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Study On the Human Gut Bacteria Under Different Ecological Conditions: Antibiotic Perturbation And Dietary Quercetin

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STUDY ON THE HUMAN GUT BACTERIA UNDER DIFFERENT ECOLOGICAL CONDITIONS : ANTIBIOTIC PERTURBATION AND DIETARY QUERCETIN

BY

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THESIS ACCEPTANCE PAGE

Shruti Menon

This thesis is approved as a creditable and independent investigation by a candidate for the master's degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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ABSTRACT

STUDY ON THE HUMAN GUT BACTERIA UNDER DIFFERENT ECOLOGICAL CONDITIONS : ANTIBIOTIC PERTURBATION AND DIETARY QUERCETIN

SHRUTI SHASHIDHARAN MENON

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The human intestine encompasses a vast community of microorganisms known as the gut microbiota that play a crucial role in maintaining health. Common perturbations such as changes in the normal diet, antibiotic treatment, and changes in environmental conditions can alter the gut microbiome. This can create dysbiosis in the gut leading to disease conditions. Therefore, it becomes important to determine the forces that influence the gut microbial ecology. In the first study, we focus on antibiotic perturbations on microbial succession and resilience in a synthetic consortium consisting of the most prevalent gut bacteria in humans. In addition, we investigated the ability of the consortium to provide colonization resistance against the gut enteric pathogen *Clostridium difficile.* The results show that the 14-species synthetic community formed after antibiotic perturbation is able to resist *C. difficile*, providing us insights for understanding the community effect against the pathogen and the possibility of using the synthetic community as a therapeutic. The bacteria *Bacteroides caccae, Bacteroides thetataimicron,* and *Parabacteroides distanosis* appear to increase significantly after antibiotic perturbation, accenting their role in inhibiting *C. difficile* growth. In the second study, we focus on the effects of supplementing quercetin on the gut microbiome. By using bioinformatic analysis, we predicted a subset of gut bacteria capable of degrading the flavonoid quercetin. From this information, we propose a set of quercetin degraders in the healthy individual that may be capable of producing antiproliferative metabolites

through quercetin biotransformation. The bioinformatic analysis showed 64 gut bacteria were predicted to have enzymes capable of degrading quercetin. The abundance of the 64 bacteria was determined by analyzing shotgun metagenomes public datasets of healthy and colorectal cancer (CRC) patients and resulted in 11 bacteria being significantly higher in the healthy population. The two studies lay the groundwork to study the gut communities under different ecological conditions in further depth. Understanding the niche an organism occupies in the gut, its survival strategies, the interactions with other microbes and advantage of certain phenotypes of gut communities under stress can provide the answer to the basic functioning of the human microbiota.

1. CHAPTER 1: POPULATION STRUCTURE, STABILITY, RESILIENCE AND THE ROLE OF SYNTHETIC GUT MICROBIOTA IN COLONIZATION RESISTANCE

1.1 Introduction

1.1.1 Core microbiota and its importance

The colon is densely populated with gut bacteria commonly known as "gut microbiota," and these bacteria play an important role in maintaining the integrity and stability of the gut [1]. The dynamic and complex interactions between the gut microbiota helps to maintain gut health. The complexity of the gut microbiota makes it challenging to study the underlying ecological concepts and the metabolic interactions taking place. The transient microorganisms add to the already existing complex community [2]. These transient microbes fluctuate depend on the environment and diet. Even with advances in sequencing technologies and culturing, a major portion of the community still remains unknown or has not been cultivated. Thus, along with mining for the microbes associated with human gut, there have been efforts to study the core microbiota [3, 4]. The core microbiota are the permanent and stable members of the gut community. The core microbiome can be defined as the set of bacteria that are conserved among individuals and are essential for better functioning of the human gut [5].Therefore, these core bacteria are the most prevalent or shared across the human gut.

1.1.2 Importance of studying synthetic microbial communities

It is difficult to carry out ecological studies in complex gut microbial systems; therefore, synthetic microbial communities are better suited for system-level studies [6].

The synthetic communities formed should be able to retain the key features of the natural environment [7]. Famous examples of synthetic communities are the Schaedler flora by Rusell W. Schaedler and the Altered Schaedler flora (ASF) developed by R.P. Orcutt, a model community of eight microorganisms [8, 9]. The synthetic microbial consortia helps to understand how the community dynamics shape the microbe-microbe interactions in the gut [10]. Thus, these communities are used as model systems to understand and study the functional, ecological and structural roles of microbiota. Such synthetic communities can be studied using *in vitro* models such as batch-culture, batch fermentation, and continuous flow, or *in vivo* models such as germ-free mice [11, 12]. *In vitro* models with defined gut communities provide controlled systems to study ecological interactions as they eliminate the multiple host-related factors influencing microbial interactions [10, 13, 14]. Also, studies have used several defined synthetic microbial communities developed from human gut microbiota to understand specific interaction patterns such as cross-feeding, syntropy and auxotrophy [10, 15]. Furthermore, using a top-down ecological perspective, we can study the overall function, resistance and resilience of the synthetic microbial communities [7]. Bottom-up ecological perspectives can be used to gain understanding in the interactions or relations between microbes and build a community from these interactions [6, 7]. Therefore, the ecological mechanistic understanding of the human gut system can be well documented using cultured synthetic communities [6]. These experiments can be performed in *in vitro* or *in vivo* systems driven by mathematics. In our study, we define a synthetic community of 14 bacteria which are known to be among the top 20 most prevalent bacterial species in the human gastrointestinal tract as identified by Forster et al. (2019) analyzing 13,490

human gastrointestinal metagenomes [16]. These 14 gut species are proposed as a model of core gut consortia for studying resistance against *Clostridium difficile* infections (CDI). We use minibioreactor arrays (MBRA) to study the ecological succession of the synthetic gut consortium and as a model for understanding CDI.

1.1.3 Importance of studying colonization resistance against enteric pathogens

The human gut plays an essential role in maintaining health. It therefore becomes crucial to study how the microbial communities confer resistance against pathogens as well as other perturbations such as antibiotics or a change in diet. Studies have shown external perturbations, such as antibiotic use, can shift the gut composition and can lead to changes in the integral human-microbe interactions needed for maintaining human physiology [17-22]. The imbalance or change of microbiota caused in the gut is known as dybiosis. Dysbiosis due to external perturbations in the gut can lead to disease-like conditions, reducing colonization resistance. Therefore, it becomes crucial to understand the key players involved in generating resistance against enteric pathogens. The next step is to understand how this resistance is achieved by studying the microbial interacting networks. These interactions can be in the form of ecological competition, production of bacteriocins, or by modulating the immune system [23]. Next-generation sequencing and metagenomics have opened new windows in the microbiome sciences. Most of the studies have performed quantitative analysis such as diversity analysis or relative abundances on composition of the microbial communities [24-26]. There have also been *in vivo* studies using ecological models to study microbial dynamics between species in the presence of perturbations such as antibiotics [23, 25, 27]. The goal of this study is to

develop a model system useful for studying integral human-microbe interactions that are required to resist enteric pathogens.

1.1.4 Choosing *Clostridium difficile* and antibiotic to study succession and resistance

C. difficile is an enteric pathogen that is known to cause antibiotic-associated diarrhea (AAD) or colitis [28] and is a major healthcare issue around the globe [29]. The treatment for Clostridium difficile infection CDI is usually metronidazole and/or vancomycin depending on the severity. However, 20% of patients develop recurrent CDI [30]. Therefore, microbial-based therapeutics such as the fecal microbiota transplant (FMT) and selected microbial mixes have emerged as treatment strategies [31-33]. However, there are concerns of infection from the donor fecal sample during FMT; thus, the use of a microbial mix as a therapeutic is being studied as substitute for stool transplant [33]. The number of bacteria in the defined mixtures used in studies to reduce or resolve CDI varies from 10 to 33 [32, 33]. Another study showed effect of defined bacterial mixtures in mice [34]. Also, there are well developed *in vitro* systems used to study *C. difficile* physiology [35]. Our study uses *C. difficile* and the antibiotic clindamycin, which is a third-generation cephalosporin and aminopenicillin associated with *C. difficile* infection CDI [36, 37].

1.1.4.1 Formulating the synthetic blend

Our hypothesis is the dominant or core members of the bacteria are important for conferring resistance against the CDI. The synthetic consortium was formulated based on a study that performed bioinformatic analysis to find 20 most prevalent species from 13,490 human gastrointestinal metagenomes [16]. Based on their study, our lab cultured 18 out of the 20 bacteria species from *Prevotella*-dominated human gut microbiota using culturomics (Table 1.1). Out of the 18 species, *Fecalibacterium praustaunzii, Bifidobacterium adolescentis* and *Dorea longicatenum* were excluded due to the pH of medium was dropped below 5.6 after 24 h growth and low pH inhibits *C. difficile* growth. *Anaerostipes hadrus* was eliminated due to issues related to culturing the species. The 14 species blend consists of both *C. difficile* inhibiting (high, low, moderate) and non-inhibiting bacteria. Apart from the different *C. difficile* inhibition efficiency, the 14 species also had slow growers (48 h) and fast growers (24 h) in the mBHI. A previous study on the substrate utilization ability of the individual bacteria also showed the differences in the utilization of these bacteria [38]. Overall, this blend consists of bacteria with different phenotypic characteristics which will help gain a better idea of the effect on *C. difficile* as a microbial community. Preliminary work on phenotypic assays such as the *C. difficile* inhibition assays, Volatile Fatty Acid (VFA) production, nutrient utilization patterns have been done for the 14 bacterial monocultures [38]. Since the 20 species were termed to be the most prevalent in the metagenome samples, we termed the blend as the “core synthetic consortium.”

Table. 1.1. The 14 species present in the *Prevotella*-dominated human gut microbiota.

Phylum	Family	Species
Bacteroidetes	*Bacteroidaceae*	*Bacteroides caccae*
		Bacteroides dorei
		Bacteroides ovatus
		Bacteroides thetaiotaomivron
		Bacteroides uniformis
		Bacteroides vulgatus
	Porphyromonadaceae	*Parabacteroides distasonis*
		Parabacteroides merdae
Actinobacteria	*Bifidobacteriaceae*	*Bifidobacterium longum*
	Coriobacteriaceae	*Collinsella aerofaciens*
Firmicutes	*Lachnospiraceae*	*Blautia obeum*
		Fusicatenibacter saccharivorans
		Roseburia faecis
Proteobacteria	*Enterobacteriaceae*	*Escherichia coli*

1.1.5 Importance of this study

This study will help us understand the ecological events occurring after perturbations and define whether the prevalent or dominant species are the keystone players involved in conferring colonization resistance against CDI. This will present a synthetic blend not only as a bacteriotherapy but also provide a model consortium to understand the integral microbe-microbe and host-microbe interactions crucial for resisting *C. difficile* and the possibility of recovering healthy gut post-CDI.

1.1.6 Objectives of this study

We aim to infer ecological "core synthetic community" dynamics under perturbations using microbiological techniques, bioinformatic analysis and mathematical modeling with the following objectives:

1. Determine the population structure and the ecological succession of core synthetic gut microbiota.

2. Determine the stability of core synthetic gut microbiota following antibiotic treatment and *C. difficile* invasion.

3. Determine the resilience of core synthetic gut microbiota following antibiotic treatment and *C. difficile* invasion.

1.2 Methodology

1.2.1 Abundance mapping on the public dataset

The bacterial abundance was mapped in order to understand the trend of the 14 species in the human gut during healthy conditions, after antibiotic perturbation and CDI. Public shotgun metagenome reads were downloaded from the study (*Milani et al.)* which consisted of 30 CDI-negative subjects not on antibiotic treatment (AB− group), 29 CDI-negative exposed to antibiotic treatment (AB+ group) and 25 CDI-positive (CDI group) [39]. The shotgun reads were converted to FASTQ format using sratoolkit [40]. Further, the raw reads were processed for quality control and to remove host reads using metawrap- readqc module [41]. Kaiju software for mapping and taxonomic classification using a custom database of the 14 bacterial protein sequences [42]. The custom database was created and indexed using the annotated protein files from the 14 bacterial whole genomes. These annotated protein sequences were generated using prokka from the assembled whole-genome sequences and the headers for each protein file were formatted to NCBI taxon identifier of each species using AWK command [43]. The kaiju2table (-r species) command was for generating .tsv format file containing abundance data and the abundance map was generated using R ggplot package [44].

1.2.2 General Workflow

Fig. 1.1. demonstrates the experimental timeline for the sampling scheme during the minibioreactor continuous flow assay. For the culturing of the bacterial isolates and minibioreactor experiment, we used the modified brain heart infusion (mBHI) media (Table 1.2). The bacterial cultures, each at 0.5 optical density $(O.D.)_{600}$ nm, were mixed equally (Mix 14) and 300 µl inoculated in each group (6 wells/ group) and allowed to stabilize for 7 days. This timeline was decided from a study that showed fecal community being stabilized after one week (~21 turnovers) [45]. The three groups included one control group which contained Mix 14 until day 23. The second group contained Mix 14 with antibiotic perturbation at day 8 and addition of *C. difficile* at day 20, which is the antibiotic treatment group. The third group contained Mix 14 with antibiotic perturbation at day 8 and addition of *C. difficile* at day 13; the CDI group. On day 8, antibiotic clindamycin was added to antibiotic and CDI treatment group s. The antibiotic treatment continued for four days and stopped on day 12. The *C. difficile* was inoculated on day 13 at 10^5 cells in CDI treatment group. For antibiotic treatment group, *C. difficile* was inoculated on day 20 at 10^5 cells in antibiotic treatment group. After addition of *C. difficile* in antibiotic treatment group and CDI treatment group, the CFU counts for *C. difficile* were performed daily by the serial dilution method on Clostridium difficile selective agar (CDSA) plates (Table 1.3).

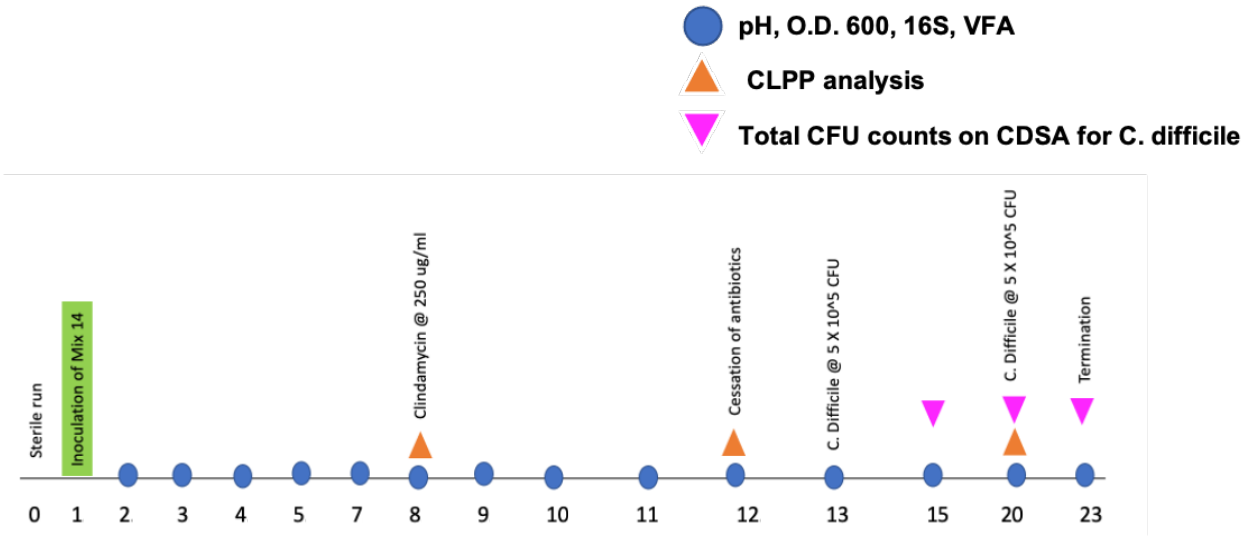

Fig. 1.1. Experimental timeline depicting the sampling scheme; the blue circles depict sampling for growth measurements, VFA and 16S samples, the orange triangle depicts the performance of the CLPP assay, and the pink triangle depicts CDSA plating. The CDSA plating was performed from day 14 to 23.

Table. 1.2. The composition of mBHI media used for this study.

Composition

Ingredients	per liter
Brain heart infusion (BHI)	37 g
Inulin	10 g
Yeast extract	5 g
L-cysteine	0.3 g
Resuarine	1 ml
Bovine bile	0.05 g
Hemin	1 ml
Menadione	1 ml
MES	1 M stock (add 100 ml to 1 L)
10N NaOH	Adjust pH to 6.8, and then bring up to 1 L

Table. 1.3. The composition of CDSA media used for this study.

Composition

Ingredients	per liter
Clostridium difficile agar (CDSA)	69 g
D-Cycloserine	0.5 g (500mg/5 ml; add 5ml in 1L)
Cefoxitin	0.016 g (100mg/ml stock; add 160 µl in 1)
L-cysteine	0.3 g
Resuarine	1 ml/L (0.25 mg/ml)

1.2.3 Bacterial culture and storage

The 14 bacterial strains obtained from our culturomics collection of 102 human gut libraries were cultured in an anaerobic chamber (Coy Lab). The cultures were grown in 3ml mBHI at 37°C. The strains, *Bacteroides caccae* (SG-0247), *Bacteroides dorei* (SG-1212), *Bacteroides ovatus* (SG-0349), *Bacteroides thetaiotaomicron* (SG-0363), *Bacteroides uniformis* (SG-0817), *Bacteroides vulgatus* (SG-0619), *Bifidobacterium longum* (SG-0552), *Blautia obeum* (SG-0764), *Collinsela aerofaciens* (SG-908), *Escherichia coli* (SG-1357), *Fusicatenibacter saccharivorans* (SG-0858), *Parabacteroides distasonis* (SG-0828), *Parabacteroides merdae* (SG-0560) and *Roseburia faecis* (SG-0935), were stocked at 0.5 $O.D_{600}$ in 10% DMSO (0.9ml culture + 0.1ml DMSO) and stored at -80°C until use. The samples were pooled (14 ml inoculum) together in equal proportion prior to the MBRA inoculation. The vegetative cells of *Clostridium difficile* strain R20291 was cultured in mBHI media, and a stock

concentration of 10^5 cells was frozen in 10% DMSO (1.8 ml + 0.2 ml), and then stored at -80°C.

1.2.4 In-vitro Bioreactor array

For the *in vitro* set-up, MBRAs were sterilized and assembled as described in the MBRA manual [35]. For the experiment, three groups of the minibioreactor were set up with the conditions as described. The input and output on the Watson Marlow pumps were set at 1 rpm and 2 rpm respectively. The rotating magnetic stirrer was set at 130 rpm. The media was allowed to flow continuously (sterile run) for 24 hours in all three group. 300 µl of the equally pooled samples was inoculated with a retention time of 16h. The minibioreactors were operated for 23 days. Samples were collected from each reactor well as per the experimental design (Fig. 1.1). The antibiotic dose was provided from day 8 to day 12. 150 µl of 25 mg/ml clindamycin antibiotic was added to each reactor of antibiotic treatment group and CDI treatment group on day 8. Each source bottle connected to antibiotic treatment group and CDI treatment group had a final clindamycin dose of 250ug/ml. On day 13, 150 µl of 10^4 *C. difficile* vegetative cells were added to CDI treatment group after 24h replacement of antibiotic-containing source media. On day 20, 150 µl of 10^4 *C. difficile* vegetative cells were added to antibiotic treatment group to check if the microbial community formed after 9 days of antibiotic treatment was able to resist the pathogen.

1.2.5 Growth measurements

Bacterial growth was determined using optical density measurements at 600 nm (Eppendorf BioPhotometer), and the pH of the culture measured with a pH meter (Mettler Toledo). The O.D. and pH values from day 2 to day 23 were plotted using Microsoft Excel. 100 µl cultures obtained from the reactor wells were used for obtaining CFU counts by the serial dilution method. The total CFU counts were obtained on days 8, 12 and 20 on mBHI plates. For the *C. difficile* CFU counts, the cultures from antibiotic and CDI treatment groups were plated on CDSA plates.

1.2.6 16S amplicon sequencing

For the time-series change in the bacterial community, 500 µl of media sample was taken from all three reactor groups from day 2 to day 23. Also, inoculum used on day 1was stored for DNA extraction. DNA from 397 samples was extracted using the Powersoil DNA isolation kit (MoBio Laboratories Inc, CA). After extraction, the quality of DNA was measured using NanoDrop™ one (Thermo Fisher Scientific, DE) and was stored at −20°C until further use. Amplicon sequencing of the samples was performed using Illumina MiSeq platform with paired-end V3 chemistry. The library was prepared using an Illumina Nextera XT library preparation kit (Illumina Inc, CA) targeting the V3-V4 regions of the 16S rDNA. The 16S data were analyzed and visualized using Qiime2 [46, 47]. Greengenes database was used to rule out contamination. The 16S data were analyzed using custom database for the 14 species and *C. difficile* 16 rDNA sequences. The representative sequences were used as query and the 14 species and *C. difficile* 16 rDNA sequences were used as database for NCBI blast tool, as better resolution at

species-level was obtained [48]. Statistical analysis was conducted for five species to determine significant differences within the three groups: control, antibiotic and CDI treatment. Kruskal wallis test and Dunn's postHoc test were performed on OTU table generated for CDI treatment group in R [49]. The Nemenyi postHoc test was also performed to check for significance [50].

1.2.7 Phenotypic measurement

1.2.7.1 VFA analysis

For the VFA analysis, 800 µl of the cultures were drawn from the reactor groups from day 2 to day 20 and mixed in 160 µl of freshly prepared 25% m-phosphoric acid. The samples were centrifuged at 20,000 g for 30 mins and stored at -80°C. 300 µl supernatant was used for injection in the TRACE1310 GC system (Thermo Scientific, USA) for VFA analysis. The analysis was performed and plotted in Microsoft Excel.

1.2.7.2 Community-level physiological profiling (CLPP) analysis

To estimate the metabolic activities of the communities formed during the course of the experiment, community-level physiological profiling was carried out using Anaerobic (AN) Biolog plates [51, 52]. Cultures obtained from random reactor wells were plated as quadruplets for days 8, 12, and 20 for each group. CLPP analysis was performed to determine the ability of the microbial community to utilize 95 carbon sources during stabilization, after antibiotic addition and after *C. difficile* inoculation. 1.5 ml culture was centrifuged at 3000G for 1 min to obtain a loose pellet, and it was washed in 500 µl PBS to remove residual media. After three PBS washes, the pellet was

resuspended in AN inoculating fluid (optical density at 650 nm [$O.D._{650}$] ~ 0.02). 100 µl of this fluid was inoculated in the AN Biolog plates and incubated at 37°C.

1.3 Results

1.3.1 Abundance mapping

The 14 species in our blend are known to be the prevalent or dominant gut species among the 20 species in the 13,490-human gastrointestinal metagenomes. In order to understand the prevalence of these species in CDI patients' gut and healthy gut, we performed abundance mapping using a custom database for the 14 species in Kaiju software. The public metagenomes downloaded contained three groups, the healthy population (AB- group), patients treated with antibiotics and negative *C. difficile* infection (AB+ group) and patients with positive CDI. The CDI group had a higher population of *E.coli and Parabacteroides distasonis* indicating that it may be supporting *C. difficile* growth. The AB+ group contained *Bacteroides caccae, Bacteroides ovatus, Bacteroides thetataiomicron* and *Bacteroides uniformis* (Fig 1.2). This correlates with the literature on the *Bacteroides spp.* being depleted in the CDI patients, indicating the importance of these species to confer resistance against *Clostridium difficile* [53].

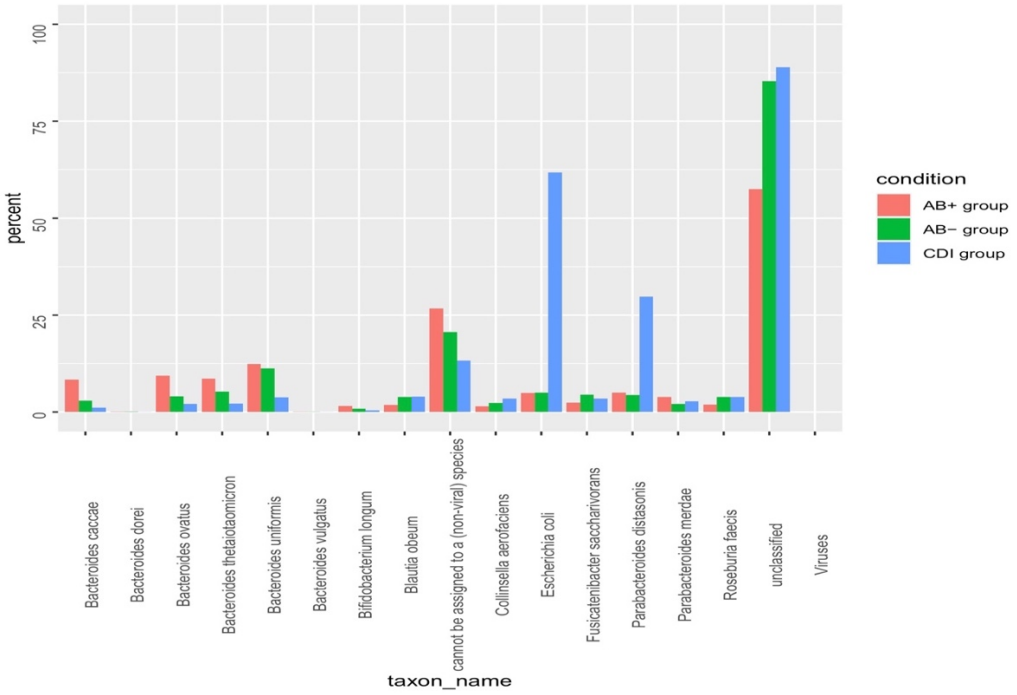

Fig. 1.2. Comparing 14- bacterial abundance in healthy (AB- group), antibiotic treated (AB+ group) and *C. difficile* infected (CDI group) individuals from a public shotgun metagenomic dataset [39]

1.3.2 Observed patterns in growth measurements

We designed a synthetic blend of 14 gut bacteria previously cultured from *Prevotella*-dominated gut. A study has shown these species to be among the 20 prevalent or dominant in the 13,490-human gastrointestinal metagenomic samples. The O.D. and pH were taken as the growth measurements for the experiment (Fig. 1.3). The optical density growth measure helps estimate bacterial load and analyze the patterns in each group. The figure shows the O.D. values observed from day2 – day 23 in the three reactor groups. The O.D. measurement in control group appears to be constant throughout all the days, while the O.D. in antibiotic and CDI treatment group appears to reduce from day 10 to 12 and starts increasing from day 14 onwards. Even after antibiotic addition at day 8, the cells appear to stabilize in the reactor groups suggesting that none or not all bacteria were killed due to clindamycin. O.D. however, cannot differentiate between live or dead cells and hence cannot be used to correlate the number of living cells in the groups [54].

The pH is the second growth measurement for the experiment which helps to determine the changes in the media conditions after the addition of synthetic blend in plain media, after antibiotic perturbation and after *C. difficile* addition. The pH measurements are all observed to be in the range of 5.5-6.4. There is an increase in pH in antibiotic and CDI treatment group at day 9, one day after the antibiotic administration. The pH increases from ~5.8 to ~6.3 in both the groups and comes back to ~5.8 on day 10. pH gives an idea about the acidity conditions of the media and the possibility of the type of bacteria growing in the medium. A low pH indicates acidophilic bacteria thriving in the medium.

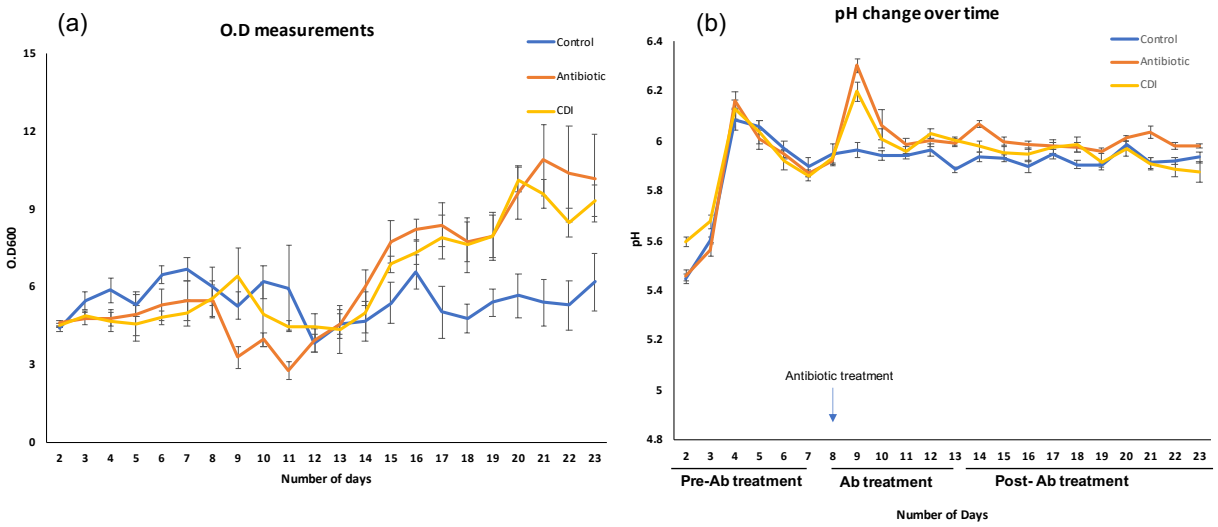

Fig. 1.3. The growth measurements for the synthetic gut consortium bioreactor samples (a) Optical density over the period of 22 days and (b) pH change over the period of 22 days.

1.3.3 Microbial succession using amplicon sequencing

Ecological succession is a time-series change or transition of a community; here a microbial community observed in a particular space [55]. The 16S analysis shows the succession of microbial patterns in the three groups under three different environmental conditions. There was no contamination in the experiment. This was ruled out using the greengenes database. The control group contains the Mix14 consortia that were allowed to grow without any perturbations in 6 wells/replicates (Fig 1.4). The consortia start to stabilize from day 5 with *E. coli* and *F. sacchanivorans* being dominant from day 2 through day 23. *R. faecais* abundance increases in from day 7 through day 23. Relative abundance of *B. obeum* appears to decrease drastically after day 4. The relative abundance of *B. dorei, B. uniformis, B. longum, P. distansonis* and *P. merdae* reduces to

zero after day 2. Overall, it appears *E. coli*, *F. sacchanivorans, B. thetataimicron, B. caccae and C. aerofaciens* are present in all the wells from day 2 to day 23. The inoculum shows only 12 out of 14 species of Mix14. It was difficult to identity *B. vulgatus* and *B. ovatus* by the 16S analysis, possibly due to close relatedness of the *Bacteroides* species. It may be challenging to obtain a species-level resolution with the amplicon sequencing.

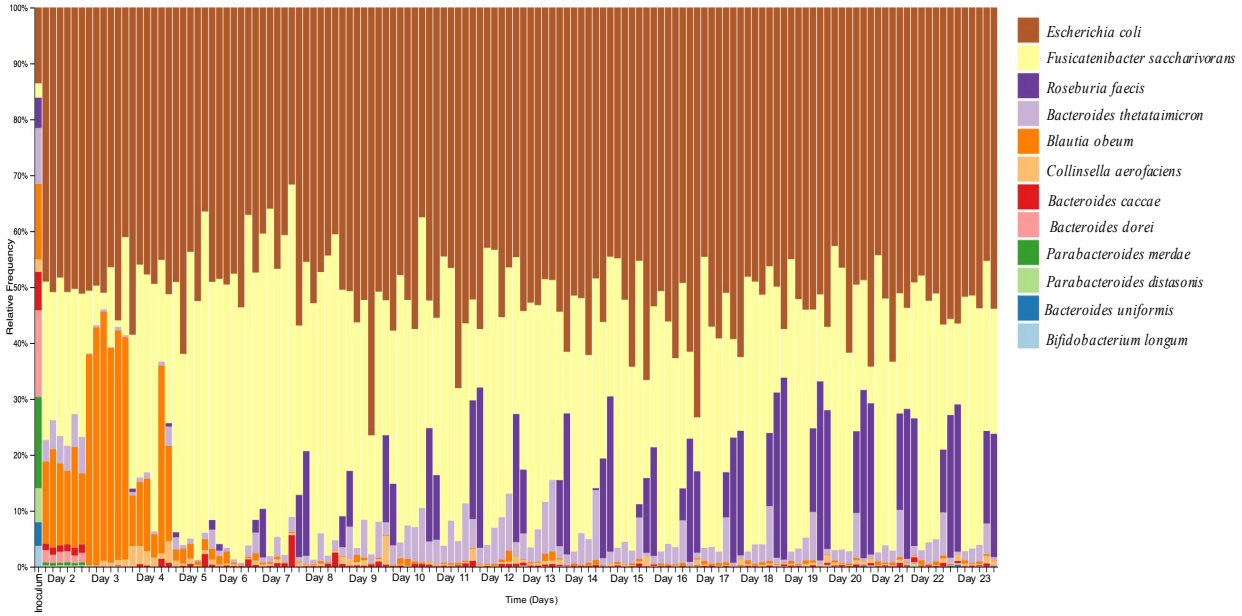

Fig. 1.4. The microbial succession of the 14 species over the period of 23 days in the control group.

Antibiotic treatment group containing the Mix14 consortia was treated with antibiotic from day 8 to 12. *C. difficile* was introduced on day 20 in 6 wells (replicates). The consortia start to stabilize from day 5 with *E. coli* having almost constant abundance from day 2 through day 23. It can be seen that *R. faecis* abundance increases in 4 wells (replicates) from day 7, but it appears to reduce after one day of antibiotic treatment and is lower until day 12. Relative abundance of *B. obeum* decreases drastically after day 4 and does not appear to be affected by antibiotics as similar conditions were observed in control group. The relative abundance of *B. dorei and P. distasonis* reduces to near zero after day 2 until day 9 and appears to increase after day 10. The relative abundance of *B. uniformis, B. longum,* and *P. merdae* appears to reduce to zero after day 2. Relative abundance of *F. saccharivorans* drastically reduces after 48 hours of antibiotic administration and appears to be zero after day 17. *C. aerofaciens* is present day 2 through day 23, but the relative abundance is very low as compared to other bacteria.

The relative abundance of *B. thetataimicron,* and *B. caccae* appear to increase after 24 hours after antibiotic administration (Fig 1.5). Overall, it appears *B. thetataimicron, B. caccae, B. dorei and P. distasonis* increase after antibiotic administration while *F. saccharivorans and R. faecis* appear to reduce in the presence of antibiotic.

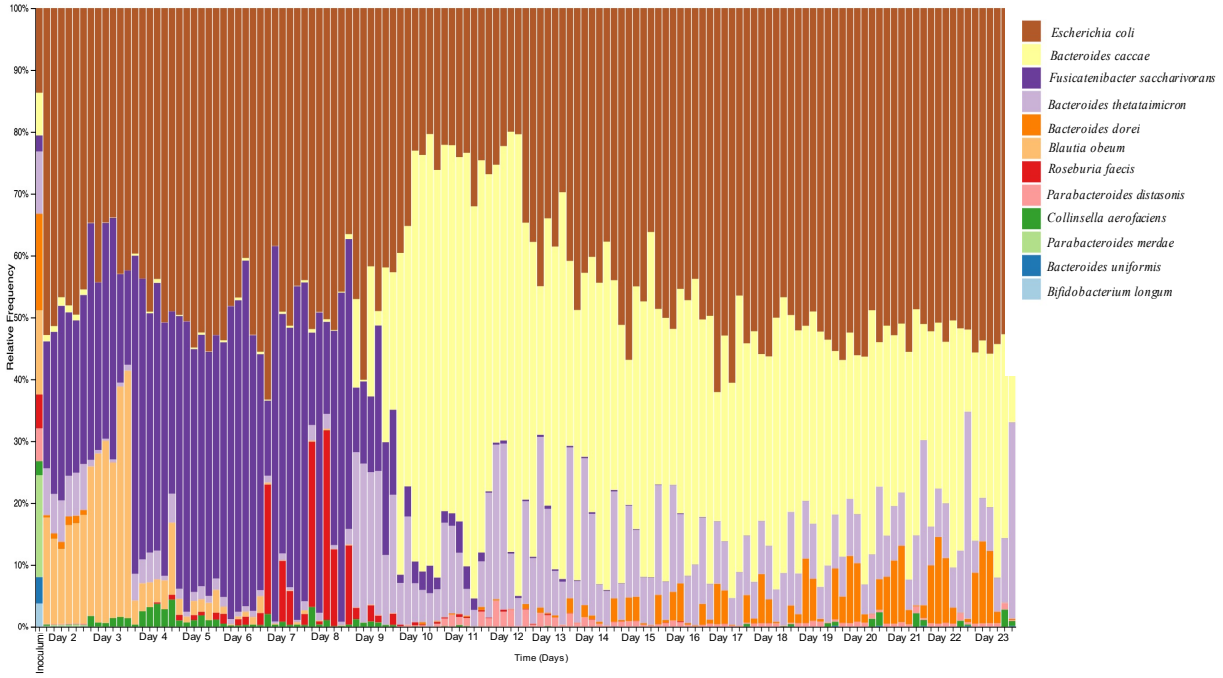

Fig. 1.5. The microbial succession of the 14 species over 23 days in the antibiotic treatment group.

CDI treatment group contains the Mix14 consortia which was treated with antibiotic from day 8 – 12. *C. difficile* was introduced on day 13 in 6 wells (replicates) (Fig 1.6). CDI treatment group results are similar to antibiotic treatment group with the exception of *R. faecis, B. dorei* and *B. thetataimicron*., appear to be low as compared to antibiotic treatment group after antibiotic treatment.

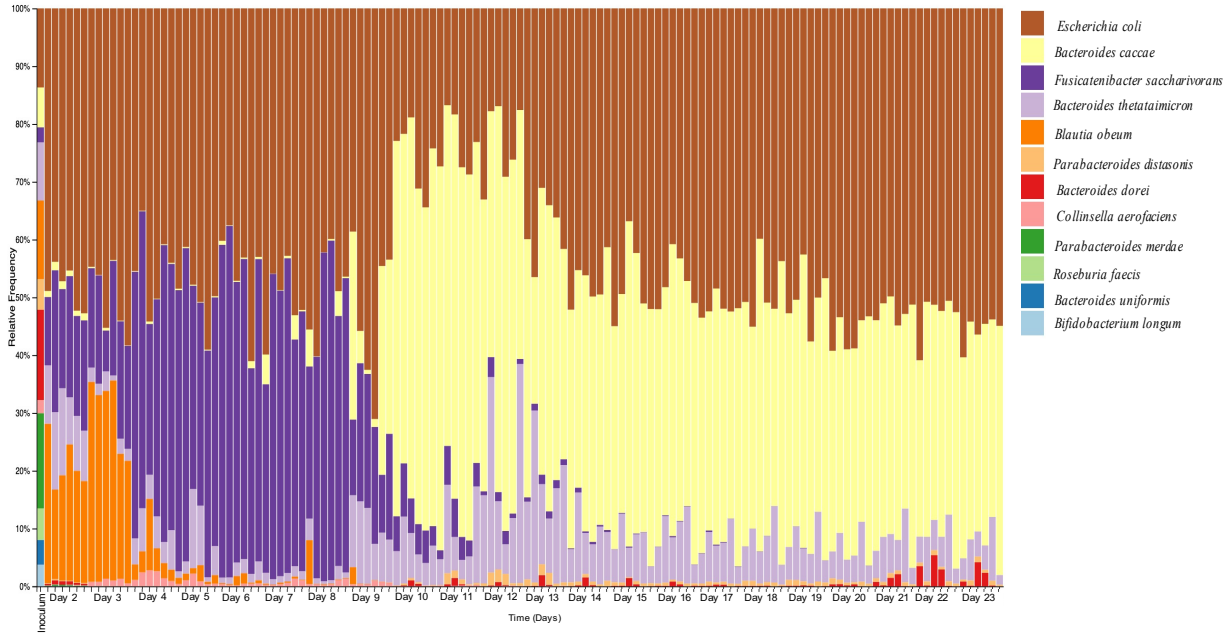

Fig. 1.6. The microbial succession of the 14 species over the period of 23 days in CDI treatment group.

Five species: *E.coli, Bacteroides caccae*, *Bacteroides thetataimicron Parabacteroides distanosis and Bacteroides dorei* appear to have a drastic increase or decrease in the antibiotic and CDI groups (Fig. 1.4, 1.5 and 1.6). Therefore, we checked whether the differences were significant among the three groups of these species. The Kruskal-Wallis test and Dunn's postHoc test revealed, *E.coli, Fusicatenibacter saccharivorans and Parabacteroides distanosis* exhibited significant differences across all three groups while the *Bacteroides caccae* and *Bacteroides thetataimicron* species had significant differences only between two groups with the antibiotic and CDI treatment groups being similar (Fig. 1.7).

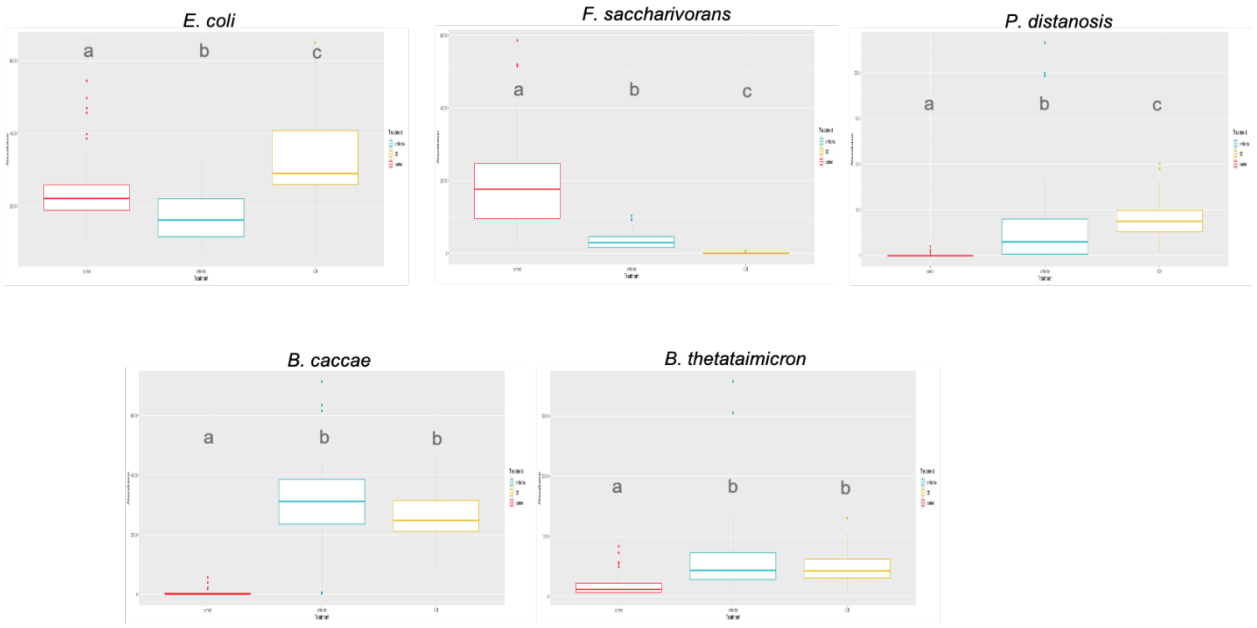

Fig. 1.7. The boxplots depicting significant groups from the postHoc Dunn's test

1.3.4 Phenotypic measurements

1.3.4.1 Volatile fatty acids (VFA) analysis

Volatile fatty acids (VFA)/ short-chain fatty acids (SCFA) are end point metabolites produced by bacteria which can confer protective effects against enteric pathogens [56]. Here, we analyze the main VFA production, acetate, propionate and butyrate. In Fig. 1.8a, the concentration of acetate appears to decrease slightly in the control group after day 12 while it decreases drastically from ~30 mM to < 10 mM in CDI treatment group. These groups were infected with *C. difficile* on day 13. In Fig 1.8b, the concentration of propionate appears to stay constant <10 mM in control and antibiotic treatment group while the propionate concentration increases significantly by ~24 mM in CDI treatment group after day 12. The concentration of butyrate increases significantly in CDI treatment group after Day 12 while overall the butyrate production appears to be at lower concentrations < 4 mM in the other groups (Fig 1.8c). Overall, the group s appear to have VFAs which can be one of the reasons *C. difficile* is not able to colonize following the antibiotic treatment in group antibiotic and CDI treatment group.

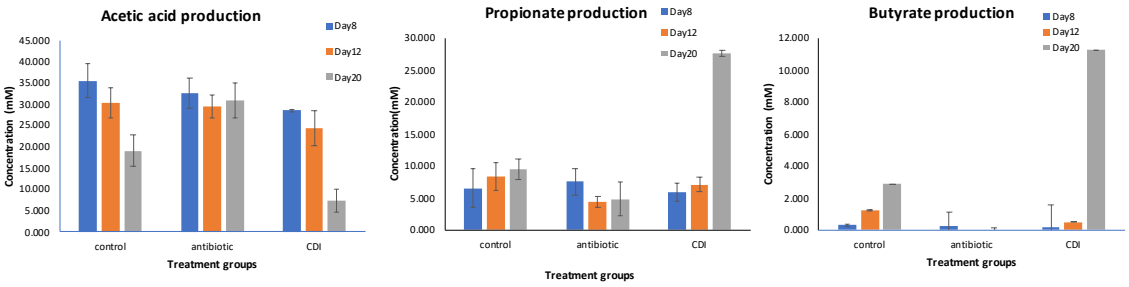

Fig. 1.8. VFA production (average ± standard error) (a) acetate, (b) propionate and (c) butyrate on days 8 (stabilization) , 12 (antibiotic cessation) and 20 (endpoint) for the bioreactor control, antibiotic and CDI treatment groups.

1.3.4.2 Community-Level Physiological Profiling (CLPP) analysis

The second phenotypic measurement performed was the community-level physiological profiling (CLPP). CLPP is a technique used to assess the sole carbon utilization patterns of a community. It is useful in understanding how microbes behave in a community via the utilization of a particular substrate over space and time [52, 57]. This information can assist in determining the function of a particular bacterial mix community. Fig. 1.9 shows the utilization patterns for 95 different carbon sources of the community formed at day 8, day 12 and day 20 in the three bioreactor groups. Previously, carbon utilization patterns for the 14 bacteria were performed from monocultures [38]. A comparative analysis of the Biolog results is displayed in Fig 1.9. The data shows the utilization patterns of the community formed on a particular day mainly the stability condition at day 8, the post-antibiotic condition at day 12 and the period after *C. difficile* inoculation at day 20. The community formed after day 12-post antibiotic addition was capable of utilizing mannitol, sorbitol and succinate [38, 58] which are the substrates that *C. difficile* use to produce infection.

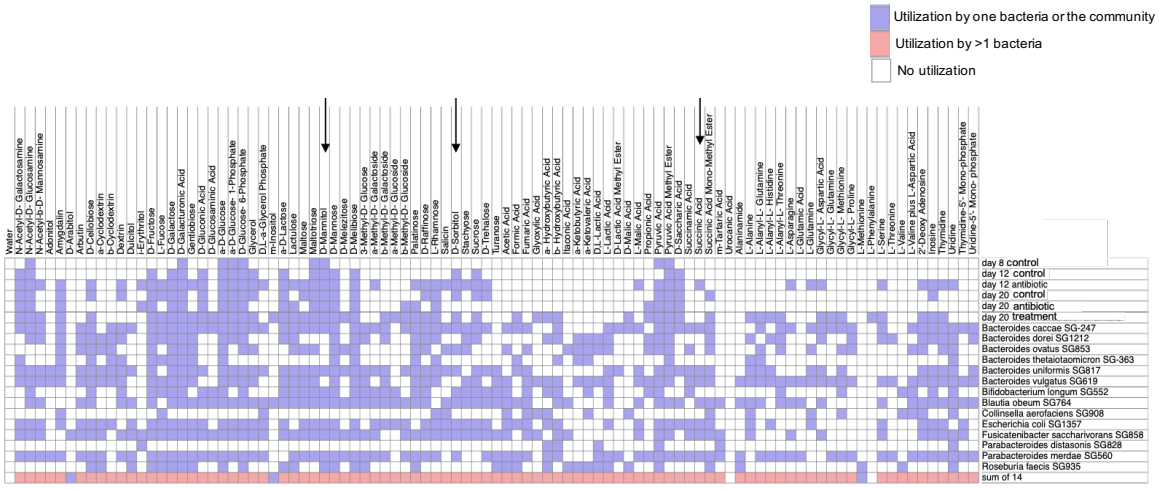

Fig. 1.9. Carbon utilization patterns of the consortia in the minibioreactor under different conditions and the utilization pattern of individual 14 bacteria forming the defined blend. The last row is the sum of utilization of all individual bacteria and the pink box denotes if the substrate is utilized by more than one bacterium.

1.4 Discussion

Enteric infections caused by *Clostridium difficile, Clostridium perfringens, Escherichia coli*, *Staphylococcus aureus,* Vancomycin-resistant enterococcus (VRE), *Vibrio cholera,* and *Salmonella typhimurium* are major health problems around the world [59]. Thus, many therapeutics such as the fecal microbiota transplant (FMT) and synthetic microbial blends have been given more importance over the past few years for treating recurring infections after antibiotic intake [60, 61]. For centuries, people have been using live microorganisms which yield health benefits when administered in the correct amounts. These are termed probiotics and have been used as a treatment to cure gastrointestinal disorders [62]. There are single organisms as well as composite probiotics such as VSL#3 [62, 63]. Many studies use selected bacterial mixes to reduce CDI [32, 33, 38]. Also, FMT has been shown to have efficacy with reducing recurrent *C. difficile* infection. However, FMT has many social and ethical issues [64]. Therefore, probiotics or a defined bacterial mix is an alternative therapeutic method for reducing CDI.

We know that the interpersonal microbiota varies and this variation can cause different resistance capabilities towards gut enteric pathogens [65]. Therefore, it becomes necessary to study the presence of the most prevalent or common bacteria across the human population and discern which organisms may confer resistance against CDI. Therefore, in our study, we developed a blend of 14 gut bacterial species, which are the most prevalent species in the human healthy gut across the *Bacteroidetes* enterotype population [16]. We hypothesize that this blend will produce a stable community and confer resistance against *C. difficile* after antibiotic perturbation in the host. We believe the presence of a few key species among these 14 species may be responsible for

inhibiting *Clostridium difficile.* In our study, we determined the population structure and succession of our mix of 14 species or blend under normal conditions, after antibiotic perturbation and following the addition of *C. difficile* after antibiotic perturbation in a continuous flow system.

The abundance of these 14 species in the public metagenome dataset corroborates with the data obtained from the 16S analysis in the minibioreactor experiment (Fig 1.4, 1.5 and 1.6). The presence of *E.coli* in higher abundance in the *C. difficile* population relates to the positive relationship between the two species while most of the *Bacteroides* species have an inverse effect on *C. difficile* growth. In the minibioreactor experiment, *Bacteroides caccae* and *Bacteroides thetataimicron* appear to be significant bacteria in the antibiotic group as well as increase in the CDI group. *Bacteroides species* may be playing a role in reducing CDI but several studies have shown a deficit in *Bacteroides* species after antibiotic treatment [32, 66-68]. However, our blend appears to have an increase in the *Bacteroides* species after the addition of antibiotics. Further study may reveal the mechanisms underlying how the *Bacteroides* species are able to persist even after antibiotic addition.

The growth measurements determined by optical density and pH were measured from day 2 to day 23 of the bioreactor experiment. These measurements relate to the bacterial succession of the blend. The sudden increase in pH after antibiotic addition can cause a favorable environment for *C. difficile* growth. Therefore, a reduced pH after day 11 may be one of the possible reasons that *C. difficile* was not able to invade the blend. Another reason may be related to the VFA production by the bacterial species. It has been known that VFAs have a protective effect on a healthy gut and VFAs are reduced in CDI

patients [25, 69]. A study showed depletion in butyrate production and *Bacteroides spp.* in CDI patients [53]. Another study in mice has shown protection against *C. difficile* by butyrate [70]. However, butyrate did not interfere with the colonization of *C. difficile* in another study, while concentrations above 50mM limited the growth. Thus, butyrate may have a protective effect by creating physiological changes in the host. The VFA propionate has been known to have resistance against *Salmonella* by *Bacteroides* spp [56, 71]. This may be one of the possible ways Mix14 inhibits *C. difficile* colonization because an increase in the *Bacteroides* spp. Further research is needed for determining the effects of VFA on CDI while using Mix14 *in vivo*, such as germ-free mice.

The CLPP analysis provides us with the carbon utilization behavior of a community formed at a particular time-point. It can be observed that for the community formed on day 12 after the antibiotic treatment, the microbes can utilize mannitol, sorbitol and succinate [38, 58]. These substrates are known to be utilized by *C. difficile* to produce infection. Utilization of these substrates by the microbial community after antibiotic perturbation could be one of the reasons for the *C. difficile* inhibition in the CDI treatment group. The behavior and function of bacteria grown as a monoculture and in a community, could be helpful for future prediction studies for the synthetic consortium.

This study provides a framework for further research work in *C. difficile* colonization resistance by the bacterial blend. One aim is to create dysbiosis conditions *in vitro* and *in vivo* and test the efficiency of this blend as a therapeutic agent. Using this bacterial blend as a method of preventing dysbiosis is also important. Mix14 can also be a model for studying the cooperation and competition between the species and elucidate

the natural community interactions for conferring colonization resistance against *C. difficile*. Studying the key ecological and evolutionary interactions in a simplified system can help simplify the understanding of colonization resistance of complex gut microbiome. The utilization of preliminary data on mono-culture assays, the 16S abundance data, growth curve, phenotypic assays such as VFA production, community-level physiological profiling and metabolomics can be useful in ecological-based modeling, genome-scale metabolomic networking, population dynamic models, prediction community functions and microbe-microbe interactions [72, 73]. The results show a promising effect of this blend as a therapeutic and as a model to study colonization resistance.

1.5 Conclusion

Our *in vitro* minibioreactor studies showed the successful inhibition of *Clostridium difficile* by the synthetic gut consortium consisting of 14 gut bacteria. The population formed after antibiotic perturbation by clindamycin was able to confer resistance against *C. difficile.* Future work on determining the mechanisms and ability of the blend to inhibit or reduce the pathogen *in vivo* mice models may be useful in deciphering host physiological and immune responses.

2. CHAPTER 2 : BIOINFORMATIC ANALYSIS REVEALS GUT MICROBES WITH POTENTIAL TO DEGRADE QUERCETIN AND THEIR POSSIBLE ROLE IN GENERATING ANTICANCER EFFECTS

2.1 Introduction

2.1.1 Flavonoids and their dietary occurrences

Flavonoids are polyphenolic compounds ubiquitously occurring in food sources from plants. Some common food and beverages with flavonoids are onion, red cabbage, celery, citrus fruits, berries, tea, and red wine [74]. There are over 6000 flavonoids identified and have been classified into six main subclasses [75]. They are mainly associated with broad spectrum of health-promoting characteristics, especially their antioxidant properties. Flavonoids occur at varying levels in fruits, beverages and vegetables and the different subclasses have been associated with different actions on the human body [76]. They have antimicrobial, anticarcinogenic, anti-inflammatory, antidiabetic, estrogenic, and antioxidant properties [77, 78]. Many animals, epidemiological and cellular studies have shown the potential positive health effects of flavonoids [79, 80]. Flavonoids have inverse effects in diabetes, mental illness, cancers and cardiovascular diseases [81-84].

Flavonoids are the largest class of dietary polyphenols, which have been used as nutraceutical ingredients due to their health benefits [85]. Their structure includes two phenyl rings and one heterocyclic ring and they are classified into six major subclasses: flavonols, flavanones, flavanols, anthocyanins, flavones and isoflavones [86, 87]. They are commonly found in plants and play a critical role in plant regulation during abiotic or

biotic stress. Plants have varying concentrations of flavonoids depending on the environmental conditions such as temperature, exposure of plants to sunlight and day length [88]. The most common subclasses found in food are flavonols, flavanones, flavanols, and flavones and they are usually found in glycoside forms in plants. Glycosylation of flavonoids increases their water and photo stability [89, 90]. The common glycosides are quercetin-3-O-rutinoside found in tea [91] while capers are high in flavonols like kaempferol 3-rhamnosyl-rutinoside, quercetin 3-rutinoside, and kaempferol 3-rutinoside [86, 92]. Apigenin-7-O-glucoside is found in chamomile [93]and apiosylglucoside malonyl conjugates were found in steam celery and parsley [94]. Citrus juices have both flavone O- and C-glycosides and flavanone-O-glycosides [95]. Berries and soybeans are known to be rich in isoflavonones and anthocyanins.

The flavonoids are metabolized in the gastrointestinal tract (GIT), liver, and the gut microbes in the large intestine. The flavonoids become available by three known possible events: 1) absorption across the GIT, 2) hepatic metabolism and 3) action of gut microbes [96]. Even with three possible ways of metabolism, the gut microbes degrade flavonoids with multiple possible enzymes and pathways. With a vast number of flavonoids in nature and many possible ways of its metabolism, it becomes important to understand the metabolism and bioavailability of flavonoids in the human body. Studying the bioavailability and mechanisms by which it induces positive effects can help us determine the optimum dosage for a favorable health effect.

2.1.2 Quercetin and its health effects

Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is the major representative of the subclass flavonol. Quercetin is a glycone and is found mostly in glycosylated form in apples, berries, capers, Brassica vegetables, nuts, onions, grapes, shallots, tea, tomatoes, flowers, leaves and bark. Medicinal plants such as *Sambucus canadensis, Gingko biloba* and *Hypercium perforatum* also contain quercetin [97-99]. The lowest concentration of quercetin occurs in tea (2mg/100g) while the highest occurs in raw capers (234mg/100g) [100]. The dietary intake of quercetin varies depending on countries and the food sources. The estimated flavonoid intake ranges from 50 to 800mg/day with 75% being quercetin [99, 101]. For example, the major source of quercetin intake in Italy is wine, while in Finland, United States and Greece, intake mostly comes from apples and onions [102]. In Australia, the main sources of quercetin are green and black tea [103]. Due to its widespread abundance in food sources, it is one of the main flavonoids studied for its properties. Quercetin has tremendous potential therapeutic effects in the medical field to improve human health [104, 105]. It is also being studied as a nutraceutical [85].

Quercetin is known to have various pharmacological usages due to its antioxidant, antiproliferative, antimicrobial, cardioprotective, and anti-estrogenic effects [106]. Quercetin has the potential to inhibit laryngeal cancer as well as ovarian cancer [107, 108]. Many pathways have been proposed to treat various cancers [109-111]. Quercetin is known to chelate metals and scavenge oxygen free radicals. The *in vivo* antioxidant activity of quercetin is mainly displayed through the signal transduction pathways, reactive oxygen species (ROS) and effects on glutathione (GSH) [105]. Oxidative stress can lead to acute and chronic disorders like inflammation, diabetes, and atherosclerosis.

In the case of atherosclerosis, quercetin modulates lipid metabolism by reducing oxidation low-density lipoprotein (LDL) which induces the condition [112]. Quercetin shows protective effects on acute myocardial infraction in rats [113]. It can also be used in alleviating depression. A study has shown that quercetin prevents neural damage and lowers hippocampal inflammatory and oxidative responses [114]. The urotoxicity induced by cyclophosphamide can also be prevented by quercetin [115]. In the context of gut health, a study has shown the potential of quercetin to be used as a prebiotic to combat gut dysbiosis after antibiotic treatment [116]. Another study showed the dietary quercetin increases gut diversity and alleviates *Citrobacter rodentium* infected mice colitis [117]. The study suggests the benefits of quercetin in restoring gut microbial balance and activating the immune system to lower inflammation. The use of quercetin for gut health and its ability to restore oxidative properties can be useful in medicinal applications.

2.1.3 Possible enzymes involved in quercetin metabolism

Quercetin can be metabolized either by hydrolysis and absorption in the stomach, the small intestine, the liver, or by the gut bacteria in the intestine [96]. Metabolism and absorption efficiency is influenced by the form of quercetin. A study showed that quercetin in its aglycone form is easily absorbed in the stomach of rats, but when it is aglucoside, it is easily absorbed in the small intestine [118]. In its glycosylated form, quercetin is easily absorbed through the sodium-dependent glucose transporter 1 (SGLT1) located in the apical membrane of small intestinal villi [119]. Quercetin glycosides can also be transported by hydrolysis from the lactase phlorizin hydrolase (LPH), a glycoside hydrolase on the outside of the brush border membrane of the small

intestine [120]. This enzyme liberates quercetin which can be absorbed across the intestine. The quercetin reaching the large intestine is metabolized by the intestinal bacteria. Degradation of quercetin by bacteria *Eubacterium ramulus, Flavinofractor plautii, Eubacterium oxidoreducens* has been reported [121-123]. Quercetin is degraded by gut bacteria into at least seven metabolites [124]. The possible metabolites produced after *Eubacterium ramulus* and *Flavinofractor plautii* degradation are phloroglucinol and 3,4-dihydroxyphenylacetic acid via taxifolin as the intermediate product [122, 125]. The enzyme quercetin 2,3-dioxygenase or quercetinases also acts on quercetin to form 3,4-dihydroxybenzoic acid, also known as protocatechuic acid (PCA) and 2,4,6-trihydroxybenzoic acid. Quercetinases have been found in fungi *Aspergillus niger, Aspergillus flavus, Fusarium oxysporum* and bacteria *Bacillus subtilis, Streptomyces* sp. FLA [126-128]. Pirin-like proteins have been identified to have quercetinase activity, however they have very weak homology to the sequences of quercetinases identified from *B. subtilis* and *Streptomyces* sp [129]. The degradation of quercetin to PCA may involve multiple pathways. One such pathway is conversion of quercetin to PCA via 4-hydroxybenzoic acid as the intermediate [124]. Phloretin hydrolase enzyme from *Flavinofractor plautii* may be able to degrade quercetin which structurally similar to phloretin [130]. Phloretin hydrolase is also characterized in *Eubacterium ramulus,* a bacteria which is also capable of degrading quercetin [131].

Although studies have shown quercetin metabolism by gut bacteria as well as determined products of quercetin after degradation in the intestine, the exact mechanism of degradation of quercetin, enzymes or complete pathways related to quercetin metabolism in the large intestine has not yet been elucidated. Degradation of quercetin

possibly involves multiple enzymes and pathways by the gut bacteria. For this study, we chose a well-characterized enzymes for sequence homology detection in known cultured gut bacteria. The three enzymes are quercetinase from *Bacillus subtilis,* and *Streptomyces* sp. FLA, phloretin hydrolase from *Flavinofractor plautii* and *Eubacterium ramulus,* and pirin-like protein known for quercetinase activity from *Pseudomonas stutzeri* [126, 127, 129, 130]. The genes and protein sequences have been used for blast and hmmer search for homology detection. Apart from the enzymes, the yxaGH operon [132]from *Bacillus subtilis* consists of qdoR, qdoI (quercetinase) and yxaH genes which were separately used for blastn analysis.

2.1.4 Gut microbial metabolism of quercetin

The dietary polyphenols are known to be majorly accumulated in the large intestine (90-95%) while 5-10% is absorbed in the small intestine [133]. Thus, the major portion of the polyphenols are subjected to microbial degradation for absorption in the human body. The absorption of these polyphenols by gut microbes may have significant effects on these polyphenols at their target site as well as the bioavailability for the body. Therefore, the ability of these polyphenols to have effects on gut microbiome becomes an important research area to study. Quercetin being the major representative of flavonols has been studied for its role in influencing gut beneficial modulation and its health effects [117, 134-137]. Many of these studies show varying effect on the microbiota, which may be due to the host-related multiple compounding effects from the *in vivo* studies. Thus, *in vitro* studies can be helpful to determine microbe-microbe interactions and study the metabolites and microbial biotransformation in an accurate manner [134, 138]. Although studying the impact of quercetin on intestinal microflora for metabolism of quercetin is

useful, there is a need to understand the species/strains involved in the metabolism as well as the enzymes and metabolic pathways for the biotransformation of flavonoids (quercetin). Many bacterial species are known to degrade glucosides of quercetin, however little is known about the bacterial species involved in quercetin transformation [96, 139]. A study was performed to isolate and identify bacteria in human gut for metabolizing quercetin in an *in vitro* model and seven bacteria from the fecal samples; *Bacteroides fragilis, Clostridium perfringens, Enterococcus gilvus, Escherichia coli, Lactobacillus acidophilus, Stretococcus lutetiensis,* and *Weissella confusa* had the ability to transform quercetin. [140]. In this study, *Bacteroides fragilis and Clostridium perfringens* were found to have the highest degrading ability. This suggests quercetin is unable to inhibit pathogen *C. perfringens*. Apart from the seven bacteria, the species; *Eubacterium ramulus, Flavinofractor plautii* have been known to degrade quercetin [122, 125, 139]. Since quercetin is such an important dietary polyphenol, it is important to isolate and identify more gut species responsible for quercetin transformation.

2.1.5 Dose-dependent, media conditions and Toxicity

Most of the studies carried out have significant variations on the effective doses as well as the model used for the studies, which may be the reason for differences in the mechanistic understanding of quercetin. One such example is the use of quercetin and its ability to protect against breast cancer [141, 142]. One study showed doses of 2% and 5% quercetin reduced tumor development in chemically induced mammary cancer in rat models [143] while another study showed quercetin was unable to confer protection against breast cancer at a dose of 2.5 g/kg food [144]. Besides being dose dependent, quercetin is degraded by bacteria differently depending on the media conditions. For

example, *E. ramulus* degraded quercetin in presence of glucose while *E. oxidoreductans* degrades in presence of formate and H2 [122, 123]. Also, *E. ramulus* exhibited cross-feeding mechanism with *B. thetataimicron* in the presence of starch indicating its strict requirement of glucose in co-metabolizing quercetin [121].

The health benefits of quercetin can be appealing to many health-conscious people but exceeding the amount of dietary quercetin or any flavonoid can lead to toxic effects in the human body. Studies have shown potential toxicity of quercetin i.e., mutagenicity, mitochondrial toxicity, inhibition of key hormone metabolism enzymes and prooxidant activity [145, 146]. These studies shed light on the use of quercetin for drug development programs as well as a need to understand the mechanisms of molecular and microbe interactions at a deeper level in order to guarantee the safety and efficacy of quercetin.

2.1.6 Importance of this study

From the literature, it is evident that *E. ramulus* and *F. plautii* transform quercetin to 3,4-dihydroxyphenylacetic acid (DOPAC), which has antiproliferative activity in colon cancer cells [147]. However, the enzymes and pathways are unknown. The second enzyme quercetinases transforms quercetin into 3,4-dihydroxybenzoic acid, also known as protocatechuic acid (PCA) and 2,4,6-trihydroxybenzoic acid. The product 2,4,6-trihydroxybenzoic acid is also known to be anti-proliferative agent [148]. However, no information on bacterial metabolism yielding these products is known. Therefore, it is important to elucidate the microbes involved and the pathway in quercetin biotransformation.

For this study, we focus on the possible metabolisms by the action of gut microbes in the large intestine for the subclass flavonols with a special focus on quercetin metabolism. One of the objectives is to determine the presence of homologs for quercetin degrading enzymes in the genomes of the known cultured gut bacterial species using bioinformatic analysis. The second objective is to find the abundance of predicted bacterial species known to have the homologs for quercetin degrading enzymes in the healthy and CRC individuals from the publicly available shotgun metagenomes. This will help us understand the enrichment and possible link of the bacteria to induce potential health effects by metabolizing quercetin to obtain beneficial products.

2.1.7 Objectives of this study

- Screen for possible enzymes and operons in the gut microbe genomes
- Screen for abundance of the bacteria predicted to have quercetin- degrading ability in the CRC and healthy datasets
- Check for significant bacteria in the healthy and CRC datasets

2.2 Methodology

2.2.1 General Workflow

Fig 2.1 depicts the general workflow carried out in this study. The goal is to predict the quercetin degrading gut-bacteria by the homology-based analysis for six enzymes and find a possible link of the bacteria with CRC or healthy conditions.

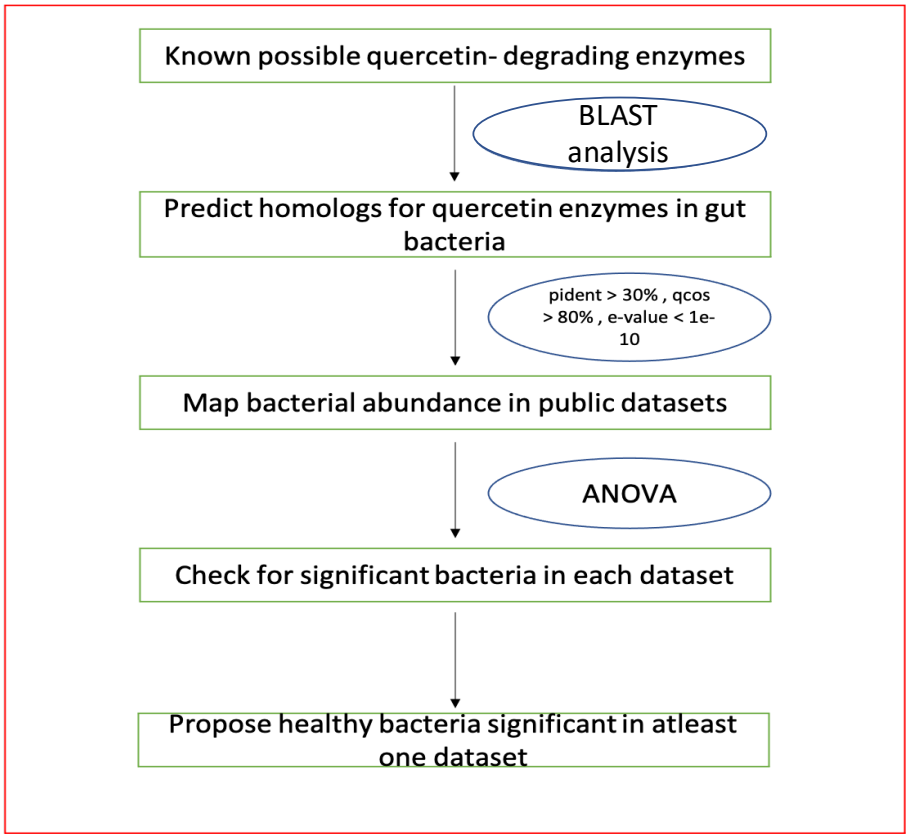

Fig. 2.1. General workflow of the bioinformatic analysis performed for this study

2.2.2 Datasets – genomes and metagenomes used for this study

In total, 1066 cultured gut bacterial genomes were chosen for this study based on a literature search and our 102-gut library [38, 149, 150]. There were 860 out of the 1066 bacterial genomes available on refseq (30th September,2020). The protein files were downloaded from NCBI-refseq via linux- command line. The protein files were concatenated to get protein query databases for blast analysis.

To calculate abundance of certain gut bacterial genomes in the CRC and healthy individuals, public shotgun metagenome datasets were downloaded from five CRC studies [151-154]. The five studies have been conducted in countries Austria, China, France, Germany and India (Table 2.1).

2.2.3 Protein sequences for homology search

To create the blast database, genes for quercetin 2,3- dioxygenase, flavone-reductase, chalcone isomerase, enoate reductase, pirin-like protein and phloretin hydrolase were selected (Table 2.2). The blast analysis for protein sequences was carried out using standalone NCBI-BLAST software. Databases was created for each enzyme using the makeblastdb command. The resulting output files were parsed in R using parameters for 30% percent identity, 80% query coverage and $1e^{-10}$ e-value, respectively [155]. In total 64 bacteria were selected to be a subclass of possible quercetin degraders. The 64 bacteria obtained from the blast hits were used for abundance mapping in the public metagenome datasets.

2.2.4 Statistical analysis for the quercetin-degrading gut bacteria in healthy and disease conditions

The public metagenomes were downloaded using the prefetch and fastq-dump commands from the Sra-toolkit software [40].The fastq files were processed to remove human host reads using metawrap read-qc module. The protein files of bacteria predicted to degrade quercetin were used for building a custom database for Kaiju [42]. The mapping of the bacteria on metagenomes was done using Kaiju software. The abundance output obtained from Kaiju were parsed in R to perform ANOVA statistical test on each bacterial abundance and condition for each country dataset. Bacterial abundance were randomly checked from each dataset for ANOVA assumptions. Some of them met the assumptions. Therefore, more analysis needs to be performed in future. After obtaining significant bacterial abundance in each country, we checked for its relative abundance in healthy or in CRC conditions. Out of the 64 bacteria, 11 bacteria were significantly ($p < 0.05$) greater in healthy individuals in either one or more countries while 44 bacteria were significantly ($p < 0.05$) greater in CRC individuals. All the heatmaps were generated using online Morpheus software (https://software.broadinstitute.org/morpheus).

Table. 2.1. Public metagenome datasets for CRC and healthy individuals based on countries.

Country	**Bioproject number**	**Number of samples**
Austria	PRJEB7774	Healthy : 63 CRC : 46
China	PRJEB10878	Healthy : 53 CRC : 75
France	PRJEB6070	Healthy : 64 CRC : 50
Germany	PRJEB6070	Healthy : 38 CRC : 5
India	PRJNA531273, PRJNA397112	Healthy : 30 CRC : 30

Table. 2.2. Reference sequences of enzymes degrading quercetin used for BLAST analysis.

Protein sequence	**Reference Species**	**Accession no.**
phloretin hydrolase	*Flavinofractor plautii* *Eubacterium ramulus*	OXE48401.1 AAQ12341.1
quercetin 2,3- dioxygenase	*Bacillus subtilis* *Streptomyces sp. FLA*	P42106 CAJ81053.1
pirin-like protein	*Escherichia coli* *Pseudomonas psutzeri*	P46852 EHY79687.1

2.3 Results

2.3.1 BLAST analysis reveals subset of bacteria capable of degrading quercetin

The literature review revealed three possible enzymes capable of quercetin degradation (Fig 2.2) [129, 130, 156]. From the literature, quercetin 2,3-dioxygenase (quercetinase) is known to metabolize quercetin into 2,4,6-trihydroxybenzoic acid and 3,4-dihydroxybenzoic acid while phloretin hydrolase is one of the proposed enzymes capable of degrading quercetin in *Flavonifractor* species [130]. The pirin-like protein is known to have quercetinase activity in *E. coli* and *P. psutzeri*. Therefore, to predict a subset of gut bacteria from the known cultured gut bacterial species we chose query sequences of the three enzymes: quercetinase, phloretin hydrolase and pirin-like protein. For the BLAST database, we selected two query protein sequences for each enzyme (quercetinase, phloretin hydrolase and pirin-like protein). Template sequences for quercetinase were taken from *B. subtilis* and *Streptomyces* sp. FLA while for phloretin hydrolase were selected from *E. ramulus* and *F. plautii*. Query sequences for pirin-like protein were selected from *E. coli* and *P. psutzeri*. The blastp analysis (p-ident >= 30%, qcovs >=80%, e-value>=$1e^{-10}$) revealed 32, 39 and 335 bacteria with quercetinase, phloretin hydrolase and pirin-like protein homologous proteins respectively (Table 2.3).

In total, 64 bacteria were chosen as the subset for further analysis for abundance mapping and statistical testing in the public datasets. Since, pirin-like protein are known to exhibit quercetin- degrading ability but the biological role is yet to be studied, we excluded the bacteria which are uniquely predicted to have this protein. Out of the 64

bacteria, 6 bacteria; *Bacillus megaterium, Clostridium butyricum, Olsenella sp., Paenibacillus polymyxa, Pseudomonas fluorescens* and *Rhodococcus erythropolis* are predicted to have all three enzymes (Fig 2.3 & 2.4). While 14 out of the 64 are known to have quercetinase and pirin-like protein and 10 out of the 64 are known to have phloretin hydrolase and pirin-like protein (Fig 2.3 & 2.4). Only one bacterium; *Enterocloster clostridioformis* commonly have both phloretin hydrolase and quercetinase enzyme homolog.

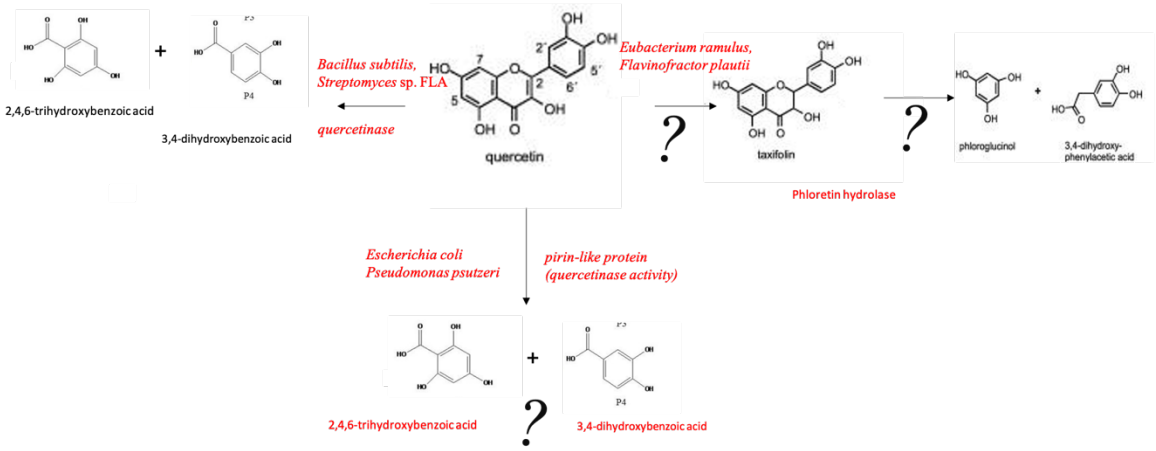

Fig. 2.2. Possible degradation pathways for quercetin.

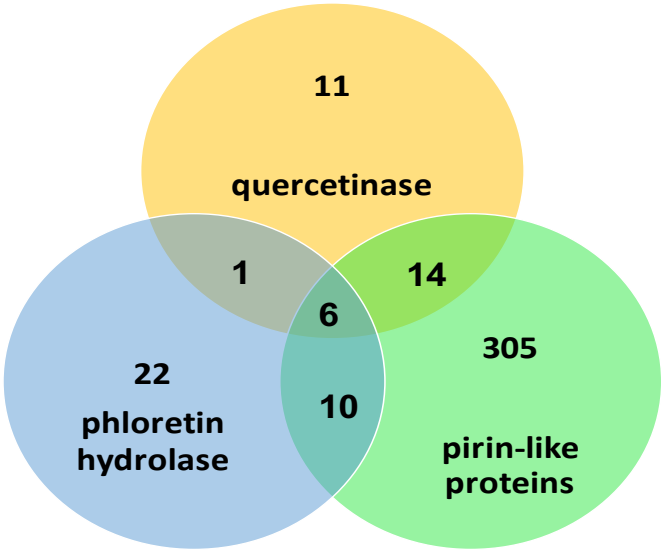

Fig. 2.3. Venn diagram depicting total number of bacteria following BLAST analysis. It shows the number of gut bacteria containing either one of the enzymes as well as number of bacteria sharing the enzymes.

2.3.2 Abundance mapping for the subset of gut microbes able to degrade quercetin

Some bacteria can degrade quercetin to produce anticancerous metabolites. However, it is not clear how many gut bacteria are capable of transforming quercetin and to what extent the metabolites produced have an impact on health. This study proposes the possibility of the subset of gut bacteria which may be capable of quercetin transformation. Up until now, quercetin metabolites are known to have anti cancerous effects, therefore we checked for the abundance of the quercetin degraders in the public

datasets of CRC patients and healthy controls (Table 2.1). Fig 2.4 shows bacterial abundances in each country and condition dataset as well as the presence of predicted homologs and the bacteria which are significant. The significance was calculated using the ANOVA statistical test in R for each country dataset based on the condition. In total, 55 bacteria were found to be significant ($p < 0.05$) in either one or more public shotgun datasets. Further, we checked if the significant bacteria were higher in the healthy or CRC conditions. Most of the bacteria showed higher abundances which were significant in CRC conditions, however overall, the data seemed to not follow a definite pattern (Fig 2.5). We focused on bacteria which were present only in healthy conditions in one or more datasets. From Fig 2.5, it can be observed that 11 bacteria are present in healthy conditions. Variations in the significance level in conditions can be due to multiple factors including geography, diet, genetic makeup, etc.

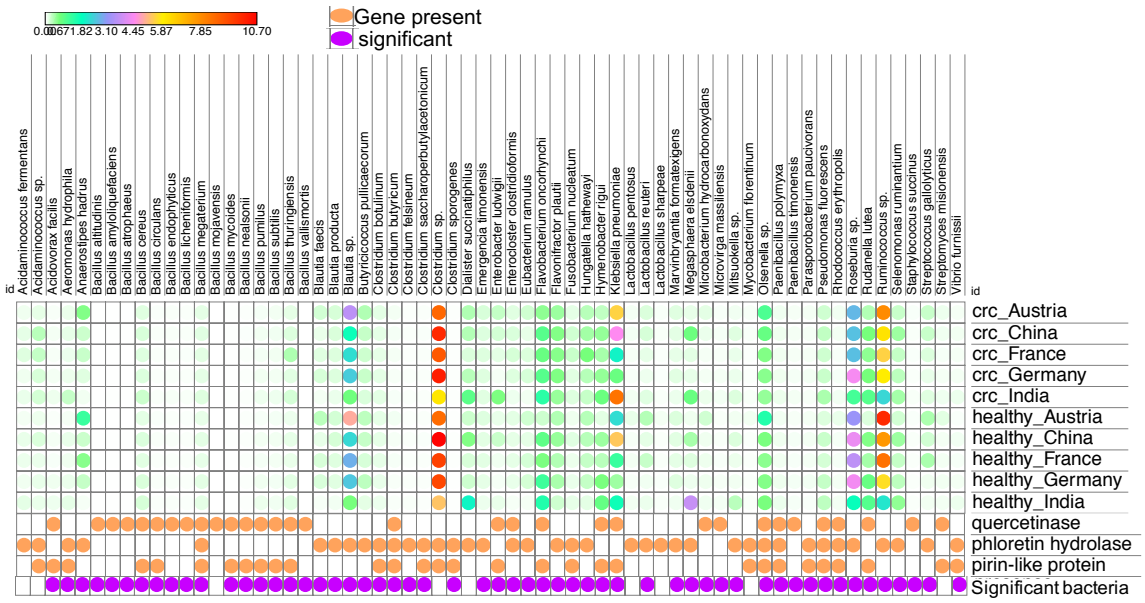

Fig. 2.4. Heatmap denoting abundance of the 64 quercetin-degraders, the presence of the three enzymes in each bacterium and the significant bacteria by ANOVA analysis ($p < 0.05$).

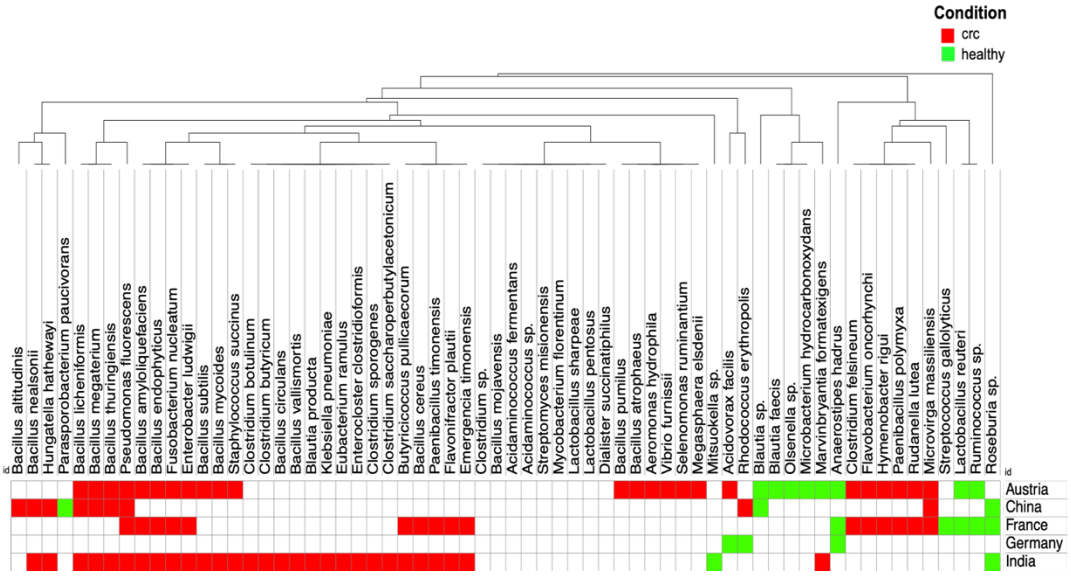

Fig. 2.5. Presence of significant bacterial abundance in each CRC country dataset where red square denotes higher in CRC condition and green denotes higher abundance in healthy condition.

2.3.3 Phloretin hydrolase gene was observed in majority of the health-related bacteria

The 11 bacteria from the analysis were observed to be higher abundance in healthy samples in at least one of the datasets. We further checked the presence of the protein homologs (quercetinase, phloretin hydrolase and pirin-like protein) in the 11 bacteria (Fig 2.6). Out of the 11 species, 10 bacterial genomes had the phloretin hydrolase enzyme from the BLAST analysis while *Microbacterium hydrocarbonyxdans* had only quercetinase enzyme homolog (Fig 2.6). *Olsenella* sp. had presence of all three enzymes while *Paraprobacterium paucivorans* had phloretin hydrolase and pirin-like protein homologs. These 11 bacteria can be used for further screening of their quercetin degrading capability and their role to produce bioactive metabolites in prevention of CRC.

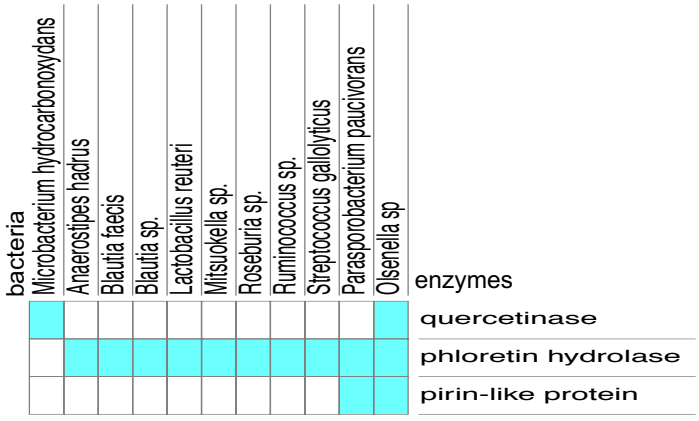

Fig. 2.6. Presence of the three enzymes in the bacteria higher in healthy individuals. The blue squares denote presence of the enzyme.

2.4 Discussion

Quercetin is the major flavonol in the flavonoid subclass and numerous researchers study its beneficial effects on the human body. However, the role of quercetin as a mediator or shaping the human gut microbiome of the regular flavonoid-consuming individuals as well as its role in abating the risks for pathologies is poorly understood. Bacteria such *Flavinofractor plautii (Clostridium orbiscindens)* and *Eubacterium ramulus* are known to degrade quercetin to produce useful metabolites. Therefore, it becomes important to study other quercetin degraders in the gut to understand and predict health outcomes. This study forms a baseline providing the possible quercetin degraders based on quercetin-degrading enzyme homologs. Apart from the role of the gut bacteria, the enzymes and pathways of quercetin degradation are not clearly understood. Few bacteria are capable of degrading quercetin through quercetinase, while bacteria like *F. plautii* and other *Flavinofractor* species are predicted to degrade quercetin by phloretin hydrolase [130]. The mechanisms of degradation, as well as the important species in the gut that are active in quercetin degradation, also varies with the dietary intakes. A study showed high abundance of *Flavinofractor* species in fecal samples when mixed with 7N minimal media (20mM sodium acetate) and quercetin, while *E. ramulus* favours media rich in glucose for co-metabolizing quercetin [121, 130]. The dietary factors therefore become one of the compounding factors while studying the effect of gut microbiome in quercetin metabolism.

This study highlights differences with respect to the abundances and significant gut species in different geographical locations of the CRC datasets. Although these studies did not include any dietary information or use of quercetin by the individuals, the overall

variations in the abundances of the quercetin degrading species might still be related to the dietary effects.

Quercetin can be studied as either a mediator in the regular flavonoid intake of individuals and its effect on the gut microbiome and overall metabolic response, or as an effect modifier [157]. For example, results of this study show that the bacteria *E. ramulus* and *F. plautii* have a significantly higher abundance in the Indian population. One of the reasons of their population increase maybe due to the elimination of their competition due to the cancer environment in the gut. Since it has been known that both *E. ramulus* and *F. plautii* can metabolize quercetin into beneficial metabolites, phloroglucinol and DOPAC [122, 125], quercetin may have some effects on the CRC condition owing to a relative high abundance of these bacteria in CRC patients. It is, however, difficult to propose from just one dataset. The datasets of the significant quercetin degraders also show variations. Therefore, it is important to conduct *in vitro* or *in vivo* experiments.

From our study, we propose 11 bacteria which are found to be higher in at least one of the datasets. It can be seen that 10 of the bacteria have phloretin hydrolase homologs, thus these bacteria may actually be helpful not only in quercetin metabolism but also phloretin metabolism, another flavonoid. The bacteria *Lactobacillus reuteri* is a probiotic which showed effectiveness in modulating pathways in the pathophysiology of diseases like CRC when pre-cultivated with mucin [158]. This bacterium can be used as a model to understand the effect of quercetin metabolism and its role in cancer prevention. Similarly, *Anaerostipes hadrus, and Roseburia sp.* are major butyrate producers in the gut [159]. These species are therefore beneficial for the gut health. It is essential to study these proposed health-related bacteria in quercetin metabolism as pure cultures as well as

in a mixed culture. The abundance of bacteria utilizing quercetin can also vary according to the dietary conditions. It therefore becomes important to study which bacteria dominate and how they interact under controlled dietary environments. These studies are essential as we know the dietary intake of quercetin varies geographically.

Although this study is limited in terms of bioinformatic analysis and predictions because of limited metadata and number of unequal samples, it gives a foundation for future research. Experiments highlighting effects of quercetin on gut microbiota in various dietary conditions can be useful. A study was performed to understand relationship between quercetin metabolism in the gut, effects on the gut microbiome structure and dietary intake in healthy elderly Japanese subjects [137]. Studying these factors can help us understand how the quercetin affects the gut microbiota ecology being a mediator. Another experiment can be performed where healthy and CRC fecal samples can be subjected to quercetin and controlled dietary condition to check the metabolic responses. This study needs further investigation to understand the microbe-microbe interactions during quercetin metabolism.

This study represents a start to study the subset of gut bacteria which maybe quercetin degraders and how their abundances vary in the healthy and CRC individuals across various geographical areas. This study provides a baseline to focus on the gut microbial role in quercetin degradation and to understand the bacterial community in health and disease condition which may be useful to understand the effects of quercetin as a mediator or an effect modifier under various dietary conditions.

2.5 Conclusion

In this study, we quantified the bacterial patterns of a subset of a total microbiome which may be capable of degrading quercetin. One of the major factors which may be driving the bacterial abundances is the diet as we know that the amount of quercetin consumed varies worldwide. The dietary differences can drive the bacterial interactions and ecology. Further investigation needs to be done to understand how the bacterial community of the gut forms and brings about a positive effect from quercetin transformation.

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