecalis	7 (39)	11 (61)
total	101(89)	13(11)

2.3.3. Characterization of Antimicrobial Activity of *S. aureus* 18D11 Against Lactobacillales

Enterococci are members of the Family Enterococcaceae within the Order Lactobacillales and were formerly classified under the *Streptococcus* genus*;* I suspected the *S. aureus* 18D11 had broader activity against members of the Lactobacillales including streptococci and lactobacilli species. To determine the specificity of the antimicrobial activity of this strain, I screened *S. aureus* 18D11 with 18 *Lactobacillus,* 10 *Streptococcus*, and 35 *Staphylococcus* isolates. The screen of *S. aureus* 18D11 with Lactobacillalles showed that the inhibitory activity was exhibited on 83% of *Lactobacillus spp.*, 20% of *Streptococcus spp*. and no activity on the *Staphylococcus spp*. (Fig. 4). The zone of clearing size varied between genus as well as, among species within the genus. The assay of 18D11 with *L. fermentum* GC 39 strain exhibited the largest zone size in this screen at 7 mm when grown in MRS media. The activity of 18D11 on *Streptococcus spp.* was the lowest among the Lactobacillales and the zone size for these assays was <2 mm. Generally, the *Streptococcus* lawn was thin and difficult to assess for phenotype. *S. aureus* 14F12 used as negative control in this study, was tested in all assays along with 18D11 and no activity was observed.

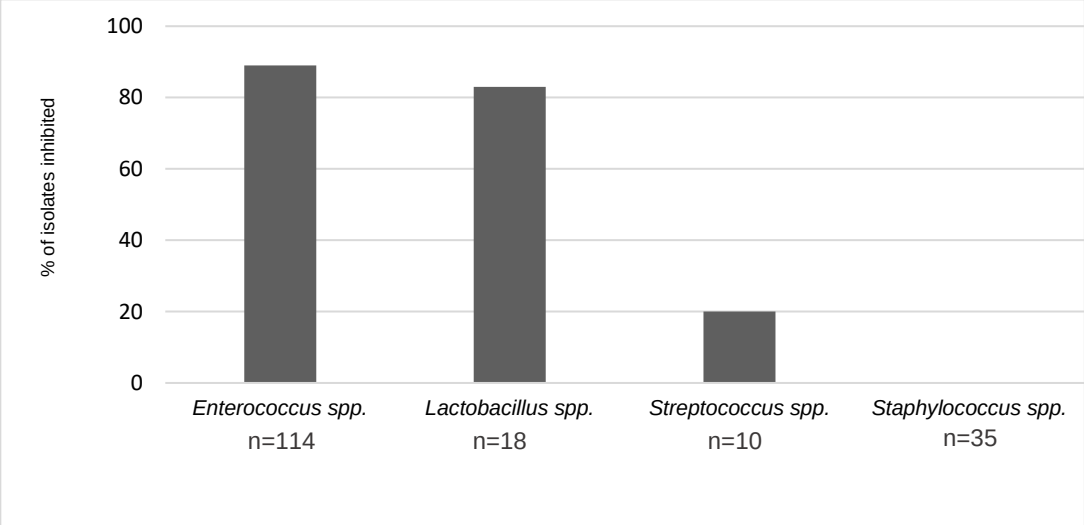

Figure 4. *S. aureus 18D11* inhibitory activity against *Enterococcus spp., Lactobacillus spp. Streptococcus spp.* and *Staphylococcus spp.* strains aerobically.

2.3.4. Assay for *S. aureus* 18D11 activity in liquid cultures

The activity assays described in the previous section were carried out on solid media (Fig. 5A). I next sought to determine whether the bioactivity was detectable in liquid cultures. *S. aureus* 18D11 was grown in BHI and MRS broth, alone or in co-culture with *E. faecium* 22H6. The CFS of 16 h cultures were filter sterilized and assayed on a lawn of *E. faecium* 22H6. No activity was detected in any of the broth-grown cultures of *S. aureus* 18D11 (Fig. 5B).

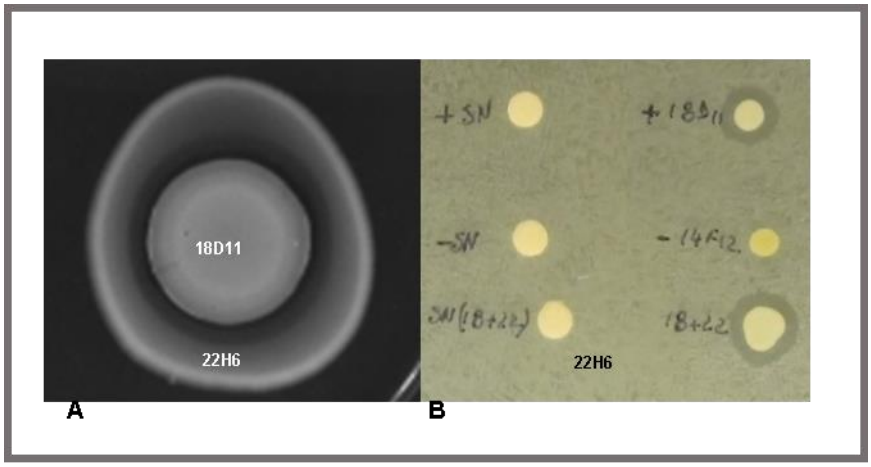

Figure 5. Inhibitory activity of *S. aureus* 18D11 against *E. faecium* 22H6.

A: Overlay of *S. aureus* 18D11 and *E. faecium* 22H6 in aerobic conditions. The ZOI is the clearing around the 18D11 growth.
B: *S. aureus* cells and supernatant disc diffusion assays on *E. faecium* 22H6 lawn. Cell supernatants are on the left and cells are on the right. From top to bottom are *S. aureus* 18D11, S*. aureus* 14F12 co-culture of *E. faecium* 22H6 and *S. aureus* 18D11, respectively.

The data suggested that the bioactivity was made exclusively when the strain was grown on solid media or that it was too dilute in broth culture to detect activity. I next attempted to optimize the production of anti-enterococcal activity of *S. aureus* 18D11 using growth in alginate beads. Alginate beads have been used to enhance bacteriocin production in liquid media (Scannell et al., 2000). This method, if successful, would facilitate larger scale production of the activity for purification. In this method the bacteriocin producing strain is entrapped in alginate beads that acted as a solid media surface. Uniform alginate beads with embedded bacteria were inoculated in different media (BHI, MRS, M9) to assess if the activity was expressed in different media types (Fig. 6A). The pores of the alginate beads are bigger than the size of the bacteriocin enabling

the bacteriocin to diffuse easily out of the beads and accumulate in the liquid (broth). The activity of the CFS generated with this method was assessed by well diffusion on a lawn seeded with the indicator strain (Fig. 6B) but inhibitory activity was not observed, regardless of the media type, indicating it was not expressed or too dilute to be detected.

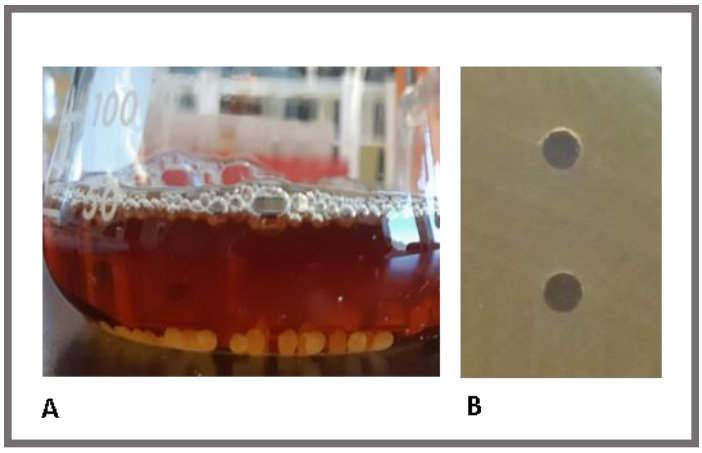

Figure 6. The putative bacteriocin activity of 18D11 was not detected from alginate beads.

A. Alginate beads enmeshed with *S. aureus* strain 18D11 suspended MRS media.
B. Negative result of the 18D11 CFS activity on well diffusion assay of the lawn seeded with 22H6.

2.3.5. Identification of Bacteriocin Gene Clusters through WGS of *S. aureus* 18D11

Whole genome sequencing and comparative genomics was undertaken as a strategy to identify the potential biochemical activity from *S. aureus* 18D11. A summary of the genome characteristics for these WGS assemblies are present in Table 3.

Table 3. Summary of *S. aureus* genome assemblies.

Contigs	*S. aureus* 18D11	*S. aureus* 14F12
# contigs	79	90
# contigs (>= 10000 bp)	34	39
# contigs (>= 25000 bp)	26	24
# contigs (>= 50000 bp)	15	16
Largest contig	437163	418666
Total length	2810762	2900400
N50	121030	144822
GC (%)	32.7	32.72

The annotated genomes were further analyzed using antiSMASH (Blin et al., 2019) and were compared to known and potential novel biosynthetic gene clusters including bacteriocins. The results of the antiSMASH predictions for *S. aureus* 18D11 are presented in Table 4. Seven clusters were predicted to encode bioactive molecules and four of them were highly conserved across *S. aureus* strains including the negative control *S. aureus* 14F12 (data not shown). The results in this study showed that the specific activity of interest was uncommon among *S. aureus* thus, we further investigated clusters 2, 6 and 7, as the source of potential activity. These three clusters were present in only a small number (≤10) of *S. aureus* strains and none of the three clusters was predicted to be in the genome of the negative control strain (*S. aureus* 14F12). The antiSMASH predicted bacteriocins were expected to be <10 kDa classified under the bacteriocin class I and II.

Table 4. AntiSmash analysis of *S. aureus* 18D11.

Name	Prediction		Prediction in other
	# of genes in cluster	Known metabolite	*S. aureus* genomes
Cluster 1	17	Unknown (terpene)	10 other *S. aureus*
Cluster 2	11	Unknown (bacteriocin)[1]	10 other *S. aureus*
Cluster 3	18	Aureusimine (NRPS)	Highly conserved
Cluster 4	13	Staphylobactin (siderophore,75% similarity)	Highly conserved
Cluster 5	4	Staphyloferrin (siderophore,100% similarity)	Highly conserved
Cluster 6	4	Unknown (bacteriocin)[2]	5 other *S. aureus* (low similarity)
Cluster 7	4	Unknown (bacteriocin)[3]	9 other *S. aureus*

[1] closest match Lactococcin_972
[2] closest match TIGR01193
[3] closest match Bacteriocin_IIc class

The predicted bacteriocin clusters should include the core genes that encodes each peptide (bacteriocin) and accessory genes required for processing and regulation of the antimicrobial peptide production (e.g. immunity gene, ABC-transport gene). The gene clusters of the predicted bacteriocins of *S. aureus* strain 18D11 are summarized on the Fig. 7. The putative bacteriocin in cluster 2 has a high similarity to Lactococcin_972. The cluster size is ≈10 kb, and includes the core gene *lcn_972* (locus tag: 18D11_Sa_00547) predicted to encode a hypothetical bacteriocin 14.5 kDa; a 642 bp- gene (locus tag: 18D11_Sa_00550) which encodes a putative ABC-transport protein 24.2 kDa; and a 603 bp- gene (locus tag: 18D11_Sa_00546) predicted to encode an Abi protein 23.3 kDa. The

Abi protein is estimated to play a role in providing imunity to the producing cell by the activity of its own bacteriocin.

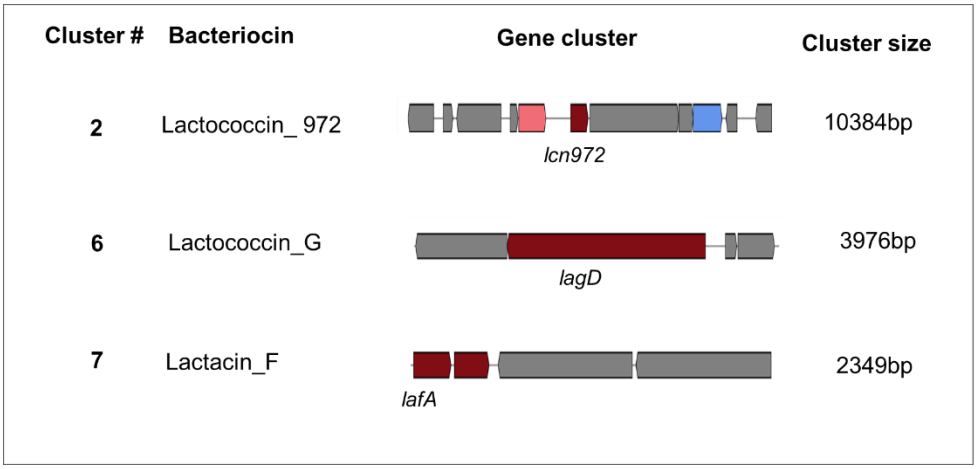

Figure 7. Bacteriocin gene clusters of *S. aureus* 18D11 predicted by antiSMASH.

The colours indicate the predicted function of these genes. The burgundy shows the bacteriocin' core gene, the coral indicates putative immunity genes and the blue colour indicates the genes that encodes proteins involved in bacteriocin transport.

The cluster 6 and 7 have 4 genes each, predicted for bacteriocin production. Both clusters are smaller compared to cluster 2 (≈2.3-4 Kb) and they lack the core gene (cluster 6) or part of the core gene (cluster 7) that encode the putative bioactive molecule. Although, the antiSMASH prediction for these clusters includes genes estimated to have a role in production or transport of the putative bacteriocins. Cluster 6 includes the 2160 bp- gene *lagD* (locus tag: 18D11_Sa_02620) predicted to encode a bacteriocin- transport protein (82.4 kDa) for the bacteriocin Lactococcin_G. The other three genes on this cluster did not have similarity to the proteins that encode Lactococcin_G or other proteins that play a role in its synthesis. Cluster 7, was predicted to encode the two-component

bacteriocin, lactacin_F. Two genes were predicted to be core genes, one is 203 bp-gene *lafA* (locus tag: 18D11_Sa_02655) which encodes subunit LafA (6.6 kDa) and the other one is 192 bp- gene (locus tag: 18D11_Sa_02656) which encodes a hypothetical protein 6.2 kDa. The other genes in cluster 7 are hypothetical proteins, without a function related to lactacin_F production or its transport.

I suspected that cluster 6 and 7 may be part of the same biosynthetic gene cluster but were predicted separately because they are found on the ends of two separate contigs in the genome assembly. A blast search with these clusters identified a plasmid which encoded a high similarly cluster comprised of these two clusters (*Staphylococcus aureus* strain FDAARGOS_159 plasmid NCBI Accession # CP014063). I conclude from the genome analysis of *S. aureus* 18D11 and comparative genomics that there are two predicted biosynthetic gene clusters (Cluster 2, Cluster 6/7) in this strain that could encode a bacteriocin with the activity characterized in this chapter.

2.4. DISCUSSION

The first aim of this study was to validate the inhibitory activity of *Staphylococcus aureus* strain 18D11 on *Enterococcus faecium* 22H6, identified previously in the study carried on by Dr. Bernier, and to characterize further its activity. The results showed that *S. aureus* 18D11 inhibits *E. faecium* 22H6 aerobically and anaerobically, its activity targets broadly Lactobacillales, and is detected in an inhibition assay only when grown on solid media. The activity of the 18D11 inhibited the growth of 89% *Enterococcus spp.*, 83% of *Lactobacillus spp.* and 20% of *Streptococcus spp*. The antimicrobial activity of *S. aureus* by bacteriocins is well studied and was evaluated to inhibit a broad range of bacteria including *M. luteus, L. lactis*, *L. monocytogenes*, *E. faecium, L. innocua, S. epidermidis*

and *S. simulans* (Navaratna et al., 1998; Netz, Bastos, et al., 2002; Netz, Pohl, et al., 2002; Potter et al., 2014; Wladyka et al., 2013). These bacteriocins are classified under classes Ia, IIb, IId and IV (Ceotto et al., 2009; Newstead et al., 2020). *S. aureus* produces aureocin A53 (class IId) which inhibits *E. faecium* via generalized membrane destruction. The range of activity of aureocin A53 is broader than the activity of the *S. aureus* strain 18D11 and target Bacillales (*S. aureus*), Lactobacillalles (*E. faecium*) and Micrococcales *(M. luteus*) (Netz, Bastos, et al., 2002). The putative bacteriocins produced by *S. aureus* in this study targeted Lactobacillalles, although it is not known e whether *S. aureus* 18D11 inhibits growth of Micrococcales as they were not included in the screen.

The expression of activity of *S. aureus* 18D11 in aerobic and anaerobic conditions for *E. faecium* 22H6 was expected, as it is a facultative anaerobe, grow well in both conditions. Bacteriocin production by facultative anaerobes in both aerobic and anaerobic conditions was previously described (Yang et al., 2012).

The phenotypic activity of *S. aureus* 18D11 on *E. faecium* 22H6 and other strains was expressed as a ZOI which suggests the activity of interest is due to a bioactive molecule that diffuses in agar. This is evidence that the inhibition is contact independent. The ZOI in contact independent inhibition fluctuates based on the size of the bioactive molecule and its charge, the amount excreted by the producing strain and susceptibility of the target strain (Li et al., 2017). The ZOI size varied for *Lactobacillus* strains and was relatively consistent with *Enterococcus* strains. Small zones were observed on *Streptococcus spp.* This may indicate a change in production of the bioactive molecule under growth conditions, change in activity of the bioactive (e.g. pH effects) or the sensitivity of the target strains. The large zone size (7 mm) on MRS assay with *L. fermentum* GC 39 led me to believe that the activity of *S. aureus* 18D11 was increased by

the pH of the media and its supplements, as MRS has a lower pH compared to BHI and TSY. However, the phenotypic assessment of 18D11 with *E. faecium* 22H6 in MRS did not affect the size of the ZOI. This implied that the increased activity expression was probably due to the susceptibility of strain *L. fermentum* GC 39.

I was unable to detect the activity of the bacteriocin when the strain was grown in liquid culture. Many studies suggest that the bacteriocin expression increases when producing strains are isolated in alginate (Scannell et al., 2000) or agarose beads (Behera, Kar, Mohanty, & Ray, 2010). Although I was able to get reproducible uniform alginate beads with embedded bacteria, I could not detect activity in the supernatants after growth. Bacteriocins are produced in small amounts and have been recovered from larger volumes of broth culture by ammonium sulfate precipitation or in small volumes of CFS (Beatriz, Suárez, & Rodrigues, 1995).

The WGS of 18D11 compared to the WGS of 14F12 identified three gene clusters that are predicted by antiSMASH that are not common in *S. aureus* and are not present in the 14F12 genome. Further bioinformatic analysis indicated that two of these likely formed a single cluster. Cluster 2 is predicted to encode bacteriocin lactococcin_972. Beatriz and colleagues have isolates and purified lactococcin_972 from a *Lactococcus lactis* strain. They assessed that the activity spectrum of this bacteriocin was narrow as it targeted only two species within the *Lactobacillus* genus: *Lactobacillus lactis subsp. cremoris* and *Lactobacillus sake* (Beatriz et al., 1995). The mechanism of action of this bacteriocin targets the cell wall septum formation of the target strain (Martínez et al., 2008).

In this study, I demonstrated the antibacterial activity of *S. aureus* 18D11 was broader compared to the activity of lactococcin_972 isolated from *L. lactis* as the activity of *S. aureus* 18D11 targets three diverse taxonomic genera within the order Lactobacillales

(*Enterococcus*, *Lactobacillus* and *Streptococcus*). Bioinformatic analysis identified two predicted bacteriocin clusters that could encode this activity. The observed activity might be attributed to one or both bacteriocins. For example, cluster 2 may be accountable for the activity on lactobacilli while cluster 6/7 for the activity enterococci/streptococci. Purification of the bioactivity from *S. aureus* 18D 11 would resolve this.

Table 5. Strains used in this study.

Genus	Species	Strain
Enterococcus	*faecalis*	C1080; C1249; C1250; C1251; C1252; C1253; C1254; C1255; C1074; C1075; C1393; C1394; C1395; C1396; C1397; C1398; C1399
Enterococcus	*faecium*	C1256
Enterococcus	*spp.*	C0497; C0498; C0499; C0500; C0501; C0502; C0503; C0504; C0505; C0506; C0507; C0508; C0509; C0510; C0511; C0512; C0513; C0514; C0515; C0516; C0517; C0518; C0519; C0520; C0521; C0522; C0523; C0524; C0525; C0526; C0527; C0528; C0529; C0530; C0531; C0532; C0533; C0534; C0535; C0536; C0537; C0538; C0539; C0540; C0541; C0542; C0543; C0544; C0545; C0546; C0547; C0548; C0549; C0550; C0551; C0552; C0553; C0554; C0555; C0556; C0557; C0558; C0559; C0560; C0561; C0562; C0563; C0564; C0565; C0566; C0567; C0568; C0569; C0570; C0571; C0572; C0573; C0574; C0575; C0576; C0577; C0578; C0579; C0580; C0581; C0582; C0583; C0584; C0585; C0586; C0587; C0588; C0589; C0590; C1257
Lactobacillus	*brevis*	GC1
Lactobacillus	*fermentum*	GC2; GC5; GC6; GC39
Lactobacillus	*gasseri*	GC36; GC37
Lactobacillus	*paracasei*	GC8; GC9
Lactobacillus	*plantarum*	GC4; GC5
Lactobacillus	*rhamnosus*	GC11; GC12; GC13; GC38
Lactobacillus	*salivarius*	GC50
Lactobacillus	*ultenensis*	GC41

Staphylococcus	*aureus*	Newman; RN6390; N315; MW2; ORF4D1; ORF4D2; ORF4F7; ORF4F11; ORF14A2; ORF14F12; ORF18B3; ORF18C9; ORF18C10; ORF18C11; ORF18C12; ORF18D1; ORF18D3; ORF18D5; ORF18D6; ORF18D7; ORF18D10; ORF18D11; ORF18D12; ORF18H5; ORF19A3; ORF19C4; ORF19C5; ORF19C6; ORF19C7; ORF20H8; C0017; C0018; C0019; C0020; C0021; C0022; C0023; C0024; C0032; C0033; C0111; C0112; C0113; C0114; C0115; C0116; C0117; C0118; C0119; C0433; C0434; C0435; C0489; C0490; C0620; C0621; C0622; C0623; C0631; C0632; C0633; C0639; C0640; C0652; C0653; C0669; C0670; C0671; C0681; C0682; C0708; C0709; C0710; C0711; C0712; C0713; C0719; C0720; C0726; C0752; C0753; C0754; C0755; C0756; C0781; C0782; C0783; C0784; C0815; C0816; C0878; C0879; C0880; C0889; C0890; C0891; C0892; C0898; C0899; C0900; C0922; C0945; C0946; C0947; C0981; C0982; C0983; C0984; C0985; C0988; C0989; C0990; C0991; C0992; C1010; C1011; C1012; C1013; C1014; C1015; C1017; C1018; C1022; C1023; C1024; C1025; C1026; C1027; C1028; C1029; C1030; C1031; C1032; C1037; C1038; C1039; C1040; C1041; C1042; C1043; C1044; C1045; C1046; C1047; C1048; C1055; C1056; C1057; C1059; C1060; C1061; C1062; C1063; C1098; C1099; C1100; C1101; C1102; C1103; C1104; C1105; C1106; C1107; C1109; C1110; C1111; C1112; C1113; C1114; C1115; C1116; C1117; C1131; C1132; C1133; C1134; C1135; C1136; C1137; C1138; C1139; C1140; C1141; C1142; C1145; C1146; C1147; C1148; C1149; C1150; C1151; C1282; C1389
Staphylococcus	*epidermidis*	ORF11F7; ORF16E10
Streptococcus	*agalactiae*	GC79; GC80
Streptococcus	*anginosus*	GC82
Streptococcus	*cristatus*	GC112
Streptococcus	*gordonii*	GC84
Streptococcus	*intermedius*	GC109; GC111
Streptococcus	*mitis*	GC108
Streptococcus	*mutants*	GC106
Streptococcus	*oralis*	GC110
Streptococcus	*parasanguinis*	GC104
Streptococcus	*rubneri*	GC105
Streptococcus	*salivarius*	GC81; GC107
Streptococcus	*sanguinis*	GC83

[1] – all strains starting with C are from the Institute for Infectious Disease Research (IIDR, McMaster University) collection of MDR pathogens and provided in kind. All strains labelled ORF or GC are Surette lab isolates. All other isolates Newman; RN6390; N315; MW2) are common laboratory strains.

CHAPTER 3. Screening the Airway Isolate Collection Strains for Inhibition of Lower Respiratory Tract Pathogens

3.1. INTRODUCTION

Respiratory diseases are a major burden for the health care system in both industrialized and non-industrialized countries. The most commonly identified etiological agent in pneumonia is *Streptococcus pneumoniae,* which can be an asymptomatic colonizer of the URT in children and adults (Troeger et al., 2018). The URT microbiota is suspected to have a protective role against LRT pathogens through colonization resistance and this has been demonstrated in some studies. These *in vivo* studies have shown that *Corynebacterium accolens* and *Streptococcus mitis* impede growth of *S. pneumoniae* (Bomar, Brugger, Yost, Davies, & Lemon, 2016; Regev-Yochay et al., 2007). Many streptococci can use the H_2O_2 production as an unsophisticated but effective weapon to inhibit (*in vitro*) the growth of *S. aureus,* another common respiratory tract pathogen (Regev-Yochay et al., 2006). *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* are pathogens that cause LRT and urinary tract infections in hospitalized and immuno-compromised individuals , including cystic fibrosis patients (Moradali et al., 2017; Podschun & Ullmann, 1998) Some commensals are recognized for their ability to inhibit *P. aeruginosa* growth. For example, H_2O_2-producing oral streptococci inhibit *Pseudomonas* in the presence nitrite, by intracell-buildup of peroxynitrite (Scoffield & Wu, 2015). The potential antagonistic interaction between URT microbiota and respiratory pathogens has not been fully explored. Bomar and colleagues showed that high levels of *C. accolens* in children's nostrils was associated with lack of colonization from *S. pneumoniae*. *In vitro,* growth inhibition of *S. pneumoniae* by *C. accolens* was attained

through generation of free fatty acids. In addition, this study established an *in vivo*-model to evaluate the impact of *C. accolens* on *S. pneumoniae* colonization (Bomar et al., 2016).

An earlier study investigated the inverse correlation of *P. aeruginosa* and *S. aureus* in the sputum of cystic fibrosis patients and isolated a bioactive molecule from *P. aeruginosa* that inhibited the growth of *S. aureus* strains (Machan et al., 1991). Bacterial isolates from sputum samples were grown on selective media for isolation of *Pseudomonas* and *Staphylococcus*. The screen was performed by the cross streaking method and well diffusion assays of the CFS of *P. aeruginosa* against *S. aureus* (Machan et al., 1991). Most screens of commensal microbiota for antimicrobial activity against pathogens have been carried out under aerobic condition or were partially screened in anaerobic conditions when isolates included in the screen grew better in the absence of O_2 (O'Sullivan et al., 2019; Zipperer et al., 2016). The screen of gastrointestinal isolates with pathogens were performed in anaerobic growth conditions as most of the microorganisms inhabiting the gut are strict anaerobes or facultative anaerobes (Raymond et al., 2019). Anaerobic growth conditions are generally not considered when screening with the respiratory pathogens although anaerobes comprise a high proportion of the URT microbiome (Brook, 2004). One of the reasons why the anaerobic growth conditions of the airway isolates are overlooked is because the airways are generally considered well-ventilated areas that do not promote extensively the anaerobic growth of its members. The other reason is that the strict anaerobes are difficult to isolate from specimens (special media, anaerobic transport conditions, longer-time growth) and their growth can easily get masked by overgrowth of the facultative anaerobes in the assays (Brook, 2006).

In this study, I assessed the microbial interaction between four LRT pathogens (*S. pneumoniae*, *S. aureus*, *P. aeruginosa* and *K. pneumoniae)* and a collection of 4652

isolates mostly isolated from the respiratory tract. The Airway Isolate Collection (AIC) includes 81 genera and encompasses bacterial isolates from the most prominent phyla present in healthy URT microbiome (Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria) (Dethlefsen et al., 2007). The screen for antimicrobial activity against four respiratory pathogens was carried out under both aerobic and anaerobic conditions. This permits assays of obligate anaerobes (which are not uncommon in the airways) and testing for anaerobic expression of activity for facultative anaerobes (that comprise most of the AIC). After the preliminary screen, I prioritized activity against *S. pneumoniae* as this is the primary pathogen of interest for the lab's ongoing studies.

3.2. MATERIALS AND METHODS

3.2.1. Bacterial Strains Growth Conditions

The targets used in this screen were *Staphylococcus aureus* 18D11, *Streptococcus pneumoniae* P1121, *Pseudomonas aeruginosa PA14* Δ*pqsR* and *Klebsiella pneumoniae* 25C9. Positive controls included in the study were *Staphylococcus lugdenensis* GC1371, *Bacillus amyloliquefaciens*, and *Pseudomonas aeruginosa* PA14 wild type (WT), which were known to inhibit respectively the growth of *S. pneumoniae* P1121 and *K. pneumoniae* 25C9, *P. aeruginosa* Δ*pqsR* PA14, and *S. aureus* 18D11. The bacterial targets and controls included in the screen, media supplements, and OD_{600} per each target bacteria are summarized in Table 6. Specific additions to the culture media were incorporated to improve the screening process. *S. pneumoniae* P1121 strain produces H_2O_2 which would likely kill many strains in the AICs and prevent any antimicrobial activity from these strains from being detected. To ensure the commensals

grew in the presence of P1121 catalase was added to the culture medium. *S. aureus* 18D11 was a strain established (Chapter 2) to inhibit the growth of Lactobacillales. *P. aeruginosa* PA14 Δ*pqsR* is a mutant strain which reduced expression of virulence factors and is less toxic towards other organisms compared to *P. aeruginosa* PA14 (WT). I predicted that the mutant strain would improve the sensitivity of the screen by allowing more commensals to grow and express their activity. *P. aeruginosa* also requires nitrate as a terminal electron acceptor to grow anaerobically and the growth medium was supplemented with 0.1 M KNO_3 as described previously (Toyofuku et al., 2007). *K. pneumoniae* 25C9 is an isolate from the Surette laboratory collection and was recovered from a respiratory tract sample. These growth conditions for the assay were optimized in preliminary experiments (data not shown).

Table 6. Bacterial strains (target and control) and medias' supplements used in this study

Target strain	OD_{600}	Media supplements	Control strains	Origin of the target strain
S. pneumoniae P1121	0.3-0.5	0.16 % catalase (aerobic)	*S. lugdenensis* (GC1371)	Bowdish laboratory, McMaster University
S. aureus 18D11	0.6	0.1M KNO_3 (anaerobic)	*P. aeruginosa* PA14	Surette Laboratory McMaster University
P. aeruginosa ΔpqsR PA14	0.1	0.1M KNO_3 (anaerobic)	*B. amyloliquefaciens*	Surette Laboratory McMaster University
K. pneumoniae 25C9	0.1	-	*S. lugdenensis* (GC1371)	Surette Laboratory McMaster University

All bacterial strains were recovered from -80°C glycerol stocks and were grown on Brain Heart Infusion (BHI) 1.5% agar for 24 hours at 37°C, under 5% CO_2 except for *S. pneumoniae* P1121 strain which was grown on BHI agar supplemented with 1000 U/ml catalase as described previously (Regev-Yochay et al., 2007). The target and control strains were then grown at 37°C for 20 h in 5 ml BHI broth, with shaking (200 rpm), with the exception of *S. pneumoniae* P1121 which was inoculated in 5 ml BHI broth and grown until it reached the required OD_{600} (0.3-0.5). The remaining target and control strains were diluted to the OD indicated in Table 6.

3.2.1.1. AIC Bacterial Strains Collection Characteristics

The AIC is a strain collection composed primarily of isolates from respiratory samples of patients and some healthy subjects (summarized in Table 7). The isolates are predominantly from the URT and LRT with a small fraction of the isolates that were not of respiratory tract origin (leg swab, urine sample, fecal sample, brain). The strain collection was not generated with rigorous purification protocols: single colonies from primary culture plates were frozen down in 96 well plates in glycerol and in most cases were not-single-colony purified. In addition, the same strain from a patient sample could be represented in the library more than once. The identification of isolates in the collection was based on partial 16S rRNA gene sequencing (using Sanger 16S primers 8 F/926 R). The isolates include facultative anaerobes, obligate anaerobes, strict aerobes, and 5% are unknown (either the 16S sequencing was poor quality and not suitable for identification or not sequenced). The most abundant predicted genus in this collection was *Streptococcus* (41%), followed by the *Staphylococcus* (8.4%). The collection was stored in standard 96 well plates and this can result in some cross-well contamination. A representative subset

of this collection (4652 bacterial isolates) was used in this study for the highly diverse taxonomic composition. The dereplication of the isolates at this stage in the screen would have been time-consuming and impractical, therefore the screen was done with the collection as was.

Table 7. The predicted taxonomy of AIC isolates used in this study

Isolate (genera)	# isolates
Streptococcus	1936
Staphylococcus	392
Actinomyces	334
No ID*	257
Veillonella	225
Neisseria	187
Rothia	157
Prevotella	108
Pseudomonas	99
Escherichia	87
Gemella	86
Corynebacterium	80
Kocuria	69
Propionibacterium	52
Fusobacterium	47
Bacillus	43
Lactobacillus	42
Enterococcus	35
Klebsiella	32
Granulicatella	28
Haemophilus	28
Moraxella	28
Lachnoanaerobaculum	26
Lachnospiraceae	25
Leptotrichia	24
Enterobacter	20
Selenomonas	17
Campylobacter	16
Capnocytophaga	14
Atopobium	13
Bifidobacterium	10
Peptostreptococcus	9
Parvimonas	9
Dolosigranulum	9
Terrahaemophilus	7
Eubacterium	6
Megasphaera	5
Oribacterium	5
Dialister	5
Microbacterium	4
Alloprevotella	4
Abiotrophia	4

Slackia	4
Arsenicococcus	3
Bacteroides	3
Citrobacter	3
Moryella	3
Scardovia	3
Solobacterium	3
Aggregatibacter	3
Micrococcus	3
Dietzia	2
Acinetobacter	2
Finegoldia	2
Kingella	2
Lautropia	2
Mogibacterium	2
Bordetella	2
Eikenella	2
Paenibacillus	2
Stenotrophomonas	2
Achromobacter	1
Afipia	1
Anaerococcus	1
Aspergillus	1
Bacteroidetes	1
Delftia	1
Helicobacter	1
Kluyvera	1
Kytococcus	1
Sanguibacter	1
Actinobaculum	1
Alloscardovia	1
Bergeyella	1
Brachybacterium	1
Catonella	1
Centipeda	1
Lactococcus	1
Mycobacterium	1
Paracoccus	1
Victivallis	1

**Note*: Isolates were not identified by16S rRNA gene sequencing.

AIC isolates were inoculated with a cryo-replicator (Enzyscreen) in 150 ul BHI broth supplemented with 0.1 M KNO_3 in 96 well plates. The plates were incubated at 37°C for 24 h in anaerobic conditions. The anaerobic growth for the isolates in this study was done

in jars (Anoxomat®) under 10% H_2, 10% CO_2 and 80% N_2, without shaking, unless stated otherwise.

3.2.2. The Competition Assay of *S. aureus* 18D11, *S. pneumoniae* P1121, *P. aeruginosa* PA14 Δ*pqsR* and *K. pneumoniae* 25C9 on Solid Medium

The following experiments were set up under ambient conditions, on the lab bench. The target strains (at the specified OD) were spread with a sterile cotton swab on BHI agar supplemented with the respective components (Table 6.) in duplicate petri plates (150 mm x 15 mm) and were left to dry in the Biosafety cabinet for 10 min. Subsequently, the 96 well isolates inoculated overnight were pinned with a 96-well pin-replicator on top of the lawn. A duplicate BHI plate with AIC bacterial strains (with no bacterial lawn) were used as a growth control. 1 µl of the *S. lugdenensis* GC1371, *P. aeruginosa* PA14 (WT) and *B. amyloliquefaciens* were spotted (separately) on top of the lawn of each duplicate plate as positive controls (Fig. 8). The four phenotypic assays and one control plate designated for aerobic growth were then incubated for 24 h at 37°C under 5% CO_2. Meanwhile, the other set of five plates (4 phenotypic assays and one control) assigned to grow in anaerobe conditions were incubated at 37°C for 72 h in anaerobic conditions. After incubation, the plates were scored by visual inspection for ZOI and other phenotypes observed on the growth lawn in the assays. The ZOI diameters were measured and the plates were photographed (Cannon PowerShot G12).

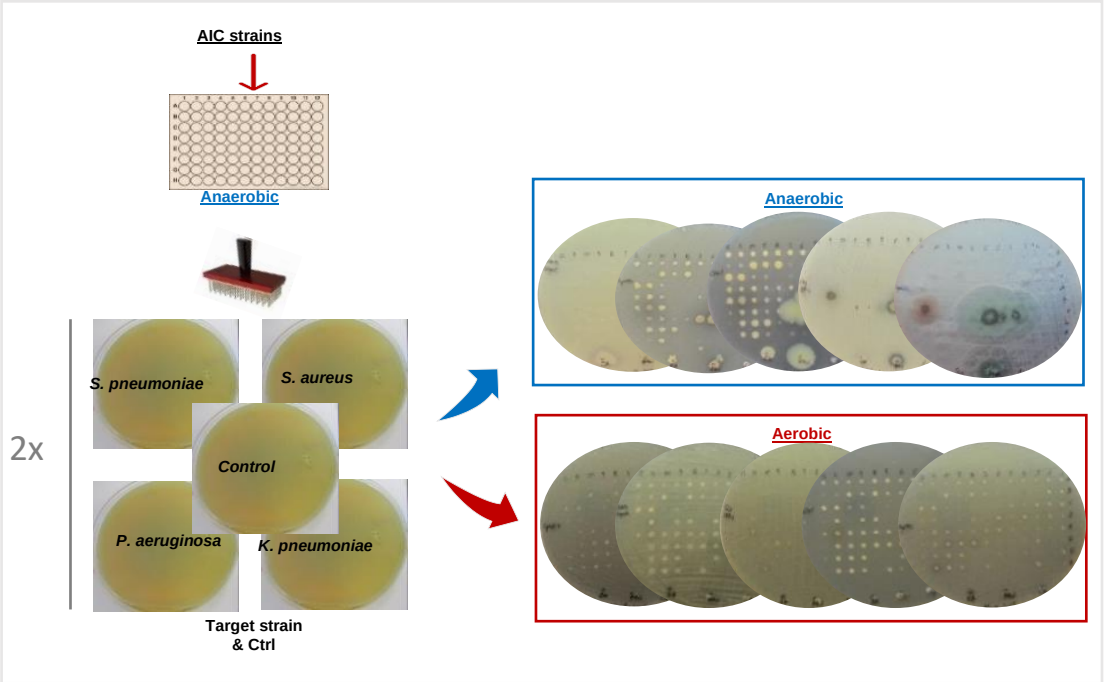

Figure 8. The screening method of AIC isolates against four pathogens.

The AIC strains (4652) were grown in BHI supplemented with 0.1 M KNO_3 for 24 h. The inoculum was then pinned on a duplicate lawn of the targets and control assay. Each 96 well plate generated 4 assays and 1 control for each condition.

3.2.3. Coding of the Phenotype Data for Cluster Analysis

Hits were considered interactions between the commensals and the targets that visibly altered the growth of the pathogen (details on 3.3.1.). Five growth changes were observed on the targets' lawn: the ZOI, H (hazy zone of inhibition), M (mutual killing), T (short-range phenotype), and V (long-range phenotype). The last two growth alteration phenotypes were observed only on the *K. pneumoniae* 25C9 lawn. Additional letter-codes were assigned for the numerical values recorded such as, ZOI sizes. Three letters (B, E, F) were designated for the ZOI phenotypes regardless of the target they were shown in. "B" represented ZOI-sizes from 0.1 mm- 1 mm, "E" (2 mm-3 mm) and F (3.5 mm-6 mm).

No-phenotype-observed was denoted as a “O”. Thus, each commensal strain was associated with a 20-character string in a text file (4 pathogens x 5 timepoints activity-assessment). A hierarchical clustering [Unweighted Pair Group Method with Arithmetic Mean (UPGMA)] tree was generated based on the Hamming distances of the phenotype string (carried out by Jake C. Szamosi). The clustering identified strains that exhibited the same or slightly different activities and help selecting the unique isolates between the replicates.

3.2.4. Dereplication of the AIC Hit Isolates

As mentioned in 3.2.1.1, the isolates in the AIC included multiple replicates of the same strain (based 16S rRNA sequencing ID) and generated from the same sample. I dereplicated the isolates that had the same ID (the same 16S rRNA sequencing ID) and derived from the same patient sample. Then, I used the phenotype (20-character-text-string) to compare and select the isolates that had the highest number of phenotypes expressed. The priority in the selection was the ZOI phenotype but not the size of the zone as small differences in ZOI size could have been affected by the amount of the commensal inoculated on the lawn. The hit isolates were reduced to a single representative isolate (referred here as unique hits).

3.2.5. The isolation, validation, and MALDI-ToF identification of 422 isolates in the screen with *S. pneumoniae* P1121 and *K. pneumoniae* 25C9.

For validation and rescreening after the preliminary screen, I focused on hits against *S. pneumoniae*. The selected isolates were re-streaked to purity (3X) on BHI (agar)

supplemented with 0.1M KNO_3 and incubated 37°C for 72 h in anaerobic jars (Anoxomat®). Single colonies were then, inoculated on BHI (broth) supplemented with 0.1 M KNO_3 in 96 well plates and grown at 37°C for 72 h in anaerobic conditions. The competition assay of *S. pneumoniae* P1121 was carried out on solid medium as described in section 3.2.2. I also included *K. pneumoniae* 25C9 in the rescreen. Isolates that generated ZOI were identified on a Bruker Biotyper Matrix Assisted Laser Desorption Ionization Time-Of-Flight (MALDI-ToF) as per manufacturer's instructions (El-Bouri et al., 2012) and 15% glycerol stocks were prepared and added to the Surette lab strain collection.

3.2.6. The screen of the 46 validated isolates with *S. pneumoniae* P1121 and reidentification by MALDI-ToF.

A set of 46 diverse isolates was selected from the validated hit isolates (in 3.2.4.), for further investigation with *S. pneumoniae* P1121, summarized in the Table 9. The 46 isolates were grown on BHI3 broth for 24 h then, the isolates were pinned (46 pin replicator). Additionally, the isolates were pinned on a duplicate set of *S. pneumoniae* P1121 lawn (BHI3). The phenotypic assays were accomplished with the same method as described in 3.2.2 with the following modification: the media used was BHI3 instead of BHI supplemented with 0.1 M KNO_3. The assays assigned to grow in anaerobe conditions were incubated at 37°C for 72 h in anaerobic jars. While the aerobic assays were incubated at 5% CO_2 at 37°C for 24 h. After incubation, the assays were visually estimated for zones of inhibition and measured and photographed (Canon PowerShot G12).

3.3. RESULTS

3.3.1 Phenotypic Interaction between Target Bacteria and Commensals

This screen aimed to assess antagonistic interaction between 4652 isolates from the AIC collection and four respiratory pathogens. During this screen I sought to monitor visible changes on the growth of the pathogen-lawn that indicated an interaction among commensals and pathogens. Every isolate that expressed a growth-change phenotype on the target's lawn was considered a hit. The hits that induced a growth-inhibition were categorized as the classic ZOI, or halo (H) (Fig. 9A). H were small, hazy zones of clearing (1 mm to 2 mm). The mutual killing (M) (Fig. 9B) represented no growth of the isolate or the lawn in an assay. It appeared as a clear oval shape, missing part from the lawn, that reaches up to 3 mm diameter. The growth-alteration-pattern was noted on the *K. pneumoniae* 25C9 assay and had two distinct growth changes on the lawn. The short-range phenotype (T) (Fig. 10A) and long-range phenotype (V) (Fig. 10B&C). The short-range phenotype appeared as an elevated, translucent, growth area of *K. pneumoniae* 25C9 lawn, around the commensal isolate, and did not exceed 8 mm. The long-range phenotype was characterized by a texture change of the growth of the *K. pneumoniae* 25C9 around the commensal isolate that extended up to 35 mm diameter on the lawn.

The hits in the screen showed different ZOI sizes, ranging from 0.1mm to 6 mm. As a reference for the ZOI and H, I used visual comparison with the positive controls on the assays. The size 0.1 mm to 0.5 mm was an arbitrary estimate by visual inspection, while every other size was measured. The H phenotype was observed mainly in *S. pneumoniae* P1121 aerobic assays.

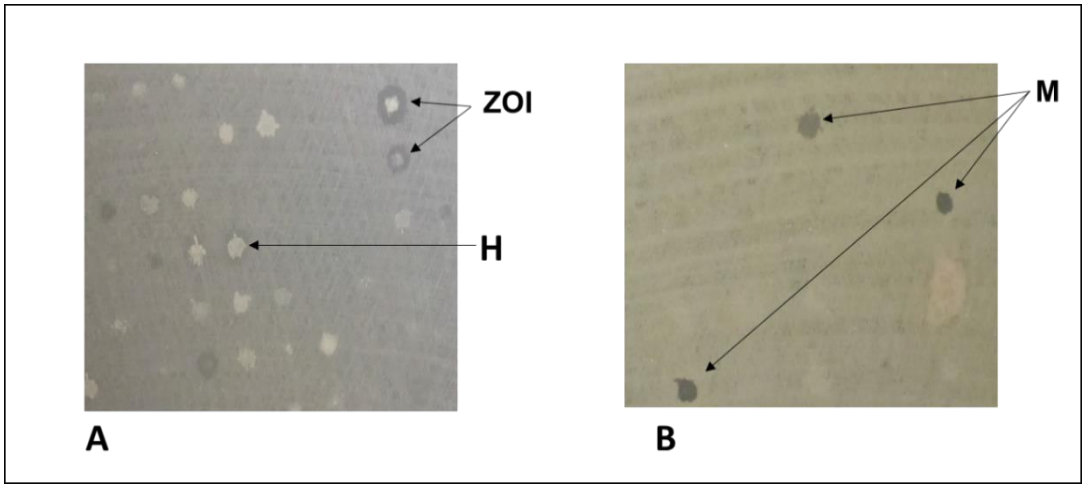

Figure 9. The growth-change phenotypes observed in the screen (ZOI, H, M).

A: *S. pneumoniae* P1121 assay: H- hazy zone of clearing, ZOI- zone of inhibition
B: *S. aureus* 18D11 assay, M- mutual killing.

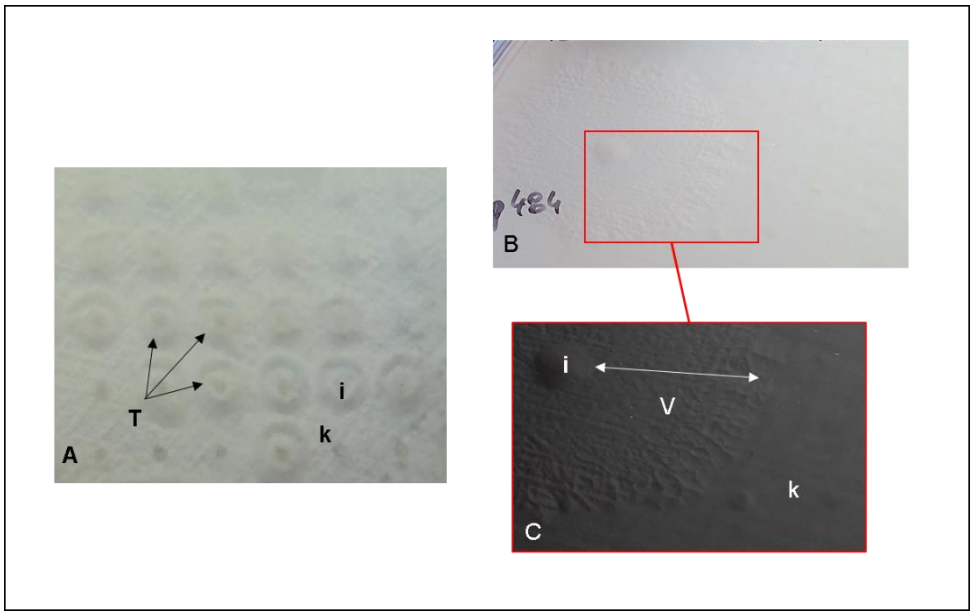

Figure 10. K. pneumoniae 25C9 growth-alteration-pattern.

A. T- short-range phenotype, k- the lawn of 25C9, i- commensal isolate
B. V- long-range phenotype
C. Modified picture B (contrast, brightness change of the enlarged image)
k-the lawn of 25C9, i- commensal isolate.

The M phenotype was frequently observed on the *S. aureus* 18D11 and *S. pneumoniae* P1121 assays, and rarely on the *P. aeruginosa PA14* Δ*pqsR* and *K. pneumoniae* 25C9 assays. T phenotype was more frequently expressed in the *K. pneumoniae* 25C9 lawn (40%) compared to V phenotype (2%).

3.3.2. A Unique Phenotype Observed with *P. aeruginosa* PA14 Δ*pqsR*

The activity of commensals on the targets was initially assessed at two time points: at 24 h and at 72 h. The aerobic assays were assessed at 24 h after incubation while anaerobic assays were evaluated at 24 h and 72 h to estimate the activity of facultative and strict anaerobes. However, early in the screening, I noticed a ZOI emerge after 48 h and 72 h incubation at room temperature [(RT) as the plates sat on the bench] on *P. aeruginosa* PA14 Δ*pqsR* anaerobic assays (Fig. 11). Consequently, an additional evaluation of activity after 48 h and 72 h at room temperature conditions was undertaken for plates initially incubated under anaerobic conditions. Also, their activity was compared to the aerobic assays. I suspect, the ZOI against the *P. aeruginosa* lawn at RT was attributed to the lethal bioactive compound, peroxynitrite. Approximately 90% of the commensals inoculated in the assay were predicted to be H_2O_2-producing *Streptococcus spp*. Likely, the H_2O_2 was generated by the *Streptococcus spp*. on the nitrate supplemented-media, when the assays were exposed to O_2. *P. aeruginosa* reduces nitrate to nitrite during the anaerobic respiration. Probably, the quantity of nitrite in the media was sufficient to react with H_2O_2 and produce peroxynitrite. Subsequently, peroxynitrite gets accumulated inside the *P. aeruginosa* PA14 Δ*pqsR* cells and kills them. However, further investigation is needed to determine whether this activity is the case. The additional observation time after the initial incubations at 37°C was applied to all the targets on this

screen. The results showed expression of inhibitory activity on all targets (data not shown here) except *S. pneumoniae* P1121. This is likely because *S. pneumoniae* P1121 growth in presence of O_2 generated H_2O_2 and limited the growth of both target and commensals.

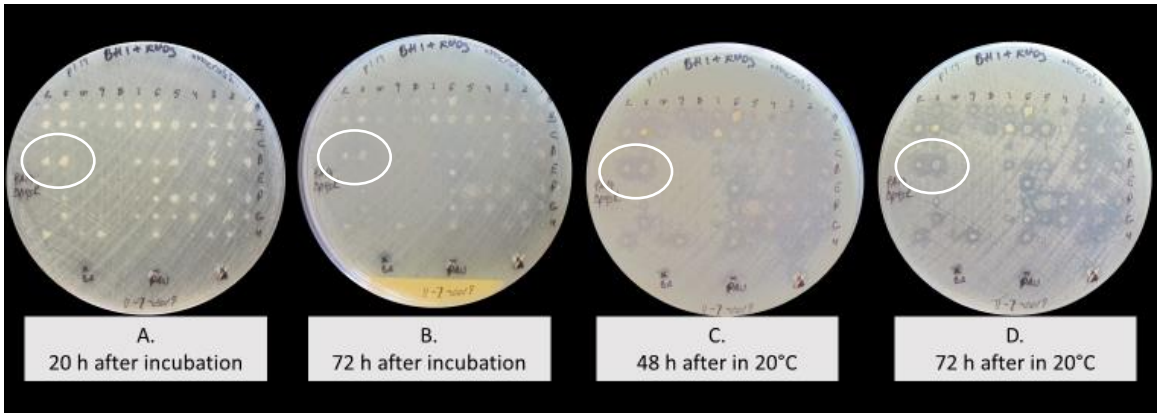

Figure 11. The growth inhibition of *P. aeruginosa* PA1 Δ*pqsR* anaerobic assay in RT.

A lawn of *P. aeruginosa PA14* Δ*pqsR* and AIC isolates from plate 19: ≈90% of the isolates are predicted to be *Streptococcus.* During anaerobic incubation (**A**&**B**); no activity is observed. The white circles indicate commensals D11 and D12 which did not show any impact on the growth of *P. aeruginosa' s* lawn. ZOI started to show 48 h and 72 h (**C**&**D**) around D11, D12 and other isolates on the plate, after the assay was stored in ambient conditions.

3.3.3. Contamination Issues in the High Throughput Screen

During the screen, an unexpected high number of contaminants skewed the results. These were predominantly *Staphylococcus* and *Enterococcus*, both known to inhibit *S. pneumoniae*. This phenomenon was suspected although, the contamination of AIC collection was confirmed during the validation and purification of a proportion of the unique hits (422 isolates). There are several sources of contamination spanning form generation protocols of the collection, to how frequent the plates were used for the projects in the lab, and the methods used to sterilize the pin replicator. As noted in the section

3.2.1.1 the AIC strain collection was not generated with rigorous protocols. Moreover, I observed that earlier plates in the collection (the most accessed plates particularly for undergraduate projects) had a higher level of contamination (2-3 organisms per well). While the sequenced based IDs for the collection represent the dominant organism in those wells, we know that even low-level contamination by fast growing strains such as, *Enterococcus* and *Staphylococcus* may outgrow a more fastidious organism.

Due to this unexpected degree of contamination in the screen, characterization of the hits in the primary screen to their original identification was considered inaccurate. For this reason, only isolates repurified in the validation screen were considered in taxonomic analysis of the distribution of hits.

3.3.4. The interaction of the Unique-hit Isolates on *S. aureus* 18D11, *S. pneumoniae* P1121, *P. aeruginosa* PA14 Δ*pqsR* and *K. pneumoniae* 25C9

The interaction of the unique isolates with the four pathogens resulted in 916 hits and among these hits 765 inhibited at least one of the targets. The hits expressed one or more growth-alteration phenotypes in at least, one target. For example, isolate P2B6 displayed the ZOI on *S. aureus* 18D11 and *S. pneumoniae* P1121 and T-phenotype on *K. pneumoniae* 25C9. The analysis below summarizes phenotypes of the screen expressed at least on one target.

The ZOI was the most frequent (≈84%) phenotype expressed by the unique hits. Among the targets *S. pneumoniae* P1121 was the most inhibited pathogen in the screen. Nearly 48% of the unique isolates expressed inhibitory activity by ZOI (≈46%), or H (≈2%) on *S. pneumoniae*. Approximately 14% of unique hits inhibited *P. aeruginosa* PA14 Δ*pqsR* by ZOI. The inhibitory activity was expressed mainly in anaerobic assays during anaerobic

incubation or at RT (section 3.2.2.). *S. aureus* 18D11 was inhibited by ≈12% of the unique hits by ZOI, and the least inhibited pathogen in this screen was *K. pneumoniae* 25C9 by ≈0.4%. The *S. aureus* 18D11 assays were characterized by less AIC isolates grown compared to the control plates. As this strain inhibited Lactobacillales (Chapter 2) and most of the AIC collection were Lactobacillales' members, *S. aureus* 18D11 most likely inhibited the growth of these isolates. The M phenotype was expressed by ≈6% of the unique hits. Lastly, the for the *Klebsiella* specific phenotypes, T phenotype was expressed by almost 40% and the V phenotype was expressed by 2% of the unique hits.

3.3.5. Secondary Screening the Unique-hit Isolates Against *S. pneumoniae* P1121

The 916 unique hit-isolates induced growth-alteration on at least one of the four targets in this screen. Although the growth-alteration phenotypes are compelling to investigate and illuminate the mechanism behind them, in this study I focused on the ZOI. The ZOI represents the classic contact independent growth inhibition of the targeted pathogen, that contributes to colonization resistance. Furthermore, I sought to investigate 422 isolates for their inhibitory activity on *S. pneumoniae* P1121. Investigating the inhibitory activity of airway commensals on *S. pneumoniae* was the priority of this project and the lab's research program, as it is the most associated pathogen, with LRT infection diseases. Additionally, I also rescreened these hits for activity on *K. pneumoniae* 25C9. This screen generated four unique isolates that were able to inhibit the growth of *Klebsiella,* moreover, these isolates also showed inhibitory activity on *S. pneumoniae*.

The 422 hits with activity against *S. pneumoniae* were recovered from the original frozen glycerol stocks (AIC collection) and were re-streaked to purity (3X) BHI agar. In

instance where two colony types were observed, both were re-assayed for phenotypic expression. I rescreened these isolates in aerobic and anaerobic conditions as described in the methods section 3.2.5. The activity was validated for 213 strains. 158 isolates were able to be identified by MALDI-ToF. Identification of all 213 by 16S sequencing is currently being carried out.

3.3.5.1. Activity Validation & Identification on the Target *K. pneumoniae* 25C9

The activity validation of the unique hits (422) resulted in four isolates that had the ability to inhibit *K. pneumoniae* 25C9. The MALDI-ToF ID and details on the condition they exhibited the activity are summarized on Table 8. The MALDI-ToF identified three strains to be *P. aeruginosa*, *E. coli, V. parvula*, and one isolate (No ID) was not able to be identified by MALDI-ToF. The inhibitory activity from the pure isolates was expressed aerobically for four isolates while, for the *E. coli*, the activity was displayed in anaerobic conditions as well. The isolate GC1314 was identified as Veillonella parvula but the activity expression was observed on aerobic conditions. Probably, this activity is expressed by a contaminant in the screen although, further investigation is needed.

Table 8. *K. pneumoniae* 25C9 validated ZOI hits.

GC #	MALDI-ToF ID	Activity expressed
-	*Pseudomonas aeruginosa*	Aer
-	No ID	Aer
GC1330	*Escherichia coli*	Aer & Ana
GC1314	*Veillonella parvula*	Aer

Note: The GC# stands for the ID of the purified strains added to the Surette lab collection.

3.3.5.2. The Analysis of the 422 Hits and the *S. pneumoniae* P1121 Inhibition; Aerobic vs Anaerobic

The rescreen for activity validation of the 422 unique hits resulted in 213 validated hits. Their activity was expressed mainly in aerobic conditions. Although, 46 isolates displayed the activity in anaerobic conditions. Eight of the 46 isolates were not identified with MALDI-ToF and hence were excluded from this summary reducing the number to 38 hits (Fig. 12). Fourteen of the remaining isolates inhibited *S. pneumoniae* in both conditions and 24 isolates expressed the activity only in anaerobic conditions. The 24 hits that expressed the activity only anaerobically comprise ≈11% of the total number of hits (213) that would not have been detected if the screen was done aerobically. The isolates that show the activity anaerobically are 50% *S. aureus* and *S. epidermidis*; 40% *Streptococcus* isolates, and the remainder were: *Cutibacterium acnes, Enterococcus faecalis, Escherichia coli* and *Gemella sanguinis*. The diversity of streptococci that inhibit *S. pneumoniae* P1121 is high among the anaerobic hits and five out of six *Streptococcus oralis* expressed the inhibitory activity in anaerobic conditions.

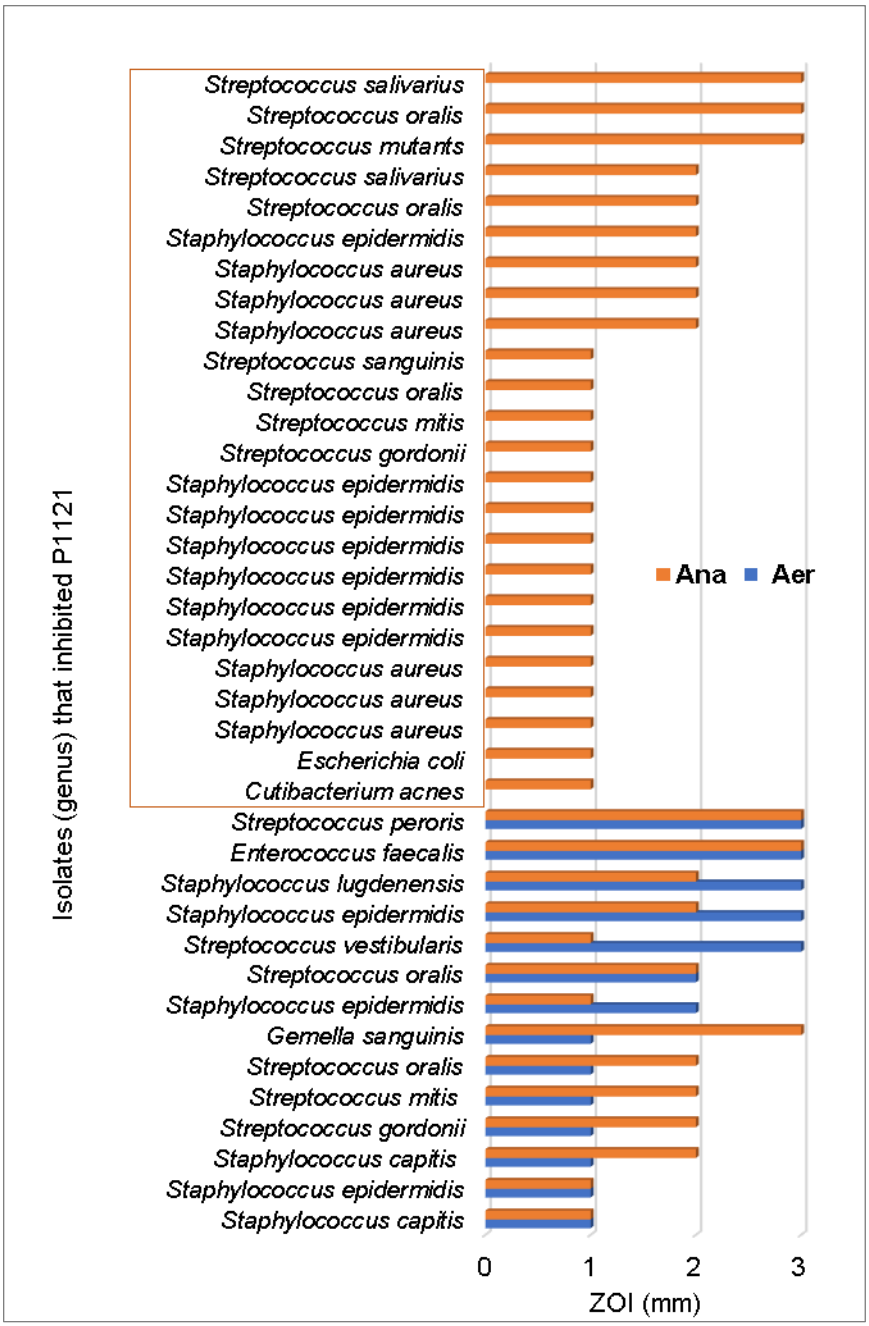

Figure 12. Inhibition of P1121 aerobic & anaerobic by validated hits.

The validated activity of the 38 unique-hit isolates that inhibited *S. pneumoniae* P1121 in anaerobic conditions. The inhibition occurred mostly anaerobic growth (orange colour). 14 isolates express the activity in both conditions (blue and orange) and 24 isolates only in anaerobic conditions. The ZOI size is indicated in the figure by the height of the bars (e.g. *E. faecalis* inhibits *S. pneumoniae* P1121 aerobically and anaerobically with a ZOI=3 mm).

3.3.7. Screening of 46 taxonomically diverse hits with *S. pneumoniae* P1121, aerobic and anaerobic

A subset of 46 hits (Table 9) out of 213 isolates was further characterized Three isolates from the GC collection (highlighted in gray in Table 9) were added to the list as strains of these species were identified as hits in the screen . The 46 isolates are a mix of Gram-positive, Gram-negative, strict anaerobes, and facultative anaerob bacteria that were expected to grow well in aerobic and anaerobic conditions.

The results showed that 17 isolates inhibited *S. pneumoniae* P1121 and the expression of inhibitory activity prevailed in anaerobic conditions. The identity of the hits from this experiment was reconfirmed by MALDI-ToF for most of them. Six isolates inhibited the growth of *S. pneumoniae* P1121 in both conditions and 11 isolates inhibited in anaerobic conditions. A summary of the isolates that inhibited *S. pneumoniae* P1121 and the condition when the inhibition was exhibited is presented in Fig. 13. *Streptococcus* species were abundant among the hits. *S. pneumoniae, S. intermedius,* and *S. agalacticae* inhibit the *S. pneumoniae* P1121 in aerobic and anaerobic conditions. The *Streptococcus* strains that express the inhibitory activity only in anaerobic condition are *S. oralis, S. mitis, S. gordonii,* and *S. mutans.* Five isolates were included in the screen without positive identification. Two of them, GC1546 and GC1657, showed activity on *S. pneumoniae P1121*, and GC1546 was subsequently identified by MALDI ToF as *S. epidermidis*. *Klebsiella* strains also exhibited the activity predominately in anaerobic conditions. One *K. pneumoniae* strain inhibited the targets anaerobically while two *K. aerogenes* strains behaved differently. One inhibited the targets in both conditions and the other one only anaerobically. *Veillonella* strains also inhibited *S. pneumoniae* P1121. The *Veillonella parvula*, inhibited the target only in anaerobic conditions while another isolate, previously predicted by MALDI ToF to be *Veillonella atypica* expressed the activity in both conditions.

As *Veillonella* are strict anaerobes, I recovered the isolate from the assay plate and reidentified it by MALDI ToF. The result showed that the isolate was *S. epidermidis,* highlighting the ongoing challenge with contamination in these experiments.

Table 9. The isolates screened with *S. pneumoniae* P1121 and their activity in aerobic and anaerobic conditions.

GC# [1]	*MALDI-ToF ID of the isolate*	Growth Inhibition	
		aer	ana
GC1599	*Actinomyces graevenitzii*	-	-
GC1551	*Actinomyces odontolyticus*	-	-
GC1600	*Actinomyces oris*	-	-
GC1281	*Cutibacterium acnes*	-	-
GC1330	*Escherichia coli*	-	-
GC1403	*Gemella sanguinis*	-	Y
GC60	*Klebsiella aerogenes*	Y	Y
GC1532	*Klebsiella aerogenes*	-	Y
GC1611	*Klebsiella pneumoniae*	-	Y
GC292	*Kocuria rhizophila*	-	-
GC1095	*Kocuria salsicia*	-	-
GC1494	*Neisseria subflava*	-	Y
GC1648	*Neisseria subflava*	-	-
GC1315	*No ID*	-	-
GC1324	*No ID*	-	-
GC1546	*No ID*	Y	Y
GC1560	*No ID*	-	-
GC1657	*No ID*	-	Y
GC1483	*Rothia aeria*	-	-
GC1555	*Rothia dentocariosa*	-	-
GC1331	*Streptococcus agalactiae*	Y	Y
GC1426	*Streptococcus constellatus*	-	-
GC1275	*Streptococcus gordonii*	-	Y
GC1285	*Streptococcus gordonii*	-	-
GC1326	*Streptococcus intermedius*	Y	Y
GC1283	*Streptococcus mitis*	-	Y
GC1402	*Streptococcus mitis*	-	Y
GC1478	*Streptococcus mitis*	-	-
GC1490	*Streptococcus mitis*	-	-
GC1266	*Streptococcus mutans*	-	-
GC1286	*Streptococcus mutans*	-	Y

GC1298	*Streptococcus mutans*	-	-
GC1129	*Streptococcus oralis*	-	Y
GC1404	*Streptococcus oralis*	-	-
GC1487	*Streptococcus oralis*	-	-
GC1605	*Streptococcus oralis*	-	-
GC1561	*Streptococcus parasanguinis*	-	-
GC1634	*Streptococcus parasanguinis*	-	-
GC1213	*Streptococcus pneumoniae*	-	-
GC1216	*Streptococcus pneumoniae*	Y	Y
GC1292	*Streptococcus sanguinis*	-	-
GC1294	*Streptococcus vestibularis*	-	-
GC1549	*Veillonella dispar*	-	-
GC1493	*Veillonella atypica*	Y	Y
GC1314	*Veillonella parvula*	-	Y
GC1430	*Yokenella regensburgei/Klebsiella*	-	-

[1]The isolates highlighted in gray are from a separate study, the others are from this study.

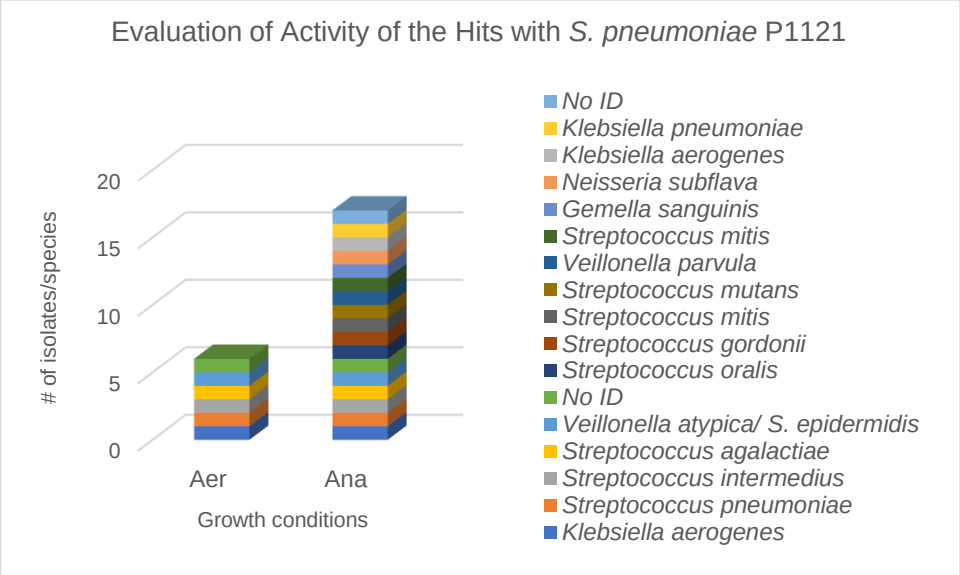

Figure 13. Reassessment of activity of the hits with *S. pneumoniae* P1121in anaerobic and aerobic conditions.

The screen with *S. pneumoniae* showed that the activity for 6 isolates is expressed in both conditions while for 11 isolates is expressed only anaerobically. Most of the hits are *Streptococcus spp.*

3.4. DISCUSSION

An important role of the human microbiome is colonization resistance applied by commensals on pathogens, one of the mechanisms is direct antimicrobial activity. In this study, I investigated the inhibitory activity of a large collection of primarily respiratory tract isolates, against four airway pathogens *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. Notably, I included anaerobic conditions for this screen with the hypothesis that this would identify more activities than screening only under aerobic conditions. These new activities could come from obligate anaerobes or facultative anaerobes that express the activity under anaerobic conditions. While the frequency of hits (916 of 4652 strains tested) likely overestimates the number of strains that can interact with the pathogens due to contamination issue that arose during the screen (discussed in detail in section 3.3.3), two aspects of my original hypothesis were validated. First, the anaerobic screening increased the number of the isolates that inhibited the respiratory pathogens and second, the diversity of bacterial species that inhibited the growth of *S. pneumoniae* P1121 were not necessarily closely related taxonomically and included hits from three main phyla: Proteobacteria, Firmicutes and Actinobacteria and these are members of the URT (Dethlefsen et al., 2007).

Results from the initial screen in this study showed that ≈16% of the bacterial isolates that were included in the screen (n=4652) inhibited the growth of at least one of the LRT pathogens. The results of the screen with airway isolates and pathogens in aerobic conditions, done in the Surette lab by Dr. Bernier, showed ≈5% hits (S. Bernier, personal communication). The difference between the number of the hits in these two

screens may be attributed partially to the addition of the anaerobic screening. These results are also likely to be skewed by the presence of contaminants in the screen. The most inhibited (≈10%) pathogen was *S. pneumoniae* P1121 and the least inhibited one, was *K. pneumoniae* 25C9 (≈0.1% of the total isolates included in the screen). This reflects the ability of the airway isolates, to inhibit the growth of the respiratory tract pathogens.

Competitive bacterial interactions between URT microbiome members are known to inhibit the growth of *S. pneumoniae* and possibly other LRT pathogens (Laufer et al., 2011). The rescreen of 46 isolates (validated *S. pneumoniae* hits) made it obvious that isolates from quite different taxonomic groups had activity against. These isolates were *Staphylococcus Streptococcus* and other genera (*Cutibacterium*, *Pseudomonas, Klebsiella, Bacillus, Veillonella, Gemella, Actinomyces* and *Escherichia*). *Cutibacterium*, previously classified as *Propionibacterium* (Corvec, 2018). Its' activity might be attributed to the production of propionic acid in anaerobic growth conditions (Brook & Frazier, 1991). Meanwhile, the pathogenic *Pseudomonas,* is well equipped with stress inducing molecules and antibacterial products (Suh et al., 1999) and it is expected to inhibit the growth of *S. pneumoniae*. However, the mechanisms utilized on the antagonistic interaction between these isolates and *S. pneumoniae* remain underexplored. The results presented here expands the list of commensals organisms that can inhibit this common pathogen. A diverse group of streptococci were also identified as hits against *S. pneumoniae*. While this was expected, and likely all mediated through bacteriocins, the diversity of these molecules within the streptococci has likely been under-estimated particularly since many of these were active only under anaerobic conditions. Future work will involve bioinformatic (whole genome sequencing and comparative genomic) and biochemical (activity guided purification) to further characterize the activities identified in this screen.

Of particular interest in this screen, is the inhibitory activity observed on *P. aeruginosa* PA14 ***ΔpqsR*** by some the commensals. Scoffield et al., have shown that streptococci can inhibit *P. aeruginosa* in aerobic conditions, by intracellular accumulation of peroxynitrite. The compound is generated when H_2O_2 and nitrite react. The H_2O_2 was produced by *Streptococcus* while nitrite was produced from reduction of nitrate by *Pseudomonas* cells (Scoffield & Wu, 2015). However, in this screen some inhibition was observed in anaerobic assays. I believe the O_2 was introduced in the nitrite supplemented media, when the assays were removed from the Anoxomat jar for activity evaluation consequently, the streptococci used O_2 to produce H_2O_2. This activity would have been greatly enhanced when the anaerobe plates were left out on the bench after the 72 h of anaerobic growth. This phenomenon highlights the complexity of microbe-microbe interactions that can occur even in a simple screen.

Altered growth phenotypes were frequently observed for *K. pneumoniae* induced by ≈8% of the AIC strains. I hypothesize that this reflects changes in expression of different stress response pathways. These may result in altered growth of the *Klebsiella* (Cortés et al., 2002) induced by the activity of the commensals (Tunçer & Karaçam, 2020). I speculate that the phenotype arises due to changes in the amount of extracellular polysaccharides production. This notion requires further investigation.

The mutual killing phenotype occurs when both the commensal and the target strain are killed resulting a clear zone where the commensal is inoculated. This has been observed in our lab in similar screens previously (S. Bernier, personal communication) and is reproducible. This was observed more often for in *S. aureus* 18D11, *S. pneumoniae* P1121 and rarely on *P. aeruginosa* PA14 ***ΔpqsR*** and *K. pneumoniae* 25C9 assays. The mechanisms underlying this phenomenon are not known but could contribute to

colonization resistance. I speculate that contact dependent killing may be one aspect of this interaction.

S. aureus 18D11 was inhibited by 2% of the AIC isolates in the screen. *S. aureus* 18D11 was shown to have inhibitory activity towards Lactobacillales (Chapter 2) and the AIC strains were predominantly in the order of Lactobacillales. Therefore, this pathogen may suppress the growth of many of the commensal strains screened here and in hindsight using another *S. aureus* strain as the target may have identified more hits. I observed less growth of the isolates in the screen that *Pseudomonas* strains inhibited *Staphylococcus* (as well as other of our target pathogens) and this has been observed in cystic fibrosis isolates (Machan et al., 1991). *Pseudomonas* produce a wide range of toxins and anti-microbial activities thus inhibitory activity was expected (Jaffar-Bandjee et al., 1995). In fact, I used a *P. aeruginosa* Pa14 *ΔpqsR* target strain that was attenuated in production of many of these activities to increase the efficacy of our screen.

As non-selective media was used to grow the strains for the screen this is an obvious source of contamination. A second related source of contaminants is from the 96 well plates used to store the AIC. Careful technique will minimize cross contamination in these plates, but it does occur. A semi-deep well plate only half filled with the stock would be less prone to cross well contamination than the standard 96 well plates filled >3/4 full. In addition, it was also observed that contamination occurred when inoculating the liquid cultures from frozen stocks (or from colonies) or from liquid cultures to the assay plates which suggest contaminants on the pin replicators. While the methods employed should have sterilized these devices between samples, this needs to be further investigated. Despite the issues of contamination, a diverse collection of organisms enriched in activity

under anaerobic conditions was identified and has provided a large collection of strains to further characterize.

The results of the screen reported in this chapter demonstrate that diverse bacteria within the respiratory tract have antimicrobial activity than can inhibit common respiratory pathogens and contribute to colonization resistance. While the screen was successful in this regard, contamination issues in the high throughput screen were problematic; however, modifications to the experiments could reduce this issue in future studies. Despite this issue, I have identified a large number of isolates from diverse taxa that inhibit the primary pathogen of interest, *S pneumoniae*, and this will be the foundation for future work.

CHAPTER 4.

4.1. CONCLUSIONS

The further characterization of the antibacterial activity of *S. aureus* 18D11 on *E. faecium* 22H6 showed that the activity was valid in anaerobic growth conditions and the activity was not limited to *Enterococcus* genera*. S. aureus* 18D11inhibited *Lactobacillus spp.*, and *Streptococcus spp.* but did not show any inhibitory activity on other *S. aureus* strains, limiting the expression of activity within the order Lactobacillales. The WGS of *S. aureus* 18D11and antiSMASH analysis predicted that the activity of strain18D11 could be due to 2 putative bacteriocins. The predicted bioactive peptides were uncommon in *S. aureus* strains and were not present in the WGS of the negative control strain (*S.aureus* 14F12) used for the screens of *S.aureus* 18D11 and Lactobacillales. The challenge on this part of the study was the isolation of the bacteriocins in liquid growth. Although, different methods were implemented to increase the production of the bacteriocins (alginate beads, isolation from the cells, and CFS) the results were all negative. These implies that the bacteriocins were produced in small quantities in liquid and it will require additional approaches to isolate them from the CFS of *S. aureus* 18D11.

The activity between commensals and pathogens in this study was observed to occur in both anaerobic and aerobic conditions and *S. pneumoniae* P1121 was the most inhibited target in the screen, by 48% of the dereplicated hits. For some isolates, the activity of interest was observed only in anaerobic conditions even though they were facultative anaerobes and could have inhibited *S. pneumoniae* in both conditions. The taxonomic composition of the hits was highly diverse with isolates form three phyla: Proteobacteria, Firmicutes and Actinobacteria. On the genus level these hits were *Gemella, Veillonella*, *Klebsiella*, *Neisseria*, *Propionibacterium, Enterococcus, Escherichia,*

Staphylococcus and *Streptococcus.* The most abundant genera were *Streptococcus* and *Staphylococcus.* This findings support what is known; that both *Staphylococcus* and *Streptococcus* possess antagonistic activity on *S. pneumoniae* and adds to the current knowledge; that taxonomically diverse members of the human airways inhibit the growth of *S. pneumoniae in vitro*. Furthermore, screening the respiratory tract microbiome in both conditions will give a more accurate estimation on, how much the airway microbiome contributes to colonization resistance of the pathogens.

The screen with respiratory tract pathogens generated hits that expressed the inhibitory activity in anaerobic conditions. These were facultative anaerobes that exclusively inhibit anaerobically although, they grew well in both conditions. Form the method used in this study the anaerobic assays were exposed to different levels of O_2/CO_2 that fairly resembles the growth conditions in the respiratory tract; where bacterial residents of the airways manage to survive the fluctuating partial pressure of O_2/CO_2. The rate of growth-inhibition on *P. aeruginosa* assays was high, and not expected. Specifically, the inhibition of *P. aeruginosa* on the anaerobic assays after they were exposed to O_2 for 48 h and 72h. The acknowledgements of these inhibitory activities displaying after the growth of the pathogens, might be useful to investigations on the behaviour of facultative anaerobes when exposed to O_2. The anaerobic metabolism may induce expression of genes that encode for antimicrobial activity that include new mechanisms or new bioactive molecules, which are still unknown.

The other phenotypes observed in the screen were also intriguing. The mutual killing (M) phenotype indicated that both the pathogen and the commensal were inhibited on the assays. And this was observed mainly on Gram-positive pathogens (*S. pneumoniae and S. aureus*) and less frequently on the Gram-negatives (*P. aeruginosa and K.*

pneumoniae). This phenomenon has been observed previously in our laboratory and further investigations of the mechanisms implemented by both bacterial isolates is warranted. The interesting phenotypes observed on the lawn of *K. pneumoniae* were growth-alterations which indicated for an interaction between *Klebsiella* and the isolates of the AIC collection and this was very frequent in this screen. Further investigation is needed to understand if this growth-change affects the susceptibility of *Klebsiella* to other antimicrobial activities or if it affects the colonization of the bacterium in the host.

The challenges on this part of the study were mostly related to the screen of the AIC collection with the four respiratory pathogens. Firstly, the density of the lawn in the assays between the targets was different. For *Streptococcus pneumoniae* P1121 growth in liquid was limited to a fixed, low, OD which sometimes resulted in a thin lawn that subsequently convoluted the interpretation of the positive results. A solution for this, would be supplementation of the liquid media with catalase. On the other hand, *K. pneumoniae* 25C9 and *P. aeruginosa* PA14 *ΔpqsR* generated thick lawns on aerobic conditions that hindered the identification of weak inhibitory activity or very small ZOIs. Taking representative photographs of the growth alteration phenotypes on *K. pneumoniae* 25C9, proved quite challenging even though they were easily spotted by naked eye. Secondly, the limited time of exposure of the anaerobic assays in room air during preparation of the assays. The approach was validated in lot of experiments but may have limited the activity of a few anaerobes. The logistics of setting up the screen in the anaerobe chamber made this impractical. Another challenge was the dereplication of the hits. Beside the fact that I ended with an overwhelming number of hits, the dereplication was done based on the predicted ID and not the actual ID of the growing colony and was confounded by contamination in many instances. I might have excluded hits that were important and could

have added valuable information to this study and I might have also selected to keep isolates that were replicates. In my opinion, the best way to do a similar screen is to isolate and identify the hits and then proceed with the dereplication. Also, the anaerobic bacterial interaction could be better assessed if were done in the anaerobic chamber by using recyclable or autoclavable pin replicator. Another important section of this screen that was left out of the attention is the collection of hits that inhibited the *S. aureus* and *P. aeruginosa*. These isolates have been identified for future experiments.

4.2. FUTURE DIRECTIONS

The future directions for the first aim of this study include the cloning and expression of the two predicted bacteriocins produced by *S. aureus* 18D11 and further investigation of their activity. Also, evaluation of the activity with Lactobacillales to identify if the spectrum of inhibition is achieved by the activity one bacteriocin or as a combination of both antimicrobial peptides.

For the second aim, the future directions include screening of the collection of 17 isolates (that inhibited the *S. pneumoniae* P1121 on section 3.3.7.) with several S. pneumoniae strains and evaluate if the activity is present in the CFS. Followed by, bioinformatic approach starting with whole genome sequencing and activity guided purification of the bioactive molecule. Another area to consider, might be to assess how the immune responds to the activity of these strains or the bioactive molecules they use to inhibit the growth of *S. pneumoniae*. Antimicrobial peptides produced by humans also have immunomodulatory activity and it is not unreasonable to predict that some commensals of the microbiota may have evolved similar dual-function bioactives. These

may be particularly beneficial in the context of restoring colonization resistance in vulnerable populations.

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