Identification of novel *Faecalibacterium* species and microbial interactions influencing *F. prausnitzii* abundance in the human gut

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Contents

1 Abstract

The human gut microbiota is composed of trillions of microorganisms inhabiting the gastrointestinal tract. Recent advances in metagenomics have revealed tremendous diversity among the microbial inhabitants, offering insight into a dense ecosystem of bacteria, archaea, viruses and eukaryotic microbes. It has also revealed a great degree of heterogeneity between individuals, such that no two people have the same gut microbial profile. One bacterial species, *Faecalibacterium prausnitzii*, has gained attention in the medical world because it has important anti-inflammatory and immunomodulatory properties. *F. prausnitzii* is also unusual in that it is found at a relatively high abundance in nearly all sampled healthy individuals. However, many individuals suffering from gastrointestinal disorders have low levels of *F. prausnitzii*. Therefore, researchers have proposed *F. prausnitzii* use as a probiotic and as the target of microbiome modification to increase the abundance *F. prausnitzii* in the gut. One challenge is the great diversity among populations ascribed to the *F. prausnitzii* species. Genetic evidence suggests that at least three unique species are currently grouped into the single species designation. The goal of this research was to better characterize the taxonomy of the genus *Faecalibacterium* and identify mechanisms behind the specific microbial interactions that promote the high abundance of the most dominant *Faecalibacterium* population in the gut, *F. prausnitzii* A2-165.

2 Introduction

2.1 Background

2.1.1 What is the gut microbiome?

The human microbiome is an ecosystem of microbial organisms that inhabit all people's bodies. Up to 10^{14} microbial cells (bacteria, archaea, fungi and other eukaryotic microbes) live on and within the human body, outnumbering the total number of human cells in an individual. There are also roughly 100 times more microbial genes than human genes in the body. [28]. The microbes are spread across nearly all surfaces of the body, even those that are within the body. While there is a diverse taxonomic array of microbes living in the nasal cavity, skin and mouth; the most biodiverse and densely populated microbial ecosystem is within the colon [9]. The human gut microbiome is a dynamic and instrumental organ in the human body, composed of trillions of microorganisms inhabiting a unique ecological habitat inside the gastrointestinal tract. Here, food particles undigested by host enzymes or microbes in the small intestine are broken down and fed upon by gut microbes. Many of them make beneficial products for the host, such as vitamins and energy sources. The gut microbiome also primes the host immune system [34] and provides colonization resistance against pathogens [24]. The composition of the gut microbiome is highly variable between individuals. This variability is partially driven by host genetics, but even

monozygotic twins have different microbial profiles. [14].

2.1.2 What is the significance to human health?

Dysbiosis in the human gut microbiome, defined as "loss of keystone taxa, loss of diversity, shifts in metabolic capacity, or blooms of pathogens" by Vangay *et al.* 2015 [21], has correlations to various diseases, especially gastrointestinal diseases like Inflammatory Bowel Disease (IBD), Colorectal Cancer (CRC) and Irritable Bowel Syndrome (IBS) [25]. A proposed mechanism for the development of IBD is that the immune system attacks resident gut microorganimsms leading to intestinal inflammation and decreased microbial diversity within the gut [18] [25]. IBD is a general term for the diseases Crohn's Disease (CD) and Ulcerative Colitis (UC), which differ mainly in where along the gastrointestinal tract the inflammation is occurring. Inflammation in UC is restricted to the distal colon, with inflammation occurring at the mucosal layer. Inflammation in CD appears in patches at any part of the GI tract, and may be transmural (inflaming all layers of the intestinal epithelial tissue). [29].

2.1.3 Why is *Faecalibacterium prausnitzii* significant?

F. prausnitzii is one of several bacteria whose abundance is altered in the guts of patients suffering from CD, UC, and some cases of CRC and IBS. The species is most commonly deficient in recurrent CD patients, and has been suggested as a biomarker for differentiating CD from UC [25]. A sepa-

rate multi-omic study found that *F. prausnitzii* is among the most depleted bacterial species in abundance and downregulated enzymatically in dysbiotic gut microbiomes in IBD [32]. These bacteria are particularly important as the species is among the most abundant and prevalent within the gut. The abundance of *F. prausnitzii* in a healthy gut is about 5% [5], but in some cases *F. prausnitzii* can constitute up to 15% of the total gut bacteria [27]. Data from a healthy human cohort of University of Michigan students reveals that *F. prausnitzii* is both highly prevalent (detectable in many different individuals) and abundant (a significant proportion of the total gut bacteria), at about 4.55% relative abundance in this dataset. The vast majority of bacterial species are neither predominant nor prevalent (the majority of dots towards the bottom left of Panel A in Fig. 1). Panel B of Fig. 1 depicts the relative abundance of *F. prausnitzii*, typically between 3-5% in this healthy college cohort, with the number of students who harbor *F. prausnitzii* in their guts, which is consistent with other datasets.

F. prausnitzii is a Gram-stain negative bacterium. It is a member of the family *Ruminococcaceae* in the phylum *Firmicutes*, which is one of the dominant bacterial phyla within the human microbiome [27]. *F. prausnitzii* is one of the few bacteria in the colon that make the short chain fatty acid (SCFA) butyrate. Butyrate is a potent anti-inflammatory metabolite, a food source for gut epithelial cells, and it aids in preventing colon cancer and IBD [25]. Butyrate also induces differentiation of colonic regulatory T cells, which adds to butyrate's immunomodulatory activity [11]. Thus, *F. prausnitzii* plays a

crucial role in maintaining gut epithelial health and immune function, which is lacking in patients suffering from IBD. This has led researchers to identify *F. prausnitzii* as a "New Generation Probiotic", targeted at restoring populations of this commensal bacterium in patients deficient in *F. prausnitzii* [26]. Given *F. prausnitzii*'s anti-inflammatory properties through its production of butyrate and other metabolites, its atypically high prevalence and abundance in healthy gut microbiotas, and decreased abundance in particular disorders of the GI tract, *F. prausnitzii* is a critical member of the human gut microbiome. When looking for mechanisms of altering a dysbiotic gut microbiome, restoring populations of *F. prausnitzii* should be an initial aim.

2.1.4 What does *F. prausnitzii* need for growth?

F. prausnitzii is an obligate anaerobe that is oxygen-sensitive, but can survive in low levels of oxygen [25]. *F. prausnitzii* also requires acetate for growth *in vitro* and in the gut, implicating cross-feeding between it and acetate-producing bacteria. Among acetate-producers, Nanditha Ravishankar described a strong preferential cross-feeding between *F. prausnitzii* and the acetate-producer *Bifidobacterium adolescentis* for her Master's thesis research. *B. adolescentis* produces acetate and other maltooligosaccharide end products as a result of its degradation of resistant starch, which are both carbon sources for *F. prausnitzii* (Fig. 2). One aim of my honors thesis research was to elucidate the mechanism behind the preferred *B. adolescentis*

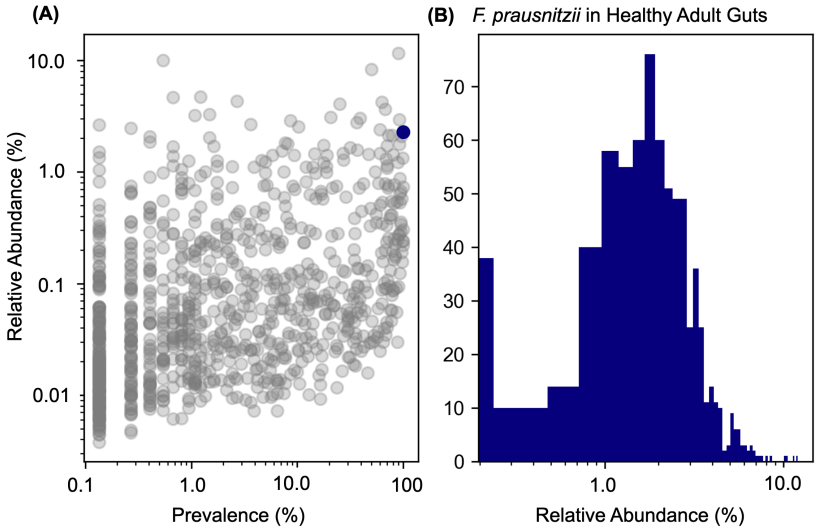


Figure 1: Abundance and prevalence of *Faecalibacterium prausnitzii*. In the panel A, each dot represents a different bacterial species that was detected through amplifying the V4 region of the 16S rRNA gene from bacteria in fecal samples. The blue dot represents ASV2, an *F. prausnitzii* sequence that is among the most abundant (y-axis) and most commonly found (x-axis). Panel B shows the frequency distribution of ASV2 abundances in different individuals. The average relative abundance of ASV2 is 4.55% in this dataset. Credit: Jonathan Golob

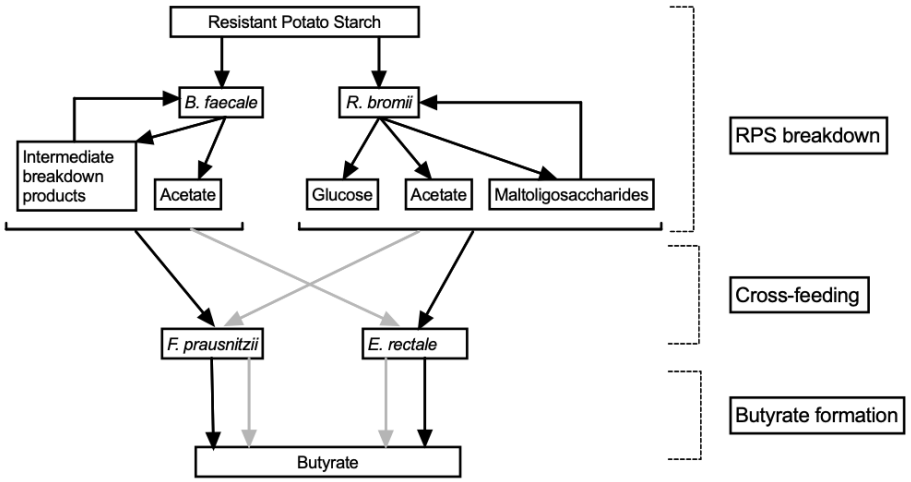


Figure 2: A proposed schematic for the preferential cross-feeding of *F. prausnitzii* with *B. adolescentis*, described here as *B. faecale*, and another abundant RPS-degrading bacteria, *R. bromii*, with the abundant and prevalent secondary degrader *E. rectale*. This image was adapted from Figure 6 of Nanditha Ravishankar's Master's thesis titled "Preferential Cross-feeding among Gut Bacteria"

and *F. prausnitzii* A2-165 cross-feeding that allows *F. prausnitzii* to outcompete *Eubacterium rectale*, its natural competitor within the gut (Fig. 3).

2.1.5 Evidence for at least three species within *F. prausnitzii*

Despite only one species in the current taxonomy of the genus *Faecalibacterium*, genetic variability of isolates of *F. prausnitzii* suggest there should be at least three species within the genus. Multiple species with the same designation may be a contributing factor to the incredibly high abundance

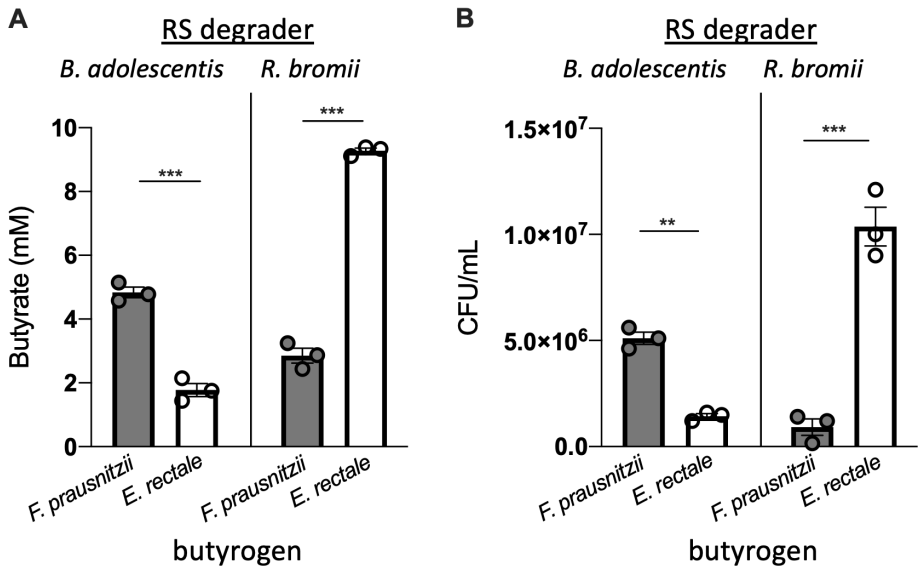


Figure 3: In tri-culture of *Bifidobacterium adolescentis* or *Ruminococcus bromii* with both *E. rectale* and *F. prausnitzii*; a preferential cross-feeding occurs between the starch-degrading bacterium *B. adolescentis* and *F. prausnitzii* and also *R. bromii* and *E. rectale* when grown on media supplemented with resistant starch from potatoes as the carbon source. There is a concurrent increase in relative butyrate production (Panel A) as the populations of butyrogens increase (Panel B) depending on the primary RS degrader. Credit: Nanditha Ravishankar

and prevalence of *F. prausnitzii*. One early and conservative method that has been used to distinguish isolates of *F. prausnitzii* is assignment into one of two Phylogroups based on data from Lopez-Siles *et al.*, 2012 [10]. Phylogroup I of *F. prausnitzii* contains isolates related to the strains ATCC 27768 and ATCC 27766. Phylogroup I strains tend to be depleted in the gut microbiome of patients with a range of gastrointestinal disorders including IBS, CRC, and both UC and CD [25]. Phylogroup II strains of *F. prausnitzii* include the most abundant population in the gut, A2-165, which is also the most extensively studied. Populations of Phylogroup II tend to be less affected by gut disorders, but can be significantly depleted in patients with CD (Fig. 4) [25][27]. While the designation of two Phylogroups is a good step in addressing the diversity within the genus *Faecalibacterium*, it still underestimates the diversity within the genus.

Fitzgerald *et al.* 2018 analyzed whole genomes of 11 *F. prausnitzii* strains and uncovered a high degree of genome plasticity and low level of average nucleotide identity among them [27]. Due to genetic and functional differences, they proposed that Phylogroup I strains should be defined as a separate species, deemed *F. moorei*, using ATCC 27768 as the type strain, which was the original type strain for the genus *Faecalibacterium*. They also propose all other *Faecalibacteria* strains will remain *F. prausnitzii*, using strain A2-165 as the new type strain. However, they do note that there is still tremendous diversity within these remaining strains, particularly in those strains related to Phylogroup II proposed by Lopez-Siles *et al*, or in the strains related to

F. prausnitzii A2-165, cited by Fitzgerald *et al* 2018 [27]. This species separation aligns closely to the separation by Phylogroup, but takes a larger step in stating that the genetic and functional differences between the strains are enough to distinguish species. However, this study still underestimates the true diversity within the genus by proposing only two separate species.

The Schmidt lab houses a bacterial metagenomic database from fecal samples of healthy undergraduates. Using this large dataset of over 5000 fecal samples, Amplicon Sequence Variant (ASV) designations were assigned to each unique Variable 4 (V4) region of the 16S rRNA gene. Each bacterial cell contains one or more copies of the 16S rRNA gene, and only one copy from each cell is ultimately sequenced after amplification. *F. prausnitzii* contains six copies of the 16S rRNA gene, and each individual copy in a particular cell may contain a unique ASV sequence [19]. Through comparing these sequences to their best match from publicly verified 16S rRNA genomic databases, best species matches were assigned to each ASV. Over 600 unique V4 regions of the 16S rRNA gene were best matched to *F. prausnitzii*, demonstrating great diversity within this gene in the genus *Faecalibacterium*. The most abundant and prevalent ASVs assigned to *F. prausnitzii* in this database were ASV2, ASV8, ASV14, ASV32, ASV78, and ASV81. When looking at ASV matches to the Basic Nucleotide Alignment Tool (BLAST) repository of known 16S rRNA genes; ASV8, ASV32, and ASV81 match closest to Phylogroup I sequences, while ASV2, ASV14, and ASV78 match closest to Phylogroup II sequences [2].

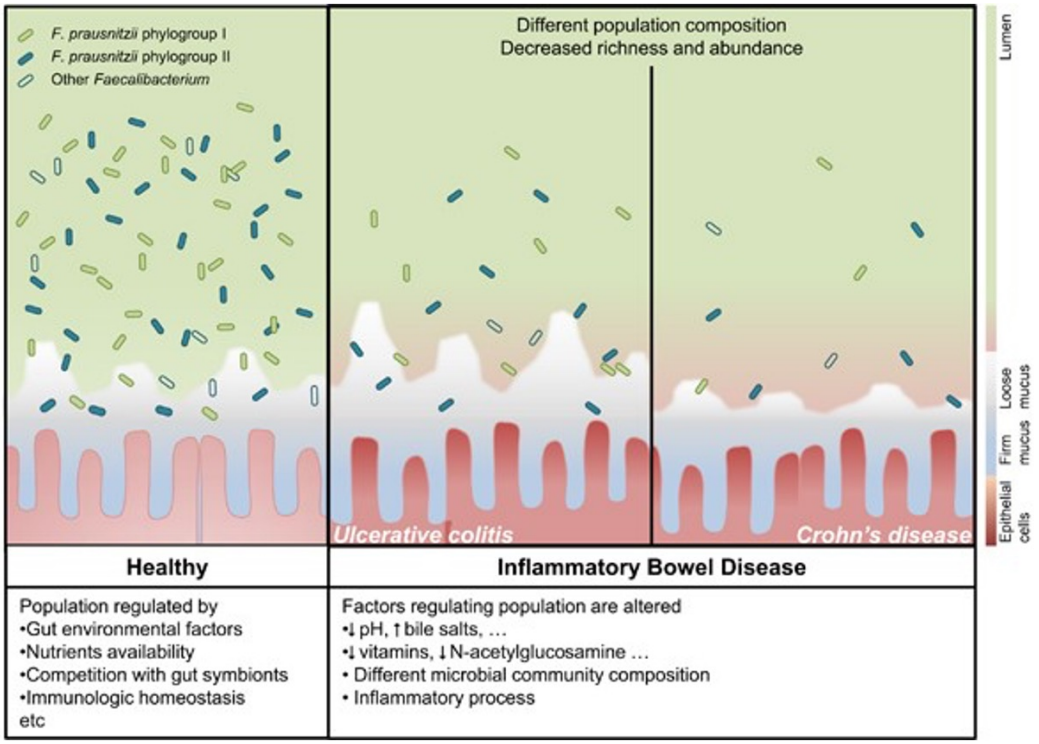


Figure 4: Differential depletion in *F. prausnitzii* Phylogroups in individuals with different manifestations of Inflammatory Bowel Disease: Ulcerative Colitis (UC) as compared to Crohn's Disease (CD). *F. prausnitzii* Phylogroup I strains tend to be depleted in most gastrointestinal disorders, whereas Phylogroup II strains tend to only be significantly depleted in individuals with Crohn's Disease [25].

2.2 Research Questions

Given the importance of a strong population of *F. prausnitzii* to overall gut health, my research set about to identify differences in genetic similarity and abundance among distinct *F. prausnitzii* ASVs and to identify a mechanism by which *F. prausnitzii* outcompetes its natural competitor, *E. rectale*, while cross-feeding with a starch-degrading *B. adolescentis*. Reporting the true diversity within the genus *Faecalibacterium* is a crucial step towards using specific strains within this genus as probiotics to reconstitute a dysbiotic gut microbiome. Addressing the cross-feeding pathway provides critical knowledge on the complex network of microbial interactions that impact the abundance of *Faecalibacteria*, and may uncover environmental conditions required to increase *Faecalibacteria* growth.

3 Materials and Methods

3.1 Cultures

The bacterial cultures used for these experiments were three *Faecalibacterium prausnitzii* strains (A2-165, ATCC 27768, and ATCC 27766), one *Eubacterium rectale* strain (A1-86), and one *Bifidobacterium adolescentis* strain (269-1). The identity of all five cultures was confirmed by sequencing the 16S rRNA gene using PCR and comparing to known sequences on the Nucleotide Basic Local Alignment Search Tool (BLAST) [2].

3.2 Media

The media used for culturing and testing during the pH experiments was SAB4 media, enriched initially for growing anaerobes, including methanogenic archaea. The SAB4 media contained per 1000 mL: 50 mL SAB3 Mineral 1 solution, 50 mL SAB3 Mineral 2 solution, 10 mL SAB3 Trace Metals, 20 mL Balch Vitamins, 1 mL 0.1% Resazurin, 10 mL 1 g/L in 20 mM NaOH Hemin Stock, 10 g Bacto-Tryptone BD211705, 2.5 g Bacto-Yeast Extract, 2.7 g Sodium Acetate, 2 g Glucose, 2 g Cysteine, 20 mL Short Chain Fatty Acid solution, 3.75 g Sodium Bicarbonate and then brought up to 1000 mL with H2O. Experimentation of the SAB4 media at the buffered pH values of 5.5, 6.2, 6.7, 7.2, and 8 was completed as these values fall within the natural pH range of the human colon. For the experiments requiring specific pH SAB4 media, 0.5 M MES, 1 M MOPS and Sodium Bicarbonate buffers were used.

The amount of 0.5 M MES and 1 M MOPS buffers for the pH values of 5.5 and 6.2 for MES and 6.7, 7.2 and 8 for MOPS were determined using the GoldBio Stock Solution measurements. The amount of Sodium Bicarbonate added to each pH buffered SAB4 media was determined using the Henderson Hasselback Equation. The Sodium Bicarbonate was added to the buffered media inside the anaerobic chamber to ensure a 5% atmospheric CO2 level. The YCFA media (yeast extract, casitone, and fatty acids) is another enriched media that was used for all other experiments, to keep standard with previous research on *F. prausnitzii* and *E. rectale* performed in the Schmidt lab. The protocol for this media was initially described by Harry Flint [4]. Studies with YCFA media were buffered with MOPS and sodium bicarbonate to a pH of 6.7 in order to facilitate growth of *F. prausnitzii* and *E. rectale* and mimic a pH found in the natural gut environment. The notation YCFAG is used to indicate YCFA media with glucose.

3.3 Biology 173 Database

The Schmidt lab houses metagenomic data from over five thousand fecal samples collected from over 900 hundred undergraduate students at the University of Michigan enrolled in the Biology 173 laboratory course. The samples were sequenced based on the Variable 4 region of the 16S rRNA gene to identify the bacteria present at a detectable limit in each fecal sample. The identified sequences were aligned to the most closely related organisms and also given a relative abundance within the fecal sample. In the database,

thousands of Amplicon Sequence Variants (ASVs) are best matched to *F. prausnitzii*, with a few highly prevalent and abundant ones. Using the multiple sequence alignment tool ClustalO [6][7][13] and RStudio (3.6.2) [33], the ASV data from the fecal samples were analyzed to identify trends in the ASVs assigned to *F. prausnitzii*, and to look for correlations between the ASVs and the three different *F. prausnitzii* strains within the lab: A2-165, ATCC 27766, and ATCC 27768. Based upon best BLAST alignment matches [2], ASV2 is most closely related to strain A2-165 in Phylogroup II. ASV8 matches closest to Phylogroup I strains, of which ATCC 27768 and ATCC 27766 are members. ASV14 matches closest to other strains in Phylogroup II that are less genetically similar to strain A2-165.

3.4 Growth Rate Measurements

The BioTek Log Phase 600 (LP600) was used to measure Optical Density (OD or absorbance at 600 nm) over a 24 or 48-hour period. Using flat bottomed 96-well plates, 5 µL of cultures of *F. prausnitzii* and *E. rectale* in exponential phase were added to 150 µL of SAB4 media buffered to a different pH, or YCFAG media with different bile salts added in four or eight replicates. The LP600 measured OD every 20 minutes, at constant incubation temperature of 37^{o}C while shaking at 800 RPM. The raw data were imported into the GrowthRates 5.0 command-line program created by the Bellingham Research Institute [15] to obtain the specific growth rate (k) and maximum OD of each replicate. Wells were blanked with sterile media.

The results were compiled in Microsoft Excel and analyzed and visualized in GraphPad Prism (9.3.1).

3.5 Co-sedimentation Experiment

Using a technique optimized by a previous lab member from a published protocol [3], I conducted a co-sedimentation experiment to determine if the primary starch degrader *Bifidobacterium adolescentis*, which the Schmidt lab has experimentally confirmed attaches to starch granules with a pilus, and the butyrogen *F. prausnitzii* physically associate while in stationary phase and in the presence of resistant starch from potatoes (RSP). The medium (YcfE) was a modified version of YCFA with no glucose, less casitone, only the branched chain fatty acids (BCFAs) and more MOPS buffer. Samples in an autoclaved hungate tube contained 2 mL of filter-sterilized deoxygenated YcfE media along with 1 mL of a stationary phase culture of *B. adolescentis* and 1 mL of a stationary phase culture of *F. prausnitzii*. The experimental samples contained 1 mL YcfE media, 1 mL of each culture, and 1 mL of sterile and deoxygenated 1 g/mL of Bob's Red Mill Resistant Potato Starch that had been washed twice with filter sterilized MilliQ water and 70% ethanol once, and then suspended in sterile MilliQ water to a concentration of 1 g/mL. The Hungate tubes were shaken for 30 minutes then allowed to settle for an hour. After the settling period, 100 µL of the supernatant of each replicate of the control and RSP tubes were serially diluted to 10^{-5} and plated on YCFC plates that contain both glucose and SCFAs necessary for

B. adolescentis and *F. prausnitzii* growth. A physical association or adhesion would be supported if there was a concurrent decrease in Colony Forming Units (CFUs) of the supernatant of tubes from the RSP samples versus from tubes from the control samples.

3.6 Yield Experiment

Early stationary phase cultures of *F. prausnitzii* A2-165 and *E. rectale* were analyzed using the Casy Cell Counter from OMNI Life Science to identify the number of cells in a mL of culture. 10 µL of growing early stationary phase cultures were added to 10 mL of Casyton solution, which was inserted into the Casy capillary for analysis.

3.7 Bioreactor Experiment

A preliminary experiment using a bioreactor system was designed to provide media to stirring wells at a constant rate while also excreting waste media to maintain a constant volume. In collaboration with the laboratory of Dr. Vincent Young, the bioreactors were used to compare the growth of *F. prausnitzii* A2-165 and *E. rectale* in both monoculture and coculture, to isolate the impact of a constant slow rate of nutrient administration on growth. This system mimics the slow rate of glucose, acetate and oligosaccharide production from a resistant starch-degrading *B. adolescentis*. The procedure followed that described in *Clostridium difficile Methods and Protocols* [22].

150 µL of exponential phase *F. prausnitzii* A2-165 and *E. rectale* were added to 15 mL of YcfE media that does not contain glucose or other carbon sources. 3 replicates were created for each monoculture. 100 µL of each exponential phase bacterium were added to 15 mL of YcfE media for the coculture bioreactors, with 6 total replicates. After inocultion, the media was changed such that YCFAG media would pump into each bioreactor at a flow rate of 0.96 mL per hour for a total of three days. 1 mL of culture from each bioreactor was extracted each day, including day 0, to analyze the relative number of cells of each species at each time point. The number of cells in each culture was inferred through a dilution series on agar YCFC plates [20]. Future efforts to compare relative abundance data in coculture will use qPCR with two primers specific to *F. prausnitzii* and *E. rectale* individually, and a third universal primer specific to copies of the 16S rRNA gene which are present in both bacteria [8]. The procedure for qPCR will follow that described for enumeration of *C. difficle* in Auchtung *et al* 2016 [22]. This experiment yielded preliminary data, but will require additional adjustments in the procedure to test the desired hypothesis.

3.8 Statistical Analyses

Analysis of the Biology 173 data was performed in RStudio (3.6.2) and ClustalO. Specific Growth Rate measurements of OD data were performed with GrowthRates 5.0 [15]. Statistical analyses and the creation of graphs involved in all other studies were performed on GraphPad Prism (9.3.1).

4 Results

4.1 Multiple Species

4.1.1 Genetic Diversity

There is only one species defined within the current taxonomy of the genus *Faecalibacterium*. However, this is because the traditional metabolic and enzymatic traits failed to separate subgroups [10]. However, recent studies of whole genome sequences demonstrate that multiple species can be distinguished within the genus [27]. Amplicon Sequence Variant (ASV) data from the Biology 173 study are consistent with multiple *Faecalibacterium* species. ASVs that best match the V4 sequence of different known strains of *F. prausnitzii* cluster into at least three groups using Uniform Manifold Approximation and Projection (UMAP) based on the bit score (Fig. 5). The three distinct clusters that appear in this analysis suggest at least three subgroups of closely related *F. prausnitzii* ASVs exist in this dataset, supporting the hypothesis that there are three or more species within the current taxonomic designation of *Faecalibacterium prausnitzii*. The most common *F. prausnitzii* ASVs are ASV2, ASV8, ASV14, AV32, ASV78, and ASV81, which are visualized in Figure 6. ASV2, ASV8 and ASV14 are the three most abundant and prevalent ASVs. ASV2 is the most abudant and prevalent and has an average relative abundance of 4.55% in healthy undergraduate students' fecal samples. The existence of three clades within the genus is also

evident in a phylogenetic tree of the six most common *F. prausnitzii* ASVs (Fig. 7). The three most common ASVs (ASV2, ASV8 and ASV14) and their related sequences represent these three branches. The cladogram also demonstrates that ASV2 and ASV8 are more genetically divergent in the V4 region of the 16S rRNA gene from the other four most common *F. prausnitzii* ASVs. Although they can co-exist in the same community, in most individuals when one *F. prausnitzii* ASV is abundant, the others are present at low levels (Fig. 8). Thus, these ASVs appear to be in competition with one another within the gut, supporting the notion that there are multiple species of *F. prausnitzii*. Previous research in the Schmidt lab found that the various ASVs that represent *F. prausnitzii* correlate in abundance differently with primary starch degrading bacteria, as shown in Figure 9. For example, while ASV2 correlates strongly with *B. adolescentis*, ASV8 has no correlation with an abundant starch degrader, ASV14 has a significant correlation with *Bifidobacterium pseudocatenalatum*, and ASV32 has its most significant correlation to *R. bromii*. These differences offer further support for separate *F. prausnitzii* species as represented by different ASVs that occupy distinct niches within the gut by cross-feeding with separate starch degrading species.

Using this large abundance and genetic dataset, I propose there are at least three different *F. prausnitzii* species, each dominated by a different ASV: ASV2, ASV8, and ASV14. Lopez-Siles *et al.* 2012 reported two Phylogroups of the same species, Fitzgerald *et al.* 2018 suggested two species, but at least three species are supported from this data.

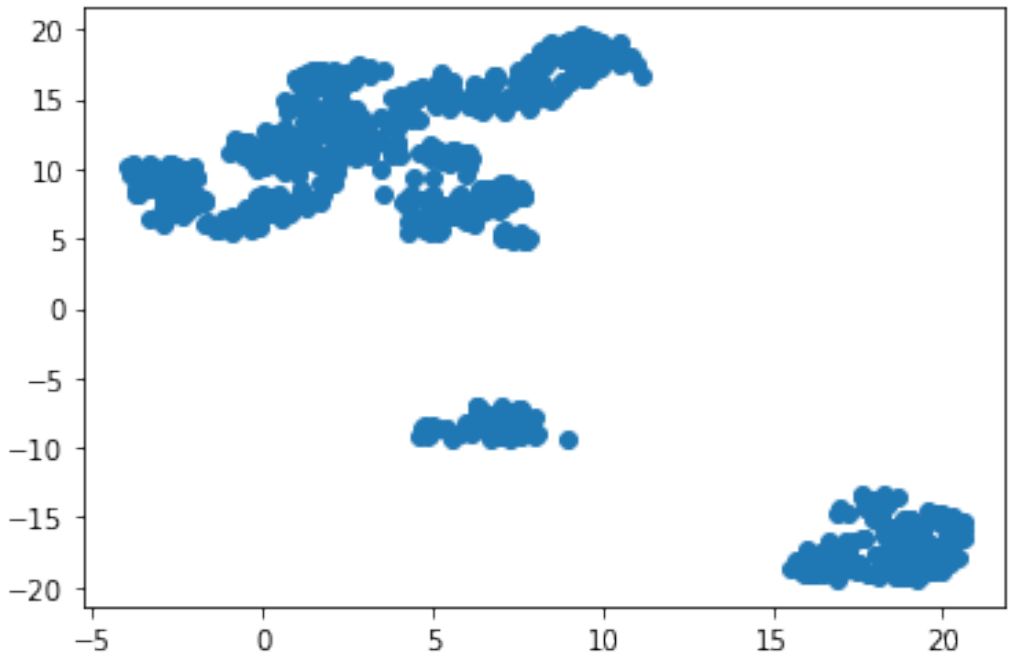


Figure 5: Uniform Manifold Approximation and Projection (UMAP) plot mapping each *F. prausnitzii* ASV by its bit score, or statistical significance of its alignment to reference genomes in BLAST. Each individual point is a different ASV that best matches to *F. prausnitzii*. Credit: Jonathan Golob

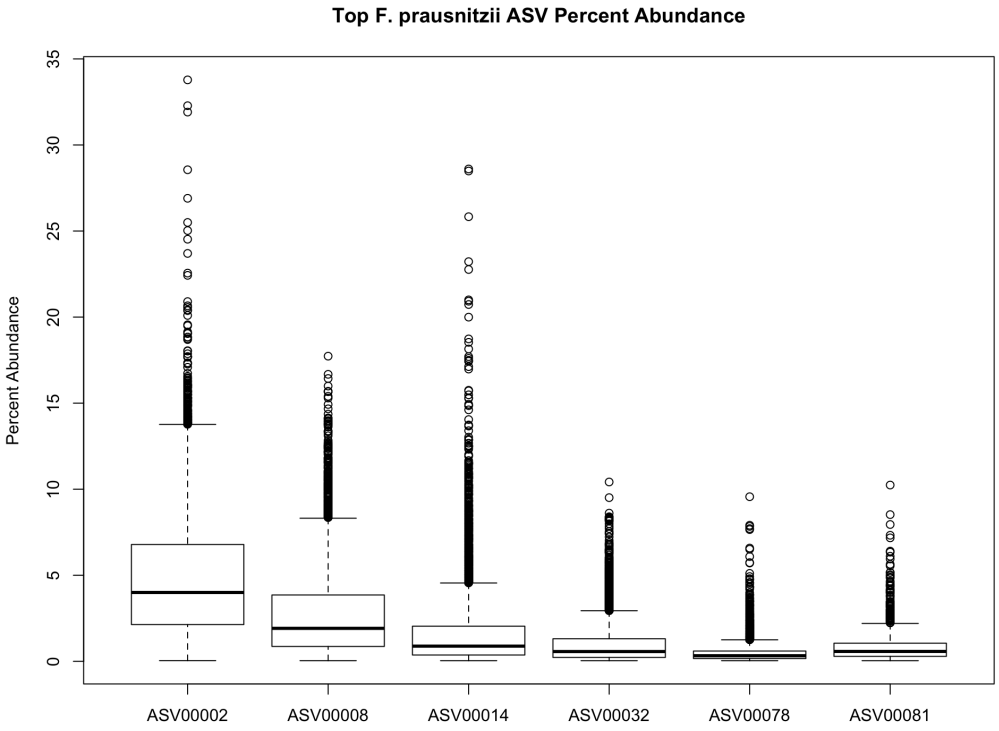


Figure 6: Relative abundance of the six most abundant *F. prausnitzii* ASVs in different healthy individuals.

Figure 7: Multiple Sequence Alignment cladogram of the 16S gene of the top six most abundant *F. prausnitzii* ASVs created with ClustalO.

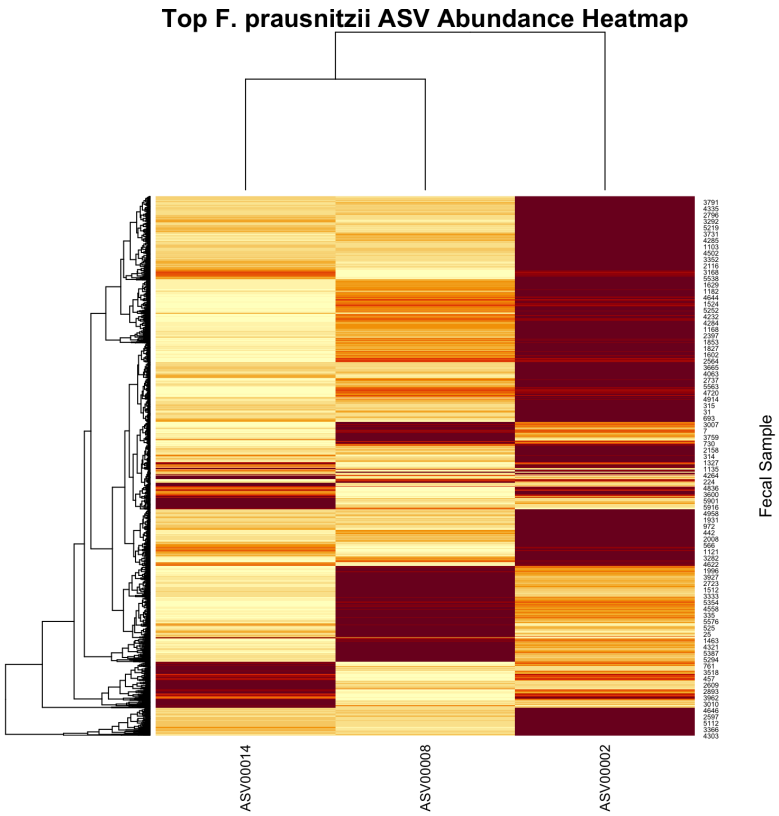


Figure 8: Heatmap of the differential abundance of the three most abundant *F. prausnitzii* ASVs. Darker colors are higher relative abundance.

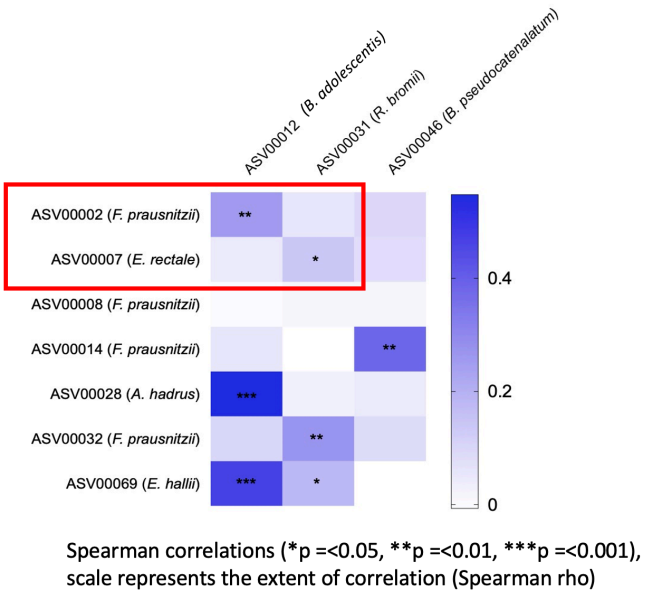


Figure 9: Spearman correlations of various secondary consumers (y-axis) with three abundant primary resistant starch degraders (x-axis) from the Biology 173 abundance dataset. Separate ASVs that best represent different populations of *F. prausnitzii* demonstrate differential correlations with primary starch degraders. The red box indicates the correlations between the two most abundant and prevalent degraders and consumers. Credit: Nanditha Ravishankar

4.1.2 Differential Response to Bile Salts

Bile acids are a potent gut environmental factor that impact the composition of the bacterial community [17]. Bile acids are produced by the degradation of cholesterol, and are synthesized in the liver. The primary bile acids, of which cholic acid (CA) and chenodeoxycholic acid (CDCA) are the major forms, can be conjugated with amino acids, typically taurine or glycine, to become primary conjugated bile salts [30]. Bacterial degradation of these primary bile acids creates secondary bile acids, such as deoxycholic acid (DCA), lithocholic acid (LCA), and many others. In Crohn's Disease (CD), patients have an elevated concentration of excreted total bile acids within their feces, and decreased serum levels of bile acids, partially driven by host bile acid malabsorption (BAM) [30]. Individuals with CD also were found to have significantly elevated levels of fecal cholic acid, taurocholic acid, and glycocholic acid, but decreased levels of secondary bile acids. This interaction implies a dysbiosis in the gut microbiome of many individuals with CD, due in part to a depletion in bile salt transforming bacteria [32]. Taurocholic acid, formed from the conjugation of CA with taurine, is of particular interest as it is a known potent germinant for spore-forming bacteria and important for isolating bacteria that are typically difficult to culture [23]. While *F. prausnitzii* is not a spore-former, *F. prausnitzii* is also difficult to isolate from fecal samples, and previous research in the Schmidt lab suggested that certain strains plate more efficiently when taurocholate is added. The impacts of taurocholate and cholate on growth of three strains of *F. praus-*

nitzii and one strain of *E. rectale* were determined. When exposed to 1.86 mM taurocholate (0.10% W/V), *F. prausnitzii* A2-165 and *E. rectale* experienced marked decrease in maximum optical density, whereas strains ATCC 27766 and ATCC 27768 did not, as shown in Figure 10. Again, Phylogroups I and II can be distinguished, this time by sensitivity to hydrophobic primary bile salts. Interestingly, the specific growth rates of all bacteria did not change greatly after introduction of bile salts, indicating the bile salts may only infer negative growth effects to *E. rectale* and *F. prausnitzii* A2-165 after they are already in their exponential phase, or at a sufficiently high population density.

One proposed hypothesis is the expression of Bile Salt Hydrolase (BSH), 7 alpha-dehydroxylase (ADH), or hydroxysteroid dehydrogenase (HSDH) enzymes by *E. rectale* and *F. prausnitzii* A2-165, but not by the two ATCC strains. BSH enzymes cleave the conjugated taurine or glycine of taurocholate or glyocholate, respectively, to release the amino acid and a cholate [16]. Then, ADH and HSDH enzymes can act on the primary unconjugated bile acid to produce secondary bile acids, like the potent anti-microbial end product deoxycholic acid. It was hypothesized that the specific growth rate of *E. rectale* and *F. prausnitzii* A2-165 were not decreased because the build up of the metabolic products of bile acid degradation did not occur until later in exponential or stationary phase, which then diminished the maximum optical density in the cultures.

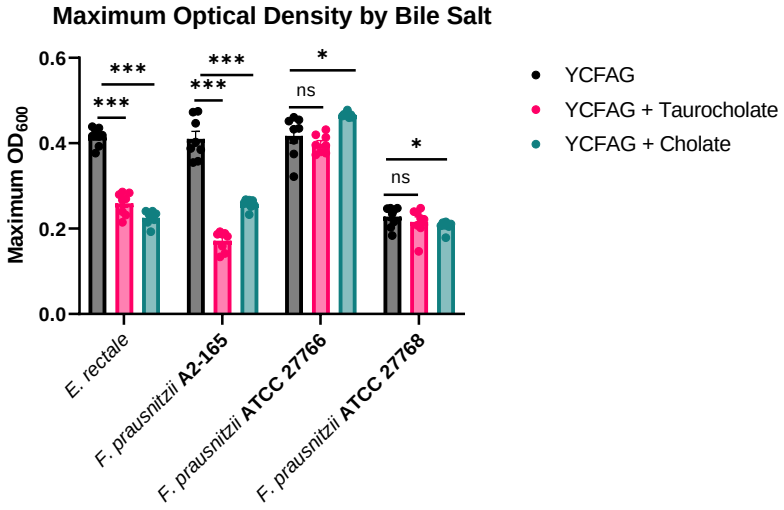
A genome search of these organisms found a Bile Salt Hydrolase (BSH)

gene (Choloylglycine hydrolase EC 3.5.1.24) in *E. rectale* A1-86 and *F. prausnitzii* A2-165, but not *F. prausnitzii* ATCC 27766 or ATCC 27768 [31][2].

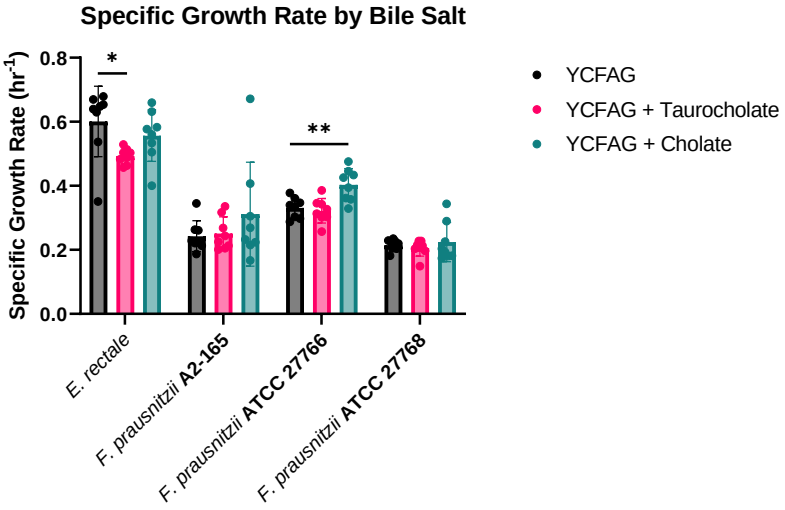
A previous metagenomic study of genes in the bile salt transformation pathway found that there is heterogeneity in the presence of genes in different *F. prausnitzii* strains [31]. For example, *F. prausnitzii* strain L2-6 likely encodes a 12-alpha-hydroxysteroid dehydrogenase (EC 1.1.1.176), or 12-α-HSDH gene, whereas strains M21/2 and SL3/3 do not [31]. This gene codes for an enzyme that inter-converts the primary bile salt, cholate, into the secondary bile acids 12-oxochenodehydrocholate and 12-epicholate [35]. Strain L2-6 is a member of the previously proposed Phylogroup II, with A2-165, and strains M21/2 and SL3/3 are members of Phylogroup I with ATCC 27766 and ATCC 27768. However, A2-165, ATCC 27766 and ATCC 27768 all do not have sequence similarity to the 12-α-HSDH gene [2].

The differential response to the addition of bile salts is another line of evidence for multiple species, as different strains of *F. prausnitzii* may or may not express key enzymes in the bile salt degradation pathway. Additionally, the decreased abundance of Phylogroup II strains (such as A2-165) in patients with Crohn's Disease is consistent with the heightened level of primary bile acids in the feces of patients with CD, as *F. prausnitzii* A2-165 expresses a BSH enzyme which can deconjugate conjugated primary bile salts into cholate, which other gut bacteria can further metabolize into the secondary forms. [25][30]. Reducing the abundance of BSH-expressing bacteria could lead to the increased concentration of excreted bile salts, as conjugated

bile salts are incapable of being passively re-absorbed by the host [30]. Additionally, BSH-expressing bacteria have been proposed as targeted probiotics for decreasing hypercholesterolemia, which is a risk factor for cardiovascular disease [12]. This offers further support for the need to differentiate species within *F. prausnitzii* for use as future probiotics and as the target of microbiome engineereing, for not all species will have the same utility for clinical application. The heterogeneity of enzymes present and absent in different *F. prausnitzii* strains offers further support to the tremendous diversity within the genus and support for multiple species.



Lines depict statistical significance as performed by multiple unpaired t-tests: ns = P > 0.05, * = P < 0.05, ** = P < 0.01, *** = P < 0.001



Lines depict statistical significance as performed by multiple unpaired t-tests: ns = P > 0.05, * = P < 0.05, ** = P < 0.01, *** = P < 0.001

Figure 10: (A) The addition of the bile salts taurocholate and cholate to YCFAG media, and its impact on the maximum optical density of *E. rectale* and *F. prausnitzii* strains A2-165, ATCC 27766, and ATCC 27768. (B) The addition of bile salts does not result in a statistically significant decrease in specific growth rate for any of the strains and species tested.

4.2 Mechanisms of *B. adolescentis* and *F. prausnitzii* cross-feeding

The preferential cross-feeding between the abundant starch-degrading bacteria *B. adolescentis* 269-1 and *F. prausnitzii* A2-165 was described by Nanditha Ravishankar in the Schmidt lab, however the mechanism was not fully elucidated. Potential hypotheses for the pathways which allow *F. prausnitzii* to outcompete *E. rectale* when *B. adolescentis* is the primary starch degrading bacteria, but not with other common starch degraders (like *Ruminococcus bromii*), relate to the physical structure and metabolism of these different species. One hypothesis proposes that *B. adolescentis* is able to physically attach to *F. prausnitzii* A2-165 cells, another is that the low pH created by a starch-degrading and SCFA-producing *B. adolescentis* would favor *F. prausnitzii* growth, and the last hypothesis is that *F. prausnitzii* would outcompete *E. rectale* under a slow rate of nutrient and resource production by the starch-degrader.

4.2.1 Lack of Physical Adhesion

When considering the mechanism of the preferred cross-feeding interaction, the first hypothesis was that a physical adhesion between *B. adolescentis* and *F. prausnitzii* enabled *F. prausnitzii* to outcompete *E. rectale*. To test this hypothesis, a co-sedimentation assay was used to determine if *F. prausnitzii* A2-165 cells adhere to RSP granules directly or to *B. adolescentis* 269-1

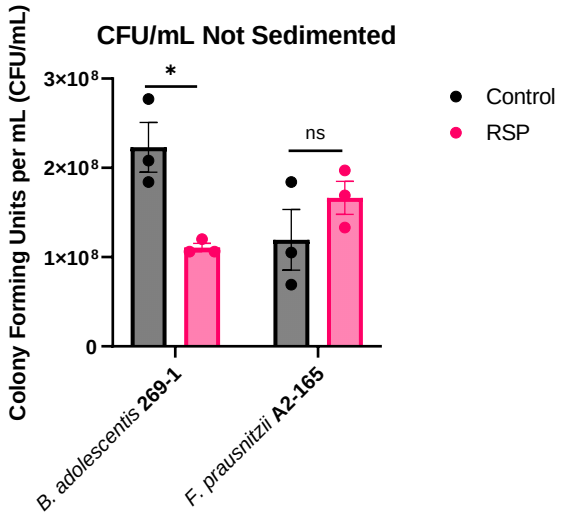


Figure 11: The number of colony forming units per mL (CFU/mL) significantly decreases for *B. adolescentis* 269-1 after exposure to resistant starch from potatoes (RSP), whereas the CFU/mL of *F. prausnitzii* A2-165 does not significantly change.

cells that bind to RSP particles. Stationary phase *F. prausnitzii* A2-165 and *B. adolescentis* 269-1 cells were cultured together in the presence of RSP, then the cells in the supernatant spread on agar medium to determine how many colongy-forming units of each species were not attached to the sedimenting RSP granules. There was a significant decrease in CFU/mL of *B. adolescentis* cells as compared to the control, but not a significant decrease in *F. prausnitzii* cells (Fig. 11). This indicates that adhesion of *F. prausnitzii* cells to starch granules or to the *B. adolescentis* cells attached to starch granules is unlikely.

4.2.2 Fast Growth Rate of *E. rectale*

pH is a component of the gut environment that has profound impacts on the resident microbial community. Sensitivity to pH can infer the capacity for preferential cross-feeding, as the end products of *B. adolescentis* fermentation are acidic SCFAs, which lower pH. Carbohydrate fermentation into SCFAs is among the largest influences on colonic pH, which can range from about 5.4-6.9 or higher depending on the location within the gut and the individual [1]. The acetate and lactate produced by *B. adolescentis* will lower pH in the immediate vicinity, which could favor the growth of one cross feeder over another. To test this hypothesis, the most dominant *F. prausnitzii* strain, A2-165, and *E. rectale* were grown in media buffered to four pH points spanning a pH range found within the colon. *E. rectale* grew at a faster specific growth rate (or shorter generation time) than *F. prausnitzii* A2-165 at every pH point (Fig. 12 & 13). Thus, sensitivity to pH does not explain the preferential *F. prausnitzii* growth over *E. rectale* in co-cultures with the starch-degrading, SCFA-producing *B. adolescentis*.

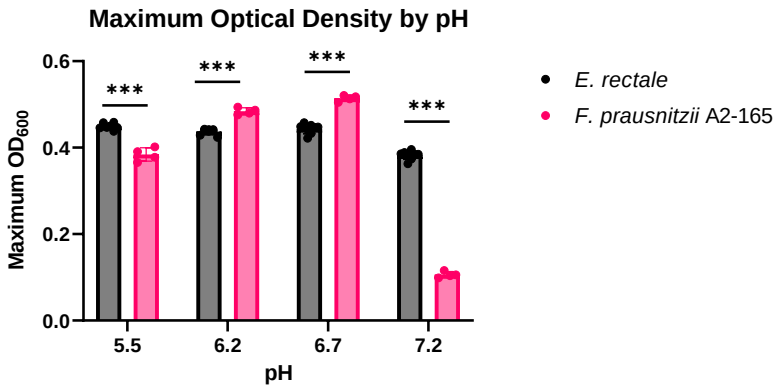


Figure 12: SAB4 media buffered to pH points mimicking a natural range that is found within the gut and its impact on the maximum optical density (OD) of the butyrogenic bacteria *E. rectale* and the most dominant *F. prausnitzii* strain, A2-165.

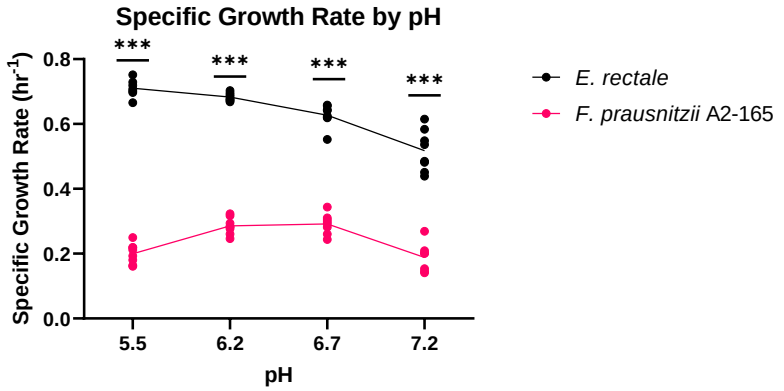


Figure 13: SAB4 media buffered to pH points mimicking a natural range that is found within the gut and its impact on the specific growth rate of *E. rectale* and *F. prausnitzii* A2-165. Specific growth rate is a measure of the fastest growth of each bacterium in its exponential phase.

4.2.3 Differing Life History Strategies

Rate of nutrient supply is another factor in reconstructing the gut environment *in vitro*. Inside the gut, cross-feeding and competition among microbes creates a nutrient-limiting environment that is also dependent on the timing of food ingestion and digestion by the host. A model was developed in order to identify the impact of nutrient administration rate on the growth of *F. prausnitzii* A2-165 and *E. rectale*. Given the faster specific growth rate of *E. rectale* than *F. prausnitzii*, the hypothesis was that a slower rate of nutrient, specifically glucose, supply would favor *F. prausnitzii* growth in co-culture, and a faster rate of nutrient supply would favor *E. rectale* growth. This hypothesis posits that *F. prausnitzii* has a metabolism that favors efficiency at the expense of rapid growth, while *E. rectale* favors the opposite.

An evolutionary tradeoff between high growth rate and low yield, as opposed to low growth rate and high yield exists among organisms. If these two species have opposing life history mechanisms such that *E. rectale* takes the fast growth approach and *F. prausnitzii* takes the slow growth approach, it would be logical that a lower rate of nutrient supply could favor *F. prausnitzii* in co-culture.

Preliminary data suggests that *F. prausnitzii* A2-165 has a higher total cellular yield when it reaches its maximal OD, than *E. rectale* (Fig. 14). Using the Casy Cell Counter, the number of bacterial cells were calculated for each bacterial species, with nearly three times as many *F. prausnitzii* cells detected. Similar data were reported by Austin Campbell. When *F.*

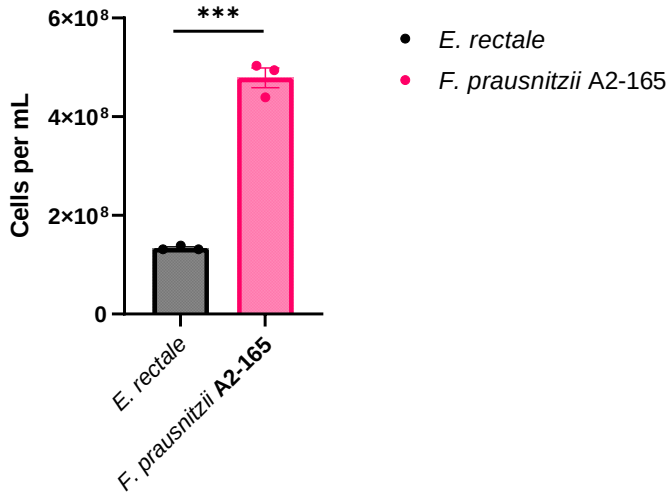


Figure 14: Bacterial cell count per milliliter of culture of *F. prausnitzii* A2-165 and *E. rectale* at their maximal optical density as measured by the Casy Cell Counter and Analyzer.

prausnitzii A2-165 and *E. rectale* were analyzed for their total protein content in culture, *F. prausnitzii* had over double the amount of protein isolated as *E. rectale*. This offers further support for *F. prausnitzii* A2-165 having a higher total cellular yield than *E. rectale* (Fig. 15).

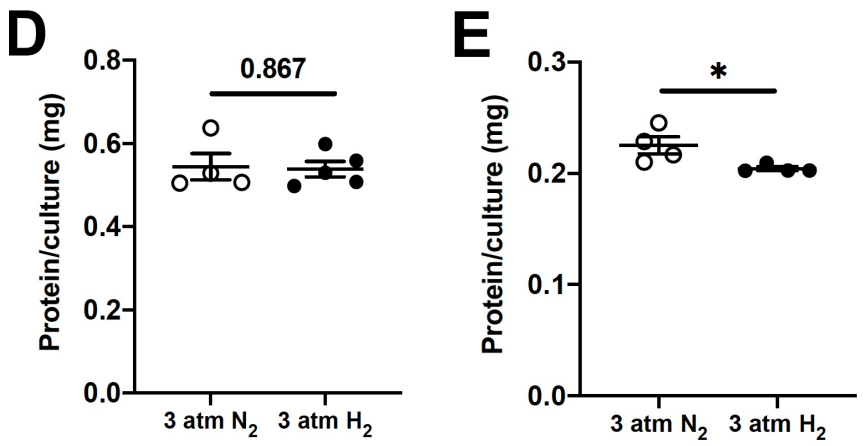


Figure 15: Panel D shows *F. prausnitzii* A2-165 protein yield in culture in mg and Panel E shows *E. rectale* protein yield. The data demonstrates a differential protein yield of *E. rectale* in response to atmospheric hydrogen gas, but not of *F. prausnitzii* A2-165. However, the data also demonstrates a significantly higher total protein yield of *F. prausnitzii* in both conditions. Credit: Austin Campbell

5 Future Directions

5.1 Limited Nutrient Rate

In order to isolate rate of nutrient supply as a potential mechanism that allows *F. prausnitzii* A2-165 to outcompete *E. rectale* in the presence of a starch-degrading *B. adolescentis*, an apparatus that continually supplies a limited supply of nutrients is needed. A designed experiment involves using a bioreactor system to constantly flow nutrients into stirring wells of either monoculture of *F. prausnitzii* A2-165, monoculture of *E. rectale*, or a co-culture of the two species. The flow of fresh media into 15 mL of culture would occur at two flow rates, one high and the other nutrient limiting, for the duration of the 3 day experiment. The slow rate of nutrient administration will mimic the release of glucose, acetate and other oligosaccharides produced by a starch-degrading *B. adolescentis*. Total and relative cellular content in each reactor can be enumerated through both plating and qPCR at each time point.

A preliminary study was performed at one flow rate (0.96 mL/hour) to assess the compatibility of the two bacteria in this system. Samples were diluted and streaked on agar plates [20], however the colony morphology of *F. prausnitzii* and *E. rectale* appear too similar to make a definitive differentiation of the two species when in coculture. In order to more accurately differentiate the proportion of *F. prausnitzii* and *E. rectale* cells within the cocultures, qPCR can be used. With primers specific to both species as in-

dicated by Bergström *et al* 2012 [8] and a universal primer targeting the 16S rRNA gene, the relative abundance of both species within the coculture can be assessed.

As the preliminary data presents (Fig. 16), the total number of cells in the *E. rectale* monoculture and the coculture bioreactors were fairly similar, and peaked after about one day of growth. The *F. prausnitzii* cultures also peaked after about one day of growth, however there was a marked decrease in total CFU/mL in the *F. prausnitzii* monocultures as compared to the other two conditions, which seems initially incompatible with the higher yield of *F. prausnitzii* than *E. rectale* hypothesis. The sharp increase in population size and subsequent overshoot of the carrying capacity suggests that the flow rate of nutrients is still too fast to create a truly nutrient-limited environment for these bacteria. Subsequent experiments will require slower flow rates in order to recreate the niche surrounding a starch-degrading *B. adolescentis*.

5.2 Conclusion

Three major hypotheses were explored in their connection between the preferential cross-feeding of *F. prausnitzii* A2-165 and *B. adolescentis* that allows *F. prausnitzii* to outcompete the faster-growing *E. rectale* (Fig. 17). Low pH and physical adhesion were explicitly denied as potential mechanisms. One hypothesis remains to be fully explored, and future experiments will be designed to isolate the impact that nutrient administration rate has on the growth of these two abundant and competing gut bacteria.

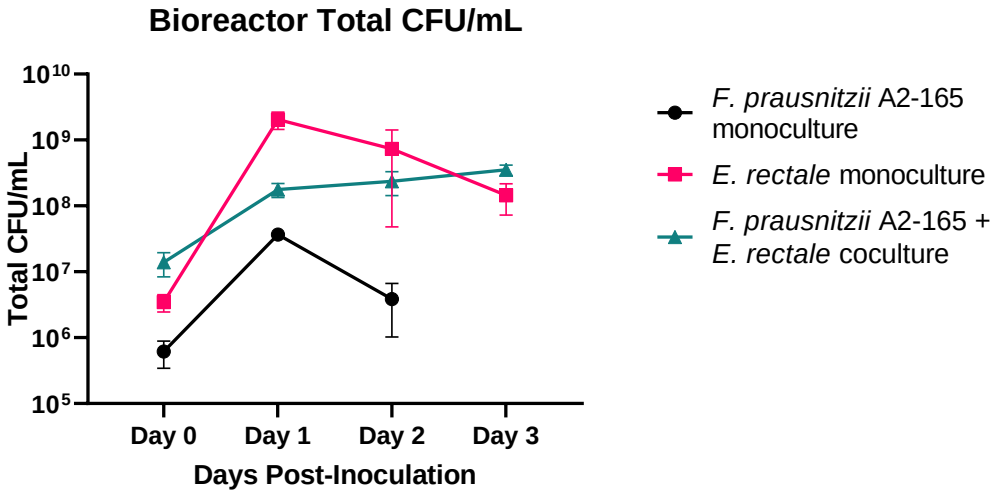


Figure 16: Total colony forming units per mL of culture within each bioreactor over time. Error bars depict standard error to the mean. Data was collected through analysis of colony growth on agar plates, and no distinction was made between *F. prausnitzii* A2-165 and *E. rectale* colonies on the coculture plates due to similarity in colony morphology. No measurement was made for the *F. prausnitzii* monoculture at day 3.

Proposed Mechanism	**Hypothesis**	**Experimental Design**	**Conclusion**
Low pH	A low pH surrounding a metabolically active *Bifidobacterium adolescentis* facilitates *F. prausnitzii* A2-165 to outcompete *E. rectale*	Measure specific growth rate at a range of pH points	A lower pH does not allow *F. prausnitzii* to outcompete *E. rectale*
Physical Adhesion	*F. prausnitzii* A2-165 cells adhere to RSP granules or to RSP-binding *B. adolescentis* 269-1 cells	Co-sedimentation experiment with *B. adolescentis* 269-1, *F. prausnitzii* A2-165 and RSP	*F. prausnitzii* does not significantly adhere to RSP granules or to *B. adolescentis* 269-1 cells
Differing Life History Strategies	*F. prausnitzii* A2-165 cells can outcompete *E. rectale* cells at a slower rate of nutrient supply	Bioreactors with coculture of *F. prausnitzii* A2-165 and *E. rectale* at a slow rate of nutrient supply	At the preliminary rate tested, *E. rectale* still outcompeted *F. prausnitzii*. Future experiments will require slower rates

Figure 17: Testable hypotheses and experimental conclusions of the mechanism which allows *F. prausnitzii* A2-165 to outcompete *E. rectale* in the presence of starch-degrading *B. adolescentis* 269-1. Future and ongoing experiments will continue to explore the slow and limiting rate of nutrient supply as a potential mechanism.

6 Discussion

The tremendous diversity within the genus *Faecalibacterium* has been underrepresented by the current taxonomy of only one species. Given the genetic and abundance data from the Biology 173 dataset, at least three species can be described. One species can be defined by containing a majority of 16S rRNA gene sequences related to ASV2, another by sequences related to ASV8, and a third by sequences related to ASV14. It is important to distinguish strains of *Faecalibacteria* by more precise species designations given the unusually high abundance and prevalence of the bacteria, which is skewed by reporting all counts as one species. Future efforts of microbiome engineering to increase *Faecalibacterium* abundance in patients suffering from gut dysbioses could require reconstitution with only a particular species. For example, if a patient has elevated concentrations of conjugated primary bile salts within the GI tract, which is a symptom of some patients with Crohn's Disease and can serve as a germinant for pathogenic spore-forming bacteria like *Clostridium difficle* [23], a probiotic of *F. prausnitzii* A2-165 which expresses a Bile Salt Hydrolase could be prescribed. Additionally, if an individual already has a strong population of a particular starch-degrading bacteria, introduction of its correlate *Faecalibacterium* species could more likely result in long-term engraftment of the bacterial population and greater yield of its beneficial products. *Faecalibacteria* use as a probiotic will require specific species and strains, and more research will be needed to compare the

colonization and health benefits of the different species of *Faecalibacteria*.

The bile salt experiments provided additional evidence for the presence of multiple species as there are stark genetic and phenotypic differences between *Faecalibacteria* strains. A genome search found a Bile Salt Hydrolase (BSH) gene in A2-165, but not ATCC 27766 or ATCC 27768. The depletion of BSH-expressing bacteria in clinical cases of Crohn's Disease is consistent with a depletion of *F. prausnitzii* Phylogroup II, including A2-165. Future research could use transcriptomics to determine alterations in gene expression among *Faecalibacteria* species when exposed to environmental stimuli, like bile salts, to better elucidate how *Faecalibacterium* populations respond differently to distinct environments in the gut.

When explaining the preferential cross-feeding of *B. adolescentis* and *F. prausnitzii* A2-165, many hypotheses were eliminated. The co-sedimentation experiment results do not support the hypothesis that a physical adhesion is occurring between *B. adolescentis* 269-1 and *F. prausnitzii* A2-165 cells in the presence of RSP. The pH experiments do not support the hypothesis that a lower pH in the microenvironment surrounding a starch-degrading *B. adolescentis* allows *F. prausnitzii* to outcompete *E. rectale*, as *E. rectale* grew at a faster specific growth rate at every pH point. More research will be needed to determine if overall yield of bacterial cells of *F. prausnitzii* and *E. rectale* indicate that *F. prausnitzii* can outcompete when administered a slower rate of nutrients.

7 Conclusion

Faecalibacterium prausnitzii is a critical member of the gut microbiome due to its unusually high abundance and prevalence, immunomodulatory activity and production of butyrate. Part of its unusual abundance can be explained by the multiple species currently all ascribed as *F. prausnitzii*. Genetic and abundance data support the existence of multiple species within the genus, and I propose at least three different species exist. Presenting the true diversity within the genus is important to future efforts of reconstituting deficient gut microbiotas with specific populations of *Faecalibacteria*.

The most abundant *Faecalibacterium* strain, A2-165, has been shown to preferentially cross-feed with the starch-degrader *B. adolescentis* 269-1 and outcompete its natural competitor *E. rectale*. While there is no support for physical adhesion or increased *F. prausnitzii* growth rate in a lowered pH, the possibility of increased yield of *F. prausnitzii* cells per mole of glucose consumed is still open as a potential mechanism. The identification of natural cross-feeding mechanisms that increase the abundance of *Faecalibacterium* is important to future interventions, as a synbiotic combination of bacterial species and prebiotic sources may maximally engraft *Faecalibacterium* populations in the gut and improve the long-term health of individuals with a dysbiotic gut microbiota.

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