Short-chain fatty acids modulate bacterial growth and airway epithelial cell inflammatory responses

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Laboratory Medicine & Pathobiology
University of Toronto

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Abstract

Short-chain fatty acids (SCFAs) are anaerobic bacterial metabolites. Cystic fibrosis (CF) lung disease is a condition caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene and is characterized by persistent lung inflammation and bacterial colonization. We measured the concentrations of SCFAs in sputum of patients with CF and tested the effect of these compounds on bacterial growth. Furthermore we found that SCFAs can influence the inflammatory protein expression and cytokine release in airway epithelial cells. SCFAs differentially alter cytokine release in CF bronchial epithelial cells (CFBE) compared to CFBE expressing wild-type CFTR. We also studied the effect of SCFAs in an acute lung injury model in BALB/cJ mice and found that intratracheally administered SCFAs can affect the inflammatory environment of the airways in vivo. We conclude that SCFAs may be important in the airways and that further investigation is warranted to understand their effects on inflammation and infection.
Acknowledgments

I wish to acknowledge the support and guidance of my supervisors, Dr. Nades Palaniyar and Dr. Hartmut Grasemann. I was privileged to be given the freedom to pursue a project outside of the major foci of their research, and I felt fortunate to be able to contribute to their ongoing projects.

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# Table of Contents

Acknowledgments ........................................................................................................ iii

Table of Contents ........................................................................................................ iv

List of Tables ................................................................................................................ vii

List of Figures .............................................................................................................. viii

List of Appendices ....................................................................................................... x

List of Abbreviations .................................................................................................. xi

1.0 Introduction ............................................................................................................. 1

1.1 Cystic Fibrosis ....................................................................................................... 1

1.2 Lung infections ....................................................................................................... 2

1.3 Anaerobic lung infections ..................................................................................... 3

1.4 Anaerobic infections in CF/Microbiome ............................................................... 3

1.5 Bacterial anaerobic metabolism .......................................................................... 4

1.6 Short chain fatty acids ........................................................................................ 6

1.6.1 Production and localization ............................................................................ 6

1.6.2 SCFAs in infections ......................................................................................... 10

1.7 Receptors for SCFAs: Ligands and expression .................................................. 12

1.8 Effects of SCFAs on host cells ............................................................................ 14

1.9 SCFAs and electrolyte transport ......................................................................... 19

1.10 SCFAs and bacterial growth .............................................................................. 19

1.11 Rationale ............................................................................................................. 20

1.12 Hypothesis .......................................................................................................... 21

1.13 Aims ..................................................................................................................... 21
2.0 Methods ................................................................................................................. 22
  2.1 Sputum samples .................................................................................................... 22
  2.2 SCFA Gas chromatography ............................................................................... 22
  2.3 Bacterial culture and strains ............................................................................... 22
  2.4 Bacterial growth assay ....................................................................................... 23
  2.5 Bacterial plating .................................................................................................. 23
  2.6 Cell lines and culture .......................................................................................... 23
  2.7 Cytomix stimulation ............................................................................................ 24
  2.8 Western blot ......................................................................................................... 25
  2.9 Cytokine analysis ................................................................................................ 25
  2.10 IL-8 enzyme-linked immunosorbent assay (ELISA) ........................................... 25
  2.11 Proliferation and viability assays ....................................................................... 26
  2.12 Lipopolysaccharide instillation ......................................................................... 26
  2.13 Histology ............................................................................................................ 27
  2.14 Statistical procedures ......................................................................................... 27
3.0 Results ................................................................................................................... 28
  3.1 SCFAs in sputum ................................................................................................ 28
  3.2 SCFA effects on bacteria .................................................................................... 32
  3.3 SCFA effect on A549 airway epithelial cells ....................................................... 39
    3.3.1 Proliferation and Viability .......................................................................... 39
    3.3.2 SCFA-mediated regulation of iNOS protein expression .............................. 42
    3.3.3 SCFA-mediated regulation of cytokine secretion ....................................... 49
  3.4 SCFA effects on CFBE ......................................................................................... 56
    3.4.1 SCFAs do not influence iNOS expression in CF epithelial cells ............... 56
    3.4.2 SCFA-mediated regulation of cytokine secretion in CF cells ..................... 56
<table>
<thead>
<tr>
<th>Section</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5</td>
</tr>
<tr>
<td>Effect of SCFAs in a model of acute lung injury</td>
</tr>
<tr>
<td>4.0</td>
</tr>
<tr>
<td>Discussion</td>
</tr>
<tr>
<td>5.0</td>
</tr>
<tr>
<td>Conclusions and Future Directions</td>
</tr>
<tr>
<td>5.1</td>
</tr>
<tr>
<td>Conclusions</td>
</tr>
<tr>
<td>5.2</td>
</tr>
<tr>
<td>Future Directions</td>
</tr>
<tr>
<td>6.0</td>
</tr>
<tr>
<td>References</td>
</tr>
<tr>
<td>7.0</td>
</tr>
<tr>
<td>Appendix</td>
</tr>
</tbody>
</table>
List of Tables

Table 1. Fatty acid receptors and their defining characteristics. ............................................14
Table 2. Statistical analysis of curves in Figure 10. .................................................................34
Table 3. Statistical analysis of curves in Figure 11. .................................................................35
Table 4. Statistical analysis of curves in Figure 12. .................................................................36
Table 5. Statistical analysis of curves in Figure 13 .................................................................38
Table 6. iNOS/ARG1 ratio in A549 cells stimulated with cytomix.................................48
Table 7. Cytokine concentrations of A549 culture supernatants incubated with SCFAs and stimulated with cytomix........................................................50
Table 8. Cytokine concentrations of CFBE41o-/F508del and CFBE41o-/corrCFTR culture supernatants incubated with SCFAs and stimulated with cytomix..................57
List of Figures

Figure 1. The denitrification cycle of *Pseudomonas stutzeri*. ........................................... 5
Figure 2. The TCA cycle and the glyoxylate shunt ................................................................. 7
Figure 3. The Wood-Ljungdahl pathway of acetogenesis ......................................................... 9
Figure 4. Chemical structure of SCFAs .................................................................................. 11
Figure 5. Proposed FFAR2 signaling pathways ....................................................................... 18
Figure 6. Total SCFA concentrations in sputum ..................................................................... 29
Figure 7. Individual SCFA concentrations in cystic fibrosis patient sputum ......................... 30
Figure 8. Correlations between SCFAs and markers of inflammation ................................... 31
Figure 9. Growth of *Pseudomonas aeruginosa* in TSB at pH 7.0 with acetate .................... 33
Figure 10. Effect of acetate concentration and pH on the growth of PAO1 ....................... 34
Figure 11. Effect of propionate concentration and pH on the growth of PAO1 .................... 35
Figure 12. Effect of butyrate concentration and pH on the growth of PAO1 ....................... 36
Figure 13. Effect of lactate concentration and pH on the growth of PAO1 ....................... 38
Figure 14. Effect of SCFAs on proliferation of A549 airway epithelial cells ...................... 40
Figure 15. Effect of SCFAs on viability of A549 airway epithelial cells ............................... 41
Figure 16. Butyrate increases or decreases iNOS expression compared to cytomix alone .......................................................... 43
Figure 17. SCFAs increase iNOS expression compared to cytomix stimulation alone in A549 cells ................................................................................................................. 44
Figure 18. Propionate increases or decreases iNOS in cytomix stimulated A549 cells. 45
Figure 19. iNOS protein expression is dependent on the concentration of L-arginine. ..46
Figure 20. Cytomix stimulation induces iNOS in a p38 MAPK and MEK1/2 dependent manner ............................................................................................................................. 48
Figure 21. Relative GM-CSF secretion profile in stimulated A549 cells. ......................... 51
Figure 22. Relative IL-1α secretion profile in stimulated A549 cells. ............................... 52
Figure 23. Relative IL-1β secretion profile in stimulated A549 cells. ............................... 53
Figure 24. Relative IL-6 secretion profile in stimulated A549 cells. ............................... 54
Figure 25. Relative MCP-1 secretion profile in stimulated A549 cells. ............................ 55
Figure 26. Relative G-CSF secretion profile in CFBE41o-/F508del and CFBE41o-/corrCFTR cell lines in response to SCFA incubation and stimulation with cytomix for 24 hours. .................................................................58
Figure 27. Relative GM-CSF secretion profile in CFBE41o-/F508del and CFBE41o-/corrCFTR cell lines in response to SCFA incubation and stimulation with cytomix for 24 hours. .................................................................59
Figure 28. Relative IL-1α secretion profile in CFBE41o-/F508del and CFBE41o-/corrCFTR cell lines in response to SCFA incubation and stimulation with cytomix for 24 hours. .................................................................61
Figure 29. Relative IL-1β secretion profile in CFBE41o-/F508del and CFBE41o-/corrCFTR cell lines in response to SCFA incubation and stimulation with cytomix for 24 hours. .................................................................62
Figure 30. Relative IL-6 secretion profile in CFBE41o-/F508del and CFBE41o-/corrCFTR cell lines in response to SCFA incubation and stimulation with cytomix for 24 hours. .................................................................63
Figure 31. Relative TNF-α secretion profile in CFBE41o-/F508del and CFBE41o-/corrCFTR cell lines in response to SCFA incubation and stimulation with cytomix for 24 hours. .................................................................64
Figure 32. Relative MCP-1 secretion profile in CFBE41o-/F508del and CFBE41o-/corrCFTR cell lines in response to SCFA incubation and stimulation with cytomix for 24 hours. .................................................................65
Figure 33. Relative IP10 secretion profile in CFBE41o-/F508del and CFBE41o-/corrCFTR cell lines in response to SCFA incubation and stimulation with cytomix for 24 hours. .................................................................66
Figure 34. Relative IL-8 secretion profile in CFBE41o-/F508del and CFBE41o-/corrCFTR cell lines in response to SCFA incubation and stimulation with cytomix for 24 hours. .................................................................68
Figure 35. BAL fluid cell infiltration from LPS-instilled mice given PBS or APB buffer intratracheally. .........................................................................................................................70
Figure 36. APB buffer reduces CitH3 in BAL of LPS-instilled mice. ........................................71
Figure 37. Proposed model of SCFA production in CF airways. ..........................................81
List of Appendices

Appendix A. Statistical analysis of SCFA effect on bacterial growth ........................................100
Appendix B. *Pseudomonas*-laden beads project .............................................................104
Appendix C. Bead preparation protocol ........................................................................107
List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABTS</td>
<td>2,2’-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)</td>
</tr>
<tr>
<td>APB</td>
<td>25 mM acetate, 5 mM propionate, 0.5 mM butyrate in PBS buffer</td>
</tr>
<tr>
<td>ARG1</td>
<td>arginase 1</td>
</tr>
<tr>
<td>BAL</td>
<td>bronchoalveolar lavage</td>
</tr>
<tr>
<td>C5a</td>
<td>complement factor 5a</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CAP</td>
<td>ventilator-associated pneumonia</td>
</tr>
<tr>
<td>CF</td>
<td>cystic fibrosis</td>
</tr>
<tr>
<td>CFBE</td>
<td>cystic fibrosis bronchial epithelial (cell)</td>
</tr>
<tr>
<td>CFTR</td>
<td>cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>Cit H3</td>
<td>citrullinated histone H3</td>
</tr>
<tr>
<td>corrCFTR</td>
<td>stable transfection of wild-type CFTR gene</td>
</tr>
<tr>
<td>CXCR2</td>
<td>(CXC-motif) chemokine receptor 2</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified essential medium</td>
</tr>
<tr>
<td>EC50</td>
<td>half (50%) maximal effective concentration</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ENaC</td>
<td>epithelial sodium channel</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>extracellular signal-related kinase 1/2</td>
</tr>
<tr>
<td>F508del</td>
<td>phenylalanine deletion at amino acid 508 of CFTR protein</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FFAR</td>
<td>free fatty acid receptor</td>
</tr>
<tr>
<td>FPR1</td>
<td>formyl peptide receptor 1</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>G-CSF</td>
<td>granulocyte colony stimulating factor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte/macrophage colony stimulating factor</td>
</tr>
<tr>
<td>GPR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
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</tr>
<tr>
<td>GTPase</td>
<td>guanosine triphosphate hydrolase</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's buffered salt solution</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon γ</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IP-10</td>
<td>interferon γ-induced protein of 10 kilodaltons</td>
</tr>
<tr>
<td>IRF1</td>
<td>interferon regulatory factor 1</td>
</tr>
<tr>
<td>JAK</td>
<td>janus kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>c-jun N-terminal kinase</td>
</tr>
<tr>
<td>LCFA</td>
<td>long-chain fatty acids</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCFA</td>
<td>medium-chain fatty acids</td>
</tr>
<tr>
<td>MCP-1</td>
<td>monocyte chemotactic protein 1</td>
</tr>
<tr>
<td>MEK1/2</td>
<td>mitogen-activated protein kinase kinase 1/2 (also p42/p44)</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>macrophage inflammatory protein 1α</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>macrophage inflammatory protein 1β</td>
</tr>
<tr>
<td>NADH</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>Nedd4-2</td>
<td>neural precursor cell expressed, developmentally down-regulated 4-2</td>
</tr>
<tr>
<td>NET</td>
<td>neutrophil extracellular trap</td>
</tr>
<tr>
<td>NFκB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>phosphate buffered saline with 0.05% Tween-20</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PIAS1</td>
<td>protein inhibitor of activated STAT1</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKB</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>RSV</td>
<td>respiratory syncytial virus</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SCFA</td>
<td>short-chain fatty acid</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>STAT1</td>
<td>signal transducer and activator of transcription 1</td>
</tr>
<tr>
<td>TCA</td>
<td>tricarboxylic acid</td>
</tr>
<tr>
<td>TLR2</td>
<td>Toll-like receptor 2</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor α</td>
</tr>
<tr>
<td>TSB</td>
<td>trypticase soy broth</td>
</tr>
<tr>
<td>VAP</td>
<td>community-acquired pneumonia</td>
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1.0 Introduction

1.1 Cystic Fibrosis

Cystic fibrosis (CF) is a genetic disorder caused by a defect in the cystic fibrosis transmembrane regulator (CFTR) gene. The CFTR gene codes for a cyclic adenosine monophosphate (cAMP)-dependent chloride ion channel [1]. CFTR is expressed throughout the body in tissues with a mucosal lining: in airways, pancreatic ducts, intestinal epithelium, colon, and sweat glands. In these organs, CFTR dysfunction may result in a number of phenotypes such as elevated sweat chloride, reduced lung function, meconium ileus, and pancreatic insufficiency. There are a large number of mutations of CFTR which result in CF, with the predominant mutation being a deletion of phenylalanine at amino acid 508 (F508del) [2].

Broadly, as CFTR-mediated chloride efflux from airway epithelial cells is impaired, so too is sodium absorption [3]. The result is increased water retention to maintain osmotic balance, leading to problems with airway clearance and an accumulation of secretions. CFTR impairment leads to increased sodium absorption through increased expression of epithelial sodium channel (ENaC). Normally there is a constant conveyor belt-like motion of the airway surface liquid, mediated by epithelial ciliary beating. Owing to the abundant mucous and reduced water content, the airway surface liquid in CF impairs ciliary function and results in reduced airway clearance [4]. The altered secretions further create a prime environment for bacteria to colonize due to the high concentrations of protein and nucleic acids released from dying immune cells, particularly neutrophils [5].

A major defining feature of CF lung disease is acute and persistent infection by opportunistic pathogens. *Staphylococcus aureus*, and *Haemophilus influenzae*, *Pseudomonas aeruginosa* are the main bacterial agents infecting CF patient lungs, the former frequently causing chronic infection in children and adults. As many as 80% of patients are colonized with *P. aeruginosa* at some point in their life [6]. As *P. aeruginosa* infections become chronic, the bacteria adopt a mucoid phenotype and generate a biofilm which further complicates the lung disease and the immune response.
CFTR transports chloride ions (Cl\(^-\)) across the cell membrane. CFTR is responsible for the bulk of Cl\(^-\) secretion in the airways epithelium, sweat glands and colon, and is regulated by PKA, PKC, calmodulin-dependent kinase and cGMP-dependent kinase. CFTR also regulates the activity of Ca\(^{2+}\)-activated Cl\(^-\) channels [7]. However compensatory chloride channels exist in the airways of mice resulting in negligible lung pathology, posing difficulty for researchers using mouse models to study CF lung disease. Most of the transgenic mice generated to knock out or impair CFTR exhibit intestinal disease ranging from mild to severe, weight reduction, and high mortality [4]. Other mouse models attempt to mimic the pathophysiology of CF lung disease by inducing the overexpression of the β-subunit of the ENaC [8,9]. Impaired CFTR is also responsible for regulating the surface expression and activity of ENaC. Together, this results in increased ENaC at the plasma membrane leading to sodium hyperabsorption. Mice with this defect exhibit increased mucous secretion and reduced ciliary clearance, as well as chronic (spontaneous) neutrophil influx in the lungs [8]. A more recent mouse model was generated to indirectly overexpress ENaC, by deleting its ubiquitin ligase, Nedd4-2, specifically in secretory airway epithelium with a surfactant protein-C promoter-linked Cre recombinase system [10]. These mice also exhibit characteristic CF lung phenotype but succumb to pulmonary complications by 3 weeks of age. While these mouse models may be useful in studying some aspects of CF lung disease, more representative CF models have also been developed in ferrets [11] and pigs [12].

1.2 Lung infections

Pneumonia is the infection of the lungs by bacteria, viruses, or fungi. Pneumonia represents a major burden in hospitals. Community-acquired pneumonia (CAP) represents a significant cause of morbidity and mortality, especially in children and the elderly [13,14]. The major causative agents of CAP include bacteria such as *S. aureus*, *H. influenzae* and viruses such as respiratory syncytial virus (RSV) and influenza.

A related form of nosocomial pneumonia is ventilator-associated pneumonia (VAP). Hospital admissions in patients experiencing breathing problems or anaesthesia often receive mechanical ventilation. A common complication of mechanical ventilation
is VAP, with an incidence between 8 and 28% of all ventilated patients [15]. Mortality associated with VAP is alarmingly high, with reports ranging between 24 and 76%. Frequent pathogens in VAP include *S. aureus*, *H. influenzae*, *P. aeruginosa* and as well as *Enterobacteriaceae* like *Escherichia coli* and *Klebsiella* spp.

1.3 **Anaerobic lung infections**

Anaerobic bacterial infections of the lung have not received the same level of attention as have aerobic bacterial infections or viral infections. A recent review of anaerobic infections by Bartlett [16] highlights the main developments in the field of anaerobic infections of the lung. The author points to a study by Levison et al. [17] where the use of a preferred antibiotic, penicillin, was compared to an antibiotic with broad coverage, clindamycin, in patients with anaerobic infections (lung abscess). Results supported the use of clindamycin and were upheld in subsequent clinical trials. This was one of the first and clearest evidences that targeting anaerobic bacteria had improved outcomes in the lungs. Anaerobic bacteria may play a larger role in pneumonia than previously thought. The origin of anaerobic bacterial lung infections is largely from aspiration of salivary fluid of the oral cavity.

1.4 **Anaerobic infections in CF/Microbiome**

Routine microbiological analysis of CF sputum involves culture of commonly known CF pathogens such as *P. aeruginosa*, *S. aureus*, *H. influenzae*, and others. Many of these pathogens are facultative anaerobes, which permits their culture and quantification using traditional aerobic culture techniques. There is no clear clinical practice for treating anaerobic infections in CF and a lack of clear understanding of the role of anaerobic bacteria in CF lung disease [18]. For this reason, antibiotic treatments are also not intentionally targeted against anaerobes. Despite this, there are some antibiotics which provide coverage for both aerobes and anaerobes (e.g. meropenem, ceftazidime) which are often used in the treatment of CF lung infections, especially in combination with tobramycin for *P. aeruginosa* infections [19]. Meropenem and ceftazidime are examples of the carbapenem and cephalosporin classes of antibiotics.
Both inhibit bacteria in a similar manner as penicillin, by interfering with bacterial cell wall synthesis and related proteins.

Recent studies have sought to characterize the extent of anaerobic bacterial presence in CF lungs. Worlitzsch et al. [20] cultured sputum onto agar plates and subcultured single colonies for biochemical identification, while Tunney et al. [21] sequenced 16S RNA by RT-PCR. Both groups found a large number of previously undetected and unreported bacterial genera such as *Gemella spp.*, *Prevotella*, *Veillonella*, and *Propionibacterium* in addition to well-known anaerobic pathogens like *Bacteroides*. Sibley et al. [22] used a technique of enriching the microbial communities obtained from patient samples by culture methods and subsequently analyzing bacterial species by sequencing. They found that almost all patients needed culture enrichment to capture the full breadth of the microbial community present. These new technologies bring insight and further reveal the limitations of measuring anaerobic bacterial presence in clinical samples.

1.5 Bacterial anaerobic metabolism

Under normoxic conditions, most bacteria preferentially oxidize glucose and other saccharides to pyruvate, and take pyruvate through the citric acid cycle. Both processes require oxygen as the final electron acceptor. Under anaerobic conditions, bacteria must use an alternate electron acceptor (e.g. sulfur), use the energy of another biochemical reaction, or bypass oxidative respiration [23,24]. The process of fermentation involves the release of energy from compounds without use of exogenous oxygen. Fermentative processes also occur in eukaryotes, such as in muscle cells during exercise through the formation of lactic acid. *Pseudomonas* species have a set of enzymes that can reduce nitrate to nitrite, nitric oxide, nitrous oxide, and finally nitrogen gas (Figure 1, [25]). In the context of CF lung infections, *P. aeruginosa* nitrate metabolism has been largely associated with its anaerobic phenotype [25–33]. The nitrate-reducing enzymes take the remaining electron generated by the reduction of NADH and regenerate the proton gradient of the cell and the pool of NAD$^+$ (Figure 1). Under normoxic conditions, *P. aeruginosa* does not require this set of enzymes because oxygen can be reduced to water. However in both cases of oxygen availability, NADH is generated from other
Figure 1. The denitrification cycle of *Pseudomonas stutzeri*. Organization and sidedness of the anaerobic electron transfer chain of *P. stutzeri*. The shaded areas represent the components of the constitutive aerobic respiratory chain consisting of an NADH dehydrogenase complex (DH), quinone cycle (Q, QH2), cytochrome *bc*1 complex (Cyt *bc*1), and the cytochrome *cb* terminal oxidase complex (Cyt *cb*). The denitrification system comprises respiratory nitrate reductase (NAR), nitrite reductase (NIR), NO reductase (NOR), and N2O reductase (N2OR). Abbreviations: FeS, iron-sulfur centers; *b*, *c*, and *d*1, heme B, heme C, and heme D1, respectively; cyt *c*, unspecified *c*-type cytochromes accepting electrons from the *bc*1 complex and acting on N2OR and NOR; cyt *c*551, cytochrome *c*551; AP, postulated nitrate/nitrite antiporter. Adapted with permission from [23]. A similar denitrification cycle can be found for *P. aeruginosa* in [26].
cycles. In normoxia NADH is generated by glycolysis and the tricarboxylic acid (TCA) cycle. In hypoxia NADH is also generated through glycolysis and the TCA cycle, with the addition of the glyoxylate shunt (Figure 2). The glyoxylate shunt is a bypass of the TCA cycle, avoiding the steps which require O$_2$ and release CO$_2$. The two important enzymes of the glyoxylate shunt are isocitrate lyase (ICL, also called aceA for acetate kinase). ICL was identified as an important virulence factor in *P. aeruginosa* [34]. *P. aeruginosa* mutants for ICL have significantly impaired virulence in alfalfa and rat infection models. The glyoxylate shunt allows for continuous assimilation of acetate for energy production. When it is impaired, *P. aeruginosa* may no longer be able to live effectively under the anaerobic conditions it creates through its own oxygen consumption. While the focus of the current text is not on alterations of bacterial genomes, the glyoxylate shunt is useful as an example to highlight the potential importance of acetate in bacterial infections.

### 1.6 Short chain fatty acids

Short chain fatty acids (SCFAs) are small carboxylic acids containing 2 to 6 carbon atoms. The most abundant SCFAs are the C2-C4: acetic acid, propionic acid and butyric acid. SCFAs also include C4 and C5 derivatives iso-butyrate, succinate, valerate, and iso-valerate, which are found in relatively lower abundance compared to the smaller SCFAs. These molecules have a pKa around 4.8, which means that at neutral pH they are almost 99% ionized and thus the anionic species is predominant [35,36]. At pH 6.0, roughly 6% of SCFA species will be protonated [36]. SCFAs are produced primarily in the colon by commensal gut bacteria through anaerobic fermentation of undigested carbohydrates and amino acids. In the gut, SCFAs have a complex set of roles, being involved in energy provision, mediating inflammation, regulating cell proliferation and apoptosis, and gene expression through histone deacetylation inhibition activity [37,38].

#### 1.6.1 Production and localization

SCFA production is highest in the colon, where the activity of commensal anaerobic bacteria converts undigested carbohydrates and amino acids. The end-product of these fermentative processes are SCFAs. The most abundant SCFA is
Figure 2. The TCA cycle and the glyoxylate shunt.
The glyoxylate shunt is represented by hollow arrows, indicating the conversion of isocitrate by isocitrate lyase to glyoxylate or succinate, and the conversion of glyoxylate by malate synthase to malate. Adapted from [34].
acetate, with concentrations as high as 70 mM in the caecum and proximal colon [39,40]. Propionate and butyrate are present between 10-30 mM. Total SCFAs in colon range from about 30-150 mM, with higher concentrations following a gradient down from the proximal colon to the distal colon [40].

Rasmussen et al. [41] examined the production of SCFAs by fecal bacteria from protein or specific amino acids. Acetate production was found to be particularly enhanced in the presence of histidine, hydroxyproline, serine and glutamate amino acids. Propionate production was primarily enhanced with aspartate supplementation. Butyrate production was enhanced in similar fashion to acetate, with hydroxyproline, serine, and glutamate supplementation increasing butyrate levels. The production of acetate and butyrate by the same amino acids likely reflects a shared degradation pathway. Interestingly, butyrate (C4) production is high with serine (C3) supplementation, suggesting either an intermediate C4 molecule before serine fermentation or, more likely, formation of butyrate from acetate. Another study looked at the formation of SCFAs from glucose using radioactive carbons. The authors concluded that glycolysis and the Wood-Ljungdahl pathway (Figure 3) were essential for acetate formation from glucose by colonic bacteria.

Some metabolic disorders also lead to the excess accumulation of SCFAs. SCFAs are intermediates in many metabolic processes such as the synthesis and breakdown of glucose, fatty acids, cholesterol, and amino acids. Acetate can accumulate in case of excess alcohol consumption, due to the conversion of ethanol to acetate (ethanoate). Increased acetate has been proposed to mediate the effects of “hangover” [42], and may be responsible for the immunosuppressive effects of alcohol consumption [43,44]. Propionate is increased in the genetic disorders affecting propionyl-CoA carboxylase [45] and methylmalonyl-CoA mutase, enzymes that are responsible for the proper conversion of propionyl-CoA to methylmalonyl-CoA. In the case of methylmalonyl-CoA mutase defects, propionate accumulation may arise from excess methylmalonyl-CoA which inhibits the activity of propionyl-CoA carboxylase. As Brown et al. [46] mention, these conditions are characterized by suppressed immunity [47,48]. Recently fatty acids were found as ligands for a number of orphan receptors, which has helped to shed light on the impact of fatty acids in disease.
Figure 3. The Wood-Ljungdahl pathway of acetogenesis.
Acetogenesis follows two branches: the carbonyl branch and the methyl branch. The carbonyl branch simply involves conversion of CO₂ to CO through acetyl-CoA synthase (and CO dehydrogenase) and then conjunction to HSCoA to get acetyl-CoA. The methyl branch incorporates a number of enzymes which take CO₂ and conjugate the carbon to tetrahydrofolate (THF) and subsequently protein. From there, Acetyl-CoA synthase forms acetyl-CoA which can go on for signal transduction and energy storage as acetyl~P by acetate kinase or incorporated into amino/fatty/nucleic acids, or polysaccharides. Adapted from [49].
1.6.2 SCFAs in infections

Early work in the area of SCFAs and infections attempted to provide a link between anaerobic bacterial presence and SCFA detection. The use of chromatographic techniques was pioneered to measure SCFAs (also called volatile fatty acids) in 1952 [50]. This pivotal method development set the stage for further analytical methods to rapidly and accurately determine SCFA concentrations in biological samples. Later studies were focused on optimizing extraction protocols in different matrices [51–53].

The first studies done to examine the utility of SCFA detection were focused on diagnosing anaerobic infections. Gas or gas-liquid chromatography (GC or GLC) was first used to diagnose anaerobic infections in abscesses [54,55] and empyema [54]. With a high degree of accuracy relative to anaerobic bacterial cultures of the same fluids, SCFA detection was able to diagnose anaerobic infection. The main benefit of GC analysis of SCFAs is turn-around time. Anaerobic bacterial species are often fastidious in growth, and require several days of culturing for proper qualification/quantitation. Furthermore, specimens containing obligate anaerobic species must be protected from oxygen exposure or they may not be accurately detected.

Botta et al. [56] looked at SCFAs in the context of gingival infection and found that gingival index, a score for determining gingival inflammation, correlated positively with SCFA concentrations, especially isobutyrate, butyrate, and isovalerate. They also found that the presence of Gram-negative rods were much more prevalent in the highest gingival index class (53% compared to 23% in the lowest class). Another study examined SCFA production by asaccharolytic Gram-positive rod bacteria (mainly from the *Eubacterium* genus) in periodontal pockets. Here, it was shown that these anaerobes can metabolize the amino acids arginine and lysine to the SCFAs acetate and butyrate and that presence of arginine and lysine can improve their growth [57].
Figure 4. Chemical structure of SCFAs.
Ball-and-stick model structures of SCFAs. Dark grey spheres – carbon, light grey spheres – hydrogen, red spheres – oxygen. Structures were designed using Avogadro software [58].
1.7 Receptors for SCFAs: Ligands and expression

The GPR40 family of G-protein coupled receptors recognize fatty acids. Free fatty acid receptors 1 (FFA1/GPR40), 2 (FFA2/GPR43), and 3 (FFA3/GPR41), GPR84, and GPR120 have different specificities for fatty acids. Many of these receptors were discovered as part of genome mining efforts to uncover pharmacological targets [59–62].

FFA1 is a receptor for medium and long chain fatty acids [63]. FFA1 has particular affinity for saturated fatty acids up to 16 carbons (with palmitic acid having the lowest EC50), and the highest affinity for certain unsaturated fatty acids (mead acid, all trans-retinoic acid, 5,8,11-eicosatriynoic acid). FFA1 is expressed primarily in the pancreas, the brain, and in primary monocytes [63]. The calculated EC50s of these ligands were performed in stably transfected HEK293 cells, and are in the low µM range. In the pancreas, FFA1 is localized to β-cells of islets. In the brain, FFA1 mRNA is most abundant in the spinal cord, substantia nigra, and medulla oblongata.

The function and expression of FFA2 has only recently been characterized and has yet to be fully explored. FFA2 is known to be highly expressed in immune cells such as monocytes, granulocytes and lymphocytes, as well as enteroendocrine cells. FFA2 expression can be upregulated in neutrophils and monocytes (as well as upon stimulation with lipopolysaccharide (LPS) or 12-phorbol-13-myristate acetate (PMA) [64]. FFA2 specifically recognizes propionate, butyrate, and acetate, with lower affinities for SCFAs with 5 or more carbons [65]. For comparison, the EC50s of propionate and acetate for FFA2 are between 14 µM to 79 µM and 35 µM to 100 µM, respectively (the difference in values are reflective of different reporter assays of receptor activation) [65].

FFA3 is another receptor for SCFAs with a slightly different specificity. FFA3 is expressed in enteroendocrine cells, sympathetic ganglia, and pancreatic β-cells. FFA3 expression in the brain is thought to regulate the sympathetic nervous system and in particular control heart rate [66]. FFA3 recognizes propionate, butyrate, valerate and caproate, but has much weaker affinity for acetate [65]. In contrast with FFA2 which has similar affinities for propionate and acetate, the EC50s of propionate and acetate for FFA3 are between 0.63 µM to 20 µM and 1 mM, respectively [65].
GPR84 was first discovered in human neutrophils and named EX33 [61]. This receptor has low similarity to other chemokine receptors and its expression on neutrophils can be reduced with GM-CSF stimulation and increased with LPS stimulation [67–70]. GPR84 expression was further determined to be high in the spleen, T lymphocytes, B lymphocytes and the RAW264.7 mouse macrophage cell line [67], as well as in microglial cells [68] and adipocytes [71]. In microglial cells, Bouchard et al. also demonstrate GPR84 induction with interleukin-1 (IL-1) and tumor necrosis factor α (TNFα) [68]. GPR84 recognizes medium-chain fatty acids (capric acid, undecanoic acid, lauric acid) [70] with EC50s in the 5 to 10 µM range. Additionally, diindolylmethane was shown to be a potent alternative agonist for GPR84. GPR84 is important in regulating the expression of cytokines: CD4⁺ T cells from GPR84−/− mice show increase IL-4 secretion in the presence of anti-CD3 and anti-CD28 antibodies [67]; GPR84 potentiates LPS-induced IL12p40 secretion in RAW264.7 cells [70]. The ligands for GPR84 suggest a relationship between inflammation and fatty acid sensing or regulation. Recent work by Nagasaki et al. [71] explored 3T3-L1 adipocytes cocultured with RAW264.7 cells to examine this potential interaction. RAW264.7 coculture increases GPR84 expression in 3T3-L1 adipocytes, and incubation with capric acid can inhibit TNFα-induced adiponectin release. Adiponectin regulates many metabolic processes associated with glucose and fatty acids, including insulin sensitivity and lipid breakdown. Furthermore, a high-fat diet can increase GPR84 expression. The authors suggest that GPR84 may explain the relationship between diabetes and obesity. As adipocytes release fatty acids in the presence of macrophages, the loop of increased GPR84 expression and its stimulation prevent the release of regulating hormones. The work on GPR84 is still very early and needs to be expanded in the context of pathophysiology and immune regulation.
Table 1. Fatty acid receptors and their defining characteristics.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligands</th>
<th>Synthetic ligands</th>
<th>Signaling</th>
<th>Tissue Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>FFA1</td>
<td>LCFA: C12+</td>
<td>GW9508 (Glaxo)</td>
<td>Undetermined</td>
<td>Pancreatic β-cells</td>
</tr>
<tr>
<td>FFA2</td>
<td>SCFA: C3&gt;C2=C4&gt;C5+</td>
<td>Phenylacetamide (Amgen), Tiglic acid, propionic acid</td>
<td>Coupled to Gi and Gq, inhibition of cAMP accumulation, intracellular Ca^{2+} release, p38 MAPK, ERK1/2</td>
<td>Granulocytes, monocytes, epithelial cells, enteroendocrine cells</td>
</tr>
<tr>
<td>FFA3</td>
<td>SCFA: C3&gt;C4&gt;C5+</td>
<td>None</td>
<td>Coupled to Gi</td>
<td>Enteroendocrine cells, sympathetic ganglia, pancreatic β-cells</td>
</tr>
<tr>
<td>GPR84</td>
<td>MCFA: C9-C14</td>
<td>Diindolylmethane</td>
<td>Undetermined</td>
<td>Granulocytes, alveolar macrophages, adipose tissue</td>
</tr>
<tr>
<td>GPR120</td>
<td>ω-3 FAs[72]</td>
<td>GW9508 (Glaxo)</td>
<td>Gαq/11</td>
<td>Macrophages adipocytes</td>
</tr>
</tbody>
</table>

Adapted and modified from [73].

1.8 Effects of SCFAs on host cells

Early research on SCFA effects looked at two main sites of endogenous SCFA production: the oral cavity and the colon. In the oral cavity, it was shown that SCFAs had an inhibitory effect on neutrophil chemotaxis, suggesting that they were potentially involved in modulating neutrophil function [74]. Researchers found that incubation of alveolar macrophages or blood granulocytes with 30 mM propionate, butyrate, or succinate (another product of anaerobic bacterial metabolism) could inhibit the phagocytosis of *S. aureus* [75]. A thorough review of the effects and influence of SCFAs in oral physiology is presented elsewhere [76].

SCFAs extracted from culture supernatants of *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, and *Prevotella loescheii* inhibited concanavalin A (Con A)-mediated mitogenic activity in splenic T cells and LPS-mediated mitogenic activity in B cells [77]. SCFAs were also tested individually and it was found that butyrate and propionate had the greatest effect on both cell types. Splenic T cells stimulated with Con
A also had reduced cytokine production profiles; IL-2, IL-4, IL-5, IL-6 and IL-10 production was inhibited in a dose-dependent manner from 1.25 mM to 5 mM by propionate and butyrate.

Butyrate increased apoptosis and potentiated LPS-mediated apoptosis in T cells isolated from blood [78]. SCFAs were also shown to inhibit proliferation and increase apoptosis in gingival keratinocytes [79,80] and in primary human gingival fibroblasts [81]. Interestingly, in neutrophils, SCFAs can inhibit (low concentrations: 1 – 5 mM) or potentiate (high concentrations: 30 mM) apoptosis. The effect of increasing apoptosis was attributed in part to caspase-3 [82]. Many oral bacteria, such as *Prevotella* spp., produce high levels of SCFAs in culture broth supernatant. Different cell lineages have different responses to these culture supernatants; epithelial cells (Ca9-22) and fibroblasts (Gin 1) have minimal sensitivity to SCFA-induced apoptosis, whereas T cells (Jurkat, U937, THP-1) have a much greater sensitivity. The prevalence of butyrate in culture supernatant appeared to increase T cell apoptosis, and butyrate alone induced apoptosis in a dose-dependent manner in all cell types [83]. A subsequent study found that higher concentrations of butyrate (10 mM) can induce apoptosis and autophagy in Ca9-22 cells [84]. Further work on the effect of butyrate revealed that apoptosis in Jurkat T cells was being induced by increasing oxidative stress, and that this effect was reversible with N-acetyl-L-cysteine treatment. The authors also found evidence of caspase-4 and caspase-10 involvement [85]. The consensus effects of SCFAs in the oral cavity appear to be influencing cell growth and maintenance. This is important because anaerobic bacteria are ever present around gingival tissue, and the presence of sugars and amino acids from food allow for SCFA production. SCFAs therefore likely influence healthy gingival epithelium maintenance, and alterations in SCFA production, such as those which promote excess propionate or butyrate, can disrupt normal epithelial integrity. The effect of SCFAs on T cells may provide an escape mechanism for pathogenic bacteria, by depleting T cell numbers through apoptosis.

Another important site of SCFA production is the colon. The colonic epithelium depends heavily on SCFA production; roughly 70% of the metabolic requirements of colonocytes are met by butyrate absorption [37]. SCFAs including butyrate have many important effects on cells in the colon. The effects of SCFAs in the colon are mediated
through several mechanisms: 1) electrolyte transport across the epithelial membrane, 2) histone deacetylase (HDAC) inhibition activity, and 3) signaling through FFAR2 and FFAR3. The next section (1.9) will outline the effects on electrolyte transport.

In studying the effects of SCFAs on colon cells, researchers first sought to identify cell types expressing FFAR2. In the rat intestine, FFAR2 was co-localized to enteroendocrine cells positive for 5-hydroxytryptamine and PYY [86]. In HT-29 colon epithelial cells, butyrate increases the expression of antimicrobial peptides such as human β defensins 1 and 2, and cathelicidin [87,88]. SCFAs reduce the electrical field stimulated contractions of isolated rat and mouse colon preparations, an effect which is not dependent on FFAR2 expression. In this study, the authors found that FFAR2 expression was highest in the proximal, mid, and distal colon [39], consistent with findings here [89]. A similar effect was observed in guinea pig tracheal rings stimulated with histamine: butyrate inhibited histamine-induced contractions of tracheal rings in a dose-dependent manner, likely as a function of its HDAC inhibitory action [90]. Bailón et al. [91] showed that butyrate can inhibit HT-29 cell proliferation. In addition to inhibiting proliferation, propionate-induced apoptosis was shown in HCT8 colon cancer cells transfected with FFAR2 vector [92]. Another study looked at the conditioning effect of butyrate on HCT15 colon epithelial cells. Incubation with butyrate lead to increased expression of acyl dehydrogenases (SCAD and MCAD) and a glucose uptake inhibitor, resistin (RETN); this leads to preferred butyrate metabolism versus glucose metabolism [93]. In addition, butyrate-conditioned cells upregulated a number of other proteins involved in differentiation, proliferation, and migration. In contrast, mice receiving oral SCFAs showed fewer apoptotic cells after infection with *Trichinella spiralis* [94]. Overall, the effects of SCFAs (mainly butyrate) in the colon results in effects on proliferation, apoptosis, and metabolism. This is likely due to the consumption of butyrate by colonic epithelial cells, the gearing of cellular metabolism to adapt to butyrate breakdown, and selection against cells which cannot metabolize butyrate.

FFAR2 is highly expressed on immune cells, especially granulocytes, lymphocytes, and macrophages [46,59,64]. The effects of SCFAs in neutrophils was studied well before the putative discovery of the receptor [60], and before the characterization of its expression and endogenous ligands [59,65,95]. Once the ligands
for FFAR2 were determined to be SCFAs, investigators began to identify the effects and signaling mechanisms of FFAR2 stimulation. Le Poul et al. [65] showed that ERK1/2 (p42/p44) phosphorylation increased in response to SCFAs in FFAR2-transfected CHO-K1 cells and that this effect was only partially inhibited by pertussis toxin (an inhibitor of G\textsubscript{i} signaling), indicating that FFAR2 is likely coupled to both the G\textsubscript{i} and G\textsubscript{q} proteins. In addition, they confirmed experiments showing the maximal chemotactic effect of SCFAs in neutrophils at concentrations of ~1 mM (acetate and propionate), and demonstrated that formyl-Met-Leu-Phe (fMLP) peptide can desensitize chemotaxis to SCFAs. SCFAs however do not desensitize against fMLP [65]. Vinolo et al. showed that PKB, p38, ERK1/2 and PI3K are also involved in mediated neutrophil chemotaxis [96]. Figure 5 shows a putative signaling pathway for FFAR2.

A useful tool in studying the one specific mechanism of SCFA stimulation is the FFAR2 knockout mouse model [97]. FFAR2\textsuperscript{−/−} mice have higher inflammation responses in models of arthritis, asthma, and colitis. Bone marrow reconstitution of wildtype mice with FFAR2\textsuperscript{−/−} donors resulted in significantly higher inflammation. FFAR2\textsuperscript{−/−} neutrophils have higher expression of signaling molecules in the MAPK and JNK pathways [97]. In wildtype neutrophils, acetate stimulation results in downregulated surface expression of several surface receptors, notably C5a, FPR1, CXCR2 and TLR2 (important chemotaxis receptors) in a similar fashion as their natural ligands. In line with this evidence, it was shown that SCFA-mediated stimulation FFAR2 can induce neutrophil chemotaxis [96]. Recently researchers have generated potent synthetic allosteric ligands for FFAR2, phenylacetamides, which will allow further refined understanding of SCFA stimulation [98–100].

SCFAs have long been known to have an anti-inflammatory role in the colon [101–103]. In dissecting the mechanism of this effect, researchers found that SCFAs result in inhibition of NFκB activation leading to reduced downstream inflammatory protein expression [104–108]. Tedelind et al. [105] first showed that SCFAs inhibited NFκB in a dose-dependent manner. They also showed that in inflamed murine \textit{ex vivo} colon cultures, high concentrations (30 mM) of SCFAs reduced the expression of a number of inflammatory proteins such as G-CSF, IL-6, and notably, iNOS. This reducing effect of SCFAs on iNOS expression was also observed in RAW264.7 cells [108].
Figure 5. Proposed FFAR2 signaling pathways.

SCFA stimulation of FFAR2 results in activation of Goi and Goq-coupled signaling. Goq recruits PLC which in turn activates PKC. PKC then phosphorylates a broad range of targets, including the Ras/Raf complex. Ras/Raf activates MEK1/2 which activate ERK1/2. Phosphorylation of ERK1/2 leads to the transcription of genes involved in regulating apoptosis, the cell cycle, and inflammation. Goi can activate PI3K which activates Src. Both Goi and Src can activate the Ras/Raf complex as well. PI3K also acts on Akt/PKB, leading to p38 MAPK activation and downstream gene transcription.

Abbreviations: PLC – phospholipase C, PKC, protein kinase C, PI3K – phosphoinositide 3-kinase, MEK1/2 – mitogen activated protein kinase kinase 1/2, ERK1/2 – extracellular signal regulated kinase 1/2, Akt/PKB – protein kinase B.
Interestingly, in intestinal epithelial cells, the opposite was observed: SCFA stimulation actually induced iNOS in IFN-γ/LPS stimulated intestinal epithelial cells [109].

1.9 SCFAs and electrolyte transport

SCFAs have been investigated as mediators of electrolyte transport in the colon [7,110–112]. SCFAs are suggested to enter cells through two mechanisms: direct diffusion of the protonated (acid) form, and antiport through bicarbonate/SCFA exchangers. As Sellin suggests, both mechanisms result in a net accumulation of SCFA ions inside the cell and protons (H⁺) outside the cell, and a consequent decrease in luminal pH [111]. Butyrate in particular has an important role in colonic epithelial health as it provides roughly 70% of the energy requirements to these cells. Butyrate transport is also suggested to be mediated by a Cl⁻/butyrate exchanger [7]. These mechanisms, which are luminal in nature, are balanced by basolateral mechanisms involving potassium channels, Na⁺/K⁺ ATPases, and calcium-dependent and –independent chloride channels.

Another important electrolyte in epithelial solute transport is bicarbonate, HCO₃⁻. CFTR is one of the important regulators of HCO₃⁻ secretion (indeed, defects in CFTR lead to impaired HCO₃⁻ secretion [113]).

1.10 SCFAs and bacterial growth

In section 1.5 we discuss where SCFAs fit in the anaerobic metabolic pathways of bacteria. SCFAs also play an important role in providing carbons as substrates for aerobic processes such as carbohydrate, amino acid, and fatty acid synthesis. Indeed SCFAs can also act as a nutrient source for Pseudomonas, as well as other microorganisms such as fungi. Acetate in particular plays other roles in bacterial metabolism; the unstable, high-energy acetyl~phosphate (ace~P) molecule is a signaling molecule which regulates various metabolic processes. A clear understanding of ace~P mechanisms of action is not available, but the phosphotransacetylase (PTA) and acetate kinase (ACKA) enzymes have been implicated [114]. These enzymes convert acetyl-CoA to ace~P and acetate, respectively. It has also been found that ace~P can influence the flagellar and biofilm phenotypes of E. coli [114].
SCFAs can preferentially inhibit the growth of oral Gram-negative bacterial species compared to Gram-positive bacteria at higher concentrations [115]. This finding was supported by other reports that found antimicrobial activity of acetate in Gram-negative bacteria, particularly *Pseudomonas* (minimum inhibitory concentration: 62.5 mg/ml; [116]). The authors also found *Bacillus subtilis*, *Escherichia coli*, *K. pneumonia*, *Serratia marcescens*, *Yersinia enterocolitica*, and the yeast *Candida albicans* to be susceptible to acetate. Another report documented the efficacy of 25 mg/ml sodium acetate solution in preventing *Pseudomonas* bacterial growth on sliced fish [117]. When compared to sodium citrate, acetate was shown to be more effective in inhibiting bacterial growth. Interestingly, while citrate is not very effective against *Pseudomonas* or other Gram-negative species, Gram-positive *Staphylococcus* species are highly susceptible to citrate with minimum inhibitory concentrations of <15.6 mg/ml [116]. The implication is that the presence of SCFAs can alter the growth of different bacterial species.

1.11 Rationale

SCFAs, being the product of microbial flora present in airway secretions, can thus influence the progression and resolution of infection and inflammation in the airways. The production of SCFAs depends on particular types of bacteria (primarily obligate anaerobes and facultative aerobes). The use of antibiotics is expected and has been shown to modify the bacterial community structure in the airways; and so too would SCFA production be expected to change with antibiotic treatment.

One condition which gives rise to persistent bacterial colonization, coupled with an anaerobic microenvironment, is CF lung disease. CF lung disease manifests itself through an altered, viscous mucous layer in the airways which is rendered hypoxic through the metabolism of host immune cells and bacteria. In this environment and under these conditions, bacteria can produce SCFAs. The reader is referred to reports in the fields of gastroenterology and dentistry, where SCFA research is prominent: here, SCFAs are said to downregulate immune cell inflammatory responses, promote neutrophil chemotaxis, induce inflammatory proteins expression in epithelial cells, inhibit
proliferation, and strengthen epithelial tight junctions [118]. This evidence is presented to support the proposal to study similar phenomena in airway epithelial cells.

As well as the reported effects on host cells, SCFAs have important effects on bacterial cells. SCFAs can inhibit the growth of certain bacteria, and thus may influence the airway microbiome. Taken together, SCFAs potentially have a significant role in the pathogenesis of chronic airway infections such as those seen in CF lung disease. To date there have been no studies of the influence of localized SCFAs in the airways or airway epithelial cells.

1.12 Hypothesis

I hypothesize that a) SCFAs are present in the airways of patients with CF and b) SCFAs modulate inflammation in the airways. I seek to provide evidence for these hypotheses with the following aims.

1.13 Aims

Aim 1: To quantify SCFAs in sputum of CF patients and controls.
Aim 2: To examine the effects of SCFAs on bacteria
Aim 3: To examine the effects of SCFAs on inflammation.
Aim 4: To examine the effects of SCFAs in a mouse model of pneumonia.
2.0 Methods

2.1 Sputum samples
Sputum samples were obtained from healthy controls and patients with cystic fibrosis and asthma at the Hospital for Sick Children in accordance with Research Ethics Board approval from 2007 to 2009. Further samples from healthy adult patients were obtained in 2011. Sputum samples were first mechanically separated to select solid mucous plugs (in order to exclude potential saliva contamination) and processed by treatment with 0.1% sputolysin (dithiothreitol) in water to dissociate mucous plugs. The homogenized sputum was then centrifuged briefly to pellet insoluble proteins and cell debris, and the supernatant was frozen at -80°C for future analysis.

2.2 SCFA Gas chromatography
Sputum supernatants were filtered through 30kDa ultrafiltration devices (Sartorius or Millipore). The filtered supernatants were then acidified with formic acid and spiked with an internal standard, 2-methylbutyric acid. Volatile compounds from the acidified filtrates were then distilled under reduced pressure and liquid nitrogen temperatures, dissolved in ultrapure water, and injected into a gas chromatography apparatus with flame ionization detector. Sample SCFA concentrations were calculated based on a standard curve of SCFAs analysed in the same fashion.

2.3 Bacterial culture and strains
Bacteria were cultured either from glycerol stocks kept at -80°C or from single colonies selected from tryptase soy agar plates. *P. aeruginosa* strains PAO1 and PA508 (a gift from the lab of Dr. Neil Sweezey, The Hospital for Sick Children) were cultured in tryptase soy broth (TSB) for most experiments.
2.4 **Bacterial growth assay**

Bacteria were grown overnight in TSB at 37°C and 225 rpm shaking in an incubator/shaker. After 16-18 hours, diluted bacterial cultures were added (25 µl) to 200 µl of modified TSB to a final dilution of 1/50 in a 96-well clear polystyrene microplate (BD Falcon). This dilution corresponded roughly to between 0.08 and 0.13 optical density (OD) at 600 nm. TSB was modified with different concentrations of SCFAs or other chemicals (sodium acetate, sodium propionate, sodium butyrate, sodium L-lactate, sodium citrate, sodium nitrate, sodium nitrite) and pH adjusted to 7.0, 6.5, 6.0, 5.5 with hydrochloric acid or HEPES, PIPES, MES, H₂SO₄, H₃PO₄ (control buffers/acids). Stock 1.5 M concentrations of SCFAs were made in sterile water. Microplates were read on an Omega plate reader for OD at 600 nm with 1 cm path correction for 225 µl volume every 30 or 60 minutes for at least 6 hours, with 10-20 seconds shaking before each read. For analysis, blank TSB corrected-OD readings were normalized to pH-matched TSB controls for each timepoint. After extended growth (> 6 hours), the pH of most conditions returned to neutral the due to accumulation of bacterial metabolites. For microaerobic conditions, the same method was used although plates were sealed with optically-clear PCR film at the start of the experiment. Due to condensation on the film, OD was read at 600 nm and subtracted by OD at 800 nm.

2.5 **Bacterial plating**

Agar plates were prepared with autoclaved trypticase soy agar plates. Bacterial cultures were streaked onto agar plates and incubated overnight at 37°C. For propagation of single colonies, agar plates were kept at 4°C wrapped in parafilm for up to two weeks. After overnight incubation at 37°C, inoculated agar plates were counted for colony forming units (CFU) and calculated to the original concentration.

2.6 **Cell lines and culture**

A549 human airway epithelial cells, RAW264.7 mouse macrophage cells, CFBE41o- immortalized cells from a patient with homozygous F508delCFTR alleles (CFBE41o-/F508del) were generated by transformation with a replication defective
pSVori- vector expressing SV40 large T antigen [119]. The counterpart control cell line used was CFBE41o- cells corrected with wild-type CFTR (CFB41o-/corrCFTR). These cells were generated by transfecting CFBE41o-/F508del with wild-type CFTR cDNA in a pCEP4 vector. A549 cells were cultured in 10% v/v fetal bovine serum (FBS) in Dulbecco’s Modified Essential Medium (DMEM, Wisent) with 4.5 g/L glucose, 110 mg/L sodium pyruvate, 2 mM L-glutamine, 100 units/ml penicillin/streptomycin at 37°C and 5% CO₂ in a humidified incubator. Cells were split at 90% confluency with 0.25% trypsin/0.53 mM EDTA and seeded in 6-, 12- or 24-well plates at 5 x 10⁴ and 1 x 10⁶ cells/ml. Volume used for plates were 2 ml, 1 ml or 0.5 ml for 6-, 12- or 24-well plates, respectively. RAW264.7 cells were cultured in similar fashion as A549 except using 5% FBS in DMEM due to more rapid cell division. CFBE/F508del cells were cultured in similar fashion as A549 cells. corrCFBE cells were cultured in similar fashion as A549 cells with the addition of 300 µg/ml hygromycin B to ensure survival of complemented cells only. Hygromycin B selects against cells not containing the hygromycin resistance gene found in pCEP4.

2.7 Cytomix stimulation

A549 cells (5 x 10⁴ cells/ml) or CFBE cells (1-2 x 10⁵ cells/ml) were seeded in 12- or 24-well plates and grown to 70-90% confluency. Plates were then washed with phosphate-buffered saline (PBS) and replenished with serum-free, phenol red-free DMEM and incubated for 24 hours. After serum starvation, cells were washed and replenished with fresh serum-free, phenol red-free DMEM and incubated for 1 hour with various SCFAs or inhibitors. After 1 hour, cytomix (IL-1β, TNF-α, IFN-γ; final concentration of 10 ng/ml each) was added to wells and incubated for different periods of time. At specified time intervals (4 hours, 8 hours, 24 hours, or as specified), cell culture supernatants were aspirated and stored at -80°C. Cells were lysed in radioimmunoprecipitation (RIPA) buffer (20 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate) with protease inhibitors (PI; Roche PI cocktail + 5 mM EDTA) on ice for 30 minutes, with intermittent vortexing. Cell lysates were stored at -80°C until further use.
2.8 Western blot

To cell lysates, 6x loading buffer (0.35 M Tris-HCl, 25% v/v glycerol, 1% v/v sodium dodecyl sulfate, 0.06% v/v bromphenol blue) was added and samples heated to 95°C for 10 minutes with shaking. Samples were loaded on 10% polyacrylamide gels and run at 70 V for 30 minutes and 120V for 60 minutes. Gels were transferred onto nitrocellulose membranes for 1 hour at 400 mA. Membranes were washed in PBS + 0.05% v/v Tween-20 (PBST) and blocked with 1% w/v non-fat milk overnight at 4°C. Membranes were washed 3 times with PBST for 5 minutes and incubated with primary antibodies for 1 hour at room temperature. Membranes were washed again and incubated for 1 hour with secondary antibodies for 1 hour at room temperature. Membranes were washed briefly with PBS and placed on plastic wrap. Enhanced chemiluminescent substrate was added directly onto each membrane and incubated for 10 minutes before being wicked off by task wipers. Membranes were developed using radiographic film on a Kodak X-Omat 2000a processor. Densitometric analysis was performed on scanned images of radiographic films with ImageJ software.

2.9 Cytokine analysis

Cell culture supernatants were analysed for cytokines using a Milliplex kit (Millipore, MPXHCYTO60K). In the first experiment, the cytokines analysed were eotaxin, G-CSF, GM-CSF, IFN-α2, IFN-γ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-15, IL-17A, IP-10, MCP-1, MIP-1α, MIP-1β, TNF-α, TNF-β. The second experiment included G-CSF, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-6, IP-10, MCP-1 and TNF-α.

2.10 IL-8 enzyme-linked immunosorbent assay (ELISA)

Cell culture supernatants were analysed for cytokines using a mini ELISA development kit (Peprotech, Human IL-8 kit). Briefly, Nunc Maxisorp 96-well plates were coated with IL-8 capture antibody overnight at room temperature. The plate was washed with 4 times with PBST and diluted cell culture supernatants and IL-8 standards were added and incubated for 1.5 hours at room temperature. Plates were washed and
biotinylated anti-IL-8 antibody was added and incubated for 1.5 hours at room temperature. Plates were washed again and avidin-conjugated horseradish peroxidase added and incubated for 30 minutes. Plates were washed and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) liquid substrate (Sigma) was added and plates read at 405 nm using a spectrophotometer. Blank-corrected sample readings were converted to concentrations using linear regression of IL-8 standards.

2.11 Proliferation and viability assays

For proliferation assays, cells were seeded at 5 x 10^4 cells/ml in 96-well plates and incubated for 1 hour with SCFAs. After SCFA incubation, plates were incubated for 1 hour or 24 hours with PrestoBlue reagent (Molecular Probes) at 1/10 dilution. Fluorescence is read with 555 nm excitation and 590 nm emission. The fluorescence signal is blank corrected and normalized to untreated cells. For viability assays, the protocol was similar except cells were allowed to grow to 80-90% confluency in 96-well plates before incubation with SCFAs. Fluorescence is then measured in the same fashion.

2.12 Lipopolysaccharide instillation

Female Balb/c mice were housed in laboratory facility and provided food and water ad libitum. Animal procedures were approved by the Research Ethics Board at the Hospital for Sick Children. Mice were anaesthetized with 50/5 mg/kg ketamizexylazine cocktail. Using an inclined plexiglass board, mice were placed in supine position and instilled with 50 µl of *E. coli* O111:B4 lipopolysaccharide in PBS or in acetate/propionate/butyrate (25 mM/5 mM/0.5 mM; APB) in PBS, with a 100 µl air purge using a catheter aimed in the trachea past the vocal cords. Mice were administered with 1 ml of 0.9% saline subcutaneously and monitored for recovery. At 24 hours post-LPS instillation, mice were intratracheally instilled with 50 µl PBS or APB buffer in the same fashion. At 48 hours post-LPS instillation, mice were anaesthetized with 100/10 mg/kg ketamine/xylazine cocktail or pentobarbitol. The trachea was exposed and a small lateral incision was performed to allow for the introduction of a
catheter tube. A 1-ml syringe was used to lavage the lungs via catheter with 1 ml HBSS three times (3 ml total). Recovery volume was between 2.4 and 2.7 ml.

2.13 Histology

BAL fluid was diluted 1/10 and total cells were counted using a hemacytometer. BAL fluid was centrifuged at 100 x g for 3 minutes using a cytospin apparatus onto microscope slides. Slides were stained using Hemacolor haematoxylin/eosin differential stain. Differential cell count was manually performed by digital light microscopy.

2.14 Statistical procedures

Statistical analysis was performed using Microsoft Excel 2010 or Graphpad Prism 5. For sputum SCFAs, unpaired and paired non-parametric t-tests were done. For bacterial growth data, repeated measures analysis of variance (ANOVA) were performed followed by Dunnett’s post test to compare growth curves against the buffer control. In these analyses, the normalized growth curves from the 2 hour timepoint to the last timepoint for each concentration of SCFAs at each pH level were compared against the pH-matched control growth curve in absence of any SCFA. For all others un-paired non-parametric t-tests were performed. Significance for all tests was taken to be p < 0.05.
3.0 Results

3.1 SCFAs in sputum

We performed vacuum distillation and gas chromatographic analysis of processed sputum supernatants from CF patients to measure the levels of SCFAs in airway secretions. Patients samples were obtained from studies where they had been recruited for the effects of antibiotic treatments for pulmonary exacerbations (n=9) at the Hospital for Sick Children, Toronto, ON. Samples from CF patients with stable disease (n=10) and asthma patients (n=10) at the same hospital were also obtained. Sputum from healthy controls (n=10) was induced by nebulized hypertonic saline. The mean age of CF patients was 13.4 years (range of 8.5 – 17.7).

We found SCFAs in millimolar concentrations in CF patient sputum. Figure 6A shows the concentrations of total SCFAs. In stable CF patients, the mean total concentration of SCFAs was 2115 µM (827 µM – 4075 µM). For patients with pulmonary exacerbation, mean total SCFA concentration before antibiotic treatment was 1280 µM (158 µM – 4570 µM) and 871 µM (166 µM – 4178 µM) (Figure 6A). In control healthy and asthmatic patients, mean total SCFA concentration were 2960 µM (1028 µM – 7854 µM) and 2399 µM (783 µM – 5773 µM), respectively (Figure 6B). There was no significant difference between the stable CF group and either of the control groups (healthy controls or asthma patients). There was also no significant difference between stable and pre-antibiotic groups, however there was a significant difference between stable and post-antibiotic groups (p = 0.0076, Mann-Whitney). There was no difference between pre- and post-antibiotic groups when including all patients (n=9; p = 0.2031, Wilcoxon paired), however when excluding the patient 58-08, who had a significant increase in SCFAs after antibiotic treatment, the groups were significantly different (n=8; p = 0.0391, Wilcoxon paired).

We calculated Spearman correlation coefficients for total SCFA concentrations and clinical parameters such as age, sex, weight, forced expiratory volume in1 sec (a measure of lung function), antibiotic regimen, bacterial culture status, or markers of inflammation (sputum cell count, IL-8 concentration, neutrophil elastase activity, nitric oxide synthase activity, arginase activity) when pooling all CF groups together.
Figure 6. Total SCFA concentrations in sputum.
A) Total SCFA concentration in CF patients with stable disease (Stable), presenting with a pulmonary exacerbation before antibiotic (Pre Abx) and after antibiotic (Post Abx) treatment. B) Total SCFA in healthy controls or patients with asthma. Total SCFA was calculated as the sum of acetate, propionate, and butyrate concentrations (µM).
Figure 7. Individual SCFA concentrations in cystic fibrosis patient sputum.
A) Acetate, B) Propionate, C) Butyrate concentrations (µM) in CF patients with stable disease (Stable), presenting with a pulmonary exacerbation before antibiotic (Pre Abx) and after antibiotic (Post Abx) treatment.
Figure 8. Correlations between SCFAs and markers of inflammation.
A) Total SCFAs in stable patients in correlation with total number of cells/g mucous. Spearman rho = 0.939, $r^2 = 0.882$, $p < 0.001$. B) Total SCFAs in patients with pulmonary exacerbation before antibiotic treatment in correlation with the fold change in NO$_x$ after antibiotic treatment. Spearman rho = 0.865, $r^2 = 0.749$, $p < 0.05$. 
3.2 **SCFA effects on bacteria**

To test the effects of SCFA on bacterial growth, we incubated *P. aeruginosa* strains with SCFAs at varying concentrations and pH levels in broth. Bacterial growth was monitored by optical density (OD) at 600 nm for aerobic growth, and OD 600 nm minus OD 800 nm for microaerobic growth. The growth was monitored for at least 6 hours, with measurements taken every 0.5 hours. The relative OD was calculated for each treatment condition by correcting to the pH-matched TSB alone treatment at each individual timepoint. Thus the growth of *P. aeruginosa* in TSB alone is taken at 1 for each timepoint, and the presence of SCFAs shifted the growth up or down depending on the concentration and pH.

3.2.1 **SCFAs increase bacterial growth at low concentrations and inhibit growth at high concentrations under aerobic conditions**

Aerobic conditions were used to monitor growth of *P. aeruginosa* strains PAO1 and PA508. The mean change over the period of measurements as well as the p-value significance levels of all the mentioned assays can be found in Table A1. in Appendix A. Figure 9 shows a representative figure of raw OD values for this assay with acetate incubation. The subsequent bacterial figures show transformations of raw OD values for SCFA-incubated conditions divided by the pH-matched broth at each timepoint.

Representative figures of growth curves for acetate (Figure 10), propionate (Figure 11), butyrate (Figure 12), and lactate (Figure 13) for PAO1 are presented. In the presence of acetate, PAO1 exhibited increased growth at pH 7.0 from 3.125 mM to 25 mM, although only statistically significant from 6.25 mM to 25 mM (Figure 10). PA508 demonstrated a less pronounced growth increase, and only at 3.125 mM acetate. The largest effect of growth increase was seen with acetate and lactate in both strains tested. Higher concentrations of acetate, propionate, butyrate, and citrate significantly inhibit the growth of *P. aeruginosa* strains at 100 mM. PAO1 had less overall susceptibility to growth inhibition, and more response to growth increase compared to PA508. PAO1 was also less susceptible to reduced growth as a function of reduced pH levels. Notably, at pH 6.5, PA508 growth was still increased with 3.125 mM (although
Figure 9. Growth of *Pseudomonas aeruginosa* in TSB at pH 7.0 with acetate. Unmodified optical density (OD) readings at 600 nm of *P. aeruginosa* strain PAO1 grown under aerobic conditions in TSB adjusted to pH 7.0 with HCl in the presence of acetate from 3.125 mM to 100 mM.
Figure 10. Effect of acetate concentration and pH on the growth of PAO1. *P. aeruginosa* PAO1 incubated with acetate at concentrations between 3.125 mM and 100 mM in TSB. pH was adjusted with HCl to A) 7.0, B) 6.5, C) 6.0, D) 5.5. p-values of repeated measures ANOVA and mean curve difference in Appendix A.

Table 2. Statistical analysis of curves in Figure 10.

<table>
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<tr>
<th>Acetate (mM)</th>
<th>pH 7.0 Mean diff.</th>
<th>Sig.</th>
<th>pH 6.5 Mean diff.</th>
<th>Sig.</th>
<th>pH 6.0 Mean diff.</th>
<th>Sig.</th>
<th>pH 5.5 Mean diff.</th>
<th>Sig.</th>
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<td>ns</td>
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Repeated measures ANOVA: *, p < 0.05; **, p < 0.01; ***, p < 0.001.
Figure 11. Effect of propionate concentration and pH on the growth of PAO1. *P. aeruginosa* PAO1 incubated with propionate at concentrations between 3.125 mM and 100 mM in TSB. pH was adjusted with HCl to A) 7.0, B) 6.5, C) 6.0, D) 5.5. p-values of repeated measures ANOVA and mean curve difference in Appendix

Table 3. Statistical analysis of curves in Figure 11.

<table>
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Repeated measures ANOVA: *, p < 0.05; **, p < 0.01; ***, p< 0.001.
Figure 12. Effect of butyrate concentration and pH on the growth of PAO1. 
*P. aeruginosa* PAO1 incubated with butyrate at concentrations between 3.125 mM and 100 mM in TSB. pH was adjusted with HCl to A) 7.0, B) 6.5, C) 6.0, D) 5.5. p-values of repeated measures ANOVA and mean curve difference in Appendix A.

Table 4. Statistical analysis of curves in Figure 12.

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<th>Acetate (mM)</th>
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<th></th>
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<td>Sig.</td>
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Repeated measures ANOVA: *, p < 0.05; **, p < 0.01; ***, p < 0.001.
not significant) and lactate at 3.125 and 6.25 mM. The growth increases seen with propionate did not achieve statistical significance in this analysis, however a spike in growth can be seen in the graphs at pH 7.0 and pH 6.5 (Figure 11), and an overall higher growth at the 7 hour timepoint. A similar situation can be seen for butyrate at pH 6.5 and pH 6.0 (Figure 12). The presence of lactate stimulates a large burst in growth early timepoints (up to 2 hours), however overall growth normalizes to control by the end of the incubation (Figure 13). We also tested the effects of citrate, nitrite and nitrate on bacterial growth. Citrate shows a similar pattern on growth as other SCFAs, but has a less pronounced inhibitory effect (no conditions led to <0.35 of maxOD600 with citrate, compared to several conditions with other SCFAs that reduced growth to <0.1 maxOD600.

It has been previously shown that acidified nitrite can inhibit the growth of *P. aeruginosa* species, and that strains adopting a mucoid phenotype become more susceptible to nitrite-mediated killing [30]. In our experimental setup, nitrite has a similar effect on PAO1 and PA508. As was the case with SCFAs, the effect of nitrite on growth inhibition correlated with decreased pH. Comparing the effect of nitrite between the two strains, PA508 growth was more significantly inhibited at all concentrations and pH values. A notable exception is the 3.125 mM concentration at pH 7.0, which saw a significant growth increase for PA508 but no significant difference (trend towards inhibition) for PAO1. Nitrate exhibited no particular trend on growth of either *P. aeruginosa* strain.
Figure 13. Effect of lactate concentration and pH on the growth of PAO1. 
*P. aeruginosa* PAO1 incubated with lactate at concentrations between 3.125 mM and 100 mM in TSB. pH was adjusted with HCl to A) 7.0, B) 6.5, C) 6.0, D) 5.5. p-values of repeated measures ANOVA and mean curve difference in Appendix A.

<table>
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<tr>
<th>Acetate (mM)</th>
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Repeated measures ANOVA: *, p < 0.05; **, p < 0.01; ***, p < 0.001.
3.3 SCFA effect on A549 airway epithelial cells

3.3.1 Proliferation and Viability

The PrestoBlue assay is a resazurin-based reagent which reacts to the reducing environment of live cells. We used PrestoBlue instead of the traditional methotrexate assay because it allows for a more rapid determination of live cells and involves fewer steps. We tested the effect of SCFAs on A549 type II airway epithelial cells by incubating cells with these compounds for 24 hours at sub-confluency (25%) or full confluency. Incubation of SCFAs at sub-confluency was taken to be a measure of proliferation; 24 hours after SCFA incubation the PrestoBlue assay was performed. Only propionate and butyrate at 25 mM and 50 mM significantly inhibited A549 cell proliferation (Figure 14). Acetate from 0.5 mM to 50 mM had no effect on proliferation. For testing viability, confluent monolayers were incubated with SCFAs for 24 hours and the PrestoBlue assay performed. In contrast to the proliferation results, propionate and butyrate at 25 and 50 mM slightly increased the signal (~10% increase above control, Figure 15). Acetate from 0.5 to 50 mM and low concentrations of propionate and butyrate had no effect on viability.
Figure 14. Effect of SCFAs on proliferation of A549 airway epithelial cells. A549 airway epithelial cells were tested for proliferation by the PrestoBlue assay. Fluorescent signal indicating live cells relative to the control media were calculated for cells incubated with acetate (Ace), propionate (Prp), or butyrate (But) at concentrations of 50 mM, 25 mM, 2.5 mM, and 0.5 mM. Symbols indicate results of un-paired, non-parametric t-test compared to control: *, p < 0.05; **, p < 0.01; ***, p < 0.001.
Figure 15. Effect of SCFAs on viability of A549 airway epithelial cells.
A549 airway epithelial cells were tested for viability by the PrestoBlue assay. Fluorescent signal indicating live cells relative to the control media were calculated for confluent monolayers of cells incubated with acetate (Ace), propionate (Prp), or butyrate (But) at concentrations of 50 mM, 25 mM, 2.5 mM, and 0.5 mM.
3.3.2 SCFA-mediated regulation of iNOS protein expression

A549 cells were incubated with SCFAs for 1 hour, followed by cytomix (IL-1β, TNF-α, IFN-γ; 10 ng/ml each) stimulation for 4, 8, or 24 hours. iNOS and GAPDH protein expression by Western blot and densitometric analysis. We used GAPDH as a loading control to normalize for potential differences in cell numbers or overall protein expression in individual experimental wells. Figure 16 shows a representative series of Western blots for each timepoint. At the 4 hour timepoint, iNOS induction can already be seen. Reports suggest that maximal stimulation occurs between 8 and 10 hours following cytomix addition. With 24 hours stimulation, iNOS protein persists in elevated quantities but less than at 8 hours. SCFAs were used at 4 different concentrations: 0.5 mM, 2.5 mM, 25 mM, and 50 mM. Two particular trends can be seen with SCFA incubation. At high concentrations (>25 mM) iNOS induction is completely inhibited by butyrate. This effect can be seen at all timepoints. At low concentrations, butyrate appears to enhance iNOS induction. These results were also observed for acetate and propionate (results not shown). A549 cells incubated with SCFAs alone without cytomix stimulation did not exhibit any iNOS expression.

Exploring the pro-inflammatory effect in more detail, SCFAs were incubated with A549 cells at concentrations found in CF patient sputum. These concentrations were found to be pro-inflammatory either individually or in combination (Figure 17). The variation in iNOS expression increases can be explained by differences in cell density from assay to assay.

Figure 18 shows the effect of propionate and butyrate on iNOS production. In this experiment butyrate did not appear to have a particular pro-inflammatory effect, but propionate showed a ~30% increase in iNOS expression. L-arginine is an important molecule for iNOS. Not only is it the primary substrate for iNOS, but some reports suggest that it also regulates iNOS expression [109]. Using L-arginine-free DMEM, we show that L-arginine is required for maximal iNOS expression (Figure 19). DMEM contains 400 µM L-arginine, whereas normal physiological levels in blood are 100 µM. At 100 µM L-arginine, iNOS expression is normal. At 0 µM L-arginine, there is roughly 50% less iNOS induction as compared to unmodified DMEM or 100 µm L-arginine in DMEM.
Figure 16. Butyrate increases or decreases iNOS expression compared to cytomix alone.
Representative Western blot for iNOS and GAPDH in A549 cells incubated with SCFAs (butyrate shown) and stimulated with cytomix (CM: IL-1β, TNF-α, IFN-γ; 10 ng/ml each) for A) 4 hours, B) 8 hours, C) 24 hours. Ascending triangles represent increasing concentration of butyrate (0.5 mM, 2.5 mM, 25 mM, 50 mM). Representative Western blots of experiments done repeated twice in duplicates.
Figure 17. SCFAs increase iNOS expression compared to cytomix stimulation alone in A549 cells.

Densitometric analysis of Western blots of A549 airway epithelial cells incubated with low concentrations of SCFAs and stimulated with cytomix (CM: IL-1β, TNF-α, IFN-γ; 10 ng/ml each). APB: acetate (Ace) 5 mM, propionate (Prp) 0.5 mM, butyrate (But) 0.1 mM. Results of t-tests are shown: *, p < 0.05. A) Cells incubated with SCFAs and stimulated with cytomix for 8 and 24 hours. B) Repeat assay with cells incubated with SCFAs with and stimulated with cytomix for 24 hours.
Figure 18. Propionate increases or decreases iNOS in cytomix stimulated A549 cells.
Densitometric analysis of Western blots of A549 airway epithelial cells incubated with low and high concentrations of propionate (Prp) and stimulated with cytomix (CM: IL-1β, TNF-α, IFN-γ; 10 ng/ml each) for 24 hours. Means +/- standard error and results of t-tests compared to control (cytomix alone): *, p < 0.05; **, p < 0.01.
Figure 19. iNOS protein expression is dependent on the concentration of L-arginine.

Densitometric analysis of Western blots of A549 cells incubated with different concentrations of L-arginine and stimulated with cytomix (CM: IL-1β, TNF-α, IFN-γ; 10 ng/ml each) for 24 hours. Results were compared to original DMEM (400 µM L-arginine). Means +/- standard error are shown. Results of unpaired, non-parametric t-test compared to control (400 µM L-arginine + CM): *, p < 0.05. Representative analysis of experiment repeated twice in triplicates (n = 5 for 400 µM L-arginine).
The signaling pathway for FFAR2 stimulation is reported to involve MEK1/2 and p38 MAP kinases. We examined whether inhibiting these kinases would interfere with the effects of SCFA incubation on iNOS induction (Figure 20). Looking at p38 MAPK, inhibition leads to almost completely reverses iNOS induction, while simultaneously increasing the level of arginase 1. In contrast, while MEK1/2 inhibiton also inhibits iNOS induction, it also decreases arginase 1 expression. Co-incubation with 25 mM propionate or either inhibitor amplifies the effects seen for each individual inhibitor. Incubation with 25 mM propionate alone leads to a reduction in iNOS and arginase 1 expression. Since we were unable to detect FFAR2 protein expression by Western blot in A549 cells (not shown), we assume that the effects of SCFAs like propionate on iNOS are unrelated to FFAR2 stimulation.
Figure 20. Cytomix stimulation induces iNOS in a p38 MAPK and MEK1/2 dependent manner.
Western blots for iNOS and ARG1 of SCFA incubation in cytomix (IL-1β, TNF-α, IFN-γ; 10 ng/ml each) stimulated A549 airway epithelial cells with and without the p38 MAPK inhibitor, doramapimod, MEK 1/2 inhibitor, U0126, or propionate. Representative Western blots of experiment repeated twice in duplicates. *Denotes cytomix with dimethyl sulfoxide vehicle control.

Table 6. iNOS/ARG1 ratio in A549 cells stimulated with cytomix.

<table>
<thead>
<tr>
<th>iNOS</th>
<th>ARG1</th>
<th>Ratio</th>
<th>Cytomix</th>
<th>MEK inh</th>
<th>p38 inh</th>
<th>25 mM Prp</th>
</tr>
</thead>
<tbody>
<tr>
<td>4183</td>
<td>542</td>
<td>7.72</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>229</td>
<td>0.04</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>3239</td>
<td>0.00</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2340</td>
<td>197</td>
<td>11.86</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>1217</td>
<td>0.01</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5182</td>
<td>1513</td>
<td>3.42</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>1158</td>
<td>0.01</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>1741</td>
<td>851</td>
<td>2.05</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>292</td>
<td>3029</td>
<td>0.10</td>
<td>+</td>
<td>+</td>
<td>-</td>
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</table>
3.3.3 SCFA-mediated regulation of cytokine secretion

Cell culture supernatants from the SCFA incubation and cytomix stimulation experiments in A549 cells were analyzed by Milliplex bead immunoassays to quantify cytokine release. In a preliminary panel of cytokines, we found that G-CSF, GM-CSF, IL-1α, IL-6, IL-8, IP-10, and MCP-1 were expressed and altered in response to SCFAs. IL-8 levels were not quantifiable in the preliminary assay due to being above the standard curve. A subsequent sandwich ELISA was performed for IL-8 and the levels were still too high. For subsequent analyses of other cytokines, the cytomix cytokines were also included.

Despite being measurable in the preliminary assay, G-CSF, IP-10 and IFN-γ were unquantifiable in the subsequent analysis. Three concentrations for each SCFA were tested in these experiments to represent low CF concentrations (0.5 mM acetate, 0.1 mM propionate, 0.005 mM butyrate), high CF concentrations (2.5 mM acetate, 0.5 mM propionate, 0.05 mM butyrate), and 10x CF concentrations (25 mM acetate, 5 mM propionate, 0.5 mM butyrate), as well as the combination of each set. Table 8 shows the protein concentrations found in culture supernatants. GM-CSF shows a dose-dependent increase in release in response to acetate, propionate and butyrate, while the combination of the three saturates at the CF high concentrations (Figure 21). IL-1α showed the greatest fold changes among all cytokines tested. The highest concentrations for each SCFA and the combination of the highest all showed a highly significant 4-5 fold increase in IL-1α secretion compared to cytomix alone. As well, 0.5 mM propionate and the high CF combination of SCFAs also showed significant 1.6 fold increases in IL-1α secretion. In contrast with IL-1α, IL-1β secretion was only significantly changed by 1.4 fold with 0.5 mM propionate incubation. Both IL-6 and MCP-1 secretion were increased with all concentrations of acetate and 0.5 mM and 0.005 mM butyrate, however MCP-1 incubation led to less of an increase (10-15%) compared to IL-6 (20-50%).
Table 7. Cytokine concentrations of A549 culture supernatants incubated with SCFAs and stimulated with cytomix.

<table>
<thead>
<tr>
<th></th>
<th>G-CSF</th>
<th>GM-CSF</th>
<th>IFN-γ</th>
<th>IL-1α</th>
<th>IL-1β</th>
<th>IL-6</th>
<th>IP-10</th>
<th>MCP-1</th>
<th>TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acetate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25 mM</td>
<td>17075.6</td>
<td>45.2</td>
<td>10899.8</td>
<td>183.8</td>
<td>6277.7</td>
<td>4285.9</td>
<td>12757.7</td>
<td>7749.9</td>
<td>3362.7</td>
</tr>
<tr>
<td>2.5 mM</td>
<td>15234.5</td>
<td>35.6</td>
<td>11144.0</td>
<td>53.5</td>
<td>6920.8</td>
<td>5167.2</td>
<td>12755.7</td>
<td>7839.3</td>
<td>3577.7</td>
</tr>
<tr>
<td>0.5 mM</td>
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<td>32.0</td>
<td>11261.4</td>
<td>42.9</td>
<td>5757.3</td>
<td>4138.5</td>
<td>12648.6</td>
<td>8042.3</td>
<td>2679.1</td>
</tr>
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<td><strong>Propionate</strong></td>
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<td></td>
</tr>
<tr>
<td>5 mM</td>
<td>27747.5</td>
<td>46.0</td>
<td>11075.9</td>
<td>208.4</td>
<td>5460.2</td>
<td>3878.2</td>
<td>12356.4</td>
<td>7840.0</td>
<td>2239.0</td>
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<td>0.5 mM</td>
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<td>39.9</td>
<td>11201.9</td>
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<td>6743.0</td>
<td>3926.4</td>
<td>12533.2</td>
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<td>0.1 mM</td>
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<td>34.2</td>
<td>4265.2</td>
<td>3629.2</td>
<td>12445.7</td>
<td>7444.6</td>
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</tr>
<tr>
<td><strong>Butyrate</strong></td>
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<td></td>
<td></td>
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<td>0.5 mM</td>
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<td>11200.5</td>
<td>157.4</td>
<td>5662.2</td>
<td>3796.2</td>
<td>12508.7</td>
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<tr>
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<td>11061.1</td>
<td>47.3</td>
<td>4823.7</td>
<td>3934.9</td>
<td>12365.6</td>
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<tr>
<td>0.01 mM</td>
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<td>34.6</td>
<td>11433.5</td>
<td>42.2</td>
<td>4938.6</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>10x</td>
<td>19983.2</td>
<td>41.2</td>
<td>11034.4</td>
<td>205.4</td>
<td>6415.0</td>
<td>3199.9</td>
<td>12245.9</td>
<td>7268.9</td>
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<td>CF High</td>
<td>19190.2</td>
<td>41.1</td>
<td>10949.3</td>
<td>69.1</td>
<td>5454.1</td>
<td>3873.4</td>
<td>12441.8</td>
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<tr>
<td>CF Low</td>
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<td>11045.6</td>
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<td>5560.7</td>
<td>3280.7</td>
<td>12232.5</td>
<td>7224.0</td>
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<td></td>
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<td></td>
<td></td>
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<tr>
<td>Naïve</td>
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<td>0.5</td>
<td>0.8</td>
<td>0.5</td>
<td>0.5</td>
<td>425.1</td>
<td>0.6</td>
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<tr>
<td>Cytomix</td>
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<td>30.5</td>
<td>10965.2</td>
<td>42.0</td>
<td>4998.6</td>
<td>3330.8</td>
<td>12134.0</td>
<td>7099.8</td>
<td>2494.6</td>
</tr>
</tbody>
</table>

§ - cytokines that registered signals below the highest standard curve value of 3.2 pg/ml and were extrapolated.
# - cytokines that registered signals above the highest standard curve value of 10000 pg/ml and are extrapolated.
Naïve controls represent cell supernatants in the absence of SCFAs or cytomix.

Cytomix (IL-1β, TNF-α, IFN-γ; 10 ng/ml each).
Figure 21. Relative GM-CSF secretion profile in stimulated A549 cells.
Abbreviations: 10X, 25 mM acetate, 5 mM propionate, 0.5 mM butyrate; CF high, 2.5 mM acetate, 0.5 mM propionate, 0.05 mM butyrate; CF low, 0.5 mM acetate, 0.1 mM propionate, 0.005 mM butyrate. Cytomix (IL-1β, TNF-α, IFN-γ; 10 ng/ml each). Naïve controls represent cell supernatants in the absence of SCFAs or cytomix. Results of unpaired, non-parametric t-tests compared to cytomix: *, p < 0.05; **, p < 0.01; ***, p < 0.001.
Figure 22. Relative IL-1α secretion profile in stimulated A549 cells.
Abbreviations: Ace, acetate; Prp, propionate; But, butyrate; 10X, 25 mM acetate, 5 mM propionate, 0.5 mM butyrate; CF high, 2.5 mM acetate, 0.5 mM propionate, 0.05 mM butyrate; CF low, 0.5 mm acetate, 0.1 mM propionate, 0.005 mM butyrate. Cytomix (IL-1β, TNF-α, IFN-γ; 10 ng/ml each). Naïve controls represent cell supernatants in the absence of SCFAs or cytomix. Results of unpaired, non-parametric t-tests compared to cytomix: *, p < 0.05; **, p < 0.01; ***, p < 0.001.
Figure 23. Relative IL-1β secretion profile in stimulated A549 cells.
Abbreviations: Ace, acetate; Prp, propionate; But, butyrate; 10X, 25 mM acetate, 5 mM propionate, 0.5 mM butyrate; CF high, 2.5 mM acetate, 0.5 mM propionate, 0.05 mM butyrate; CF low, 0.5 mM acetate, 0.1 mM propionate, 0.005 mM butyrate. Cytomix (IL-1β, TNF-α, IFN-γ; 10 ng/ml each). Naïve controls represent cell supernatants in the absence of SCFAs or cytomix. Results of unpaired, non-parametric t-tests compared to cytomix: *, p < 0.05; **, p < 0.01; ***, p < 0.001.
Figure 24. Relative IL-6 secretion profile in stimulated A549 cells.
Abbreviations: Ace, acetate; Prp, propionate; But, butyrate; 10X, 25 mM acetate, 5 mM propionate, 0.5 mM butyrate; CF high, 2.5 mM acetate, 0.5 mM propionate, 0.05 mM butyrate; CF low, 0.5 mM acetate, 0.1 mM propionate, 0.005 mM butyrate. Cytomix (IL-1β, TNF-α, IFN-γ; 10 ng/ml each). Naïve controls represent cell supernatants in the absence of SCFAs or cytomix. Results of unpaired, non-parametric t-tests compared to cytomix: *, p < 0.05; **, p < 0.01; ***, p < 0.001.
Figure 25. Relative MCP-1 secretion profile in stimulated A549 cells.
Abbreviations: Ace, acetate; Prp, propionate; But, butyrate; 10X, 25 mM acetate, 5 mM propionate, 0.5 mM butyrate; CF high, 2.5 mM acetate, 0.5 mM propionate, 0.05 mM butyrate; CF low, 0.5 mM acetate, 0.1 mM propionate, 0.005 mM butyrate. Cytomix (IL-1β, TNF-α, IFN-γ; 10 ng/ml each). Naïve controls represent cell supernatants in the absence of SCFAs or cytomix. Results of unpaired, non-parametric t-tests compared to cytomix: *, p < 0.05; **, p < 0.01; ***, p < 0.001.
3.4 SCFA effects on CFBE

3.4.1 SCFAs do not influence iNOS expression in CF epithelial cells

Initially we intended to assay for iNOS in CF cell lines as a better representative model of CF patient airways. We were however unable to detect iNOS protein expression in either cytomix-stimulated CFBE41o-, bronchial epithelial cells immortalized from a patient homozygous for the F580del mutation of the CFTR protein, or in CFBE41o- stably transfected with a vector expressing wild-type full length CFTR. Pre-incubation with SCFAs did not induce otherwise absent iNOS expression.

3.4.2 SCFA-mediated regulation of cytokine secretion in CF cells

CFBE41o- F508del and CFBE41o- corrected CFTR cell lines were incubated with or without SCFAs and stimulated with cytomix. In a preliminary panel of cytokines, we found considerable secretion in at least one of the two cell lines for G-CSF, GM-CSF, IFN-α2, IL-1α, IL-6, IL-7, IL-8, IP-10, MCP-1, and MIP-1α (not shown). We tested but did not find significant for eotaxin, IL-2, IL-3, IL-4, IL-5, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-15, IL-17A, MIP-1β, or TNF-β. For subsequent analyses, we examined G-CSF, GM-CSF, IL-1α, IL-6, IL-8, IP-10 and MCP-1 secretion, as well as the levels of cytomix cytokines. We only tested high (2.5 mM acetate, 0.5 mM propionate, 0.05 mM butyrate) and low (0.5 mM butyrate, 0.1 mM propionate, 0.005 mM butyrate) CF concentrations of SCFAs, as well as in combination (APB high and low) in CF cell lines.

G-CSF and GM-CSF were secreted at about 67 pg/ml and 194 pg/ml, respectively, by CFBE41o-/F508del cells in response to cytomix (Table 8). SCFA pre-incubation did not significantly affect this. In CFBE41o-/corrCFTR cells, G-CSF and GM-CSF secretion was similar (108 pg/ml and 128 pg/ml, respectively) but both cytokines responded positively to SCFAs. G-CSF in particular showed up to 1.6 fold increase in secretion with 0.5 mM propionate, and to a lesser extent acetate and 0.1 mM propionate, in CFBE41o-/corrCFTR (Figure 26). GM-CSF secretion was also significantly increased by 0.5 mM propionate in corrected cells (Figure 27, 1.44 fold, p < 0.05), but in the mutant cells there was no change. IL-1α secretion was below the
Table 8. Cytokine concentrations of CFBE41o-/F508del and CFBE41o-/corrCFTR culture supernatants incubated with SCFAs and stimulated with cytomix.

<table>
<thead>
<tr>
<th>CFBE41o-/F508del</th>
<th>CFBE41o-/corrCFTR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acetate</strong></td>
<td></td>
</tr>
<tr>
<td>2.5 mM</td>
<td>58.7  183.7  1.7  2818.5  1007.9  838.8  2717.3  2530.8</td>
</tr>
<tr>
<td>0.5 mM</td>
<td>53.9  144.4  0.5  1815.3  760.8   672.5  2196.8  1741.0</td>
</tr>
<tr>
<td><strong>Propionate</strong></td>
<td></td>
</tr>
<tr>
<td>0.5 mM</td>
<td>61.1  201.4  1.9  2160.8  1160.8  739.2  3479.5  2117.8</td>
</tr>
<tr>
<td>0.1 mM</td>
<td>57.3  179.9  0.9  2228.0  1015.7  777.2  3001.9  1849.0</td>
</tr>
<tr>
<td><strong>Butyrate</strong></td>
<td></td>
</tr>
<tr>
<td>0.05 mM</td>
<td>69.1  210.5  0.9  2935.9  1085.3  835.3  3235.8  2590.4</td>
</tr>
<tr>
<td>0.005 mM</td>
<td>60.7  169.9  1.2  2391.5  906.6   733.6  2708.3  2045.2</td>
</tr>
<tr>
<td><strong>Combo</strong></td>
<td></td>
</tr>
<tr>
<td>APB high</td>
<td>58.2  200.7  1.1  2646.7  983.4   754.1  3121.5  2049.7</td>
</tr>
<tr>
<td>APB low</td>
<td>68.9  204.7  0.9  2625.8  970.2   793.9  2763.3  2100.9</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
</tr>
<tr>
<td>naïve</td>
<td>7.4   7.5    0.5  0.5     87.2    140.5  99.8    0.5</td>
</tr>
<tr>
<td>cytomix</td>
<td>67.8  194.8  0.6  3063.6  953.2   700.7  2731.2  2362.3</td>
</tr>
<tr>
<td><strong>Acetate</strong></td>
<td></td>
</tr>
<tr>
<td>2.5 mM</td>
<td>164.0 114.8  2.2  2779.8  2729.1  14699.9  2.9  2139.8</td>
</tr>
<tr>
<td>0.5 mM</td>
<td>135.1 118.3  3.3  2528.8  2119.0  13476.1  2.4  2105.3</td>
</tr>
<tr>
<td><strong>Propionate</strong></td>
<td></td>
</tr>
<tr>
<td>0.5 mM</td>
<td>174.6 184.6  6.5  2883.0  7454.6  12927.2  2.8  2312.2</td>
</tr>
<tr>
<td>0.1 mM</td>
<td>138.2 140.4  3.0  2377.7  2793.9  14921.2  2.6  2262.1</td>
</tr>
<tr>
<td><strong>Butyrate</strong></td>
<td></td>
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<tr>
<td>0.05 mM</td>
<td>131.8 125.8  3.1  2531.0  2169.8  14402.0  2.7  2477.7</td>
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<tr>
<td>0.005 mM</td>
<td>136.6 119.4  3.0  2322.9  2450.2  14312.8  2.3  2290.0</td>
</tr>
<tr>
<td><strong>Combo</strong></td>
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<td>117.6 143.7  3.5  2477.3  2451.9  13508.3  2.5  2191.3</td>
</tr>
<tr>
<td>APB low</td>
<td>163.8 149.6  2.7  2947.4  2863.5  15497.7  2.7  2307.6</td>
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<tr>
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<td></td>
</tr>
<tr>
<td>naïve</td>
<td>9.6   15.9   0.5  0.5     402.1   161.6  1.5    0.8</td>
</tr>
<tr>
<td>cytomix</td>
<td>109.0 127.8  3.4  2707.2  2089.0  13242.5  2.6  2340.4</td>
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</tbody>
</table>

§ - cytokines that registered signals below the lowest standard curve value of 3.2 pg/ml and were extrapolated.

# - cytokines that registered signals above the highest standard curve value of 10000 ng/ml and were extrapolated.

Naïve controls represent cell supernatants in the absence of SCFAs or cytomix. Cytomix (IL-1β, TNF-α, IFN-γ; 10 ng/ml each).
Figure 26. Relative G-CSF secretion profile in CFBE41o-/F508del and CFBE41o-/corrCFTR cell lines in response to SCFA incubation and stimulation with cytomix for 24 hours.

Abbreviations: Ace, acetate; Prp, propionate; But, butyrate; APB hi, 2.5 mM acetate, 0.5 mM propionate, 0.05 mM butyrate; APB low, 0.5 mM acetate, 0.1 mM propionate, 0.005 mM butyrate. Cytomix (IL-1β, TNF-α, IFN-γ; 10 ng/ml each). Naïve controls represent cell supernatants in the absence of SCFAs or cytomix. Results of unpaired, non-parametric t-tests compared to cytomix for each respective cell type: *, p < 0.05; **, p < 0.01; ***, p < 0.001.
Figure 27. Relative GM-CSF secretion profile in CFBE41o-/F508del and CFBE41o-/corrCFTR cell lines in response to SCFA incubation and stimulation with cytomix for 24 hours.

Abbreviations: Ace, acetate; Prp, propionate; But, butyrate; APB hi, 2.5 mM acetate, 0.5 mM propionate, 0.05 mM butyrate; APB low, 0.5 mM acetate, 0.1 mM propionate, 0.005 mM butyrate. Cytomix (IL-1β, TNF-α, IFN-γ; 10 ng/ml each). Naïve controls represent cell supernatants in the absence of SCFAs or cytomix. Results of unpaired, non-parametric t-tests compared to cytomix for each respective cell type: *, p < 0.05; **, p < 0.01; ***, p < 0.001.
detection limit in CFBE41o-/F508del (fold change was calculated relative to extrapolated values which were considered unreliable) and very low in CFBE41o-/corrCFTR, but did show an increase in CFBE41o-/corrCFTR by 0.5 mM propionate (Figure 28, 1.94 fold, p < 0.001). IL-1β secretion was notable in that in CFBE41o-/F508del, 0.5 mM acetate and 0.5 mM propionate decreased secretion by 30-40% (Figure 29, p < 0.001), and CFBE41o-/corrCFTR showed a ~15% decrease with 0.1 mM propionate and 0.005 mM butyrate (p < 0.05). IL-6 was increased by 0.5 mM propionate (1.22 fold, p < 0.05) in the CFBE41o-/F508del, and also by SCFAs CFBE41o-/corrCFTR (Figure 30). In the corrected cells although there appeared to be a large increase (3.5 fold) for IL-6 by 0.5 mM propionate, the difference was not statistically significant. IL-6 was also secreted at the highest levels by naïve CFBE41o-/corrCFTR, at 402 pg/ml. TNF-α secretion was decreased only in CFBE/F508del with 0.5 mM acetate and APB low.

We observed a dichotomy with both IP-10 and MCP-1 (Figure 32, Figure 33). IP-10 secretion is modest in CFBE41o-/F508del, and not significantly altered by SCFAs (Figure 33). In contrast, IP-10 secretion by CFBE41o-/corrCFTR was above the detection limit of the assay (> 10000 pg/ml), and thus SCFA-mediated changes were undetectable. Both cell types secreted IP-10 at similar levels (140 pg/ml vs 162 pg/ml) in the absence of cytomix stimulation. MCP-1 exhibited the opposite trend, where CFBE41o-/F508del secreted the cytokine at high levels (2731 pg/ml in cytomix alone) compared to being under the detection limit in CFBE41o-/corrCFTR. In CFBE41o-/F508del, 0.5 mM propionate increased MCP-1 by ~25% (p < 0.05).
Figure 28. Relative IL-1α secretion profile in CFBE41o-/F508del and CFBE41o-/corrCFTR cell lines in response to SCFA incubation and stimulation with cytomix for 24 hours.

Abbreviations: Ace, acetate; Prp, propionate; But, butyrate; APB hi, 2.5 mM acetate, 0.5 mM propionate, 0.05 mM butyrate; APB low, 0.5 mM acetate, 0.1 mM propionate, 0.005 mM butyrate. Cytomix (IL-1β, TNF-α, IFN-γ; 10 ng/ml each). Naïve controls represent cell supernatants in the absence of SCFAs or cytomix. Results of unpaired, non-parametric t-tests compared to cytomix for each respective cell type: *, p < 0.05; **, p < 0.01; ***, p < 0.001.
Figure 29. Relative IL-1β secretion profile in CFBE41o-/F508del and CFBE41o-/corrCFTR cell lines in response to SCFA incubation and stimulation with cytomix for 24 hours.

Abbreviations: Ace, acetate; Prp, propionate; But, butyrate; APB hi, 2.5 mM acetate, 0.5 mM propionate, 0.05 mM butyrate; APB low, 0.5 mm acetate, 0.1 mM propionate, 0.005 mM butyrate. Cytomix (IL-1β, TNF-α, IFN-γ; 10 ng/ml each). Naïve controls represent cell supernatants in the absence of SCFAs or cytomix. Results of unpaired, non-parametric t-tests compared to cytomix for each respective cell type: *, p < 0.05; **, p < 0.01; ***, p < 0.001.
Figure 30. Relative IL-6 secretion profile in CFBE41o-/F508del and CFBE41o-/corrCFTR cell lines in response to SCFA incubation and stimulation with cytomix for 24 hours.

Abbreviations: Ace, acetate; Prp, propionate; But, butyrate; APB hi, 2.5 mM acetate, 0.5 mM propionate, 0.05 mM butyrate; APB low, 0.5 mM acetate, 0.1 mM propionate, 0.005 mM butyrate. Cytomix (IL-1β, TNF-α, IFN-γ; 10 ng/ml each). Naïve controls represent cell supernatants in the absence of SCFAs or cytomix. Results of unpaired, non-parametric t-tests compared to cytomix for each respective cell type: *, p < 0.05; **, p < 0.01; ***, p < 0.001.
Figure 31. Relative TNF-α secretion profile in CFBE41o-/F508del and CFBE41o-/corrCFTR cell lines in response to SCFA incubation and stimulation with cytomix for 24 hours.

Abbreviations: Ace, acetate; Prp, propionate; But, butyrate; APB hi, 2.5 mM acetate, 0.5 mM propionate, 0.05 mM butyrate; APB low, 0.5 mM acetate, 0.1 mM propionate, 0.005 mM butyrate. Cytomix (IL-1β, TNF-α, IFN-γ; 10 ng/ml each). Naïve controls represent cell supernatants in the absence of SCFAs or cytomix. Results of unpaired, non-parametric t-tests compared to cytomix for each respective cell type: *, p < 0.05; **, p < 0.01; ***, p < 0.001.
Figure 32. Relative MCP-1 secretion profile in CFBE41o-/F508del and CFBE41o-/corrCFTR cell lines in response to SCFA incubation and stimulation with cytomix for 24 hours.

Abbreviations: Ace, acetate; Prp, propionate; But, butyrate; APB hi, 2.5 mM acetate, 0.5 mM propionate, 0.05 mM butyrate; APB low, 0.5 mM acetate, 0.1 mM propionate, 0.005 mM butyrate. Cytomix (IL-1β, TNF-α, IFN-γ; 10 ng/ml each). Naïve controls represent cell supernatants in the absence of SCFAs or cytomix. Results of unpaired, non-parametric t-tests compared to cytomix for each respective cell type: *, p < 0.05; **, p < 0.01; ***, p < 0.001.
Figure 33. Relative IP10 secretion profile in CFBE41o-/F508del and CFBE41o-/corrCFTR cell lines in response to SCFA incubation and stimulation with cytomix for 24 hours.

Abbreviations: Ace, acetate; Prp, propionate; But, butyrate; APB hi, 2.5 mM acetate, 0.5 mM propionate, 0.05 mM butyrate; APB low, 0.5 mM acetate, 0.1 mM propionate, 0.005 mM butyrate. Cytomix (IL-1β, TNF-α, IFN-γ; 10 ng/ml each). Naïve controls represent cell supernatants in the absence of SCFAs or cytomix. Results of unpaired, non-parametric t-tests compared to cytomix for each respective cell type: *, p < 0.05; **, p < 0.01; ***, p < 0.001.
Possibly the most important cytokine in the context of CF lung disease is IL-8. Initially we included IL-8 in our Milliplex assay however the expression was above the detection limit in both CF cell lines (similar to A549 cells). We performed a sandwich ELISA to detect IL-8 in culture supernatants of CFBE41o-F508del and CFBE41o-/corrCFTR and found that F508del cells secreted considerably more IL-8 with cytomix alone (1600 pg/ml vs 400 pg/ml). We also found that acetate at 2.5 mM and 0.5 mM significantly increased the level of IL-8 in CFBE41o-/F508del (Figure 34: 47%, p < 0.01 and 35%, p < 0.05, respectively). There was no significant change in IL-8 secretion by SCFAs in CFBE41o-/corrCFTR.
Figure 34. Relative IL-8 secretion profile in CFBE41o--/F508del and CFBE41o-/corrCFTR cell lines in response to SCFA incubation and stimulation with cytomix for 24 hours.

Abbreviations: Ace, acetate; Prp, propionate; But, butyrate; APB hi, 2.5 mM acetate, 0.5 mM propionate, 0.05 mM butyrate; APB low, 0.5 mM acetate, 0.1 mM propionate, 0.005 mM butyrate. Cytomix (IL-1β, TNF-α, IFN-γ; 10 ng/ml each). Naïve controls represent cell supernatants in the absence of SCFAs or cytomix. Results of unpaired, non-parametric t-tests compared to cytomix for each respective cell type: *, p < 0.05; **, p < 0.01; ***, p < 0.001.
3.5 Effect of SCFAs in a model of acute lung injury

We used a model of acute lung injury to test the effects of a high concentration of SCFAs on inflammation. We instilled BALB/cJ mice intratracheally with 5 µg LPS in 50 µl PBS buffer or PBS with 25 mM acetate, 5 mM propionate, 0.5 mM butyrate (APB) buffer. We performed a second intratracheal administration of PBS or APB buffer 24 hours after LPS instillation to maintain the levels of SCFAs in the lungs. The mice were sacrificed at the 48 hour timepoint and BAL fluid collected.

We measured total cell density and differential cell count as markers of cell infiltration in the lungs. There was a trend toward higher cell numbers in the APB treated mice but the difference was not statistically significant. We measured the differential cell count and found a small but significant increase (0.3% vs 5.4%, p < 0.01) in macrophage cell percentage in the APB buffer-treated group.

We measured the levels of two protein markers of airway inflammation. Citrullinated histone H3 (CitH3) is a marker of neutrophil extracellular traps (NETs), an antimicrobial-protein coated DNA matrix expelled from dying neutrophils upon activation [120]. Surfactant protein-D (SP-D) is an innate immune collectin[121], upregulated in response to inflammatory stimuli and responsible for immunomodulatory effects as well as antimicrobial activity [122–126]. We found a marginally significant decrease (30.8%, p = 0.044) in CitH3 band intensity in BAL of APB-treated animals. There was no difference in the expression of SP-D between the two groups (data not shown).
Figure 35. BAL fluid cell infiltration from LPS-instilled mice given PBS or APB buffer intratracheally.

BAL fluid A) absolute cell density and B) different cell count in mice intratracheally instilled with LPS and treated with PBS or APB (acetate 25 mM, propionate 5 mM, butyrate 0.5 mM) buffer. Means +/- SE are presented. Results of unpaired, nonparametric t-test: *, p < 0.05. n=3 per group. In panel A, the comparison between PBS and APB groups is not significant (p = 0.477).
Figure 36. APB buffer reduces CitH3 in BAL of LPS-instilled mice.
A) Citrullinated histone H3 Western blot of BAL samples. Equal protein was loaded in each lane (10 µg). B) Citrullinated histone H3 (CitH3) band intensity by Western blot densitometric analysis on BAL samples from mice instilled with LPS. Results of unpaired, non-parametric t-test: * , p < 0.05. n=3 per group.
4.0 Discussion

The anaerobic bacterial metabolites SCFAs play a major role in regulating epithelial health and inflammatory responses in the colon. The goal of this work was to shed light on the contribution of SCFAs in the context of lung infections. For this we used the disease model of CF, which involves neutrophilic inflammation and persistent bacterial colonization. We were able to quantify SCFAs in airway secretions of CF patients and found that SCFAs can influence bacterial growth as well as the inflammatory responses of normal and CF airway epithelial cells.

We first determined that SCFAs were present in CF patient sputum. We found acetate and propionate between \(10^{-4}\) M and \(10^{-3}\) M, and butyrate at \(10^{-5}\) M. For reference, the concentrations of SCFAs in the colon are in the range of \(10^{-2}-10^{-1}\) M (30-150 mM), while in blood concentrations are at \(10^{-4}\) M. Despite a limited number of samples, we were able to show that in the majority (7/9) of CF patients presenting with a pulmonary exacerbation, antibiotic treatment led to a reduction in SCFAs (Figure 6). A likely explanation for this outcome is that the antibiotics given to the patients for treatment of their infections may have resulted in considerable reduction in anaerobic bacterial load. Evidence for this line of reasoning can be found in several reports where oral antibiotics resulted in reduced colonic production of SCFAs [127–129].

The patient samples used for SCFA analysis were collected as part of studies aimed at discerning the changes in lung physiology and inflammation following antibiotic treatment. Thus we were able to correlate several other parameters cross-sectionally and longitudinally with SCFA measurements. We expected that since there is evidence of direct SCFA effects on immune cells [83,95,106,130], that a similar effect would be observed in the lungs when higher SCFAs are present. However when looking at all we found no significant correlations between SCFAs and markers of inflammation: cell count, neutrophil percentage, arginase activity, nitric oxide metabolites (NO\(_x\)), NOS activity, neutrophil elastase, or IL-8. We also did not find any correlation between SCFAs and age, weight, sex, or lung function (forced expiratory volume in 1 second, FEV\(_1\) % of predicted). Upon subgroup analysis, we found a significant correlation (Spearman \(r^2 = 0.882, p = 0.002\)) between total SCFAs and cell counts/g mucous in stable CF patients (Figure 8A); this was not found in samples from patients before or
after antibiotic treatment. This follows because literature suggests that SCFAs can promote the recruitment of immune cells, particularly neutrophils [76,106,131,132], and CF airways have pronounced persistent neutrophilic inflammation [4,133]. In addition, we found a significant negative correlation (Spearman $r^2 = 0.749$, $p = 0.024$) between the fold change in sputum NO$_x$ concentrations after antibiotic treatment and total SCFAs before treatment (Figure 8B). This relationship indicates that SCFAs may be a potential biomarker for improvement following antibiotic treatment, as patients with increased SCFAs before treatment showed the smallest increase in NO$_x$. These relationships suggest that the presence of SCFAs may have some effect on the inflammatory environment of the airways. It remains to be determined whether the causative agent of these changes is the presence of SCFAs, or the airway environment conducive to SCFA production, or if anaerobic bacteria are responsible for modifying the inflammatory response. Pairing SCFA measurement with bacterial microbiome cataloguing [18,22,134] could shed light on this question.

SCFAs are involved in a number of metabolic processes as both degradative products and building blocks for macromolecules. Bacteria generally produce SCFAs as waste under anaerobic conditions to restore NAD$^+$ and the reducing environment. Although anaerobic bacteria cannot reincorporate SCFAs, bacteria or other organisms in the surrounding environment can take up and use SCFAs. It is now well understood that the CF airways are polymicrobial in nature and harbour multiple bacterial species exploiting different niches [18,22,134–136]. To study the effect of SCFAs on a prominent CF pathogen, *P. aeruginosa*, we developed a simple 96-well absorbance based plate assay of bacterial growth. We incubated the laboratory strain (PAO1) or a mucoid CF clinical isolate (PA508) with SCFAs from 3.125 mM to 100 mM in TSB and monitored bacterial growth by OD under aerobic or microaerobic conditions. We found that SCFAs at the lower concentrations increased bacterial growth compared to TSB alone. We found that this effect was abrogated by reducing the pH of the media. *P. aeruginosa* growth was actually increased in HCl-acidified TSB, however it was more susceptible to influence by SCFAs.

A possible explanation for the changes in bacterial growth with SCFA incubation could be that at low concentrations, the presence of SCFAs acts as an additional source
of assimilable nutrients. This would help the bacteria grow more quickly by bypassing
the breakdown of more complex nutrients like glucose or amino acids. As the bacteria
consume SCFAs in the broth over time, the advantage compared to control broth is lost
and the effect of increased growth is diminished. This might explain why the highest
growth increases we see are in the early timepoints between 1 and 3 hours. Bacterial
growth curves involve an initial lag phase, a log phase with rapid population doubling,
and a stationary phase where bacterial growth slows down and the rate of dividing
bacteria and dying bacteria begin to equalize. After 4-5 hours in our assay, bacteria
begin entering the stationary phase of their growth. SCFA-mediated growth-promoted
cultures have an initial boost that wanes after the likely depletion of SCFAs in the broth,
and would thus be expected to reach the stationary phase earlier than the control broth.
Our analysis shows the relative OD per timepoint, so it may be that the advantage given
to SCFA-treated bacteria is masked by the fact that their growth saturates at a time
when the control cultures are still growing rapidly. A more sensitive assay may be useful
to see whether SCFAs can positively influence the growth of bacteria at lower densities.

We further demonstrated that SCFAs at higher concentrations, similar to those
found in the colon, lead to significantly reduced bacterial growth. Of note is that this
effect was not related to killing of bacteria, but appeared to be an arrest of metabolism.
We confirmed this by serially diluting and plating bacteria treated with SCFAs following
the incubation period where OD readings were unchanged from the start of the
experiment. We saw that there was still a very high CFU count (>10^8 CFU/ml),
indicating that the bacteria were still viable but did not grow or divide as rapidly. We did
not detect any overt differences when comparing PAO1 and PA508 responses to
SCFAs. We postulate that the growth inhibitory effects of SCFAs could be due to
overwhelming the cellular machinery involved in glucose breakdown and fatty acid
synthesis. As mentioned in the introduction, acetate is a crucial intermediate molecule
and is usually bound to co-enzyme A (CoA). The presence of high concentrations of
SCFAs may overload the cells with intermediary products which disrupt the ratio of
substrate to products, limiting the ability, for example, of TCA cycle enzymes to continue
the cycle.
The mechanism behind the pH dependency of SCFA-mediated bacterial growth inhibition is unclear. We were able to rule out a SCFA-independent role of pH by incubating bacteria in nutrient broth buffered with various Good’s buffers (PIPE for pH 7.0 and 6.5, MES for pH and 5.5) and adjusted with HCl. We found no difference between bacteria grown in broth buffered at low pH compared to the condition at neutral pH. In contrast with these control experiments, our SCFA incubation experiments were not buffered and the adjusted pH of the broth represents the initial conditions. We measured the pH of the broth during and after the assay for certain experiments and found that when bacteria grew robustly, the pH approached 7.0-7.5, even if the initial pH was adjusted to 5.5. For bacteria grown with high concentrations of SCFAs, we observed smaller changes in pH. It is likely that these observations are not due to a buffering effect of SCFAs, but rather a consequence of lower bacterial metabolic activity and byproducts that would contribute to pH alterations. Earlier we discussed a possible mechanism for growth inhibition by overloading the metabolic cycles. At low pH a larger percentage of SCFAs will be protonated, and thus would have a much higher diffusion rate through cell membranes.

Higher intracellular concentrations of SCFAs may be responsible for increased growth of bacteria. One way this idea can be tested by titrating CoA, the major chaperone molecule for SCFAs, to bacterial cultures incubated with SCFAs. This might relieve some of the burden of SCFA presence. Another option would be to add exogenous NAD\(^+\) to restore the reducing environment to see whether this is cause of growth inhibition.

Our experiments on the influence of SCFAs on bacterial growth were limited to the Gram-negative bacterium \textit{P. aeruginosa}. We chose to study this bacterial species due to its preponderance in CF lung disease and its association with severe morbidities in CF patients. Nevertheless there are a number of other Gram-negative and Gram-positive bacteria important in CF lung disease, such as \textit{Stenotrophomonas maltophilia} and \textit{S. aureus}. Reports suggest that Gram-negative species grow slower in response to high concentrations of SCFAs, but that Gram-positives do not [116]. Although there is not much documented, extrapolating from this and our data we might infer that other Gram-negative species would experience a similar growth increase as we have shown
for *P. aeruginosa*. Furthermore, we speculate that since Gram-positive *S. aureus* is not inhibited by high concentrations of SCFAs, even low concentrations may induce significant growth increases. The observation that growth of Gram-positives is not inhibited by high SCFA concentrations along with our findings could possibly implicate SCFAs as a factor in the maintenance of bacterial communities in CF lung disease [18,134,135].

We took the concentrations of SCFAs found in sputum of CF patients as reference for our subsequent experiments. We picked concentrations at the high and low ends of the ranges found in CF patients, and in other experiments we used concentrations similar to those found in the colon. Since we study the innate immune system, a logical application of SCFAs was to test their effects on airway epithelial cells, which are important players in the innate immune response of the airways. We used A549 type II airway epithelial cells to model the effects of SCFAs on induction of iNOS [137]. iNOS is a relevant and important protein in the context of lung infections, because its expression is directly linked to the inflammatory state of the airways [138]. The iNOS promoter region includes multiple NFκB response elements [139,140], and iNOS expression is thus controlled by many cytokines and inflammatory stimuli including IFN-γ, TNF-α, IL-1β, and LPS. Important mediators of the interferon signaling cascade controlling iNOS expression are p38 MAPK, ERK1/2, JAK/STAT1, and interferon response factor 1 (IRF1) [137,140–142]. The link between SCFAs and iNOS can be found in numerous studies of immune cells such as neutrophils and macrophages [105,108,132,143,144], but comparatively few studies were done on epithelial cells. Among the reports of the latter, mammary epithelial cells have been studied because of increases in blood SCFA concentrations during lactation. It was found that both FFAR2 and FFAR3 are expressed in mammary epithelial cells and that SCFA stimulation resulted in activation of p38 MAPK [145,146]. Also it was shown that SCFA stimulation could reduce the internalization of *S. aureus* bacteria in mammary epithelial cells [147].

As alluded to in section 1.8, effects of SCFAs on iNOS have also been studied in intestinal epithelial cells. There, butyrate induced higher iNOS expression after IFN-γ/LPS stimulation [109]. In our experiments in A549 airway epithelial cells, we found that SCFAs could result in increased or decreased iNOS expression depending on the
concentrations used. At low concentrations, similar to those found in sputum from CF patients, SCFAs induce increased iNOS expression. Acetate at 2.5 mM (not presented), propionate from 0.1 mM to 1.0 mM, and butyrate at 0.1 mM to 0.5 mM resulted in a significant increase in iNOS protein expression compared to cytomix stimulation alone. When using 10-fold higher concentration of SCFAs, approaching SCFA concentrations normally found in colon, iNOS protein expression was reduced. This effect was most pronounced with propionate and butyrate at 25 and 50 mM, followed by acetate at 25 mM and 50 mM (not shown). The mechanism of SCFA reduction in iNOS expression following cytomix stimulation is still unclear, however experiments using MAPK inhibitors suggested that p38 MAPK or MEK1/2 (kinases upstream of ERK1/2) may be involved. Based on published reports, p38 and ERK1/2 activity can be influenced by SCFAs depending on the cell type. As yet, it is unclear whether SCFAs signal through FFAR2 or FFAR3 in airway epithelial cells. We tested for FFAR2 protein expression in A549 cells by Western blot and immunofluorescence but did not reliably detect expression of the receptor (data not shown).

We tested the effect of SCFAs on the inflammatory state of CFBE41o- cells to model the interaction expected in CF patient airways. We were unable to detect any changes in iNOS expression in these cells. Other researchers have reported that there is deficient iNOS expression in CF airway epithelium [148–152]. Whether iNOS dysregulation may be one of the factors mediating inefficient bacterial clearance in CF is unclear and therefore represents an important question. This finding included CF mouse and human primary airway epithelial cells, as well as several immortalized CF airway epithelial cell lines. An analysis of iNOS regulation in airway epithelial cells indicated that IRF1 and protein inhibitor of activated STAT1 (PIAS1) influenced iNOS expression and these proteins were dysregulated in CFTR<sup>-/-</sup> murine epithelial cells [153]. STAT1 regulates IRF1, which acts as a transcription factor to drive iNOS gene expression. PIAS1 inhibits STAT1 activation, and can thus regulate IRF1 and iNOS. IRF1 is downregulated and PIAS is upregulated in CF [153,154]. Further investigation showed that STAT1 activation is controlled by Rho GTPase [155], an enzyme important in regulating cholesterol homeostasis. iNOS expression in CF cells was rescued by statin
treatment [154], suggesting that dysregulated cholesterol in CF [156] plays a role in suppressing iNOS induction.

Another tool to study the impact of CFTR deficiency is the CFBE41o- cell line stably transfected to express wild-type CFTR (described in [157]). This cell line exhibits normal transepithelial resistance when compared to CFBE41o-, and thus represents a useful control. We used these cells to test for iNOS induction and the effect of SCFAs; again, we did not detect any iNOS expression. We postulate that the mechanisms by which iNOS is dysregulated in CF cells stem from an abundance of improperly trafficked CFTR/F508del protein, which is not changed when wild-type CFTR is co-expressed to normal levels. Recent pharmaceutical developments have resulted in drugs that can correct the defects in CFTR (the G551D mutation is potentiated by VX-770/lvacaftor [158–160]). It remains to be seen whether this drug can also restore cholesterol homeostasis, STAT1 signaling, and iNOS expression, and whether new drugs or drug combinations targetting the F508del can act similarly. While we were unable to study the effect of SCFAs on iNOS expression in CFBEs, our data obtained from experiments using A549 cells suggest that SCFAs do have an effect on iNOS expression in cells that express this NOS isoform upon stimulation. As neutrophils, macrophages, and other cells are thought to express iNOS in CF, the effects of SCFAs of these cells need to be investigated further.

Though we could not employ iNOS as a marker of the inflammatory state in CF cells, we measured the levels of a number of cytokines affected by cytomic stimulation. We used concentrations of SCFAs at the upper and lower range found in CF sputum. Looking at A549 cells, we found that a number of pro-inflammatory cytokines such as GM-CSF, IL-1α, IL-6, MCP-1 were induced by cytomic stimulation and were significantly upregulated by SCFA pre-incubation. The changes in cytokine secretion were similar in CFBE41o-/corrCFTR cells and A549 cells. SCFAs upregulated G-CSF, GM-CSF, IL-1α, IL-6, and MCP-1 secretion in CFBE41o-/corrCFTR. In CFBE41o-/F508del cells however, SCFAs increased IL-6, IL-8, and MCP-1 secretion, but decreased IL-1β and TNF-α. We used these cytokine profiles to compare between control cells and CFBE41o-/-F508del. We found that important TH2 cytokines are upregulated and TH1 cytokines are downregulated in CF cells, whereas both TH1 and TH2 cytokines are
upregulated by SCFAs in A549 and CFBE41o-/corrCFTR. At the same time, the chemokines MCP-1 and IL-8, recruiters of monocytes and neutrophils, respectively, were upregulated mainly in CFBE41o-/F508del.

These data are in accordance with recently published reports suggesting that CF epithelial cells have impaired T\(_H\)1 response due to diminished type I interferon signaling [161]. Likewise, the inflammatory response of airways of patients with CF has been described as predominantly T\(_H\)2 [162]. In addition, a report suggests that patients infected with \textit{P. aeruginosa} have a predominant T\(_H\)2 response, whereas patients without \textit{P. aeruginosa} had a higher T\(_H\)1 response [163]. Furthermore the authors found that IL-4 and IL-13, important T\(_H\)2 cytokines, correlated negatively with forced expiratory volume in 1 second, a measure of lung function. In contrast however, we found that the ratio of GM-CSF to G-CSF was higher in CFBE41o-/F508del compared to CFBE41o-/corrCFTR. Incubation with SCFAs further skewed the ratio towards GM-CSF in F508del but towards G-CSF in corrCFTR cells. It was reported that in peripheral blood monocytes of patients with CF infected with \textit{P. aeruginosa}, the ratio of GM-CSF to G-CSF correlated positively with lung function and the ratio of T\(_H\)1 over T\(_H\)2 response [164]. Taken together this suggests that SCFAs may influence the inflammatory state of the airways, but that further experiments are necessary to determine the ultimate impact of these changes on lung function and airway inflammation.

We did a preliminary study testing the effects of SCFAs in an acute lung injury model. We instilled LPS intratracheally in BALB/cJ mice and administered PBS or SCFAs in PBS (APB buffer) at 0 hours and 24 hours after LPS. We found that the percent of macrophages in APB-treated mice were significantly higher than in PBS-treated mice and there was a trend toward higher total cell count. We also found that NET formation was reduced as indicated by CitH3 blots. Our \textit{in vitro} epithelial cell experiments suggest that SCFAs may increase the expression of chemoattractant cytokines MCP-1 and IL-8 which may result in an increase in monocyte and neutrophil numbers, respectively, in the airways. In addition SCFAs themselves may be acting as chemotactic agents through FFAR2 directly on these immune cells. If the effect we observed is verified, an interesting study would be to use FFAR2 global knockout mice or immune cell specific FFAR knockouts to see whether the increase in airway immune
cells is due to SCFA-mediated chemotaxis or increased cytokine release. This suggests that SCFAs may influence the progression of inflammation in the airways and should be investigated in greater detail.

Integrating our findings of SCFAs in the context of the airways with evidence from the literature, we propose a model where anaerobic bacteria produce SCFAs which diffuse through the airway surface liquid (Figure 37). In anaerobic pockets, SCFA production and concentration is presumed to be high, mediating anti-inflammatory effects on host cells and growth suppression on bacterial cells. As SCFAs diffuse into more oxygenated airway surface liquid, lower concentrations can promote the growth of other bacterial species such as P. aeruginosa. In addition to increasing bacterial growth, lower concentrations of SCFAs can promote the inflammatory condition of the airway epithelium. Overall, SCFAs may also promote the infiltration of neutrophils and monocytes into the airways.
Figure 37. Proposed model of SCFA production in CF airways.
A model of the CF airways is depicted where anaerobic bacteria (red rods) produce SCFAs (acetate, blue rectangles, propionate, white triangles, butyrate, yellow squares) in airway surface liquid/mucous layer with low oxygen, possibly presenting a biofilm phenotype. In this microenvironment SCFAs may act to suppress the inflammatory response of airway epithelial cells as well as immune cells such as neutrophils. As SCFAs diffuse away to areas with more oxygen, lower concentrations exacerbate inflammation mediated by epithelial cells and promote the growth of other bacteria in aerobic and planktonic form. AEC, alveolar epithelial cell.
5.0 Conclusions and Future Directions

5.1 Conclusions
From the work presented here, we can conclude that SCFAs are present in the airways secretions of CF patients. We further show that SCFAs have dichotomous effects on bacteria and mammalian cells. In bacteria, SCFAs promote growth at concentrations of 3.125-12.5 mM at neutral pH and aerobic conditions. At concentrations from 25-100 mM, SCFAs inhibit growth in both type and clinical mucoid strains of Gram-negative *P. aeruginosa*. The inhibitory effect of SCFAs was observed in both aerobic and microaerobic conditions. Given the anaerobic nature of airways secretions in CF patients and the presence of anaerobic bacterial species, this suggests that SCFA production in the airways may contribute to disease progression and establishment of bacterial communities in CF lung disease.

In A549 airway epithelial cells, we found that SCFAs, at concentrations found in CF patient sputum, can increase the expression of pro-inflammatory iNOS protein in response to cytomix. We also found that SCFAs can upregulate the secretion of pro-inflammatory cytokines like GM-CSF, IL-1α, IL-6 and MCP-1. In CF epithelial cells, we found SCFA-mediated upregulation of IL-6 and MCP1 secretion and downregulation of IL-1β and TNF-α. In contrast wild-type CFTR-corrected CF epithelial cells showed SCFA-mediated increase of G-CSF, GM-CSF, IL-1α, IL-1β, and IL-6 as well as very high expression of IP-10. These results suggest that SCFAs modulate inflammation in CF epithelium differently than in normal epithelium, and shifting the balance towards a **T**H2 response over **T**H1.

There are some limitations to our airway epithelial cell inflammatory response results. The A549 cell line is an adenocarcinomatous cell line was derived from the type II airway epithelial cell. Although this cell line has been widely used to study general phenomena pertaining to airway epithelial cells, primary epithelial cell cultures likely yield results that better represent *in vivo* conditions. Furthermore, our study of only one cell line limits our claims since patient to patient variability is well documented. Similarly,
our experiments with CFBE cells could be strengthened by using multiple CF cell lines which have been generated.

In a preliminary acute lung injury model we found that a high dose of SCFAs administered topically to the lung resulted in increased number of macrophages and reduced neutrophil extracellular trap formation. We conclude that SCFAs in the airways can influence the inflammatory state of the lungs, but further investigation is warranted to identify the effects of lower concentrations and over longer timeframes.

In summary, we identified the presence of bacterial metabolites in the airways and documented their effects on bacterial growth and airway epithelial cells. There are a number of future experiments that can be designed around this hypothesis-generating work.

5.2 Future Directions

We correlated some clinical parameters with SCFA concentrations in CF patients. Since SCFA concentrations are presumed to follow the level of anaerobic bacteria in the lungs, we would consider that SCFAs may be a useful biomarker of anaerobic bacterial activity. Therefore measuring SCFAs and quantifying anaerobic bacterial load or number of anaerobic bacterial species may provide insight into the dynamics of SCFA production.

We showed that SCFAs influence the growth of the Gram-negative bacteria *Pseudomonas*. There are many other bacteria in the CF airways which may be influenced by SCFAs as well. We postulate that Gram-positive bacterial growth can be similarly increased by SCFAs at low concentrations, but have a less pronounced growth inhibition at higher concentrations. Completing these experiments will give a better understanding of the broader effects of SCFAs on bacterial communities in CF.

The effects of SCFAs on airway epithelial cells is not yet completely understood. Further work should be done to characterize the influence of SCFAs on apoptosis, tight junctions, CFTR expression, and bacterial invasion. In addition, the effect of SCFAs on bacterial growth is rudimentary. A co-culture system of CF-relevant SCFA-producing bacteria with SCFA-consuming bacteria would provide important information on the interaction between members of bacterial communities. These results could be
corroborated by performing them using sputum from CF patients as a matrix, or synthetic airway growth medium [165].

The effect of SCFAs on neutrophils has been documented in the literature to some extent, but not exhaustively. A particular feature of neutrophil-dominated CF lung disease is NET formation. Though preliminary data hinted at an SCFA-mediated effect on this cell death mechanism, we did not include it in this manuscript. Further experiments should be done exposing neutrophils to SCFAs prior to activation with various factors and monitor the progression of NET formation.

In our in vivo model, we used high concentrations of SCFAs in combination to alter the inflammation associated with LPS instillation. Though limited, it suggested that it is possible for SCFAs to modify the inflammatory profile of the airways. Further work needs to be carried out to determine whether lower concentrations of SCFAs can mediate similar or opposite effects. In addition, the dosing of SCFAs was not optimized. We treated with SCFAs every 24 hours over two days, but the rate of absorption of SCFAs is likely to be much faster. Additional work should be done to determine the effect of different dosing regimens and different concentrations of SCFAs in inflammatory models like LPS instillation and infection models like P. aeruginosa infection.
6.0 References


### 7.0 Appendix

Appendix A. Statistical analysis of SCFA effect on bacterial growth

**Table A1. Effect of SCFAs on bacterial growth.**

Statistical analysis was performed using repeated measures analysis of variance (ANOVA) with a post-hoc Dunnett test comparing growth curves with SCFAs to their pH-matched controls. A positive mean difference refers to increased growth and a negative mean difference refers to reduced growth. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

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Appendix B. *Pseudomonas*-laden beads project

At the start of my Master’s project I was working on optimizing a *Pseudomonas*-laden bead model of infection in mice. There is a lack of a good model for studying CF-related *Pseudomonas* infection and my goal was to define a better protocol with greater reliability and reduced variability in the size and bacterial density of bead preparations. The full, finalized protocol can be found in section 0.

The beads model of infection has been routinely used to study *Pseudomonas* pneumonia *in vivo*, but has traditionally been associated with great variability over laboratories, operators, and time. The most glaring problems inherent to this model are a lack of defined preparation methods and characterization of the inoculum to be administered to animals. This variability caused either insufficient infection (rapid clearance by mice) or high mortality.

My goal was to optimize and define specific steps to address the variability in size and bacterial density of the bead preparations. Briefly, I optimized the actual bead forming steps to maintain a similar number of beads formed with a similar size distribution. Next I ensured that a specific amount of bacteria are used to prepare the beads. Lastly I excluded the sizes of the beads to between 102 µm and 203 µm using stainless steel mesh swatches. I verified the size distribution of the bead preparations by measuring them using light microscopy (Figure B1) and ImageJ software (Figure B2). This optimized preparation was validated by infecting mice and calculating the bacterial load after 3 days of infection.

This optimized protocol was used to study the effect of arginase inhibition in pneumonia. With Dr. Anne Mehl and Dr. Hailu Huang I prepared *Pseudomonas*-laden beads and infected mice at $2 \times 10^6$ CFU/ml intratracheally. After 3 days the mice were sacrificed and organs were harvested for analysis. We measured NO$_x$, concentrations of amino acids, and cytokines in lung homogenate. We found that arginase inhibition can increase the amount of NO$_x$ in the lungs, which suggests the potential for this approach as a treatment to increase NO in CF patients. This work is being prepared for submission.
Figure B1. Microscopic images of *Pseudomonas*-laden beads.
Light microscopic images taken using a phase contrast ring with A) 20X objective and B) 40X objective. Faint grey spheres are TSA beads, laden with *P. aeruginosa* (black dots).
Figure B2. Bead size distribution without and with mesh size exclusion.
Beads prepared and sized by light microscopic evaluation. A) Beads prepared without using 102 µm mesh. B) Beads prepared using 102 µm mesh. Mean bead size for the preparations were A) 66 µm and B) 162 µm. Anecdotal evidence suggests that very small beads correlate with increased mortality in the mouse model of infection.
Appendix C. Bead preparation protocol

*Pseudomonas aeruginosa* Culture:
- Seed a small scraping of frozen mPAO1 strain stock (-80°C) in 100 mL trypticase soy broth at 37°C for 18 hours.
- Seed the stationary culture into fresh TSB to 1:50. Incubate the culture for 2-3 hours. Measure the OD at 600 nm, and grow until OD reaches ~ 1.0 (corresponds to roughly $3 \times 10^9$ CFU/mL).
- Centrifuge 50 mL of culture at 2000xg for 10 minutes and resuspend with phosphate-buffered saline pH 6.8-7.4 (PBS) to 1.5 mL. This will give approximately $1 \times 10^{11}$ CFU/mL.

Prepare 1.5% trypticase soy agar (TSA):
- dissolve 750 mg of TSA into 50 mL PBS (note: normal TSA is prepared as 4%)
- boil or heat the solution to 85°C+ to ensure all the agar is melted and dissolved
- (optional) autoclave the agar solution
- maintain the agar solution at 50-55°C in a water bath until further use

Prepare mineral oil solution:
- add 100 µL Span-80 (final 0.25%) to 40 mL of heavy mineral oil in a 100 mL beaker
- stir with a 3 cm magnetic stir bar for several minutes to ensure adequate dispersion
- maintain the mineral oil at 50-55°C in a water bath until further use

Preparation of beads:
- place the beaker of mineral oil on a magnetic stir plate and stir at ~100 rpm
- mix 1 mL of *Pseudomonas* cell suspension with 9 mL of heated 1.5% agar
  - this will result in ~$1 \times 10^{10}$ CFU/mL
- withdraw 2 mL of *Pseudomonas*-agar mixture into a 5 or 10 mL syringe with an 18G needle
- dispense the mixture slowly into the oil, such that large droplets form at the opening of the needle
- stir for 5 minutes at room temperature once all the *Pseudomonas*-agar mixture is added
- surround the beaker with wet ice (ie. place the beaker inside a 1000 mL beaker and place ice around it) and stir for 15 minutes

Washing beads:
- aliquot the oil-bead mixture into 15 mL conical centrifuge tubes by pouring or by pipette (note: 50 mL tubes are less efficient in separation)
- centrifuge the beads at 2000xg for 5-10 minutes at 2-8°C, discard the supernatant and resuspend in 5-10 mL of PBS
- (optional) combine the beads into one or two tubes
- repeat washing step at least 3 times
Filtering beads:
- place a swatch of 203 µm stainless steel mesh over a 250 mL beaker
- dispense 2-4 mL of bead suspension onto the mesh and carefully tease the underside of the mesh with a pipette tip or transfer pipette to wick the water through
  - repeat until the bead suspension is all filtered
  - wash the retained beads with 2-4 mL of PBS through the mesh
- place a swatch of 102 µm mesh over another 250 mL beaker
  - repeat the process using the 203 µm-filtered bead suspension
- take the 102 µm mesh with its retained beads and place it in a 100 mL beaker
- wash the beads off of the mesh (place it vertically) with 6-10 mL of PBS
- transfer the filtered bead suspension into a 15 mL tube
- centrifuge to remove excess PBS, leaving roughly 1:1 volume of PBS:bead slurry
- store at +4°C until required

Quantifying bacteria in beads:
- using a sterile glass dounce homogenizer, crush 100-300 µL of bead slurry
- serially dilute the homogenized beads 10-fold
- plate 100 µL of each dilution in duplicate on TSA plates to quantify *P. aeruginosa* CFU/mL in beads