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Optimized production and prebiotic uses of sulfonolipids

Nick Ahrends

08/07/2025 -

01/06/2026



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STUDENT REPORT

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Title: Optimized production and prebiotic uses of sulfonolipids

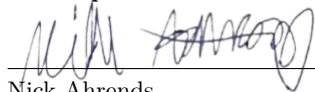
Theme: Medical biotechnology

Project Period: July 8th 2025 to June 1st 2026

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Page Numbers: 74

Appendices: Page 75-121

Date of Completion: June 1st

Abstract:

Inflammatory bowel disease (IBD) affects 2.5–3 million individuals in Europe and is associated with chronic inflammation of the gastrointestinal tract. Although its etiology is not fully understood, both genetic factors and shifts in the gut microbiome's microbial diversity, sulfur metabolism, and the presence of specific bacterial taxa play an important role in IBD. Modulating the microbiome through diet or prebiotics is therefore a promising strategy for improving gut health. Sulfonolipids (Sols) have emerged as potential anti-inflammatory compounds, but their production and effects on the microbiome remain unclear. In this study, Sol production was optimized using *Flavobacterium johnsoniae* (Fjoh), with CYE medium supplemented with 0.5 g/L cysteate yielding increased biomass and Sol production. Fjoh was shown to produce a Sol profile similar to that of the gut-associated genus *Alistipes*. The effects of Sols were tested by incubating pooled human stool samples with extracted lipids. Sol supplementation significantly increased hydrogen sulfide (H₂S) production but did not consistently affect short-chain fatty acid (SCFA) levels, as measured by capillary electrophoresis and quantitative NMR. Furthermore, 16S rRNA gene amplicon sequencing showed no significant changes in microbial diversity, and quantitative PCR analysis indicated no effect on the absolute abundance of *Alistipes*. Overall, while Sols can influence microbial metabolism, particularly by increasing H₂S production, they do not substantially alter microbiome composition or SCFA production under the tested conditions.

Preface

This thesis was written by Nick Ahrends as part of the 9th–10th semester of the Biotechnology program at Aalborg University’s Department of Chemistry and Bioscience, in collaboration with the Division of Microbial Ecology at the University of Vienna. The project was carried out from July 2025 to June 2026 and focused on optimizing sulfonolipid production and assessing its potential prebiotic effects on the gut microbiome. The author would like to thank the supervisors Jeppe Lund Nielsen, Alexander Loy, Tomohisa Sebastian Tanabe, Lærke Valsted Bak Nielsen, and Søren Østergaard Gertsen for their guidance throughout the project period.

*The project was carried out during the 9-10 semester
July 8th, 2025 – June 1st, 2026*

Nomenclature

Abbreviations

A		M	
ANOVA	Analysis of Variance	MS	Mass spectrometry
C		m/z	Mass to charge
CCA	Canonical Correspondence Analysis	N	
CYE	Casitone Yeast Extract (media)	NMR	Nuclear magnetic resonance
CE	Capillary electrophoresis	NGS:	Next Generation Sequencing
D		O	
DNTP	Deoxynucleotide triphosphates	OD	Optical Density
DNA	Deoxyribonucleic acid	OTU	Operational taxonomic units
dsDNA	Double stranded Deoxyribonucleic acid	P	
E		PCR	Polymerase chain reaction
ESI	Electron spray ionization	Q	
<i>E. coli</i>	<i>Escherichia coli</i>	qNMR:	Quantitative nuclear magnetic resonance
F		qPCR:	Quantitative polymerase chain reaction
Fjoh	<i>Flavobacterium johnsoniae</i>	R	
G		RPC	Reversed-phase chromatography
GI	Gastrointestinal	S	
H		sd	Standard deviations
H₂S	Hydrogen sulfide	SDS	Sodium dodecyl sulphate-
HPLC	High-Performance Liquid-chromatography	-PAGE	polyacrylamide gel electrophoresis
I		SDS	Sodium dodecyl sulphate
IBD	Inflammatory bowel disease	Sol	Sulfonolipid
L		SRB	Sulfate-reducing bacteria
LC	Liquid chromatography	SCFA	Short-chain fatty acids
LC-MS	Liquid chromatography–mass spectrometry	slit	wavelength selector
LPS	Lipopolysaccharide	T	
		TLR4	Toll-like receptor 4
		U	
		UV-Vis	Ultraviolet-Visible

Symbols

<u>Symbol:</u>	<u>Quantity:</u>	<u>Unit:</u>
c	Concentration	[mol/L]
l	Length, cuvette width	[mm, cm]
h	Height	[m]
m	Mass	[μ g, mg, kg, Da]
n	Molar amount	[mol]
r	Rate	[kg/min, kg/h, kg/y]
t	Time	[s, min, h]
A	Absorbance	[1]
T	Temperature	[$^{\circ}$ C, K]
T	Transmittance	[1]
V	Volume	[L, dm ³]
OD	Optical density	[1]
RT	Retention Time	[s, min, h]

Sub-script

<u>Symbol:</u>	<u>Quantity:</u>	<u>Unit:</u>
0	Initial	[-]
m	Molar (per mole)	[mol ⁻¹]
rel	Relative	[-]

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Introduction

1

Around the world, major health challenges continue to affect millions of individuals and compromise quality of life. In response, the United Nations established the 17 Sustainable Development Goals, including Goal 3, Good Health and Well-being, which aims to ensure healthy lives and promote well-being for all [1].
5 Despite these efforts, chronic health conditions remain widespread and continue to place a significant burden on global populations [2].

One condition that significantly contributes to the disease burden is inflammatory bowel disease (IBD), a group of chronic inflammatory disorders affecting the gastrointestinal (GI) tract [3]. IBD causes symptoms such as diarrhea, often accompanied by blood and mucus, abdominal pain, constipation, nausea, and vomiting [3]. The prevalence of IBD has increased worldwide, with approximately 4.9 million cases reported in 2019 [4]. In Europe alone, IBD affects approximately 2.5-3 million individuals and results in healthcare costs of 4.6-5.6 billion euros annually [5]. The incidence of IBD has been strongly linked to industrialization, with rates below 0.1% in low-income regions but increasing to approximately 1-1.5% in industrialized countries such as Canada, Denmark, and Scotland, where the incidence rate appears to plateau [6]. A study conducted in India, tracking IBD rates in villages over 10 years, showed a 2.5-fold increase in the most urbanized villages compared with the least urbanized. This suggests that the global burden of IBD may continue to increase with urbanization. [7].

IBD most commonly develops between the ages of 15 and 30; however, approximately 25% of cases occur during adolescence, while 10% to 15% develop after the age of 60 [3]. IBD primarily encompasses two diseases: ulcerative colitis and Crohn's disease. Ulcerative colitis, the most common form, is characterized by inflammation of the colon, leading to mucosal damage, friability, and superficial erosions that can cause bleeding [8]. Crohn's disease, in contrast, can affect the entire GI tract and is characterized by discontinuous transmural inflammation, resulting in symptoms such as diarrhea, abdominal pain, nausea, vomiting, fatigue, and weight loss [9, 10]. IBD can also lead to severe complications, including colon cancer, dehydration, and thrombotic events [11]. Current treatments, including 5-aminosalicylic acid, corticosteroids, and immunomodulators, primarily target inflammatory symptoms rather than the underlying causes of the disease [12]. Although the exact etiology of IBD remains unclear, it is believed to involve complex interactions between genetic susceptibility, environmental factors, immune responses, and the gut microbiome [13].

The gut microbiome refers to the community of microorganisms, including bacteria, archaea, and fungi, that inhabit the intestinal tract [14]. In healthy individuals, the gut microbiome consists of approximately 10^{14} microbial cells belonging to numerous species and genera, forming a relatively stable ecosystem over time [14, 15]. These microorganisms influence a wide range of physiological and cellular processes, including metabolism, vitamin synthesis, immune regulation, regulation of inflammatory responses, and, in particular, microbial fermentation of dietary compounds into metabolites such as short-chain fatty acids (SCFA) [15]. The composition of the microbiome is initially shaped by host genetics and early-life diet and typically stabilizes during adulthood [16]. However, factors such as illness, medication, and dietary changes can disrupt this balance and lead to dysbiosis, a condition characterized by reduced microbial diversity and altered community composition, which has been associated with several diseases, including IBD [17, 18].

Diet is considered one of the most important environmental factors influencing the composition and activity of the gut microbiome, as habitual dietary intake continuously supplies nutrients that selectively promote specific microbial taxa and shape the community's metabolic output. [19]. For example, fiber-rich diets have been associated with a reduced risk of developing IBD and with decreased inflammatory activity in affected individuals [20]. Certain dietary compounds can also act as prebiotics, defined as substrates that are selectively utilized by host microorganisms to confer a health benefit [21]. These compounds stimulate the growth and activity of beneficial bacteria, often leading to increased production of SCFAs such as acetate, propionate, and butyrate, which play important roles in maintaining gut health and regulating inflammation [22].

50 In addition to SCFAs, other microbial metabolites may influence host health. One such compound is hydrogen sulfide (H_2S), a gaseous signaling molecule produced by both host cells and gut microorganisms that plays a complex, concentration-dependent role in the intestinal environment, where it affects an array of processes, including cellular stress responses, apoptosis, and inflammation. For inflammation, at high concentrations, typically in the millimolar range, H_2S can have harmful effects and contribute
55 to inflammation, while lower concentrations in the micromolar range may exert protective physiological effects [23]. The production of H_2S in the gut is largely attributed to sulfate-reducing bacteria and the metabolism of sulfur-containing compounds such as the amino acids methionine and cysteine [24]. Therefore, maintaining a balanced production of microbial metabolites, including H_2S , is essential for intestinal homeostasis, as both excessive and insufficient levels may contribute to disease development
60 [25].

Among potential dietary compounds that may influence the microbiome are sulfonolipids (Sol). Sols are a class of sulfur-containing lipids found in plants, algae, and bacteria, where they function as important components of cellular membranes [26]. In bacteria such as the model organism *Flavobacterium johnsoniae* (Fjoh), where Sols account for up to 23.2% of membrane lipid, Sols have been linked to key cellular
65 processes, including gliding motility; Fjoh lacking Sols also lacks the ability to glide [27]. Certain Sols, such as sulfobacin A and B, have attracted increasing attention due to their potential biological activity. These molecules have been reported to exert anti-inflammatory effects through interactions with host receptors, including antagonism of the blood glycoprotein, von Willebrand factor receptor, and lipopolysaccharide-induced inflammation, which was reduced in an *in vivo* mouse model by sulfobacin B [28], and competition
70 with lipopolysaccharide for binding to Toll-like receptor 4 (TLR4), which has been discovered through the use of multi-omics methods and mouse models that found that with the decrease in Sols was the severity of IBD increased [29]. Within the human gut microbiome, the bacterial genera *Alistipes* and *Odoribacter* are producers of Sols. Notably, these genera have been negatively associated with the occurrence of IBD, suggesting a potential protective role of Sols in maintaining intestinal health [29]. Despite these
75 promising indications, the extent to which Sols can modulate gut microbial composition and metabolic activity remains poorly understood. However, it could be expected that Sols have similar effects on the microbiome to those of sphingolipids, a lipid group that shares many of Sols characteristics, which are metabolized by *Bacteroides* and *Prevotella* [30]. It may be possible that the sulfur group could be used similarly to the sulfur-containing sugar Sulfoquinovose, which has a sulfur group that has mainly been
80 found to be metabolized and produced into H_2S by *Bilophila wadsworthia* [31].

Given the growing interest in diet-based strategies for modulating the gut microbiome, Sols represent a potential candidate for influencing microbial composition and host health. Therefore, finding methods for Sol production and effective extraction. Understanding how these lipids interact with the gut microbiome and how they may alter its composition and microbial metabolism could provide insight
85 into new approaches for managing inflammatory diseases such as IBD.

Problem Analysis 2

2.1 The human gastrointestinal tract

The Gastrointestinal tract (GI) performs essential functions, including the ingestion, digestion, and absorption of nutrients, as well as the excretion of waste. These processes occur along the esophagus, stomach, small intestine, large intestine, and rectum, with support from accessory glandular organs such as the salivary glands, liver, gallbladder, and pancreas (Figure 2.1) [32]. The GI tract is supported by four histologic layers: the mucosa, submucosa, muscularis externa, and serosa, each of which contributes to its overall structure and function (Figure 2.1) [32].

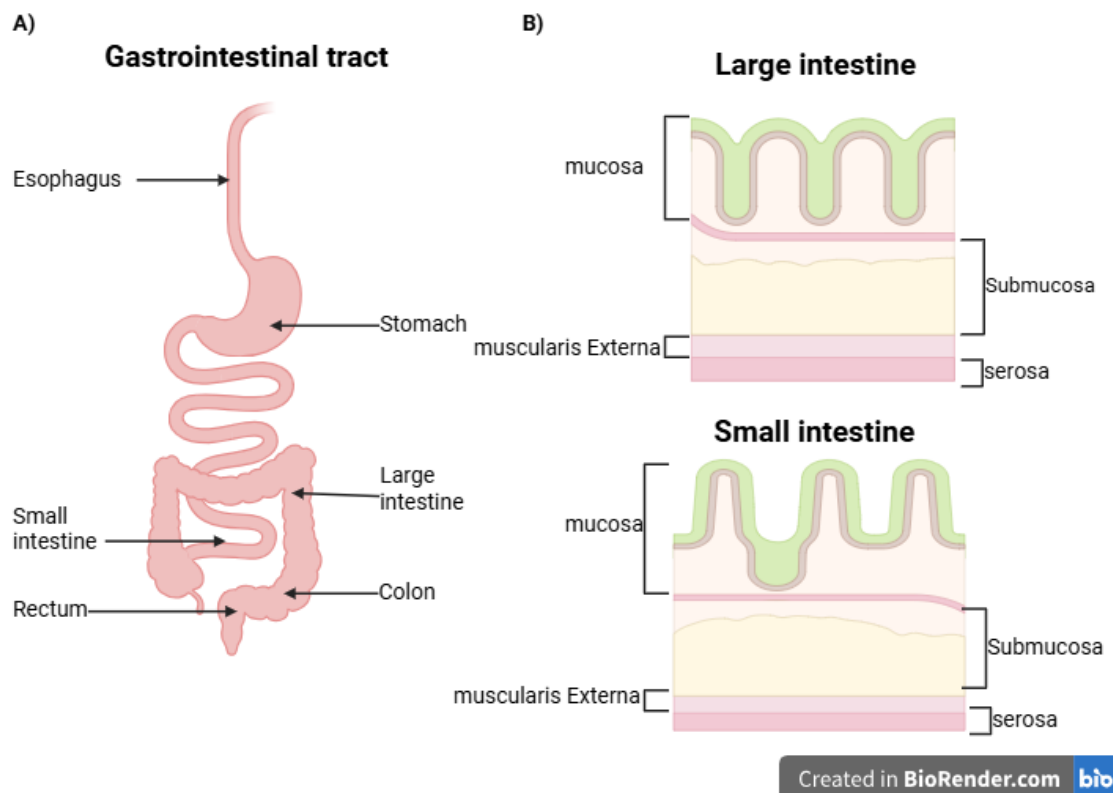


Figure 2.1. The anatomy of the GI tract and the layer that coats it. A) The GI tract starts at the esophagus, leading into the stomach, where it further goes to a stretched-out version of the small intestine, going to the large intestine, ending with the colon and rectum. B) the large and small intestine layers, mucosa, submucosa, muscularis externa, and serosa [32, 33].

The GI tract can be subject to many diseases, including inflammatory bowel disease (IBD), hemorrhoids, acute and chronic pancreatitis, and various forms of hepatitis. which can cause various symptoms [34]. The intestinal immune system plays a central role in the pathogenesis of IBD. Under normal conditions, the intestinal epithelium acts as a barrier, preventing bacteria and antigens from entering systemic circulation through tightly sealed intercellular junctions. In IBD, this barrier function is compromised, leading to increased permeability, immune activation, and inflammation. Additionally, reduced intrinsic antimicrobial activity in the gut further increases microbial exposure, exacerbating inflammatory responses [3].

Ulcerative colitis is characterized by continuous mucosal inflammation that originates in the rectum and

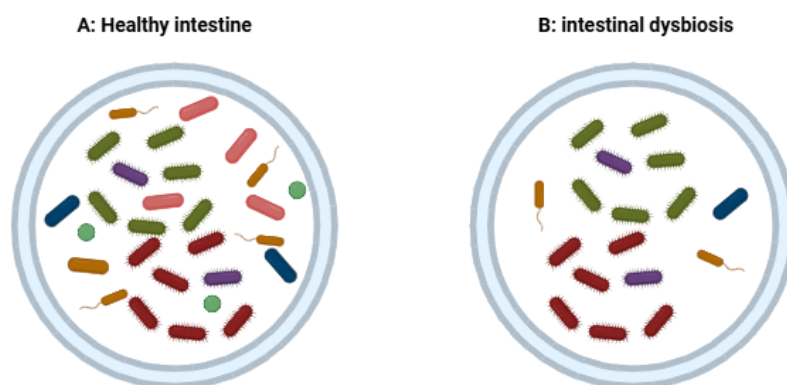
extends proximally through the colon, often leading to edema, ulceration, and bleeding. In contrast, Crohn's disease can affect the entire GI tract and involves transmural inflammation, impacting all layers of the bowel wall. In advanced stages, this may result in characteristic cobblestoning of the mucosa due to linear ulcers [3].

The etiology of intestinal inflammation is multifactorial, involving genetic susceptibility, environmental factors such as smoking, and alterations in the gut microbiome. The gut microbiome is a complex and dynamic community of microorganisms that exists in symbiosis with the host and plays essential roles in digestion, immune regulation, pathogen defense, and broader physiological processes. Disruptions to this community, known as dysbiosis, can impair these functions and contribute to the development and progression of diseases such as IBD [35].

2.2 The human gastrointestinal microbiome

The gut microbiome is the collection of bacteria, archaea, fungi, and bacteriophages inhabiting the human gut, with an estimated 10^{14} individual cells [14]. To date, 2172 bacterial species have been identified in the GI tract, classified into 12 different phyla, with 93.5% belonging to *Proteobacteria*, *Bacillota*, *Actinobacteria*, or *Bacteroidetes*. These species have evolved to establish mutually beneficial relationships with the host, supporting immune and metabolic homeostasis under normal physiological conditions [15].

There is increasing evidence that the development of the microbiome begins before birth, with the maternal microbiome serving as the microbial inoculum for the child. After birth, microbial diversity increases progressively until adulthood, at which point the gut microbiome becomes relatively stable throughout adult life [16]. The development of the microbiome throughout life depends on a multitude of environmental factors, including the host's genetics and diet [15]. It must be noted that functional redundancy is present within the gut microbiome, meaning that despite compositional differences, the system serves the same functions, in producing similar proteins and metabolic profiles [17]. It is noted that healthy individuals can exhibit large variations in microbiome composition [19]. Disturbances in the microbiome can derive from a variety of sources, such as diet, as previously mentioned, but also antibiotic treatment, illness, and infection. If disturbances become too large, dysbiosis is created within the microbiome [17]. Dysbiosis is the imbalance of the bacterial composition, which can occur in three distinct ways: loss of beneficial bacteria, loss of bacterial diversity, and gain of pathogenic bacteria (Figure 2.2) [18].



Created in BioRender.com 

Figure 2.2. Comparison of a healthy intestine (A) and a dysbiotic intestine (B). A) depicts a diverse microbial community with balanced bacterial populations. B) illustrates dysbiosis characterized by reduced microbial diversity and dominance of a single bacterial species [18].

130 Dysbiosis of the GI tract has been linked to conditions such as IBD, Obesity, and Type 2 Diabetes, and certain GI cancers [18]. In patients with IBD, a pattern of decreased commensal bacterial diversity has been observed, with the main decrease occurring in the *Bacillota* phylum, while an increase occurs in the *Proteobacteria* [18, 36]. For Crohn's disease, a decrease in *Faecalibacterium prausnitzii*, *Bifidobacterium adolescentis*, *Dialister invisus*, and an unknown Clostridium cluster XIVa. There is also an increase in
 135 *Ruminococcus gnavus* [37]. For ulcerative colitis, a decrease in *Bacillota* and an increase in *Gamma-proteobacteria* and *Enterobacteriaceae* have been found [38]. In IBD, there is generally a decrease in bacterial species with anti-inflammatory effects and an increase in those with pro-inflammatory effects [36].

140 Diet is a major shaper of the microbiome, with high-fiber diets from whole grains, fruits, and vegetables associated with greater GI microbial diversity. Individuals who partake in these diets are less likely to develop IBD [18]. Diets characterized by a high intake of animal-derived proteins and fats, commonly referred to as Western diets, are associated with an increased predisposition to IBD [39].

2.3 Diet impact on the gut microbiome

145 Life is connected with diet, both as a source of vital nutrients and a major cultural aspect. Diets have changed over time, with developments in urbanization and food availability. In modern times, this has led to increased consumption of highly processed foods high in unhealthy fats, free sugars, and salt, while fruit and vegetable consumption has decreased [40]. These dietary changes are suspected to have led to an increase in diseases such as IBD by affecting multiple bodily systems, such as the GI microbiome [13],

150 Diet is a major determinant of the microbiome community found in the GI tract. Diets have the greatest impact on the microbiome in the first three years of development. Though the impact of breastfeeding and the introduction of solid food affects the foundational formation of the microbiome [41].

The microbiome responds to habitual diets, in which a consistent supply of nutrients and substrates is introduced, thereby shaping its composition. Certain habitual diets have been linked to enterotype, with personalized differences in the composition [19]. Short-term, dramatic dietary changes can rapidly alter
 155 the human gut microbiome; however, these changes are transient and typically return to baseline within a few days [42]. Generally, diet accounts for 44% of the variation in the microbiome, while other factors, such as gender, BMI, and age, account for 34% [19]. Following dietary changes, such as a shift from an animal-based to a plant-based diet or the addition of nutrient supplements, compositional changes in the microbiome occur [19]. Generally, diets with a large variation in food intake have been associated with
 160 stability and variation in the microbiome, while monotonous diets have not [19].

Diet influences the risk of developing conditions such as IBD. Protective dietary factors, including breastfeeding during the first year of life, a high intake of n-3 polyunsaturated fatty acids, and a high-fiber diet, have been associated with a reduced risk of IBD. These diets promote a symbiotic relationship between the gut microbiome and the host, thereby lowering the likelihood of IBD development and limiting
 165 inflammatory responses [20]. Diets falling into the westernized character with high sucrose, saturated fat, monosaccharide intake, and processed foods indicate an increased likelihood of developing IBD [20].

Prebiotics are nutrients that can be used to alter the microbiome in favorable ways. This includes nutrients such as SCFAs, dietary fibers like inulin, fructooligosaccharides, and galactooligosaccharides [42]. Prebiotics help improve the intestinal barrier, alter the composition of the microbiome, and activate
 170 microbes to produce metabolites that are beneficial to the host. As with diet, it is difficult to estimate the effects of prebiotics; however, certain prebiotics have been shown to benefit specific conditions [36]. Supplements can also be needed when certain nutrient demands are not met, like for sulfur, where certain parts of the population are receiving insufficient amounts of sulfur through their diets, and supplementation could be of benefit [43].

175 Prebiotics for IBD are expected to react in different ways to mediate the conditions. Fructans and resveratrol mediate IBD in mice by increasing the amount of *Bifidobacterium* and *Lactobacillus* [36]. This hinders IBD by keeping the colon's pH low, producing large amounts of butyrate, and thereby inhibiting the growth of harmful bacteria. In humans, there are currently no established guidelines for the use of prebiotics to prevent the development of IBD [36]. However, several candidates may have beneficial effects
 180 on IBD. One group of interest for lessening the impact of IBD is Sols, a lipid group produced by *Alistipes* and *Odoribacter*. Sols have been found to be upregulated by high-fat diets in mice [44], and, with their potential anti-inflammatory effects in the gut [29], they are a candidate for combating IBD.

2.4 *Flavobacterium johnsoniae*

Flavobacterium johnsoniae (Fjoh), formerly *Cytophaga johnsonae*, is a well-studied gram-negative bacterium, part of the phylum *Bacteroidetes* that was first described in 1947 [45, 27]. Fjoh produces yellow colonies, is chemoorganotrophic and aerobic, with an optimum growth occurring between 20 and 30 degrees. In nature, it is found in soil and freshwater habitats. Some species within the genus are also found to be pathogenic to fish, with Fjoh found in superficial lesions on diseased fish [46]. Fjoh has been used as a model organism for its gliding ability, gliding up to 2 to $10 \mu\text{m} \times \text{s}^{-1}$. Fjoh cells are rod-shaped bacteria about $6 \mu\text{m}$ long that do not have flagella or pili [47]. Fjoh is notable for producing Sols, making it a valuable model organism for studying Sol biosynthesis.

2.4.1 Sulfonolipids

Sols are a group of molecules found in photosynthetic plants, algae, and bacteria, where they constitute one of the most abundant lipid classes in both prokaryotic and eukaryotic cells, accounting for up to 29% of total lipid content. Sols have been reported to exert beneficial biological effects, including modulation of DNA polymerase and viral reverse transcriptase activities, as well as anti-inflammatory properties [26], which have been particularly associated with sulfobacins, a specific subclass of Sols. Sulfobacins are Sols found in the membrane of gram-negative bacteria that are bioactive. Sulfobacins have multiple major formations in sulfobacins [48], which are produced by *N*-acylation of capnine with different iso-fatty acids. Other forms of sulfobacins are derived from sulfobacin A or B (Figure 2.3) and differ in their length of carbon chains or contain double bonds at different locations on the chains. Structurally, sulfobacins are analogs of sphinganine [29].



Figure 2.3. Sulfobacin A and B have a chemical structure, both being lipids with two carbohydrate chains, each bearing either a hydroxide or an oxygen group at the head region; the chains are connected by an NH group. Both sulfobacins contain a sulfonated head group SO_3^- . The difference between the compounds is the addition of a hydroxide group on one of the sulfobacin A carbohydrate chains.

Sulfobacins A and B have been directly linked to human physiological and immunological functions. Sulfobacin B has previously been identified as a von Willebrand factor receptor antagonist and as an inhibitor of DNA polymerase α . Additionally, it has been shown *in vivo* to suppress TNF- α production, thereby reducing symptoms associated with lipopolysaccharide (LPS)-induced acute inflammation [28]. In contrast, sulfobacin A primarily acts as a dual immunomodulator through specific interactions with Toll-like receptor 4 (TLR4). Specifically, sulfobacin A binds to the TLR4/myeloid differentiation factor 2 complex and competes with LPS, the natural ligand of myeloid differentiation factor 2, by occupying the reactive site without triggering the conformational changes necessary for receptor activation (Figure 2.4). This competitive binding effectively creates an inactive complex that prevents productive TLR4 dimerization, thereby lessening LPS-induced inflammatory signaling through suppression of both MyD88-dependent and TRIF-dependent pathways, ultimately reducing pro-inflammatory cytokine expression and NF- κ B-mediated inflammatory gene transcription. Furthermore, sulfonolipids, including sulfobacin A, are negatively correlated with IBD and are proposed to exert a protective effect through modulation of TLR4-mediated immune responses, potentially involving microbiome modulation, enhanced intestinal barrier function, and promotion of immune homeostasis through regulatory T cell differentiation while suppressing pathogenic Th1/Th17 responses [29].

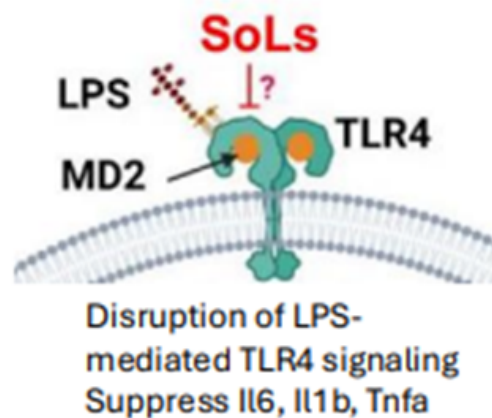


Figure 2.4. TLR4 depicted in the membrane being interacted by a LPS in the myeloid differentiation factor 2, where Sol is shown to possibly be able to interact with the TLR4 to hinder the interaction with LPS [29, 49].

In the gut microbiome, two *Bacteroidetes* genera, *Alistipes* and *Odoribacter*, are the most prominent producers of Sol. These bacteria in the microbiome have been linked with a negative correlation to IBD. Other bacteroidetes species do not have this association. This factor indicates that *Alistipes* and *Odoribacter* production Sol might be the factor limiting IBD effects [29]. It has been shown that individuals with IBD have lower levels of Sulfobacin B and F in their metabolome, and that piroxicam-treated mice, which are prone to IBD, have lower levels of sulfobacin than control mice. Increased expression of NF – kB – regulated inflammatory markers was also found in the piroxicam-treated mice, further indicating sulfobacins' anti-inflammatory effects [29].

The bacteroides *Alistipes*, *Odoribacter*, and their produced sulfobacins have been indicated in limiting the harmful effects of IBD. Sulfobacins interact with cells to produce anti-inflammatory effects. For Bacteroides, a negative correlation with IBD has been shown [29]; however, *Alistipes* and *Odoribacter* have also been linked to increased risk of cardiovascular disease [50, 51]. Therefore, it is necessary to gain a better understanding of the bacteria and how sulfobacins might influence them to increase benefits and reduce risks if they are to be used as prebiotics.

2.4.2 Sulfonolipid production in *Flavobacterium johnsoniae*

The lipid profile of Fjoh shows a plurality of ornithine lipids composing 35% of the profile, followed by phosphatidylethanolamine at 25%, then Sols at 19 % with glycine lipids, serineglycine lipids, fatty acids, and unknown lipids making up the rest. Fjoh gliding has been linked to the lipid profile, with Sol being a necessary component. A Fjoh lacking its Fjoh_2419 gene was found to lack both the Sols synthesis and its gliding ability, indicating an entwined aspect of gliding to Sols in the membrane [27]. The absence of gene Fjoh_2419 prevents Sol production, thereby halting gliding on solid surfaces and increasing bacterial sensitivity to antibiotics. The gene Fjoh_2419 is identified as a cysteate-fatty acyl transferase by producing 3-Keto-capnine, a precursor to capnine that is used in the synthesis of Sols [27]. The conversion from capnine to sulfobacin B has been proposed to involve Fjoh_2421 as a candidate Sol synthase (Figure 2.5) [49].

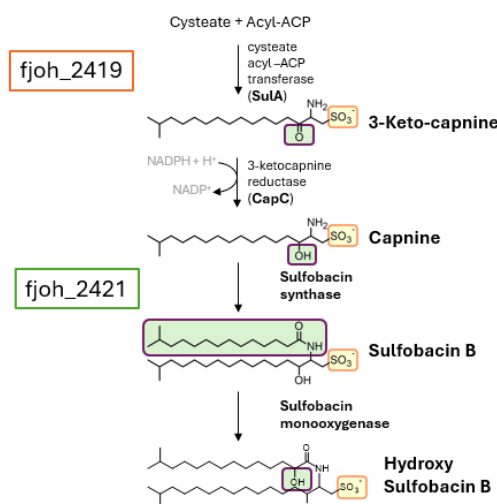


Figure 2.5. Pathway for the synthesis of sulfobacin B from cysteate and Acyl-ACP and the enzymes needed. Cysteate+Acyl-ACP interacts with cysteate acyl-ACP transferase into 3-Keto-capnine, which then gets synthesized into capnine by 3-ketocapnine reductase. Capnine is then synthesized into sulfobacin B by sulfobacin synthase [49].

Fjoh, with the knocked-out genes not synthesizing sulfobacin B, has been shown not to produce any other Sols, showing that sulfobacin B is vital for all other Sol production, and that in Fjoh, knocking this pathway out hinders all Sol production [49]. This makes Fjoh a particularly useful model organism for studying Sol production, as its biosynthetic pathway is well defined and can be genetically manipulated. However, the conditions required to maximize Sol yield in Fjoh remain to be fully characterized, making optimization of its cultivation conditions a necessary step towards harnessing Sols as potential prebiotic candidates for IBD intervention.

2.5 Intestinal hydrogen sulfide

Hydrogen sulfide (H₂S) is a gas produced in the gut by both human cells and microbiome. At higher concentrations, it is extremely hazardous, possibly leading to death at exposure above 700 ppm. At lower concentrations, H₂S has been shown to have multiple positive health effects, such as combating inflammation, oxidative stress, and cancer [23, 25].

H₂S is the third gasotransmitter, with nitric oxide and carbon monoxide being the others. As a gasotransmitter, knowledge of H₂S function remains far from complete; however, it is known that H₂S is involved in many physiological and pathological processes, such as inflammation and ulcer healing. The active use of H₂S as a medical treatment has its challenges with the difficulty of measuring the highly reactive molecule, though animal studies have shown promise that H₂S releasing drugs are safe and can be used for a variety of disorders, including inflammation, where linking H₂S with anti-inflammatory drugs can lead to better health outcomes [52].

The host's endogenous H₂S originates from various tissues, including the central nervous system, liver, and GI tract. The H₂S is synthesized enzymatically by a reverse transsulfuration pathway or by non-enzymatic production, which reduces elemental sulfur to H₂S [53]. The microbiome have been found to account for 50% of the H₂S production in fecal samples, showing that the microbiome is a major component of the gut's H₂S production [54]. The most prominent producer of bacterial H₂S is the sulfate-reducing bacteria (SRB), which belong to the δ - Proteobacteria with the most predominant genus being *Desulfovibrio*. Other prevalent genera include *Desulfobacter*, *Desulfomonas*, *Desulfobulbus*, and *Desulfotomaculum*. The SRB uses sulfate as a terminal electron acceptor, leading to the production of H₂S [23].

Higher SRB counts in the gut have been linked to Crohn's disease and ulcerative colitis, and H₂S levels in the feces of ulcerative colitis patients have also been reported to be 3-4-fold higher than in controls [55]. SRB and H₂S have generally been shown at increased levels in inflammatory conditions. However, H₂S has also been shown to have anti-inflammatory effects and help with ulcer healing, with H₂S being able to enhance the neutrophil apoptosis and reduce leukocyte migration, mitigating inflammation. H₂S also has

a protective effect against $IFN - \gamma$ and $TNF - \alpha$ – induced injury to the epithelial barrier [23].

The difference between beneficial and detrimental H_2S can be the donor that releases the molecule with GYY4137 as a slow H_2S donor, leading to decreases in inflammation in rats [56]. H_2S as a molecule appears dichotomous, with both possible beneficial and detrimental effects on host health depending on the amount of H_2S present and the donor from which the H_2S is derived [23].

2.6 Gut-derived short-chain fatty acids

SCFAs are a group of saturated fatty acids containing six or fewer carbon atoms that are produced in the gut by fermentation of dietary fibers by gut bacteria. Major SCFAs produced in the gut are acetate, propionate, butyrate, lactate, valerate, and formate. These SCFAs have been linked to cellular and molecular systems, and a deficiency in them may affect the pathogenesis of a diverse range of diseases [22].

The production of SCFA in the gut can vary based on factors such as available substrates, microbiome composition, gut pH, gut transit time, and the location of fermentation. These factors also vary depending on the specific SCFA, with butyrate mainly serving as an energy source for colonocytes, propionate largely used in the liver, and acetate metabolized by peripheral tissues. SCFAs can also have reciprocal effects on each other, with acetate increasing cholesterol synthesis and propionate decreasing it. Therefore, having the right SCFA at the right concentration and location is paramount to achieving a healthy outcome [57].

The application of prebiotics to increase SCFA production has been widely tested and shown to be effective. Specifically, oligosaccharides that are more rapidly fermented and produce a greater SCFA yield compared to other compounds, such as polysaccharides. Prebiotics might also affect the production of SCFAs by altering transit time or gut pH, creating more favorable conditions for SCFA-producing bacteria [21].

SCFAs have shown biological effects on a multitude of processes, including anti-inflammatory, anti-obesity, anti-diabetic, anticancer, and cardiovascular-protective effects. The anti-inflammatory process of Acetate, Propionate, and Butyrate has been shown in *in vivo* and *in vitro* studies to enhance the production of IL-10 and $IFN - \gamma$, and decrease the expression of IL-6 and reactive oxygen species [58, 59], leading to reduced inflammation [60]. Butyrate and sodium propionate have also been found to limit the inflammation induced by lipopolysaccharide, for butyrate by up-regulating IL-10, and for sodium propionate by protection of cells, promotion of angiogenesis, and reduction in the response from $NF - \kappa B$ pathway, all of which lead to a reduced inflammation caused by lipopolysaccharides [60].

IBD is driven by a complex interplay between host genetics, environmental factors, and dysbiosis of the gut microbiome, characterized by reduced microbial diversity, altered sulfur metabolism, and shifts in key anaerobic taxa. While the anti-inflammatory potential of Sols, particularly sulfobacins produced by *Alistipes* and *Odoribacter*, has been demonstrated through their interaction with TLR4 and negative correlation with IBD, the mechanisms by which Sols modulate the gut microbial community remain poorly understood. Specifically, it is unknown whether Sols supplementation can alter microbiome composition, microbial diversity, or the production of key metabolites such as SCFAs and H_2S . Furthermore, the conditions required to optimize Sols production in Fjoh have not been fully established.

Scope and Problem Statement 3

3.1 Scope

315 With the increasing prevalence of IBD, identifying new therapeutic and preventive strategies has become increasingly important. Prebiotics represent a relatively inexpensive approach for modulating the gut microbiome and potentially mitigating the effects of IBD. One potential prebiotic candidate is Sols, as reduced abundances of Sol-producing genera such as *Alistipes* and *Odoribacter* have been associated with IBD. However, the functional role of Sols in shaping gut microbial composition, diversity, and metabolic activity remains poorly understood. In addition, no standardized method for Sol production has been 320 established, although *Fjoh* represents a promising model organism for Sol biosynthesis.

This study therefore addresses two key research gaps: (1) the cultivation conditions required for optimal *Fjoh* Sol production have not been fully established, and (2) the downstream effects of exogenous Sol-containing lipids on stool-derived microbial communities remain largely uncharacterized.

325 3.2 Problem Statement

Thereby, the main problem statement formed is:

Do sulfonolipids modulate gut microbial composition or metabolism in ways associated with reduced inflammatory potential relevant to IBD?

Hypothesis

- 330
1. Incubation of stool samples with Sols increases the production of H_2S .
 2. Sols increase the production of specific short-chain fatty acids in stool samples.
 3. Sols alter the composition and diversity of the gut microbiome.
 4. Specific bacterial taxa are upregulated in response to Sol supplementation.

Method Theory 4

4.1 Plasmid construction

4.1.1 Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) is a molecular biology technique used to amplify a specific DNA sequence. For a PCR reaction to occur, several components are required, including template DNA, primers, deoxynucleotide triphosphates (dNTPs), and a thermostable DNA polymerase, most commonly Taq polymerase. Primers are short, single-stranded DNA sequences that bind to complementary regions flanking the target sequence. dNTPs serve as the nucleotide building blocks that are incorporated into the newly synthesized DNA strand. Taq polymerase catalyzes the extension of the primers by adding dNTPs in the 5' to 3' direction, resulting in the synthesis of double-stranded DNA copies of the target sequence [61].

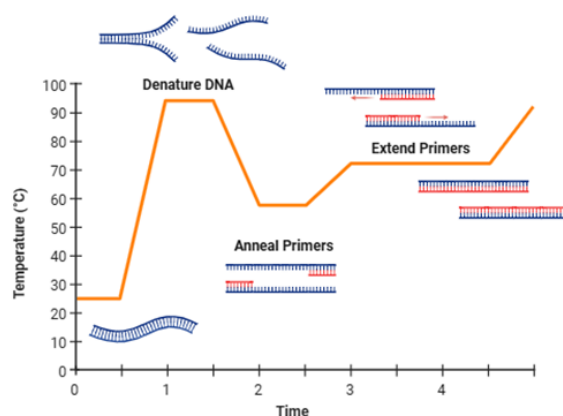


Figure 4.1. The reaction consists of repeated cycles of denaturation, annealing, and extension. During denaturation, double-stranded DNA separates into single strands. In the annealing step, primers bind to complementary sequences on the template strands. During extension, DNA polymerase synthesizes new DNA strands by extending the primers [61].

PCR proceeds through a series of thermal cycles. The first step is denaturation, typically carried out at approximately 95 °C, during which hydrogen bonds between complementary DNA strands dissociate, resulting in single-stranded DNA. The temperature is then lowered to the annealing temperature, usually between 50 and 65 °C, allowing primers to hybridize to their complementary sequences on the template DNA. Following annealing, the temperature is increased to 72 °C, which is the optimal temperature for Taq polymerase activity, allowing the extension of primers through the incorporation of dNTPs to form new double-stranded DNA molecules. Each PCR cycle approximately doubles the target DNA, leading to exponential amplification over successive cycles [61].

Quantitative polymerase chain reaction (qPCR) is a PCR-based method used to quantitatively assess gene presence, often employed to detect changes in expression resulting from various treatments in a microbiome culture. The method involves growing cells under varying conditions, harvesting the cells, isolating DNA, removing co-purified DNA using enzymes such as Deoxyribonuclease I, and then performing qPCR [62, 63].

In qPCR, a fluorescent dye, such as SYBR Green, is used to quantify double-stranded DNA (dsDNA). This is possible since when the dye is excited by a light source, it fluoresces about 1000 times brighter than

the unbound type. Therefore, the fluorescence generated is proportional to the dsDNA formed during the PCR. The increase in dsDNA can be followed across four phases: a baseline phase during the first PCR cycles, where fluorescence is below the detection limit, followed by a geometric phase in which fluorescence steadily increases as dsDNA accumulates. This transitions into the linear phase, characterized by a stable increase in fluorescence. In the end, the fluorescent signal remains steady during the plateau phase. The relative expression can then be determined by the number of cycles required for the sample to reach the detection limit or the plateau phase [64].

4.1.2 Digestion, Ligation, Transformation, and Conjugation

DNA digestion is performed using restriction enzymes, which cleave double-stranded DNA at specific recognition sequences or at defined positions relative to these sequences [65]. Depending on the enzyme used, cleavage can generate either blunt ends, which lack single-stranded overhangs, or sticky ends, which contain short single-stranded overhangs. Sticky ends facilitate the directional ligation of DNA fragments due to complementary base pairing, enabling the precise insertion of DNA fragments into plasmid vectors [66].

Ligation is the enzymatic process by which two or more DNA fragments are covalently joined through the formation of phosphodiester bonds between a 5'-phosphate and a 3'-hydroxyl group [67]. DNA ligases catalyze this reaction, such as *Escherichia coli* (*E. coli*) DNA ligase, which utilizes NAD^+ as an energy source, or T4 DNA ligase, which requires Adenosine triphosphate [68]. While both enzymes efficiently ligate DNA fragments with complementary sticky ends, T4 DNA ligase is uniquely effective at joining blunt-ended DNA fragments and is therefore commonly used in molecular cloning workflows [69].

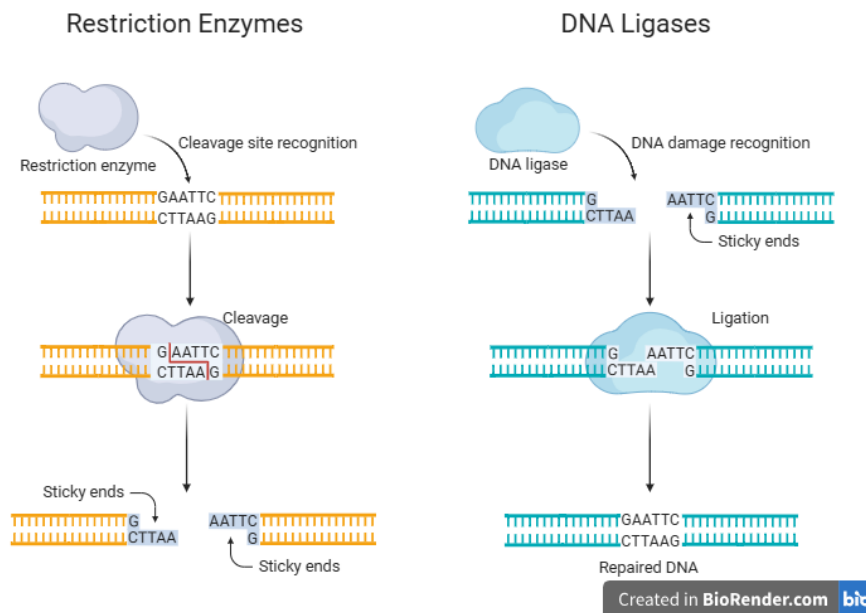


Figure 4.2. restriction enzyme cloning. Restriction enzymes recognize and cleave specific double-stranded DNA sequences, generating fragments with complementary sticky ends. DNA ligase subsequently rejoins the sticky ends, enabling insertion of the target fragment into a vector.

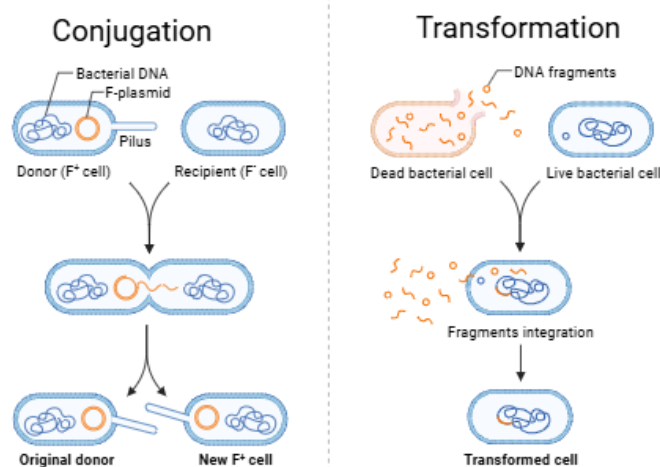
Created in BioRender.com 

Figure 4.3. Two mechanisms of horizontal gene transfer. Conjugation involves direct cell-to-cell contact via a pilus, through which DNA is transferred from a donor to a recipient cell. Transformation involves uptake of free environmental DNA by a competent cell.

Transformation is the process by which foreign DNA is introduced into a bacterial cell from its surrounding environment. For transformation to occur, bacterial cells must be competent, allowing uptake of extracellular DNA [70]. In *E. coli*, competence is typically induced artificially by incubating cells on ice in the presence of plasmid DNA, followed by a brief heat shock at 42 °C. This treatment transiently increases membrane permeability, facilitating the entry of plasmid DNA into the cytoplasm. Following heat shock, the cells are returned to ice and subsequently allowed to recover under optimal growth conditions. Transformed bacteria are then plated on selective media containing an antibiotic resistance marker encoded by the plasmid, ensuring that only cells that have successfully taken up and maintained the plasmid survive and proliferate [71].

Bacterial conjugation is a form of horizontal gene transfer in which genetic material, most commonly a plasmid, is transferred from a donor cell to a recipient cell through direct physical contact. This process is mediated by a sex pilus, which establishes a connection between the two cells. The donor cell carries a conjugative plasmid containing a fertility factor that enables plasmid transfer. During conjugation, a copy of the plasmid is transferred to the recipient cell, often conferring advantageous traits such as antibiotic resistance and thereby providing a selective benefit [72].

4.2 Liquid Chromatography Mass Spectrometry

4.2.1 Lipid extraction

Lipid extraction is the procedure of isolating lipids from all other components of a cell. Different methods are used depending on the desired purity and quantity of the lipids. A simple extraction method is methanol extraction, in which a pellet of cellular material is suspended in methanol, thereby solubilizing hydrophobic lipids [73]. To disrupt the cells and release intracellular lipids, sonication is employed. This technique uses ultrasound to generate alternating high- and low-pressure cycles in the liquid, leading to the formation of cavitation bubbles that collapse violently under high pressure. The collapse of these bubbles produces micro-jets and intense turbulence, generating mechanical stress that disrupts cell-surface barriers and effectively lyses the cells, thereby releasing lipids into the methanol [74]. To separate cellular debris from

the extracted lipids, centrifugation is used to partition the liquid and solid phases by applying a centrifugal force that exploits density differences. Denser material sediments away from the axis of rotation, while lighter components remain closer to the axis of rotation. This process accelerates separation by applying forces approximately 4000–14000 times greater than gravity [75]. The methanolic supernatant can then be collected and evaporated, as methanol has a low boiling point, leaving the desired lipids.

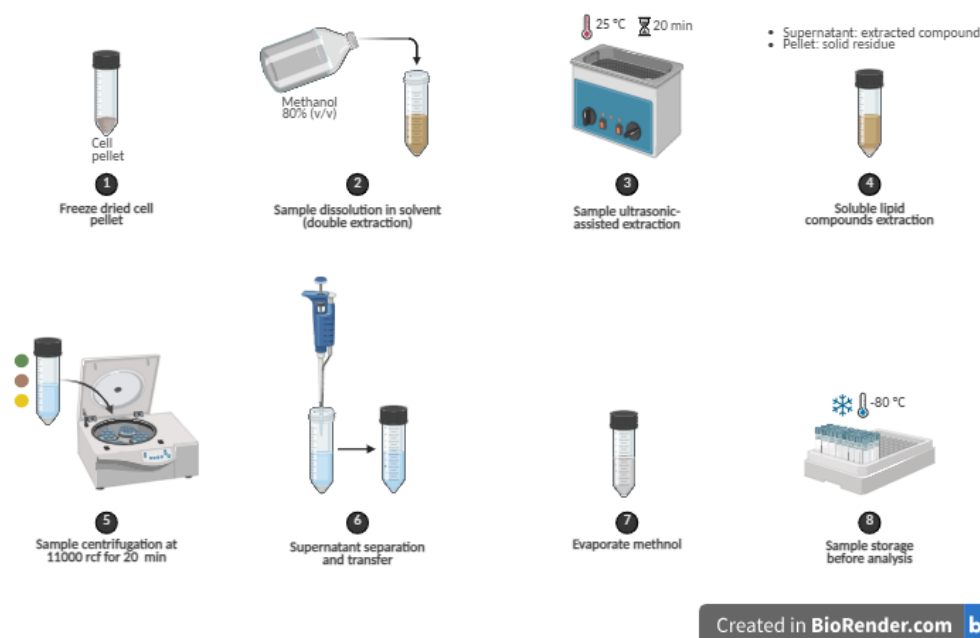


Figure 4.4. Methanol extraction is performed in multiple steps. First, the cells are pelleted, then methanol is added to the sample to resuspend the pellet, and the cells are sonicated for 20 min. Solid particles and liquid are then separated through centrifugation. The liquid is then pipetted into new tubes, and methanol is then evaporated by nitrogen flow, pellets are then stored at -80 °C.

Methanol evaporation is performed using a nitrogen blowdown evaporator, which removes solvent by continuously sweeping methanol vapor away, thereby lowering the vapor pressure above the sample. Replacing saturated air with dry nitrogen increases effective surface exposure and maintains a concentration gradient that promotes further evaporation. Using this method, evaporation rates of up to 2 mL of methanol per hour can be achieved, leaving the lipid extract upon completion [49].

4.2.2 Liquid Chromatography

Liquid chromatography (LC) is a separation technique in which components of a mobile phase are separated based on their interactions with a stationary phase. High-performance liquid chromatography (HPLC) is the most widely used form of LC, in which the mobile phase is pumped through the column under high pressure, enabling faster and more efficient separations. Four major types of stationary phases are commonly employed. Normal-phase chromatography relies on adsorption and desorption to a polar stationary phase, causing polar molecules to be retained longer while nonpolar molecules elute more rapidly. Reversed-phase chromatography utilizes a hydrophobic stationary phase, resulting in greater retention of nonpolar compounds. Ion-exchange chromatography employs a charged stationary phase that selectively retains molecules with opposite charges. Size-exclusion chromatography uses a porous matrix to separate molecules by size, with larger molecules eluting faster due to limited access to the pores [76].

Reversed-phase chromatography (RPC) is based on the separation of analytes using a polar mobile phase and a hydrophobic stationary phase. Common stationary phases include C8 alkyl groups bonded to silica particles, which provide structural support and enable hydrophobic interactions with analytes, forming a liquid-like layer at the surface of the stationary phase. The mobile phase typically consists of a mixture of water with methanol or acetonitrile. RPC is the most widely used HPLC technique, accounting for approximately 70% of all HPLC analyses [76].

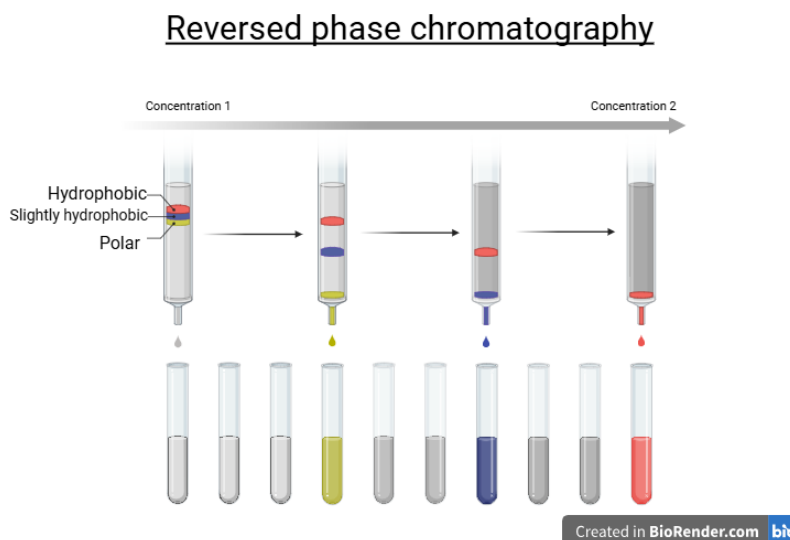


Figure 4.5. The figure illustrates RPC at different stages of the separation process. From left to right, the progression represents increasing time after sample injection. The yellow compound elutes first, as it is the least hydrophobic and therefore interacts least with the stationary phase. The blue compound elutes next, followed by the red compound, which is the most hydrophobic and thus is retained the longest in the column. This process separates the sample components based on their relative hydrophobicity.

Detection of analytes following chromatographic separation is commonly performed using ultraviolet–visible (UV–Vis) spectroscopy. This technique quantifies compounds based on their absorption of UV or visible light, where absorbance is directly proportional to analyte concentration according to the Beer–Lambert law. Conversely, transmitted light decreases with increasing concentration. Despite its widespread use, UV–Vis detection has limitations, including relatively low specificity and precision, as compounds eluting at the same wavelength may interfere with accurate quantification [77].

4.2.3 Mass Spectrometry

Mass Spectrometry (MS) is an analytical technique that, by ionizing a molecule, then traveling through a mass analyzer where molecules interact differently based on their mass to charge (m/z) ratio, signals are then created when the molecules make contact with a detector, which provides information on the specific molecule's abundance and formation graphically on a mass spectrum [78]. MS consists of three main components: an ionization source, a mass analyzer, and an ion detection system [79].

There are multiple ionization sources that aim to ionize molecules, causing them to transition from the solid phase to the gas phase, which can then be directed into a mass analyzer [80]. One of the most common methods for ionizing is Electron Spray Ionization (ESI), which consists of three steps: 1. Nebulization of the sample into charged droplets. This process is dependent on droplet formation and subsequent charging. Droplet formation depends on the flow rate, surface tension, and electrolyte concentration. Positively charged droplets are made by the removal of negatively charged droplets by the discharge of negative ions shot against the spray capillary metal wall 2. Ion formation begins as charged droplets undergo successive fission into smaller droplets when electrostatic repulsion exceeds surface tension. As the droplets shrink, the electric field at their surface increases, and once it becomes sufficiently strong, ions are emitted into the surrounding gas phase. These gas-phase ions are then transported into the vacuum system and guided toward the mass analyzer [81].

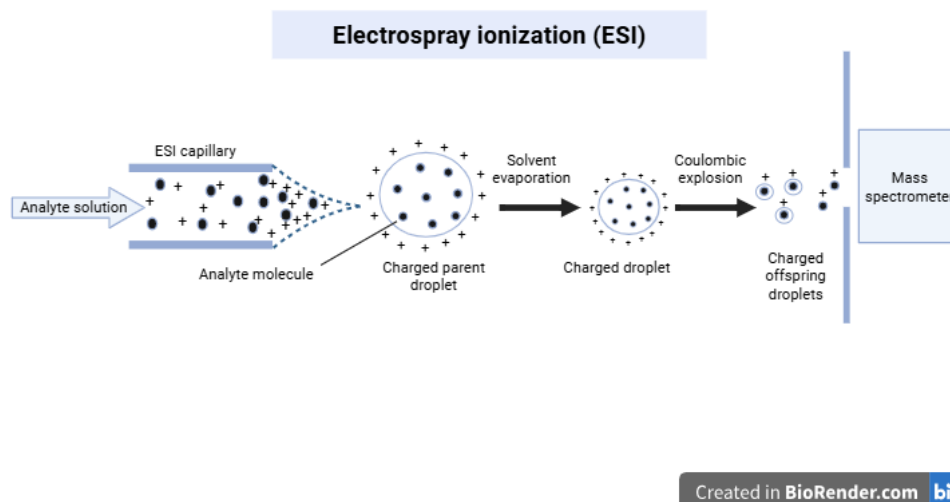


Figure 4.6. The analyte solution is introduced into the ESI capillary, where a high voltage is applied, causing the liquid to become electrically charged. The solution is then dispersed as fine, highly charged droplets. As the solvent evaporates, the droplets decrease in size, increasing surface charge density. When the Rayleigh limit is reached, Coulombic repulsion causes the droplets to undergo fission, forming smaller charged droplets. This process continues until gas-phase ions are released. These ions are subsequently transferred into the mass spectrometer, where they are analyzed to generate a mass spectrum [78].

The ionized samples are transported into a mass analyzer, where ions are separated according to their m/z ratio. A commonly used mass analyzer is the quadrupole, which consists of four parallel rods positioned equidistant from one another. The rods are arranged in two opposing pairs, with one pair connected to a direct-current voltage and the other to a radio-frequency voltage. The resulting electric field causes ions to oscillate along the x and y axes as they travel through the quadrupole. Ions with unstable trajectories collide with the rods and are eliminated, while ions with stable trajectories pass through the quadrupole and reach the detector, where they are recorded to generate a mass spectrum [80].

After the molecules have traveled through the mass analyzer, the ions reach the detector, where a mass spectrum is generated. A detector often used for proteomics and metabonomics is an Orbitrap. The analysis of the samples derived from the Orbitrap creates an electric field that the ions orbit around. The electric field is made from an outer barrel-like electrode and a central spindle-like electrode. The m/z value is then derived from the frequency of harmonic ion oscillations along the axis of the Orbitrap [82, 83].

To gain further insight into specific molecules, such as novel bioactive lipids in the samples, tandem mass spectrometry is utilized, as it provides more structural and mass information about these molecules [84]. This is achieved through multiple steps in mass spectrometry, in which an ion of interest is first selected and then fragmented, yielding product ions that reveal sequence-specific information [85]. Beam-type collisional activation dissociation is a method used to fragment ions, in which the ion of interest is actively injected into a collision cell containing an inert gas. The collisions between the ion and gas then fragment the ion into smaller parts that can help determine the precise structure of the original ion [86].

4.3 Spectrophotometry

Spectrophotometry measures the interaction of light with a substance, indicating absorption, transmission, or reflection, which shows concentration, purity, or color change within a liquid [87]. A spectrophotometer consists of four main components. The first is a light source that emits a beam toward the second component, the monochromator. The monochromator, often a prism or diffraction grating, disperses the light into its component wavelengths, allowing a specific, preselected wavelength to pass through the sample. This wavelength is selected by a wavelength selector (slit); the slit excludes all other wavelengths,

so only the desired wavelength passes through the sample. The light then reaches a detector, which measures the amount of light it receives and provides a digital readout [87].

A common application of spectrophotometry is measuring optical density at 600 nm (OD_{600}) to estimate microbial concentration. In this method, a wavelength of 600 nm is chosen because bacterial pigments minimally absorb light at this wavelength, ensuring that light scattering by microbial cells is the primary factor measured. As microbial concentration increases, more light is scattered, allowing a non-destructive estimation of cell density with minimal interference from pigments [88].

Spectrophotometry can also be used to determine the concentration of specific substances within a sample. For example, H_2S can be measured using Cline's reagent, which changes color in proportion to the amount of H_2S present. By measuring absorbance at a wavelength of 617 nm, the concentration of H_2S in a sample can be determined by comparing the observed color change to a pre-established calibration curve [89].

4.4 Capillary electrophoresis

Capillary electrophoresis (CE) is an analytical separation technique that separates analytes based on their charge-to-size ratio under the influence of a strong electric field. Separation is carried out in a narrow fused-silica capillary with an inner diameter of 25–75 μm , filled with an electrolyte buffer. The inner capillary wall contains silanol groups that become deprotonated at neutral to alkaline pH, resulting in a negatively charged surface, which generates an electroosmotic flow when an electric field is applied [90].

Upon application of a high voltage, typically in the range of 10–30 kV, a current in the μA range is generated, causing the bulk buffer solution to migrate toward the cathode. The overall migration velocity of an analyte is determined by the sum of its electrophoretic mobility and the electroosmotic flow [91]. As a result, cationic species migrate fastest, followed by neutral compounds that are transported solely by electroosmotic flow, while anionic species migrate more slowly, though they are still carried toward the detector by the flow. Small, highly charged electrolytes therefore reach the detector earlier than larger or less charged analytes. Neutral compounds exhibit no intrinsic electrophoretic mobility and are thus poorly separated under standard CE conditions [90].

Before sample analysis, the capillary is conditioned by flushing it with a filtered and degassed buffer for approximately 4–5 capillary volumes to ensure stable electroosmotic flow, remove residual contaminants, and improve run-to-run reproducibility. Samples, typically in the nL range, are introduced at the anode end of the capillary using either hydrodynamic or electrokinetic injection. Under the applied electric field, analytes migrate through the capillary toward the cathode, where a detector is positioned [90].

Detection is commonly performed using UV–visible spectrophotometry, which allows monitoring of analytes based on changes in absorbance as they pass the detector. This method is well-suited for the analysis of secondary metabolites that possess UV-active functional groups. To enable accurate identification of target compounds, pure reference standards are analyzed before sample runs to determine characteristic migration times. These retention times are then used to assign peaks observed in the resulting electropherogram, with migration time used for qualitative identification and peak area or height for quantitative analysis [90].

Despite its high separation efficiency and low sample consumption, CE has limitations, including relatively low sensitivity when using UV detection due to the short optical path length and variability in electroosmotic flow, which can affect reproducibility if buffer conditions are not carefully controlled.

4.5 Nuclear Magnetic Resonance

Nuclear Magnetic Resonance (NMR) spectroscopy is a nondestructive characterization method that provides indirect information about molecular structure, purity, and conformational changes. The fundamental principle behind NMR is that certain atomic nuclei possess intrinsic magnetic properties that can be exploited to obtain chemical information. Subatomic particles such as protons and neutrons possess spin angular momentum. In atoms with an even number of both protons and neutrons, these spins cancel each other, resulting in a net nuclear spin of zero, rendering such nuclei NMR inactive. In contrast, atoms with an odd total number of protons and neutrons exhibit a non-zero nuclear spin, typically a half-integer value, making them detectable by NMR spectroscopy. If a nucleus has both an odd number of protons and an odd number of neutrons, it will have an integer spin, which also enables NMR analysis [92].

NMR analysis is based on exposing a sample to a strong external magnetic field, which causes the magnetic moments of the nuclei to align either parallel or antiparallel to the applied field. This alignment results in two distinct energy states: a low-energy alpha state and a high-energy beta state. Upon application of a radiofrequency pulse, nuclei in the alpha state can absorb energy and transition to the beta state. After
535 excitation, the nuclei return to thermal equilibrium through relaxation processes. Spin-lattice relaxation T1 describes the transfer of energy from the excited nuclei to the surrounding lattice, while spin-spin relaxation T2 describes the loss of phase coherence between spins. These relaxation processes depend on both the type of nucleus and its molecular environment. During relaxation, energy is emitted, typically in the radio frequency range, and the resulting resonance frequency is influenced by the local environment
540 of the nucleus, giving rise to the chemical shift. Together, relaxation times and chemical shifts enable the elucidation of molecular structure and composition [93]. Commonly analyzed nuclei include hydrogen-1, phosphorus-31, carbon-13, and fluorine-19, as they are abundant in organic molecules and possess half-integer nuclear spin values [94].

The energy associated with nuclear spin transitions is relatively small and depends on the strength of
545 the applied magnetic field. For example, a hydrogen-1 nucleus in a magnetic field of 4.7 Tesla exhibits an energy difference of approximately 0.08 J mol^{-1} between spin states. This small energy separation necessitates highly sensitive and precisely tuned instrumentation, as the nuclear energy levels are closely spaced [93].

Several NMR techniques exist, each offering distinct advantages and limitations depending on the analytical
550 objective. One such technique is quantitative Nuclear Magnetic Resonance (qNMR), which is used to determine the absolute quantity and purity of compounds. In qNMR, the integrated area of an NMR signal is directly proportional to the number of resonating nuclei contributing to that signal. Unlike many other analytical techniques, qNMR does not rely on compound-specific response factors, allowing for direct quantification based on fundamental physical principles. By comparing the integrated signal of an analyte
555 to that of a reference compound with known concentration and purity, accurate quantitative information can be obtained. Due to its high accuracy, reproducibility, and broad chemical compatibility, qNMR is increasingly used as a reference method for purity determination and calibration in analytical chemistry [95].

qNMR commonly employs an internal standard with a known concentration and high chemical purity.
560 The internal standard must be chemically inert, stable under experimental conditions, and produce well-resolved signals that do not overlap with the analyte's signals. By comparing the integrated signal areas of the analyte and the internal standard, the absolute amount of the analyte can be calculated. Accurate quantification further requires that all nuclei fully relax between successive radio frequency pulses. Therefore, qNMR experiments are typically conducted using relaxation delays significantly longer
565 than the longest spin lattice relaxation time T1 present in the sample, ensuring that signal intensities accurately reflect the number of contributing nuclei [95].

The linear relationship between signal integral and number of nuclei assumes uniform excitation and consistent experimental parameters across all resonances. When these conditions are met, qNMR enables
570 highly reproducible and traceable measurements. As a result, qNMR is often regarded as a primary ratio method and is widely used for the certification of reference materials and purity assessment in pharmaceutical and analytical chemistry. Despite its advantages, qNMR is limited by spectral overlap, relatively long acquisition times, and lower sensitivity compared to mass spectrometry-based techniques, which can restrict its application to samples with sufficient concentration and spectral resolution [95]

4.6 Amplicon sequencing of 16S rRNA gene

575 16S rRNA is a universally conserved prokaryotic gene encoding a structural component of the 30S subunit of the bacterial ribosome. Due to its essential role in protein synthesis, the gene is present in all prokaryotic cells and is highly conserved across taxa. This conservation enables culture-independent microbial community analysis by allowing the identification of bacteria in complex samples without cultivation. The gene contains nine hypervariable regions (V1–V9) that are flanked by conserved regions [96, 97].

580 The conserved regions enable the design of near-universal primers for PCR amplification. By targeting the hypervariable regions, taxonomic information can be obtained to characterize microbial communities without cultivation [98]. The V1–V8 regions or the V4 region alone are commonly used for sequencing, as they provide a balance between taxonomic resolution and compatibility with short-read sequencing platforms. Bacterial community analysis is typically performed by grouping sequences into operational
585 taxonomic units (OTUs), based on sequence similarity thresholds, where >95% identity often corresponds

to the same genus and >97% to the same species [98].

Following PCR amplification, the targeted regions are sequenced using next-generation sequencing (NGS), which can sequence millions of DNA fragments in parallel, making it faster and more cost-effective than traditional Sanger sequencing [99]. Nanopore sequencing is a third-generation sequencing technology that
590 determines DNA sequences by measuring changes in ionic current as a DNA molecule passes through a nanopore protein embedded in a membrane. An electric potential is applied across the membrane, creating a current through the nanopore. Due to the negatively charged phosphate backbone of DNA, the strand is driven through the pore toward the anode. As the DNA is pulled through the nanopore, each nucleotide causes characteristic disruptions in the ionic current. These current changes are detected and interpreted
595 to determine the DNA strand's nucleotide sequence [100].

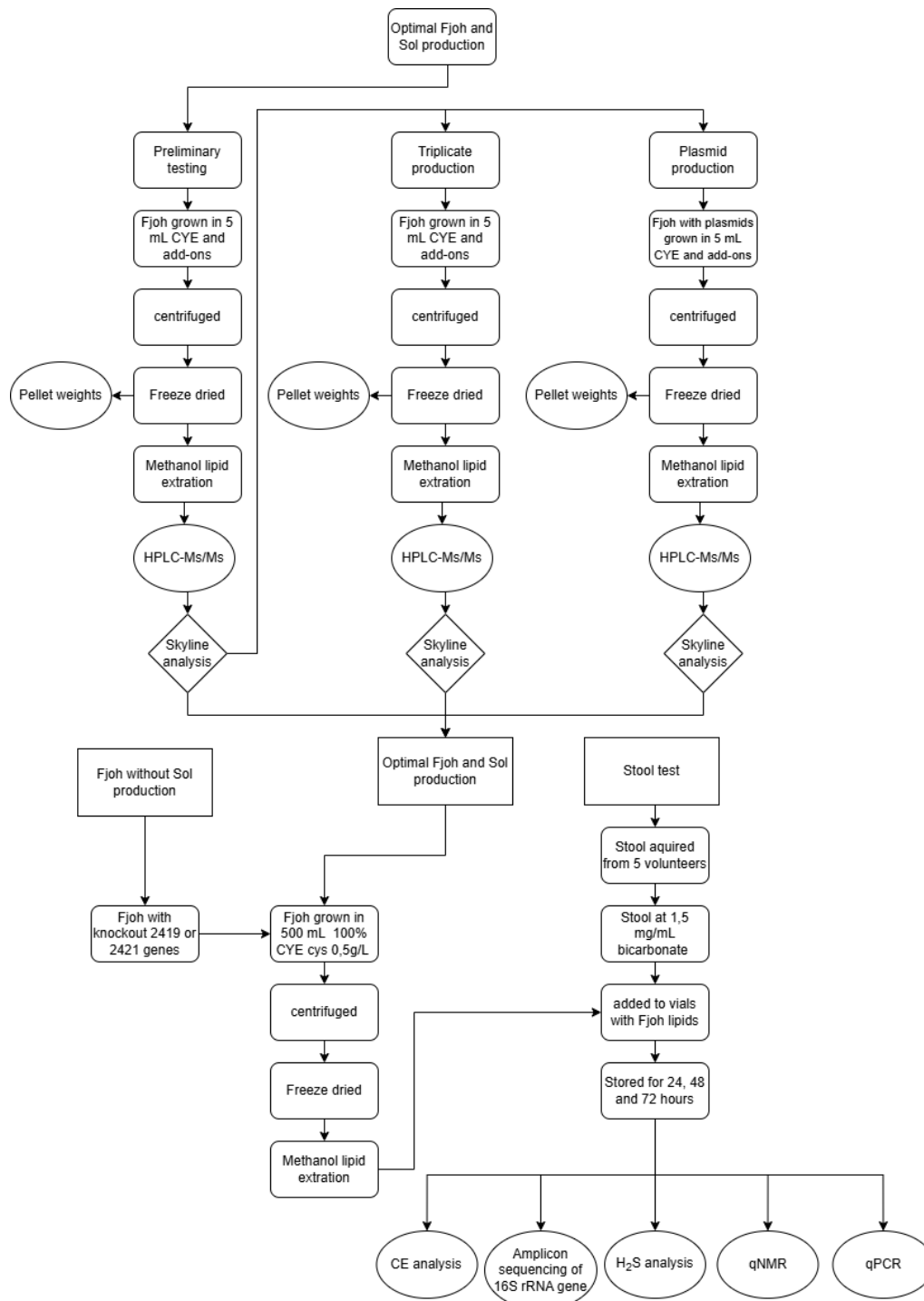
During library preparation, barcodes are unique DNA sequences that are added to individual samples. This enables multiple samples to be pooled and sequenced simultaneously in a process known as multiplexing [101, 102].

The raw sequencing data are then processed through a bioinformatic pipeline that includes quality filtering,
600 removal of chimeric sequences, and clustering into OTUs. Taxonomic classification is performed by comparing sequences to reference databases such as SILVA or Greengenes [103].

Although 16S rRNA gene sequencing is a widely used and reliable method for profiling bacterial communities, it has several limitations. These include limited taxonomic resolution at the species level, biases introduced during PCR amplification, and dependence on the completeness and accuracy of reference
605 databases.

Materials and Methods

5



5.1 Sulfonolipid production

Flavobacterium johnsoniae was used as a model organism for Sol production. To determine optimal conditions, Fjoh was inoculated in 5 mL of liquid or 20 mL of solid CYE medium (Appendix A.3) under a laminar flow hood (Lamsystems SAVVY, Germany, Spetec). Cultures were incubated overnight at 27°C. In addition to standard CYE, growth was evaluated in media supplemented with various carbon sources and metabolic additives at a final concentration of 15 mM. Multiple concentrations of cysteate were tested in both liquid and solid media to assess its effect on growth and Sol synthesis. (Appendix A.5).

Fjoh cultures were grown overnight. Solid-state cultures were harvested by scraping. Liquid cultures were centrifuged (VWR, United States, MicroStar 12) at maximum speed for 1 min, after which the supernatant was discarded. The cell pellets were freeze-dried (Alpha 1-4 LSCplus, Martin Christ, Germany) at -30 °C and 0.380 bar for 24 hours. Afterward, the pellets were weighed to determine cell biomass.

Lipids were extracted with methanol: 0.5 mL was added to each pellet, and the suspension was sonicated for 20 min (Sonorex, Bandelin, Germany). The samples were then centrifuged (Centrifuge 5804 R, Eppendorf, Germany) at 14000xg for 20 min. The resulting supernatants were transferred to HPLC vials, and the methanol was evaporated under a stream of nitrogen. The remaining lipid extracts were weighed and subsequently re-dissolved in 0.5 mL of methanol, of which 200 µL was used for HPLC-MS/MS analysis (Appendix A.3). The analytical run included four methanol blanks and one pooled sample. Sol profiles were generated from the relative abundances. To normalize the mass spectrometry data, all samples were adjusted to the same lipid mass. (Appendix A.7, A.8, A.16).

The growth conditions yielding the highest cell mass and Sol production were selected for further optimization. Fjoh were subsequently grown in 5 mL CYE medium with variations in concentration, additives, cysteate supplementation, or combinations thereof (Appendix A.13). After overnight growth, pellets were collected, freeze-dried (Appendix A.13), and lipid extracts (chapter 5.1) diluted to a final concentration of 50 µg/mL prior to HPLC-MS/MS analysis (Appendix A.14,A.3).

5.2 Plasmid construction

To investigate the role of specific plasmid insertion genes in Sol production, the genes Fjoh_2419 and Fjoh_2421 were cloned into Fjoh, using the *E.coli-Flavobacterium* shuttle vector pCP11 [104]. Primers were designed to amplify the target genes by PCR using Q5 High-Fidelity DNA Polymerase. Successful amplification of the target genes was verified by agarose gel electrophoresis using a 0.5% agarose gel. Both the PCR products and the vector were digested with XbaI and SalI, and the fragments were ligated using T4 DNA ligase to produce the plasmids. This lead to a Fjoh_2419+pCP11 plasmid (Appendix A.4) and a Fjoh_2421+pCP11 plasmid (Appendix A.5).

Plasmids were propagated in 10-beta competent *E. coli* by heat-shock transformation at 42°C, for 90 sec, and selection on LB agar supplemented with ampicillin. Correct plasmid identity was confirmed by EcoRI/XbaI restriction digest and gel electrophoresis. Verified plasmids were subsequently transferred into *E. coli* strain S17-1 for conjugation into Fjoh. Conjugation was carried out by mixing S17-1 and Fjoh cultures on CYE – CaCl₂ plates overnight at 27°C. Fjoh transconjugants were selected on CYE agar supplemented with erythromycin and confirmed by growth in liquid CYE with tetracycline.

5.3 Lipid profile of *Alistipes*

To compare the Sol profile of Fjoh with that of gut-associated Sol producers, *Alistipes timonensis* and *Alistipes putredinis* were cultivated in an anaerobic atmosphere consisting of 85% N₂, 10% CO₂, and 5% H₂, with hydrogen levels maintained between 1.5% and 2.5%. (Coy Laboratory Products, United States; Vinyl Anaerobic Chambers) using liquid Brain Heart Infusion (BHI) medium. *Alistipes timonensis* was grown for either 24h or 72h. All *Alistipes putredinis* cultures were incubated for 72 hours. Cysteates effect on both species was assessed at concentrations ranging from 0.25 g/L to 4 g/L (chapter A.6). Following incubation, cultures were centrifuged (VWR, United States, MicroStar 12) The resulting cell pellets. The method described in section (Chapter 5.1) was applied to analyze the samples.

To characterize Sol production dynamics, cultures of both species were inoculated from 5 mL pre-cultures into 50 mL BHI flasks. Optical density was then measured at 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 24, 26, 28, 30, and 48 h after inoculation. OD was measured in a handheld Biochrom spectrophotometer (Biochrom, United States; UniTrospec 10 cell density meter), blanked with BHI medium. Samples for HPLC-MS/MS were taken at 0, 2, 4, 6, 8, 10, 24, 28, 48 hours after inoculation. which was centrifuged to create a pellet,

and the pellet was prepared for HPLC-MS/MS as described in section (Chapter 5.1). The HPLC-MS/MS was run with these settings (Appendix A.3).

5.4 Large lipid extraction

For wild-type Fjoh and the Sol-deficient knockout mutant Fjoh $\Delta 2421$, biomass was produced in parallel for use in downstream stool incubation experiments. 5 mL vials of CYE media were inoculated, and after overnight growth, the culture was inoculated into four 1 L Erlenmeyer flasks, each containing 500mL CYE medium with 0.5 g/L cysteate which was stored shaken at 120 rpm, at 27°C overnight. The media was then pelleted by centrifugation (Eppendorf, Germany; centrifuge 5910Ri) for 40 min at $1000 \times g$. the pellets were transferred into a 50mL Falcon tube, where they were centrifuged (Eppendorf, Germany; centrifuge 5804 R) for 20 min at $10000 \times g$. This was then freeze-dried (Martin Christ Germany, Alpha 1-4 LSCplus) at -30°C at 0.380 bars for 48 hours.

Lipids were extracted from the freeze-dried samples using methanol extraction (Chapter 5.1), the supernatant was transferred to HPLC vials, and the methanol was evaporated under nitrogen flow. The lipids were then weighed. All of one type of lipids were then suspended in 1.5 mL, which were then separated into HPLC vials, both for Fjoh lipids and Fjoh $\Delta 2421$ lipids.

5.5 Prebiotic sulfonolipid stool tests

5.5.1 Exploratory stool test

An initial exploratory incubation was conducted with stool from a single individual (Appendix A.18). cultivated under an anaerobic atmosphere consisting of 85% N₂, 10% CO₂, and 5% H₂, with hydrogen levels maintained between 1.5% and 2.5%. (Coy Laboratory Products, United States; Vinyl Anaerobic Chambers). The stool was suspended in 30 mM bicarbonate buffer (pH 6.8) to a final concentration of 1.5 mg/mL fecal slurry. 1.5 mL of fecal slurry was then added to HPLC vials (Appendix A.3), with either Fjoh lipids, $\Delta 2421$ lipids, or no lipids as a control. These were then stored for 24 hours at 37°C.

H₂S production was quantified using Cline's reagent. H₂S was bound to zinc acetate to trap dissolved sulfide, then incubated with Cline's reagent (Solve 2 g dimethyl-4-phenylenediamine (DMPD; T042) for 20 min in the dark. where it was centrifuged for 3 min at 14000xg, absorbance was measured at 670 nm in a plate reader (Tecan Switzerland, INFINITE M NANO). A standard curve was prepared from serial dilutions of Na₂S – 9H₂O

5.5.2 Expanded stool test

A stool test was performed on five individuals' stool (Appendix A.19). Fecal slurry was prepared and handled as described above. The 1.5 mL fecal slurry was then added to 4mL tubes, with either Fjoh lipids, Fjoh $\Delta 2419$ and Fjoh $\Delta 2421$ lipids, and 3 control vials with no lipids (Appendix A.20). These were then stored for 72 hours at 37°C. with samples taken every 24 hours. For H₂S was measured as (Chapter 5.5.1)

CE was commenced on a (Agilent Technologies, United States, 7100 capillary electrophoresis) run over a (Agilent Technologies, United States, G1600-62311 - HPCE standard cap 75um id, 72cm lght 2/PK). The capillary was filled with an organic acid buffer at pH 5.6 for CE. A calibration curve was made with a mix standard composed of formate, acetate, propionate, butyrate, valerate, and lactate at concentrations (25–1000 μ M) with an internal standard of 2 μ L succinate 0.1 mM. Samples were injected at 50 mbar for five seconds. Measurements were performed at –25kV, 100 μ A, and 6W.

Samples for CE were prepared with a 98 μ L sample as done in the (Appendix A.1.5) method and 2 μ L of 10 mM succinate. They were then loaded into the CE.

5.6 Quantitative nuclear magnetic resonance

SCFA concentrations in the stool incubation samples were additionally quantified by quantitative NMR. The control, Fjoh, and $\Delta 2419$ Fjoh 5 mg/mL samples were selected at 24 and 72 hours.

qNMR was commenced on the stool samples, where they were homogenized. The stool was weighed (Appendix A.36) and transferred to 1 mL of buffer. The samples were then vortexed on a (Scientific Industries, United States, vortex genie 2). The samples were then centrifuged at $16160 \times g$ for 15 min.

Then, 200 μ L supernatant was transferred to a new 2 mL Eppendorf tube. The samples are then centrifuged at $16160 \times g$ for 15 min, and 100 μ L of the supernatant and 55 μ L of the NMR standard are transferred to an NMR tube, vortexed 3 times, and then centrifuged before analysis on an NMR spectrometer (Bruker, Switzerland, AVIII-600 MHz spectrometer). The instrument was equipped with a cryogenically cooled
 710 CPP-TCI 5 mm probe. Quantitative ^1H NMR spectra were acquired using the automated PR-PLUCON pulse program with water suppression at 298.1 K, with 32 scans collected per sample.

5.7 DNA 16S rRNA gene Sequencing

For sequencing, the control, Fjoh, and Fjoh Δ 2419 conditions at 5 mg/mL were selected at 48 and 72 h, yielding 18 samples in total for DNA extraction, amplification, and library preparation. DNA was
 715 extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals, United States). Cell disruption was achieved by bead beating in four rounds of 40 sec with 2 min intervals on ice, followed by centrifugation at $14000 \times g$ for 7 min. Protein precipitation was performed by the addition of 250 μ L PPS reagent, mixing, and centrifugation at $14000 \times g$ for 5 min.

DNA was bound to the supplied silica spin columns by incubation with Binding Matrix Solution, washed with SEWS-M buffer, and eluted in 100 μ L DES Elution Solution. DNA concentration was quantified
 720 using a Qubit 4 Fluorometer (Invitrogen, Thermo Fisher Scientific, United States) with the $1 \times$ dsDNA High Sensitivity Assay Kit. The V1-V8 region of the 16S rRNA gene was amplified by PCR using the primers 27F and 1392R at a working concentration of 1 μ M, in 50 μ L reactions containing 25 μ L PCR BIO 2 $\times$

725 UltraMix and 5 μ L template DNA. Each sample was amplified in duplicate, and the products were pooled prior to purification. Amplicons were purified using AMPure XP beads at a 0.7 bead-to-sample ratio, washed twice with 80% ethanol, and eluted in 25 μ L nuclease-free water. Successful amplification and fragment size were confirmed by Qubit fluorometry and Agilent TapeStation 4150 analysis.

Sequencing libraries were prepared using the Ligation Sequencing Kit SQK-LSK114 with PCR Barcoding
 730 Expansion EXP-PBC096 (Oxford Nanopore Technologies, United Kingdom). Purified amplicons were normalized to 200 fmol, and individual samples were barcoded by PCR using LongAmp Taq 2 $\times$

Master Mix, followed by AMPure XP bead purification. The pooled barcoded library was subjected to end repair and dA-tailing using the NEBNext Ultra II End-prep Module, purified with AMPure XP beads, and ligated to the Ligation Adapter using NEBNext Quick T4 DNA Ligase for 10 min at room temperature.
 735 The final library was purified with AMPure XP beads using Long Fragment Buffer washes, eluted in Elution Buffer, and quantified by Qubit prior to loading. The library was diluted to 10-20 fmol in 32 μ L and loaded onto a PromethION R10.4.1 flow cell, which was sequenced for 24 h.

5.7.1 qPCR for *Alistipes*

The abundance of *Alistipes* in stool-derived DNA samples was quantified by qPCR using a standard curve
 740 generated from a purified PCR product targeting the *Alistipes communis* gene A5CBH24_09830. All reagents were UV-treated for 15 min before use. The standard was amplified from the Fjoh 5 mg/mL 72 h replicate sample in 50 μ L reactions containing 25 μ L PCR BIO 2 $\times$

UltraMix, 20 μ L primer working solution (1 μ M), and 5 μ L template, split across duplicate wells and amplified according to the conditions in (Appendix A.52). The pooled PCR product was purified by a
 745 two-step AMPure XP bead purification. In the first step, beads were added at a ratio of 0.56 $\times$ and the supernatant transferred to a new well for a second purification at 0.29 $\times$.

Following magnetic separation, the pellet was washed with 175 μ L of 80% ethanol, air-dried for 5 min, and eluted with 25 μ L of nuclease-free water. DNA concentration and fragment size were confirmed by Qubit fluorometry and TapeStation analysis as described in sections (Appendix A.1.7, A.1.7). The purified
 750 product was serially diluted across ten 10-fold steps to generate the qPCR standard curve. Reactions were prepared in 15 μ L volumes containing 10 μ L Brilliant III 2 $\times$

qPCR Master Mix, 2 μ L primers, and 0.3 μ L ROX reference dye (1:500), with 5 μ L of standard, sample, or no-template control added per well. The control, Fjoh, and Fjoh Δ 2419 samples at 48 and 72 h were included alongside the standards and run on a Stratagene Mx3005P (Agilent Technologies, United States).

755 Amplification specificity was confirmed by dissociation curve analysis, which showed a single fluorescence derivative peak at 88-90 $^{\circ}\text{C}$, consistent with a single amplicon from the A5CBH24_09830 target (Appendix

A.9). Most samples crossed the detection threshold between cycles 16 and 22. The standard curve exhibited high linearity across the dilution series ($R^2 = 0.979$) (Appendix A.10), and absolute copy numbers were determined by interpolation from this curve.

760 **5.7.2 Data analysis**

Data analysis was conducted using R v4.5.1 and RStudio 2025.5.1.513. All microbiome data were visualised using the ampvis2 package v.2.8.9 and the ggplot2 package v.4.0.0. Alpha diversity was investigated using the Operational Taxonomic Unit (OTU) and Shannon diversity index, and beta diversity using Non-metric MultiDimensional Scaling (NMDS) and canonical correspondence analysis (CCA).

Results and Discussion

6

The production of Sols by Fjoh was initially investigated through exploratory experiments designed to identify cultivation conditions that increased Sol production and to determine whether the Sol profile remained consistent between conditions. Based on these findings, the most promising growth conditions were selected for more focused experiments to evaluate whether the observed increases in Sol production were systematic and reproducible. Finally, the selected cultivation conditions were tested using Fjoh strains containing plasmids carrying genes from the Sol biosynthetic pathway to assess whether overexpression of pathway-associated genes could further enhance Sol production.

6.1 Exploratory assessment of sulfonolipid production

To identify a possible optimized production method for Sols, exploratory tests were conducted in 5 mL of medium to determine the optimal CYE concentration and potential add-ons that could enhance both Fjoh growth and Sols production.

Fjoh end pellet weight was influenced by the different CYE concentrations and add-ons tested (Appendix A.5). Higher CYE concentration positively affected pellet formation, with 150% of the base CYE making up (Appendix A.3), resulting in a pellet weight of 3 mg. The addition of most add-ons resulted in low pellet weights, indicating that they hindered Fjoh growth. In contrast, most other additives showed no clear effect on Fjoh growth. The only addition that increased Fjoh growth was 15 mM acetate, which resulted in a pellet weight of 3.6 mg, higher than that observed in CYE 100% and CYE 150% media. Cysteate was also tested at concentrations of 0.25, 0.5, 1, 2, and 4 g/L (Appendix A.5). Cysteate showed increased growth at the lower concentrations: 0.25 g/L had a pellet weight of 4.6mg, 0.5 g/L had 4.4mg, and 1 g/L had 4.2mg. These data indicate enhanced Fjoh growth at lower cysteate concentrations. Cysteate at the higher concentrations of 2g/L had a pellet weight of 1.2mg, and 4 g/L had 0.1mg pellet weights. This suggests that cysteate becomes inhibitory above a certain threshold (Figure 6.1).

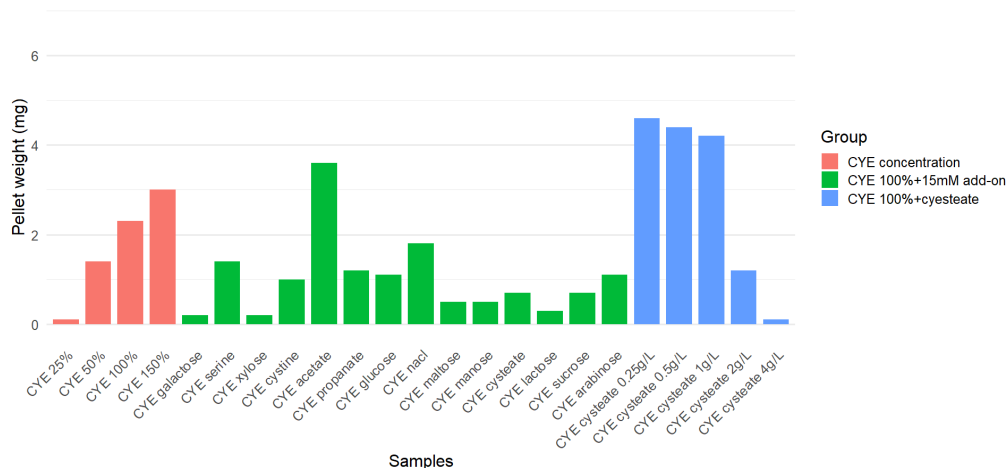


Figure 6.1. Pellet weights (mg) of Fjoh grown in 5 mL CYE medium were measured across samples with varying media makeups. In (red) was CYE media with different concentrations measured from 25% to 150% (Appendix A.1, A.2, A.2, A.3, A.4), in (green) was media tested with CYE 100% (Appendix A.3) and 15 mM of add-ons, in (blue) was media tested with CYE 100% (Appendix A.3) and varying concentrations of cysteate from 0.25 g/L to 4g/L

As shown in (Figure 6.1), the CYE 150% sample produced the highest pellet mass among the CYE concentrations, having a pellet mass of 3 mg, while the lower concentration of CYE 100% had a pellet mass of 2.3 mg, CYE 50% had 1.4 mg pellet weight, and CYE 25% had a pellet mass of 0.1 mg. These data show that as the concentration increases, the pellet mass increases. This follows the more nutrient-rich environment of the higher CYE concentrations.

All the add-ons have been described as Utilizable energy sources for Fjoh [45]. Only acetate showed an increased pellet mass relative to CYE 100%, whereas the other additives reduced or did not improve growth. The specific reason for the observed increase in growth with acetate, compared to the other additives, remains unclear at this stage. Cysteate concentrations of 1 g/L or lower resulted in the highest pellet weights, whereas concentrations above 2 g/L reduced growth, indicating a concentration-dependent inhibitory effect at higher levels.

Overall, the CYE concentration that supported the largest pellet formation was CYE 150%. Supplementation with 15 mM acetate enhanced growth, and cysteate at or below 1 g/L significantly increased pellet mass compared to the control.

The profile of Sols (Figure 6.2) in the samples was determined by the percentages each lipid represented of the total. The profile for all samples is largely similar, with a plurality of sulfobacin B and SL 604.4608 in all samples, with other Sols mainly sulfobacin A, sulfobacin B variant01, Sulfobacin D, and SL 590.4451 making up a lesser extent of the profile, with the rest of the Sols making up an even lesser part of the profile. This profile is consistent across all samples, with only minor variations.

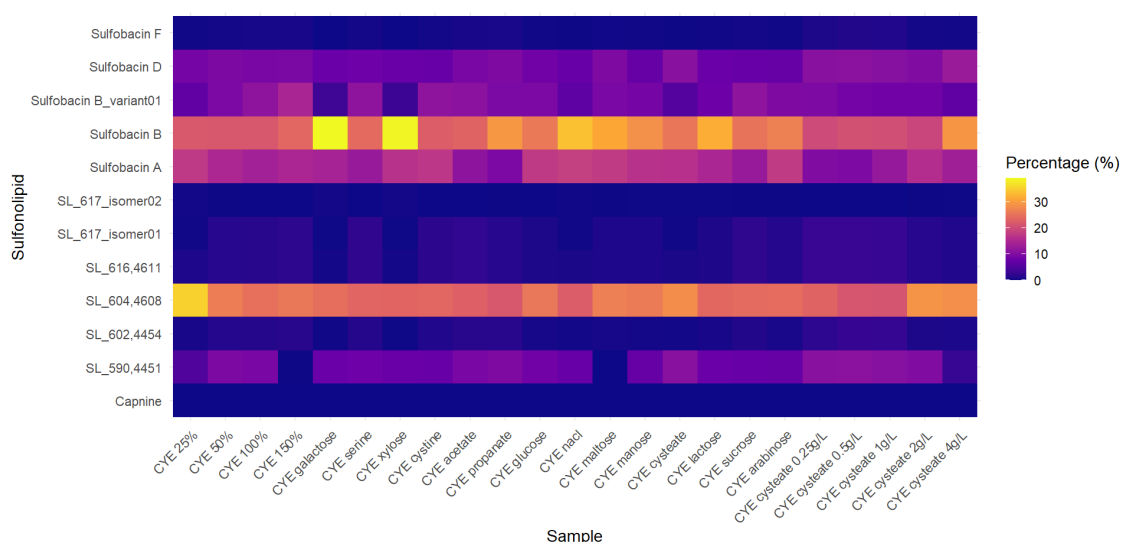


Figure 6.2. Heatmap over liquid Fjoh samples and their Sols content. The color varies from deep purple, indicating that the Sol makes up a low percentage of the total Sols presence, to yellow, indicating it makes up a large percentage. All samples show a similar profile dominated by SL 604.4808 and sulfobacin A and B, with lesser amounts of sulfobacin D, SL 590.4451, and the sulfobacin B variant01.

The galactose- and xylose-supplemented samples differed slightly from the other conditions, with sulfobacin B constituting a larger proportion of the total profile. However, in these samples, sulfobacin B variant01 accounted for a smaller fraction, resulting in overall sulfobacin B-type proportions comparable to those observed in the other samples (Figure 6.2) demonstrates that variations in CYE concentration and the tested add-ons did not substantially affect the Sols profile in liquid-grown Fjoh.

All media concentrations and additives were also evaluated on solid CYE medium. The Sols profiles were stable between samples (Appendix A.1), indicating that Fjoh maintains a stable Sol composition regardless of changes in media concentration or additive supplementation.

The Sols profiles of both liquid and solid Fjoh samples were dominated by sulfobacin B, SL 604.4608, with sulfobacin A, sulfobacin B variant01, sulfobacin D, and SL 590.4451 present at lower abundances. In the solid samples, the major Sols, sulfobacin B, SL 604.4608, and sulfobacin D, constituted a larger proportion of the total profile. In contrast, the liquid samples exhibited a relatively greater contribution

from less-abundant Sols, resulting in a more diverse overall Sol composition.

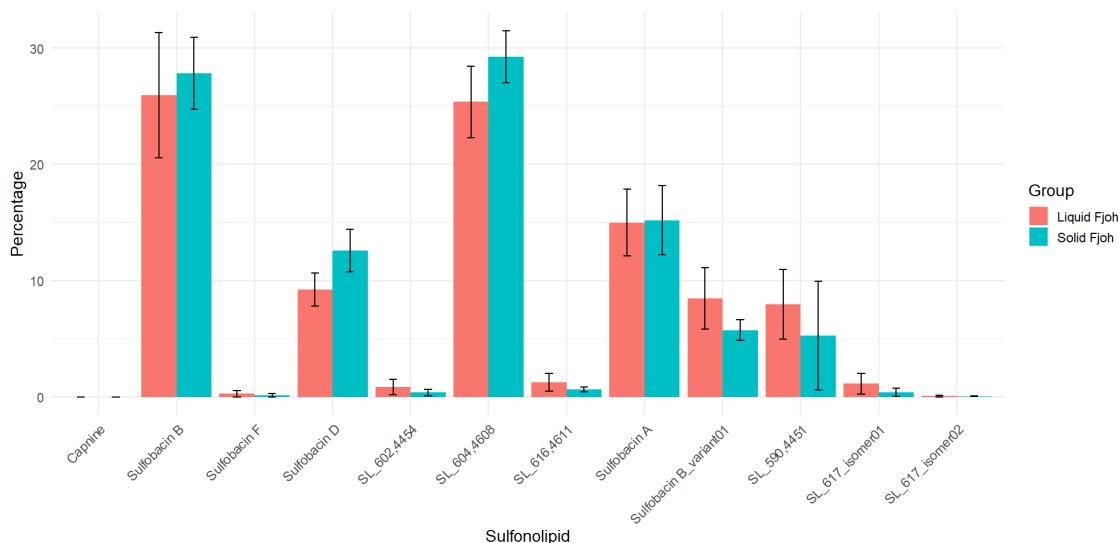


Figure 6.3. Mean $\pm$ SD relative abundance of individual Sol components in liquid (red) and solid (blue) Fjoh samples. Sol species are shown and their relative abundance as a percentage of total Sols. Bars represent group means.

820 The results demonstrate that under both solid- and liquid-growth conditions, Fjoh exhibits a similar, consistent Sols profile (Figure 6.3). This observation contrasts with previous findings, which reported that sulfobacin D and 2-hydroxy sulfobacin A (SL 604.4608) were detected exclusively in Fjoh grown on solid media [105, 106]. Sulfobacin D and 2-hydroxy sulfobacin A were also detected in the liquid-grown samples, indicating that Fjoh is capable of producing the full range of Sols under both growth conditions. This suggests that Sol biosynthesis may not be strictly dependent on growth on solid media. This indicates that upscaling production in liquid media is feasible without compromising the overall Sol profile.

830 The Sol profile identified in Fjoh shows similarities to previously described Sol profiles from other bacterial species, particularly through the dominance of sulfobacin B and sulfobacin D [107]. However, a notable difference is the high abundance of SL 604.4608 in Fjoh, which was not reported in the previously studied species, indicating potential species-specific variation in Sol composition.

835 The total area for each Sol type in every sample was graphed to determine whether the amounts of Sol produced would differ from sample to sample (Figure 6.4). The amount of Sols in the liquid samples was normalized to the total amount of Fjoh lipids detected after extraction. Sols appear at different amounts, with some, such as capin, at the lower end, showing 2000, and sulfobacin B at the higher end, showing 4×10^9 .

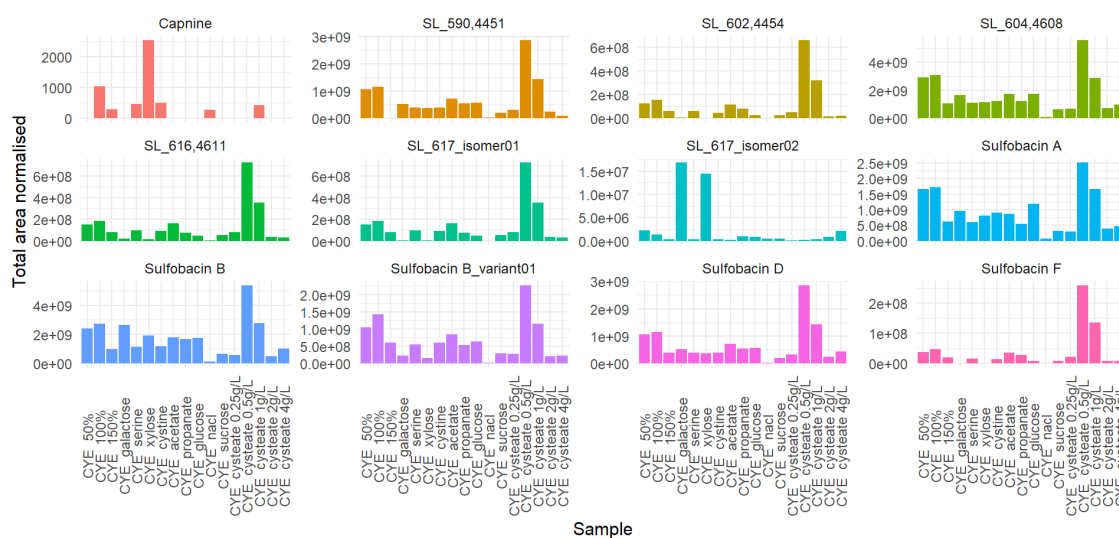


Figure 6.4. Normalized total ion areas of individual Sol species in liquid Fjoh samples. Each subplot represents a single Sol species, with the total ion area shown. Normalization was performed to account for differences in biomass between samples.

All samples were normalized to total extracted lipid content before comparison. Some samples showed irregular pellet weights and were removed from the graphical analysis. Therefore, the results should be interpreted conservatively and regarded as indicative of general trends rather than precise quantitative differences (Appendix A.7).

Across most Sols, CYE supplemented with 0.5 g/L cysteine showed the highest total area, followed by 1 g/L cysteine. This indicates that cysteine at moderate concentrations increases the production of most Sols in Fjoh. The effect was observed broadly across the lipid profile and not limited to a single compound, suggesting an overall enhancement of Sol biosynthesis under these conditions. However, SL 617 isomer02 did not follow this general trend. These Sol seem to respond to the supplementation of galactose and xylose. This may suggest that different carbon sources can influence specific parts of the Sol profile, although this effect was not consistent across all lipid species.

For the solid-grown samples, the total areas varied more across conditions, and a clear overall trend was less apparent than in liquid cultures. This could reflect differences in growth dynamics or nutrient availability between solid and liquid media (Appendix A.2).

Overall, both solid and liquid growth conditions resulted in similar Sol profiles, indicating that Sol production in Fjoh is relatively stable across cultivation formats. This differs from earlier studies, in which sulfobacin D and SL 604.4608 were found only in solid samples [105]. This new information enables liquid cultivation by allowing easier upscaling and process control. It was selected as the preferred production method for further optimization.

Although CYE 150% produced the largest pellet weights (Figure 6.1), higher biomass did not consistently correspond to higher total areas. In fact, CYE 100% showed relatively high Sol signal intensities compared to most other conditions, suggesting that increased nutrient concentration primarily enhances growth rather than Sol production.

In contrast, cysteine at low concentrations 0.25–1 g/L resulted in both increased pellet formation and the highest total areas. Given that cysteine is a substrate in Sol biosynthesis (Figure 2.5), and that cysteine has been found to be vital for gliding in certain mutant Fjoh [105]. This supports the idea that Sol production may be increased by precursor availability under standard conditions. However, the decrease in growth and total area observed at concentrations of 2 g/L or higher suggests that cysteine is inhibitory at these levels.

Taken together, these results indicate that liquid cultivation supplemented with low concentrations of cysteine represents the most promising approach for increasing Sol production in Fjoh.

6.2 Optimization of sulfonolipids expression

The purpose was to determine whether the trends observed in the exploratory experiments were systematic, and whether increased cysteine supplementation and higher CYE medium concentrations could enhance both Fjoh growth and the internal production of Sols. Optimization of Sol production experiments was performed using Fjoh grown under selected conditions: CYE 100%, CYE 150%, CYE 100% supplemented with 0.5 g/L cysteine, and CYE 150% supplemented with 0.5 g/L of both cysteine and acetate (Chapter A.7).

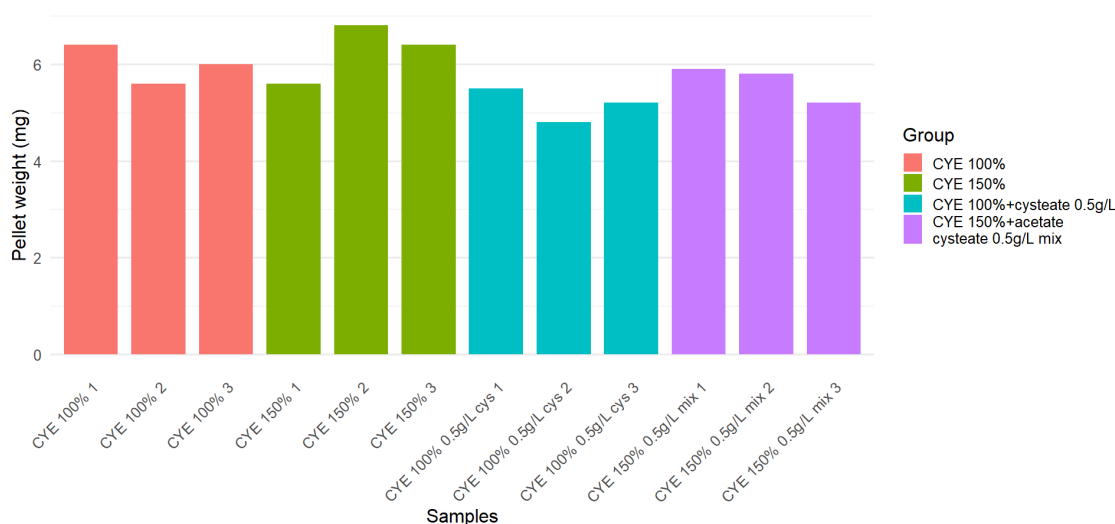


Figure 6.5. Pellet weights (mg) of three Fjoh replicates grown in 5 mL media under four conditions: CYE 100% (red), CYE 150% (green), CYE 100% with 0.5 g/L cysteine (blue), and CYE 150% with 0.5 g/L cysteine/acetate mix (purple). All groups yielded pellet weights between 4 and 6 mg, indicating comparable biomass across conditions.

All groups produced pellet weights, ranging between 4 and 6 mg, which were higher than those found in the exploratory test (Figure 6.1). The highest pellet weights were from the CYE 100% and CYE 150% groups, both of which had samples at or above 6mg of pellet. The samples supplemented with cysteine or cysteine and acetate showed a lower pellet production, with all samples being below 6mg and one CYE 100% 0.5 g/L cys 2 being below 5 mg.

For HPLC-MS/MS analysis, lipid extracts were normalized to 50 µg/mL before injection, thereby establishing a comparable baseline across samples. Total areas for the detected Sols were mapped using jitter plots to evaluate differences in expression between the sample groups. For each molecule, a different y-axis scale was used because expression levels can differ substantially between molecules.

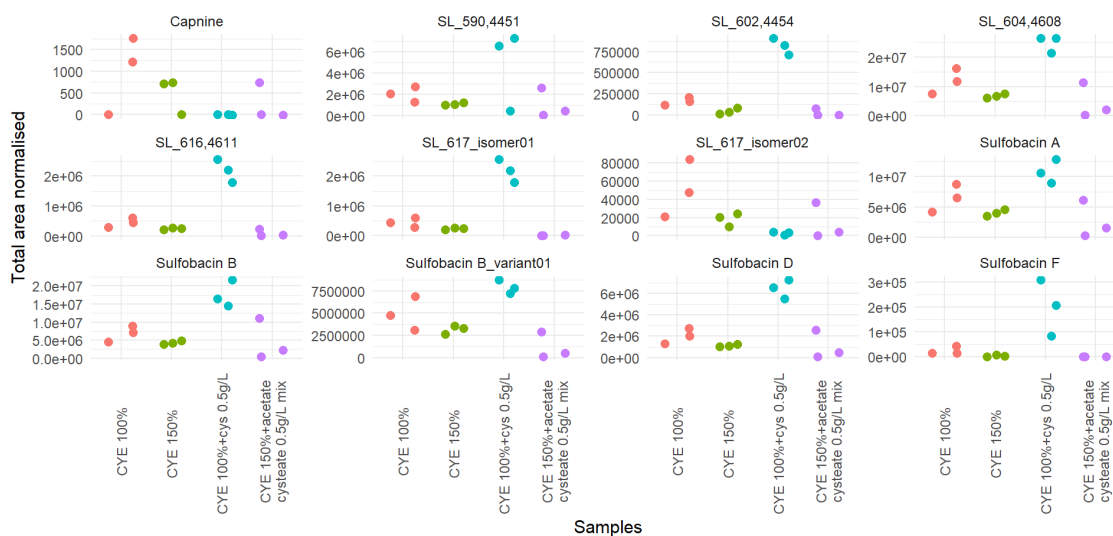


Figure 6.6. Four sample groups were made: CYE 100% (red), CYE 150% (green), CYE 100% cys 0.5 g/L (blue), CYE 150% acetate cysteate 0.5 g/L mix (purple), where the total area of all found Sols is shown in individual graphs. Each graph represents a singular Sol. The 12 Sols shown are the major Sols, their isomers, and variants.

Across most Sol species, a general trend was observed: CYE 100% 0.5 g/L cysteate exhibited the highest total areas, while the remaining conditions showed similar but lower signal intensities. This trend was consistent for the majority of the major Sols and their isomers. However, for capnine, the CYE 100% 0.5 g/L cysteate condition was below the detection limit, whereas detectable levels were observed in the other sample groups. As capnine is a precursor in Sol biosynthesis, the presence of additional cysteate may promote its conversion to downstream Sols, resulting in reduced detectable precursor levels. In contrast, SL 617 isomer02 did not follow this trend and exhibited the highest total area under the same conditions. This deviation suggests that its biosynthesis may not follow the same pathway or be regulated in the same way as the other Sols. The results from (Figure 6.6) show that the addition of cysteate raises the production of most Sols.

To assess if the increases in Sol production are statistically significant, three named sulfobacins A, B, and D were selected for analysis using an ANOVA with a significance threshold of $p < 0.05$. These sulfobacins were chosen due to their prior chemical characterization and reported biological relevance [29, 107].

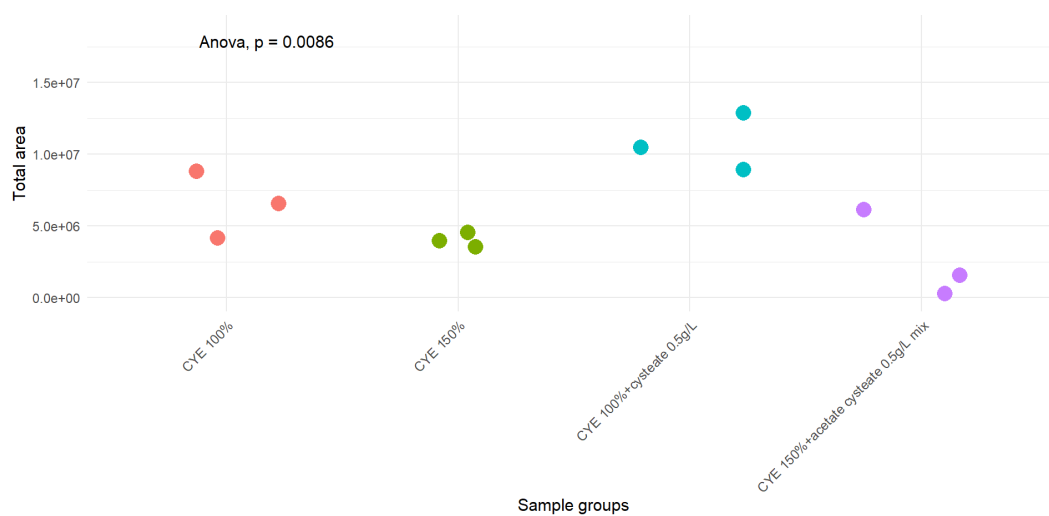
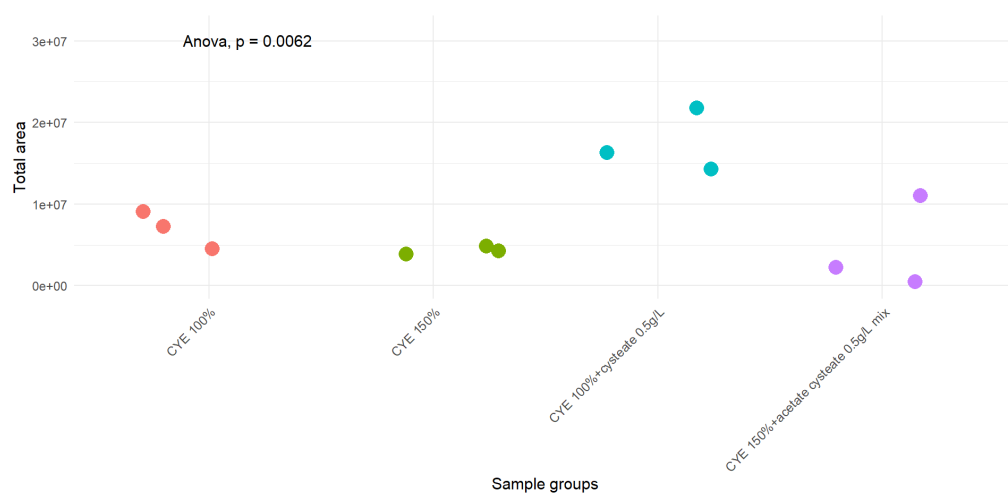
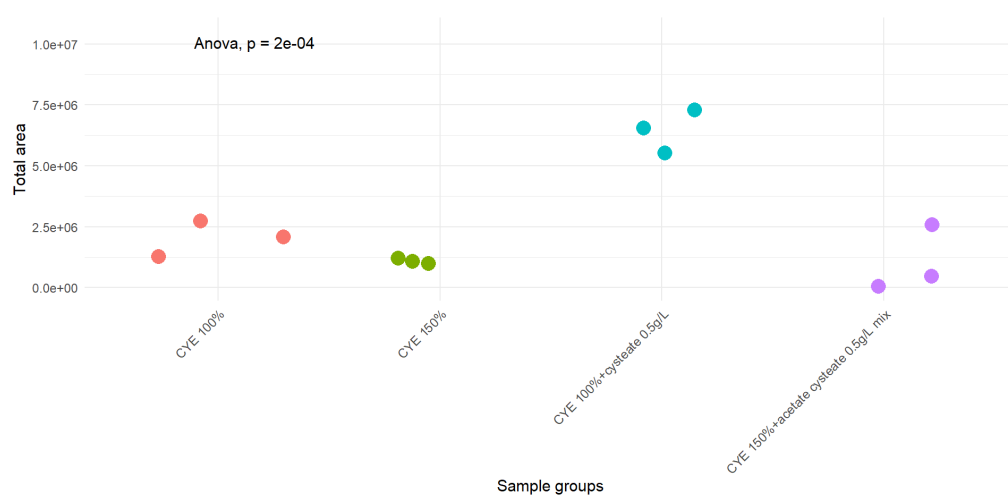
(a) *Sulfobacin A*(b) *Sulfobacin B*(c) *Sulfobacin D*

Figure 6.7. Statistical comparison of major sulfobacin production across growth conditions using an ANOVA test. CYE 100% with 0.5 g/L cysteate showed significantly higher production of all sulfobacins compared to other conditions, except for sulfobacin A versus CYE 100% ($p = 0.073$).

The ANOVA results showed statistically significant differences ($p < 0.05$) between the groups, indicating that the increase in Sulfobacin A, B, and D production in CYE 100% cys 0.5 g/L is systematic. The observed trend of a significant increase in the major sulfobacins, combined with the consistent increases seen across multiple Sol species, supports the conclusion that cystate supplementation enhances overall Sol production. The specific increase in sulfobacins A and B production is of interest, because of their previously described anti-inflammatory actions [29].

Pellet weights were comparable across conditions, indicating that cystate may not be a limiting factor in Fjoh growth. However, among the tested conditions, CYE 100% + 0.5 g/L cystate resulted in the highest and most consistent Sol production. This aligns with the proposed pathway, in which cystate serves as a precursor for 3-keto-capnine (Figure 2.5), supporting the hypothesis that precursor availability partially limits Sol production under standard conditions. These findings provide a potential production method that results in increased Sol expression compared to Fjoh grown under the other tested cultivation conditions.

6.2.1 Optimization of sulfonolipid expression with plasmids

To further optimize Sol production, Fjoh strains containing plasmids either Fjoh 2419 or Fjoh 2421 were constructed (Appendix A.4, A.5). The aim was to evaluate whether overexpression of these genes could enhance Fjoh growth or increase Sol production. Plasmid strains were cultivated under three conditions: CYE 100%, CYE 150%, and CYE 100% 0.5 g/L cystate in 5 mL of media.

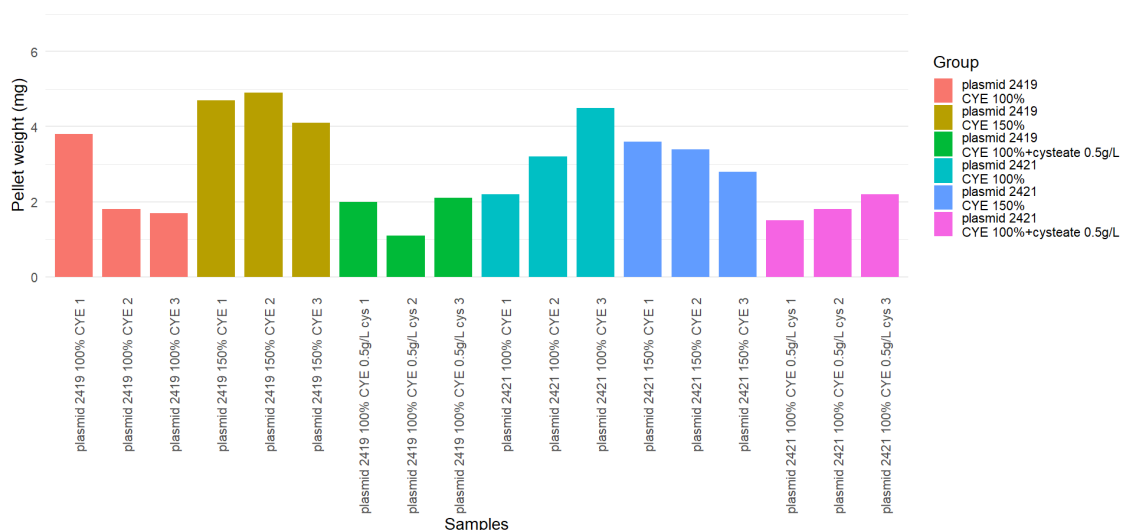


Figure 6.8. Fjoh 2419 and Fjoh 2421 samples CYE 100%, CYE 150%, CYE 100% cystate 0.5 g/L. The pellet weights vary between 1 and 5 mg, with the Fjoh 2419 CYE, 150% having the highest stable pellet growth, and Fjoh 2419, Fjoh 2421 CYE 100% cystate 0.5 g/L having the lowest growth.

Pellet weights from triplicate cultures ranged between 1 and 5 mg. The lowest biomass was observed for both plasmid strains grown in CYE 100% supplemented with 0.5 g/L cystate, where pellet weights were at or below 2 mg. In contrast, Fjoh 2419 grown in CYE 150% showed the highest and most consistent growth, with all replicates exceeding 4 mg.

Notably, all plasmid-containing strains exhibited lower pellet weights compared to Fjoh grown without plasmids under similar conditions (Figure 6.5). This reduction in growth is likely attributable to the metabolic burden associated with plasmid maintenance and gene expression. Additionally, cultivation in tetracycline-containing media may further reduce growth, even in resistant strains, due to physiological stress. These results suggest that plasmids impose a measurable energetic cost on Fjoh, reflected in reduced biomass accumulation [108]. The lower pellet weights indicate that, for future larger-scale production of Fjoh, plasmids might hinder lipid production.

Chromatograms from Fjoh control and plasmid-containing strains grown in CYE 100% revealed highly similar Sol profiles. Across all samples, the dominant peak corresponded to SL 602.4454, followed by

sulfobacin B, sulfobacin A, sulfobacin D, and sulfobacin F. Capnine was not detected above the detection limit in any sample.

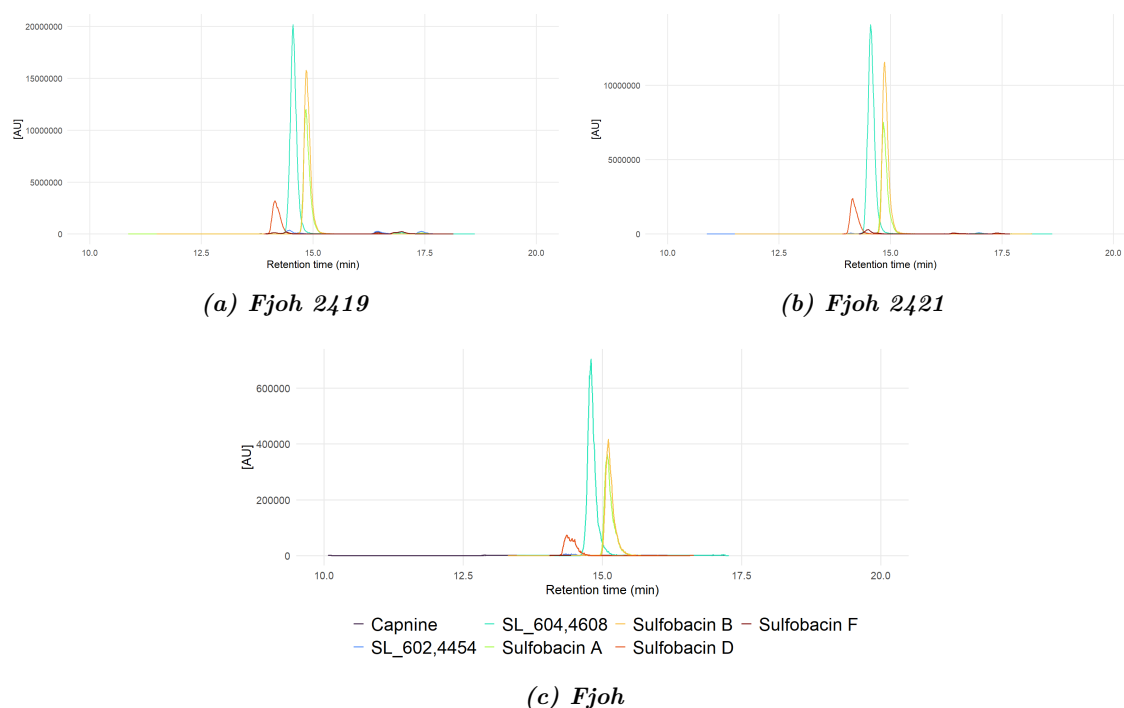


Figure 6.9. Chromatograms over the most present Sols. Capnine depicted (black), SL 602.4454 (dark blue), SL 604.4608 (light blue), sulfobacin A (light green), sulfobacin B (yellow), sulfobacin D (red), sulfobacin F (maroon). The three graphs show the Fjoh pCP 11 plasmid with the 2419 gene, the Fjoh pCP 11 plasmid with the 2421 gene, and the Fjoh profile. Fjoh profile were grown in 5 mL CYE 100%.

Compared with Fjoh without plasmids, the plasmid-containing Fjoh strains showed differences in their Sol profiles, particularly with increased signals of sulfobacin B and sulfobacin D. The elevated sulfobacin B levels in the plasmid-containing strains resulted in a profile more strongly dominated by sulfobacin B, whereas in Fjoh without plasmids, sulfobacin A and sulfobacin B were expressed at more comparable levels. There are slight differences in the sulfobacin profile between Fjoh 2419 and Fjoh 2421 in the minor Sols, such as SL 602.4454 being more expressed in Fjoh 2419 and sulfobacin D being more expressed in Fjoh 2421. The overall Sol composition remained highly consistent. This indicates that introducing either Fjoh 2419 or Fjoh 2421 does not substantially alter the qualitative Sol profile.

Because the plasmid strains were grown in tetracycline-containing media, it cannot be determined whether the differences arise from the inserted gene being expressed, the presence of the plasmid alone, or antibiotic resistance, which has been seen to change the lipid profile [109]. To determine whether the expression of the inserted gene or the presence of the plasmid alone drives the observed changes, inclusion of a pCP11 empty vector control would be necessary.

The total area of the major Sol species and their isomers further confirmed the similarity between the Fjoh control and plasmid strains. Substantial overlap was observed between all sample groups, indicating that plasmid insertion does not significantly enhance total Sol production.

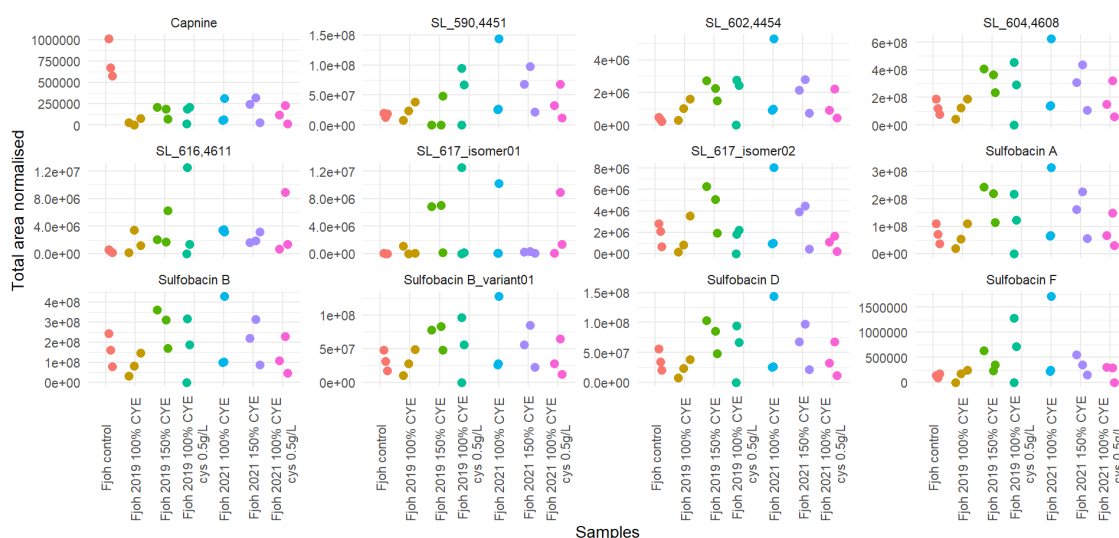


Figure 6.10. The total areas of seven sample groups, Fjoh control (red), Fjoh 2419 CYE 100% (brown), Fjoh 2419 CYE 150% (green), Fjoh 2419 CYE 100% cys 0.5 g/L (Turquoise), Fjoh 2421 CYE 100% (blue), Fjoh 2421 CYE 150% (purple), Fjoh 2421 CYE 100% cys 0.5 g/L (magenta), each sample from these groups is represented as a dot of the color described. The 12 Sols shown are the major Sols and their isomers and variants.

945 The total areas of the Sol species show substantial overlap between the Fjoh control and the plasmid-containing strains. Likewise, there is considerable overlap between the Fjoh 2419 and Fjoh 2421 sample groups, indicating that overall Sol production remains comparable across all strains.

Capnine represents the main deviation, with the Fjoh control exhibiting higher total areas than the plasmid-containing strains. As capnine is a precursor in Sol biosynthesis (Figure 2.5), this difference may suggest altered pathway synthesis in the plasmid strains [110]. Either capnine is being converted more efficiently downstream, or its synthesis is reduced due to the burdens imposed by plasmid maintenance and antibiotic selection. However, since total Sol levels do not increase proportionately, the lower capnine levels in plasmid strains are more likely due to reduced synthesis rather than enhanced downstream conversion.

For optimized Sol production, biomass yield and lipid output were considered together. Fjoh grown without plasmids under CYE 100%, CYE 150%, and CYE 100% 0.5 g/L cysteate all produced pellet weights exceeding 5 mg (Figure 6.5), outperforming all plasmid-containing strains. The lesser pellet weights in plasmid-containing Fjoh align with the energetic and fitness cost that is accompanied by the plasmid in bacteria, which leads to reductions in the reproductive rate for plasmid-carrying bacteria [108]. Therefore, the higher pellet weights of the non-plasmid-containing Fjoh are expected as they do not carry this extra energetic burden.

Among the Fjoh strains without plasmid, CYE 100% supplemented with 0.5 g/L cysteate consistently yielded higher Sol total areas compared to the other growth conditions (Figure 6.6). This increase in Sol production fits with cysteate use as a precursor in the production of Sols as described in (Figure 2.5) [49]. Since this increase in production of Sols occurred without a substantial reduction in biomass (Figure 6.5), cysteate supplementation appears to enhance the overall production of Sols. Based on these combined findings, CYE 100% supplemented with 0.5 g/L cysteate was selected as the optimal medium for upscaling Sol production.

Before upscaling, the Fjoh Sol profile was compared with those of *Alistipes timonensis* and *Alistipes putredinis* to evaluate whether Fjoh produces a Sol profile similar to that of known gut-associated Sol producers [29]. This comparison was performed to assess whether Fjoh is a suitable model organism for Sol production and for investigating the potential prebiotic effects of Sols.

6.2.2 *Alistipes* sulfonolipids profile and production

Alistipes timonensis and *Alistipes putredinis* were analyzed to gain insight into Sol profiles represented in the gut environment. *Alistipes* is one of the two known Sol-producing genera in the human gut. The *Alistipes* genera growth was also tested to determine if the Sol profiles changes deepend on which part of the growth cycle it is in.

Chromatograms for *Alistipes timonensis* and *Alistipes putredinis* were generated for both species and compared with Fjoh control samples. The detected Sol species included capnine, SL 602.4454, SL 604.4608, sulfobacin A, sulfobacin B, sulfobacin D, and sulfobacin F.

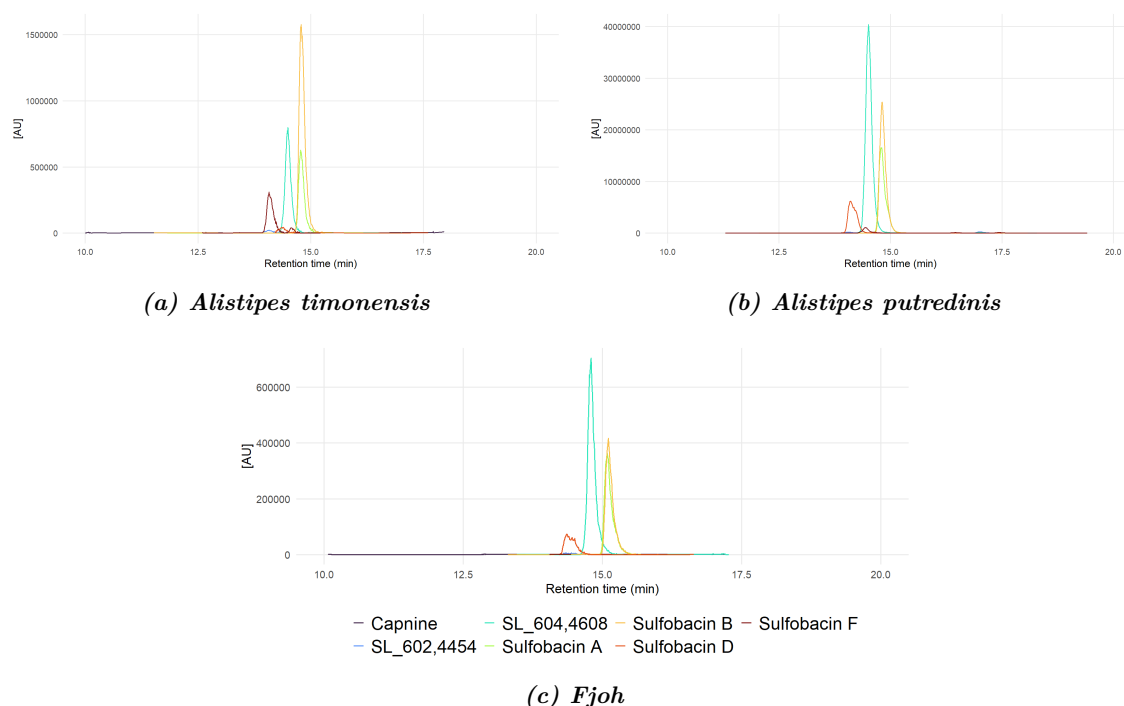


Figure 6.11. Chromatograms over *Alistipes timonensis* and *Alistipes putredinis* and Fjoh control. The Sols are depicted as lines with Capnine in (black), SL 602.4454 in a (dark blue), SL 604.4608 in a (light blue), sulfobacin A in a (light green), sulfobacin B in (yellow), sulfobacin D in (red), sulfobacin F in a (maroon).

The Sol profiles of the two *Alistipes* species differ from each other. In *Alistipes timonensis*, sulfobacin B is the dominant Sol, followed by SL 604.4608 and sulfobacin A, with lower contributions from sulfobacin F and D. In contrast, *Alistipes putredinis* shows SL 604.4608 as the most prevalent Sol, followed by sulfobacin B and A, and then D and F. Although the same major Sol species are present in both strains, their relative abundances differ.

The chromatograms indicate that Fjoh is a suitable model organism for Sol production, as it produces the same major Sol species as the tested gut strains that have been linked with anti-inflammatory effects [28, 111]. Notably, both Fjoh and *Alistipes* predominantly produce sulfobacin A and B, which have been associated with anti-inflammatory activity [29].

To further determine if Fjoh is a good model organism for Sol production, a chromatogram over *Odoribacter* Sols would have been able to determine if it also produces a similar profile to that of *Alistipes* and Fjoh, and thereby if Fjoh Sol matches the full picture of the major Sols that exist within the human gut.

Most *Alistipes* samples show relatively stable profiles under the tested conditions (Appendix A.3). Notably, one *Alistipes timonensis* sample grown for 24 hours differed from the 72-hour profile, suggesting that Sol composition may depend on growth phase. To investigate this further, both species were sampled at multiple time points across their growth curves.

A growth curve was made over both *Alistipes* strains to determine at which time point there were

certain growth phases. The growth curve showed that *Alistipes timonensis* exhibited a lag phase until approximately 5 hours, followed by exponential growth until around 24 hours, after which it entered the stationary phase and began declining by 48 hours. *Alistipes putredinis* displays a slightly longer lag phase, 6 hours, and enters exponential growth later than *Alistipes timonensis*, reaching the stationary phase around 28 hours. These differences indicate that *Alistipes timonensis* has a longer lag phase than *Alistipes putredinis*.

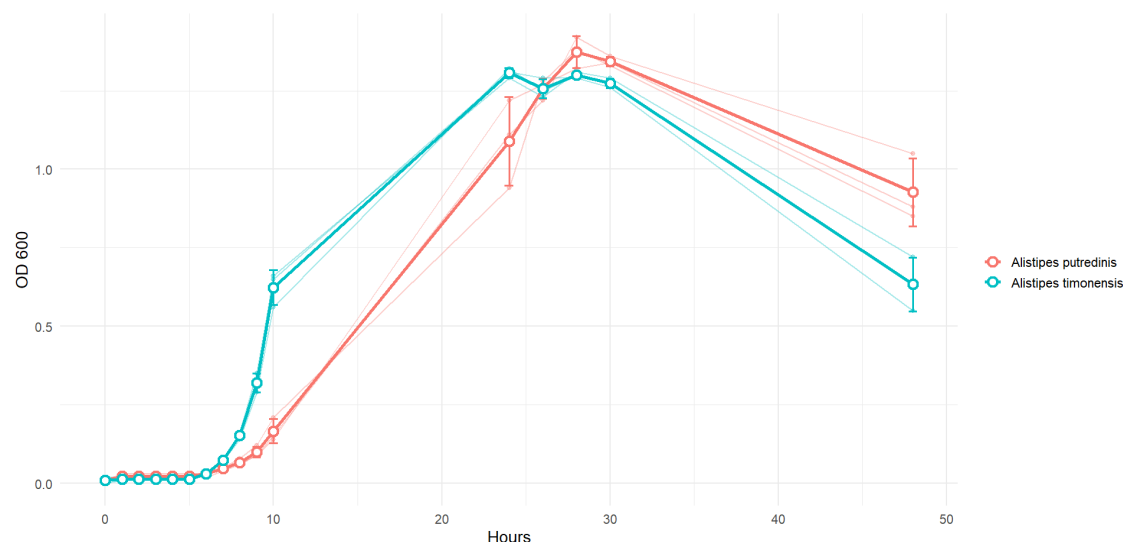


Figure 6.12. Growth curve for *Alistipes putredinis* in (red) and *Alistipes timonensis* in (blue) based on three at each timepoint. *Alistipes timonensis* enters the exponential growth phase more quickly. At 24 hours, it has reached the stationary phase; at 48 hours, it is declining. *Alistipes Putredinis* starts its exponential phase later, after 24 hours, is still growing, reaches the stationary phase around 28 hours, and decreases at 48 hours.

The growth curves for *Alistipes timonensis* and *Alistipes putredinis* followed each other, indicating that the bacteria entered the specific growth phases at similar time frames.

The growth curve shows that the *Alistipes* samples that differed in their Sol profiles in (Appendix A.3) were tested at different growth phases. Growth phases have major impacts on the specific lipid profiles [112]; therefore, the differences in Sol profile most likely derive from this factor.

Sol total areas were analyzed at 4, 10, 24, 28, and 48 hours. Overall, the majority of Sol species in *Alistipes* were detected at lower total areas compared to Fjoh, with several Sol types falling near or below the detection limit. Only a single time point for each strain showed all Sol species in detectable amounts: a 48-hour sample for *Alistipes timonensis* and a 24-hour sample for *Alistipes putredinis*. Since comprehensive detection occurs only at these isolated time points, it is not possible to draw robust conclusions about systematic growth-phase-dependent shifts in Sol composition.

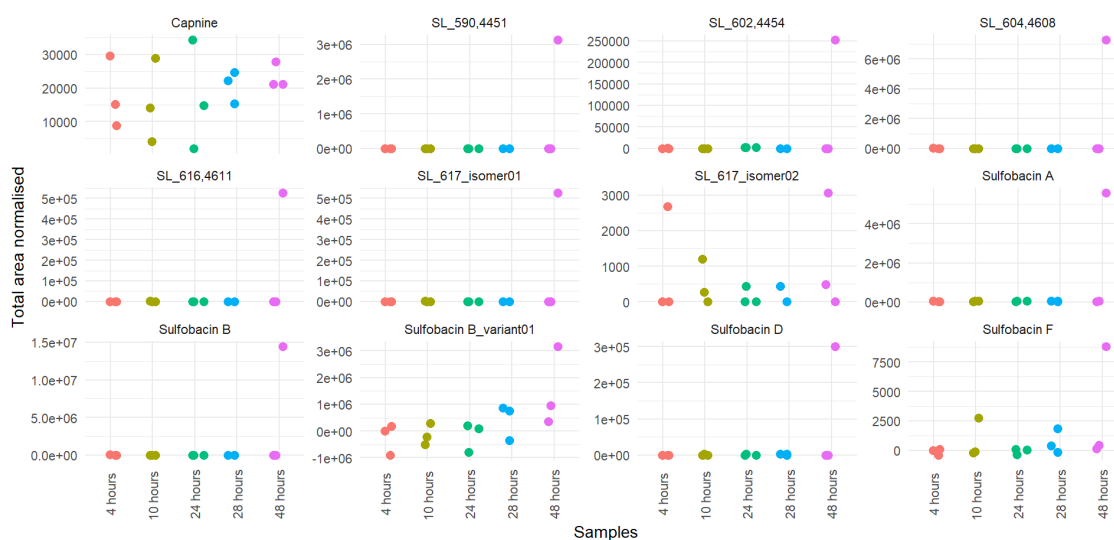


Figure 6.13. Total ion areas of Sols detected in *Alistipes timonensis* across five time points: 4 hours (red), 10 hours (brown), 24 hours (green), 28 hours (blue), and 48 hours (purple). Results are displayed across 12 plots representing the major Sol species, their isomers, and variants.

The figure shows that only one 48-hour sample had detectable concentrations of all Sols. Therefore, it can be noted that the general amount of Sols is low in *Alistipes timonensis*. All samples had detectable amounts of Caprine and Sulfobacin B variant01, and some samples had detectable amounts of SL 617 isomer02 and Sulfobacin F.

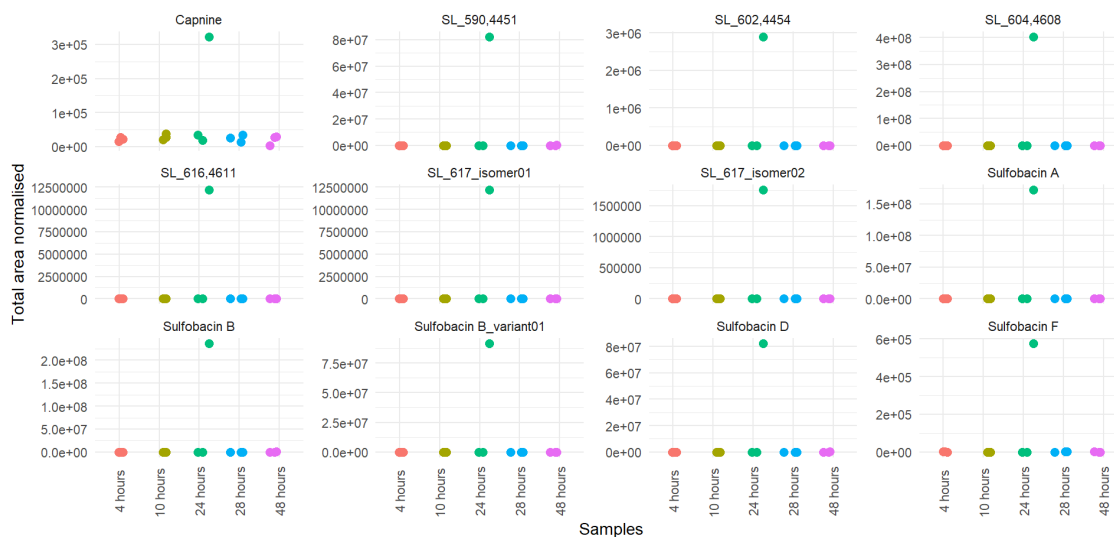


Figure 6.14. Total ion areas of Sols detected in *Alistipes putredinis* across five time points: 4 hours (red), 10 hours (brown), 24 hours (green), 28 hours (blue), and 48 hours (purple). Results are displayed across 12 plots representing the major Sol species, their isomers, and variants.

For *Alistipes putredinis*, only one sample from 24 hours showed any detectable level of Sols, while all samples showed low amounts of Caprine, and the 24-hour sample showed a high amount. These data show that *Alistipes putredinis* has a low amount of Sols, so much so that it is below the detection limit for most samples. Compared to the *Alistipes timonensis* (Figure 6.13) samples, *Alistipes putredinis* appears to have an even lower overall Sol content since there are some Sols for *Alistipes timonensis* where all samples have above detection level amounts of the Sol, and this is not true for *Alistipes putredinis* where only the 24-hour samples have any Sols.

When comparing Fjoh with *Alistipes*, the same major Sol species are present across bacteria (Figures 6.11). However, the overall production of Sols is substantially higher in Fjoh (Figures 6.6, 6.13, 6.14). This shows that Fjoh is a valuable model organism for the production of Sols, and that the anti-inflammatory impacts of the Sols can be studied out from Fjoh-produced Sols [29, 28, 111].

6.3 Prebiotic sulfonolipid stool test

Stool samples were incubated either without added lipids (control) or supplemented with lipids extracted from Fjoh to evaluate whether Sols influence microbial metabolic activity. The lipid-supplemented groups consisted of Sol producing Fjoh extracts and extracts from $\Delta 2419$ and $\Delta 2421$ Fjoh strains, in which key genes in the Sol biosynthetic pathway were knocked out (Figure 2.5) [49]. This allowed assessment of whether any metabolic effects could be specifically attributed to the presence of Sols rather than to background lipid components.

To evaluate metabolic changes, two methods were performed at 24, 48, and 72 hours of incubation. H_2S production was measured to determine whether the sulfur from Sols could be utilized by the stool microbiome and metabolized to H_2S . In parallel, SCFA profiles were analyzed using CE and qNMR to assess whether Sol supplementation altered fermentative metabolism.

By comparing control, Sol-positive, and Sol-lacking lipid extracts across time points, the experiment was designed to determine whether Sols modulate sulfur metabolism, and SCFA production.

6.3.1 Hydrogen sulfide testing

Lead hydrogen sulfide experiment

H_2S can lead to a variety of health outcomes based on its concentration found in the gut [23]. Up to 50% of H_2S is produced by the microbiome, which Sols might modulate.

To initially assess whether adding Sols would influence the microbiome, a lead experiment was conducted using stool from a single individual (Appendix A.18). The stool samples were inoculated either without lipids or with lipids extracted from Sol-producing Fjoh grown with cysteate, or from Fjoh with the 2419 gene knocked out, which impairs Sol production. This approach enabled comparison of the effects of Sol-containing lipid extracts with the remaining Fjoh lipid profile, which lacked Sols.

Two concentrations were tested: 5 mg/mL and 1.5 mg/mL lipids; the stool was inoculated for 24 hours (Figure 6.15). The H_2S testing did not show any significant difference in the amount of H_2S between the samples. However, the samples with Sol are slightly higher. However, all the samples had such low absorbance that the calibration curve could not detect the micromolar concentration of H_2S .

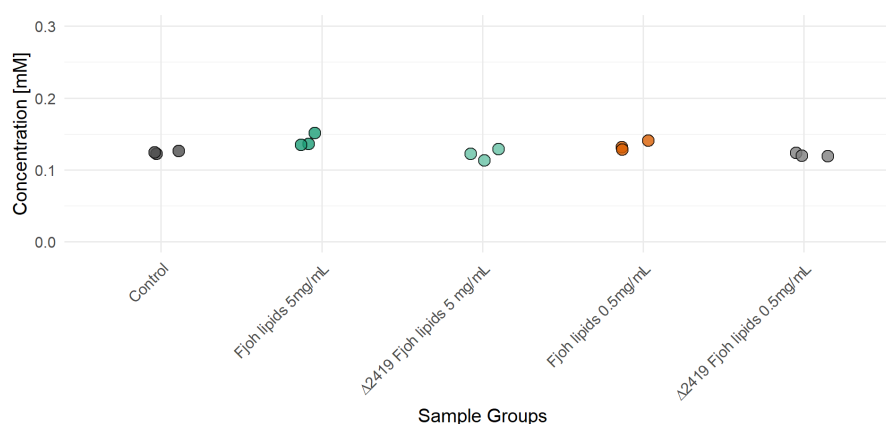


Figure 6.15. H_2S absorbance measured at 24 hours for five sample groups: control, Fjoh $\Delta 2419$ Fjoh lipids 0.5 mg/mL, 5 mg/mL, and Fjoh lipids 0.5 mg/mL, 5 mg/mL. The control and $\Delta 2419$ Fjoh groups showed absorbance values of approximately 0.12, while Fjoh Sol-containing samples exhibited slightly higher absorbance at both concentrations.

The H₂S assay did reveal slight differences between the groups. The control and Fjoh Δ 2419 Fjoh lipid samples, both 0.5 mg/mL and 5 mg/mL, showed absorbance values around 0.12. The Fjoh samples containing Sols exhibited slightly higher absorbance values at both concentrations; however, the increase was minimal. Additionally, all absorbance values were low and fell below the calibration curve's reliable quantification range, preventing accurate determination of micromolar H₂S concentrations.

Although no major differences were observed after 24 hours, the slight increase in absorbance in the Fjoh 5 mg/mL sample compared to the Fjoh Δ 2419 and control samples suggests that Sols may contribute to H₂S production. The results indicate that a longer incubation time may be required for the stool microbiome to metabolize the added Sols and generate measurable amounts of H₂S.

Expanded cohort Hydrogen sulfide experiment

The main H₂S experiment was conducted using two different lipid profiles: Fjoh Sol-producing, Fjoh Δ 2419, and Fjoh Δ 2421 non-Sol-producing, each tested at two concentrations, 1.5 mg/mL and 5 mg/mL. A 1.5 mg/mL fecal slurry was used for all conditions. Samples were incubated for 24, 48, and 72 hours.

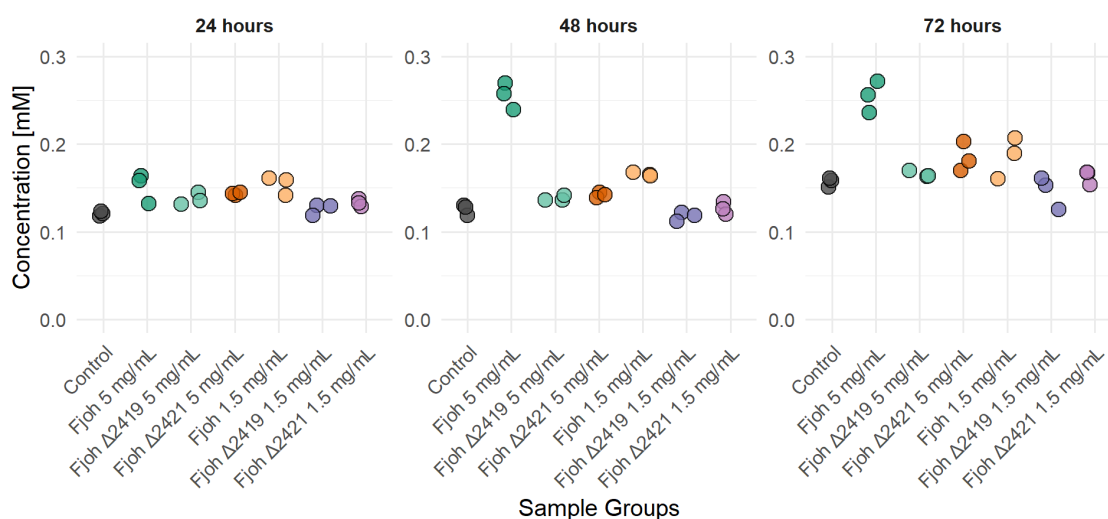


Figure 6.16. Three different time points: 24, 48, and 72 hours, for a control without lipids. Fjoh Δ 2419, Fjoh Δ 2421, and Fjoh profile with Sols. After 24 hours, there was only a slight difference between the samples, with the Fjoh samples containing Sols showing higher absorbance. For 48 hours, the control, Fjoh Δ 2419, and Fjoh Δ 2421 are around the same as they were at 24 hours, while Fjoh with Sols are significantly higher, and there can also be seen between the 5 mg/mL and 1.5 mg/mL. For 72 hours, all sample absorbances have risen, and the controls, Fjoh Δ 2419 and Fjoh Δ 2421, are closer to the Fjoh with Sols; however, the 5 mg/mL still has a significantly higher absorbance.

After 24 hours, Fjoh samples containing Sols showed significantly higher absorbance compared to several Fjoh Δ 2419, Fjoh Δ 2421, and control samples ($p < 0.05$). The Fjoh 5 mg/mL and 1.5 mg/mL groups exhibited absorbance values of approximately 0.14–0.16, whereas the knockout and control groups exhibited absorbance values of approximately 0.12–0.14. However, no significant difference was observed between the Fjoh 5 mg/mL and 1.5 mg/mL groups at the 24-hour time point ($p = 0.354$), suggesting that Sol concentration was not yet a determining factor for H₂S production within 24 hours (Appendix A.22).

At 48 hours, the differences became markedly more pronounced. Fjoh 5 mg/mL displayed a substantial increase in absorbance, 0.23–0.26, while Fjoh 1.5 mg/mL reached approximately 0.16. In contrast, Fjoh Δ 2419, Fjoh Δ 2421, and control samples remained around 0.13–0.14. Multiple comparisons demonstrated highly significant differences between Fjoh 5 mg/mL and all other groups (****, $p < 0.0001$). Additionally, a clearer distinction emerged between the two Fjoh concentrations, indicating a concentration-dependent effect of Sols on H₂S production after prolonged incubation (Appendix A.23).

By 72 hours, absorbance values increased in all groups, including control and knockout samples, 0.16–0.20, indicating background microbial sulfur metabolism in the fecal slurry. Nevertheless, Fjoh 5 mg/mL

remained significantly higher than all other conditions ($p < 0.001$). This suggests that although Sol supplementation accelerates and enhances H_2S production, extended incubation allows endogenous stool metabolism to partially diminish relative differences between groups (Appendix A.24).

1085 The absence of a 0-hour sample for all groups makes it difficult to determine whether H_2S concentrations initially increased or decreased during the first 24 hours of incubation.

1090 These results demonstrate that Sol containing Fjoh lipids significantly enhances H_2S production in fecal slurry, with the strongest and most concentration-dependent effect observed after 48 hours of incubation. The similar behavior of Fjoh $\Delta 2419$, Fjoh $\Delta 2421$, and control samples indicates that the presence of Sols is the primary factor driving the observed increase in H_2S production. This was expected because Sols contain sulfur groups that could potentially serve as substrates for sulfur-metabolizing and H_2S producing microorganisms. H_2S production has been shown in the context of the sulfur-containing sugar sulfoquinovose, to upregulate *Desulfovibrio*, which is a H_2S producer [113].

1095 The upregulation of SRB could lead to the unwanted effect of enhancing the likelihood of developing IBD [55]. However, a controlled rise in H_2S to preferable levels could lead to lessening the impacts of IBD [23]. Therefore the shown increase in H_2S production from the addition of Sols to the stool (Figure A.23) indicate that there might be use cases in the future for Sols as a prebiotic to raise the H_2S level in the gut to preferable levels which then would lead to positive health outcomes [52, 25].

6.3.2 Short chain fatty acids quantification

1100 Capillary electrophoresis

1105 CE was used to quantify SCFAs using peak areas ($\text{mAU} \cdot \text{s}$) and compound-specific retention times. Six calibration curves were generated to convert $\text{mAU} \cdot \text{s}$ values into micromolar concentrations using compound-specific linear equations (Appendix A.25). The concentrations of various SCFAs were analyzed to determine whether Sols affect SCFA production in stool. This factor is important since SCFAs have been linked to a wide variety of health outcomes [58, 59].

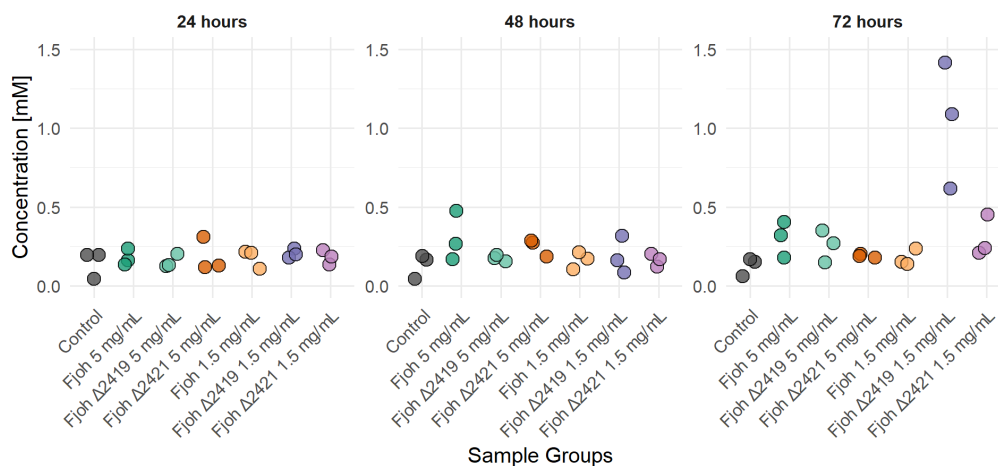


Figure 6.17. Formate concentration in micromolar is shown at three time points of 24, 48, and 72 hours. At each time point, three samples have been tested for seven sample groups: a control of just 1.5 mg/mL stool, and six groups with added material Fjoh, Fjoh $\Delta 2419$, and Fjoh $\Delta 2421$ at either 5 mg/mL or 1.5 mg/mL added material.

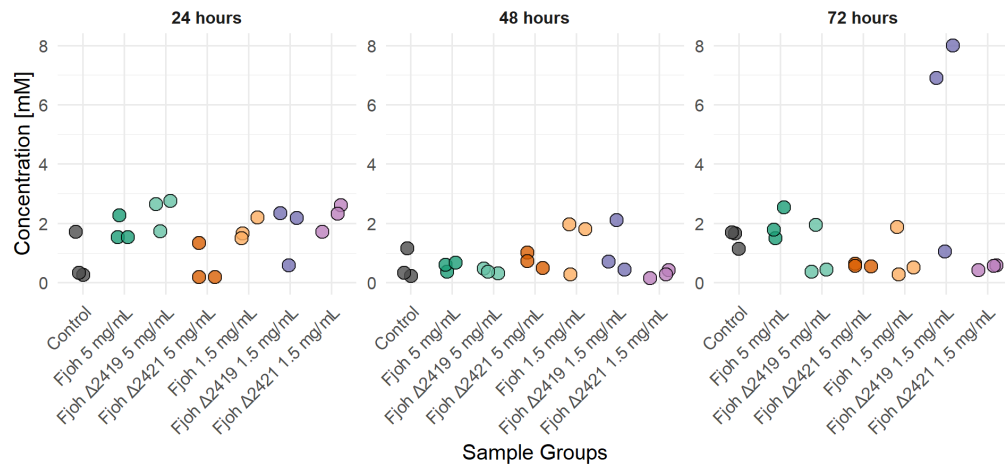


Figure 6.18. Acetate concentration in micromolar is shown at three time points of 24, 48, and 72 hours. At each time point, three samples have been tested for seven sample groups: a control of just 1.5 mg/mL stool, and six groups with added material Fjoh, Fjoh Δ 2419, and Fjoh Δ 2421 at either 5 mg/mL or 1.5 mg/mL added material.

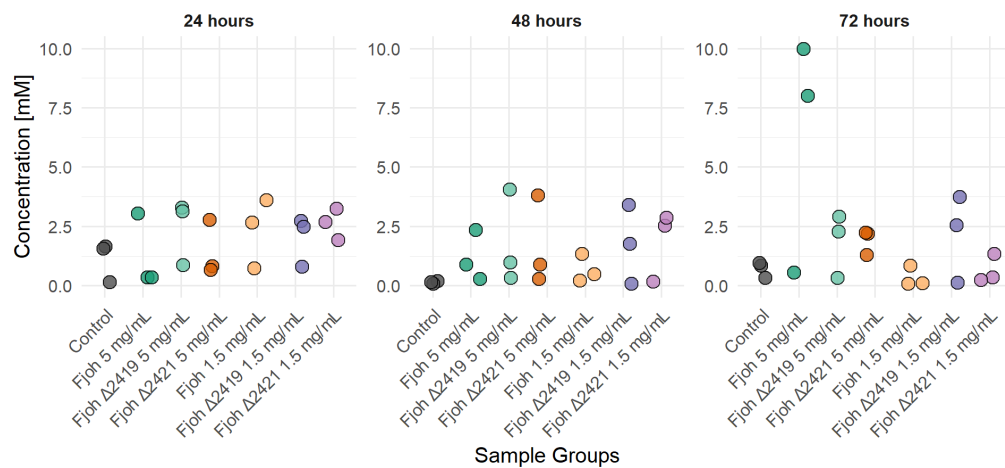


Figure 6.19. Lactate concentration in micromolar is shown at three time points of 24, 48, and 72 hours. At each time point, three samples have been tested for seven sample groups: a control of just 1.5 mg/mL stool, and six groups with added material Fjoh, Fjoh Δ 2419, and Fjoh Δ 2421 at either 5 mg/mL or 1.5 mg/mL added material.

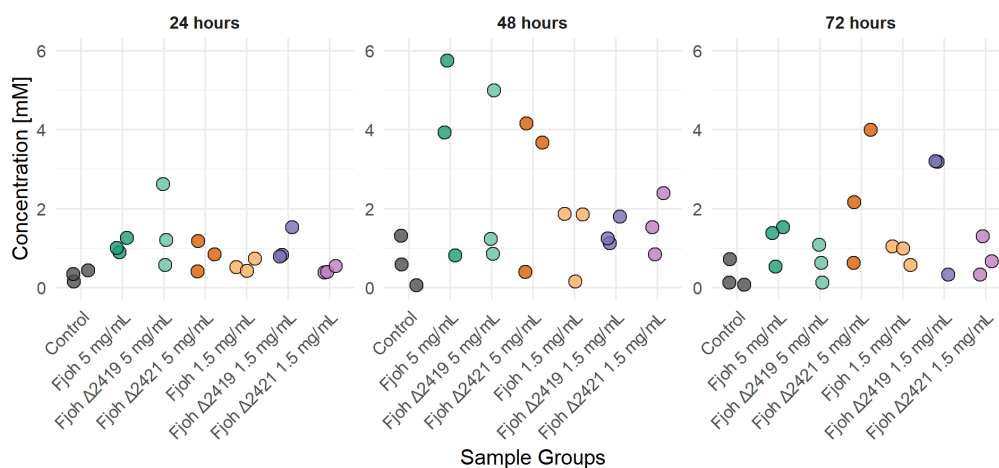


Figure 6.20. Propionate concentration in micromolar is shown at three time points of 24, 48, and 72 hours. At each time point, three samples have been tested for seven sample groups: a control of just 1.5 mg/mL stool, and six groups with added material Fjoh, Fjoh Δ 2419, and Fjoh Δ 2421 at either 5 mg/mL or 1.5 mg/mL added material.

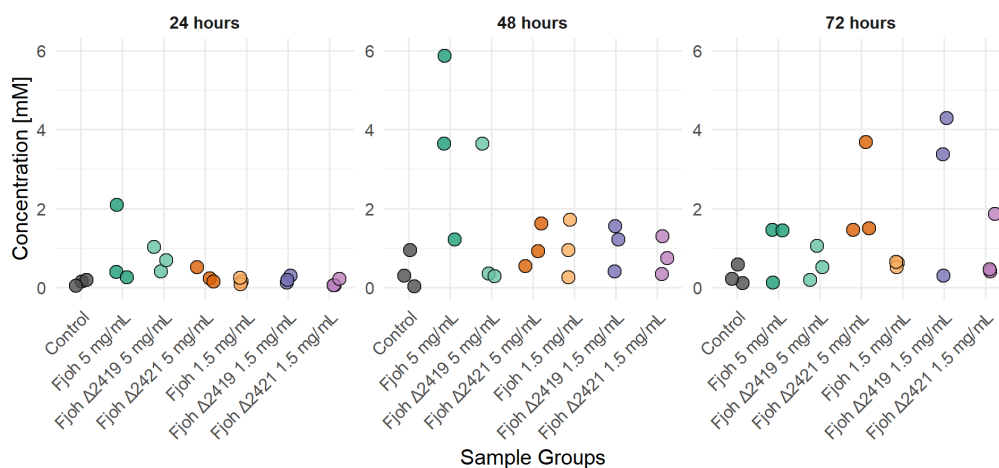


Figure 6.21. Butyrate concentration in micromolar is shown at three time points of 24, 48, and 72 hours. At each time point, three samples have been tested for seven sample groups: a control of just 1.5 mg/mL stool, and six groups with added material Fjoh, Fjoh Δ 2419, and Fjoh Δ 2421 at either 5 mg/mL or 1.5 mg/mL added material.

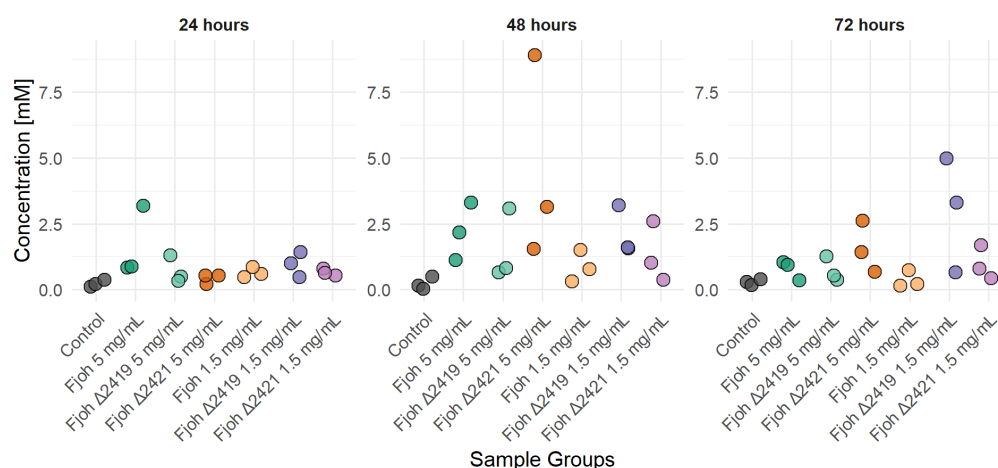


Figure 6.22. Valerate concentration in micromolar is shown at three time points of 24, 48, and 72 hours. At each time point, three samples have been tested for seven sample groups: a control of just 1.5 mg/mL stool, and six groups with added material Fjoh, Fjoh Δ 2419, and Fjoh Δ 2421 at either 5 mg/mL or 1.5 mg/mL added material.

Across all measured SCFAs, lipid supplementation generally resulted in higher concentrations compared to control, particularly at 48 hours. After 24 hours, differences between groups were limited, with concentrations often overlapping. By 48 hours, clearer increases were observed for lactate (Figure 6.19), propionate (Figure 6.20), butyrate (Figure 6.21), and valerate (Figure 6.22), whereas formate (Figure 6.17) and acetate (Figure 6.18) remained more comparable across conditions.

At the 48 and 72-hour time points, isolated increases were observed in specific groups; however, these were neither consistently concentration-dependent nor reproducibly associated with the presence of Sols. While Fjoh samples containing Sols showed higher lactate concentrations at 72 hours and higher butyrate in selected replicates at 48 hours, these effects were not systematic across all replicates. If Sol did have specific increases in these SCFAs, this could be associated with positive health outcomes, as butyrate has been shown to have anti-inflammatory properties that could help individuals with IBD [60]. Lactate has also been tested and shows promise, as it reduces local epithelial damage in models of acute intestinal inflammation [114].

All sample groups were t-tested to determine whether any group was significantly different from any other. This was done to determine whether both the Fjoh 5 mg/mL and 1.5 mg/mL samples were statistically higher than their corresponding lipid-supplemented samples lacking the Sol lipids. If this were the case, it would be appropriate to link the increase in that SCFA to Sols. The t-test shows that this was not the case and that there is a high variability in all samples, and no clear signs were able to be determined. (Appendix A.30, A.31, A.26, A.27, A.32, A.33, A.34, A.35, A.28, A.29, A.39, A.40). The lack of a universally statistically significant addition to the stool samples shows that a larger sample size would be necessary to determine if only subtle differences in expression occur, which would be extra relevant for butyrate (Figure 6.21) and lactate (Figure 6.19) since they showed an increase.

Overall, the CE data indicate that the addition of the Fjoh lipid profile influences SCFA production over time. In contrast, the specific contribution of Sols does not produce a consistent or statistically supported metabolic signature across conditions.

Quantitative nuclear magnetic resonance for metabolites

qNMR provides a second view of the SCFAs produced by lipid and Sols supplementation. qNMR also provides insight into other metabolites, such as ethanol, methanol, and trimethylamine, which together provide a broader picture of the impacts of lipid supplementation. QNMR analysis was performed on control, Fjoh, and Δ 2419 Fjoh samples at 24 and 72 hours. The metabolites quantified included SCFAs, ethanol, methanol, and trimethylamine.

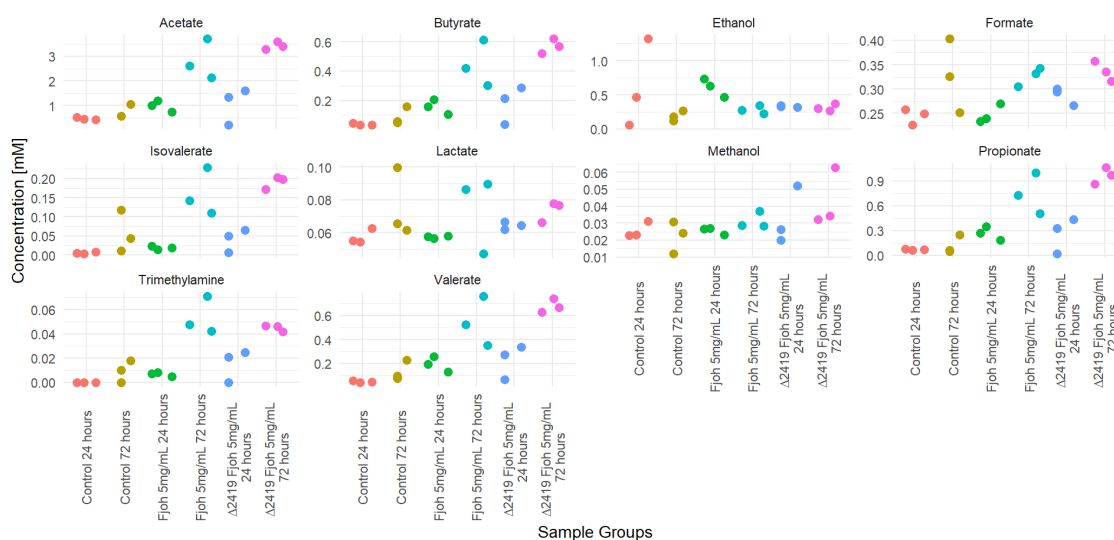


Figure 6.23. qNMR-derived concentrations (mM) shown as jitter plots across sample groups: control, Fjoh, and Fjoh $\Delta 2419$ Fjoh, at 24 and 72 hours of incubation. Each point represents an individual replicate (Appendix A.37,A.38.)

For acetate, butyrate, isovalerate, propionate, valerate, and trimethylamine, a consistent trend was observed in which both 24-hour and 72-hour control samples exhibited lower concentrations compared to the corresponding Fjoh and $\Delta 2419$ Fjoh samples. This indicates that the addition of lipid extracts enhances the production of several SCFAs and metabolites.

At the 24-hour time point, Fjoh and $\Delta 2419$ Fjoh samples showed comparable metabolite concentrations across acetate, butyrate, isovalerate, propionate, valerate, and trimethylamine, suggesting that the presence or absence of added lipids does not markedly influence early-stage metabolite production. However, at 72 hours, a trend emerged in which one of the Fjoh replicates displayed similar concentrations to the $\Delta 2419$ Fjoh samples, while two replicates showed a lower concentration. This pattern may be related to differences in lipid composition. Since $\Delta 2419$ Fjoh lacks Sols, which make up 19% of the profile in a non-knockout [27], the relative enrichment in other lipid classes in $\Delta 2419$ Fjoh may provide substrates that are more efficiently metabolized and produced into SCFA, thereby promoting increased SCFA production in the $\Delta 2419$ Fjoh samples.

Trimethylamine did not fully follow this pattern. At 72 hours, Fjoh samples exhibited two concentrations similar to, and one higher than, those observed in $\Delta 2419$ Fjoh. This suggests that Sols may be equally effective, or potentially more effective, at facilitating Trimethylamine production. This observation is notable, as Trimethylamine is a precursor to trimethylamine N-oxide, a metabolite associated with inflammatory and cardiovascular conditions [115]. Thus, lipid supplementation appears to enhance not only beneficial metabolites such as SCFAs but also metabolites with potential adverse physiological effects. This highlights the importance of monitoring a broader panel of metabolites, including potentially harmful compounds, when evaluating prebiotic candidates, as a net beneficial effect cannot be assumed from SCFA data alone.

In contrast, ethanol, formate, lactate, and methanol did not display a consistent increase following the addition of either Fjoh or $\Delta 2419$ Fjoh lipid profiles. Concentrations across sample groups remained relatively stable at both time points, with only minor variability and occasional outliers such as ethanol in the 24-hour control. This stability suggests that the added lipids do not significantly influence the production of these metabolites under the tested conditions.

Attempts were also made to quantify additional metabolites with potential health impacts, such as secondary bile acids [116]. However, due to the limited amount of stool material available for analysis, these compounds were below the detection threshold in the NMR spectra and could therefore not be reliably quantified.

Overall, the qNMR data demonstrate that lipid supplementation increases the production of several SCFAs. However, the presence of Sols in the Fjoh profile appears to moderate this increase for most metabolites,

except for trimethylamine at 72 hours.

The qNMR data are largely consistent with the CE results, showing that control samples cluster more closely and exhibit lower inter-sample variability than the Fjoh and $\Delta 2419$ Fjoh samples. Furthermore, metabolite concentrations in control samples remain relatively similar between 24 and 72 hours, whereas greater divergence over time is observed in the lipid-supplemented groups.

Some differences between the two analytical methods are evident. In the qNMR data, metabolite trends appear more consistent, particularly at 72 hours, where Fjoh samples tend to show one replicate with concentrations comparable to $\Delta 2419$ Fjoh and two replicates with lower concentrations. This pattern is less apparent in the CE data, where inter-sample variability obscures a clear directional trend between Fjoh and $\Delta 2419$ Fjoh.

When integrating results from both techniques, it becomes evident that lipid supplementation has a limited impact at 24 hours, as metabolite concentrations are generally comparable between control and lipid-treated samples. At later time points, however, lipid addition is associated with increased concentrations of several metabolites. At the same time, both datasets highlight that inter-sample variability is a substantial contributor to the observed differences in metabolite levels and must therefore be considered when interpreting the metabolic effects of lipid supplementation.

The absence of significant changes in SCFA concentrations suggests that Sol supplementation alone was insufficient to substantially alter fermentative metabolic activity within the stool microbiome. Although increased levels of acetate, propionate, and butyrate are commonly associated with beneficial prebiotic activity and improved intestinal health [58, 59], their production depends on factors such as substrate accessibility, microbial composition, and incubation conditions. Therefore, the lack of detectable changes does not necessarily exclude biological activity of Sols, but may instead indicate that their effects are directed toward alternative metabolic pathways, including sulfur metabolism.

6.3.3 Microbiome 16S rRNA gene sequencing

To analyze the microbial community composition, near-full-length 16S rRNA gene amplicon sequencing was performed on three sample groups: control, Fjoh, and $\Delta 2419$ Fjoh at two incubation time points: 24 and 72 hours. To determine whether the addition of Sols influenced the microbiome composition, the bacterial genera present in the samples were first identified. To more clearly evaluate differences between the sample groups, both alpha and beta diversity analyses were conducted.

To assess sequencing quality and depth, rarefaction curves were generated by plotting sequencing depth (reads) against the number of observed OTUs.

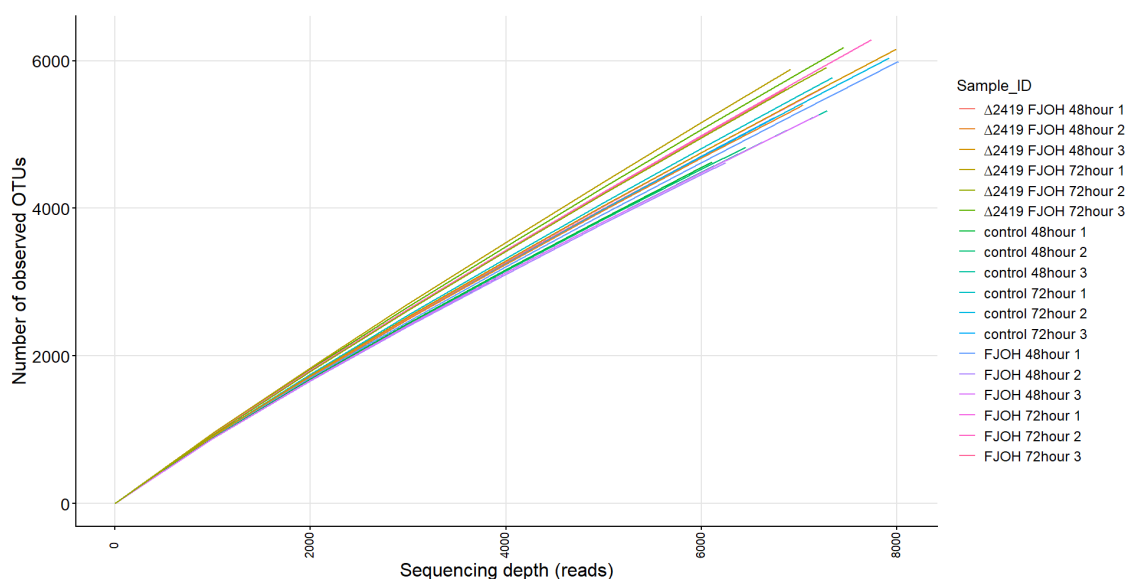


Figure 6.24. Rarefaction curve for three sample groups: control, Fjoh, and $\Delta 2419$ Fjoh at 2 different time points, 49 hours and 72 hours, the sequencing depth shows that all samples are between 6000 and 8000 with an observed OTUs between 4000 and 6500.

The rarefaction curves indicate that none of the samples reached a clear horizontal plateau. Instead, the number of observed OTUs continues to increase with sequencing depth across all groups. This suggests that the sequencing depth did not capture the full microbial diversity present in the samples, and that additional sequencing would likely reveal further OTUs. This leaves some information not present in the sequencing data, such as *H₂S* produces *Desulfovibrio*. *Desulfobacter*, *Desulfomonas*, *Desulfobulbus*, and *Desulfotomaculum*, which if present, would have been able to be linked to the increase in *H₂S* in (Figure 6.16), where this bacterial family is known to have a major role [23]. The Sol producer *Odoribacter* genus was also not spotted, making it harder to determine if Sols can be used by the strain as an energy source. Not finding these genera makes conclusions around Sols microbiome impact more spurious.

Microbiome genera relative abundance

Relative abundance analysis provides insight into the bacterial genera present in the samples and the dominance of specific genera within the microbiome. To visualize these patterns, a heatmap was generated for the control, Fjoh, and $\Delta 2419$ Fjoh sample groups at two incubation time points: 48 and 72 hours.

	$\Delta 2419$ Fjoh 48H			$\Delta 2419$ Fjoh 72H			Control 48H			Control 72H			Fjoh 48H			Fjoh 72H		
Bacteroidota; Prevotella	13.5	14.1	13.2	6.1	10.4	8.0	15.0	16.5	17.2	14.1	15.1	16.4	16.1	17.2	16.0	14.9	6.6	10.7
Bacillota; Gemmiger	3.6	3.3	3.7	1.8	2.3	1.6	4.1	3.5	3.0	2.3	2.3	2.2	3.5	3.8	4.2	2.1	2.5	1.6
Bacillota; Blautia_A	1.0	1.4	1.6	2.9	2.9	2.7	1.8	1.6	1.7	2.3	2.5	2.2	1.4	1.2	1.1	2.4	2.8	2.7
Bacillota; Lachnospira	1.0	0.9	0.9	0.6	0.6	0.5	1.2	1.0	0.9	0.6	0.6	0.8	0.9	1.1	1.0	0.8	0.5	0.6
Bacillota; Agathobacter	0.7	0.9	0.7	0.6	0.6	0.5	1.0	0.9	0.9	0.7	0.8	0.6	0.6	0.8	1.0	0.5	0.5	0.5
Bacillota; Ruminococcoides	0.5	0.5	0.5	0.3	0.2	0.3	0.5	0.5	0.4	0.4	0.3	0.3	0.3	0.4	0.5	0.2	0.3	0.2
Bacillota; Anaerostipes	0.0	0.0	0.1	0.6	0.6	0.5	0.1	0.0	0.1	0.4	0.4	0.5	0.1	0.0	0.0	0.6	0.6	0.6
Bacillota; Anthropogastromicrobium	0.1	0.2	0.3	0.4	0.3	0.4	0.2	0.3	0.2	0.4	0.3	0.3	0.2	0.2	0.2	0.3	0.5	0.4
Bacillota; Fusicatenibacter	0.1	0.2	0.2	0.3	0.2	0.3	0.3	0.2	0.3	0.4	0.4	0.4	0.3	0.2	0.1	0.4	0.5	0.5
Bacillota; Anaerostipes	0.1	0.1	0.1	0.3	0.4	0.3	0.2	0.1	0.2	0.3	0.4	0.3	0.2	0.1	0.1	0.4	0.3	0.5
Bacillota; Faecalibacillus	0.2	0.3	0.3	0.2	0.2	0.2	0.3	0.3	0.3	0.2	0.2	0.2	0.3	0.2	0.3	0.2	0.2	0.1
Bacillota; Oscillibacter	0.3	0.3	0.2	0.2	0.1	0.2	0.3	0.3	0.2	0.2	0.2	0.1	0.2	0.4	0.3	0.1	0.2	0.1
Bacteroidota; Bacteroides	0.2	0.2	0.2	0.2	0.2	0.2	0.1	0.3	0.2	0.1	0.1	0.1	0.3	0.2	0.3	0.1	0.5	0.1
Bacillota; Vescimonas	0.2	0.3	0.2	0.1	0.2	0.1	0.4	0.2	0.2	0.1	0.2	0.2	0.2	0.3	0.4	0.2	0.1	0.1
k_Bacteria_OTU_8971; k_Bacteria_OTU_8971	0.3	0.2	0.2	0.0	0.1	0.1	0.3	0.2	0.3	0.1	0.1	0.2	0.4	0.4	0.1	0.3	0.1	0.2
Bacillota; Dorea_A	0.0	0.1	0.2	0.3	0.2	0.3	0.1	0.1	0.2	0.3	0.1	0.3	0.1	0.0	0.1	0.3	0.3	0.2
Bacillota; o_Oscillospirales_OTU_24568	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.2	0.3	0.1	0.2	0.1	0.1	0.2	0.2	0.1	0.1	0.1
Bacillota; Enterocloster	0.1	0.1	0.1	0.3	0.2	0.3	0.0	0.0	0.0	0.1	0.1	0.0	0.2	0.2	0.1	0.3	0.3	0.4
k_Bacteria_OTU_9388; k_Bacteria_OTU_9388	0.1	0.4	0.2	0.1	0.1	0.1	0.1	0.2	0.2	0.2	0.2	0.1	0.2	0.1	0.2	0.2	0.1	0.1
Bacteroidota; Phocaeicola	0.2	0.2	0.2	0.1	0.1	0.1	0.2	0.3	0.2	0.1	0.1	0.1	0.1	0.2	0.3	0.1	0.2	0.0
	$\Delta 2419$ Fjoh 48hour 1	$\Delta 2419$ Fjoh 48hour 2	$\Delta 2419$ Fjoh 48hour 3	$\Delta 2419$ Fjoh 72hour 1	$\Delta 2419$ Fjoh 72hour 2	$\Delta 2419$ Fjoh 72hour 3	control 48hour 1	control 48hour 2	control 48hour 3	control 72hour 1	control 72hour 2	control 72hour 3	Fjoh 48hour 1	Fjoh 48hour 2	Fjoh 48hour 3	Fjoh 72hour 1	Fjoh 72hour 2	Fjoh 72hour 3

Figure 6.25. Heatmap showing taxonomic abundance across control, Fjoh, and $\Delta 2419$ Fjoh sample groups at 48 and 72 hours of incubation. Color intensity reflects relative abundance, with darker shading indicating higher values. Rows represent an individual genus, and columns represent sample groups.

The relative abundance of bacterial genera was analyzed across the control, Fjoh, and $\Delta 2419$ Fjoh samples at both 48 and 72 hours. Across all conditions, the microbial community was dominated by members of the *Bacteroidota* and *Bacillota* phyla. Genera including *Prevotella*, *Gemmiger*, *Blautia*, *Lachnospira*, and *Roseburia* were consistently detected across most samples, suggesting that the core microbial community structure remained relatively stable.

The high relative abundance of *Prevotella* observed in the samples is commonly associated with non-Western or plant-rich diets, including vegetarian diets [117]. This is consistent with the composition of the pooled stool samples used in this study, which were obtained from a cohort containing a substantially higher proportion of vegetarians (40%) compared to the general Western population, where vegetarianism is estimated at approximately 5 to 6% [118].

Several taxa showed time-dependent shifts in abundance. Most notably, *Prevotella* exhibited a marked decrease in the Fjoh 72H and $\Delta 2419$ Fjoh 72H samples compared with their corresponding 48-hour samples and the control conditions. A different trend was observed for *Gemmiger*, which decreased in relative abundance at 72 hours at similar levels across all conditions.

Differences between the Fjoh and $\Delta 2419$ Fjoh samples were not present, with similar trends appearing in both Fjoh and $\Delta 2419$ Fjoh samples. These observations suggest that the absence of Sols in the $\Delta 2419$ Fjoh strain did not produce large shifts in dominant microbial taxa.

Overall, the taxonomic profiles indicate that incubation time had a stronger influence on genus-level community composition than the presence or absence of Sols, with the most pronounced changes occurring between 48 and 72 hours.

To focus on the five most abundant genera, boxplots were generated for the control, Fjoh, and $\Delta 2419$ Fjoh sample groups at two incubation time points: 48 and 72 hours.

1235

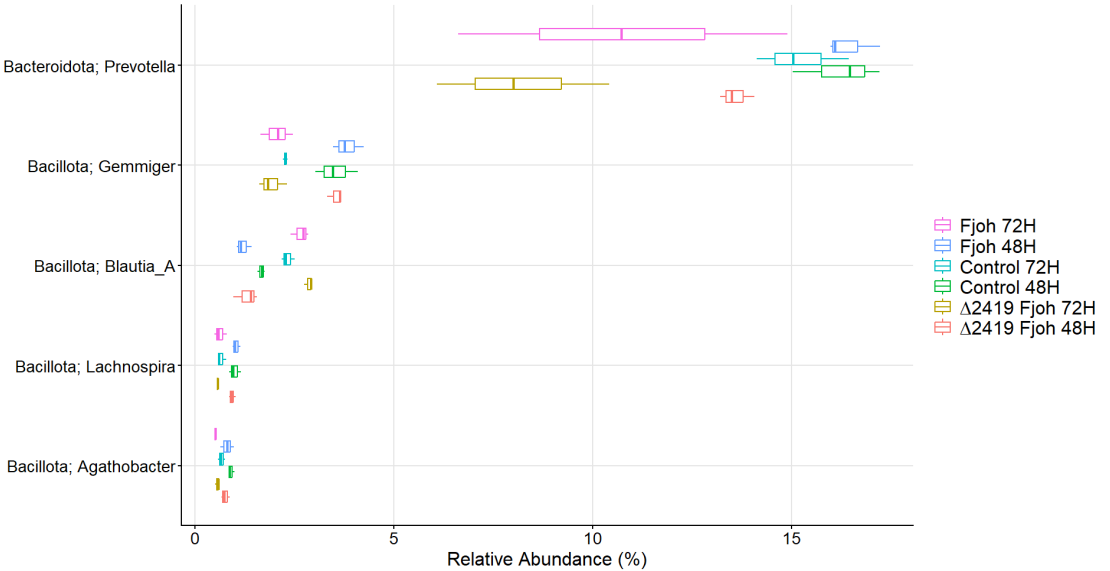


Figure 6.26. Relative abundance of the five most prevalent genera across sample groups control, Fjoh, and $\Delta 2419$ Fjoh at 48 and 72 hours of incubation.

The relative abundance of the dominant bacterial genera was compared across the control, Fjoh, and $\Delta 2419$ Fjoh samples at 48 and 72 hours. A general trend is that several major genera show slightly lower relative abundances at 72 hours than at 48 hours. This pattern suggests that less relatively abundant taxa may increase in relative abundance during the later stages of incubation, thereby reducing the proportional representation of the dominant genera, or that the reverse might occur: more relatively abundant taxa might decrease, thereby increasing the relative abundance of less abundant taxa.

1240

This change in relative abundance is particularly evident for *Prevotella*. In the Fjoh and $\Delta 2419$ Fjoh samples, the relative abundance of *Prevotella* decreases by approximately 5% between 48 and 72 hours, whereas the control samples show a smaller reduction over the same time period. This difference suggests that the addition of lipids may promote the growth or activity of lower-abundance taxa, thereby reducing the relative abundance of *Prevotella* within the microbial community.

1245

At 48 hours, the Fjoh samples also showed a higher relative abundance of *Prevotella* than the $\Delta 2419$ Fjoh samples. This may indicate that Sols are taken up and utilized by *Prevotella* as an energy source. Such an interaction would be consistent with previous findings showing that structurally similar sphingolipids can be metabolized by *Prevotella* [119]. The uptake of Sols could therefore help explain the higher relative abundance observed in the Sol-containing Fjoh samples.

1250

Not all genera follow this pattern. For example, *Blautia A* shows an increase in relative abundance from 48 to 72 hours across all sample groups. The increase appears to be most pronounced in the lipid-supplemented samples, indicating that some taxa may expand during longer incubation periods, particularly when additional lipid substrates are present.

1255

Overall, these results indicate that incubation time can alter the balance between dominant and lower-abundance genera within the microbial community, while the overall set of dominant taxa remains largely consistent across samples. The observed differences in *Prevotella* abundance also suggest that Sols may be utilized by this genus, indicating a possible role for Sols in modulating specific microbial interactions within the gut microbiome.

1260

Box plots for genera were used to evaluate how specific experimental variables influenced the microbial community composition. The three constraints tested were the presence of Sols (Figure 6.27a), inoculation

time (Figure 6.27b), and the addition of the lipid profile (Figure 6.27c).

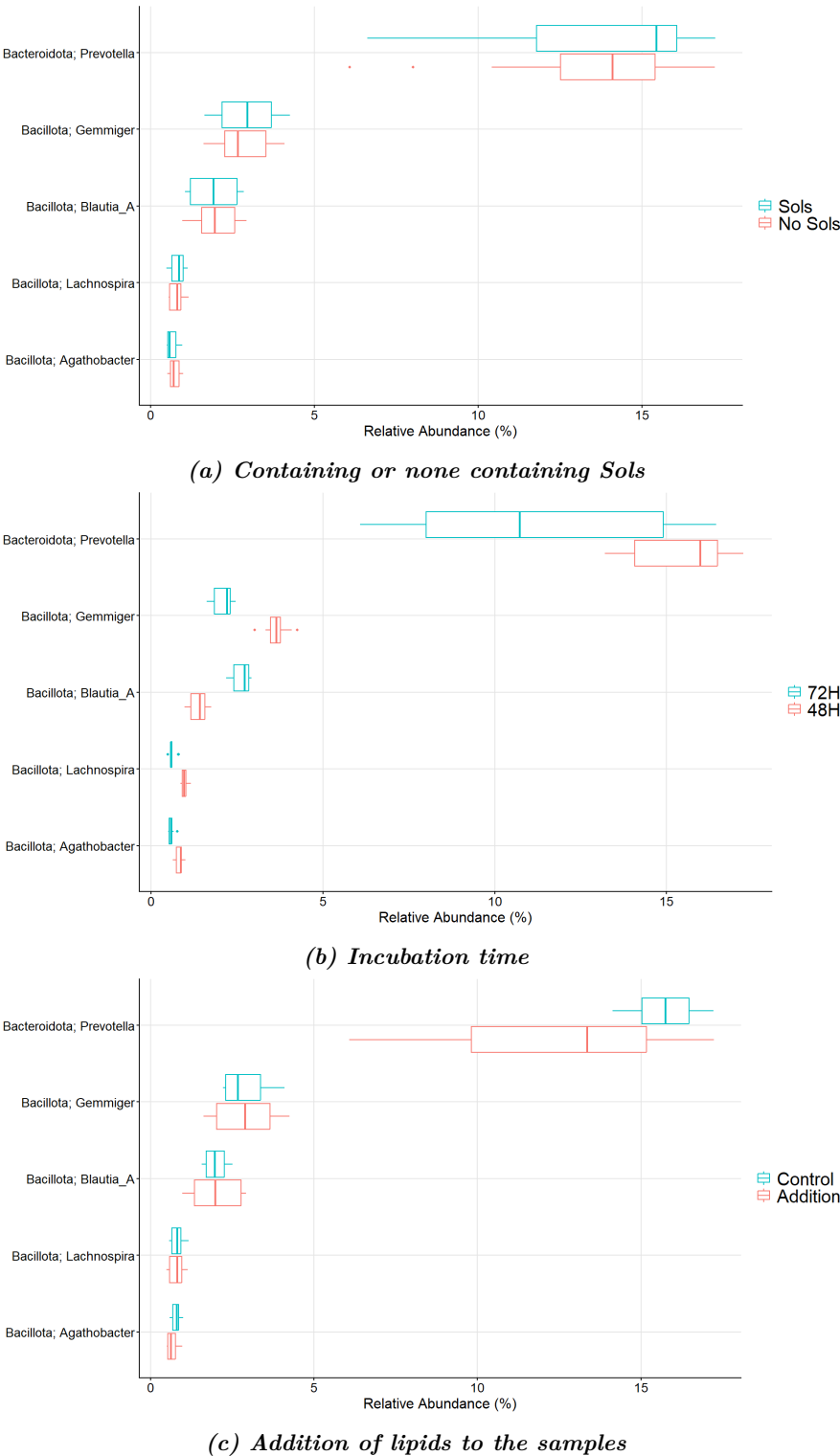


Figure 6.27. Relative abundance of the five most prevalent genera across in Sol-containing Fjoh and Sol-lacking $\Delta 2419$ Fjoh and control samples, over incubation time 48 to 72 hours, and lipid-added Fjoh and $\Delta 2419$ Fjoh against control samples.

The influence of Sols on the most abundant genera appears to be minimal, as most of the box plots overlap substantially between the groups. This strong overlap indicates that the presence of Sols does not

significantly up- or downregulate any of the dominant genera in the samples. These findings complicate the indication observed in the Fjoh 48H samples compared to the $\Delta 2419$ Fjoh samples (Figure 6.26), where *Prevotella* appeared more abundant in the presence of Sols. Therefore, a more targeted analysis of *Prevotella*, including determination of its absolute abundance, would be beneficial to clarify whether Sols directly influence this genus.

In contrast, inoculation duration exerts a clearer effect on microbial composition. The relative abundances of four of the five dominant genera decrease when comparing 48 h and 72 h incubation times, while *Blautia A* is the only genus that increases in relative abundance over time. These shifts indicate that inoculation duration is a key factor shaping microbiome structure. Similar effects of incubation or storage time on bacterial composition have been reported [120], so the major factor of incubation time was to be expected.

The addition of lipids to the samples appears to have only minor effects on the five most prevalent genera. For *Prevotella*, a difference can be observed where samples with added lipids exhibit a lower relative abundance compared to the control samples. However, for the remaining major genera, the relative abundance distributions largely overlap between the lipid-treated and control groups. This indicates that lipid supplementation does not produce consistent or systematic changes in the abundance of these dominant genera.

To evaluate if *Alistipes* was affected by the presence of Sols, the relative abundance of *Alistipes* was examined due to its reported anti-inflammatory properties and its role as a producer of Sols.

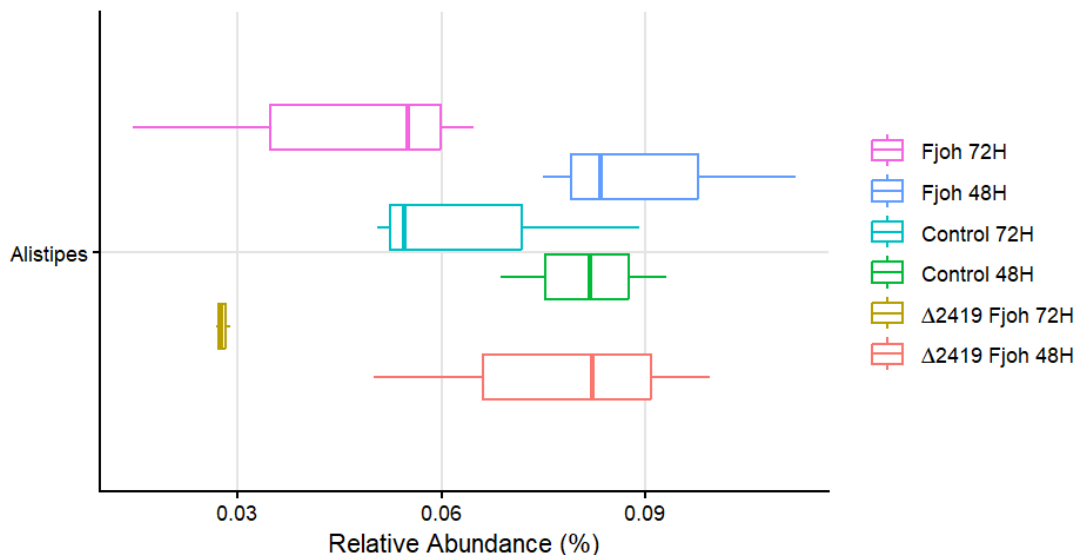


Figure 6.28. Relative abundance of *Alistipes* across sample groups control, Fjoh, and $\Delta 2419$ Fjoh at 48 and 72 hours of incubation. Each point represents an individual replicate, with bars indicating group medians.

At 48 hours, all sample groups exhibit similar *Alistipes* abundances, with median values just below 0.09%. This suggests that neither lipid addition nor Sol presence markedly influences *Alistipes* at this early time point. At 72 hours, a clearer divergence between sample groups is observed. The control samples show a reduction to approximately 0.06%, while Fjoh samples with Sol display a comparable median but increased variability. In contrast, the $\Delta 2419$ Fjoh samples exhibit a more pronounced decrease to below 0.03% with low variance.

This pattern suggests that the presence of Sols may help maintain *Alistipes* abundance over time, potentially by serving as a substrate or by supporting its persistence in the microbial community. However, given the overall low relative abundance and variability across samples, this interpretation should be made with caution; if Sols is used as an energy source by *Alistipes*, that could lead to beneficial health outcomes in IBD patients [29]. Further analysis of the presence of *Alistipes* through qPCR would provide a more solid basis for conclusions.

Alpha diversity

The number of observed OTUs was investigated to estimate the species richness of each treatment group was used to investigate the species richness and evenness of each treatment group. Alpha diversity analysis was performed on three sample groups: control, Fjoh, and $\Delta 2419$ Fjoh at two time points: 48 and 72 hours.

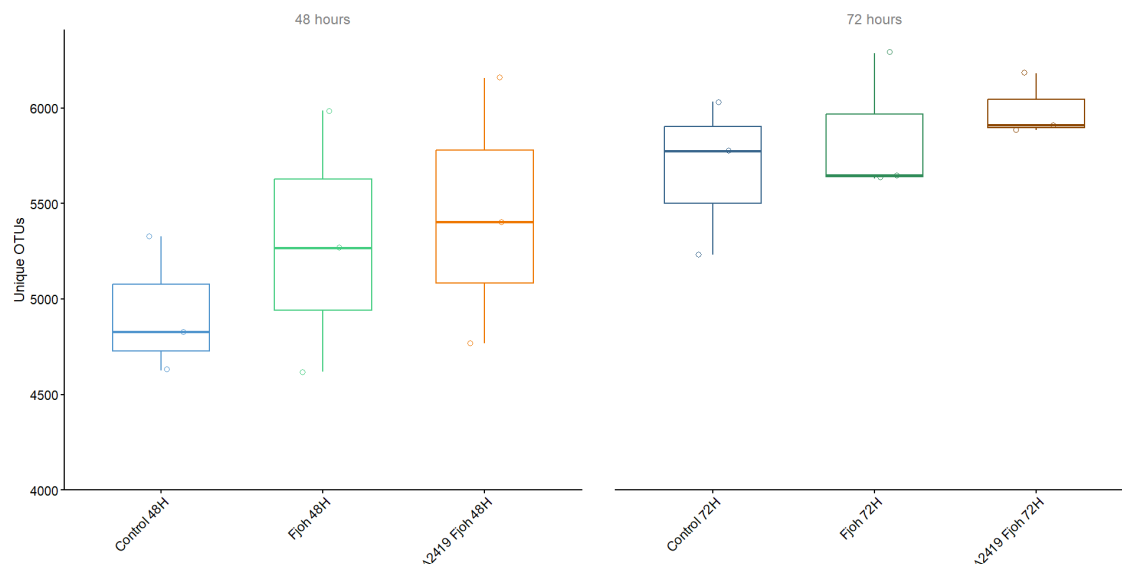


Figure 6.29. Alpha diversity for observed OTUs across the three sample groups: control, Fjoh, and $\Delta 2419$ Fjoh at two time points, 48 hours and 72 hours. The sequencing depth indicates that all samples have between 4000 and 6500 OTUs.

1300 The alpha diversity results indicate two general patterns. The 48-hour samples show greater inter-sample variability and tend to have fewer observed OTUs compared to the 72-hour samples. In contrast, the 72-hour samples cluster more closely and exhibit higher numbers of observed OTUs.

At 48 hours, the control samples display fewer reads and slightly lower observed OTU counts compared to the lipid-supplemented groups. This could suggest that lipid addition supports either bacterial growth or the maintenance of a broader microbial community, leading to increased detectable diversity. However, the t-test indicated that these differences were not significant, limiting the strength of this interpretation.

By 72 hours, all sample groups exhibit similar levels of observed OTUs, suggesting comparable community richness across treatments. This indicates that, at this later time point, lipid supplementation does not appear to substantially alter overall community complexity.

1310 The generally modest differences in alpha diversity between groups likely reflect the shared origin of all samples from the same stool. Since the primary variables are incubation time and lipid supplementation, and given the relatively short incubation period, major shifts in overall richness may not yet have occurred. The slight increase in OTUs for the 72-hour samples can stem from the lower relative abundance of major genera (Figure 6.26), heightening the chances of sequencing a less abundant genus, which increases the amount of OTUs.

The alpha diversity for observed OTUs for samples containing or not containing Sols (Figure 6.30a), inoculation time (Figure 6.30b), and the addition of lipids, and the control without the addition of lipids (Figure 6.30c).

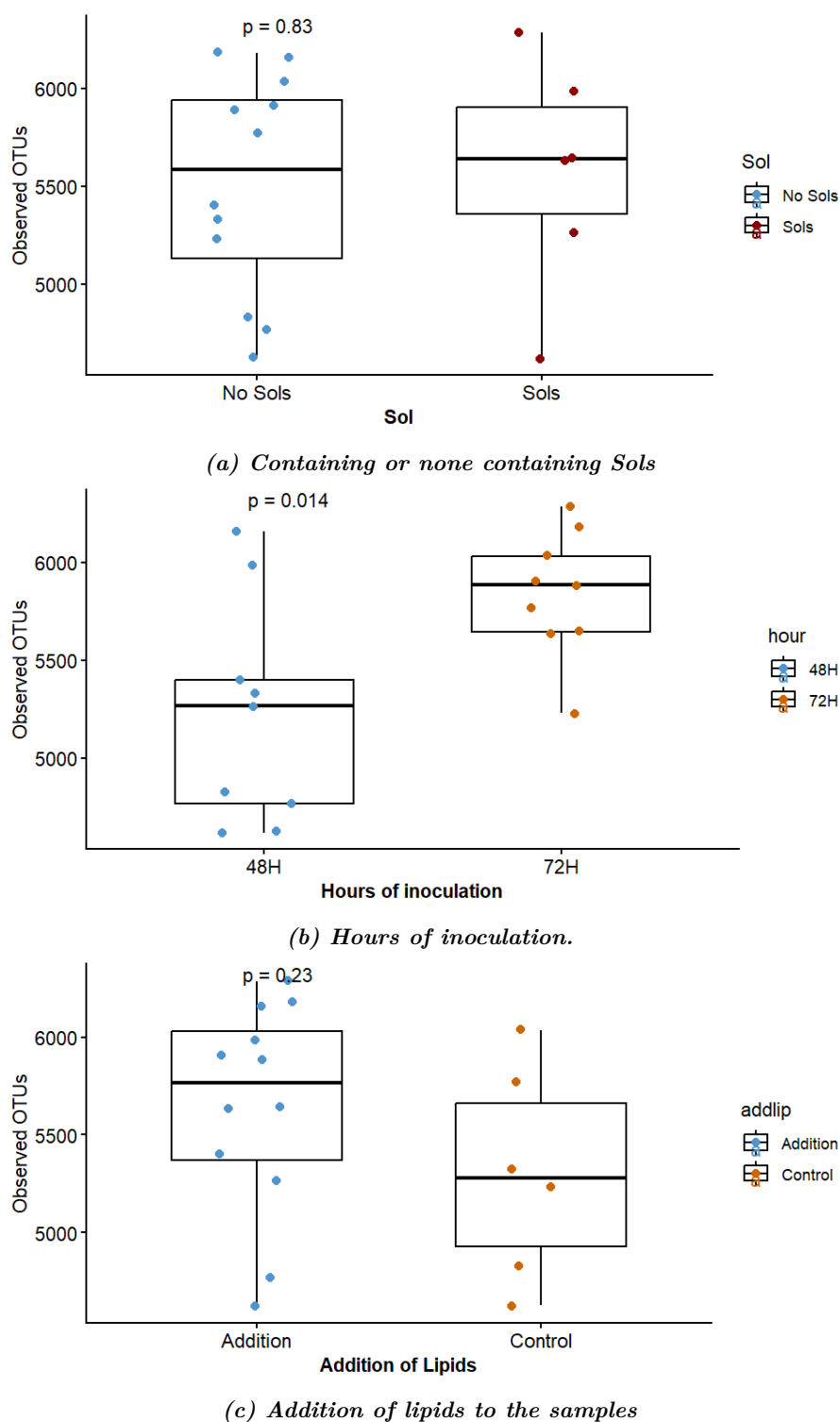


Figure 6.30. Alpha diversity observed OTUs a) Sol-containing Fjoh and Sol-lacking $\Delta 2419$ Fjoh and control samples, b) incubation time 48 to 72 hours, c) lipid added Fjoh and $\Delta 2419$ Fjoh against control samples. Each dot represents a single sample, and a box plot shows the quantiles and the median. Each plot includes an ANOVA p-value indicating whether the data differ significantly.

Alpha diversity analysis showed no significant difference in observed OTUs between samples containing Sols and those without ($p = 0.83$), indicating that the presence of Sols does not directly influence overall community richness.

In contrast, incubation time had a significant effect on alpha diversity. Samples incubated for 72 hours exhibited a significantly higher number of observed OTUs compared to 48-hour samples ($p = 0.014$). This increase in richness may reflect time-dependent developments of the community during incubation.

No statistically significant difference in observed OTUs was found between lipid-supplemented samples and controls ($p = 0.23$). However, the median richness in samples with added lipids had approx 500 more observed OTUs, compared to the control samples. Suggesting a potential trend toward increased or maintained diversity following lipid addition, or an increase in less prevalent genera, although this effect was not statistically significant.

Overall, alpha diversity appears to be primarily influenced by incubation time, contributing to differences in detected richness. Lipid supplementation may have a modest effect on community complexity, whereas Sols specifically do not appear to alter overall richness. These findings should be interpreted cautiously due to the limited sequencing depth, which may underestimate rare OTUs and true community diversity.

To evaluate the diversity composition of the genera present in the samples, alpha diversity analysis using the Shannon index (Figure 6.31) was conducted for the control, Fjoh, and $\Delta 2419$ Fjoh sample groups at two incubation time points: 48 and 72 hours.

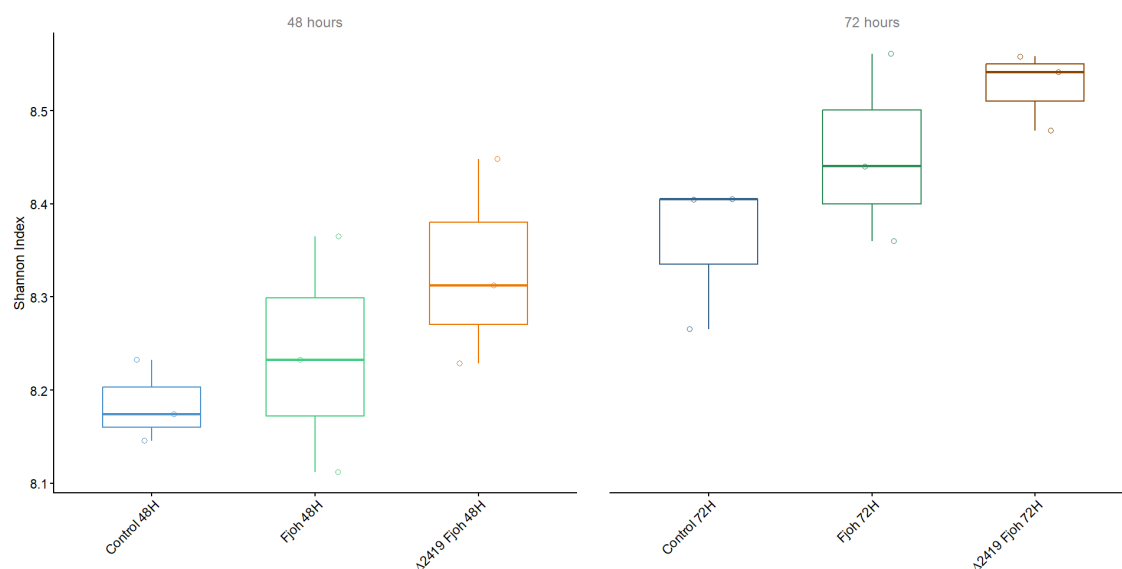


Figure 6.31. Alpha diversity expressed as the Shannon index for three sample groups: control, Fjoh, and $\Delta 2419$ Fjoh at 48 and 72 hours of incubation. Shannon index values ranged between 7.5 and 8.0 across all groups, indicating consistently high microbial diversity with significant differences between conditions, Control 72H and $\Delta 2419$ Fjoh 72H.

The Shannon diversity index largely follows the same pattern observed for the number of observed OTUs. In general, the 48-hour samples exhibit lower alpha diversity compared to the 72-hour samples. This difference may be influenced by the longer inoculation time in the 72-hour samples. It was seen that most of the abundant genera decreased in abundance over the incubation time (Figure 6.26), which makes for a better weighted microbiome at the 72-hour samples, which increases the 72-hour samples' Shannon scores.

When comparing the control samples with the Fjoh and $\Delta 2419$ Fjoh groups at the same time point, there are no statistically significant differences in Shannon diversity. A possible explanation for this increased diversity is the presence of additional lipids that may serve as nutrients, thereby supporting a broader range of microbial taxa and increasing overall community diversity. In contrast, no significant difference is observed between the control and Fjoh samples at 72 h. This may be related to the composition of the lipid profile, in which Sols account for approximately 19% of the lipids [27]. These lipids may be less readily utilized by the microbiome or metabolized only by specific bacterial groups, thereby limiting their overall impact on the Shannon diversity index.

The alpha diversity for the Shannon index for samples containing or not containing Sols (Figure 6.32a), inoculation time (Figure 6.32b), and the addition of lipids, and the control without the addition of lipids (Figure 6.32c).

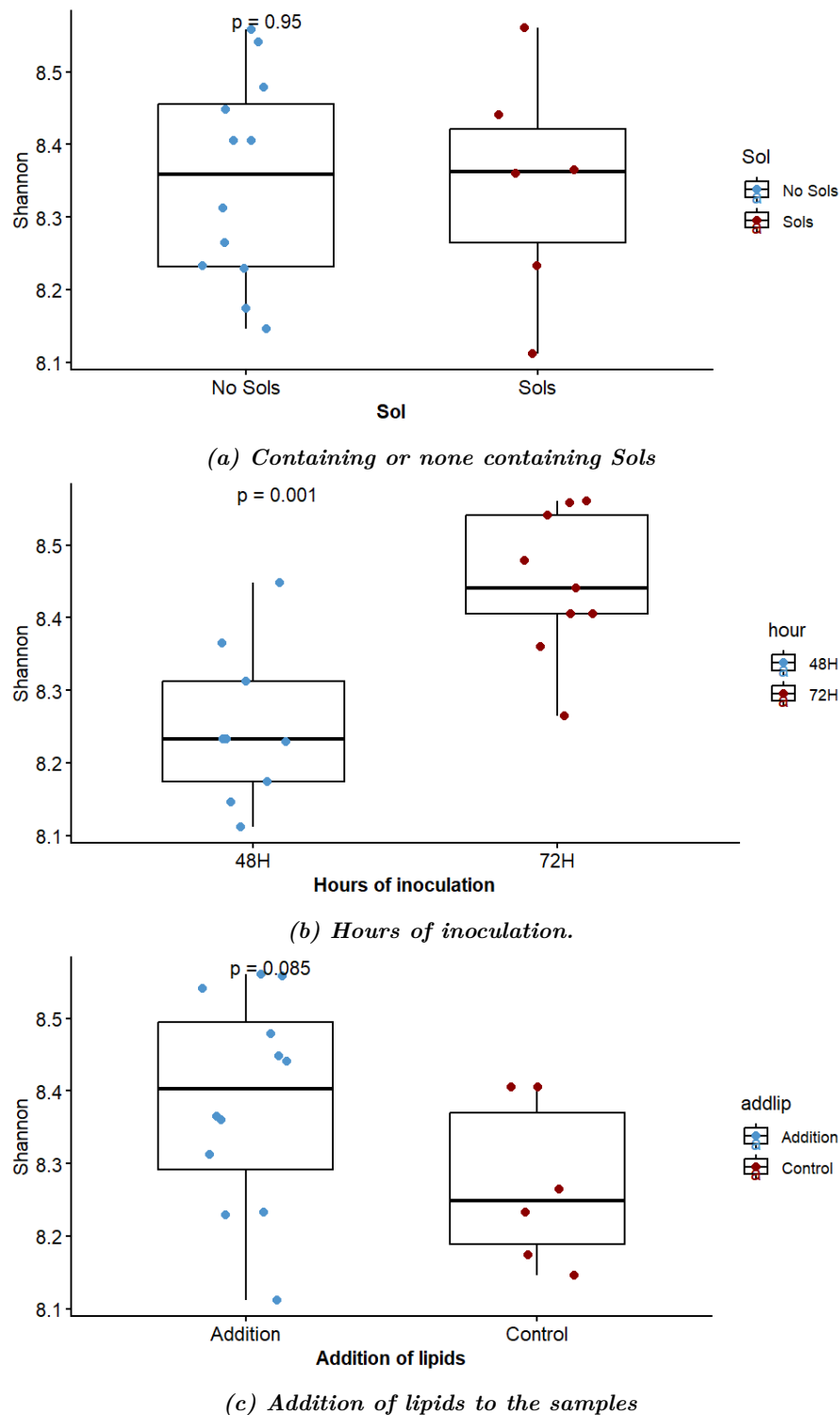


Figure 6.32. Alpha diversity as Shannon index in samples Sol-containing Fjoh and Sol-lacking $\Delta 2419$ Fjoh and control samples, over incubation time 48 to 72 hours, and lipid added Fjoh and $\Delta 2419$ Fjoh against control samples. Each dot represents a single sample, and a box plot shows the quantiles and the median. Each plot includes an ANOVA p-value indicating whether the data differ significantly.

The Shannon diversity index follows a similar pattern to the number of observed OTUs shown in (Figure 6.30). When comparing samples containing Sols with those without Sols, no significant differences in diversity are observed. However, the Shannon index indicates less variation between samples than the OTU counts do, suggesting that the evenness of the microbial communities results in more similar diversity

values between the Sol- and non-Sol-containing groups.

In contrast, the two inoculation time points, 48H and 72H, show a significant difference in the Shannon index. The increased diversity observed in the 72H samples may be explained by the longer inoculation time, which allows for further microbial growth and diversification. Compared with the OTU analysis (Figure 6.30b), the Shannon index indicates a more pronounced difference between the time points, with a statistically significant p-value ($p = 0.001$). This suggests that the microbial communities at 72H are more diverse than those at 48H.

In the lipid supplementation experiments, adding lipids yields a slightly higher median Shannon index. Although the difference is not statistically significant, the p-value ($p = 0.085$) approaches the significance threshold, indicating a potential trend toward increased microbial diversity in the lipid-supplemented samples. Compared to the OTU results (Figure 6.30c), the Shannon index suggests somewhat larger differences between the groups than those observed when considering OTU counts alone.

Beta diversity

Beta diversity reflects differences in microbiome composition between samples and, therefore, can indicate how the overall composition of the samples changes. NMDS over the sample groups control, Fjoh, and $\Delta 2419$ Fjoh at two time points: 48 and 72 hours.

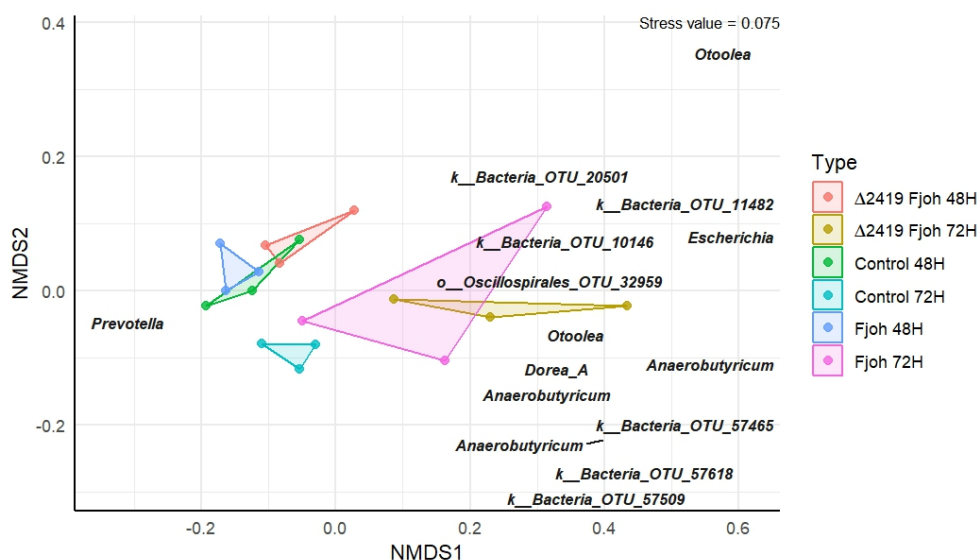


Figure 6.33. Beta diversity shown as NMDS ordination plots for three sample groups: control, Fjoh, and $\Delta 2419$ Fjoh at 48 and 72 hours of incubation. Each point represents an individual sample. Clustering of points indicates similarity in microbial community composition between replicates within each group.

The NMDS shows a stress value of 0.075, indicating that the data shown on the plot is trustworthy. The NMDS plot revealed substantial overlap among control, Fjoh, and $\Delta 2419$ Fjoh at 48 hours, indicating similar microbial community composition across treatments at this time point. This suggests that lipid supplementation, with or without Sols, had a limited impact on overall community structure after 48 hours of incubation. This aligns with the alpha diversity data, where the 48-hour samples were a closer match both in OTUs (Figure 6.29) and the Shannon index (Figure 6.31) at 48 hours, showing that all sample groups overlap in both alpha and beta diversity at this time point.

At 72 hours, all sample groups shifted along the NMDS1 axis and did not overlap with the 48-hour samples, demonstrating a clear temporal effect on community composition. The Fjoh and $\Delta 2419$ Fjoh samples at 72 hours continued to overlap, indicating similar microbial profiles between the lipid-treated sample groups. However, their dispersion increased compared to 48 hours, reflecting greater inter-sample variability within these groups over time.

In contrast, 72-hour control samples formed a tighter cluster and did not overlap with the 72-hour lipid-supplemented samples, indicating a distinct community composition and lower within-group variability. The difference between the samples with and without lipid addition could also be seen in the Shannon

index (Figure 6.32c), where the samples with added lipids both have a higher variability but also have a higher median Shannon index, indicating that the samples are more diverse both within samples and between samples.

Overall, the NMDS analysis shows that community divergence increases from 48 to 72 hours, with lipid-treated samples exhibiting greater inter-sample variability than controls.

The beta diversity NMDS for samples containing or not containing Sols (Figure 6.34a), inoculation time (Figure 6.34b), the addition of lipids, and the control without the addition of lipids (Figure 6.34c).

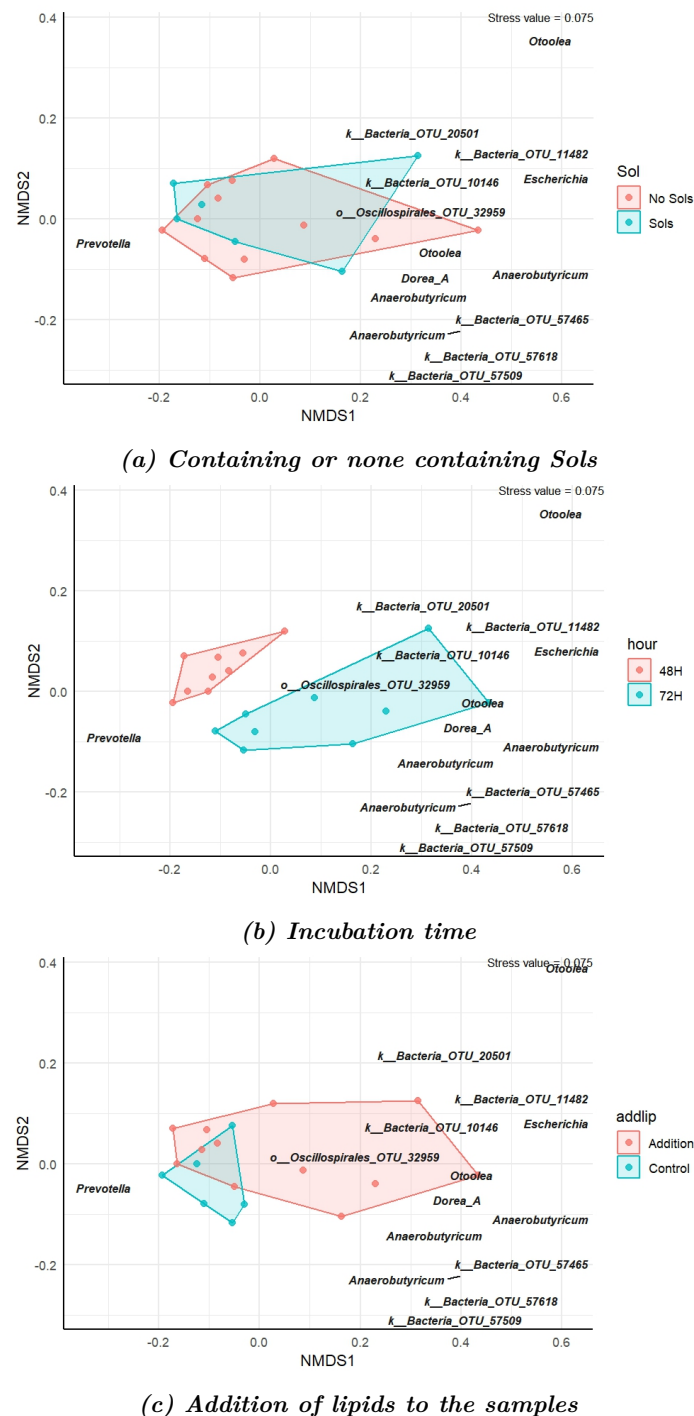


Figure 6.34. Beta diversity shown as NMDS ordination comparing Sol-containing Fjoh and Sol-lacking $\Delta 2419$ Fjoh and control samples, over incubation time 48 to 72 hours, and lipid-added Fjoh and $\Delta 2419$ Fjoh against control samples. Each point represents an individual sample.

For containing and non-containing Sols, NMDS shows that most of the samples overlap and that those that do not overlap do not have a unidirectional pattern in which they do not overlap, showing that the addition of Sols does not facilitate one specific kind of change within the samples.

The NMDS for inoculation time shows that the 48H and 72H samples do not overlap, and that there is a clear shift to the right on NMDS1 for the 72H samples. A clear structural change can therefore be inferred in the 48H samples compared to the 72H samples. The samples of the 48H group appear tightly clustered in the NMDS, showing less inter-sample variability compared to the 72H samples, which show a larger spread, indicating that over the 24 hours, the samples' compositions drifted further from each other.

The NMDS for the addition of lipids shows that the controls are all located in one corner of the plot, overlapping with some samples with added lipids. The samples with lipids can be seen to cover a larger area, showing that they have a larger inter-sample variability. However, the shift to the right on NMDS1 indicates that the addition of lipids appears to cause a general change across the samples.

The NMDS for these factors shows that the presence or absence of Sols does not significantly change the composition in a controlled manner, and that the passage of time and the addition of the general lipid profile have the greatest impact on the microbiome composition of the samples. This fits with the data from the relative abundances (Figure 6.27), OTUs (Figure 6.30), and Shannon index (Figure 6.32), where the major impacts were time, addition of lipids, and that Sols impact was negligible.

To evaluate the contribution of different factors to variation between samples, a Canonical Correspondence Analysis (CCA) was conducted (Figure 6.35). This approach allowed for quantification of how much of the observed variation in microbial composition could be explained by each factor.

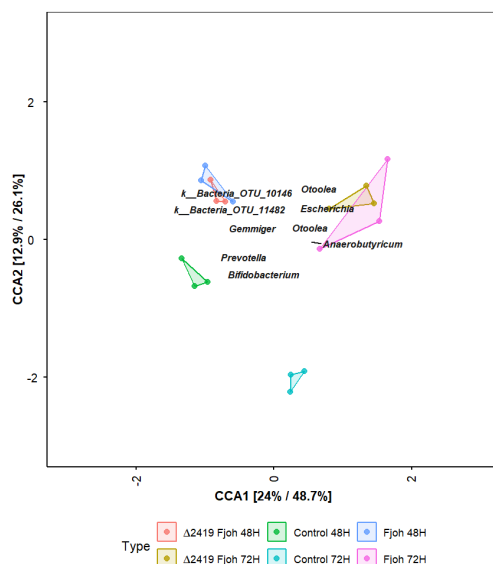


Figure 6.35. CCA of beta diversity comparing sample groups. CCA axes reflect constrained variation associated with the experimental variables.

CCA revealed a clear separation between control and lipid-supplemented samples at both 48 and 72 hours. Control samples clustered distinctly from Fjoh and $\Delta 2419$ Fjoh along the CCA2 axis, with the separation increasing at 72 hours, indicating that the compositional difference between control and lipid-treated communities becomes more pronounced over time. A general shift along the CCA1 axis was observed from 48 to 72 hours across all groups, suggesting a temporal effect of incubation on community structure. Fjoh and $\Delta 2419$ Fjoh samples largely overlapped in the ordination space, indicating similar compositions despite the absence of Sols in $\Delta 2419$ Fjoh. Minor separation between these groups was observed; however, given the small sample size, it is unclear whether this reflects true structural differences or inter-sample variability. The CCA plot largely depicts a similar picture as the NMDS plot (Figure 6.33) as expected, though with more pronounced differences in control sample groups to the ones with added lipids.

The CCA was used to evaluate how specific experimental variables influenced the microbial community composition. The three constraints tested were the presence of Sols (Figure 6.36a), the inoculation time (Figure 6.36b), and the addition of the Fjoh lipid profile (Figure 6.36c).

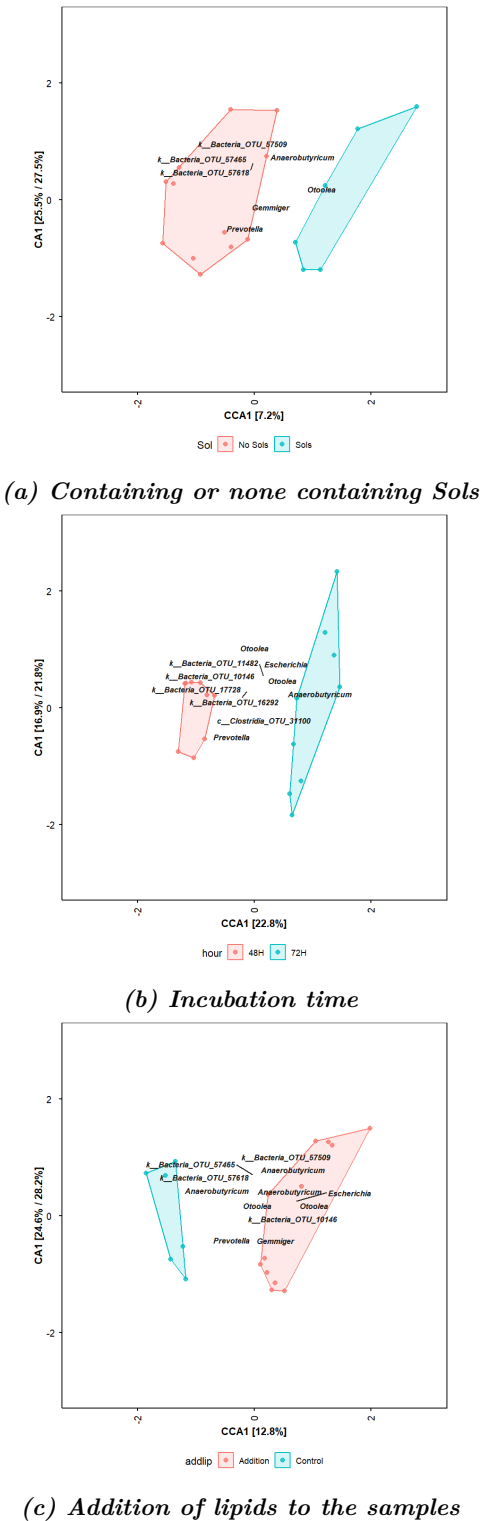


Figure 6.36. Beta diversity CCA over samples Sol-containing Fjoh and Sol-lacking $\Delta 2419$ Fjoh and control samples, over incubation time 48 to 72 hours, and lipid added Fjoh and $\Delta 2419$ Fjoh against control samples.

When constraining the analysis by the presence or absence of Sols, the samples separate along the CCA1 axis, which explains 7.2% of the variation. While this indicates that Sols contribute to differences in microbiome composition, the spread of the samples along the CA1 axis (25.5–27.5%) suggests that inter-sample variability accounts for a larger proportion of the observed differences than the presence of Sols

alone.

Constraining the samples by inoculation time shows a much clearer separation between the 48-hour and 72-hour samples along the CCA1 axis, which explains 22.8% of the variation. This indicates that incubation time is a major factor influencing microbial community structure. The clear clustering of samples by time point suggests that microbial composition shifts substantially during the additional 24 hours of incubation.

When the addition of lipids constrains the analysis, the samples also separate along the CCA1 axis, which explains 12.8% of the variation. This indicates that the addition of the Fjoh lipid profile alters the microbial community composition compared with the control samples. Although this effect is smaller than that of incubation time, it still represents a measurable shift in the microbiome structure.

Overall, the constrained analyses indicate that incubation time has the strongest influence on microbiome composition, followed by lipid addition, whereas the presence of Sols accounts for a smaller proportion of the observed variation. The remaining variation likely reflects biological differences between replicates and the limited sequencing depth observed in the rarefaction analysis. The CCA data align with the NMDS data (Figure 6.34), where both sets indicate inoculation time as the major factor, followed by lipid addition and then Sols present. In the CCA plot, Sols are shown to account for 7.2% of the differences, which is a substantial amount, while in the NMDS plot (Figure 6.34a) is no systematic change in the microbiome from the addition of Sols. Therefore, a deeper investigation of Sols' precise effects on the microbiome would be necessary, specifically in genera that are expected to be impacted by the presence of the Sols, as the Sol producers *Alistipes* and *Odoribacter* [29] or H₂S producing genera [23].

16S rRNA gene sequencing overview

Taxonomic analysis of the most abundant bacterial genera shows that most of the dominant genera decrease in relative abundance over time, while *Blautia* A increases in relative abundance across the samples (Figure 6.27). The largest shifts in relative abundance differences are associated with inoculation time (Figure 6.27b), followed by the addition of lipids (Figure 6.27c). While the presence of Sols (Figure 6.27a) appears to have only a minor influence on the major genera present in the stool microbiome, this could be due to Sols accounting for only a minority of the inoculated lipid fraction [27]. Alternatively, it may indicate that detecting prebiotic effects requires longer incubation periods or more sustained exposure to the prebiotic compound, as long-term dietary changes are often necessary to induce stable shifts in microbiome composition [42].

Sols share structural similarities with sphingolipids, which have previously been shown to be preferentially taken up by genera such as *Bacteroides* and *Prevotella* [119]. The high relative abundance of *Prevotella* observed in the samples could therefore indicate that Sols may have promoted the growth or activity of this genus during the early stages of incubation. However, because no 0h and a 24h sample was included, it is not possible to determine whether *Prevotella* increased during the first hours of incubation. One observation arguing against a strong Sol-specific effect is that the control samples also exhibited high relative abundances of *Prevotella*. Nevertheless, the Sol-containing sample groups showed a higher relative abundance of *Prevotella* at 48 hours compared to the sample group lacking Sols (Figure 6.26), which could suggest uptake or utilization of Sols by *Prevotella*. However, the less distinct pattern observed in (Figure 6.27a) suggests that *Prevotella* was not substantially increased in relative abundance by the presence of Sols.

Alistipes showed a similar relative abundance across all sample groups at 48 hours, with median values just below 0.09%. In the 72-hour samples, the median relative abundance began to differ, with the control and Fjoh samples remaining just below 0.06%, while the Δ 2419 Fjoh samples decreased to just below 0.03%. These findings suggest that the presence of Sols may contribute to maintaining a higher relative abundance of *Alistipes* during prolonged incubation (Figure 6.28). If this effect is indeed linked to Sols, it could indicate that Sols may help sustain *Alistipes*, a genus associated with anti-inflammatory effects and reduced IBD incidence [29]. However, determining the absolute abundance using qPCR provides a more precise indication of whether Sols truly influence the total amount of *Alistipes* present in the samples.

The sequencing data indicate that alpha diversity, assessed by both observed OTUs and the Shannon index, was significantly influenced by inoculation time (Figures 6.30b, 6.32b). While the addition of lipids did not result in statistically significant differences in observed OTUs, an increase in the median of 500 OTUs suggests a trend toward higher diversity in lipid-supplemented samples (Figure 6.30c). In contrast, the Shannon index revealed a significant increase in diversity following lipid supplementation (Figure 6.32c), indicating effects on both richness and evenness. The presence of Sols did not produce significant changes in either metric, suggesting a limited impact on overall microbial diversity (Figures 6.30a, 6.32a). This

may indicate that Sols influence specific metabolic pathways or microbial functions such as H₂S production (Figure 6.16) rather than broadly reshaping community diversity.

Analysis of beta diversity similarly shows that the main differences between sample groups are associated with inoculation time. At 48 hours, the control, Fjoh, and Δ 2419 Fjoh samples cluster relatively closely together, indicating similar microbial community composition. At 72 hours, the samples become more separated, suggesting that microbial communities diverge over time. The Fjoh and Δ 2419 Fjoh samples largely overlap, whereas the control samples form a distinct cluster that does not overlap with the lipid-treated samples (Figure 6.33, 6.35). Overall, the beta diversity analyses indicate that inoculation time is the strongest driver of community differences, followed by lipid addition, while the presence of Sols has the smallest effect. This is further supported by the NMDS plots, which show no clear systematic change based on Sols, and the CCA analysis, where Sols explain 7.2% of the variation along the CCA1 axis (Figure 6.34, 6.36). This suggests that Sols impact on the stool microbiome remained relatively small, with limited despite measurable metabolic changes in H₂S production. Such resilience has previously been described in gut microbiome systems exposed to short-term dietary interventions [42].

The sequencing data suggest that Sol supplementation does not induce major shifts in overall microbiome composition. This indicates that Sols may primarily affect microbial metabolism rather than broadly restructuring the bacterial community. Focusing analysis on specific genera such as *Alistipes* that might have a higher relative abundance because of the incubation with Sols (Figure 6.28) or the H₂S producing genera [23], that might use Sols as a possible energy source and turn it into H₂S (Figure 6.16), suggest that Sols may still influence selected metabolic or ecological interactions within the microbiome. Future analyses focusing on SRBs and specific Sol-associated taxa may therefore provide a more detailed understanding of how Sols interact with gut microbial communities.

6.3.4 Quantitative PCR for *Alistipes*

qPCR was performed to determine whether the total abundance of *Alistipes* was affected by the presence of Sols, thereby assessing the potential of Sols as a compound capable of modulating *Alistipes* abundance in the gut microbiome.

qPCR was conducted on *Alistipes communis* A5CBH24 09830 gene to determine if Sols would increase the total amount of *Alistipes* present within the samples. qPCR was done on the sample groups Control, Fjoh, and Δ 2419 Fjoh for both 48 and 72 hours.

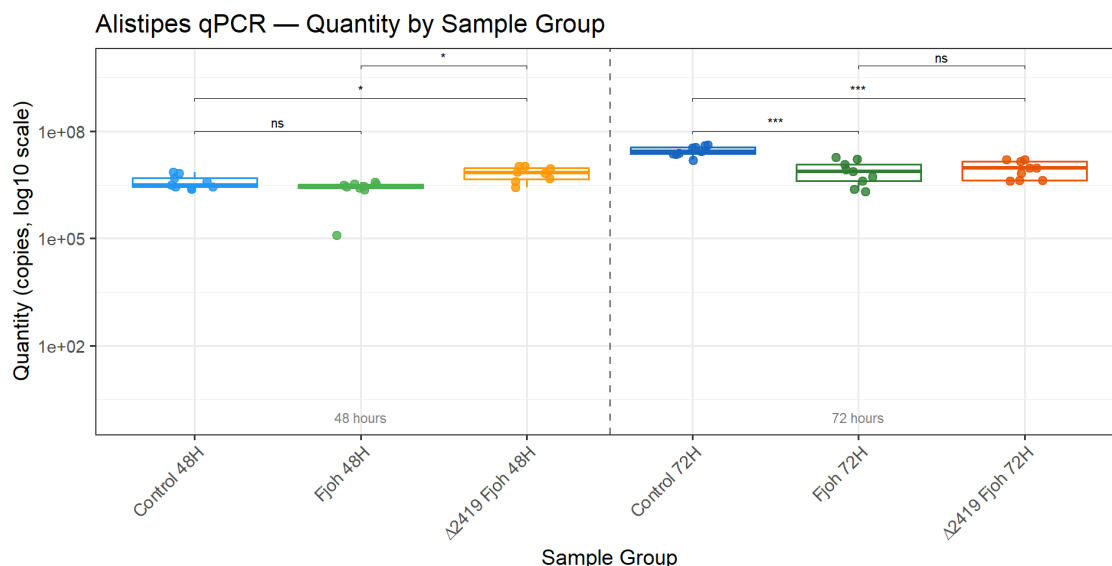


Figure 6.37. qPCR analysis was performed on three sample groups: control, Fjoh, and Δ 2419 Fjoh at two time points, 48 and 72 hours. At 48 hours, the control and Fjoh samples showed similar median copy numbers; however, the control exhibited higher upper-quartile and maximum values. The Δ 2419 Fjoh samples displayed the highest overall copy numbers at this time point. At 72 hours, the control samples showed the highest copy numbers, while the Fjoh and Δ 2419 Fjoh samples exhibited comparable levels.

qPCR analysis of *Alistipes* at 48 hours showed that the control and Fjoh with Sols sample groups had comparable gene copy numbers. Although the control exhibited a higher upper quartile and maximum value, no significant difference was observed between the groups based on Analysis of Variance (ANOVA) with Tukey's HSD post hoc testing. In contrast, both the control and Fjoh samples had significantly lower gene copy numbers than the $\Delta 2419$ Fjoh without the Sols group. At 72 hours, the Fjoh and $\Delta 2419$ Fjoh sample groups showed similar gene copy numbers, with no significant difference between them. However, the control samples displayed significantly higher gene copy numbers compared to both Fjoh groups.

Between 48 and 72 hours, a substantial increase in gene copy number is observed in the control sample group, indicating a marked rise in *Alistipes* abundance over time. The Fjoh sample group also shows an increase and greater inter-sample variability. In contrast, the $\Delta 2419$ Fjoh group exhibits only a minor increase, maintaining relatively stable copy numbers across the two time points. Overall, these results suggest that *Alistipes* abundance increases over time in the control and Fjoh-treated samples, whereas the $\Delta 2419$ Fjoh condition maintains a more constant population level.

The qPCR data indicate that the presence of Sols does not increase the absolute abundance of *Alistipes* in the stool samples at either 48 or 72 hours. This provides important context for the sequencing results (Figure 6.28), where a higher relative abundance of *Alistipes* is observed in the Fjoh 72H samples compared to the $\Delta 2419$ Fjoh 72H group. This apparent increase can be explained by differences in overall community composition: a lower total bacterial load in the Fjoh 72H samples leads to a higher relative abundance, despite comparable absolute *Alistipes* levels across groups.

6.3.5 Sulfonolipid prebiotic potential

Sols' impact on the stool microbiome is most clearly reflected in the H₂S data, where a distinct increase in H₂S production was observed at 48 hours in both sample groups containing Sols compared to those without Sols (Figure 6.16). This suggests that sulfur-reducing bacteria (SRB) may utilize the sulfur-containing groups present in Sols (Figures 2.3a, 2.3b) and convert them into H₂S.

These findings indicate that Sols may influence sulfur metabolism within the gut microbiome. Potentially, this could be relevant in situations where increasing H₂S production may be beneficial for individuals with unusually low physiological levels. Conversely, because elevated H₂S concentrations have been associated with harmful effects and intestinal inflammation, the results may also suggest that individuals with already high H₂S levels should avoid excessive Sol exposure. However, further studies are required to determine whether Sol-induced changes in H₂S production are beneficial or detrimental under physiological conditions.

In contrast, the effect on SCFAs is less clear. The CE data show substantial inter-sample variability across all groups, making consistent trends difficult to identify. Some indications of increased lactate at 72 hours (Figure 6.19) and butyrate at 48 hours (Figure 6.21) are observed in Sol-containing samples; however, these patterns are not consistent. When combined with qNMR data, Sol-containing samples generally exhibit comparable or lower SCFA levels relative to lipid-only controls, with lactate as a possible exception. Overall, these findings do not provide strong evidence that Sols systematically influence SCFA production. Therefore, obtaining pure Sols would be of benefit to determine whether Sols do indeed upregulate butyrate and lactate production beyond what is observed with the Fjoh lipid fraction alone.

Similarly, 16S rRNA gene sequencing indicates that inoculation time and lipid addition are the primary drivers of microbiome variation. No consistent or significant effects specifically attributable to Sols were observed in either alpha diversity (Figures 6.30, 6.31) or beta diversity (Figures 6.33, 6.36), suggesting that Sols do not induce major shifts in overall community structure under the tested conditions. However, smaller changes in specific genera may still occur, with *Prevotella* and potentially SRB could be influenced by the presence of Sols. Therefore, a more targeted and deeper analysis of these taxa would be beneficial to better determine the possible impacts of Sols on the gut microbiome.

qPCR analysis further supports this conclusion, showing that Sol addition does not increase the absolute abundance of *Alistipes* at either 48 or 72 hours. This indicates that any potential role of Sols in modulating gut health is unlikely to occur through enrichment of this genus alone [29]. This may also be beneficial, as *Alistipes* has been associated with an increased risk of cardiovascular disease [50]. Therefore, the lack of Sol-driven enrichment of *Alistipes* may reduce concerns regarding potential adverse health effects linked to increased abundance of this genus.

These results were obtained using pooled stool samples from five donors. Pooling reduces inter-individual variation and can make larger systematic changes in the microbiome easier to identify. However, this

1570 approach also limits the ability to detect donor-specific microbial interactions or individualized responses to Sol supplementation, which may vary substantially between individuals due to differences in baseline microbiome composition.

To better understand the role of Sols as potential prebiotic compounds, future studies should include the other Sol-producing genus *Odoribacter*, to assess whether its abundance is influenced by Sol availability.
1575 Additionally, targeted investigation of *Prevotella* and SRB would be valuable, particularly given the observed increase in H₂S production (Figure 6.16), to determine whether Sols indirectly affect microbial sulfur-metabolism pathways. Additionally, determining the time point at which Sols are depleted in the samples would be valuable, as similar approaches have been applied to compounds such as sulfoquinovose [113].

Conclusion 7

This study investigated the optimization of *Flavobacterium johnsoniae* cultivation for Sol production and evaluated the effects of extracted *Flavobacterium johnsoniae* lipids on stool microbiome composition and metabolism.

Liquid cultivation revealed that sulfobacin D and 2-hydroxy sulfobacin A, previously reported only in solid cultures, were also detected in liquid samples (Figure 6.3). This demonstrates that liquid cultivation can yield a complete Sol profile, in contrast to earlier reports [105].

Optimization experiments indicated that CYE 100% medium supplemented with 0.5 g/L cysteine provides favorable conditions for both *Flavobacterium johnsoniae* growth and Sol production. In contrast, *Flavobacterium johnsoniae* strains carrying plasmids containing *Flavobacterium johnsoniae*_2419 or *Flavobacterium johnsoniae*_2421 exhibited reduced growth and no increase in normalized Sol production (Figures 6.8, 6.10), suggesting that either plasmid burden or expression conditions negatively impact Sol biosynthesis. Comparison of the *Flavobacterium johnsoniae* Sol profile with that of *Alistipes*, a known gut Sol producer, showed substantial overlap in major Sol species, supporting the relevance of *Flavobacterium johnsoniae*-derived Sols as a model for gut-associated Sol lipids (Figure 6.11).

Application of extracted *Flavobacterium johnsoniae* lipids to stool samples resulted in increased H₂S production (Figure 6.16), suggesting that sulfate-reducing bacteria may utilize Sol-derived sulfur groups. In contrast, no consistent relationship between Sol presence and SCFA production was observed (Figures 6.17–6.23). Microbiome sequencing revealed that under the tested experimental conditions, Sol supplementation did not induce detectable alpha or beta diversity changes. For specific genera, The genus *Prevotella* might be increased by Sols (Figure 6.26).

Analysis of *Alistipes* showed that Sols did not increase either relative or absolute abundance at 48 or 72 hours (Figures 6.28, 6.37). Although the relative abundance of *Alistipes* was higher in Sol-containing samples at 72 hours, qPCR data demonstrated that absolute abundance remained comparable. Rarefaction analysis indicated insufficient sequencing depth (Figure 6.24), limiting detection of low-abundance taxa, including key sulfate-reducing bacteria such as *Desulfovibrio*, *Desulfobacter*, *Desulfomonas*, *Desulfobulbus*, *Desulfotomaculum*. This constraint restricts conclusions regarding the relationship between Sol metabolism and H₂S production.

Overall, the results demonstrate that *Flavobacterium johnsoniae* can be cultivated under optimized conditions to produce Sols resembling those found in the gut microbiome. While Sols appear to increase H₂S production, they do not substantially alter the overall microbiome composition under the tested conditions. The effects of Sols on SCFA production were less clear, although butyrate and lactate may be influenced by Sol supplementation. However, larger and more targeted studies are required to determine whether these trends are biologically significant.

Future perspective 8

1615 With the rising rate of IBD, it is paramount to find possible ways to combat both the development and symptoms of the disease; therefore, the study of possible prebiotics is needed to lessen the personal and health care costs. It is evident that Sols are a potential prebiotic compound, but more information is needed regarding their effects before they can be used. Future studies would benefit from larger sample sizes to test SCFA and sequencing, which could highlight smaller differences between samples containing
1620 Sols and those lacking Them. Having a more diverse group of stool donors could provide greater insight into the general population, since the current donor population is not representative; this study population skews younger and healthier than the general population. Therefore, expanding the scope of participants and the number of samples collected from mixed stool could yield clearer answers about the effects of Sols on the environment and on bacteria in the GI tract. Also, studying the effects of Sol on non-mixed stool
1625 from a large cohort could provide insight into the unique effects of Sol on specific microbiome composition or other factors, such as age, disease conditions, or diet.

Procurement of pure Sols would provide a clearer picture of their specific effects in the gut, independent of background lipid components. A possible chemical pathway to produce pure Sols, so that the inoculation of Sols into stool does not include a large amount of non-Sol lipids, would be able to give a clearer picture
1630 of Sols impact in the gut.

A deeper analysis of specific taxa associated with H₂S, and the specific Sol producers would be of interest. The sequencing did not find these specific taxa. This could be done through qPCR, where looking for the specific genes associated with the bacteria and their H₂S and Sol production would determine whether the presence of Sols increases the abundance of these genes and, thereby, the presence of the genera. Another
1635 suitable target for qPCR analysis could be *Prevotella*, which would provide a clearer indication of whether this genus takes up and utilizes Sols like its uptake of sphingolipids.

Having larger stool pellets for qNMR analysis would benefit future studies by allowing a deeper look into the metabolites present and any possible changes after an inoculation period with Sols. This could allow analysis of bile acids and assess whether they are affected in the presence of Sols.

1640 A general limitation of the stool incubation experiments was the absence of 0-hour baseline samples. Consequently, the analyses primarily reveal relative differences between sample groups and incubation times, rather than the absolute temporal changes occurring from the initial stool composition. Therefore, should future studies include 0-hour baseline samples.

Future studies into Sols hope to lead to a new prebiotic compound that can be used to lessen symptoms
1645 of conditions like IBD and other conditions. Care should be taken to ensure that any future prebiotic application does not cause adverse effects, particularly given the observed increase in trimethylamine production.

8.1 Hormones impact on *Alistipes timonensis* growth and Sol expression

1650 The effects of hormones on *Alistipes timonensis* were also investigated, focusing on both bacterial growth and the Sol profile. The three tested hormones dopamine, cortisol, and melatonin are naturally present in the gut and may therefore interact with gut bacteria [121, 122], potentially influencing bacterial growth or lipid composition.

Hormonal effects on *Alistipes timonensis* were evaluated over 48 hours. A growth curve similar to (Figure
1655 6.12) was generated, with corresponding growth stages occurring at comparable time points.

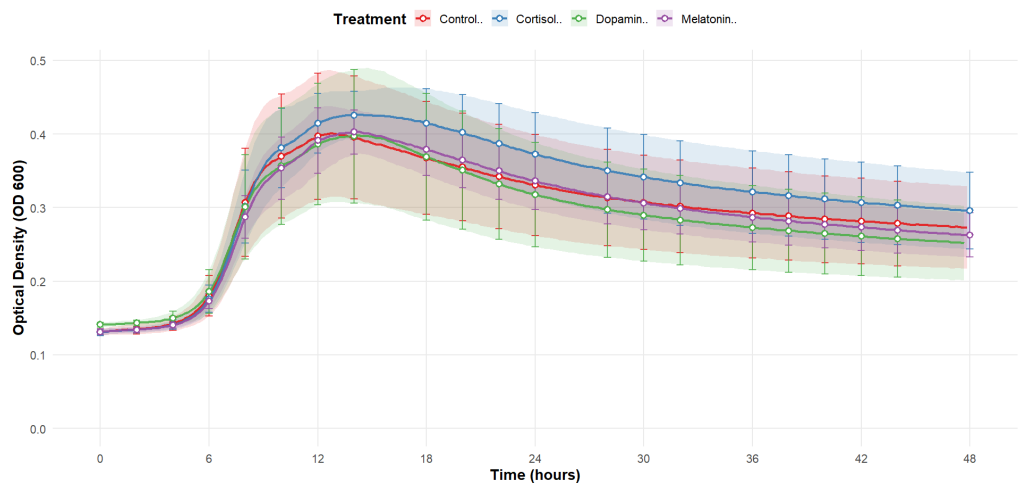


Figure 8.1. Growth curve over *Alistipes timonensis* inoculated in BHI media either inculcated with cortisol, dopamin, melatonin, or without any added hormones as a control.

The growth curves indicate that the tested hormones do not substantially affect the growth of *Alistipes timonensis*. Among the tested conditions, only cortisol showed a noticeable effect, with cultures exhibiting a slightly higher average OD after approximately 9 hours that persisted throughout the remaining 48-hour incubation period. Overall, the results suggest that these hormones have a limited influence on bacterial growth under the tested conditions.

To investigate whether the hormones influenced Sol production, the lipid profiles of the samples were analyzed. The relative abundance of each lipid within the total Sol profile was calculated and visualized as a heatmap. The profiles were primarily dominated by sulfobacin B, sulfobacin A, and SL 604.4608, allowing comparison of whether hormonal exposure altered the proportional composition of the major Sol species.

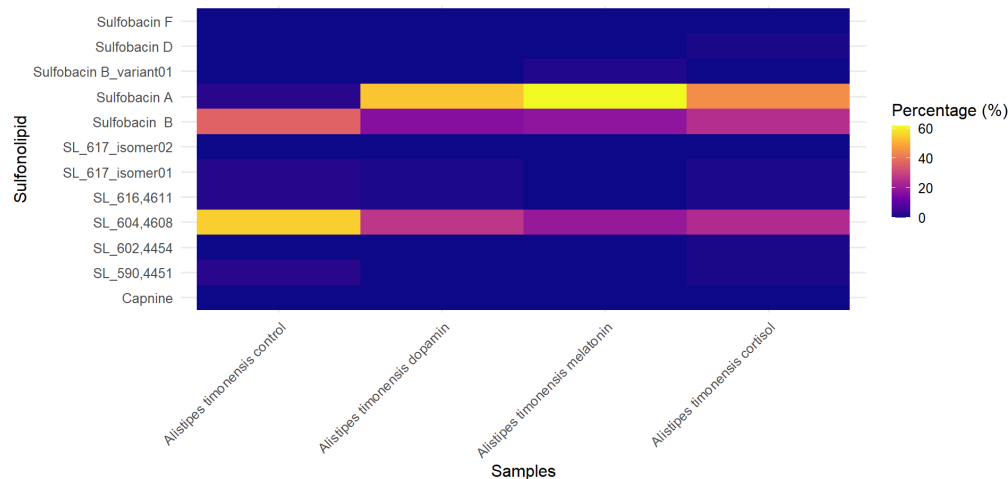


Figure 8.2. Heatmap over the major Sols in *Alistipes timonensis* with a control, and samples exposed to dopamin, melatonin, and cortisol.

The heatmap demonstrates that all tested hormones altered the Sol profile of *Alistipes timonensis*. In the control samples, the profile was primarily dominated by sulfobacin B and SL 604.4608, whereas hormone-treated samples showed a shift toward dominance by sulfobacin A. These findings provide evidence that hormones can influence the Sol composition of *Alistipes*, suggesting that Sol profiles observed under standard laboratory conditions may differ from those present in the gut environment. Therefore,

the chromatograms shown in (Figures 6.11a, 6.11b) may not fully represent *in vivo* Sol production. Nevertheless, the Fjoh profile (Figure 6.11c) still overlaps with the major Sol species identified in *Alistipes*.

These findings highlight that the gut environment is more complex than standard laboratory media and suggest that host-associated factors, such as hormones, may influence bacterial lipid metabolism. This should be considered in future studies investigating Sols as potential prebiotic compounds.

8.2 Sulfonolipid producing proteins structure and possible active sites.

To identify potential mutation sites for improving Sol production, several *in silico* approaches were used to locate conserved amino acids within predicted active sites of Sol-associated proteins.

Four genes involved in Sol biosynthesis were selected for analysis: Fjoh_2419 (SulA), Fjoh_2421 (sulfobacin synthase), Fjoh_2557 (cysteate synthase), and Fjoh_3782 (capC). Amino acid sequences for these genes were obtained from the Kyoto Encyclopedia of Genes and Genomes. Paralogs and orthologs of each protein were identified, and their amino acid sequences were aligned using Clustal Omega to determine conserved residues (Figure 8.3). In parallel, AlphaFold was used to predict the three-dimensional structures of the proteins, and the resulting structures were analyzed in CHARMM-GUI to identify potential active sites. Conserved amino acids identified through sequence alignment were then compared with the predicted active-site regions to identify residues likely important for enzymatic activity.

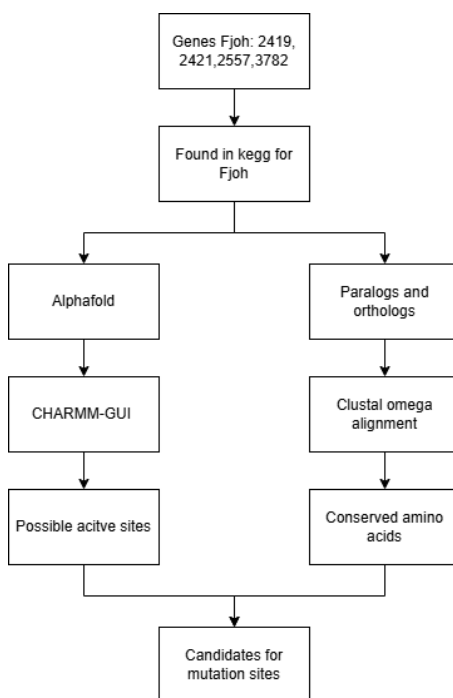


Figure 8.3. Workflow over the chosen proteins and how the conserved amino acids were found by comparing paralogs and orthologs in Clustal Omega, and how possible active sites were found from AlphaFold and CHARMM-GUI. The data were then combined to identify conserved amino acids located within the predicted active sites.

The workflow used for identifying conserved residues and predicted active sites is shown in (Figure 8.3). Conserved amino acids located within the predicted active sites are highlighted in (Figures 8.4, 8.5, 8.6, 8.7). For SulA, the residues (Y55), (T224), (W309), and (T380) were identified within the predicted active site. In sulfobacin synthase, the residues (L105), (P123), (L197), and (Y223) were identified. Cysteate synthase contained the conserved residues (T75) and (S76), while CapC showed conserved active-site residues at (I166), (Y181), and (K185).



Figure 8.4. Sula protein in Pymol with the conserved amino acids in the active site shown in red: (Y, 55), (T, 224), (W, 309), (T, 380).



Figure 8.5. Sulfobacin synthase protein in Pymol with the conserved amino acids in the active site shown in red: (L, 105) (L, 197) (Y, 223) (P, 123).



Figure 8.6. Cysteate synthase protein in Pymol with the conserved amino acids in the active site shown in red: (T, 75) (S, 76).



Figure 8.7. CapC protein in Pymol with the conserved amino acids in the active site shown in red: (I, 166) (Y, 181) (K, 185).

The identification of conserved residues within predicted active sites provides targets for future mutational studies aimed at clarifying the enzymatic functions of these proteins and potentially improving Sol biosynthesis efficiency.

1695

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Appendix A

A.1 Protocol

A.1.1 Sulfonolipid production

Flavobacterium johnsoniae was used as the production bacterium for Sols. To determine optimal production conditions, Fjoh was inoculated into 5 mL of liquid or 20 mL of solid CYE medium (Appendix A.3) under a laminar flow hood (Lamsystems SAVVY, Germany, Spetec). Cultures were incubated overnight at 27°C. In addition to standard CYE media, growth was tested in CYE supplemented with various additives at a final concentration of 15 mM. For liquid cultures, the additive was directly incorporated into the media. For solid media, 1 mL of Milli-Q water containing the additive was applied to the agar plates. Three standard liquid cultures were prepared, in which Fjoh was incubated in 100% CYE media for six hours (Appendix A.3). Multiple concentrations of cysteate were tested in both liquid and solid media to assess its effect on growth and Sol synthesis. (Appendix A.5).

Fjoh cultures were grown overnight from approximately 15:00–16:00 until the following morning at 08:00–09:00. Solid-state cultures were harvested by scraping the biomass and transferring it into 2 mL Eppendorf tubes. Liquid cultures were centrifuged in 2 mL Eppendorf tubes (VWR, United States, microstar 12) at maximum speed for 1 min, after which the supernatant was discarded. The resulting cell pellets were stored at -20°C until further analysis.

The cell pellets were freeze-dried (Alpha 1-4 LSCplus, Martin Christ, Germany) at -30 °C and 0.380 bar for 24 hours to remove residual liquid. Following lyophilization, the pellets were weighed to determine cell biomass. Lipids were extracted with methanol: 0.5 mL was added to each Eppendorf tube containing the dried pellets, and the mixture was sonicated for 20 min (Sonorex, Bandelin, Germany). The samples were then centrifuged (Centrifuge 5804 R, Eppendorf, Germany) at 14000xg for 20 min. The resulting supernatants were transferred to HPLC vials, and the methanol was evaporated under a stream of nitrogen. The remaining lipid extracts were weighed and subsequently re-dissolved in 0.5 mL of methanol, of which 200 µL was used for HPLC–MS/MS analysis (Appendix A.3). The analytical run included four methanol blanks and one pooled sample containing aliquots from all extracts. Sol profiles were generated based on the relative abundance of individual lipid species. To normalize the mass spectrometry data, all samples were adjusted to the same lipid mass, allowing for consistent comparison across samples. (Appendix A.7, A.8, A.16).

The growth conditions yielding the highest cell mass and Sol production were selected as the basis for further optimization. Cultures were subsequently grown in 5 mL CYE medium with variations in concentration, additives, cysteate supplementation, or combinations thereof (Appendix A.13). Inoculations were performed between 15:00–16:00, and cultures were incubated until 08:00–09:00 the following morning. After incubation, cells were pelleted by centrifugation, and the supernatant was discarded. The pellets were freeze-dried (Alpha 1–4 LSCplus, Martin Christ, Germany) at -30 °C and 0.380 bar for 24 h and subsequently weighed (Appendix A.13). Lipids were extracted using methanol as previously described (chapter A.1.1). Before HPLC–MS/MS analysis (Appendix A.3), lipid extracts were diluted to a final concentration of 50 µg/mL. (Appendix A.14).

A.1.2 Plasmid construction

To clone the genes Fjoh_2419 and Fjoh_2421 into Fjoh, two plasmids were constructed using the *E.coli*-*Flavobacterium* shuttle vector pCP11 [104]. Primers were designed to amplify the target genes by PCR. PCR reactions were prepared using 5 µL of 5 Q5 reaction buffer, 0.5 µL of 11 mM dNTPs, 1.25 µL of each forward and reverse primer (10 µM), 1 µL of template DNA, and 0.25 µL of Q5 High-Fidelity DNA Polymerase, with nuclease-free water added to a final volume of 25 µL. Successful amplification of the

target genes was verified by agarose gel electrophoresis using a 0.5% agarose gel.

To insert the genes into the pCP11, the vector was first digested together with the genes, 10 μ L Fjoh_2419 or Fjoh_2421 or pCP 11, 2 μ L cut smart buffer, 1 μ L XbaI, 1 μ L SalI, 6 μ L milli-Q water. It was then stored for 1 hour at 37 °C. The vector and genes were then ligated together using 2 μ L T4 DNA ligase buffer, 13 μ L milli-Q water, 5 μ L pCP 11 cut, 1 μ L insert DNA Fjoh_2419, Fjoh_2421, 1 μ L T4 DNA ligase, to generate sticky ends, the mixture was stored for 10 min at room temperature, followed by inactivation of T4 ligase at 10 min at 65 °C to inactivate the T4 ligase, this led to a Fjoh_2419+pCP11 plasmid (Appendix A.4) and a Fjoh_2421+pCP11 plasmid (Appendix A.5).

Competent 10-beta *E. coli* and s-17 *E. coli* were grown in mL LB media overnight. The culture was then inoculated into 70 mL LB medium and grown until reaching an OD600 of 0.6 (Thermo Fisher Scientific, United States, Genesys 40). The cells were subsequently transferred to 50 mL Falcon tubes and pelleted by centrifugation (Eppendorf, Germany, Centrifuge 5804 R) at 4000 $\times$ g-10min-4°C. The supernatant was discarded, and the pellet was resuspended in 10.5 mL MgSO₄/CaCl₂, composed of 14.8 mL CaCl₂ and 4.4 mL MgSO₄, where it was placed on ice for 40min. Afterwards, it was centrifuged (Eppendorf Germany, centrifuge 5804 R) at 4000 $\times$ g-10min-4°C again, and the supernatant was discarded, and the pellet was resuspended in 3.5 mL MgSO₄/CaCl₂ where it was stored on ice for 40 min. Subsequently, 875 μ L glycerol was added. The liquid was aliquoted by 200 μ L into 0.5 mL Eppendorf tubes.

The plasmids were then transformed into 10-beta Competent *E. coli* [123] to be produced, this is commenced by 10 μ L plasmids mixed with 100 μ L 10-beta Competent *E. coli* [123]. The cells were then on ice for 30 min, where they were then heat shocked (LLG Labware Germany, uniBLOCKTHERM) at 42°C for 90 sec, then moved back on ice for 2 min, then 800 μ L LB media was added, the stored at 37 °C for 1 hour the sample was then centrifuged (VWR United States, microstar 12) to make a pellet and the supernatant were discarded, the pellet were then resuspend and spread over a plate with AMP, this then grows overnight where a single colony was then picked and inoculated into 5 mL LB media with 5 μ L 10 μ g/mL AMP to growth overnight.

The LB media with 10-beta Competent *E. coli* [123] that has the extra plasmid were then centrifuged to form a pellet. To obtain the plasmid, the (New England BioLabs, United States, Monarch) kit was used [124]. To confirm the presence of a plasmid in the sample, a nanodrop was used. After digestion with EcoRI and XbaI, the sample is then subjected to gel electrophoresis (Bsb11, Germany, Mini) to determine if the exact vectors have been obtained by their unique pattern and size of DNA fragments found by scanning the gel (BIO RAD, United States, Gel Doc XR+) (Appendix A.6).

Once confirmed, the plasmids are transformed (chapter A.1.2) into *E. coli* strain S17-1 [125], where it were then grown in 5 mL LB with 5 μ L 10 μ g/mL AMP liquid media. After overnight growth, the conjugation was performed by mixing 1 mL of LB+AMP medium containing the *E. coli* strain S17-1 with 1 mL of CYE medium containing Fjoh. This was then centrifuged to create a pellet, and the supernatant was discarded. The pellet is washed with 1 mL of CYE media, centrifuged, and the supernatant is discarded. This is done to remove the AMP. The pellet is then resuspended in 50 μ L CYE media. Then, 25 μ L of the CYE-Fjoh and *E. coli* mixture is spread onto a CYE-CaCl₂ plate. This is then grown overnight at 27°C.

The bacterial layer from the CYE-CaCl₂ plate is then scraped into a 2 mL Eppendorf tube containing 1 mL of CYE media. After transferring the bacterial layer, 1 mL of extra CYE media was added to the tube and mixed. Then 20 μ L of CYE media was transferred to a separate Eppendorf tube containing 180 μ L of CYE media. This dilution pattern was done five times. From these, a dilution of 100 μ L, which was spread over a CYE plate with erythromycin 1mL 10 μ g pr 100mL CYE, and then grown over 4 days at 27°C.

To confirm the presence of the pCP11 plasmid within the Fjoh, a colony from the CYE+erythromycin plate was inoculated into 5 mL liquid CYE with 5 μ L 10 μ g/mL tetracycline to grow overnight at 27°C. When the plasmids' presence was confirmed, the Fjoh+plasmids were inoculated into 5 mL CYE+5 μ L tetracycline 10 μ g/mL with varying concentrations and add-ons. These were then grown overnight from 15:00-16:00 till 08:00-09:00, where they were pelleted and then freeze-dried (Martin Christ Germany, Alpha 1-4 LSCplus) to remove the last liquid from the samples at -30°C at 0.380 bars for 24 hours (Appendix A.15).

A.1.3 Lipid profile of *Alistipes*

Alistipes timonensis and *Alistipes putredinis* were cultivated under anaerobic conditions in an anaerobic chamber (Coy Laboratory Products, United States; Vinyl Anaerobic Chambers) using liquid Brain Heart

Infusion (BHI) medium. The chamber atmosphere consisted of 85% N₂, 10% CO₂, and 5% H₂, with hydrogen levels maintained between 1.5% and 2.5%. *Alistipes timonensis* was grown for varying durations: one sample (BHI timonensis 24 hours) was incubated for 24 hours, while all other *Alistipes timonensis* cultures were grown for 72 hours. All *Alistipes putredinis* cultures were incubated for 72 hours. Cysteates effect on both species was assessed at concentrations ranging from 0.25 g/L to 4 g/L (chapter A.6). Following incubation, cultures were removed from the anaerobic chamber by centrifugation (VWR, United States, MicroStar 12) at maximum speed for 1 min, after which the supernatant was discarded. The resulting cell pellets were stored at -20°C until further analysis. The method described in section 5.1 was applied to analyze the samples.

Alistipes timonensis and *putredinis* were each inoculated in three vials with 5 mL BHI media inside an anaerobic tent for 24 hours. After this, each vial was inoculated into its own flask with 50 mL BHI. Optical density was then measured at 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 24, 26, 28, 30, and 48 hours after inoculation. OD was measured in a 1mL sample in a semi-micro cuvette using a handheld Biochrom spectrophotometer (Biochrom, United States; UniTrospec 10 cell density meter), blanked with BHI medium. Samples for HPLC-MS/MS were taken at 0, 2, 4, 6, 8, 10, 24, 28, 48 hours after inoculation. Then 2 mL was taken from the flask into a 2 mL Eppendorf tube, which was centrifuged to create a pellet, and the pellet was prepared for HPLC-MS/MS as described in section 5.1 (Appendix A.3).

A.1.4 Large lipid extraction

Four 5 mL vials of CYE media were inoculated and then stored shaking at 120 rpm, at 27°C overnight. After overnight growth, the culture was inoculated into four 1 L Erlenmeyer flasks, each containing 500mL CYE medium with 0.5 g/L cysteate. These flasks were then stored shaking at 120 rpm at 27°C overnight. The media was then pelleted by centrifugation (Eppendorf, Germany; centrifuge 5910Ri) for 40 min at 1000×g. The supernatant was discarded, and the pellets were transferred into a 50mL Falcon tube, where they were centrifuged (Eppendorf, Germany; centrifuge 5804 R) for 20 min at 10000×g to further pellet the Fjoh. This was then freeze-dried (Martin Christ Germany, Alpha 1-4 LSCplus) to remove the last liquid from the samples at -30°C at 0.380 bars for 48 hours.

Fjoh Δ2421 that does not produce Sols was cultivated in two 5 mL vials that grew shaking at 120 rpm at 27°C overnight. Each vial was then inoculated into 500mL CYE+0.5g/L cysteate in a 1 L Erlenmeyer flask that then grew overnight at 27°C. The media was then pelleted by centrifuging (Eppendorf Germany, centrifuge 5910 Ri) for 40 min at 1000xg, the supernatant was then discarded, the pellets were then filled into a 50mL Falcon, where they were centrifuged (Eppendorf Germany, centrifuge 5804 R) for 20 min at 10000xg to pellet the Fjoh further. This was then freeze-dried (Martin Christ Germany, Alpha 1-4 LSCplus) to remove the last liquid from the samples at -30°C at 0.380 bars for 24 hours.

Lipids were extracted from the freeze-dried samples using methanol extraction, in which 5 mL of methanol was added to 15 mL Falcon tubes containing pellets up to 100 mg. These were then sonicated for 20 min, and the Eppendorf tubes were then centrifuged (Eppendorf Germany, centrifuge 5804 R) at 14000xg for 20 min. After this, the supernatant was transferred to HPLC vials, and the methanol was evaporated under nitrogen flow. The lipids were then weighed. All of one type of lipids were then suspended in 1.5 mL, which were then separated into three 5 mg/mL and three 0.5 mg/mL HPLC vials, both for Fjoh lipids and Fjoh Δ2421 lipids.

A.1.5 Prebiotic sulfonolipid stool tests

Exploratory stool test

An exploratory stool test was done with one individual (Appendix A.18). The stool was sampled and placed in an anaerobic tent (Coy Laboratory Products, United States, Vinyl Anaerobic Chambers) with a gas mixture of 85% N₂, 10% CO₂, and 5% H₂. The H₂ levels in the tent atmosphere ranged between 1.5 and 2.5%. The stool was then mixed with 30 mM bicarbonate buffer (pH 6.8) to a final concentration of 1.5 mg/mL fecal slurry. 1.5 mL of fecal slurry was then added to HPLC (Appendix A.3), three 5 mg/mL, and three 0.5 mg/mL, with either Fjoh lipids and Fjoh Δ2421 lipids, and three control vials with no lipids. These were then stored for 24 hours at 37°C.

After 24 hours, 150 µL was added to 1 mL of zinc acetate taken for sulfide testing. A calibration curve for sulfide testing was made with 250 µL 36g/mL zinc-acetate added to a 2 mL Eppendorf tube in a dark environment. Into the tubes where 0, 1, 2, 5, 10, 20 µL Na₂S – 9H₂O, 900 µL milli-Q water, then 100 µL Cline's reagent (Solve 2 g dimethyl-4-phenylenediamine (DMPD; T042) and 4.5 g FeCl₃ – 6H₂O (F112) in 25 % HCl. Fill with HCl until the volume reaches 100 ml). The tubes are then stored in the dark for

20 min. From min 13 to 16, they were centrifuged at 14000xg, then 200 μ L mixed solution was pipetted to a 96-well plate, and measured at 670 nm in a plate reader (Tecan Switzerland, INFINITE M NANO).

Then, 1 mL of zinc acetate was added to a 2 mL Eppendorf tube and 150 μ L of the sample. Out of this was 500 μ L mixed with 750 μ L of milli-Q water and 100 μ L of Cline's reagent. Then stored in the dark for 20 min, from min 13 to 16, it was centrifuged, then 200 μ L was pipetted to a 96-well plate, and measured at 670 nm in a plate reader (Tecan Switzerland, INFINITE M NANO)

Expanded stool test

A stool test was performed on five individuals' stool (Appendix A.19). The stool was sampled, placed in an anaerobic tent (Coy Laboratory Products, United States, Vinyl Anaerobic Chambers) with a gas mixture of 85% N₂, 10% CO₂, and 5% H₂. The H₂ levels in the tent atmosphere ranged from 1.5% to 2.5%. The stool was then mixed and suspended in 30 mM bicarbonate buffer (pH 6.8) to 1.5 mg/mL fecal slurry. The 1.5 mL fecal slurry was then added to 4mL tubes, three 5 mg/mL, and three 1.5 mg/mL, with either Fjoh lipids, Fjoh Δ 2419 and Fjoh Δ 2421 lipids, and 3 control vials with no lipids (Appendix A.20). These were then stored for 72 hours at 37°C. with samples taken every 24 hours, used directly for H₂S analysis and stored at -20°C until use for CE .

For H₂S 250 μ L, 36g/mL zinc-acetate was added to an Eppendorf tube. Then 100 μ L fecal slurry was added to the Eppendorf, followed by 900 μ L milli-Q water, and the tubes are then transported to a dark environment where 100 μ L Cline's reagent The tubes are then stored in the dark for 20 min. From minute 13 to 16, they were centrifuged, then 200 μ L mixed solution was pipetted to a 96-well plate, and measured at 670 nm in a plate reader (Tecan, Switzerland, INFINITE M NANO).

CE was commenced first on formate, acetate, propionate, butyrate, valerate, and lactate samples at 1mM and 1M to determine the retention time on a CE (Agilent Technologies, United States, 7100 capillary electrophoresis) run over a (Agilent Technologies, United States, G1600-62311 - HPCE standard cap 75um id, 72cm lght 2/PK). The capillary was pre-washed with milli-Q water and then filled with an organic acid buffer at pH 5.6 for CE, which was used as the running buffer. After the retention times had been determined, a calibration curve was made with a mix standard composed of formate, acetate, propionate, butyrate, valerate, and lactate at concentrations of 1 mM, 500 μ M, 250 μ M, 100 μ M, 50 μ M, and 25 μ M with an internal standard of 2 μ L succinate 0.1 mM, this was then run over the CE. Samples were then injected at 50 mbar for five seconds. Measurements were performed at -25kV, 100 μ A, and 6W.

Samples for CE were prepared with a 98 μ L sample as done in the A.1.5 method and 2 μ L of 10 mM succinate, then vortexed to mix. They were then loaded into the CE. Samples were then injected at 50 mbar for five seconds. Measurements were performed at -25kV, 100 μ A, and 6W.

A.1.6 Quantitative nuclear magnetic resonance

For qNMR, the control, Fjoh, and Δ 2419 Fjoh 5 mg/mL samples were selected at 24 and 72 hours, for a total of 18 samples.

qNMR was commenced on the stool samples in a 1.5 mL Eppendorf tube, where they were homogenized. The stool was weighed (Appendix A.36) and transferred to a 2 mL Eppendorf tube with 1 mL of buffer. The samples were then vortexed on a (Scientific Industries, United States, vortex genie 2). The samples were then centrifuged at 16160xg for 15 min. Then, 200 μ L supernatant was transferred to a new 2 mL Eppendorf tube. The samples are then centrifuged at 16160 g for 15 min, and 100 μ L of the supernatant and 55 μ L of the NMR standard are pipetted into a 3 mm tube. The NMR tube was vortexed 3 times and then centrifuged before analysis on an NMR spectrometer (Bruker, Switzerland, AVIII-600 MHz spectrometer). The instrument was equipped with a cryogenically cooled CPP-TCI 5 mm probe. Quantitative ¹H NMR spectra were acquired using the automated PR-PLUCON pulse program with water suppression at 298.1 K, with 32 scans collected per sample.

A.1.7 DNA 16S rRNA gene Sequencing

For sequencing, the control, Fjoh, and Δ 2419 Fjoh 5 mg/mL samples were selected at 48 and 72 hours, for a total of 18 samples for DNA extraction, PCR, barcoding, and sequencing.

For DNA extraction, an MP Bio kit (MP Biomedicals, United States, FastDNA SPIN Kit for Soil) was used. First, the stool samples were defrosted and transferred to a fume hood (Labflex Group, Denmark, Fume Hood model 1000), where 20 μ L was pipetted to the lysing matrix E, then 478 μ L sodium phosphate buffer

and 122 μL MT buffer were added to the bead matrix. The bead matrix was then shaken for 40 seconds in four rounds using a bead beater (MP Biomedicals, United States, FastPrep-24 Classic bead beating grinder and lysis system), with 2 min on ice between rounds. The lysing matrix E is then centrifuged (sigma laborzentrifugen, Germany, Minizentrifuge Sigma 1-14) for 7 min at 14000xg, and afterward is transferred to a 2 mL Eppendorf tube where 250 μL PPS was added to it, the Eppendorf tube is then mixed 10 times and centrifuged for 5 min at 14000xg. The supernatant is then pipetted to a new 2 mL Eppendorf tube with 1 mL Binding Matrix Solution, and the Eppendorf tube is then lightly shaken for 2 mins and place the tube on a rack for 3 mins. The volume is then reduced to 500 μL in the tube, and the contents are transferred to a spin column, which is centrifuged for 1 min at 14000xg into a catch tube. Empty the catch tube and add 500 μL of the prepared SEWS-M Solution to the spin column and centrifuge for 1 min at 14000xg. Empty the catch tube, centrifuge for 2 min at 14000xg, let the spin tube air-dry for 5 min at room temperature, then add 100 μL DES Elution Solution to the spin column. Centrifuge this for 1 min at 14000xg into a 1.5 mL Eppendorf tube.

To check DNA concentration, the Qubit fluorometer (Thermo Fisher Scientific, United States, Invitrogen Qubit 4 Fluorometer) was used. First, a calibration curve was made with (Thermo Fisher Scientific, United States, Invitrogen Qubit 1x double stranded DNA (dsDNA) High Sensitivity and Broad Range analysis set), where 190 μL of the Qubit 1x dsDNA High Sensitivity was added to 2 tubes and 10 μL of each Qubit 1x dsDNA Broad Range Assay was added to the appropriate tube, then mix by vortexing. The Qubit 3.0 was then set to "dsDNA", "High Sensitivity", then "read standards". Standard 1 reads 100 RFU, and standard 2 reads 18000 RFU.

For Qubit was 198 μL of the Qubit 1x dsDNA High Sensitivity and 2 μL of the sample were then added to each sample tube; these were vortexed and incubated for 2 min at room temperature. Then, load the sample into Qubit 3.0 at "dsDNA", "High Sensitivity", then "original sample amount" to 2 μL , then read the samples (Appendix A.45) .

All non-DNA-containing materials are first UV for 15 min inside a LAF bench (Holm & Halby, Denmark, Labogene Mars silence), then 10 μL of both primers (Appendix A.46) and 980 μL UV-treated nuclease-free water. In an Eppendorf tube, there was 25 μL PCRBIO 2x UltraMix and 20 μL Primer working solution (1uM) (Appendix A.46), multiplied by the number of samples prepared. 45 μL aliquots are then transferred to PCR tubes. 5 μL sample DNA is pipetted to the PCR tubes that are then covered with PCR lids, vortexed, and spun down (VWR, United States, PCR plate spinner). 25 μL of each PCR reaction is transferred to a duplicate in a different PCR tube and then spun down. The PCR reactions are run on two PCR machines (VWR, United States, UNO96/Doppio) (Appendix A.47).

The PCR product was purified. First, AMPure XP beads were equilibrated to room temperature, then vortexed for 1 min, and 40 μL pr sample to a reservoir. Then the PCR duplicates were pooled, and in a new tube, a 0.7 bead-to-sample ratio was added, and the mixture was pipetted up and down 10 times. The sample/bead mixture was incubated for 2 min, and the tubes were then placed on a magnet rack (Thermo Scientific, United States, Magjet Rack 96-well) for 5 min until the liquid was clear. The supernatant was then discarded, and the tubes were removed from the magnet rack. The pellet was then washed using 175 μL 80% ethanol, and stood for 30 sec. The tubes were then placed on a magnet rack for 5 min until the liquid became clear, after which the supernatant was discarded. The pellet was then air dried for 5 min, removed from the magnet rack, and suspended in 25 μL UV-treated nuclease-free water. Then, it stood for 2 min, was placed back on the magnet rack until the liquid was clear, and then 20 μL was transferred to new tubes that were stored at -20°C .

To confirm successful PCR amplification, a Qubit analysis was performed. where 190 μL of the Qubit 1x dsDNA High Sensitivity was added to 2 wells, and 10 μL of each Qubit 1x dsDNA Broad Range Assay was added to the appropriate well. Then, 198 μL of the Qubit 1x dsDNA High Sensitivity and 2 μL of the sample were added to a well, and it was analyzed in a spectrometer (Tecan group, Switzerland, Infinite 200Pro) (Appendix A.48). To further confirm the presence of the 16SRNA, a TapeStation was used (Agilent Technologies, United States, TapeStation 4150). 10 μL Genomic ScreenTape buffer was pipetted into each tube; then, in one tube, 1 μL Genomic ScreenTape ladder was added to the remaining 1 μL sample pipetted per tube. The tubes were then spun down; the ScreenTape device was inserted into the tape station, followed by the tubes, and the tape station was then run (Appendix A.7).

With nuclease-free water and the DNA samples, all adjusted to 200 fmol (Appendix A.49) and spun down. Then a unique barcode was chosen for each sample, where a barcoding PCR reaction was made in a 96-well plate, 1 μL barcode, 24 μL sample, 25 μL LongAmp Taq 2X master mix in each well, which was mixed by pipetting and spun down, and then PCR (Appendix A.50). Then the AMPure XP beads were resuspended by vortex, and in each well, 20 μL AMPure XP beads were added. This was incubated for 5 min at room

temperature, then placed on a magnet rack until a pellet formed and the supernatant could be discarded. then washed with 200 μ L 80% ethanol, and the ethanol was then removed and discarded. This was done twice. The tubes were then spun down and reintroduced to the magnet rack, where the residual ethanol was removed, and the pellet was air-dried for 30 sec. The tubes were removed from the magnet rack, and 25 μ nuclease-free water was introduced into each well, where it was incubated for 2 min. The tubes were reintroduced to the magnet rack until a clear liquid could be transferred to a clean 0,2 mL PCR tube.

First, AMPure XP beads were equilibrated to room temperature, then vortexed for 1 min. DNA Control Sample store on ice. In a 0.2 mL PCR tube, 1 μ L DNA Control Sample, 49 μ L sample, 7 μ L Ultra II End-prep Reaction Buffer, 3 μ L Ultra II End-prep Enzyme Mix. Mix it by pipetting, then incubate at 20 °C for 5 min, then at 65 °C for 5 min. transfer to 1.5 mL Eppendorf DNA LoBind tube, add 60 μ L AMPure XP Beads, Incubate on a Hula mixer for 5 min at room temperature. Take tubes on a magnet rack until the supernatant was clear and colorless, then discard. Wash the pellet twice with 200 μ L 80% ethanol and discard the ethanol. The tubes are then spun down and reintroduced to the magnet rack, where the residual ethanol was removed, and the pellets were air-dried for 30 seconds. The tubes were removed from the magnet rack, and 61 μ L of nuclease-free water was added to each. where it was incubated for 2 min. The tubes were reintroduced to the magnet rack until a clear liquid could be transferred to a clean 1.5 ml Eppendorf DNA LoBind tube.

The Ligation Adapter and Quick T4 Ligase are spun down and placed on ice. The Ligation Buffer was thawed at room temperature, spun down, and mixed by pipetting. The Elution Buffer and Long Fragment Buffer were thawed at room temperature, centrifuged, and vortex-mixed. Then was 1.5 ml Eppendorf DNA LoBind tube, 60 μ L DNA sample, 25 μ L Ligation Buffer, 1 μ L NEBNext Quick T4 DNA Ligase, and 5 μ L Ligation Adapter were added, and mixed by pipetting. It was then incubated for 10 min at room temperature. 40 μ L of resuspended AMPure XP Beads was added to each tube. Incubate on a Hula mixer at room temperature for 5 min. The tubes on a magnet rack until the supernatant was clear and colorless, then discard. The tubes were removed from the magnet rack, and the pellet was washed with 250 μ L Long Fragment Buffer. The tubes were placed on a magnet rack until the supernatant was clear and colorless, and then discarded. This was done twice. The tubes are then spun down and reintroduced to the magnet rack, where the residual ethanol was removed, and the pellet was air dried for 30 sec. The tubes were removed from the magnet rack, and 25 μ L Elution Buffer was introduced and incubated for 10 min at room temperature. The tubes were reintroduced to the magnet rack until a clear liquid was obtained, where the supernatant was removed, and the Qubit was analysed. The library was then diluted to 32 μ L at 10-20 fmol, using Elution Buffer.

The PromethION Flow Cell was primed and loaded with the Sequencing Buffer, Library Beads, and Flow Cell Tether, and one tube of Flow Cell Flush was thawed at room temperature, vortexed, and then stored on ice. 1170 μ L Flow Cell Flush, and 30 μ L Flow Cell Tether were mixed in an Eppendorf tube. Load the PromethION flow cell. Load 500 μ L of the priming mix into the flow cell via the inlet port, let it stand for 5 min. In a 1.5 ml Eppendorf DNA LoBind tube, mix 100 μ L Sequencing Buffer, 68 μ L Library Beads, and 32 μ L DNA library. Load 500 μ L of the priming mix into the flow cell inlet port. A P1000 pipette tip was inserted into the inlet port, and 200 micro L of the library was loaded. The inlet was closed, and the experiment was initiated after 10 min.

qPCR for *Alistipes*

A standard curve was generated to quantify the abundance of *Alistipes* in DNA extracted from stool samples. The standard was produced by PCR amplification using primers targeting the *Alistipes communis* A5CBH24_09830 gene (Appendix A.51). All non-DNA-containing reagents were UV-treated for 15 min in a LAF bench (Holm & Halby, Denmark, Labogene Mars Silence) before use.

The PCR master mix was prepared in a 96-well plate, consisting of 25 μ L PCRBIO 2x UltraMix, 20 μ L primer working solution (1 μ M), and 1 μ L nuclease-free water per reaction. To this, 4 μ L of the Fjoh 5 mg/mL, 72-hour sample (replicate 3) was added. The reaction mixture was split into two wells containing 25 μ L in each, then centrifuged (VWR, United States, PCR plate spinner) and amplified using a thermal cycler (VWR, United States, UNO96/Doppio) using (Appendix A.52) conditions.

Following PCR, the products were pooled and purified using AMPure XP beads. Initially, 28 μ L beads were added to the pooled PCR product and mixed thoroughly by pipetting. After a 2-minute incubation, the plate was placed on a magnetic rack (Thermo Scientific, United States, Magjet Rack 96-well) for 5 min, and the supernatant was transferred to a new well. A second purification step was performed by adding 14.5 μ L beads, followed by mixing, incubation, and magnetic separation. The supernatant was discarded, and the beads were washed with 175 μ L of 80% ethanol, incubated for 30 seconds, and placed

on the magnetic rack for 5 min. After removing the ethanol and allowing residual solvent to evaporate for 5 min, DNA was eluted in 25 μ L nuclease-free water. The eluate was separated on the magnetic rack and transferred to a tube. DNA concentration and fragment size were assessed using a Qubit 4 Fluorometer (Thermo Fisher Scientific, United States, Invitrogen Qubit 4 Fluorometer) and Tapestation (Agilent Technologies, United States, Tapestation 4150), done with the same methodology as in Chapter A.1.7, A.1.7 (Appendix A.8).

The purified *Alistipes* PCR product was used to generate a standard curve through a 10-step, 10-fold serial dilution. For qPCR analysis, a master mix was prepared containing 10 μ L 2 $\times$ Brilliant III qPCR Master Mix, 2 μ L primers (Appendix A.51), 0.3 μ L ROX (1:500 dilution), and 2.7 μ L nuclease-free water per reaction. To each well, 5 μ L of either standard, sample (control, Fjoh, and Δ Fjoh at 48 and 72 hours), or negative control was added. Reactions were mixed by pipetting, centrifuged (VWR, United States, PCR plate spinner), and analyzed by qPCR (Agilent Technologies, United States, Stratagene Mx3005P). The resulting amplification data were used to quantify the abundance of *Alistipes* in the samples.

A dissociation curve analysis was performed to verify the specificity of the qPCR amplification. The curve shows a distinct peak in fluorescence derivative ($-R'(T)$) at 88–90 $^{\circ}$ C (Appendix A.9), indicating that both samples and standards consist of a single, specific amplicon corresponding to the *Alistipes communis* A5CBH24_09830 gene. The amplification plot further demonstrates that most samples amplified as expected, crossing the detection threshold between 16 and 22 cycles (Appendix A.10). Absolute copy numbers were determined using a standard curve generated from serial dilutions, which exhibited a high linearity with an R^2 value of 0.979 (Appendix A.10).

A.1.8 Data analysis

Data analysis was conducted using R v4.5.1 and RStudio 2025.5.1.513. All microbiome data were visualised using the ampvis2 package v.2.8.9 and the ggplot2 package v.4.0.0. Alpha diversity was investigated using the Operational Taxonomic Unit (OTU) and Shannon diversity index, and beta diversity using Non-metric MultiDimensional Scaling (NMDS) and canonical correspondence analysis (CCA).

A.2 Growth of Bacteria

CYE 25% media made of 2.5g casitone, 1.25g yeast extract, 0.4925g $MgSO_4$, 2.5 mL tris buffer, and if the CYE is to be solid, 15g agar is added, pr L milliQ water.

Table A.1. composition of CYE 25% pr L. liquid media does not contain agar, while solid media uses agar to solidify

CYE 25%	amount
Casitone	2.5g
Yeast extract	1.25g
$MgSO_4$	0.4925g
1M tris buffer	2.5mL
Agar	15g

CYE 50% media made of 5g casitone, 2.5g yeast extract, 0.985g $MgSO_4$, 5 mL tris buffer, and if the CYE is to be solid, 15g agar is added, pr L milliQ water.

Table A.2. composition of CYE 50% pr L. liquid media does not contain agar, while solid media uses agar to solidify

CYE 50%	amount
Casitone	5g
Yeast extract	2.5g
$MgSO_4$	0.985g
1M tris buffer	5mL
Agar	15g

CYE 100% media made of 10g casitone, 5g yeast extract, 1.97g $MgSO_4$, 10 mL tris buffer, and if the CYE is to be solid, 15g agar is added, pr L milliQ water.

Table A.3. Composition of CYE 100% media pr L. liquid media does not contain agar, while solid media uses agar to solidify

CYE 100%	amount
Casitone	10 g
Yeast extract	5 g
$MgSO_4$	1.97 g
1M tris buffer	10 mL
Agar	15 g

CYE 150% media made of 15g casitone, 7.5g yeast extract, 2.955g $MgSO_4$, 15 mL tris buffer, and if the CYE is to be solid, 15g agar is added, pr L milliQ water.

Table A.4. Composition of CYE 150% media pr L. liquid media does not contain agar, while solid media uses agar to solidify

CYE 150%	amount
Casitone	15g
Yeast extract	7.5g
$MgSO_4$	2.955g
1M tris buffer	15mL
Agar	15g

Table A.5. Different concentrations of CYE media, and 15 mM add-ons, cysteate was of extra interest since it's a precursor in the sulfobacin pathway. The pellets made from the liquid media then showed their corresponding conditions in the liquid media.

Sample name	Pellet weights in mg
CYE standard	0.6
CYE standard 2	0.6
CYE standard 3	2.2
CYE 25%	0.1
CYE 50%	1.4
CYE 100%	2.3
CYE 150%	3
CYE media+add-on 15mM	
CYE galactose	0.2
CYE serine	1.4
CYE xylose	0.2
CYE cystine	1
CYE acetate	3.6
CYE propanate	1.2
CYE glucose	1.1
CYE nacl	1.8
CYE maltose	0.5
CYE manose	0.5
CYE cysteate	0.7
CYE lactose	0.3
CYE sucrose	0.7
CYE arabinose	1.1
CYE media+cysteat	
CYE cysteate 0.25g/L	4.6
CYE cysteate 0.5g/L	4.4
CYE cysteate 1g/L	4.2
CYE cysteate 2g/L	1.2
CYE cys 4g/L	0.1

Cysteate as an add-on was of extra interest since it has been shown to enhance the growth of *Flavobacterium johnsoniae* and therefore was both solid and liquid CYE media tested with 0.25g/L, 0.5g/L, 1g/L, 2g/L, 4g/L cysteate tested in the liquid media, for the solid media was 1mL milliQ water with these concentrations of cysteate spread over a plate.

Alistipes timonensis and *putredinis* were tested for their sulfobacin production, as they are relevant gut bacteria that are the main producers of sulfobacines in humans. *Alistipes* was tested with BHI media only in liquid form; it was only tested with the add-on cysteate at 0.25g/L, 0.5g/L, 1g/L, 2g/L, and 4g/L.

Table A.6. *Alistipes* in BHI media was tested under differing conditions with the pellets gained through centrifuging the samples after 3 days of incubation for *Alistipes timonensis* and 1 day of incubation for *Alistipes putredinins*.

Sample name	Pellet weight in mg
<i>Alistipes timonensis</i>	
BHI tim standard 1	0
BHI tim standard 2	0
BHI tim standard 3	0
BHI tim 24 hours	0.1
BHI tim cysteate 0.25g/L	4.2
BHI tim cysteate 0.5g/L	0.5
BHI tim cysteate 1g/L	0.3
BHI tim cysteate 2g/L	0
BHI tim cysteate 4g/L	0
<i>Alistipes putredinins</i>	
BHI put micheal	2
vl6 put	0.9
BHI put	2.1
BHI put 0.25g/L cysteate	0.5
BHI put 0.5g/L cysteate	1.3
BHI put 1g/L cysteate	1.7
BHI put 2g/L cysteate	0.5
BHI put 4g/L cysteate	2.5

To confirm the amount of Sols in each sample where the lipids were inserted into an HPLC-MS/MS. Some of the lipid fractions were in the minuses or unreasonably high; these data were excluded from the graph (6.4) and marked with an x in the table below.

Table A.7. The liquid samples and their lipid pellet weights, which shows if it was possible to normalize the weights of them

Sampel name	Pellet lipids weight mg	x not normalised
CYE concentration		
CYE standard 1	0.7	
CYE standard 2	0.5	
CYE standard 3	0.8	
CYE 25%	5.1	x
CYE 50%	0.1	
CYE 100%	0.2	
CYE 150%	1	
CYE media + 15mM add-on		
CYE galactose	0.1	
CYE serine	0.6	
CYE xylose	0.1	
CYE cystine	0.5	
CYE acetate	0.7	
CYE propanate	0.4	
CYE glucose	0.6	
CYE nacl	1.3	
CYE maltose	1.6	x
CYE manose	-0.5	x
CYE cysteate	-14	x
CYE lactose	-0.5	x
CYE sucrose	0.5	
CYE arabinose	-0.1	x
CYE media + cysteate		
CYE cysteate 0.25g/L	1.3	
CYE cysteate 0.5g/L	0.3	
CYE cysteate 1g/L	0.7	
CYE cysteate 2g/L	0.6	
CYE cys 4g/L	0.3	

To confirm the amount of Sols in each sample where the lipids were inserted into an HPLC-MS/MS. Some of the lipid fractions were in the minuses or unreasonably high; these data were excluded from the graph (A.2) and marked with an x in the table below.

Table A.8. The solid samples and their pellet weights, which shows if it was possible to normalize the weights of them

Sampel name	Pellet lipids weight mg	x not normalised
CYE media solid concentrations		
CYE 100% solid standard 1	0.1	
CYE 100% solid standard 2	2.6	x
CYE mm solid	0.4	
CYE 25% solid	0.5	
CYE 50% solid	0.8	
CYE 100% solid	0.1	
CYE 150% solid	0.7	
CYE media solid + 15mM add-on		
CYE serine solid	0.3	
CYE cysteate solid	0.5	x
CYE maltose solid	0.6	
CYE manose solid	0.4	
CYE sucrose solid	-9.4	x
CYE arabinose solid	0.1	
CYE xylose solid	0.3	
CYE glucose solid	0.6	
CYE cystine solid	15.8	x
CYE lactose solid	-0.7	x
CYE acetate solid	6.7	x
CYE propanate solid	0.3	
CYE galactose solid	0.1	
CYE media solid + cysteate		
CYE cysteate 0.5g/L solid	4.9	x
CYE cysteate 1g/L solid	1.3	x
CYE cysteate 4g/L solid	-0.2	x

A.3 MS settings

The setting for the HPLC-MS/MS is as follows

Table A.9. Parameters over the MS machine and under which factors the samples were run

Overall Method Settings (Q Exactive)	
Parameter	Value
Use lock masses	Off
Lock mass injection	–
Chrom. peak width (FWHM)	15 s
Method duration	25.00 min
Customized tolerances	–
Lock masses	–
Inclusion	–
Exclusion	–
Neutral loss	–
Mass tags	–
Dynamic exclusion	–
Experiment: Full MS / dd-MS² (TOPN) – General	
Runtime	0–25 min
Polarity	Negative
In-source CID	0.0 eV
Default charge state	2
Inclusion	–
Exclusion	–
Tags	–

Table A.10. Parameters over the MS machine and under which factors the samples were run

Full MS Settings	
Parameter	Value
Microscans	1
Resolution	70
AGC target	3.00E+06
Maximum IT	100 ms
Number of scan ranges	1
Scan range	100–1500 m/z
Spectrum data type	Profile
dd-MS² / dd-SIM Settings	
Minimum AGC target	8.00e3
Intensity threshold	1.6e5
Apex trigger	–
Charge exclusion	All
Multiple charge states	Preferred
Peptide match	On
Exclude isotopes	On
Dynamic exclusion	10.0 s
If idle	Do not pick others

Table A.11. Parameters over the MS machine and under which factors the samples were run

Tune Files	
Parameter	Value
Switch count	0
Base tune file	C:\Xcalibur\methods\Yuntao PG.mstune
Contact Closure	
Used	FALSE
Start in closed	TRUE
Switch count	0
Syringe Settings	
Used	FALSE
Start in OFF	TRUE
Stop at end of run	
Switch count	0
Syringe type	Hamilton
Flow rate	3.000 $\mu\text{L}/\text{min}$
Inner diameter	2.303 mm
Volume	250 μL
Divert Valve A	
Used	TRUE
Start in 1-2	TRUE
Switch count	2
Element 1	1.50 min $\rightarrow$ switch to 1-6
Element 2	18.97 min $\rightarrow$ switch to 1-2
Divert Valve B	
Used	FALSE
Start in 1-2	TRUE
Switch count	0

Table A.12. Parameters over the MS machine and under which factors the samples were run

Lock Masses			
m/z	Polarity	Start (min)	End (min)
21.408.963	Positive	0	20.00
16.298.240	Negative	0	20.00

A.4 Solid preliminary Profiles

Pellet weights of the freeze-dried samples.

Table A.13. Second batch samples and their pellet weights in mg

Sample name	Pellet weight mg
100% CYE 1	6.4
100% CYE 2	5.6
100% CYE 3	6
150% CYE 1	5.6
150% CYE 2	6.8
150% CYE 3	6.4
100% CYE cys 0.5 g/L 1	4.9
100% CYE cys 0.5 g/L 2	6.3
100% CYE cys 0.5 g/L 3	5.8
150% 0.5 g/L mix 1	4
150% 0.5 g/L mix 2	5.6
150% 0.5 g/L mix 3	5.5
150% 0.25g/L mix	7.4
150% 15mM mix	2.7

The Solid samples were tested for their Sols profile.

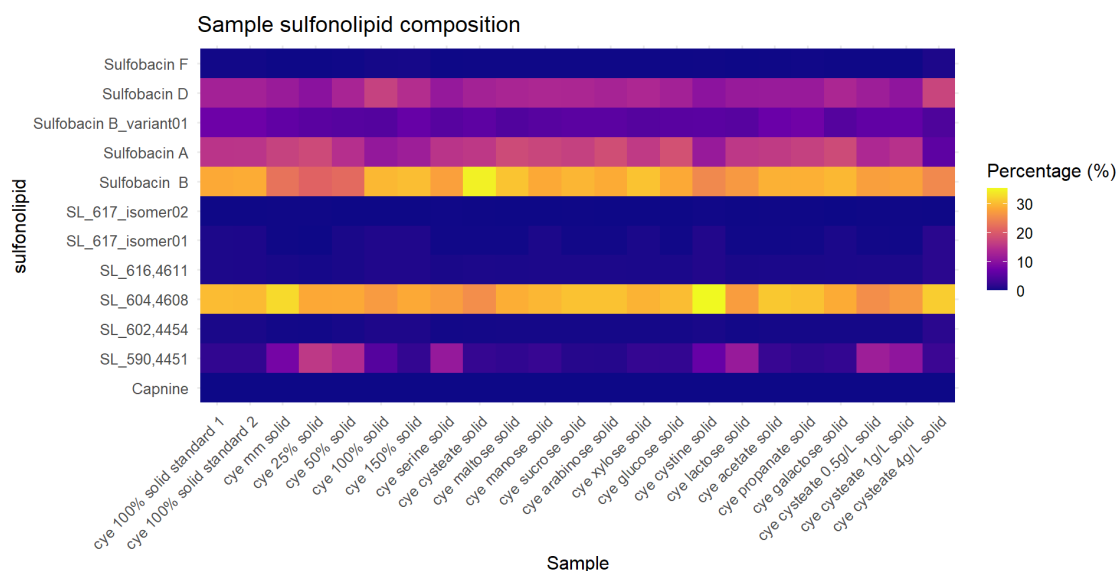


Figure A.1. Heat map over solid Fjoh samples and their Sols content. The Y axis shows the different Sol's, and the X axis shows the sample. The color varies from deep purple, indicating that the Sol accounts for a small fraction of the total Sol's presence, to yellow, indicating that it accounts for a large fraction. All samples show a similar profile dominated by SL 604.4808 and sulfobacin A and B, with lesser extents of sulfobacin D.

The profile for solid Fjoh samples shows a similar profile for all samples, indicating that concentrations and add-ons do not alter the general profile of Sols. However, there is some variability between the samples for SL 590.4451. This is clear with some samples, such as CYE 25% solid having above 10% of its profile be SL 590.4451, while its near 0% for CYE sucrose solid.

Total area normalized shows the presence of Second Sols at varying amounts in all samples except capnine and SL 686.4645, which is not seen in all samples. As the profile indicates, there is a difference between the amounts of ions found for each lipid, with the dominant lipids from the profile having orders of magnitude greater amounts than less expressed Sols.



Figure A.2. normalized total area of Sol's for solid Fjoh samples, with each graph representing a singular Sol.

CYE standard and CYE 100% are for most Sols; the samples with the highest amounts of CYE arabinose also show high amounts in lipids such as sulfobacin B and sulfobacin A. CYE serine varies in having a middling amount of sulfobacin A and B, while having some of the highest amounts of SL 686.4645 and SL 658.4325, showing that serine might upregulate these Sols.

Dilution was made to have a normalized amount of lipids per sample.

Table A.14. Second MS batch samples and their pellet lipid weights in mg and their dilution so that all samples have the same concentration of lipids

Smaple name	Lipid weight	Weight in µg/mL	1.5 mL dilution µL needed
100% CYE 1	25.7	25700	2.918288
100% CYE 2	34.3	34300	2.186589
100% CYE 3	28.8	28800	2.604167
150%CYE 1	33.1	33100	2.265861
150%CYE 2	29.7	29700	2.525253
150%CYE 3	26.9	26900	2.788104
100% CYE cys 0.5 g/L 1	26.9	26900	2.788104
100% CYE cys 0.5 g/L 2	32.6	32600	2.300613
100% CYE cys 0.5 g/L 3	23.6	23600	3.177966
150% 0.5 g/L mix 1	29.8	29800	2.516779
150% 0.5 g/L mix 2	37.3	37300	2.010724
150% 0.5 g/L mix 3	31.1	31100	2.411576
150% 0.25g/L mix	36.4	36400	2.06044
150% 15mM mix	35	35000	2.142857

The last batch of Fjoh samples to optimize the production of Sols. The weight of the Fjoh after freeze drying.

Table A.15. Third batch samples and their pellet weights in mg

Sample name	Pellet in mg
2419 100% CYE 1	3.8
2419 100% CYE 2	1.8
2419 100% CYE 3	1.7
2419 100% CYE 0.5g/L cys 1	2
2419 100% CYE 0.5g/L cys 2	1.1
2419 100% CYE 0.5g/L cys 3	2.1
2419 150% CYE 1	4.7
2419 150% CYE 2	4.9
2419 150% CYE 3	4.1
2419 150% CYE mix 0.5g/L 1	3.3
2419 150% CYE mix 0.5g/L 2	3.1
2419 150% CYE mix 0.5g/L 3	2.9
2419 100% CYE 15mm ace 1	4.7
2419 100% CYE 15mm ace 2	2.8
2419 100% CYE 15mm ace 3	2.3
2421 100% CYE 1	2.2
2421 100% CYE 2	3.2
2421 100% CYE 3	4.5
2421 100% CYE 0.5g/L cys 1	1.5
2421 100% CYE 0.5g/L cys 2	1.8
2421 100% CYE 0.5g/L cys 3	2.2
2421 150% CYE 1	3.6
2421 150% CYE 2	3.4
2421 150% CYE 3	2.8
2421 150% CYE mix 0.5g/L 1	7.2
2421 150% CYE mix 0.5g/L 2	2.7
2421 150% CYE mix 0.5g/L 3	2.3
2421 100% CYE 15mm ace 1	3.1
2421 100% CYE 15mm ace 2	3.2
2421 100% CYE 15mm ace 3	2.5

A.5 Alistipes

Alistipes samples were tested to determine if Fjoh was a good model organism for Sol production.

Table A.16. Alistipes samples and their pellet weights, which show whether it was possible to normalize their weights

Sampel name	Pellet lipids weights mg	x not normalised
BHI tim		
BHI standard tim 1	-1.7	x
BHI standard tim 2	5.7	x
BHI standard tim 3	0.1	
BHI tim 24 hours	1.3	x
BHI cysteate 0.25g/L tim	26.2	x
BHI cysteate 0.5g/L tim	0.6	
BHI cysteate 1g/L tim	0.2	
BHI cysteate 2g/L tim	0.6	x
BHI cysteate 4g/L tim	0.4	x
BHI put		
BHI micheal put	-0.1	x
vl6 ALI put	0.8	
ALI put	0.7	
ALI put 0.25g/L cysteate	29.4	x
ALI put 0.5g/L cysteate	0.9	
ALI put 1g/L cysteate	-3.9	x
ALI put 2g/L cysteate	1.4	x
ALI put 4g/L cysteate	1.7	

Alistipes profiles heatmap over the most major types of Sols.

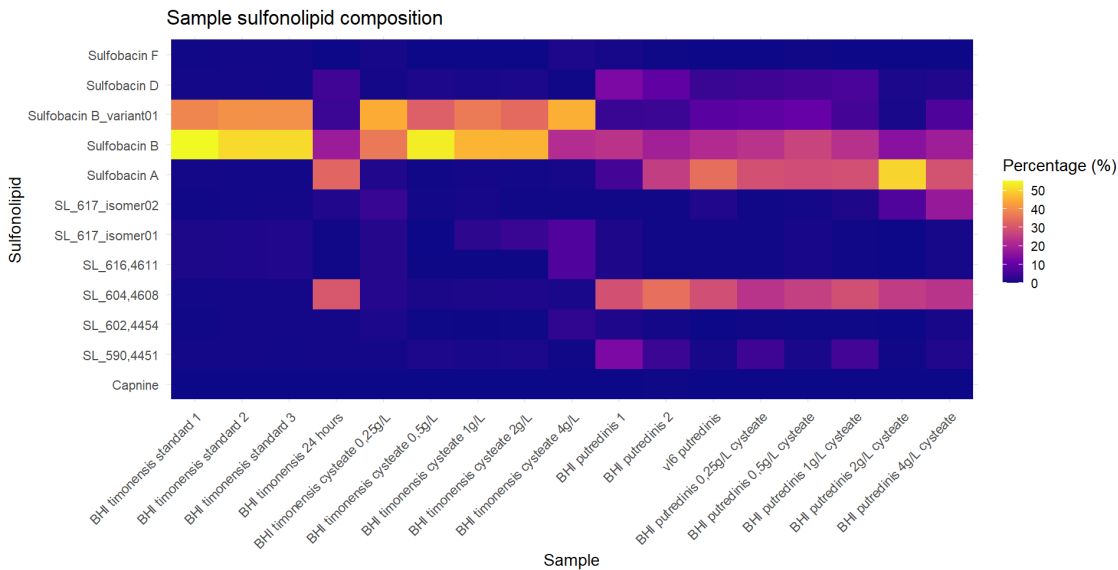


Figure A.3. Heat map over Sol profile of liquid *Alistipes timonensis* and *Alistipes putredinis* samples, where 12 different SoL were found.

A.6 plasmids

Primers that was used to amplify the genes that was inserted into the plasmids.

Table A.17. Primers used for the copying of Fjoh 2419 and Fjoh 2421

Primers	Nucleotide code
Fjoh 2419 upstream	GCGTCTAGAAAGCCAGAGTTAAATCTTG
Fjoh 2419 downstream	GCGGTCGACATAAAACACTTCATTTATTCTACTTTAC
Fjoh 2421 upstream	GGCGGTCTAGAAATATAGTACATTTGAATTGCG
Fjoh 2421 downstream	GCGCGTCTGACTTTATATTATAACAAAGTGTTTAGTG

plasmids that where made and inserted into Fjoh.

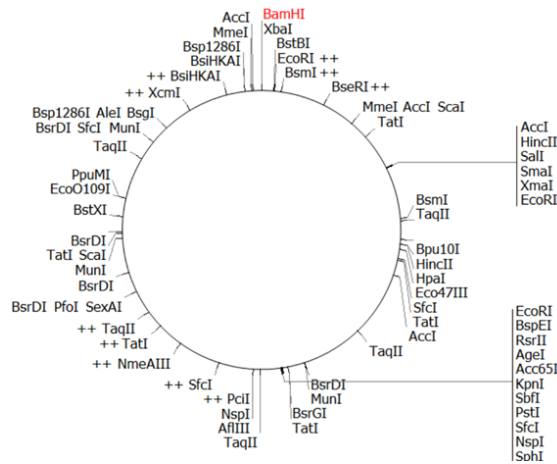


Figure A.4. Overview of the plasmid 2419+pcp11 plasmid that is inserted into the 2419 Fjoh

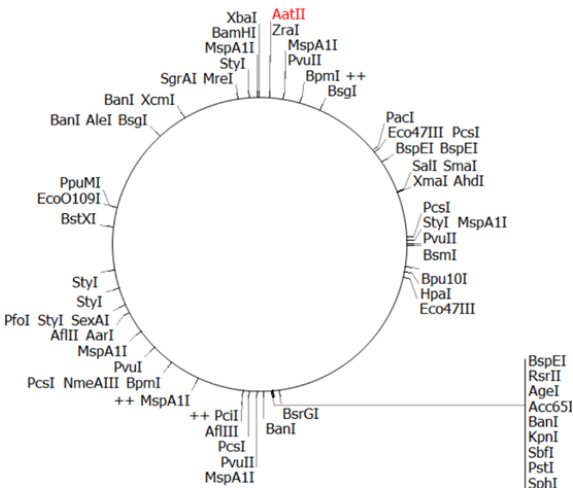


Figure A.5. Overview of the plasmid 2421+pcp11 plasmid that is inserted into the 2421 Fjoh

SDS page with the digested plasmids showing a different digestion for the plasmids than the pcp11 vector, showing an insertion of the plasmids into Fjoh

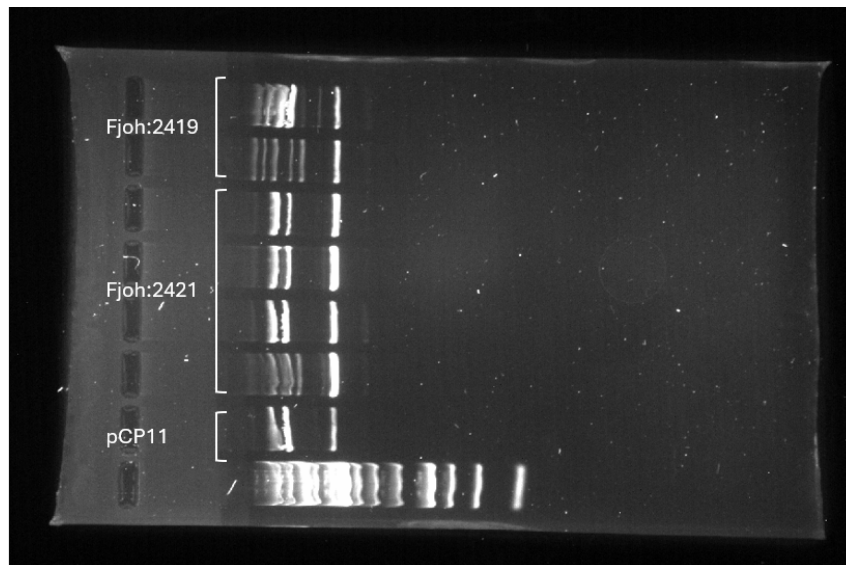


Figure A.6. SDS gel over digested pCP11, 2419+pCP11 plasmid and 2421+pCP11 plasmid. with a ladder in the first well, a digested pCP11 in well two that is digested into three parts, from well 3 to 6 are 2421+pCP11 plasmid digested that shows that well 3 was digested and the rest was just a pCP11 vector, in 7 and 8 was 2419+pCP11 plasmid where both wells shows a digested 2419+pCP11 plasmid

A.7 Stool test

A donor was used for a preliminary stool test to see if the Sols would have a measurable interaction with the microbiome.

Table A.18. The individual characteristics of the stool deliverer for the preliminary stool experiment.

Questions	Individual 1
Age	23
Height	180
Weight	76 kg
Gender	Male
Smoker	No
Diet	Vegetarian
Meat, egg, dairy	Several times per week
Exercise/ perfrom sport	Serveral times per week
Antibiotics	No
Average stool frequency	Once per day
Constipation or diarrhea	No
Digestive disease	No
Digestive disease in the past	No
Medication	No
Medication in the last 14 days	No
Medical test with contrast material	No

Stool donors for a more comprehensive stool experiment that included the mixed stool from these donors.

Table A.19. The individuals characteristics of the stool deliverer for the stool experiments.

Questions	Individual 1	Individual 2	Individual 3	Individual 4	Individual 5
Age	23	28	22	28	24
Height	180	164	173	190	172
Weight	75	56	64	85	66
Gender	Male	Female	Male	Male	Female
Smoker	No	No	No	Ex smoker stopped 8 years ago	No
Diet	Vegetarian	Omnivore	Omnivore	Omnivore	Vegetarian
Meat, egg, dairy	Several times a week	Every day	Several times a week	Several times a week	Once per week
Excercise/ Perform sport	Several times a week	Several times a week	Once per week	Every day	Several times a week
Antibiotics	No	No	No	No	No
Average stool frequency	Once per day	Once every 2 days	Once per day	Twice per day	Once per day
Constipation or diarrhea	No	No	No	No	No
Digestive disease	No	No	No	No	No
Digestive disease in the past	No	No	No	No	No
Medication	No	No	No	No	No
Medication in the past 14 days	No	Ibuprofen	No	paracetamol	No
Medical test with contrast material	No	No	No	No	No

concentration of lipids in the fecal slurry and the amount of fecal slurry.

Table A.20. Stool experiment and the precise content of the fecal slurry.

sample	lipid content	mL fecal slurry	concentration
standard 1	0	4	0
standard 2	0	4	0
standard 3	0	4	0
Sol 5 mg/mL 1	22.2	4.2	5.285714
Sol 5 mg/mL 2	21.8	4.2	5.190476
Sol 5 mg/mL 3	22.7	4.2	5.404762
Sol 1.5 mg/mL 1	6.2	4.1	1.512195
Sol 1.5 mg/mL 2	6	4	1.5
Sol 1.5 mg/mL 3	6.4	4.2	1.52381
Δ 2419 5 mg/mL 1	16.3	3.3	4.939394
Δ 2419 5 mg/mL 2	22.8	4.2	5.428571
Δ 2419 5 mg/mL 3	18.2	3.6	5.055556
Δ 2419 1.5 mg/mL 1	4.4	3	1.466667
Δ 2419 1.5 mg/mL 2	4.3	3	1.433333
Δ 2419 1.5 mg/mL 3	4.3	3	1.433333
Δ 2421 5 mg/mL 1	13	3	4.333333
Δ 2421 5 mg/mL 2	13.4	3	4.466667
Δ 2421 5 mg/mL 3	12.3	3	4.1
Δ 2421 1.5 mg/mL 1	5.4	3.5	1.542857
Δ 2421 1.5 mg/mL 2	5.3	3.5	1.514286
Δ 2421 1.5 mg/mL 3	4.7	3.1	1.516129

Table A.21. H₂S calibration curve.

H ₂ S concentration	0 micro molar	50 micro molar	100 micro molar	250 micro molar	500 micro molar	1000 micro molar	1500 micro molar	H ₂ S calibration
Absorbance	0.1141	0.1438	0.2206	0.3499	0.5697	0.9588	1.2495	1287.4*x-177.56

Table A.22. Comparison between sample groups to determine P value and if the differences between the groups are significant by the use of a T-test. H₂S at 24 hours

compound	group 1	group 2	n1	n2	p	p.sig
24 hours	control	Fjoh Δ2419 5mg/mL	3	3	6.25e-04	***
24 hours	control	Fjoh Δ2421 1.5mg/mL	3	3	3.51e-04	***
24 hours	Fjoh Δ2421 1.5mg/mL	Fjoh 5mg/mL	3	3	1.63e-03	**
24 hours	Fjoh Δ2419 5mg/mL	Fjoh 5mg/mL	3	3	2.96e-03	**
24 hours	control	Fjoh Δ2421 5mg/mL	3	3	6.04e-03	**
24 hours	Fjoh Δ2421 1.5mg/mL	Fjoh 1.5mg/mL	3	3	1.09e-02	*
24 hours	Fjoh Δ2419 5mg/mL	Fjoh 1.5mg/mL	3	3	1.98e-02	*
24 hours	control	Fjoh Δ2419 1.5mg/mL	3	3	3.23e-02	*
24 hours	Fjoh Δ2421 5mg/mL	Fjoh 5mg/mL	3	3	2.88e-02	*
24 hours	Fjoh Δ2419 1.5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	3.67e-02	*
24 hours	Fjoh Δ2419 5mg/mL	Fjoh Δ2419 1.5mg/mL	3	3	6.44e-02	ns
24 hours	control	Fjoh 1.5mg/mL	3	3	1.02e-01	ns
24 hours	Fjoh Δ2419 1.5mg/mL	Fjoh 5mg/mL	3	3	1.36e-01	ns
24 hours	Fjoh Δ2421 5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	1.68e-01	ns
24 hours	Fjoh Δ2421 5mg/mL	Fjoh 1.5mg/mL	3	3	1.61e-01	ns
24 hours	Fjoh Δ2419 5mg/mL	Fjoh Δ2421 5mg/mL	3	3	2.68e-01	ns
24 hours	Fjoh 5mg/mL	Fjoh 1.5mg/mL	3	3	3.54e-01	ns
24 hours	Fjoh Δ2419 1.5mg/mL	Fjoh Δ2421 5mg/mL	3	3	4.07e-01	ns
24 hours	control	Fjoh 5mg/mL	3	3	4.41e-01	ns
24 hours	Fjoh Δ2419 1.5mg/mL	Fjoh 1.5mg/mL	3	3	5.43e-01	ns
24 hours	Fjoh Δ2419 5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	7.67e-01	ns

Table A.23. Comparison between sample groups to determine P value and if the differences between the groups are significant by the use of a T-test. H₂S at 48 hours

compound	group 1	group 2	n1	n2	p	p.sig
48 hours	control	Fjoh Δ2419 5mg/mL	3	3	3.29e-12	****
48 hours	Fjoh Δ2419 5mg/mL	Fjoh 5mg/mL	3	3	1.45e-12	****
48 hours	Fjoh Δ2419 5mg/mL	Fjoh 1.5mg/mL	3	3	3.78e-12	****
48 hours	Fjoh Δ2419 5mg/mL	Fjoh Δ2419 1.5mg/mL	3	3	1.30e-11	****
48 hours	Fjoh Δ2419 5mg/mL	Fjoh Δ2421 5mg/mL	3	3	2.11e-11	****
48 hours	Fjoh Δ2419 5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	4.70e-10	****
48 hours	Fjoh Δ2421 1.5mg/mL	Fjoh 5mg/mL	3	3	1.22e-06	****
48 hours	control	Fjoh Δ2421 1.5mg/mL	3	3	9.64e-06	****
48 hours	Fjoh Δ2421 1.5mg/mL	Fjoh 1.5mg/mL	3	3	1.38e-05	****
48 hours	Fjoh Δ2419 1.5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	3.89e-04	***
48 hours	Fjoh Δ2421 5mg/mL	Fjoh 5mg/mL	3	3	9.99e-04	***
48 hours	Fjoh Δ2421 5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	1.49e-03	**
48 hours	Fjoh Δ2419 1.5mg/mL	Fjoh 5mg/mL	3	3	3.91e-03	**
48 hours	control	Fjoh Δ2421 5mg/mL	3	3	1.44e-02	*
48 hours	Fjoh Δ2421 5mg/mL	Fjoh 1.5mg/mL	3	3	2.22e-02	*
48 hours	control	Fjoh Δ2419 1.5mg/mL	3	3	5.45e-02	ns
48 hours	Fjoh Δ2419 1.5mg/mL	Fjoh 1.5mg/mL	3	3	8.12e-02	ns
48 hours	Fjoh 5mg/mL	Fjoh 1.5mg/mL	3	3	1.39e-01	ns
48 hours	control	Fjoh 5mg/mL	3	3	1.98e-01	ns
48 hours	Fjoh Δ2419 1.5mg/mL	Fjoh Δ2421 5mg/mL	3	3	5.00e-01	ns
48 hours	control	Fjoh 1.5mg/mL	3	3	8.29e-01	ns

Table A.24. Comparison between sample groups to determine P value and if the differences between the groups are significant by the use of a T-test. H₂S at 72 hours

compound	group 1	group 2	n1	n2	p	p.sig
72 hours	Fjoh Δ2419 5mg/mL	Fjoh 5mg/mL	3	3	4.99e-07	****
72 hours	control	Fjoh Δ2419 5mg/mL	3	3	1.61e-06	****
72 hours	Fjoh Δ2419 5mg/mL	Fjoh 1.5mg/mL	3	3	3.37e-06	****
72 hours	Fjoh Δ2419 5mg/mL	Fjoh Δ2419 1.5mg/mL	3	3	4.70e-06	****
72 hours	Fjoh Δ2419 5mg/mL	Fjoh Δ2421 5mg/mL	3	3	5.71e-05	****
72 hours	Fjoh Δ2419 5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	6.84e-05	****
72 hours	Fjoh Δ2421 1.5mg/mL	Fjoh 5mg/mL	3	3	7.24e-03	**
72 hours	Fjoh Δ2421 5mg/mL	Fjoh 5mg/mL	3	3	8.88e-03	**
72 hours	control	Fjoh Δ2421 1.5mg/mL	3	3	3.65e-02	*
72 hours	control	Fjoh Δ2421 5mg/mL	3	3	4.43e-02	*
72 hours	Fjoh Δ2421 1.5mg/mL	Fjoh 1.5mg/mL	3	3	8.99e-02	ns
72 hours	Fjoh Δ2421 5mg/mL	Fjoh 1.5mg/mL	3	3	1.08e-01	ns
72 hours	Fjoh Δ2419 1.5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	1.31e-01	ns
72 hours	Fjoh Δ2419 1.5mg/mL	Fjoh Δ2421 5mg/mL	3	3	1.55e-01	ns
72 hours	Fjoh Δ2419 1.5mg/mL	Fjoh 5mg/mL	3	3	1.47e-01	ns
72 hours	Fjoh 5mg/mL	Fjoh 1.5mg/mL	3	3	2.09e-01	ns
72 hours	control	Fjoh 5mg/mL	3	3	4.22e-01	ns
72 hours	control	Fjoh Δ2419 1.5mg/mL	3	3	4.92e-01	ns
72 hours	control	Fjoh 1.5mg/mL	3	3	6.31e-01	ns
72 hours	Fjoh Δ2419 1.5mg/mL	Fjoh 1.5mg/mL	3	3	8.32e-01	ns
72 hours	Fjoh Δ2421 5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	9.20e-01	ns

Table A.25. SCFA calibration curve for formate, acetate, lactate, propionate, butyrate, and valerate.

SCFA	50 Micro molar	100 Micro molar	250 Micro molar	500 Micro molar	1000 Micro molar	CE calibration
formate	3.3	5.97	3.511	22.08	44	$21.78 \cdot x - 38.388$
acetate	3.2	no data	8.61	17.61	33.33	$31.114 \cdot x - 38.105$
lactate	3.4	5.25	7.46	24.1645	53.7	$21.485 \cdot x - 55.531$
propionate	4.9	12.06	12.48	25.77	57.3005	$18.288 \cdot x + 33.392$
butyrate	4.9	12.12	10.1237	22.4452	47.0899	$18.383 \cdot x - 33.645$
valerate	3.6	6.581	14.3396	31.2317	45.625	$22.717 \cdot x - 59.241$

Table A.26. Comparison between sample groups to determine P value and if the differences between the groups are significant by the use of a T-test. For acetate samples at 24 and 48 hours

Time	Compund	Group 1	Group 2	n1	n2	p.val	p.sig
24 hours	acetate	Fjoh Δ2421 5mg/mL	Fjoh 5mg/mL	3	3	1.75e-03	**
24 hours	acetate	Fjoh 5mg/mL	Fjoh Δ2419 1.5mg/mL	3	3	3.05e-03	**
24 hours	acetate	Fjoh 5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	2.89e-03	**
24 hours	acetate	Fjoh 5mg/mL	Fjoh 1.5mg/mL	3	3	1.61e-03	**
24 hours	acetate	control	Fjoh 5mg/mL	3	3	7.77e-03	**
24 hours	acetate	Fjoh Δ2419 5mg/mL	Fjoh 5mg/mL	3	3	1.58e-02	*
24 hours	acetate	Fjoh Δ2419 5mg/mL	Fjoh Δ2421 5mg/mL	3	3	2.86e-01	ns
24 hours	acetate	Fjoh Δ2419 5mg/mL	Fjoh 1.5mg/mL	3	3	2.68e-01	ns
24 hours	acetate	control	Fjoh Δ2421 5mg/mL	3	3	4.66e-01	ns
24 hours	acetate	Fjoh Δ2419 5mg/mL	Fjoh Δ2419 1.5mg/mL	3	3	4.21e-01	ns
24 hours	acetate	Fjoh Δ2419 5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	4.06e-01	ns
24 hours	acetate	control	Fjoh 1.5mg/mL	3	3	4.41e-01	ns
24 hours	acetate	control	Fjoh Δ2419 5mg/mL	3	3	7.25e-01	ns
24 hours	acetate	control	Fjoh Δ2419 1.5mg/mL	3	3	6.46e-01	ns
24 hours	acetate	Fjoh Δ2421 5mg/mL	Fjoh Δ2419 1.5mg/mL	3	3	7.83e-01	ns
24 hours	acetate	control	Fjoh Δ2421 1.5mg/mL	3	3	6.27e-01	ns
24 hours	acetate	Fjoh Δ2421 5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	8.04e-01	ns
24 hours	acetate	Fjoh Δ2419 1.5mg/mL	Fjoh 1.5mg/mL	3	3	7.51e-01	ns
24 hours	acetate	Fjoh Δ2421 1.5mg/mL	Fjoh 1.5mg/mL	3	3	7.71e-01	ns
24 hours	acetate	Fjoh Δ2419 1.5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	9.79e-01	ns
48 hours	acetate	Fjoh Δ2421 5mg/mL	Fjoh 5mg/mL	3	3	1.75e-03	**
48 hours	acetate	Fjoh 5mg/mL	Fjoh Δ2419 1.5mg/mL	3	3	3.05e-03	**
48 hours	acetate	Fjoh 5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	2.89e-03	**
48 hours	acetate	Fjoh 5mg/mL	Fjoh 1.5mg/mL	3	3	1.61e-03	**
48 hours	acetate	control	Fjoh 5mg/mL	3	3	7.77e-03	**
48 hours	acetate	Fjoh Δ2419 5mg/mL	Fjoh 5mg/mL	3	3	1.58e-02	*
48 hours	acetate	Fjoh Δ2419 5mg/mL	Fjoh Δ2421 5mg/mL	3	3	2.86e-01	ns
48 hours	acetate	Fjoh Δ2419 5mg/mL	Fjoh 1.5mg/mL	3	3	2.68e-01	ns
48 hours	acetate	control	Fjoh Δ2421 5mg/mL	3	3	4.66e-01	ns
48 hours	acetate	Fjoh Δ2419 5mg/mL	Fjoh Δ2419 1.5mg/mL	3	3	4.21e-01	ns
48 hours	acetate	Fjoh Δ2419 5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	4.06e-01	ns
48 hours	acetate	control	Fjoh 1.5mg/mL	3	3	4.41e-01	ns
48 hours	acetate	control	Fjoh Δ2419 5mg/mL	3	3	7.25e-01	ns
48 hours	acetate	control	Fjoh Δ2419 1.5mg/mL	3	3	6.46e-01	ns
48 hours	acetate	Fjoh Δ2421 5mg/mL	Fjoh Δ2419 1.5mg/mL	3	3	7.83e-01	ns
48 hours	acetate	control	Fjoh Δ2421 1.5mg/mL	3	3	6.27e-01	ns
48 hours	acetate	Fjoh Δ2421 5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	8.04e-01	ns
48 hours	acetate	Fjoh Δ2419 1.5mg/mL	Fjoh 1.5mg/mL	3	3	7.51e-01	ns
48 hours	acetate	Fjoh Δ2421 1.5mg/mL	Fjoh 1.5mg/mL	3	3	7.71e-01	ns
48 hours	acetate	Fjoh Δ2419 1.5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	9.79e-01	ns
48 hours	acetate	Fjoh Δ2421 5mg/mL	Fjoh 1.5mg/mL	3	3	9.66e-01	ns

Table A.27. Comparison between sample groups to determine P value and if the differences between the groups are significant by the use of a T-test. For acetate samples at 72 hours

Time	Compound	Group 1	Group 2	n1	n2	p.val	p.sig
72 hours	acetate	Fjoh Δ 2421 5mg/mL	Fjoh 5mg/mL	3	3	1.75e-03	**
72 hours	acetate	Fjoh 5mg/mL	Fjoh Δ 2419 1.5mg/mL	3	3	3.05e-03	**
72 hours	acetate	Fjoh 5mg/mL	Fjoh Δ 2421 1.5mg/mL	3	3	2.89e-03	**
72 hours	acetate	Fjoh 5mg/mL	Fjoh 1.5mg/mL	3	3	1.61e-03	**
72 hours	acetate	control	Fjoh 5mg/mL	3	3	7.77e-03	**
72 hours	acetate	Fjoh Δ 2419 5mg/mL	Fjoh 5mg/mL	3	3	1.58e-02	*
72 hours	acetate	Fjoh Δ 2419 5mg/mL	Fjoh Δ 2421 5mg/mL	3	3	2.86e-01	ns
72 hours	acetate	Fjoh Δ 2419 5mg/mL	Fjoh 1.5mg/mL	3	3	2.68e-01	ns
72 hours	acetate	control	Fjoh Δ 2421 5mg/mL	3	3	4.66e-01	ns
72 hours	acetate	Fjoh Δ 2419 5mg/mL	Fjoh Δ 2419 1.5mg/mL	3	3	4.21e-01	ns
72 hours	acetate	Fjoh Δ 2419 5mg/mL	Fjoh Δ 2421 1.5mg/mL	3	3	4.06e-01	ns
72 hours	acetate	control	Fjoh 1.5mg/mL	3	3	4.41e-01	ns
72 hours	acetate	control	Fjoh Δ 2419 5mg/mL	3	3	7.25e-01	ns
72 hours	acetate	control	Fjoh Δ 2419 1.5mg/mL	3	3	6.46e-01	ns
72 hours	acetate	Fjoh Δ 2421 5mg/mL	Fjoh Δ 2419 1.5mg/mL	3	3	7.83e-01	ns
72 hours	acetate	control	Fjoh Δ 2421 1.5mg/mL	3	3	6.27e-01	ns
72 hours	acetate	Fjoh Δ 2421 5mg/mL	Fjoh Δ 2421 1.5mg/mL	3	3	8.04e-01	ns
72 hours	acetate	Fjoh Δ 2419 1.5mg/mL	Fjoh 1.5mg/mL	3	3	7.51e-01	ns
72 hours	acetate	Fjoh Δ 2421 1.5mg/mL	Fjoh 1.5mg/mL	3	3	7.71e-01	ns
72 hours	acetate	Fjoh Δ 2419 1.5mg/mL	Fjoh Δ 2421 1.5mg/mL	3	3	9.79e-01	ns
72 hours	acetate	Fjoh Δ 2421 5mg/mL	Fjoh 1.5mg/mL	3	3	9.66e-01	ns

Table A.28. Comparison between sample groups to determine P value and if the differences between the groups are significant by the use of a T-test. For butyrate samples at 24 and 48 hours

Time	Compound	Group 1	Group 2	n1	n2	p.val	p.sig
24 hours	butyrate	control	Fjoh Δ2421 5mg/mL	3	3	3.99e-02	*
24 hours	butyrate	control	Fjoh 5mg/mL	3	3	1.47e-02	*
24 hours	butyrate	Fjoh Δ2419 5mg/mL	Fjoh 5mg/mL	3	3	7.22e-02	ns
24 hours	butyrate	Fjoh Δ2421 5mg/mL	Fjoh Δ2419 1.5mg/mL	3	3	7.43e-02	ns
24 hours	butyrate	Fjoh 5mg/mL	Fjoh Δ2419 1.5mg/mL	3	3	2.84e-02	*
24 hours	butyrate	Fjoh Δ2421 5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	7.61e-02	ns
24 hours	butyrate	Fjoh 5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	2.91e-02	*
24 hours	butyrate	Fjoh 5mg/mL	Fjoh 1.5mg/mL	3	3	5.83e-02	ns
24 hours	butyrate	Fjoh Δ2421 5mg/mL	Fjoh 1.5mg/mL	3	3	1.44e-01	ns
24 hours	butyrate	Fjoh Δ2419 5mg/mL	Fjoh Δ2421 5mg/mL	3	3	1.75e-01	ns
24 hours	butyrate	control	Fjoh Δ2419 5mg/mL	3	3	4.17e-01	ns
24 hours	butyrate	Fjoh Δ2421 5mg/mL	Fjoh 5mg/mL	3	3	6.14e-01	ns
24 hours	butyrate	control	Fjoh Δ2419 1.5mg/mL	3	3	7.41e-01	ns
24 hours	butyrate	Fjoh Δ2419 5mg/mL	Fjoh Δ2419 1.5mg/mL	3	3	6.25e-01	ns
24 hours	butyrate	control	Fjoh Δ2421 1.5mg/mL	3	3	7.31e-01	ns
24 hours	butyrate	Fjoh Δ2419 5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	6.34e-01	ns
24 hours	butyrate	control	Fjoh 1.5mg/mL	3	3	4.84e-01	ns
24 hours	butyrate	Fjoh Δ2419 1.5mg/mL	Fjoh 1.5mg/mL	3	3	7.08e-01	ns
24 hours	butyrate	Fjoh Δ2421 1.5mg/mL	Fjoh 1.5mg/mL	3	3	7.18e-01	ns
24 hours	butyrate	Fjoh Δ2419 5mg/mL	Fjoh 1.5mg/mL	3	3	9.08e-01	ns
24 hours	butyrate	Fjoh Δ2419 1.5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	9.89e-01	ns
48 hours	butyrate	control	Fjoh Δ2421 5mg/mL	3	3	3.99e-02	*
48 hours	butyrate	control	Fjoh 5mg/mL	3	3	1.47e-02	*
48 hours	butyrate	Fjoh Δ2419 5mg/mL	Fjoh 5mg/mL	3	3	7.22e-02	ns
48 hours	butyrate	Fjoh Δ2421 5mg/mL	Fjoh Δ2419 1.5mg/mL	3	3	7.43e-02	ns
48 hours	butyrate	Fjoh 5mg/mL	Fjoh Δ2419 1.5mg/mL	3	3	2.84e-02	*
48 hours	butyrate	Fjoh Δ2421 5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	7.61e-02	ns
48 hours	butyrate	Fjoh 5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	2.91e-02	*
48 hours	butyrate	Fjoh 5mg/mL	Fjoh 1.5mg/mL	3	3	5.83e-02	ns
48 hours	butyrate	Fjoh Δ2421 5mg/mL	Fjoh 1.5mg/mL	3	3	1.44e-01	ns
48 hours	butyrate	Fjoh Δ2419 5mg/mL	Fjoh Δ2421 5mg/mL	3	3	1.75e-01	ns
48 hours	butyrate	control	Fjoh Δ2419 5mg/mL	3	3	4.17e-01	ns
48 hours	butyrate	Fjoh Δ2421 5mg/mL	Fjoh 5mg/mL	3	3	6.14e-01	ns
48 hours	butyrate	control	Fjoh Δ2419 1.5mg/mL	3	3	7.41e-01	ns
48 hours	butyrate	Fjoh Δ2419 5mg/mL	Fjoh Δ2419 1.5mg/mL	3	3	6.25e-01	ns
48 hours	butyrate	control	Fjoh Δ2421 1.5mg/mL	3	3	7.31e-01	ns
48 hours	butyrate	Fjoh Δ2419 5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	6.34e-01	ns
48 hours	butyrate	control	Fjoh 1.5mg/mL	3	3	4.84e-01	ns
48 hours	butyrate	Fjoh Δ2419 1.5mg/mL	Fjoh 1.5mg/mL	3	3	7.08e-01	ns
48 hours	butyrate	Fjoh Δ2421 1.5mg/mL	Fjoh 1.5mg/mL	3	3	7.18e-01	ns
48 hours	butyrate	Fjoh Δ2419 5mg/mL	Fjoh 1.5mg/mL	3	3	9.08e-01	ns
48 hours	butyrate	Fjoh Δ2419 1.5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	9.89e-01	ns

Table A.29. Comparison between sample groups to determine P value and if the differences between the groups are significant by the use of a T-test. For butyrate samples at 72 hours

Time	Compound	Group 1	Group 2	n1	n2	p.val	p.sig
72 hours	butyrate	control	Fjoh Δ 2421 5mg/mL	3	3	3.99e-02	*
72 hours	butyrate	control	Fjoh 5mg/mL	3	3	1.47e-02	*
72 hours	butyrate	Fjoh Δ 2419 5mg/mL	Fjoh 5mg/mL	3	3	7.22e-02	ns
72 hours	butyrate	Fjoh Δ 2421 5mg/mL	Fjoh Δ 2419 1.5mg/mL	3	3	7.43e-02	ns
72 hours	butyrate	Fjoh 5mg/mL	Fjoh Δ 2419 1.5mg/mL	3	3	2.84e-02	*
72 hours	butyrate	Fjoh Δ 2421 5mg/mL	Fjoh Δ 2421 1.5mg/mL	3	3	7.61e-02	ns
72 hours	butyrate	Fjoh 5mg/mL	Fjoh Δ 2421 1.5mg/mL	3	3	2.91e-02	*
72 hours	butyrate	Fjoh 5mg/mL	Fjoh 1.5mg/mL	3	3	5.83e-02	ns
72 hours	butyrate	Fjoh Δ 2421 5mg/mL	Fjoh 1.5mg/mL	3	3	1.44e-01	ns
72 hours	butyrate	Fjoh Δ 2419 5mg/mL	Fjoh Δ 2421 5mg/mL	3	3	1.75e-01	ns
72 hours	butyrate	control	Fjoh Δ 2419 5mg/mL	3	3	4.17e-01	ns
72 hours	butyrate	Fjoh Δ 2421 5mg/mL	Fjoh 5mg/mL	3	3	6.14e-01	ns
72 hours	butyrate	control	Fjoh Δ 2419 1.5mg/mL	3	3	7.41e-01	ns
72 hours	butyrate	Fjoh Δ 2419 5mg/mL	Fjoh Δ 2419 1.5mg/mL	3	3	6.25e-01	ns
72 hours	butyrate	control	Fjoh Δ 2421 1.5mg/mL	3	3	7.31e-01	ns
72 hours	butyrate	Fjoh Δ 2419 5mg/mL	Fjoh Δ 2421 1.5mg/mL	3	3	6.34e-01	ns
72 hours	butyrate	control	Fjoh 1.5mg/mL	3	3	4.84e-01	ns
72 hours	butyrate	Fjoh Δ 2419 1.5mg/mL	Fjoh 1.5mg/mL	3	3	7.08e-01	ns
72 hours	butyrate	Fjoh Δ 2421 1.5mg/mL	Fjoh 1.5mg/mL	3	3	7.18e-01	ns
72 hours	butyrate	Fjoh Δ 2419 5mg/mL	Fjoh 1.5mg/mL	3	3	9.08e-01	ns
72 hours	butyrate	Fjoh Δ 2419 1.5mg/mL	Fjoh Δ 2421 1.5mg/mL	3	3	9.89e-01	ns

Table A.30. Comparison between sample groups to determine P value and if the differences between the groups are significant by the use of a T-test. For formate samples at 24 and 48 hours

Time	Compund	Group 1	Group 2	n1	n2	p.val	p.sig
24 hours	formate	control	Fjoh 5mg/mL	3	3	1.44e-05	****
24 hours	formate	Fjoh Δ2421 5mg/mL	Fjoh 5mg/mL	3	3	3.04e-05	****
24 hours	formate	Fjoh 5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	2.55e-05	****
24 hours	formate	Fjoh 5mg/mL	Fjoh Δ2419 1.5mg/mL	3	3	6.92e-05	****
24 hours	formate	Fjoh Δ2419 5mg/mL	Fjoh 5mg/mL	3	3	1.22e-04	***
24 hours	formate	Fjoh 5mg/mL	Fjoh 1.5mg/mL	3	3	1.21e-04	***
24 hours	formate	control	Fjoh Δ2419 5mg/mL	3	3	2.39e-01	ns
24 hours	formate	control	Fjoh 1.5mg/mL	3	3	2.40e-01	ns
24 hours	formate	Fjoh Δ2419 5mg/mL	Fjoh Δ2421 5mg/mL	3	3	4.45e-01	ns
24 hours	formate	control	Fjoh Δ2419 1.5mg/mL	3	3	3.76e-01	ns
24 hours	formate	Fjoh Δ2419 5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	3.89e-01	ns
24 hours	formate	Fjoh Δ2421 5mg/mL	Fjoh 1.5mg/mL	3	3	4.46e-01	ns
24 hours	formate	Fjoh Δ2421 1.5mg/mL	Fjoh 1.5mg/mL	3	3	3.90e-01	ns
24 hours	formate	control	Fjoh Δ2421 5mg/mL	3	3	6.64e-01	ns
24 hours	formate	Fjoh Δ2419 5mg/mL	Fjoh Δ2419 1.5mg/mL	3	3	7.57e-01	ns
24 hours	formate	Fjoh Δ2421 5mg/mL	Fjoh Δ2419 1.5mg/mL	3	3	6.45e-01	ns
24 hours	formate	control	Fjoh Δ2421 1.5mg/mL	3	3	7.39e-01	ns
24 hours	formate	Fjoh Δ2419 1.5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	5.75e-01	ns
24 hours	formate	Fjoh Δ2419 1.5mg/mL	Fjoh 1.5mg/mL	3	3	7.59e-01	ns
24 hours	formate	Fjoh Δ2421 5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	9.19e-01	ns
24 hours	formate	Fjoh Δ2419 5mg/mL	Fjoh 1.5mg/mL	3	3	9.98e-01	ns
48 hours	formate	control	Fjoh 5mg/mL	3	3	1.44e-05	****
48 hours	formate	Fjoh Δ2421 5mg/mL	Fjoh 5mg/mL	3	3	3.04e-05	****
48 hours	formate	Fjoh 5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	2.55e-05	****
48 hours	formate	Fjoh 5mg/mL	Fjoh Δ2419 1.5mg/mL	3	3	6.92e-05	****
48 hours	formate	Fjoh Δ2419 5mg/mL	Fjoh 5mg/mL	3	3	1.22e-04	***
48 hours	formate	Fjoh 5mg/mL	Fjoh 1.5mg/mL	3	3	1.21e-04	***
48 hours	formate	control	Fjoh Δ2419 5mg/mL	3	3	2.39e-01	ns
48 hours	formate	control	Fjoh 1.5mg/mL	3	3	2.40e-01	ns
48 hours	formate	Fjoh Δ2419 5mg/mL	Fjoh Δ2421 5mg/mL	3	3	4.45e-01	ns
48 hours	formate	control	Fjoh Δ2419 1.5mg/mL	3	3	3.76e-01	ns
48 hours	formate	Fjoh Δ2419 5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	3.89e-01	ns
48 hours	formate	Fjoh Δ2421 5mg/mL	Fjoh 1.5mg/mL	3	3	4.46e-01	ns
48 hours	formate	Fjoh Δ2421 1.5mg/mL	Fjoh 1.5mg/mL	3	3	3.90e-01	ns
48 hours	formate	control	Fjoh Δ2421 5mg/mL	3	3	6.64e-01	ns
48 hours	formate	Fjoh Δ2419 5mg/mL	Fjoh Δ2419 1.5mg/mL	3	3	7.57e-01	ns
48 hours	formate	Fjoh Δ2421 5mg/mL	Fjoh Δ2419 1.5mg/mL	3	3	6.45e-01	ns
48 hours	formate	control	Fjoh Δ2421 1.5mg/mL	3	3	7.39e-01	ns
48 hours	formate	Fjoh Δ2419 1.5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	5.75e-01	ns
48 hours	formate	Fjoh Δ2419 1.5mg/mL	Fjoh 1.5mg/mL	3	3	7.59e-01	ns
48 hours	formate	Fjoh Δ2421 5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	9.19e-01	ns
48 hours	formate	Fjoh Δ2419 5mg/mL	Fjoh 1.5mg/mL	3	3	9.98e-01	ns

Table A.31. Comparison between sample groups to determine P value and if the differences between the groups are significant by the use of a T-test. For formate samples at 72 hours

Time	Compound	Group 1	Group 2	n1	n2	p.val	p.sig
72 hours	formate	control	Fjoh 5mg/mL	3	3	1.44e-05	****
72 hours	formate	Fjoh Δ 2421 5mg/mL	Fjoh 5mg/mL	3	3	3.04e-05	****
72 hours	formate	Fjoh 5mg/mL	Fjoh Δ 2421 1.5mg/mL	3	3	2.55e-05	****
72 hours	formate	Fjoh 5mg/mL	Fjoh Δ 2419 1.5mg/mL	3	3	6.92e-05	****
72 hours	formate	Fjoh Δ 2419 5mg/mL	Fjoh 5mg/mL	3	3	1.22e-04	***
72 hours	formate	Fjoh 5mg/mL	Fjoh 1.5mg/mL	3	3	1.21e-04	***
72 hours	formate	control	Fjoh Δ 2419 5mg/mL	3	3	2.39e-01	ns
72 hours	formate	control	Fjoh 1.5mg/mL	3	3	2.40e-01	ns
72 hours	formate	Fjoh Δ 2419 5mg/mL	Fjoh Δ 2421 5mg/mL	3	3	4.45e-01	ns
72 hours	formate	control	Fjoh Δ 2419 1.5mg/mL	3	3	3.76e-01	ns
72 hours	formate	Fjoh Δ 2419 5mg/mL	Fjoh Δ 2421 1.5mg/mL	3	3	3.89e-01	ns
72 hours	formate	Fjoh Δ 2421 5mg/mL	Fjoh 1.5mg/mL	3	3	4.46e-01	ns
72 hours	formate	Fjoh Δ 2421 1.5mg/mL	Fjoh 1.5mg/mL	3	3	3.90e-01	ns
72 hours	formate	control	Fjoh Δ 2421 5mg/mL	3	3	6.64e-01	ns
72 hours	formate	Fjoh Δ 2419 5mg/mL	Fjoh Δ 2419 1.5mg/mL	3	3	7.57e-01	ns
72 hours	formate	Fjoh Δ 2421 5mg/mL	Fjoh Δ 2419 1.5mg/mL	3	3	6.45e-01	ns
72 hours	formate	control	Fjoh Δ 2421 1.5mg/mL	3	3	7.39e-01	ns
72 hours	formate	Fjoh Δ 2419 1.5mg/mL	Fjoh Δ 2421 1.5mg/mL	3	3	5.75e-01	ns
72 hours	formate	Fjoh Δ 2419 1.5mg/mL	Fjoh 1.5mg/mL	3	3	7.59e-01	ns
72 hours	formate	Fjoh Δ 2421 5mg/mL	Fjoh Δ 2421 1.5mg/mL	3	3	9.19e-01	ns
72 hours	formate	Fjoh Δ 2419 5mg/mL	Fjoh 1.5mg/mL	3	3	9.98e-01	ns

Table A.32. Comparison between sample groups to determine P value and if the differences between the groups are significant by the use of a T-test. For lactate samples at 24 and 48 hours

Time	Compund	Group 1	Group 2	n1	n2	p.val	p.sig
24 hours	lactate	control	Fjoh Δ2419 5mg/mL	3	3	6.51e-03	**
24 hours	lactate	Fjoh Δ2419 5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	4.29e-03	**
24 hours	lactate	Fjoh Δ2419 5mg/mL	Fjoh 1.5mg/mL	3	3	6.07e-03	**
24 hours	lactate	Fjoh Δ2419 5mg/mL	Fjoh Δ2421 5mg/mL	3	3	2.61e-02	*
24 hours	lactate	Fjoh Δ2419 5mg/mL	Fjoh Δ2419 1.5mg/mL	3	3	2.40e-02	*
24 hours	lactate	Fjoh Δ2419 5mg/mL	Fjoh 5mg/mL	3	3	3.34e-02	*
24 hours	lactate	control	Fjoh Δ2421 5mg/mL	3	3	4.92e-01	ns
24 hours	lactate	control	Fjoh 5mg/mL	3	3	4.18e-01	ns
24 hours	lactate	control	Fjoh Δ2419 1.5mg/mL	3	3	5.19e-01	ns
24 hours	lactate	Fjoh Δ2421 5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	3.75e-01	ns
24 hours	lactate	Fjoh 5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	3.14e-01	ns
24 hours	lactate	Fjoh Δ2419 1.5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	3.98e-01	ns
24 hours	lactate	Fjoh Δ2421 5mg/mL	Fjoh 1.5mg/mL	3	3	4.71e-01	ns
24 hours	lactate	Fjoh 5mg/mL	Fjoh 1.5mg/mL	3	3	3.99e-01	ns
24 hours	lactate	Fjoh Δ2419 1.5mg/mL	Fjoh 1.5mg/mL	3	3	4.97e-01	ns
24 hours	lactate	Fjoh Δ2421 5mg/mL	Fjoh 5mg/mL	3	3	9.00e-01	ns
24 hours	lactate	Fjoh Δ2421 5mg/mL	Fjoh Δ2419 1.5mg/mL	3	3	9.66e-01	ns
24 hours	lactate	Fjoh 5mg/mL	Fjoh Δ2419 1.5mg/mL	3	3	8.66e-01	ns
24 hours	lactate	control	Fjoh Δ2421 1.5mg/mL	3	3	8.37e-01	ns
24 hours	lactate	control	Fjoh 1.5mg/mL	3	3	9.72e-01	ns
24 hours	lactate	Fjoh Δ2421 1.5mg/mL	Fjoh 1.5mg/mL	3	3	8.64e-01	ns
48 hours	lactate	control	Fjoh Δ2419 5mg/mL	3	3	6.51e-03	**
48 hours	lactate	Fjoh Δ2419 5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	4.29e-03	**
48 hours	lactate	Fjoh Δ2419 5mg/mL	Fjoh 1.5mg/mL	3	3	6.07e-03	**
48 hours	lactate	Fjoh Δ2419 5mg/mL	Fjoh Δ2421 5mg/mL	3	3	2.61e-02	*
48 hours	lactate	Fjoh Δ2419 5mg/mL	Fjoh Δ2419 1.5mg/mL	3	3	2.40e-02	*
48 hours	lactate	Fjoh Δ2419 5mg/mL	Fjoh 5mg/mL	3	3	3.34e-02	*
48 hours	lactate	control	Fjoh Δ2421 5mg/mL	3	3	4.92e-01	ns
48 hours	lactate	control	Fjoh 5mg/mL	3	3	4.18e-01	ns
48 hours	lactate	control	Fjoh Δ2419 1.5mg/mL	3	3	5.19e-01	ns
48 hours	lactate	Fjoh Δ2421 5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	3.75e-01	ns
48 hours	lactate	Fjoh 5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	3.14e-01	ns
48 hours	lactate	Fjoh Δ2419 1.5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	3.98e-01	ns
48 hours	lactate	Fjoh Δ2421 5mg/mL	Fjoh 1.5mg/mL	3	3	4.71e-01	ns
48 hours	lactate	Fjoh 5mg/mL	Fjoh 1.5mg/mL	3	3	3.99e-01	ns
48 hours	lactate	Fjoh Δ2419 1.5mg/mL	Fjoh 1.5mg/mL	3	3	4.97e-01	ns
48 hours	lactate	Fjoh Δ2421 5mg/mL	Fjoh 5mg/mL	3	3	9.00e-01	ns
48 hours	lactate	Fjoh Δ2421 5mg/mL	Fjoh Δ2419 1.5mg/mL	3	3	9.66e-01	ns
48 hours	lactate	Fjoh 5mg/mL	Fjoh Δ2419 1.5mg/mL	3	3	8.66e-01	ns
48 hours	lactate	control	Fjoh Δ2421 1.5mg/mL	3	3	8.37e-01	ns
48 hours	lactate	control	Fjoh 1.5mg/mL	3	3	9.72e-01	ns
48 hours	lactate	Fjoh Δ2421 1.5mg/mL	Fjoh 1.5mg/mL	3	3	8.64e-01	ns

Table A.33. Comparison between sample groups to determine P value and if the differences between the groups are significant by the use of a T-test. For lactate samples at 72 hours

Time	Compound	Group 1	Group 2	n1	n2	p.val	p.sig
72 hours	lactate	control	Fjoh Δ 2419 5mg/mL	3	3	6.51e-03	**
72 hours	lactate	Fjoh Δ 2419 5mg/mL	Fjoh Δ 2421 1.5mg/mL	3	3	4.29e-03	**
72 hours	lactate	Fjoh Δ 2419 5mg/mL	Fjoh 1.5mg/mL	3	3	6.07e-03	**
72 hours	lactate	Fjoh Δ 2419 5mg/mL	Fjoh Δ 2421 5mg/mL	3	3	2.61e-02	*
72 hours	lactate	Fjoh Δ 2419 5mg/mL	Fjoh Δ 2419 1.5mg/mL	3	3	2.40e-02	*
72 hours	lactate	Fjoh Δ 2419 5mg/mL	Fjoh 5mg/mL	3	3	3.34e-02	*
72 hours	lactate	control	Fjoh Δ 2421 5mg/mL	3	3	4.92e-01	ns
72 hours	lactate	control	Fjoh 5mg/mL	3	3	4.18e-01	ns
72 hours	lactate	control	Fjoh Δ 2419 1.5mg/mL	3	3	5.19e-01	ns
72 hours	lactate	Fjoh Δ 2421 5mg/mL	Fjoh Δ 2421 1.5mg/mL	3	3	3.75e-01	ns
72 hours	lactate	Fjoh 5mg/mL	Fjoh Δ 2421 1.5mg/mL	3	3	3.14e-01	ns
72 hours	lactate	Fjoh Δ 2419 1.5mg/mL	Fjoh Δ 2421 1.5mg/mL	3	3	3.98e-01	ns
72 hours	lactate	Fjoh Δ 2421 5mg/mL	Fjoh 1.5mg/mL	3	3	4.71e-01	ns
72 hours	lactate	Fjoh 5mg/mL	Fjoh 1.5mg/mL	3	3	3.99e-01	ns
72 hours	lactate	Fjoh Δ 2419 1.5mg/mL	Fjoh 1.5mg/mL	3	3	4.97e-01	ns
72 hours	lactate	Fjoh Δ 2421 5mg/mL	Fjoh 5mg/mL	3	3	9.00e-01	ns
72 hours	lactate	Fjoh Δ 2421 5mg/mL	Fjoh Δ 2419 1.5mg/mL	3	3	9.66e-01	ns
72 hours	lactate	Fjoh 5mg/mL	Fjoh Δ 2419 1.5mg/mL	3	3	8.66e-01	ns
72 hours	lactate	control	Fjoh Δ 2421 1.5mg/mL	3	3	8.37e-01	ns
72 hours	lactate	control	Fjoh 1.5mg/mL	3	3	9.72e-01	ns
72 hours	lactate	Fjoh Δ 2421 1.5mg/mL	Fjoh 1.5mg/mL	3	3	8.64e-01	ns

Table A.34. Comparison between sample groups to determine P value and if the differences between the groups are significant by the use of a T-test. For propionate samples at 24 and 48 hours

Time	Compund	Group 1	Group 2	n1	n2	p.val	p.sig
24 hours	propionate	control	Fjoh Δ2421 5mg/mL	3	3	2.64e-02	*
24 hours	propionate	control	Fjoh 5mg/mL	3	3	2.75e-02	*
24 hours	propionate	Fjoh Δ2421 5mg/mL	Fjoh Δ2419 1.5mg/mL	3	3	5.58e-02	ns
24 hours	propionate	Fjoh 5mg/mL	Fjoh Δ2419 1.5mg/mL	3	3	5.80e-02	ns
24 hours	propionate	Fjoh Δ2421 5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	9.93e-02	ns
24 hours	propionate	Fjoh 5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	1.03e-01	ns
24 hours	propionate	Fjoh Δ2421 5mg/mL	Fjoh 1.5mg/mL	3	3	7.88e-02	ns
24 hours	propionate	Fjoh 5mg/mL	Fjoh 1.5mg/mL	3	3	8.18e-02	ns
24 hours	propionate	Fjoh Δ2419 5mg/mL	Fjoh Δ2421 5mg/mL	3	3	1.81e-01	ns
24 hours	propionate	Fjoh Δ2419 5mg/mL	Fjoh 5mg/mL	3	3	1.87e-01	ns
24 hours	propionate	control	Fjoh Δ2419 5mg/mL	3	3	3.01e-01	ns
24 hours	propionate	Fjoh Δ2419 5mg/mL	Fjoh Δ2419 1.5mg/mL	3	3	5.09e-01	ns
24 hours	propionate	control	Fjoh Δ2421 1.5mg/mL	3	3	4.86e-01	ns
24 hours	propionate	control	Fjoh 1.5mg/mL	3	3	5.68e-01	ns
24 hours	propionate	control	Fjoh Δ2419 1.5mg/mL	3	3	6.98e-01	ns
24 hours	propionate	Fjoh Δ2419 5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	7.26e-01	ns
24 hours	propionate	Fjoh Δ2419 1.5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	7.53e-01	ns
24 hours	propionate	Fjoh Δ2419 5mg/mL	Fjoh 1.5mg/mL	3	3	6.33e-01	ns
24 hours	propionate	Fjoh Δ2419 1.5mg/mL	Fjoh 1.5mg/mL	3	3	8.52e-01	ns
24 hours	propionate	Fjoh Δ2421 1.5mg/mL	Fjoh 1.5mg/mL	3	3	8.98e-01	ns
24 hours	propionate	Fjoh Δ2421 5mg/mL	Fjoh 5mg/mL	3	3	9.83e-01	ns
48 hours	propionate	control	Fjoh Δ2421 5mg/mL	3	3	2.64e-02	*
48 hours	propionate	control	Fjoh 5mg/mL	3	3	2.75e-02	*
48 hours	propionate	Fjoh Δ2421 5mg/mL	Fjoh Δ2419 1.5mg/mL	3	3	5.58e-02	ns
48 hours	propionate	Fjoh 5mg/mL	Fjoh Δ2419 1.5mg/mL	3	3	5.80e-02	ns
48 hours	propionate	Fjoh Δ2421 5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	9.93e-02	ns
48 hours	propionate	Fjoh 5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	1.03e-01	ns
48 hours	propionate	Fjoh Δ2421 5mg/mL	Fjoh 1.5mg/mL	3	3	7.88e-02	ns
48 hours	propionate	Fjoh 5mg/mL	Fjoh 1.5mg/mL	3	3	8.18e-02	ns
48 hours	propionate	Fjoh Δ2419 5mg/mL	Fjoh Δ2421 5mg/mL	3	3	1.81e-01	ns
48 hours	propionate	Fjoh Δ2419 5mg/mL	Fjoh 5mg/mL	3	3	1.87e-01	ns
48 hours	propionate	control	Fjoh Δ2419 5mg/mL	3	3	3.01e-01	ns
48 hours	propionate	Fjoh Δ2419 5mg/mL	Fjoh Δ2419 1.5mg/mL	3	3	5.09e-01	ns
48 hours	propionate	control	Fjoh Δ2421 1.5mg/mL	3	3	4.86e-01	ns
48 hours	propionate	control	Fjoh 1.5mg/mL	3	3	5.68e-01	ns
48 hours	propionate	control	Fjoh Δ2419 1.5mg/mL	3	3	6.98e-01	ns
48 hours	propionate	Fjoh Δ2419 5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	7.26e-01	ns
48 hours	propionate	Fjoh Δ2419 1.5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	7.53e-01	ns
48 hours	propionate	Fjoh Δ2419 5mg/mL	Fjoh 1.5mg/mL	3	3	6.33e-01	ns
48 hours	propionate	Fjoh Δ2419 1.5mg/mL	Fjoh 1.5mg/mL	3	3	8.52e-01	ns
48 hours	propionate	Fjoh Δ2421 1.5mg/mL	Fjoh 1.5mg/mL	3	3	8.98e-01	ns
48 hours	propionate	Fjoh Δ2421 5mg/mL	Fjoh 5mg/mL	3	3	9.83e-01	ns

Table A.35. Comparison between sample groups to determine P value and if the differences between the groups are significant by the use of a T-test. For propionate samples at 72 hours

Time	Compund	Group 1	Group 2	n1	n2	p.val	p.sig
72 hours	propionate	control	Fjoh Δ 2421 5mg/mL	3	3	2.64e-02	*
72 hours	propionate	control	Fjoh 5mg/mL	3	3	2.75e-02	*
72 hours	propionate	Fjoh Δ 2421 5mg/mL	Fjoh Δ 2419 1.5mg/mL	3	3	5.58e-02	ns
72 hours	propionate	Fjoh 5mg/mL	Fjoh Δ 2419 1.5mg/mL	3	3	5.80e-02	ns
72 hours	propionate	Fjoh Δ 2421 5mg/mL	Fjoh Δ 2421 1.5mg/mL	3	3	9.93e-02	ns
72 hours	propionate	Fjoh 5mg/mL	Fjoh Δ 2421 1.5mg/mL	3	3	1.03e-01	ns
72 hours	propionate	Fjoh Δ 2421 5mg/mL	Fjoh 1.5mg/mL	3	3	7.88e-02	ns
72 hours	propionate	Fjoh 5mg/mL	Fjoh 1.5mg/mL	3	3	8.18e-02	ns
72 hours	propionate	Fjoh Δ 2419 5mg/mL	Fjoh Δ 2421 5mg/mL	3	3	1.81e-01	ns
72 hours	propionate	Fjoh Δ 2419 5mg/mL	Fjoh 5mg/mL	3	3	1.87e-01	ns
72 hours	propionate	control	Fjoh Δ 2419 5mg/mL	3	3	3.01e-01	ns
72 hours	propionate	Fjoh Δ 2419 5mg/mL	Fjoh Δ 2419 1.5mg/mL	3	3	5.09e-01	ns
72 hours	propionate	control	Fjoh Δ 2421 1.5mg/mL	3	3	4.86e-01	ns
72 hours	propionate	control	Fjoh 1.5mg/mL	3	3	5.68e-01	ns
72 hours	propionate	control	Fjoh Δ 2419 1.5mg/mL	3	3	6.98e-01	ns
72 hours	propionate	Fjoh Δ 2419 5mg/mL	Fjoh Δ 2421 1.5mg/mL	3	3	7.26e-01	ns
72 hours	propionate	Fjoh Δ 2419 1.5mg/mL	Fjoh Δ 2421 1.5mg/mL	3	3	7.53e-01	ns
72 hours	propionate	Fjoh Δ 2419 5mg/mL	Fjoh 1.5mg/mL	3	3	6.33e-01	ns
72 hours	propionate	Fjoh Δ 2419 1.5mg/mL	Fjoh 1.5mg/mL	3	3	8.52e-01	ns
72 hours	propionate	Fjoh Δ 2421 1.5mg/mL	Fjoh 1.5mg/mL	3	3	8.98e-01	ns
72 hours	propionate	Fjoh Δ 2421 5mg/mL	Fjoh 5mg/mL	3	3	9.83e-01	ns

Table A.36. Stool pellet weights in mg used for qNMR.

Name	Pellet weight in mg
Control 1 72 hours	0.0142
Control 2 72 hours	0.0093
Control 3 72 hours	0.0104
Fjoh 5mg/mL 1 72 hours	0.0186
Fjoh 5mg/mL 2 72 hours	0.0386
Fjoh 5mg/mL 3 72 hours	0.0391
Δ 2419 Fjoh 5mg/mL 1 72 hours	0.0254
Δ 2419 Fjoh 5mg/mL 2 72 hours	0.0397
Δ 2419 Fjoh 5mg/mL 3 72 hours	0.0378
Control 1 24 hours	0.0133
Control 2 24 hours	0.0083
Control 3 24 hours	0.0183
Fjoh 5mg/mL 1 24 hours	0.0247
Fjoh 5mg/mL 2 24 hours	0.0056
Fjoh 5mg/mL 3 24 hours	0.0177
Δ 2419 Fjoh 5mg/mL 1 24 hours	0.0062
Δ 2419 Fjoh 5mg/mL 2 24 hours	0.0189
Δ 2419 Fjoh 5mg/mL 3 24 hours	0.0094

Table A.37. Concentration in millimolar found from qNMR, formate, acetate, lactate, propionate, butyrate, valerate.

name	Formate	Acetate	Lactate	Propionate	Butyrate	Valerate
control 1 72 hours	0.883803	3.719718	0.216549	0.860563	0.560211	0.797535
control 2 72 hours	2.171505	3.159677	0.536559	0.133871	0.304839	0.499462
control 3 72 hours	1.563462	2.824038	0.314904	0.309135	0.118269	0.352404
Fjoh 5mg/mL 1 72 hours	0.922312	5.723387	0.240591	1.36371	0.812903	0.944355
Fjoh 5mg/mL 2 72 hours	0.430311	4.80013	0.06114	1.295596	0.795984	0.987047
Fjoh 5mg/mL 3 72 hours	0.389514	3.327366	0.110358	0.929156	0.537852	0.667775
Δ 2419 Fjoh 5mg/mL 1 72 hours	0.660236	7.065354	0.152953	2.08937	1.221654	1.455709
Δ 2419 Fjoh 5mg/mL 2 72 hours	0.44937	4.11335	0.083501	1.08665	0.657305	0.789169
Δ 2419 Fjoh 5mg/mL 3 72 hours	0.418915	4.491402	0.101323	1.285317	0.751058	0.882407
control 1 24 hours	0.796127	1.582394	0.191901	0.217254	0.118662	0.148944
control 2 24 hours	1.206452	2.293548	0.336559	0.359677	0.188172	0.227957
control 3 24 hours	1.237981	2.503365	0.265385	0.368269	0.223558	2.644231
Fjoh 5mg/mL 1 24 hours	0.642204	3.235215	0.151613	0.872849	0.549731	0.678495
Fjoh 5mg/mL 2 24 hours	0.350648	0.95272	0.07513	0.242746	0.138342	0.169819
Fjoh 5mg/mL 3 24 hours	0.298721	1.290921	0.073913	0.342967	0.203453	0.245652
Δ 2419 Fjoh 5mg/mL 1 24 hours	0.578346	2.644882	0.121457	0.637992	0.425	0.53563
Δ 2419 Fjoh 5mg/mL 2 24 hours	0.335516	2.023048	0.080982	0.544836	0.359446	0.425693
Δ 2419 Fjoh 5mg/mL 3 24 hours	0.396693	0.292593	0.088228	0.025397	0.047487	0.084524

Table A.38. Concentration in millimolar found from qNMR, ethanol, isovalerate, methanol, trimethylamine.

name	Ethanol	Isovalerate	Methanol	Trimethylamine
control 1 72 hours	0.947535	0.153169	0.084859	0.063732
control 2 72 hours	0.671505	0.058602	0.064516	0.053763
control 3 72 hours	0.875	0.5625	0.148077	0
Fjoh 5mg/mL 1 72 hours	0.611559	0.295968	0.076613	0.11371
Fjoh 5mg/mL 2 72 hours	0.452979	0.296762	0.047798	0.092228
Fjoh 5mg/mL 3 72 hours	0.353325	0.180946	0.036701	0.061125
Δ 2419 Fjoh 5mg/mL 1 72 hours	0.519094	0.399409	0.067717	0.090551
Δ 2419 Fjoh 5mg/mL 2 72 hours	0.374433	0.216373	0.04068	0.586902
Δ 2419 Fjoh 5mg/mL 3 72 hours	0.493386	0.262037	0.832275	0.055556
control 1 24 hours	1.631338	0.01338	0.081338	0
control 2 24 hours	7.108065	0.041935	0.167204	0
control 3 24 hours	0.285481	0.023558	0.109135	0
Fjoh 5mg/mL 1 24 hours	1.702151	0.036559	0.072043	0.022043
Fjoh 5mg/mL 2 24 hours	0.606477	0.024093	0.029793	0.003109
Fjoh 5mg/mL 3 24 hours	0.938107	0.030946	0.034015	0.009335
Δ 2419 Fjoh 5mg/mL 1 24 hours	0.674803	0.097441	0.051772	0.040945
Δ 2419 Fjoh 5mg/mL 2 24 hours	0.402141	0.083375	0.065365	0.030982
Δ 2419 Fjoh 5mg/mL 3 24 hours	0.434259	0.008466	0.026455	0

Table A.39. Comparison between sample groups to determine P value and if the differences between the groups are significant by the use of a T-test. For valerate samples at 24 and 48 hours

Time	Compound	Group 1	Group 2	n1	n2	p.val	p.sig
24 hours	valerate	control	Fjoh 5mg/mL	3	3	4.48e-03	**
24 hours	valerate	Fjoh 5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	5.51e-03	**
24 hours	valerate	Fjoh Δ2419 5mg/mL	Fjoh 5mg/mL	3	3	1.50e-02	*
24 hours	valerate	Fjoh 5mg/mL	Fjoh Δ2419 1.5mg/mL	3	3	1.36e-02	*
24 hours	valerate	Fjoh 5mg/mL	Fjoh 1.5mg/mL	3	3	2.41e-02	*
24 hours	valerate	Fjoh Δ2421 5mg/mL	Fjoh 5mg/mL	3	3	1.00e-01	ns
24 hours	valerate	control	Fjoh Δ2421 5mg/mL	3	3	1.28e-01	ns
24 hours	valerate	Fjoh Δ2421 5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	1.52e-01	ns
24 hours	valerate	Fjoh Δ2419 5mg/mL	Fjoh Δ2421 5mg/mL	3	3	3.29e-01	ns
24 hours	valerate	Fjoh Δ2421 5mg/mL	Fjoh Δ2419 1.5mg/mL	3	3	3.06e-01	ns
24 hours	valerate	control	Fjoh 1.5mg/mL	3	3	4.08e-01	ns
24 hours	valerate	Fjoh Δ2421 5mg/mL	Fjoh 1.5mg/mL	3	3	4.56e-01	ns
24 hours	valerate	Fjoh Δ2421 1.5mg/mL	Fjoh 1.5mg/mL	3	3	4.67e-01	ns
24 hours	valerate	control	Fjoh Δ2419 5mg/mL	3	3	5.53e-01	ns
24 hours	valerate	control	Fjoh Δ2419 1.5mg/mL	3	3	5.86e-01	ns
24 hours	valerate	Fjoh Δ2419 5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	6.22e-01	ns
24 hours	valerate	Fjoh Δ2419 1.5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	6.57e-01	ns
24 hours	valerate	Fjoh Δ2419 5mg/mL	Fjoh 1.5mg/mL	3	3	8.11e-01	ns
24 hours	valerate	Fjoh Δ2419 1.5mg/mL	Fjoh 1.5mg/mL	3	3	7.73e-01	ns
24 hours	valerate	Fjoh Δ2419 5mg/mL	Fjoh Δ2419 1.5mg/mL	3	3	9.61e-01	ns
24 hours	valerate	control	Fjoh Δ2421 1.5mg/mL	3	3	9.18e-01	ns
48 hours	valerate	control	Fjoh 5mg/mL	3	3	4.48e-03	**
48 hours	valerate	Fjoh 5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	5.51e-03	**
48 hours	valerate	Fjoh Δ2419 5mg/mL	Fjoh 5mg/mL	3	3	1.50e-02	*
48 hours	valerate	Fjoh 5mg/mL	Fjoh Δ2419 1.5mg/mL	3	3	1.36e-02	*
48 hours	valerate	Fjoh 5mg/mL	Fjoh 1.5mg/mL	3	3	2.41e-02	*
48 hours	valerate	Fjoh Δ2421 5mg/mL	Fjoh 5mg/mL	3	3	1.00e-01	ns
48 hours	valerate	control	Fjoh Δ2421 5mg/mL	3	3	1.28e-01	ns
48 hours	valerate	Fjoh Δ2421 5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	1.52e-01	ns
48 hours	valerate	Fjoh Δ2419 5mg/mL	Fjoh Δ2421 5mg/mL	3	3	3.29e-01	ns
48 hours	valerate	Fjoh Δ2421 5mg/mL	Fjoh Δ2419 1.5mg/mL	3	3	3.06e-01	ns
48 hours	valerate	control	Fjoh 1.5mg/mL	3	3	4.08e-01	ns
48 hours	valerate	Fjoh Δ2421 5mg/mL	Fjoh 1.5mg/mL	3	3	4.56e-01	ns
48 hours	valerate	Fjoh Δ2421 1.5mg/mL	Fjoh 1.5mg/mL	3	3	4.67e-01	ns
48 hours	valerate	control	Fjoh Δ2419 5mg/mL	3	3	5.53e-01	ns
48 hours	valerate	control	Fjoh Δ2419 1.5mg/mL	3	3	5.86e-01	ns
48 hours	valerate	Fjoh Δ2419 5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	6.22e-01	ns
48 hours	valerate	Fjoh Δ2419 1.5mg/mL	Fjoh Δ2421 1.5mg/mL	3	3	6.57e-01	ns
48 hours	valerate	Fjoh Δ2419 5mg/mL	Fjoh 1.5mg/mL	3	3	8.11e-01	ns
48 hours	valerate	Fjoh Δ2419 1.5mg/mL	Fjoh 1.5mg/mL	3	3	7.73e-01	ns
48 hours	valerate	Fjoh Δ2419 5mg/mL	Fjoh Δ2419 1.5mg/mL	3	3	9.61e-01	ns
48 hours	valerate	control	Fjoh Δ2421 1.5mg/mL	3	3	9.18e-01	ns

Table A.40. Comparison between sample groups to determine P value and if the differences between the groups are significant by the use of a T-test. For valerate samples at 72 hours

Time	Compound	Group 1	Group 2	n1	n2	p.val	p.sig
72 hours	valerate	control	Fjoh 5mg/mL	3	3	4.48e-03	**
72 hours	valerate	Fjoh 5mg/mL	Fjoh Δ 2421 1.5mg/mL	3	3	5.51e-03	**
72 hours	valerate	Fjoh Δ 2419 5mg/mL	Fjoh 5mg/mL	3	3	1.50e-02	*
72 hours	valerate	Fjoh 5mg/mL	Fjoh Δ 2419 1.5mg/mL	3	3	1.36e-02	*
72 hours	valerate	Fjoh 5mg/mL	Fjoh 1.5mg/mL	3	3	2.41e-02	*
72 hours	valerate	Fjoh Δ 2421 5mg/mL	Fjoh 5mg/mL	3	3	1.00e-01	ns
72 hours	valerate	control	Fjoh Δ 2421 5mg/mL	3	3	1.28e-01	ns
72 hours	valerate	Fjoh Δ 2421 5mg/mL	Fjoh Δ 2421 1.5mg/mL	3	3	1.52e-01	ns
72 hours	valerate	Fjoh Δ 2419 5mg/mL	Fjoh Δ 2421 5mg/mL	3	3	3.29e-01	ns
72 hours	valerate	Fjoh Δ 2421 5mg/mL	Fjoh Δ 2419 1.5mg/mL	3	3	3.06e-01	ns
72 hours	valerate	control	Fjoh 1.5mg/mL	3	3	4.08e-01	ns
72 hours	valerate	Fjoh Δ 2421 5mg/mL	Fjoh 1.5mg/mL	3	3	4.56e-01	ns
72 hours	valerate	Fjoh Δ 2421 1.5mg/mL	Fjoh 1.5mg/mL	3	3	4.67e-01	ns
72 hours	valerate	control	Fjoh Δ 2419 5mg/mL	3	3	5.53e-01	ns
72 hours	valerate	control	Fjoh Δ 2419 1.5mg/mL	3	3	5.86e-01	ns
72 hours	valerate	Fjoh Δ 2419 5mg/mL	Fjoh Δ 2421 1.5mg/mL	3	3	6.22e-01	ns
72 hours	valerate	Fjoh Δ 2419 1.5mg/mL	Fjoh Δ 2421 1.5mg/mL	3	3	6.57e-01	ns
72 hours	valerate	Fjoh Δ 2419 5mg/mL	Fjoh 1.5mg/mL	3	3	8.11e-01	ns
72 hours	valerate	Fjoh Δ 2419 1.5mg/mL	Fjoh 1.5mg/mL	3	3	7.73e-01	ns
72 hours	valerate	Fjoh Δ 2419 5mg/mL	Fjoh Δ 2419 1.5mg/mL	3	3	9.61e-01	ns
72 hours	valerate	control	Fjoh Δ 2421 1.5mg/mL	3	3	9.18e-01	ns

Table A.41. Comparison between sample groups to determine P value and if the differences between the groups are significant by the use of a T-test. For Acetate, butyrate, ethanol, samples.

Time	Compound	Group 1	Group 2	n1	n2	p.val
Acetate	Control 72 hours	Fjoh Δ 2419 72 hours	3	3	0.000422	***
Acetate	Fjoh Δ 2419 72 hours	Control 24 hours	3	3	0.000416	***
Acetate	Fjoh Δ 2419 72 hours	Fjoh 24 hours	3	3	0.000256	***
Acetate	Control 72 hours	Fjoh 72 hours	3	3	0.036	*
Acetate	Fjoh 72 hours	Control 24 hours	3	3	0.037	*
Acetate	Fjoh Δ 2419 72 hours	Fjoh Δ 2419 24 hours	3	3	0.026	*
Acetate	Fjoh 72 hours	Fjoh 24 hours	3	3	0.05	*
Acetate	Fjoh 72 hours	Fjoh Δ 2419 24 hours	3	3	0.05	*
Acetate	Control 24 hours	Fjoh 24 hours	3	3	0.057	ns
Acetate	Control 72 hours	Control 24 hours	3	3	0.214	ns
Acetate	Control 72 hours	Fjoh 24 hours	3	3	0.314	ns
Acetate	Fjoh 72 hours	Fjoh Δ 2419 72 hours	3	3	0.324	ns
Acetate	Control 24 hours	Fjoh Δ 2419 24 hours	3	3	0.298	ns
Acetate	Control 72 hours	Fjoh Δ 2419 24 hours	3	3	0.547	ns
Acetate	Fjoh 24 hours	Fjoh Δ 2419 24 hours	3	3	0.881	ns
Butyrate	Control 72 hours	Fjoh Δ 2419 72 hours	3	3	0.000574	***
Butyrate	Fjoh Δ 2419 72 hours	Fjoh 24 hours	3	3	0.000501	***
Butyrate	Fjoh Δ 2419 72 hours	Control 24 hours	3	3	0.002	**
Butyrate	Fjoh Δ 2419 72 hours	Fjoh Δ 2419 24 hours	3	3	0.023	*
Butyrate	Control 72 hours	Fjoh 72 hours	3	3	0.045	*
Butyrate	Fjoh 72 hours	Control 24 hours	3	3	0.046	*
Butyrate	Control 24 hours	Fjoh 24 hours	3	3	0.05	*
Butyrate	Fjoh 72 hours	Fjoh 24 hours	3	3	0.075	ns
Butyrate	Fjoh 72 hours	Fjoh Δ 2419 24 hours	3	3	0.088	ns
Butyrate	Control 72 hours	Fjoh 24 hours	3	3	0.209	ns
Butyrate	Control 24 hours	Fjoh Δ 2419 24 hours	3	3	0.199	ns
Butyrate	Control 72 hours	Control 24 hours	3	3	0.296	ns
Butyrate	Fjoh 72 hours	Fjoh Δ 2419 72 hours	3	3	0.304	ns
Butyrate	Control 72 hours	Fjoh Δ 2419 24 hours	3	3	0.354	ns
Butyrate	Fjoh 24 hours	Fjoh Δ 2419 24 hours	3	3	0.8	ns
Ethanol	Control 72 hours	Fjoh 24 hours	3	3	0.016	*
Ethanol	Fjoh 72 hours	Fjoh 24 hours	3	3	0.035	*
Ethanol	Fjoh Δ 2419 72 hours	Fjoh 24 hours	3	3	0.045	*
Ethanol	Control 72 hours	Fjoh Δ 2419 72 hours	3	3	0.092	ns
Ethanol	Control 72 hours	Fjoh Δ 2419 24 hours	3	3	0.077	ns
Ethanol	Fjoh 24 hours	Fjoh Δ 2419 24 hours	3	3	0.067	ns
Ethanol	Control 72 hours	Fjoh 72 hours	3	3	0.169	ns
Ethanol	Fjoh 72 hours	Fjoh Δ 2419 24 hours	3	3	0.327	ns
Ethanol	Control 72 hours	Control 24 hours	3	3	0.374	ns
Ethanol	Fjoh 72 hours	Control 24 hours	3	3	0.469	ns
Ethanol	Fjoh Δ 2419 72 hours	Control 24 hours	3	3	0.501	ns
Ethanol	Control 24 hours	Fjoh Δ 2419 24 hours	3	3	0.524	ns
Ethanol	Fjoh 72 hours	Fjoh Δ 2419 72 hours	3	3	0.606	ns
Ethanol	Fjoh Δ 2419 72 hours	Fjoh Δ 2419 24 hours	3	3	0.623	ns
Ethanol	Control 24 hours	Fjoh 24 hours	3	3	0.994	ns

Table A.42. Comparison between sample groups to determine P value and if the differences between the groups are significant by the use of a T-test. For formate, iosvalerate, lactate samples.

Time	Compound	Group 1	Group 2	n1	n2	p.val
Formate	Fjoh 72 hours	Control 24 hours	3	3	0.006	**
Formate	Fjoh Δ 2419 72 hours	Control 24 hours	3	3	0.004	**
Formate	Fjoh Δ 2419 72 hours	Fjoh 24 hours	3	3	0.006	**
Formate	Fjoh 72 hours	Fjoh 24 hours	3	3	0.008	**
Formate	Fjoh Δ 2419 72 hours	Fjoh Δ 2419 24 hours	3	3	0.034	*
Formate	Control 24 hours	Fjoh Δ 2419 24 hours	3	3	0.038	*
Formate	Fjoh 72 hours	Fjoh Δ 2419 24 hours	3	3	0.061	ns
Formate	Fjoh 24 hours	Fjoh Δ 2419 24 hours	3	3	0.067	ns
Formate	Control 72 hours	Control 24 hours	3	3	0.198	ns
Formate	Control 72 hours	Fjoh 24 hours	3	3	0.21	ns
Formate	Control 72 hours	Fjoh Δ 2419 24 hours	3	3	0.463	ns
Formate	Fjoh 72 hours	Fjoh Δ 2419 72 hours	3	3	0.585	ns
Formate	Control 72 hours	Fjoh Δ 2419 72 hours	3	3	0.851	ns
Formate	Control 24 hours	Fjoh 24 hours	3	3	0.815	ns
Formate	Control 72 hours	Fjoh 72 hours	3	3	0.999	ns
Isovalerate	Fjoh Δ 2419 72 hours	Control 24 hours	3	3	0.002	**
Isovalerate	Fjoh Δ 2419 72 hours	Fjoh 24 hours	3	3	0.001	***
Isovalerate	Fjoh Δ 2419 72 hours	Fjoh Δ 2419 24 hours	3	3	0.005	**
Isovalerate	Control 72 hours	Fjoh Δ 2419 72 hours	3	3	0.041	*
Isovalerate	Fjoh 72 hours	Control 24 hours	3	3	0.049	*
Isovalerate	Fjoh 72 hours	Fjoh 24 hours	3	3	0.057	ns
Isovalerate	Fjoh 72 hours	Fjoh Δ 2419 24 hours	3	3	0.059	ns
Isovalerate	Control 24 hours	Fjoh 24 hours	3	3	0.035	*
Isovalerate	Control 72 hours	Fjoh 72 hours	3	3	0.097	ns
Isovalerate	Control 24 hours	Fjoh Δ 2419 24 hours	3	3	0.186	ns
Isovalerate	Control 72 hours	Control 24 hours	3	3	0.241	ns
Isovalerate	Control 72 hours	Fjoh 24 hours	3	3	0.346	ns
Isovalerate	Fjoh 24 hours	Fjoh Δ 2419 24 hours	3	3	0.343	ns
Isovalerate	Fjoh 72 hours	Fjoh Δ 2419 72 hours	3	3	0.483	ns
Isovalerate	Control 72 hours	Fjoh Δ 2419 24 hours	3	3	0.678	ns
Lactate	Fjoh Δ 2419 72 hours	Control 24 hours	3	3	0.027	*
Lactate	Fjoh Δ 2419 72 hours	Fjoh 24 hours	3	3	0.045	*
Lactate	Fjoh 24 hours	Fjoh Δ 2419 24 hours	3	3	0.031	*
Lactate	Fjoh Δ 2419 72 hours	Fjoh Δ 2419 24 hours	3	3	0.11	ns
Lactate	Control 24 hours	Fjoh Δ 2419 24 hours	3	3	0.102	ns
Lactate	Control 72 hours	Control 24 hours	3	3	0.271	ns
Lactate	Control 72 hours	Fjoh 24 hours	3	3	0.273	ns
Lactate	Fjoh 72 hours	Control 24 hours	3	3	0.339	ns
Lactate	Fjoh 72 hours	Fjoh 24 hours	3	3	0.339	ns
Lactate	Control 72 hours	Fjoh Δ 2419 24 hours	3	3	0.449	ns
Lactate	Fjoh 72 hours	Fjoh Δ 2419 24 hours	3	3	0.536	ns
Lactate	Control 72 hours	Fjoh 72 hours	3	3	0.948	ns
Lactate	Control 72 hours	Fjoh Δ 2419 72 hours	3	3	0.884	ns
Lactate	Fjoh 72 hours	Fjoh Δ 2419 72 hours	3	3	0.959	ns
Lactate	Control 24 hours	Fjoh 24 hours	3	3	0.991	ns

Table A.43. Comparison between sample groups to determine P value and if the differences between the groups are significant by the use of a T-test. For methanol, propionate, trimethylamine samples.

Time	Compund	Group 1	Group 2	n1	n2	p.val
Methanol	Control 72 hours	Fjoh 72 hours	3	3	0.239	ns
Methanol	Control 72 hours	Fjoh Δ 2419 72 hours	3	3	0.158	ns
Methanol	Fjoh 72 hours	Control 24 hours	3	3	0.215	ns
Methanol	Fjoh 72 hours	Fjoh 24 hours	3	3	0.154	ns
Methanol	Fjoh Δ 2419 72 hours	Control 24 hours	3	3	0.212	ns
Methanol	Fjoh Δ 2419 72 hours	Fjoh 24 hours	3	3	0.213	ns
Methanol	Control 72 hours	Control 24 hours	3	3	0.626	ns
Methanol	Control 72 hours	Fjoh 24 hours	3	3	0.626	ns
Methanol	Control 72 hours	Fjoh Δ 2419 24 hours	3	3	0.417	ns
Methanol	Fjoh 72 hours	Fjoh Δ 2419 72 hours	3	3	0.354	ns
Methanol	Fjoh Δ 2419 72 hours	Fjoh Δ 2419 24 hours	3	3	0.493	ns
Methanol	Control 24 hours	Fjoh Δ 2419 24 hours	3	3	0.547	ns
Methanol	Fjoh 24 hours	Fjoh Δ 2419 24 hours	3	3	0.535	ns
Methanol	Fjoh 72 hours	Fjoh Δ 2419 24 hours	3	3	0.904	ns
Methanol	Control 24 hours	Fjoh 24 hours	3	3	0.959	ns
Propionate	Control 72 hours	Fjoh Δ 2419 72 hours	3	3	0.000597	***
Propionate	Fjoh Δ 2419 72 hours	Fjoh 24 hours	3	3	0.000859	***
Propionate	Fjoh Δ 2419 72 hours	Control 24 hours	3	3	0.004	**
Propionate	Fjoh Δ 2419 72 hours	Fjoh Δ 2419 24 hours	3	3	0.016	*
Propionate	Control 72 hours	Fjoh 72 hours	3	3	0.033	*
Propionate	Fjoh 72 hours	Control 24 hours	3	3	0.042	*
Propionate	Control 24 hours	Fjoh 24 hours	3	3	0.049	*
Propionate	Fjoh 72 hours	Fjoh 24 hours	3	3	0.067	ns
Propionate	Fjoh 72 hours	Fjoh Δ 2419 24 hours	3	3	0.063	ns
Propionate	Control 72 hours	Fjoh 24 hours	3	3	0.135	ns
Propionate	Fjoh 72 hours	Fjoh Δ 2419 72 hours	3	3	0.259	ns
Propionate	Control 24 hours	Fjoh Δ 2419 24 hours	3	3	0.264	ns
Propionate	Control 72 hours	Fjoh Δ 2419 24 hours	3	3	0.39	ns
Propionate	Control 72 hours	Control 24 hours	3	3	0.503	ns
Propionate	Fjoh 24 hours	Fjoh Δ 2419 24 hours	3	3	0.948	ns
Trimethylamine	Fjoh Δ 2419 72 hours	Fjoh 24 hours	3	3	6.23e-05	****
Trimethylamine	Fjoh Δ 2419 72 hours	Control 24 hours	3	3	0.001	***
Trimethylamine	Control 72 hours	Fjoh 72 hours	3	3	0.02	*
Trimethylamine	Control 72 hours	Fjoh Δ 2419 72 hours	3	3	0.016	*
Trimethylamine	Fjoh 72 hours	Control 24 hours	3	3	0.026	*
Trimethylamine	Fjoh 72 hours	Fjoh 24 hours	3	3	0.032	*
Trimethylamine	Fjoh 72 hours	Fjoh Δ 2419 24 hours	3	3	0.031	*
Trimethylamine	Control 24 hours	Fjoh 24 hours	3	3	0.022	*
Trimethylamine	Fjoh Δ 2419 72 hours	Fjoh Δ 2419 24 hours	3	3	0.056	ns
Trimethylamine	Control 24 hours	Fjoh Δ 2419 24 hours	3	3	0.186	ns
Trimethylamine	Control 72 hours	Control 24 hours	3	3	0.215	ns
Trimethylamine	Fjoh 24 hours	Fjoh Δ 2419 24 hours	3	3	0.388	ns
Trimethylamine	Fjoh 72 hours	Fjoh Δ 2419 72 hours	3	3	0.421	ns
Trimethylamine	Control 72 hours	Fjoh Δ 2419 24 hours	3	3	0.572	ns
Trimethylamine	Control 72 hours	Fjoh 24 hours	3	3	0.671	ns

Table A.44. Comparison between sample groups to determine P value and if the differences between the groups are significant by the use of a T-test. For valerate samples.

Time	Compound	Group 1	Group 2	n1	n2	p.val
Valerate	Fjoh Δ 2419 72 hours	Fjoh 24 hours	3	3	0.000585	***
Valerate	Control 72 hours	Fjoh Δ 2419 72 hours	3	3	0.001	***
Valerate	Fjoh Δ 2419 72 hours	Control 24 hours	3	3	0.002	**
Valerate	Fjoh Δ 2419 72 hours	Fjoh Δ 2419 24 hours	3	3	0.02	*
Valerate	Control 72 hours	Fjoh 72 hours	3	3	0.058	ns
Valerate	Fjoh 72 hours	Control 24 hours	3	3	0.053	ns
Valerate	Control 24 hours	Fjoh 24 hours	3	3	0.054	ns
Valerate	Fjoh 72 hours	Fjoh 24 hours	3	3	0.087	ns
Valerate	Fjoh 72 hours	Fjoh Δ 2419 24 hours	3	3	0.1	ns
Valerate	Control 24 hours	Fjoh Δ 2419 24 hours	3	3	0.165	ns
Valerate	Control 72 hours	Control 24 hours	3	3	0.224	ns
Valerate	Control 72 hours	Fjoh 24 hours	3	3	0.365	ns
Valerate	Control 72 hours	Fjoh Δ 2419 24 hours	3	3	0.395	ns
Valerate	Fjoh 72 hours	Fjoh Δ 2419 72 hours	3	3	0.383	ns
Valerate	Fjoh 24 hours	Fjoh Δ 2419 24 hours	3	3	0.748	ns

Table A.45. DNA content of DNA extracted stool from three sets of stool samples at two different time points

	sample 1 48 hours ng/uL	sample 2 48 hours ng/uL	sample 3 48 hours ng/uL	sample 1 72 hours ng/uL	sample 2 72 hours ng/uL	sample 3 72 hours ng/uL
control	0.772	1.15	1.43	16.5	6.25	4.44
Fjoh	1.06	0.965	0.908	1.24	1.41	1.41
Δ 2419 Fjoh	0.227	too low	1.9	6.64	11.1	9.65

Table A.46. Primers used for the v1-v8 region of the 16S rRNA amplification.

Primers	Nucleotide code
27F (V1)	TTTCTGTTGGTGCTGATATTGCAGAGTTTGATCCTGGCTCAG
1392R (V8)	ACTTGCCTGTCGCTCTATCTTCGACGGGCGGTGWGTRCA

Table A.47. PCR settings used for the amplification of the 16S rRNA region.

Step	Temperature	Time
Initial denaturation	95C	2 minutes
35 cycles		
Denaturation 15 seconds	95C	15 seconds
Annealing	55C	15 seconds
Extension	72C	90 seconds
Cycle end		
Final extension	72C	5 minutes
Storage	8-12C	Forever

Table A.48. DNA content of the cleaned 16SRNA PCR.

Well	Sample	Value	nano gram pr micro liter
A1	Control 48hour 1	35291	81.84
B1	Control 48hour 2	32604	75.60
C1	Control 48hour 3	27007	62.60
F1	Control 72hour 1	32155	74.56
G1	Control 72hour 2	33382	77.40
H1	Control 72hour 3	33945	78.71
A2	Fjoh 48hour 1	40837	94.71
B2	Fjoh 48hour 2	37341	86.60
C2	Fjoh 48hour 3	36539	84.73
F2	Fjoh 72hour 1	36026	83.54
G2	Fjoh 72hour 2	38689	89.72
H2	Fjoh 72hour 3	38713	89.78
A3	Δ 2419 Fjoh 48hour 1	28711	66.56
B3	Δ 2419 Fjoh 48hour 2	27616	64.02
C3	Δ 2419 Fjoh 48hour 3	23420	54.28
D3	Positiv control	20623	47.78
E3	Negativ control	4680	10.77
F3	Δ 2419 Fjoh 72hour 1	17446	40.41
G3	Δ 2419 Fjoh 72hour 2	21177	49.07
H3	Δ 2419 Fjoh 72hour 3	24397	56.54
G12	Low calibration curve	41	0.00
H12	High calibration curve	43115	100.00

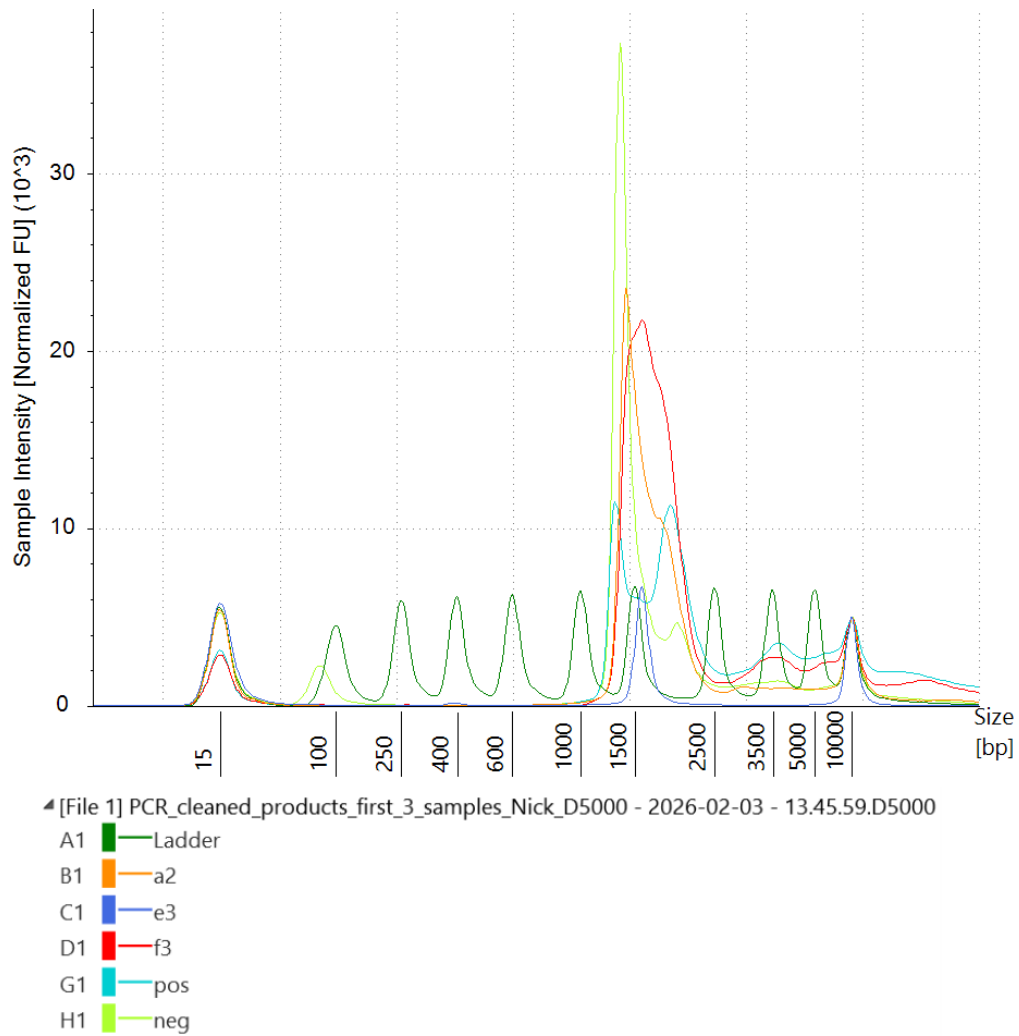


Figure A.7. A tape station overview of contents of multiple tests. A1 is a ladder over DNA lengths, B1 is Fjoh 48hour 1, C1 is $\Delta 2419$ Fjoh 72hour 1, D1 is $\Delta 2419$ Fjoh 72hour 2, G1 is the positive control, and H1 is the negative control.

Table A.49. Dilutions needed to reach 200 fmol with 42 μ L volume for the cleaned 16SRNA PCR

ID	PCR clean Qubit conc. [ng/ μ L]	fmol DNA/ μ L	μ L in Pool	nfw 48ul
control 48hour 1	81.83591029	95.13189	4.2046889	43.7953111
control 48hour 2	75.59780842	87.88028	4.55164708	43.44835292
control 48hour 3	62.60389098	72.77522	5.49637632	42.50362368
Fjoh 48hour 1	74.55541626	86.66852	4.61528567	43.38471433
Fjoh 48hour 2	77.40400241	89.97992	4.44543607	43.55456393
Fjoh 48hour 3	78.71105539	91.49934	4.37161644	43.62838356
Δ 2419 Fjoh 48hour 1	94.71142685	110.0993	3.63308373	44.36691627
Δ 2419 Fjoh 48hour 2	86.59516181	100.6644	3.9736001	44.0263999
Δ 2419 Fjoh 48hour 3	84.73324976	98.49996	4.06091523	43.93908477
control 72hour 1	83.54227608	97.11549	4.11880739	43.88119261
control 72hour 2	89.72465989	104.3023	3.83500528	44.16499472
control 72hour 3	89.78037795	104.3671	3.83262526	44.16737474
Fjoh 72hour 1	66.55987371	77.37394	5.16969947	42.83030053
Fjoh 72hour 2	64.01773692	74.41878	5.37498763	42.62501237
Fjoh 72hour 3	54.27636161	63.09471	6.33967594	41.66032406
Δ 2419 Fjoh 72hour 1	40.4072062	46.97221	8.51567273	39.48432727
Δ 2419 Fjoh 72hour 2	49.06904397	57.04135	7.01245666	40.98754334
Δ 2419 Fjoh 72hour 3	56.54455124	65.73141	6.0853705	41.9146295

Table A.50. PCR program for barcoding the cleaned 16SRNA

Step	Temperature	Time
Initial denaturation	95C	3 minutes
15 cycles		
Denaturation 15 seconds	95C	15 seconds
Annealing	62C	15 seconds
Extension	65C	90 seconds
Cycle end		
Final extension	65C	5 minutes
Storage	4C	Forever

A.7.1 qPCR

Table A.51. The primers used to find a quantitative amount of *Alistipes* within the samples Control, Fjoh, and Δ Fjoh at 48 and 72 hours.

Primers	Nucleotide code
<i>Alistipes communis</i> F qPCR	GAGGGCFTCTTCGGCATGA
<i>Alistipes communis</i> R qPCR	GATGCCGGCCATCGACT

Table A.52. PCR program for primers to create a *Alistipes* standard curve

Step	Temperature	Time
Initial denaturation	95C	3 minutes
15 cycles		
Denaturation 15 seconds	95C	15 seconds
Annealing	62C	15 seconds
Extension	65C	90 seconds
Cycle end		
Final extension	65C	5 minutes
Storage	4C	Forever

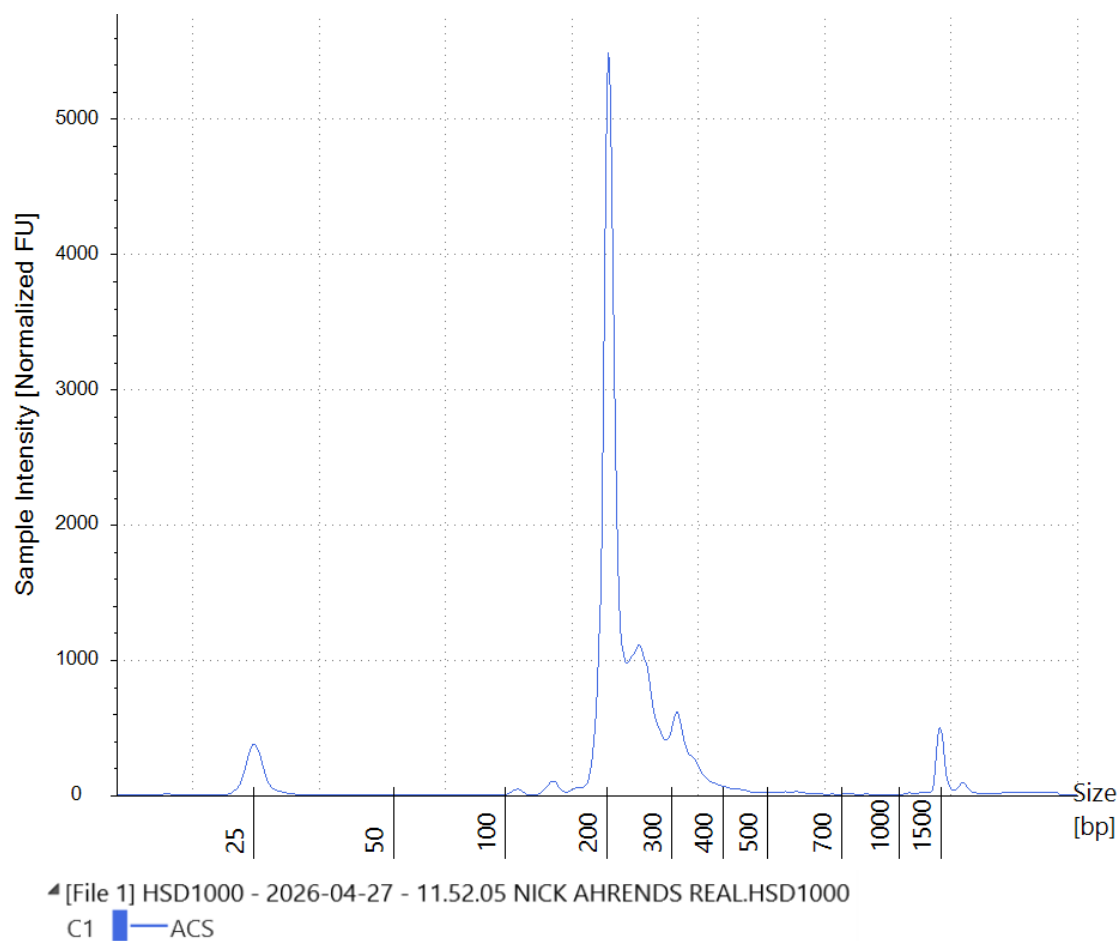


Figure A.8. Graph over the sizes of the DNA fragments found within the *Alistipes Communis* small (ACS) DNA fragment purification. The graph depicts a large amounts of DNA at 203 base pairs which align with the expected size for the primer sequenced made by the used primer pair.

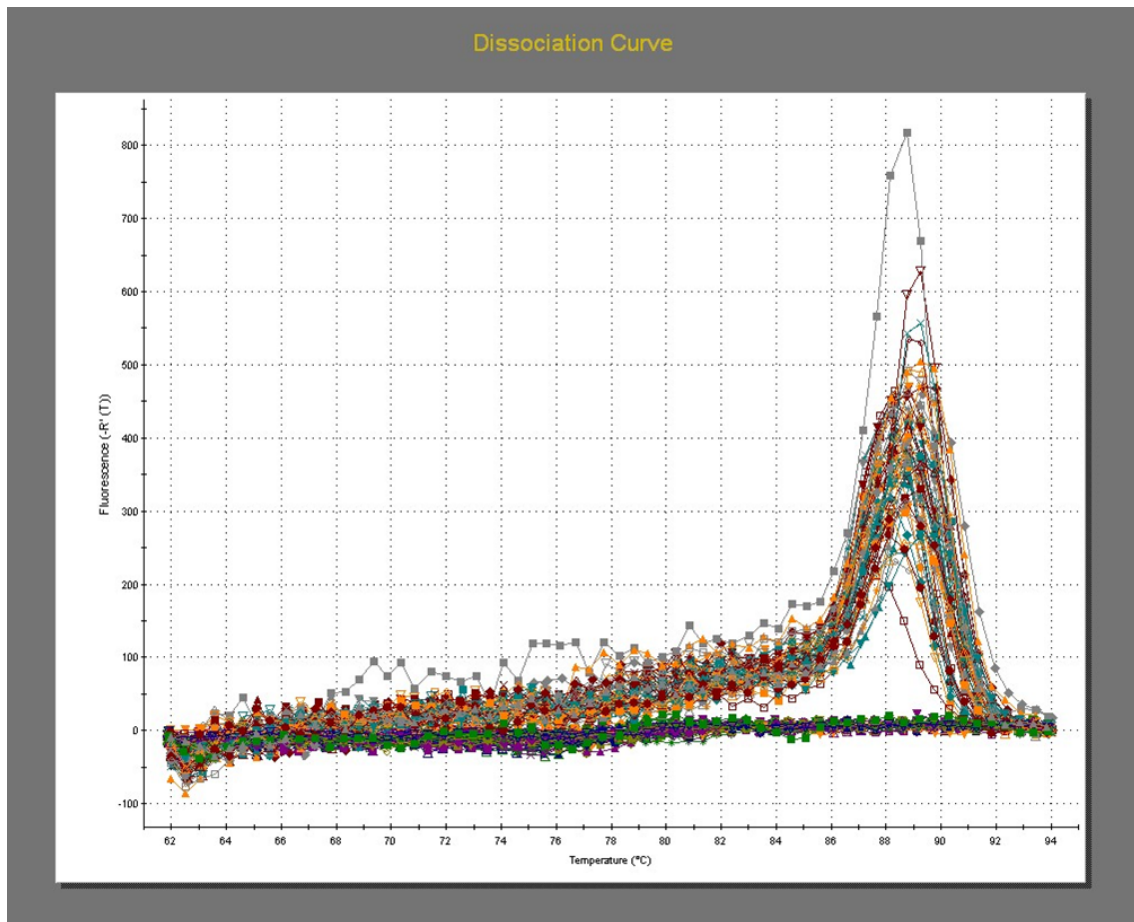


Figure A.9. Dissociation curve over all samples and standards tested in the qPCR. The graphs peak at 88 to 90 °C show that all samples and standards contain a similar amplified region.

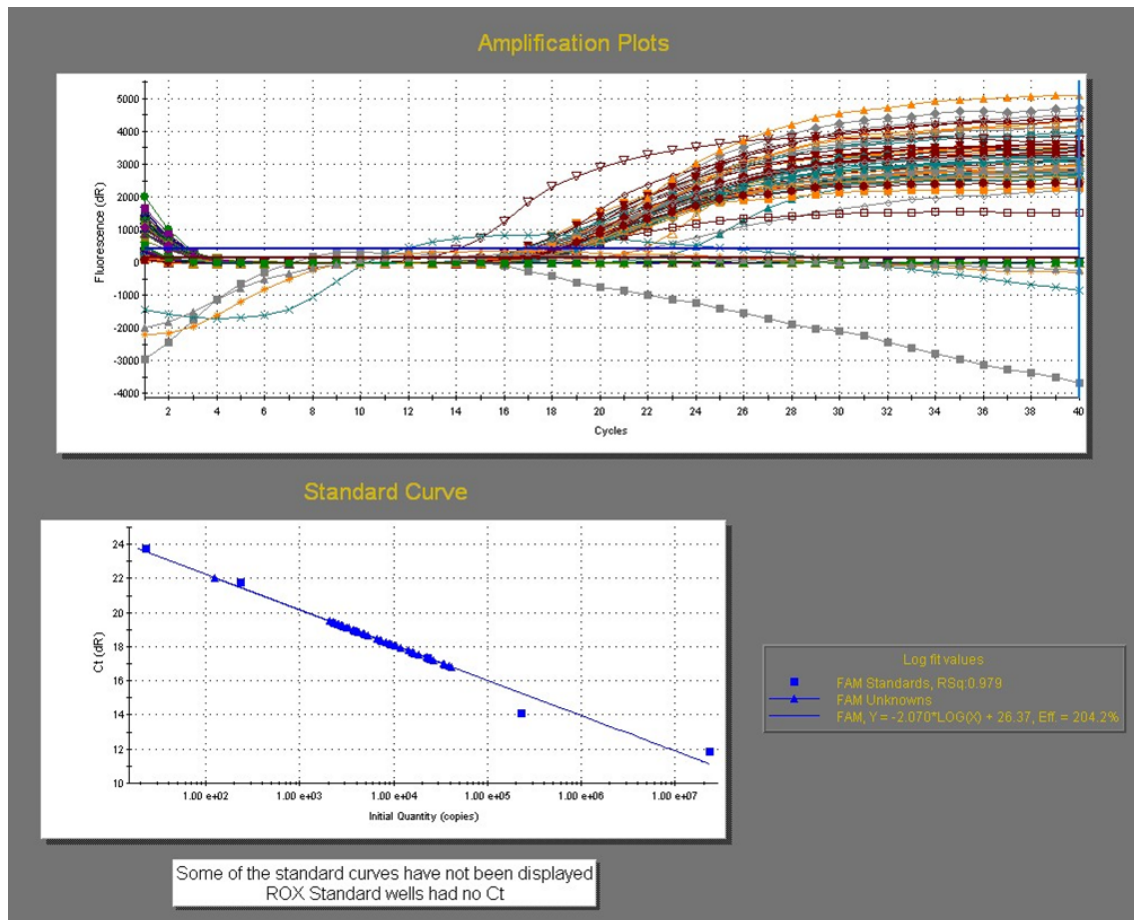


Figure A.10. Amplification plot and Standard curve over qPCR *Alistipes communis* A5CBH24 09830 gene. The amplification plot shows that most samples follow a similar amplification that rises above the threshold between 16 and 22 PCR cycles. The standard curve contains both standards as squares and samples as triangles. The standard curve has an R^2 value of 0.979.