Characterizing specific glycan metabolism by gut bacteria using metabolic labelling coupled to fluorescence-activated cell sorting and 16S rDNA sequencing

Suraya Yasmine
Master of Science
Pharmacology and Therapeutics

McGill University
Montreal, Quebec
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TABLE OF CONTENTS

ABSTRACT

Background: The diverse microbial community residing in our gut, termed the "gut microbiota", is an ecosystem with multiple functions in human physiology, including nutrition, immune system development, and protection against enteric pathogens. Any alteration in this microbial composition (dysbiosis) can potentially influence diseases such as inflammatory bowel disease, cancer, and obesity. Prebiotics are non-digestible dietary fibers, which can be used to modulate the microbiota composition with beneficial outcomes for the host health. Indeed, complex glycans in our diet are an essential source of carbon for the gut microbiota, which collectively encodes the thousands of carbohydrate-active enzymes (CAZymes) necessary to metabolize those complex glycans. While promising, prebiotic approaches have shown mitigated results in the clinic. This, we believe, is owing to a lack of knowledge on glycan metabolism in the gut microbiota, as well as the fact that only a limited number of structures have been used in the clinic. Hence, we aim to understand better the metabolism of specific complex glycan structures by the gut bacteria.

Hypothesis: We hypothesize that understanding which bacteria are responsible for the metabolism of specific glycans ("who eats what?") will inform the design of future prebiotic approaches. Therefore, we aim to identify bacterial consumers of specific glycans in the context of the human gut microbiota.

Methods: To identify gut bacteria that take up specific glycan structures in human microbiota samples, a functional and culture-independent method has been developed in our laboratory (Dridi et al., *unpublished*). This method combines metabolic labelling of bacteria using fluorescently labelled glycans with fluorescence-activated cell sorting (FACS) and 16S rDNA sequencing, which will be abbreviated as "metFACSseq" henceforth.

Results: Metabolic labelling experiments of one human microbiota sample (PY31) with a fluorescein cyclodextrin conjugate (CD-F) resulted in between 2 to 4 % of glycan$^+$ cells, in line with previous results with this probe. Metabolic labeling with a new fluorescein-maltodextrin (MAL-F) probe was also observed with almost 2% of glycan$^+$ cells. We then applied metFACSeq on ten different microbiota samples from healthy volunteer with a total of nine glycan probes (β-cyclodextrin, maltodextrin, mannotetraose, arabinoxylotetraose, fructo-oligosaccharide, xylotetraose, galactomannopentaose, galactooligosaccharides, and nystose). Around 400K fluorescent cells were sorted via FACS from the labelled samples (0.3-4 % glycan$^+$ cells). The sorted cells (glycan$^+$) from a total of 60 different samples were then lysed, and their DNA was extracted. In addition, DNA was also collected from the starting stool samples and the non-fluorescent sorted cell population (without probe) to compare the diversity and abundance of different bacterial species presented in the microbiota samples before and after the metabolic labelling. This protocol significantly increased the metFACSseq throughput, which was previously performed on 1-3M sorted cells.

Future Direction: The sequencing data of the V4 region of the 16S rDNA will be analyzed in collaboration with Dr. Emmanuel Gonzalez (collaborator). This will give us a network of specific glycans and their putative primary consumers in the gut microbiota that will then be validated with cultured isolates.

Conclusion: The metabolic labelling of the stool samples with CD-F was replicated. Metabolic labelling with nine probes and DNA extraction of 400K sorted cells has also been successful. Beyond contributing to the basic understanding of metabolism, this work has the potential to guide rational prebiotic approaches in various diseases.

Résumé

Contexte : La communauté microbienne diverse qui réside dans notre intestin, appelée le microbiote intestinal est un écosystème qui a de multiples fonctions dans la physiologie humaine incluant la nutrition, le développement du système immunitaire et la protection contre les pathogènes entériques. Toute altération de cette composition microbienne (dysbiose) peut avoir une influence potentielle sur des maladies telles que les maladies inflammatoires de l'intestin, le cancer et l'obésité. Les prébiotiques sont des fibres alimentaires non digestibles, qui peuvent être utilisées pour moduler la composition du microbiote avec des résultats bénéfiques pour la santé de l'hôte. En effet, les glycanes complexes contenus dans notre alimentation sont une source importante de carbone pour le microbiote intestinal, qui code collectivement les milliers d'enzymes actives en carbohydrates (CAZymes) nécessaires pour les métaboliser. Bien que prometteuses, les approches prébiotiques ont montré des résultats mitigés en clinique. Nous pensons que cela est dû à un manque de compréhension sur le métabolisme des glycanes dans le microbiote intestinal, et le fait que seul un nombre très limité de structures a été utilisé en clinique. Nous visons à mieux comprendre le métabolisme de glycanes complexes spécifiques par les bactéries intestinales.

Hypothèse: Nous émettons l'hypothèse que comprendre quelles bactéries sont responsables du métabolisme de glycanes spécifiques (« qui mange quoi? ») aidera à la conception de futures approches prébiotiques. Par conséquent, nous visons à identifier les consommateurs bactériens de glycanes spécifiques dans le contexte du microbiote intestinal humain.

Méthodes: Pour identifier les bactéries intestinales qui prennent des structures glycanes spécifiques dans des échantillons de microbiotes humains, une méthode fonctionnelle et indépendante de la culture a été développée dans notre laboratoire (Dridi et al., *non publié*). Cette

méthode combine l'étiquetage métabolique des bactéries utilisant des glycanes fluorescents avec le tri cellulaire induit par fluorescence (FACS) et le séquençage 16S rDNA, qui sera désormais abrégé en «metFACSseq».

Résultats: Les expériences d'étiquetage métabolique d'un échantillon humain de microbiote (PY31) avec une cyclodextrine conjugué à la fluorescéine (CD-F) ont donné lieu à entre 2 et 4 % de cellules glycanes^{+}, conformément aux résultats antérieurs de cette sonde. L'étiquetage métabolique avec une nouvelle sonde de fluorescéine-maltodextrine (MAL-F) a également été observé avec près de 2% de cellules glycanes^{+}. Nous avons ensuite appliqué metFACSeq sur dix échantillons différents de microbiote provenant de volontaires en bonne santé avec un total de neuf sondes glycanes (β-cyclodextrine, maltodextrine, mannotétraose, arabinoxylotétraose, fructo-oligosaccharide, xylotétraose, galactomannopentaose, galactooligosaccharides et nystose). Environ 400K cellules fluorescentes ont été triées par FACS à partir des échantillons étiquetés (0,3-4 % de cellules glycanes^{+}). Les cellules triées (glycanes^{+}) à partir d'un total de 60 échantillons différents ont ensuite été lysées, et leur ADN a été extrait. De plus, l'ADN a également été prélevé à partir des échantillons de selles de départ et de la population de cellules triées non fluorescentes (sans sonde) afin de comparer la diversité et l'abondance des différentes espèces bactériennes présentées dans les échantillons de microbiote avant et après l'étiquetage métabolique. Ce protocole a augmenté de manière significative le débit metFACSseq, qui a été précédemment effectué sur les cellules triées 1-3M.

Orientation future: Les données de séquençage de la région V4 de l'ADN ribosomal 16S seront analysées en collaboration avec Dr Emmanuel Gonzalez (collaborateur). Cela nous donnera un réseau de glycanes spécifiques et leurs consommateurs primaires présumés dans le microbiote intestinal qui sera ensuite validé avec des isolats cultivés.

Conclusion: L'étiquetage métabolique des échantillons de selles avec CD-F a été reproduit. L'étiquetage métabolique avec neuf sondes et l'extraction de l'ADN des cellules triées 400K a également été un succès. Au-delà de contribuer à la compréhension basique du métabolisme, ce travail a le potentiel de guider des approches prébiotiques rationnelles pour diverses maladies.

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Contribution of authors

This work is part of a larger (currently unpublished) collaborative project.

Suraya Yasmine: stool labelling assays and Flow cytometry of bacteria labelled with β-cyclodextrin-Fl, maltodextrin-Fl, fructooligosaccharides-Fl mannotetraose-Fl, xylotetraose-Fl, and arabinoxylotetraose-Fl, DNA extraction, and quantification of sorted bacteria, labelled with β-cyclodextrin-Fl, maltodextrin-Fl, mannotetraose-Fl, xylotetraose-Fl, arabinoxylotetraose-Fl, fructooligosaccharides-Fl galactomannopentaose-Fl, Galactooligosaccharide-Fl, castalagin-Fl, Nystose-Fl. Physiology analysis of the JX94 and PY31. DNA extraction and quantification of sorted cells. Writing of this thesis.

Bastien Castagner: experimental design and project supervision.

Lharbi Dridi: method optimization and experimental pipeline from stool labelling up to DNA extraction.

Corinne Maurice: project advising (microbiology).

Emmanuel Gonzalez: project advising (bioinformatics).

Fernando Altamura: synthesis, purification, and characterization of the old batches of β-cyclodextrin-Fl, maltodextrin-Fl, mannotetraose-Fl, xylotetraose-Fl, arabinoxylotetraose-Fl, fructooligosaccharides-Fl galactomannopentaose-Fl, galactooligosaccharide-Fl, and nystose-Fl. Section 2.2 of this thesis was adapted from Fernando's thesis.

Ryszard Kubinski and Olivia Lui: synthesis, purification, and characterization of the new batches of matodextrin-Fl and fructooligosaccharides-Fl

Reilly Pidgeon: synthesis, purification, and characterization of the new batches of galactooligosaccharide-Fl and castalagin-Fl

LIST OF FIGURES

1. INTRODUCTION & LITERATURE REVIEW

1.1. Microbiome

The human body, the earth's soils, seawater, and all other scientifically studied life sources are colonized by microorganisms, including: bacteria, archaea, fungi, and viruses (Flemming & Wuertz, 2019). This vast community of microorganisms is termed “microbiota.” Microorganisms are often defined as “microbiome” as well. Classically the microbiome refers to the collection of genes contained within a community of microbes. However, Susan L. Prescott (2017) reviewed the origin of the terms and clarified that “microbiome” is a combination of microbe and biome, describing not only genomes, but also the entire microbial ecosystem and all its inhabitants(Prescott, 2017). Thus, both terms “microbiome” and “microbiota” can be used interchangeably.

1.2. Human microbiome

The human microbiome consists of a thousand different bacterial species residing in the mouth, gut, vagina, and skin(Turnbaugh et al., 2007). In 2007, the National Institutes of Health (NIH) funded an investigation into the characterization of the human microbiome called the Human Microbiome Project. This project revealed that the human body contributes to one-fifth of the 100,000 genes found in the microbiome(Turnbaugh et al., 2007). The number of cells within the human microbiome was estimated to be ten times greater than human somatic and germ cells (Turnbaugh et al., 2007). However, according to Sender *et al*. (2016), the amount of bacteria in the body is roughly equal to the number of human cells, and thus contradicting the widely held 10:1 rule (Sender et al., 2016). Despite the ongoing debate over the exact human-microbiome ratio, there is widespread consensus that the microbiome is important, dynamic, and fundamental to the functioning of the human body. It fulfills an important symbiotic relationship

that is vital to both the host and inhabitant microorganisms(Bakken et al., 2011; Spor et al., 2011).

1.3. Human gut physiology and composition of healthy gut microbiome

While bacteria reside all over the human body, the majority of bacteria, 10^{14} bacteria/mL, reside in the colon. (Berg, 1996; Savage, 1977; Tannock, 1995). In the human body, the gastrointestinal (GI) tract is one of the most significant interfaces between the host and the environment (Thursby & Juge, 2017). A lifetime's worth of food (around 60 tonnes), along with a variety of microorganisms move through the human GI tract (Thursby & Juge, 2017). The microorganisms colonizing the GI tract is termed the 'gut microbiome' and forms a profound and mutually advantageous relationship with the host (Bäckhed et al., 2005). The gut microbiome includes the stomach, the small intestine (SI) and the colon. The low pH of the stomach and the fast flow rate of the content through the stomach and SI do not create a hospitable environment for bacteria(Sender et al., 2016). The concentration of bacteria in the stomach, and the duodenum and jejunum of the small intestine is 10^3–10^4 bacteria/mL(Savage, 1977; Sender et al., 2016; Tannock, 1995).

The bacterial population in the colon maintains a profound anaerobic state (Byndloss & Bäumler, 2018; Friedman et al., 2018). Colonic epithelial cells (colonocytes) play a significant role in creating this anaerobic state (Byndloss & Bäumler, 2018; Shelton & Byndloss, 2020). Colonocyte maturation and differentiation requires a nuclear receptor called peroxisome proliferator-activated receptor– γ (PPAR -γ)(Shelton & Byndloss, 2020). PPAR -γ causes physiological epithelial hypoxia by facilitating mitochondrial β -oxidation of long-chain and short-chain fatty acids. Therefore, mature colonocytes' highly oxidative metabolism restricts the amount of O_2 diffused from the mucosal surface, creating an anaerobic environment in the

colonic lumen (Byndloss & Bäumler, 2018; Shelton & Byndloss, 2020). The colonic epithelium preserves gut homeostasis by ensuring the dominance of beneficial anaerobic microorganisms (Byndloss & Bäumler, 2018; Shelton & Byndloss, 2020).

The bacterial ecosystem in the colon is the most extensively studied organ of the human body, due to its high cell count and diversity amongst individuals. Two of the largest projects currently investigating the microbiome are: the METAgenomics of the Human Intestinal Tract Project (MetaHit) (Ehrlich, 2011) and the Human Microbiome Projects(Turnbaugh et al., 2007). Both projects have determined that the "core" human microbiome is in the colon and have characterized the diversity of the microbial composition as well as describe the potential health benefits and risks they could pose to host-health (Sender et al., 2016). These projects have given researchers the most thorough representation of human gut microbial repertoire to date (Hugon et al., 2015; Thursby & Juge, 2017). A healthy gut microbiome is generally dominated by two phyla: *Bacteroidetes* and *Firmicutes* (90% of gut microbiota). Other important phyla include *Actinobacteria, Proteobacteria, Fusobacteria,* and *Verrucomicrobia*. More than 200 different genera comprise the *Firmicutes* phylum, including *Lactobacillus, Bacillus, Clostridium, Enterococcus,* and *Ruminococcus* (Rinninella et al., 2019). The major genera representing *Bacteroidetes* phylum are *Bacteroides, Prevotella, Alistipes, Paraprevotella, Parabacteroides,* and *Odoribacter*. The *Actinobacteria* phylum is less prevalent and is primarily expressed by the *Bifidobacterium* genus (Hugon et al., 2015; Lloyd-Price et al., 2016; Rinninella et al., 2019).

1.4. Contribution of healthy gut microbiome in host metabolism

Most of the contributions made by the gut microbiome to the physiology of the human gut are related to microbial metabolism of host glycans and complex dietary fibers (Vernocchi et al., 2020). An important part of human diet is composed of plant-based foods in the form of fruits,

vegetables and cereals. These foods include simple carbohydrates, complex polysaccharides, and glycans (El Kaoutari et al., 2013).

1.4.1. Glycan metabolism by gut microbiome

Most dietary fibres are composed of plant cell wall polysaccharides, glycans, and starch (Vernocchi et al., 2020). Many structurally diverse sugar moieties are linked together by glycosidic bonds to form chains, and branches. The more complex a polysaccharide is, the more enzymes are required to break it down (Cockburn & Koropatkin, 2016; Hehemann et al., 2019). The enzymes that break down complex polysaccharides are called carbohydrate-active enzymes (CAZymes). Remarkably, the human genome encodes very few of these enzymes, and the only polysaccharide humans can digest is starch(Cockburn & Koropatkin, 2016; Grondin et al., 2017). As a result, all other dietary polysaccharides remain undigested in the stomach, and small intestine, reaching the colon intact. However, since the bacteria residing in the colon encode a large variety of CAZymes, gut bacteria consequently facilitate hosts digestion of these complex polysaccharides (El Kaoutari et al., 2013).

Digestion of a specific complex polysaccharides requires multiple proteins, which are usually encoded in a multigene locus known as a polysaccharide utilization locus (PUL). A PUL contains genes required for sensing, depolymerizing, transporting, and coordinating the breakdown of a specific glycans (Hehemann et al., 2019). PUL-encoded proteins comprise Sus-like systems, named after the starch utilization system. In Gram-negative bacteria (i.e., *B. thetaiotaomicron*), SusC/D-like TonB-dependent transporters can accommodate large polysaccharides (i.e., yeast α-mannan) for cellular metabolism. Those polysaccharides are further processed to monosaccharides for transporting into the cytoplasm (Figure 1.1).

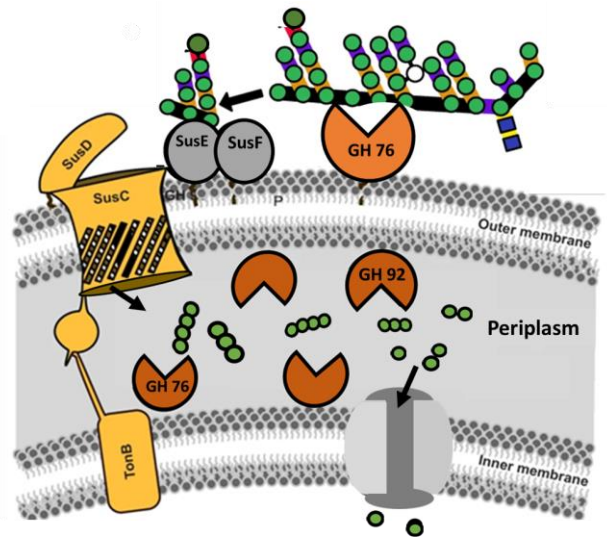

Figure 1.1. Complex glycan metabolism by gut microbiome. (Adapted from Hehemann *et al*., 2019)

In contrast to Gram-negative bacteria, Gram-positive bacteria (i.e., *Firmicutes*) rely far less on extracellular degradation of polysaccharides. Instead, they utilize ABC transporters to import smaller oligosaccharides for bacterial fermentation or intracellular processing(Cockburn & Koropatkin, 2016; El Kaoutari et al., 2013; Hehemann et al., 2019).

The major end products of bacterial fermentation of indigestible dietary fibers and complex glycans are the short-chain fatty acids (SCFAs): acetate, propionate, and butyrate(Mohajeri et al., 2018). These SCFAs are generated in the large intestine under anaerobic conditions, and have the potential to modulate the intestinal barrier as well as to enter the bloodstream to affect systemic health (Mohajeri et al., 2018; Oliphant & Allen-Vercoe, 2019). SCFAs regulate gut homeostasis through G-protein-coupled-receptors found in intestinal epithelial cells, macrophages, dendritic cells, and mast cells (Kim et al., 2013). Further, acetate also protects against infection, whereas butyrate is the main energy source for colonocytes and has anti-inflammatory properties (Kim et al., 2013; Parada Venegas et al., 2019). This improves

gut barrier function, and reduces the incidence of both diabetes (Priyadarshini et al., 2016; Sanna et al., 2019) and obesity(Kobyliak et al., 2016). Moreover, butyrate increases colonic mucus secretion and provides protection against the development of colorectal cancer (CRC) (Cockburn & Koropatkin, 2016; Rios-Covian et al., 2016; Turnbaugh et al., 2006). Therefore, the multifaceted functions of SCFAs demonstrate how the gut microbiome helps humans to receive nutrients from otherwise indigestible food sources.

1.5. Disease State of Gut Microbiome Composition and its Impact on the Host

A link between the gut microbiome and diseases has been confirmed in a large number of human observational studies. The gut microbiota has now been implicated in chronic inflammatory and autoimmune pathologies, as well as metabolic diseases(Zheng et al., 2020).

1.5.1. Inflammatory Bowel Disease and its Connection to the Gut Microbiome

Inflammatory bowel disease (IBD) is a chronic gastrointestinal disease that is induced by an dysregulated immune response (Kaistha & Levine, 2014). Various types of IBD, including Crohn's disease (CD) and ulcerative colitis (UC) have been associated to an altered gut microbiota composition (Khan et al., 2019). IBD induced altered gut microbial composition exhibits loss of enteric bacterial diversity with decreased abundances of *Bifidobacteriaceae, Erysipelotrichaceae, Clostridiales,* and *Bacteroidales,* and an increase abundance of *Enterobacteriaceae, Pasteurellaceae, Fusobacteriaceae, Neisseriaceae, Veillonellaceae*, and *Gemellaceae* (Shreiner et al., 2015). In 2016, Schaubeck and colleagues reported that transferring the pro-inflammatory bacteria from diseased mice into healthy mice can induce inflammation, and thus they concluded that chronic inflammation with IBD depends on microbial triggers(Schaubeck et al., 2016). Furthermore, in 2019 Britton *et al.* demonstrated that colonization of mice with intestinal microbiome from human donors with IBD exacerbates colitis

by altering the immune response in the mouse model(Britton et al., 2019).These studies demonstrate the strong phenotypic association between the gut microbiome and IBD.

1.5.2. Imbalance in Gut microbiome and *C. difficile* Infection

Clostridioides difficile (C. difficile) is an anaerobic, spore-forming, gram-positive bacillus found in the human gut microbiome (Theriot & Young, 2015). *Clostridioides difficile* infection (CDI) is most often associated with antibiotic administration (Gu et al., 2016). Following antibiotic administration, patients have been detected to have increased endotoxin - producing pathogens and lactate producing phylotypes, and a decreased butyrate-producing anaerobic bacterium (Gu et al., 2016). This disturbed intestinal homeostasis then promotes the progression of CDI by increasing susceptibility to *C. difficile* colonization. Improved view of the role of antibiotic-induced microbiome dysbiosis in the pathogenesis of CDI and its recurrence has led to the creation of a new promising therapeutic strategy that involves gut microbiota restoration(Kho & Lal, 2018).

1.5.3. Gut Microbiome and Obesity

Obesity is associated with a significantly reduced bacterial diversity (decrease in *Bacteroidetes* and a rise in *Firmicutes*), which could lead to adiposity by increasing energy harvest (Turnbaugh et al., 2009). A growing number of *in vivo* and human studies suggest that associations between the gut microbiota and the host genotype, as well as dietary changes, may be important factors in obesity and associated metabolic disorders (Turnbaugh et al., 2009; Turnbaugh et al., 2006). Changes in microbiota composition can encourage the development of obesity and other metabolic diseases through increased gut permeability with subsequent metabolic inflammation, impaired short-chain fatty acid synthesis, and altered bile acid metabolism (Festi et al., 2014). In a study published in 2006, Jeffery I. Gordon and his

colleagues discovered that colonizing germ-free mice with microbiota derived from obese mice results in a large increase in total body fat compared to colonizing germ-free mice with microbiota derived from lean mice (Turnbaugh et al., 2006). Further research has shown that microbiota from lean co-twin's microbiota can prevent adiposity gain in obese mice if those obese mice are fed an adequate diet (Ridaura et al., 2013). The above-mentioned evidence indicates that manipulating the composition of the intestinal microbial population, for the purpose of regulating energy balance and metabolism, may be a useful therapeutic method for treating obesity.

1.6. Modulation of the Gut Microbiome as a Therapeutic Approach

Alteration of the gut microbiome composition is associated with loss of intestinal bacteria. This disrupted gut microbiome can trigger intestinal inflammation and metabolic diseases. These consequences, further suggest that manipulating the microbiome to restore depleted microbial functions in diseased patients, could provide novel therapeutic interventions. There are three intriguing approaches to modulate gut microbiome: fecal microbiota transplantation (FMT), probiotics and prebiotics (Altamura et al., 2020).

1.6.1. Fecal Microbiota Transplantation

The method of transferring a faecal solution from a healthy donor into the gastrointestinal tract of a recipient to obtain a health advantage by restoring microbial homeostasis is known as fecal microbiota transplantation (FMT) (Gupta et al., 2016). The protocol requires choosing a donor who does not have a family history of autoimmune, metabolic, or malignant disorders, as well as screening for potential pathogens(Gupta et al., 2016; Nicco et al., 2020).

FMT was first proposed by a Chinese medicine doctor from the 4th century AD (Nicco et al., 2020). The first successful report of treating pseudomembranous colitis with FMT was in

1958, by Eiseman and his colleagues (Eiseman et al., 1958; Nicco et al., 2020). Since then, FMT's clinical experience includes mostly the treatment of recurrent *Clostridioides difficile* infections (CDI) (Gupta et al., 2016). Unlike antibiotics, which deplete bacterial diversity while treating CDI, FMT not only eradicates CDI but also replaces missing microbiota components to increase bacterial diversity, similar to that of healthy donors (Gupta et al., 2016; Smits et al., 2013). FMT may also have therapeutic potential for other metabolic disorders including obesity, and cancer immunotherapy, according to some ongoing studies (Lee et al., 2019; Routy et al., 2018; Tanoue et al., 2019).

Although FMT has proven to be a successful therapeutic approach to treat CDI, it remains uncertain which microorganisms should be administered to patients via FMT as the exact microbial composition of a healthy human gut remains unknown. This uncertainty may cause severe long-term effects on the patient's health, and thus drives investigations into different therapeutic approaches that target the gut microbiome in a more precise manner.

1.6.2. Probiotics

Probiotics are described by the United Nations' Food and Agriculture Organization and the World Health Organization as "living microorganisms that confer health benefits on the host when administered in adequate quantities" (Hotel & Cordoba, 2001; Mack, 2005). Elie Metchnikoff, a Nobel Laureate, was the first to note that Bulgarians who consumed fermented milk products containing viable *Lactobacilli* lived longer(Hemarajata & Versalovic, 2013). This indicated that ingesting such microbes may be beneficial to human health. The most widely used probiotics so far are *Lactobacillus* and *Bifidobacterium* (Islam, 2016).

In 2012, Ki Cha *et al.* reported that a probiotic mixture of *Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus rhamnosus, Bifidobacterium breve, Bifidobacterium*

lactis, *Bifidobacterium longum*, and *Streptococcus thermophilus* provided symptomatic relief in patients with diarrhea-dominant IBS (Ki Cha et al., 2012). *Lactobacillus* strain have also been shown in studies to improve the integrity of the intestinal barrier, which may result in the maintenance of immune tolerance and pathogen suppression (Lee & Bak, 2011). In recent years, *Akkermansia muciniphila*, a mucin-degrading bacterium, has piqued researchers' interest as a promising therapy for several metabolic diseases, and cancer immunotherapy (Plovier et al., 2017; Routy et al., 2018; Zhang et al., 2019; Zhou, 2017). For example: supplementing HFD-induced obese mice with *A. muciniphila* lowered circulating serum lipopolysaccharides (LPS), increased glucose tolerance, and decreased systemic inflammation(Shin et al., 2014). Routy *et al.* (2018) profiled samples from lung and kidney cancer patients and discovered that patients who do not respond to immune checkpoint inhibitors (ICIs) had low levels of *A. muciniphila*. They also showed that antibiotic-treated mice colonized with non-responder stool, receiving oral *A. muciniphila* supplementation, restored the immunotherapy response(Routy et al., 2018). Recently, Tanoue *et al.* (2019), proposed that in syngeneic tumour models, colonization of mice with a consortium of 11 bacterial strains (4 non-*Bacteroidales* and 7 *Bacteroidales* species) provides resistance to *Listeria monocytogenes* infection, and improves the therapeutic efficacy of immune checkpoint inhibitors (Tanoue et al., 2019).

Undoubtedly, there are several other advantages and health benefits associated with probiotics or probiotic food products, but probiotic therapy often has risks. When we are giving probiotics to a patient, we are introducing foreign organisms into the gut microbiome. This raises the question of how probiotics will interact with the commensal bacteria which are already present in the gut, and what the consequences will be for the host health. Future research will

focus on better understanding these interactions and their modes of action, as well as determining which probiotic strains can provide which specific health benefits(Teshale et al., 2017).

1.6.3. Prebiotics

Prebiotics and their relationship to human overall health have sparked people's interest in recent years. Prebiotics are a type of nutrient that the gut microbiome degrades and then uses as a source of nutrients for both the host and the microbiome (Davani-Davari et al., 2019). The prebiotic concept was first introduced by Glenn Gibson and Marcel Roberfroid in 1995. They defined prebiotic as 'a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health'(Hutkins et al., 2016). A food ingredient is considered a prebiotic if it has been clinically proven to meet the following characteristics (Davani-Davari et al., 2019; Slavin, 2013):

1. Stable: Resistant to gastric acidity, mammalian enzymes, and gastrointestinal absorption (Slavin, 2013).
2. Fermented: Can be fermented by intestinal microflora. (Slavin, 2013).
3. Selective: Selectively promotes the growth of intestinal bacteria (mainly *bifidobacteria* and *lactobacilli*) potentially associated with host health and well-being (Slavin, 2013).

However, following the observation of "cross-feeding" by Scott *et al.* (2013), the term selective in the above criteria has been brought into question (Davani-Davari et al., 2019; Scott et al., 2013). This is because cross-feeding describes the process by which one species consumes the by-product of another species (Hehemann et al., 2019). For example, resistant starch is degraded by *Ruminococcus bromii*, and several other species can take advantage of the degraded products of this reaction(Patnode et al., 2019; Ze et al., 2012).

1.6.3.1. Variation of Prebiotics

A nominee prebiotic is any non-digestible dietary substance that reaches the large intestine undigested. Polysaccharides, oligosaccharides, resistant starch, and dietary fibre, as well as proteins and lipids, fall into this category. However, some research suggests that prebiotics are more than mere carbohydrates (Davani-Davari et al., 2019). The following are brief descriptions of some of the most common prebiotics:

1.6.3.1.1. Galacto-Oligosaccharides

Galacto-oligosaccharides (GOS) are a key component of human breast-milk or human milk oligosaccharides (HMOS) and the first prebiotic that humans encounter after birth (R.G. Crittenden, 1996). These oligosaccharides in combination with glycoproteins and glycolipids lead to a *Bifidobacterium*-dominated intestinal community in breastfed infants(Pacheco et al., 2015). Nowadays, many infant formulas are supplemented with GOS (Cockburn & Koropatkin, 2016). Davis *et al*., found that increasing doses of GOS increased the abundance of certain species of *Bifidobacterium,* including *Bifidobacterium adolescentis* and *B. longum*, largely at the expense of *Bacteroides* species(Davis et al., 2011). GOS also stimulate *Enterobacteria,* and *Firmicutes*, but to a lesser extent than *Bifidobacteria* (Davani-Davari et al., 2019).

1.6.3.1.2. Inulin and Fructo-Oligosaccharides (FOS)

FOS are small chain oligosaccharides made up of fructose units connected by (2->1)-glucosidic bonds and a single D-glucosyl unit at the chain's nonreducing end (Tymczyszyn et al., 2013). Although longer-chain inulin is more selective in vitro than short-chain FOS, the ability to metabolize FOS is more widespread within the microbiota. (Scott et al., 2014). FOS has long been known to be beneficial for the growth of *Bifidobacterium* species in the gut, and it is used as a prebiotic in special milk for infants and the elderly (Harmsen et al., 2002). Moreover,

increased *bifidobacteria* levels are thought to underlie human health by developing compounds that suppress possible pathogens, and produce vitamins and digestive enzymes (Chow, 2002). FOS has been shown to have anti-cancer properties, diabetes regulation, uremia reduction, and a systemic effect on hepatic lipid metabolism (Tymczyszyn et al., 2013).

1.6.3.1.3. Resistant Starch (RS)

Resistant starch (RS) is a form of starch that is resistant to digestion in the upper gut (Davani-Davari et al., 2019). Since RS produces a lot of butyrate, it's been proposed that it should be treated as a prebiotic (Fuentes-Zaragoza et al., 2011). RS is also an effective low glycemic replacement for regular starches. Unlike the previously discussed prebiotics, it stimulates the growth of butyrate-producing organisms such as *E. rectale* in addition to *Bifidobacterium* species (Cockburn & Koropatkin, 2016). Various groups of *Firmicutes* display the highest integration with a large amount of RS (Fuentes-Zaragoza et al., 2011). In an in vitro study, *Ruminococcus bromii* and *Bifidobacterium adolescentis* were found to degrade RS, as were *Eubacterium rectale* and *Bacteroides thetaiotaomicron* to a lesser degree. In the absence of *R. bromii*, however, RS degradation is unlikely in mixed bacterial and faecal incubations, which represents an example of cross-feeding strategy among the gut microbiome(Ze et al., 2012).

1.6.3.1.4. Polyphenols

The microbiota of the human gut is also involved in the breakdown of non-carbohydrates, such as polyphenols (phenolic compounds) ingested in the diet (Cardona et al., 2013). Flavonoids and flavonoids sub-families are the most common polyphenolic secondary metabolites, and can be found in a wide range of plants, fruits, tea, cocoa, and wine, among other things (Jandhyala et al., 2015). In most cases, polyphenols in the diet are inactive. By extracting

the sugar moiety, the colonic microflora convert polyphenols into bioactive compounds. The bioactivated compound then can affect human health by influencing intestinal ecology phytochemical profile due to its strong antioxidant and anti-inflammatory potential(Jandhyala et al., 2015; Kumar Singh et al., 2019; Ouyang et al., 2020). Recently it was demonstrated that a crude extract of camu camu (CC), a phytochemical-rich fruit, can trigger the expansion of A. *muciniphila* and other potentially beneficial bacteria in the gut microbiome(Anhe et al., 2019; Langley et al., 2015). It has been also demonstrated that CC prevented obesity and metabolic syndrome in diet-induced obese mice through increasing energy expenditure by preventing visceral and liver fat deposition (Anhe et al., 2019).

1.6.4. Diet and Prebiotics

In humans, a higher bacterial richness and diversity is linked to a diet that is rich in fibre or polyphenol-rich foods. Studies have revealed a strong connection between the metabolic benefits of prebiotic diets and significant improvements in the gut microbiota (David et al., 2014; Koropatkin et al., 2012). The main source of prebiotics is undoubtedly natural foods; such as: leeks, asparagus, chicory, jerusalem artichokes, garlic, onions, wheat, oats, dandelion greens, bananas, and seaweed as well as soybeans (Cockburn & Koropatkin, 2016). As a result, fortifying more commonly consumed foods with prebiotic ingredients is an efficient way to achieve a health-promoting intake (Cockburn & Koropatkin, 2016). These foods contain GOS, FOS, RS and numerous other non-digestible glycans, which have all the potential properties of an ideal prebiotic. Despite having such an ample collection of prebiotic compounds surrounding us, we have very few in clinical trial or even fewer that are commercially available as prebiotics. The reason behind this gap is that we still lack proper knowledge on specific glycan metabolism of each bacterium in our gut. To address this, we must gain the proper genotypic knowledge on

gut bacterial glycan metabolism, which now seems possible with the advancement of modern technology in the research field of gut microbiome.

1.7. Advancement in the Gut Microbiome Research

Cultivation of individual bacteria (Gibbons RJ, 1964) and studies of interactions through co-culture of microbial consortia were used in early gut microbiome research (R.B. Parker, 1961). These initial microbiota studies were solely concerned with composition and function. Early methods for determining microbiome composition revealed differences between microbial communities but provided few details about taxonomic composition of communities. The use of gnotobiotic animals and anaerobic environments, such as anaerobic chambers (Speers et al., 2009), greatly facilitates the cultivation of difficult-to-grow microbes when compared to conventional microbiology approaches. Thanks to technological advancements, many previously uncultivable microbes can now be cultured in a laboratory environment(Connon & Giovannoni, 2002).

Furthermore, the study of microbe behaviour within microbial communities has become increasingly interesting as sequencing technology and bioinformatics research have advanced. (Arnold et al., 2016). The advent of next-generation sequencing (NGS), also known as high throughput sequencing (HTS), facilitated massively parallel sequencing approaches that allowed characterization of the human microbiome a feasible task within reasonable time and effort. HTS technologies can sequence several DNA molecules in parallel, allowing for the sequencing of hundreds of millions of DNA molecules at once (Arnold et al., 2016). Because of this advantage, HTS can be used to produce large data sets, allowing researchers to better understand the cellular genomic and transcriptomic signatures of various diseases and stages of development (Churko et al., 2013; Di Bella et al., 2013).

Currently, isolation of total DNA from samples, PCR amplification of regions within uniformly conserved 16S/18S rRNA genes, and HTS of these amplicons are the gold standard methods for determining the gut microbiome composition. This technology has removed the need for cloning individual genes, blotting for particular RNA, or cultivating individual microbes to identify the members of a bacterial community (Arnold et al., 2016; Di Bella et al., 2013). Although sequencing may provide information on microbiota taxonomy, it has a tendency to overestimate microbial diversity in the complex microbial ecosystem. To understand the microbiome-host relationship, single cell level identification is important. Microbiome analysis using high-resolution flow cytometry and fluorescence-activated cell sorting (FACS) is a fast and powerful tool that allows researchers to separate specific cells of interest from a heterogeneous cell mixture for further molecular and functional analysis (Maurice & Turnbaugh, 2013; Wang et al., 2014; Zimmermann et al., 2016).

1.7.1. Visualization of Selective Glycan Metabolism

Despite the significant progress in characterizing microbiome composition from a variety of sources and identifying the molecular basis of PUL functions, advanced technologies for assigning metabolic phenotypes to genotypes in microbial communities are still needed. Maurice & Turnbaugh (2013) showed us that the use of fluorescent dyes and FACS coupled with 16S rDNA sequencing is a powerful method to analyze the physiology of individual cells within complex bacterial communities, allowing the identification of bacteria labeled according to their nucleic acid content or cell membrane integrity (Maurice & Turnbaugh, 2013).

In addition to the protocol of using fluorescent dyes to identify bacteria (Maurice & Turnbaugh, 2013), fluorescent glycan conjugates (FGCs) has become another powerful tool for rapid and selective study of glycan–microbe interactions within a complex bacterial

community(Hehemann et al., 2019; Reintjes et al., 2017). Reintjes *et al*. (2017) used epifluorescence microscopy to demonstrate that marine bacteria selectively introduce fluorescently labelled polysaccharides into their periplasm. In these experiments, to identify specific cells, substrate-based staining was combined with fluorescence in situ hybridization. Later, Hehemann *et al.* (2019) further demonstrated that glycan uptake in gut bacteria can be visualized with fluorescent glycan conjugates (FGCs) and epifluorescence microscopy. They fed *Bacteroides thetaiotaomicron* VPI-5482 fluorescently labelled yeast-mannan and rhamnogalacturonan-II (Figure 1.2). Wild-type cells consumed the FGCs readily and were

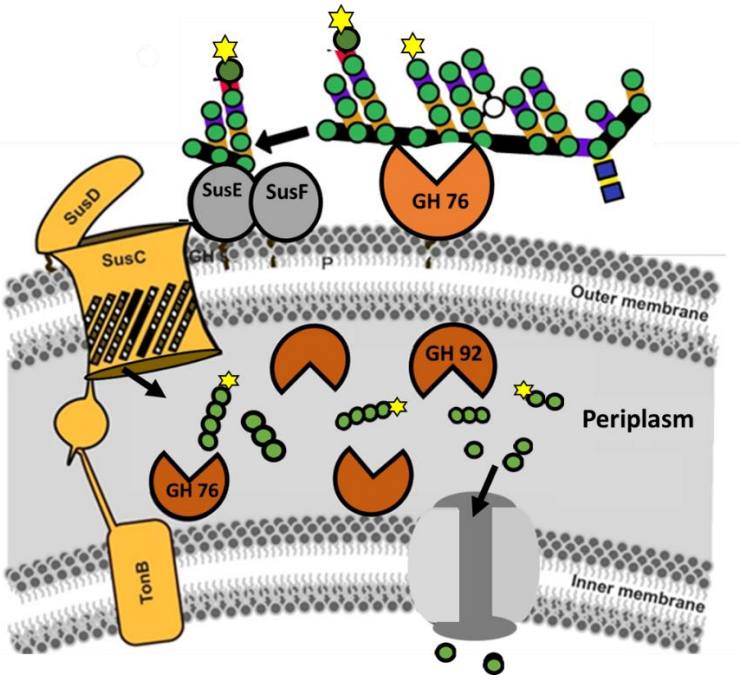

Figure 1.2. Visualizing complex glycan metabolism of gut microbiome (Yellow stars represents the fluorescence tag attached to the glycan) (Adapted from Hehemann *et al.*, 2019)

fluorescent, while strains lacking glycan degradation and transport pathways were non-fluorescent. Therefore, FGC uptake is indeed a direct method for assessing precise glycan metabolism in intestinal bacteria at the single-cell level.

1.7.2. MetFACSeq

Maurice & Turnbaugh (2013), Reintjes *et al*. (2017), and Hehemann *et al.* (2019) showed us that how fluorescent dyes and fluorescent conjugates can be used to label bacteria at the single cell level based on physiological or functional parameters. In addition to that, Castagner and colleagues have developed a functional and culture-independent method that identifies gut bacteria which take up specific glycan structures in human microbiota samples. The method is called metFACSseq that combines metabolic labelling of bacteria using fluorescently labelled oligosaccharides with fluorescence-activated cell sorting (FACS) and 16S rDNA sequencing (Dridi et al., *unpublished*). A similar strategy has been utilized in marine environment to identify consumer of polysaccharides (Martinez-Garcia et al., 2012).

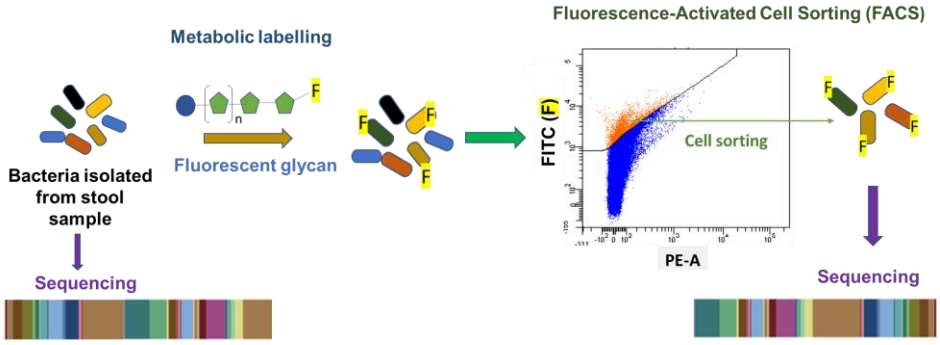

Figure 1.3. Illustration of MetFACSeq. A functional and culture-independent method developed in the Castagner lab, combines metabolic labelling of bacteria using fluorescently labelled glycans with fluorescence-activated cell sorting (FACS) and 16S rDNA sequencing.

1.7.2.1. Preliminary Data Showing the Effectiveness of MetFACSeq

Before applying "MetFACSeq" on human microbiota samples, the uptake of fluorescein conjugated glycans (e.g., β-cyclodextrin and nystose) was investigated in cultured isolates of gut bacteria (e.g., *Klebsiella oxytoca* and *Lactobacillus acidophilus*). After demonstrating significant uptake of fluorescent glycans in *K. oxytoca* and *L. acidophilus*, microbial samples derived from stool samples were metabolically labelled with three fluorescent glycans (e.g., β-cyclodextrin, nystose and galactosyl-mannopentaose). Bacteria isolated from a frozen stool aliquot were resuspended in minimum media lacking carbon sources and incubated with fluorescent β-cyclodextrin for 1h under anaerobic conditions. The optimum incubation time was fixed at 1 hour, as experiments with prolong incubation time did not significantly increase the glycan uptake (Figure 1.4_A). To demonstrate whether the transport of fluorescein conjugated glycans was active or not, experiments were done with free fluorescein and the bacteria were treated with a heat shock of 10 min at 65^{o}C prior to uptake. Flow cytometry data showed no uptake in both conditions (Figure 1.4_B). Furthermore, performing the incubation at 0˚C abolished uptake (data not shown), hence proving the specific and active transport of glycans in bacteria.

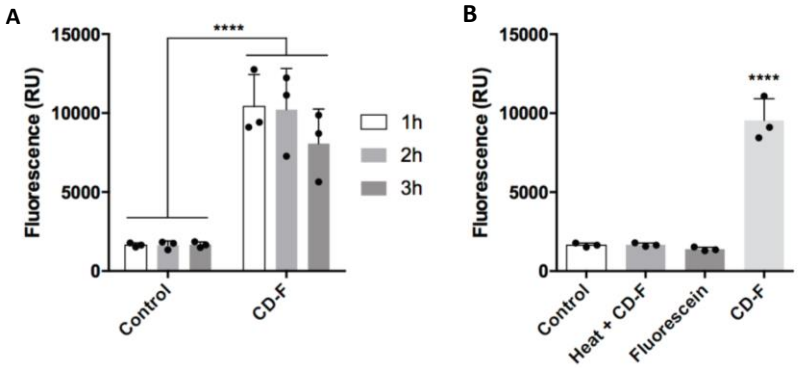

Figure 1.4. Specific and active transport of glycans in bacteria. Fluorescence quantification of bacteria isolated from stool samples after incubation with CD-F(A), free fluorescein for 1h with or without a pre-treatment of the microbiota with a heat shock of 10 min at 65°C (B). Data are mean ± SD (n = 3). ****p < 0.0001 (Dridi *et al.*, *unpublished*).

Finally, the “MetFACSeq” method was applied on microbiota samples from three healthy volunteers using three different above mentioned glycans. After successful metabolic labelling, glycan^{+} cells were sorted by using FACS. Sorted cells were then lysed before DNA extraction and amplification of the V4 region of the bacterial 16S rDNA gene using 515F / 806R V4 primer with custom barcodes. The method was notably able to identify glycan preferences of gut bacterial species, such as *C. aerofaciens* for nystose, or *P. distasonis* for β-cyclodextrin, different *firmicutes* for galactomannan. Thus, proving the effectiveness by identifying assumed consumers of this glycan. This thesis applies this method to a larger number of microbiota samples and glycans to improve our understanding of glycan metabolism.

1.8. Rationale and Hypothesis

The emerging field of gut microbiome has enlightened us with the importance of maintaining a healthy and diversified gut microbial composition. Prebiotic is one of the major therapeutic approaches to modulate the gut microbiome for the host health's beneficial outcome. To identify effective prebiotics, we must understand better the glycan structures and their metabolism by the gut microbiome.

However, the question which specific bacterium and which genes are responsible for metabolizing a particular glycan remains unresolved. So, we hypothesize that identifying bacteria consuming specific glycans in the gut by our unique method “MetFACSeq” will help us to understand specific bacterial metabolism and guide us to design better prebiotic approaches.

2. METHODS AND MATERIALS

2.1.Collection and storage of human stool samples

All stool samples were collected by our collaborator, Dr. Corinne Maurice following the McGill Committee on Human Research Protocol (protocol A04-M27-15B). Screening criteria for stool donors included adult age (18 to 60 years old), health status (no history of gastrointestinal diseases), and antibiotic abstinence in the previous 6 months. After collection, the samples were aliquoted in the anerobic chamber as fast as possible and stored at -80 ^{o}C.

2.2. Selection, synthesis, purification and characterization of fluorescent-glycans

In this thesis, 9 different fluorescent glycans: β-cyclodextrin-Fl (Cyc-Fl), nystose-Fl (Nys-Fl), maltodextrin-Fl (Mal-Fl), mannotetraose-Fl (Man-Fl), xylotetraose-Fl (Xyl-Fl), arabinoxylotetraose-Fl (Ara-Fl), fructo-oligosaccharides-Fl (Fos-Fl), galactomannopentaose-Fl (Gal-Fl), and β-galactosyl-oligosaccharides-Fl (Gos-Fl), and one polyphenol: castalagin (Cast-Fl) were used (Figure 2.1).

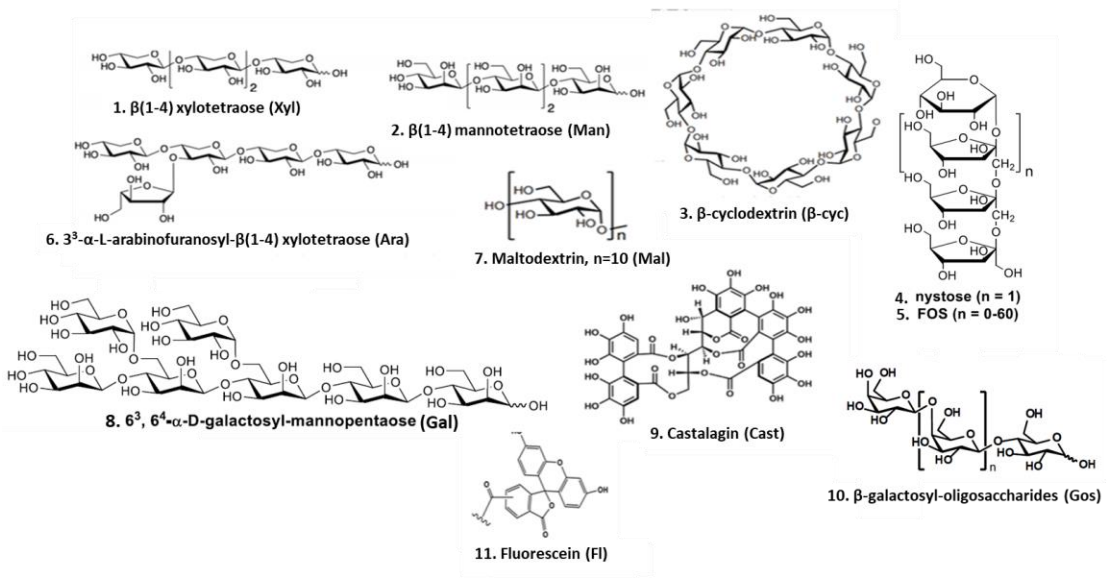

Figure. 2.1. Chemical structures of the selected probes for fluorophore conjugation (1-10) and the fluorophore compound (11)

All the glycans were synthesized, purified and characterized by members of Castagner lab. The selection of the probes was done on the basis of their previously reported prebiotic properties or potential, and the availability of commercial sources with well-defined structures. The reactions for the conjugation of the probes to fluorescein was carried out in basic conditions to allow for the deprotonation of the sugar's alcohol groups. In general, the negatively charged hydroxide ion forms a new ester bond between the sugar and the fluorophore by nucleophilic substitution at the carbonyl carbon of the Fl-NHS. Carbohydrates can conjugate to fluorescein at multiple sites since they include a lot of alcohol functional groups. To benefit the bacterial assays and to prevent the interference of bacterial identification patterns for sugar uptake with a specific location of fluorescein, a random mix of regio-isomeric mono-functionalized sugars was used. All crude reactions have been purified via liquid chromatographic techniques. Each reaction's end product was analyzed using LCMS, and purity was calculated using peak integrations at 280

nm absorbance. Only the products having the purity of greater than 95% were used as chemical probes for bacterial labelling.

2.3. Labelling human stool with fluorescent glycan probe

One day before starting the labelling experiments, 50 mL of phosphate buffered saline (PBS) (1x) and 50 mL of minimum medium (MM) [6.6 mM KH_2PO_4 (pH 7.2), 15 mM NaCl, 100 µM $MgCl_2$, 175 µM $CaCl_2$, 50 µM $MnSO_4$, 5 mM $(NH4)_2SO_4$, 15 µM $FeSO_4$, 24 µM $NaHCO_3$, 1 g/L L-cysteine, 1.9 µM hematin, 6 µM Hemin and 200 ng/ ml vitamin B_{12}] were prepared and filtered through a 0.2 µm filter. The filtered PBS and MM solution were reduced in the anaerobic chamber overnight.

To start the labelling experiment, frozen stool aliquots were quickly introduced in the anaerobic chamber (87% N_2, 10% CO_2 and 3% H_2) and suspended in minimum medium (1 mL per 0.1 g of stool) until a homogeneous suspension was obtained by vortexing thoroughly. The homogenous suspension was centrifuged 3 minutes at 700 g. The supernatant (containing bacteria) was transferred to a new tube, and the pellet (mostly containing undigested particles) was discarded. The new tube was centrifuged 5 minutes at 6500 g, and this time the supernatant was discarded, and the bacterial pellet was kept. The bacterial pellet was then washed (to remove all possible residual glycans) with the same volume of MM added previously and centrifuged again at 6500 g for 5 minutes. Finally, the supernatant was discarded, and the final pellet was resuspended with appropriate volume of MM (195 uL per 0.1 g of stool). For each labelling experiment 195 uL of the stool solution was transferred to an Eppendorf tube. The appropriate volume of probe (5.36 µM) or no probe (control) was added to the appropriate Eppendorf tube and incubated at 37° C for 1 hour. After 1 hour, the sample was washed twice in PBS by centrifuging at 6500 g for 5 minutes. The supernatant (containing possible traces of fluorescence

derived from the MM) was discarded and, each pellet was resuspended in 500 μL of PBS for flow cytometry or cell sorting analysis.

2.4. Quantifying the level of probe uptake with flow cytometry

Each labelling sample was diluted in PBS from 1/30 to 1/50 (depending on the optimal bacterial density for sorting) for a total volume of 1 mL in a plastic test tube. Flow cytometry analysis was performed on a 5 lasers LSR Fortessa, 20 parameters analyzer. Cell sorting was performed on 3 lasers, 13 detector FACSAria-III or 4 lasers, 18-detector FACSAria Fusion. Data were analyzed using BD FACSDiva or FlowJo software. ~10000 (FlowCytometry) to ~500,000 (Sorting) events per sample were analyzed and FITC fluorescence was measured using the FITC channel with excitation at 488 nm and emission at 535 nm. A PBS sample was used to determine the background fluorescence that was later subtracted from the bacterial samples. Subsequently, a sample of non-labelled bacteria was employed to determine the basal fluorescence (Figure. 3.1. A). The gate was determined by FITC-A vs. Side Scatter height (SSC-H) area (Figure. 3.1 & 3.3). The events above this basal point were considered positive in fluorescence due to the uptake of probe. (Figure 3.1.B-C). For MetFACSeq, the labelled bacteria were sorted according to gates designed to only include the fluorescent positive cells that were not auto- fluorescence (based on the B530/30 FITC: B585/42 PE detector filters) (Figure 3.5. A-F). Both the positive (400k) and negative (2M) cells were sorted through a 70 μm nozzle at 70 psi.

2.5. DNA extraction, quantification and 16S rDNA sequencing

DNA extraction of all the sorted cells was performed with the AllPrep PowerFecal DNA/RNA Kit by Qiagen. The positive sorted cells were treated according to the manufacturer instructions. The negative (not labelled) cells were collected in 5 mL tubes, and the serial

dilution was done to get a final volume 200uL to 500uL before starting the extraction procedures. We also extracted DNA from the starting stool samples following the protocols by Qiagen.

The concentration of the extracted DNA was obtained with the Qubit dsDNA HS Assay Kit method by Thermo Fisher Scientific.

For DNA sequencing, the V4 region of the 16S rDNA gene were amplified. DNA amplification and sequencing were performed by Génome Québec.

3. RESULT

3.1. Scatterplots of labelled bacteria

Before embarking on a large-scale MetFACSeq experiment, two stool samples which were used in previous experiments (PY31 and JX94) were labeled with only β-cyclodextrin (Cyc-F and Mal-F) to re-validate the method. Bacteria were isolated from stool samples by successive steps of washing and centrifugation and stained with Cyc-F and Mal-F (at 4.36 uM) under anaerobic conditions for 1 hr. All samples were dissolved in 0.5 mL of PBS, and different dilutions were tested to individualize the optimal bacterial density for sorting. The labelled bacteria were then analyzed and quantified by Flow Cytometry. The basal fluorescence level was determined using the PBS and negative control (unlabelled bacteria) (Figure 3.1A & 3.3A) where the percentage of uptake should be 0%. In PY31, we observed a shift in fluorescence when the labelled bacteria were administered. This shift was the result of bacterial uptake, and the values are 0.6% for Cyc-F and 0.3% for Mal-F (Figure 3.1B & 3.1C). This experiment was repeated for three more times to assure the successful bacterial uptake of both β-Cyc-F and Mal-F in PY31(Figure 3.2). However, we did not observe any uptake in JX94 (Figure 3.3).

Flow cytometry data of stool bacteria (PY31) labelled with β-cyclodextrin-Fl and maltodextrin-Fl

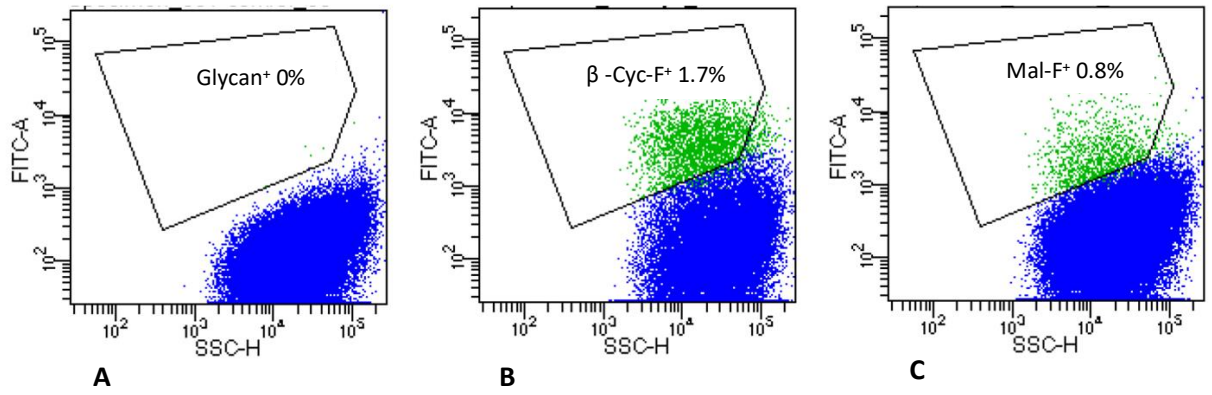

Figure 3.1. Flow cytometry of stool bacteria labelled with β-cyclodextrin-Fl and maltodextrin-Fl. A Negative control (unlabelled bacteria). **B.** bacteria labelled with β-cyclodextrin-Fl (in green). **C.** bacteria labelled with maltodextrin-Fl (in green).

PY31 stool bacteria labelled with with β-cyclodextrin-Fl and Maltodextrin-Fl

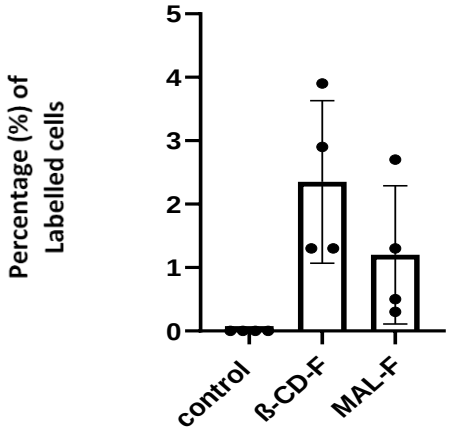

Figure 3.2 PY31 stool labelled with β-cyclodextrin-Fl and maltodextrin-Fl. Both β-CD-Fl and Mal-Fl showed positive uptake compared to the control. Each of the black data points represents each individual labelling experiment (N=4)

JX94 stool bacteria were not labelled with β-cyclodextrin-Fl and maltodextrin-Fl

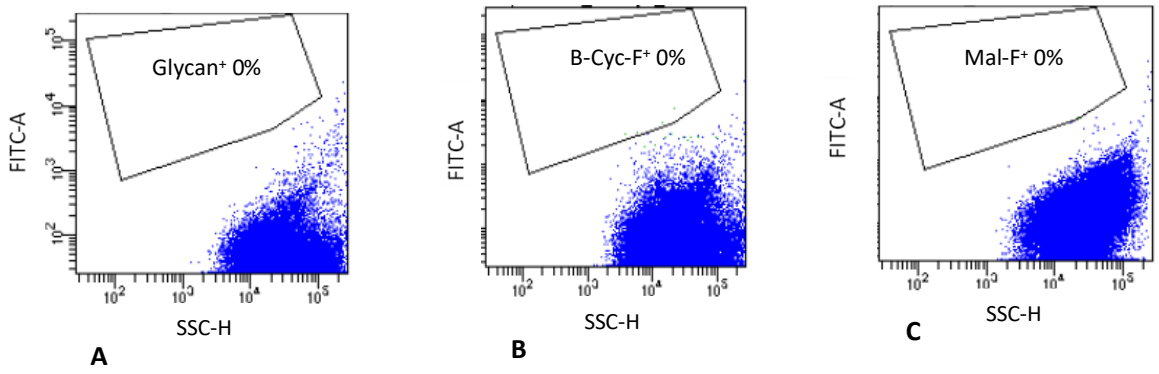

Figure 3.3 JX94 stool bacteria were not labelled with β-cyclodextrin-Fl and maltodextrin-Fl. A Negative control (unlabelled bacteria). **B-C.** Bacteria labelled with **B.** β-cyclodextrin-Fl and **C**. maltodextrin-Fl (No uptake)

3.2. Investigating microbial physiology of PY31 and JX94

To clarify that the low uptake (<0.1%) of β-Cyc-F and Mal-F in JX94 is not because of the metabolic inactivity of the bacteria, the microbial physiology of both PY31 and JX94 was investigated. The experiments were done following the protocol established by our collaborator Dr. Corine Maurice and her lab, using dyes indicative of microbial physiology(Maurice & Turnbaugh, 2013). The bacteria from the stool sample were stained with SybrGreen I (SYBR) for high nucleic acid content and Propidium Iodide (Pi) bacterial damage or dead cells. $SYBR^+$ cells and Pi^+ cells were quantified and analyzed using FlowCytometry and FlowJo software (Figure 3.4). Saining with Propidium Iodide (Pi) revealed that 39.4 % cells of PY31 (Figure 3.4 A), and 46.2% cells of JX94 (3.4B) were damaged. Staining with SybrGreen I (SYBR) revealed that 52.8 % of PY31 (3.4C**),** and 51.1 % of JX94 (3.4D) were metabolically active. Thus, we found no substantial difference between PY31 and JX94 in terms of percentage of dead cells and

high nucleic acid content, yet we see a big difference in the glycan uptake (Figure. 3.1 & 3.3). This shows that the lack of uptake is not correlated with the physiological activity of the samples.

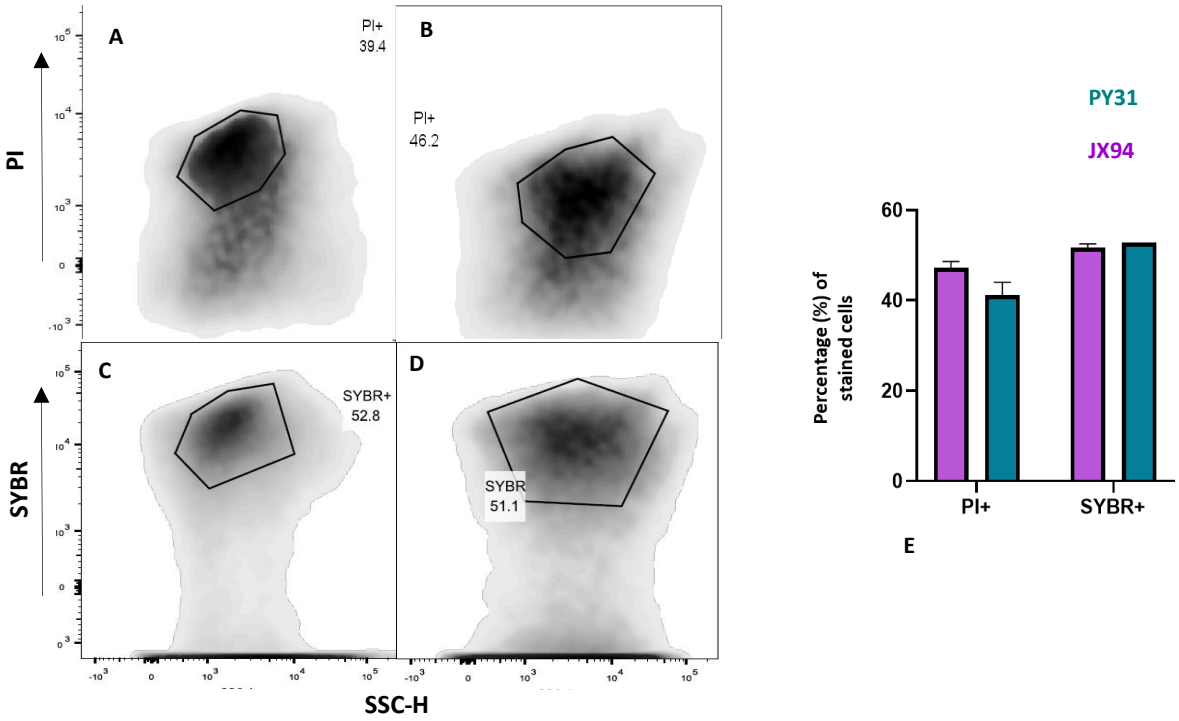

Figure 3.4. Quantifying microbial activity and cell damage. Density plots of human fecal sample PY31 **(A,C)** and JX94 **(B,D).** Staining with Propidium Iodide (Pi) revealed that 39.4 % cells of PY31 **(A),** and 46.2% cells of JX94 **(B)** were damaged. Staining with SybrGreen I (SYBR) revealed that 52.8 % of PY31 (**C),** and 51.1 % of JX94 **(D)** were metabolically active**.** Bar graph showing the mean percentage of total stained cells (**E**) (N=2).

3.3. MetFACSeq:

After the demonstration of labelling with the two probes above, we proceeded with a total 10 different human stool samples and 8 to10 fluorescent probes. Figure 3.5 is showing the FlowCytometry data for one sample: MI44, which was labelled with five fluorescent glycans (Arabinoxylotetraose-Fl, Maltotetraose- Fl,Fructo-oligosaccharide-Fl, Xylotetratose-Fl, Mannotetraose-Fl) in one experiment. At first, the basal fluorescence was adjusted by running unlabelled bacteria in the cytometer (Figure 3.5A). Bacteria were gated on a FITC (530/30 bandpass filter) vs PE (582/42 bandpass filter) to exclude auto fluorescent cells. After adjusting the gate, the labelled bacteria were ran in the cytometer. We observed bacterial uptake for all five fluorescent glycans (Figure 3.5.B-F). Both the labelled (Figure 3.5) and unlabelled (Figure 3.6) cells were sorted through a 70 μm nozzle at 70 psi. DNA was extracted from all the sorted cells of total 80 different glycan^{+}, glycan^{-} and starting stool samples by following AllPrep PowerFecal DNA/RNA Kit by Qiagen. All the uptake and DNA conc value has been shown in Table 1 to Table 11. The sorted samples were sent to Génome Québec for sequencing, and the sequencing data was analyzed by our collaborator bio-informatician (Emmanuel Gonzalez). Figure 3.7 represents the preliminary data of sequencing showing the number of observed operational taxonomic units (OTUs) as a measure of alpha-diversity (diversity of the bacterial community within one sample) differences between the stool, control, and glycan^{+} samples.

Sorting of positive samples

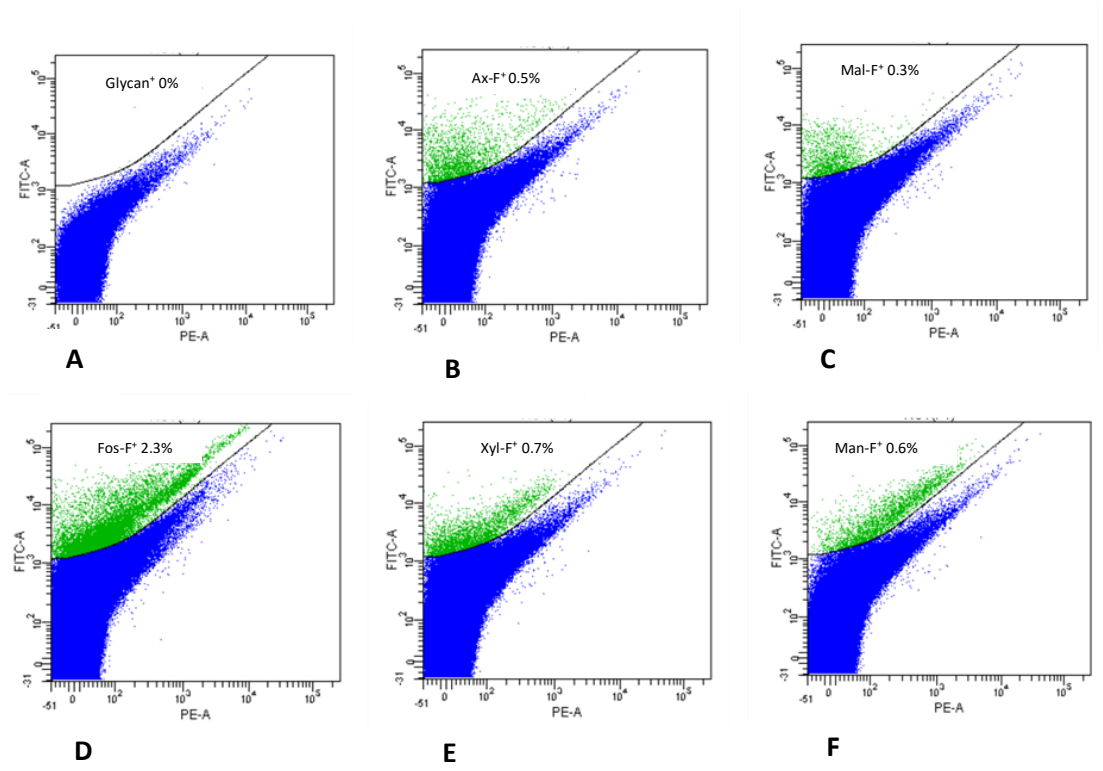

Figure 3.5. Sorting of stool (MI44) bacteria labelled with five different fluorescent glycans.

A Negative control (unlabelled bacteria). **B-F.** Bacteria (in green) labelled with **B.** Arabinoxylotetraose-Fl **C.** Maltotetraose- Fl **D.** Fructo-oligosaccharide-Fl **E.** Xylotetratose-Fl **and F.** Mannotetraose-Fl

Sorting of Negative samples

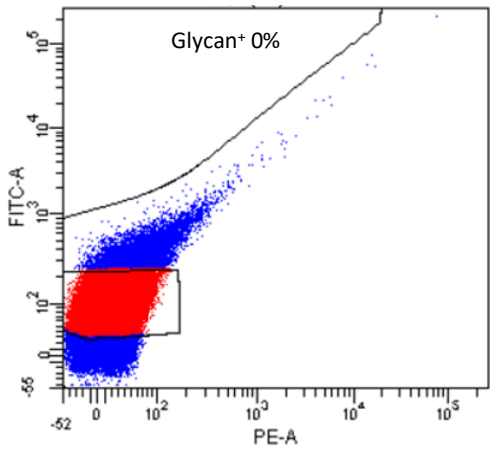

Figure 3.6. Sorting of stool (MI44) unlabelled bacteria (Negative). Negative control (unlabelled bacteria). Red box represents the unlabelled bacteria that were sorted for subsequent steps.

3.4. List of all values of fluorescent probes uptake and concentration of extracted DNA of 10 stool samples:

Table 1. Glycan: Maltodextrin

Sample	Uptake (%)	DNA Conc. (ng/uL)
MI44	0.3	0.0423
YM54	0.4	0.017
VF74	2.7	0.0577
FI87	0.2	0.0263
DT34	0.7	0.0777
TR06	0.6	0.0317
JD98	1.9	0.037
JL73	1	0.023
MX73	0.7	0.0194

Table 2. Glycan: Galacto-mannopentaose

Sample	Uptake (%)	DNA Conc. (ng/uL)
MI44	0.4	0.019
YM54	0.5	0.0727
VF74	0.5	0.0266
FI87	0.3	0.0237
DT34	1.5	0.044
TR06	0.7	0.0977
JD98		
JL73		
MX73	0.4	0.0134

Table_3. Glycan: Xylotetraose

Sample	Uptake (%)	DNA Conc. (ng/uL)
MI44	0.7	0.0277
YM54	0.2	0.018
VF74	0.7	0.0723
FI87	1	0.039
DT34	0.6	0.0217
TR06	0.9	0.0436
JD98	1.5	0.019
JL73	0.9	0.038
MX73		

Table_4. Glycan: Arabinoxylotetraose

Sample	Uptake (%)	DNA Conc. (ng/uL)
MI44	0.6	0.0227
YM54	1.6	0.0148
VF74	1.3	0.0427
FI87	1.2	0.0243
DT34	3.4	0.062
TR06	2.1	0.0187
JD98	1.3	0.021
JL73	0.3	0.033
MX73		

Table_5. Glycan: Fructo-oligosaccharide

Sample	Uptake (%)	DNA Conc. (ng/uL)
MI44	2.3	0.0283
YM54	1.8	0.0283
VF74	0.8	0.035
FI87	0.2	0.018
DT34	0.3	0.02
TR06		
JD98	2	0.0503
JL73		

Table_6. Glycan: Mannotetraose

Sample	Uptake (%)	DNA Conc. (ug/uL)
MI44	0.4	0.0247
YM54	2	0.028
VF74	1.3	0.0467
FI87	0.3	0.0247
DT34	1	0.0623
TR06	0.8	0.027
JD98		
JL73		

Table_7. Glycan: Galacto-oligosaccharides

Sample	Uptake (%)	DNA Conc. (ng/uL)
MI44		
YM54		
VF74		
FI87	0.4	0.0457
DT34	0.4	0.024
TR06		
JD98	0.3	0.0203
JL73	0.7	0.0178
MX73		

Table_8. Polyphenol: Castalagin

Sample	Uptake (%)	DNA Conc. (ng/uL)
MI44	1.2	0.0263
YM54		
VF74	1.1	0.0224
FI87		
DT34	0.7	0.0134
TR06		
JD98	1.4	0.017
JL73	4.7	0.05
MX73	2.1	0.0193

Table_9. Glycan: Cyclodextrin

Sample	Uptake (%)	DNA Conc. (ng/uL)
MI44	0.9	0.0253
YM54	1.1	0.0247

Table_10. Glycan: Nystose

Sample	Uptake (%)	DNA Conc. (ng/uL)
MI44	0.6	0.024
YM54	0.4	0.0933

Table_11: Concentration of extracted DNA of both negative controls and starting stool samples.

Sample	Control (with FACS) DNA Conc. (ng/uL)	Control (starting) DNA Conc. (ng/uL)
MI44	0.0183	251.5
YM54	0.0243	203.5
VF74	0.0198	338.5
FI87	0.017	600
DT34	0.033	515
TR06	0.022	235
JD98	0.0196	132
JL73	0.024	126
MX73	0.013	150.67

3.5. Preliminary Data of Sequencing

α-diversity (Observed OTUs) values of initial stool samples (Stool), negative controls and glycan$^+$ samples:

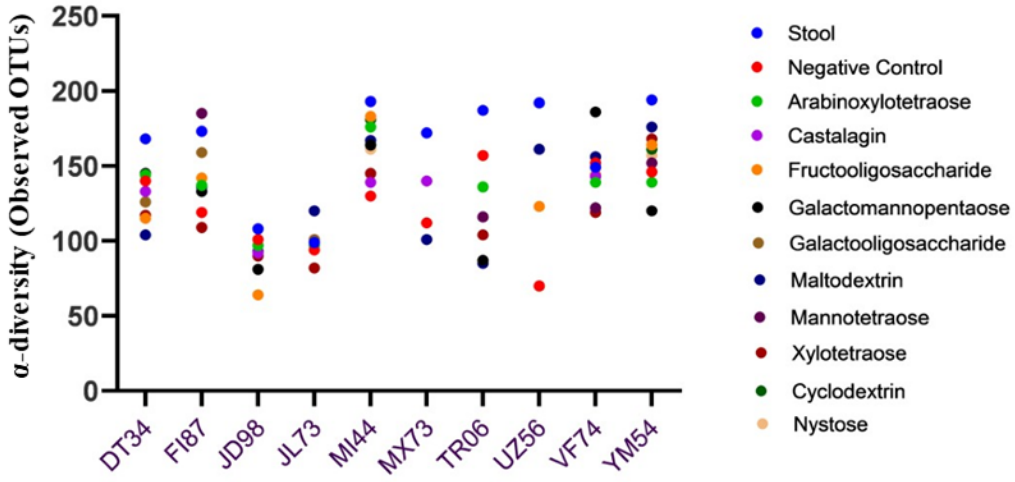

Figure 3.7. α-diversity (observed OTUs) values of initial stool samples (Stool), negative controls, and glycan$^+$ samples. Preliminary data of sequencing showing the number of OTUs as a measure of alpha-diversity differences between the stool, control, and glycan$^+$ samples.

α- diversity (observed OTUs) of initial stool samples and negative controls

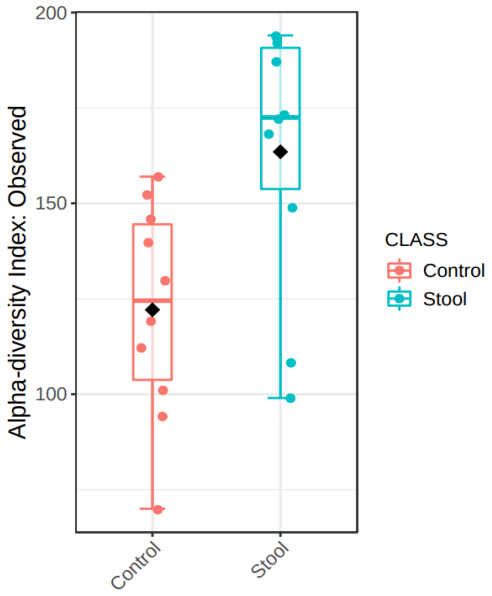

Figure 3.8. α- diversity (observed OTUs) of initial stool samples and negative controls. Significant lower α-diversity (observed OTUs) was found in negative control samples than in starting stool samples. p-value: 0.0091632; [T-test]. The plot was generated using Microbiome Analyst.(Dhariwal et al., 2017)

4. DISCUSSION

Labelling experiments were started with Cyc-Fl and Mal-Fl, and were done on one specific stool sample (PY31) four times to ensure reproducibility of the method: MetFACSeq, and also to replicate the results from our previous study (Dridi et al., *unpublished)*. The presence of uptake (Figure. 3.1 & 3.2) confirmed that our labelling experiments were successful with both Cyc-Fl and Mal-Fl. Other glycans (Ara-Fl, Man-Fl and Xyl-Fl) were also tested and showed that our labelling experiments were working with different glycans and stool samples (Figure 3.5. A-F).

Although most of our labelling experiments were successful, but we were surprised to see that there were some stool samples, which were inadequately labelled (uptake ≤ 0.1) with our fluorophore conjugates (Figure. 3.3). In stool JX94, the uptake was zero after labelling with β-cyclodextrin-Fl and maltodextrin-Fl. Indeed, for a successful labelling experiment, the bacteria in the stool samples need to be metabolically active since the uptake is energy-dependent. Therefore, we tried to investigate whether this low uptake was connected with the overall physiology of the bacteria or not. We followed the protocol established by our collaborator Dr. Corinne Maurice and her lab, using dyes indicative of microbial physiology(Maurice & Turnbaugh, 2013).We tried to correlate the metabolic activity or high nucleic acid content ($SYBR^+$ cells) with bacterial damage or dead cells (Pi^+ cells) (Figure. 3.4). We found no substantial difference between two stool samples in terms of percentage of dead cells and high nucleic acid content, yet we see a big difference in the glycan uptake (Figure. 3.1 & 3.3). This might be explained by the fact that the percentage of dead or live cells does not tell which cells are consuming which glycans. One possible reason for no or low uptake could be that stool does not have the bacterial species that are competent at consuming the specific glycan or that bacterial metabolism is inhibited by an unknown mechanism.

Interestingly, we also observed different glycans labelled different proportions of the overall stool sample. In MI44, FOS-Fl labelled by far the most bacteria (2.3% of the community), followed by Xyl-Fl (0.7%), Man-Fl (0.5%), Ara-Fl (0.5%), and Mal-Fl (0.3%) (Figure 3.5. B-F). Different uptake values of different glycans suggests that specific bacteria in that stool sample (MI44) have specific interest for each of those glycans, and indeed, the presence of those particular bacteria could determine the uptake value.

We then moved forward with the large-scale experiment and started to apply MetFACSeq on 10 different stool samples (MI44, YM54, VF74, FI87, DT34, TR06, JD98, JL73 and MX73) with 10 different fluorescent glycans. Table 1 to 10 shows the uptake level and DNA Concentration of our all experiments. Each table is for each glycan. We consider uptake level of 0.3% or above is sufficient for our experiment. We can see from Table 1 to 10 that few stool samples are missing the DNA Conc. value, as we did not continue the sorting of those samples, because of the low uptake during the labelling experiments. Sorting of the samples having low uptake (0.1 to 0.2) is exceedingly time consuming which can cause the sorting process to be unnecessarily expensive or yields poor amounts of DNA. Therefore, we focused on sorting only those samples which have enough fluorescent labelled cells (Uptake value ≥ 0.3) to get 400K cells after sorting and enough DNA concentration (e.g., ≥ 0.002 ng/ml) to make the DNA sequencing successful.

We opted to sort 400K labelled cells, as we must balance the time to get a reasonable number of cells to get the optimum amount of DNA concentration that would be enough for PCR and DNA sequencing process. Sorting 400K cells after successful labelling experiments (Uptake $\geq$ 0.3%) of the stool samples takes around 30 minutes to 3 hours for each glycan+ samples depending on the uptake level.

Table 11 shows us the DNA concentration of starting stool samples and sorted cells from negative control. Previously sequencing data of only starting stools were compared with the glycan positive population, and it was found that the diversity and number of reads of the glycan positive cells were less compared to the stool (Dridi et al., *submitted*). In the starting stools we expect to see all the bacteria, including bacteria that are agglomerated together, or attached to particles and fibers of the stools, and thus a high amount of DNA concentration is observed. Whereas, in the glycan positive samples, we see a low amount of uptake and DNA concentration compared to the starting stools. We know that the uptake level depends on the presence of specific and metabolically active bacteria that are competent to consume our glycans. Moreover, we also must consider the fact that after the centrifugation and dilution process of the labelling experiments, we may lose some bacteria that may never go through the FACS. Hence, comparing both the starting stools and negative samples with the positive samples is a better way to see which bacteria are labelled successfully, and which are not. Figure. 3.6 shows how we selected 2 million negative cells for sorting. The gating was done to avoid auto fluorescent cells. Figure. 3.8 shows that indeed, the α-diversity in terms of observed OTUs is lower in the negative controls compared to the stool samples, demonstrating that not all bacteria are detected after cytometry. The sequencing data analysis, and the biological interrelation of which bacteria is consuming which of the glycans will be done in the future by others.

However, it is worth mentioning that "metFACseq" has some limitations as well. Since our method is capable of detecting only the oligosaccharide uptakes, we are missing information on the species that utilize glycans by cross feeding (Hehemann et al., 2019; Patnode et al., 2019). There is also strain level variability on glycan utilization which we cannot detect through metFACseq using 16S sequencing (Klassen et al., 2021; Patnode et al., 2019). Despite these

limitations, identifying the primary consumers of specific glycans is undoubtedly an important step in attempting to predict the complex microbial response to glycans.

5. CONCLUSION

More than two millennia ago Hippocrates stated, “All disease begins in the gut”. This statement now seems timelier than ever, as advanced genomics and bioinformatics shed new light on the complexity and importance of the complex microbial community residing in our gut. Our novel and culture-independent method: “metFACSeq” indeed has the potentiality to be an invaluable contribution to the ongoing advanced gut microbial studies. Our future investigations on the biological interrelations of the sorted glycan positive bacteria will certainly lead us to reveal the detail on gut microbial metabolism and precise prebiotic approaches.

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Abbreviations

- ABC transporter: ATP Binding Cassette transporter
- Ara-Fl: Arabinoxylotetraose-fluorescein conjugate
- β-Cyc-Fl : β-Cyclodextrin-fluorescein conjugate
- CAZymes: Carbohydrate-active enzymes
- CD: Crohn's disease
- CDI: *Clostridioides difficile* infection
- CAZymes: Carbohydrate-active enzymes
- CRC: Colorectal cancer
- CC: Camu camu
- FMT: Fecal microbial transplant
- FOS: Fructooligosaccharaides
- Fos-Fl: Fructooligosaccharides-fluorescein conjugate
- FACS: Fluorescence-activated cell sorting
- FGCs: Fluorescent glycan conjugates
- GIT: Gastrointestinal tract
- Gal-Fl: Galactomannopentaose-fluorescein conjugate
- GH: Glycoside hydrolase
- GOS: Galactooligosaccharides
- GOS-Fl: β-galactosyl-oligosaccharides-Fl
- HMOS: Human milk oligosaccharides
- HFD: High-fat diet
- HITS: High throughput sequencing

- IBD: Inflammatory bowel disease
- IBS: Irritable bowel syndrome
- ICIs: Immune checkpoint inhibitors
- LPS: Lipopolysaccharides
- MetaHit: METAgenomics of the Human Intestinal Tract Project
- Mal-Fl: Maltodextrin fluorescein conjugate
- Man-Fl: Mannnotetraose fluorescein conjugate
- Nys-Fl: Nystose fluorescein conjugate
- NIH: National Institutes of Health
- NGS: Next-generation sequencing
- PPAR -γ: peroxisome proliferator-activated receptor– γ
- PUL: Polysaccharide utilization locus
- RS: Resistant starch
- SI: small intestine
- SCFAs: Short chain fatty acids
- SUS-like system: Starch-utilization system
- UC: Ulcerative colitis
- WHO: World Health Organization
- Xyl-Fl: Xylotetraose-fluorescein conjugate

Materials

Instruments

▪ BD FACSCanto II and BD FACSAria III from BD Biosciences for cell analyzer and sorter respectively.

▪ Vinyl anaerobic chamber from Coy Lab Products for stool manipulation and labelling.

▪ Qubit 3 Fluorometer from Thermo Fisher Scientific for quantification of DNA concentration.

Software

▪ Chem3D Ultra (v.20.1.0.110) for the drawing and analysis of all chemical structures.

▪ EndNote (v.X9.3.3) for the bibliography compilation.

▪ GraphPad (v.9.0.1.151) for the analysis of different uptake values of probes.

▪ FACS DIVA (v.8) for the analysis of cell analyzer data.

▪ FACS DIVA (v.6) for the analysis of cell sorter data.

▪ FlowJo (v.10) for the analysis of physiology data.

▪ Office 365 (v.2105) for the writing of this thesis.