Microbiome After Bariatric Surgery and Microbial Insights into Surgical Weight Loss

by

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ABSTRACT

Obesity is a worldwide epidemic accompanied by multiple comorbidities. Bariatric surgery is currently the most efficient treatment for morbid obesity and its comorbidities. The etiology of obesity is unknown, although genetic, environmental, and most recently, microbiome elements have been recognized as contributors to this rising epidemic. The role of the gut microbiome in weight-loss or weight-gain warrants investigation, and bariatric surgery provides a good model to study influences of the microbiome on host metabolism. The underlying goals of my research were to analyze (i) the factors that change the microbiome after bariatric surgery, (ii) the effects of different types of bariatric surgeries on the gut microbiome and metabolism, (iii) the role of the microbiome on the success of bariatric surgery, and (iv) temporal and spatial changes of the microbiome after bariatric surgery.

Roux-en-Y gastric bypass (RYGB) rearranges the gastrointestinal tract and reduces gastric acid secretions. Therefore, pH could be one of the factors that change microbiome after RYGB. Using mixed-cultures and co-cultures of species enriched after RYGB, I showed that as small as 0.5 units higher gut pH can aid in the survival of acid-sensitive microorganisms after RYGB and alter gut microbiome function towards the production of weight loss-associated metabolites. By comparing microbiome after two different bariatric surgeries, RYGB and laparoscopic adjustable gastric banding (LAGB), I revealed that gut microbiome structure and metabolism after RYGB are remarkably different than LAGB, and LAGB change microbiome minimally. Given the distinct RYGB alterations to the microbiome, I examined the contribution of the microbiome to weight loss. Analyses revealed that *Fusobacterium* might lessen the success of RYGB by

producing putrescine, which may enhance weight-gain and could serve as biomarker for unsuccessful RYGB.

Finally, I showed that RYGB alters the luminal and the mucosal microbiome. Changes in gut microbial metabolic products occur in the short-term and persist over the long-term. Overall, the work in this dissertation provides insight into how the gut microbiome structure and function is altered after bariatric surgery, and how these changes potentially affect the host metabolism. These findings will be helpful in subsequent development of microbiome-based therapeutics to treat obesity.

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COD	Chemical oxygen demand
GC-MS	Gas chromatography mass spectrometry
HPLC	High pressure liquid chromatography
LAGB	Laparoscopic adjustable gastric banding
LC-MS	Liquic chromatography mass spectrometry
NMR	Nuclear magnetic resonance
OTU	Operational taxonomic unit
PICRUSt	Phylogenetic investigation of communities by reconstruction of unobserved states
PreB-Ob	Pre-bariatric morbidly obese
PCA	Principal coordinate analysis
PCoA	Principal component analysis
QPCR	Quantitative real-time polymerase chain reaction
QIIME	Quantitative insights into microbial ecology
RYGB	Roux-en-Y gastric bypass

CHAPTER 1

INTRODUCTION AND SIGNIFICANCE

1.1 Obesity pandemic and bariatric surgery as a management strategy

Defined as body mass index (BMI) of 30 kg/m^2 or higher, obesity is a condition that affects multiple organs and causes severe health issues in children and adults. Comorbidities of obesity can be physiological or psychological and range in severity from depression to diabetes, hyperlipidemia, hypertension, and increased risks of cancers and strokes (Musich *et al.*, 2016). Obesity-related diseases escalate obesity to an expensive health condition: obese individuals incur ~US$1500 higher annual medical expenses than normal weight individuals (Musich *et al.*, 2016).

A global rise in obesity rates, especially in developed countries such as U.S.A., has boosted research into its etiology and treatment. Obesity is often been associated with an imbalance between energy intake and energy expenditure (Serra-Majem *et al.*, 2013). However, this over-simplified definition has been abandoned due to recent evidence showing that behavioral, neurological, hormonal, and genetic factors contribute to the development of obesity and related disorders (Mitchell & Shaw, 2015). More recently, the gut microbiota has shown to be a contributing factor to the development of obesity (Ley *et al.*, 2005; Turnbaugh *et al.*, 2006).

A number of diet, fitness, and behavioral improvement programs have been developed for the management of obesity and diabetes (Yumuk *et al.*, 2015). However, these strategies have limited success, especially on morbidly obese individuals with BMIs >40 (Buchwald & Oien, 2013). Bariatric surgery is currently considered the most

effective treatment for morbid obesity (Buchwald & Oien, 2013; Dixon, Zimmet, Alberti, Rubino, & Int Diabet Federation Taskforce, 2011). Numerous open and laparoscopic bariatric surgeries are available, with Roux-en-Y gastric bypass (RYGB), vertical sleeve gastrectomy (VSG), and adjustable gastric banding (LAGB) being the most commonly performed surgeries (Buchwald & Oien, 2013). LAGB and VSG are restrictive surgeries, which limit food intake by decreasing the stomach size, but leave the digestive system otherwise intact. RYGB is considered a restrictive and malabsorptive process (Aron-Wisnewsky, Dore, & Clement, 2012). RYGB reduces the size of the gastric pouch, bypasses the duodenum, reduces acid secretions, and rearranges the gastrointestinal tract (Aron-Wisnewsky, *et al.*, 2012).

In general, RYGB patients experience higher weight loss and remission of metabolic syndrome versus LAGB patients (Carlin *et al.*, 2013). Researchers have proposed that the gut microbiome is modified due to altered gastrointestinal tract anatomy after bariatric surgery (Tremaroli *et al.*, 2015; Zhang *et al.*, 2009), and it was demonstrated in mice that these changes have an important effect on the surgery's success (Liou *et al.*, 2013).

1.2 Introduction to the gut microbiome world

The trillions of microorganisms inhabiting our guts have many functions; they produce vitamins or metabolites that act as signaling molecules to the brain and convert undigested food into energy resources (Macfarlane & Macfarlane, 1997; Neuman, Debelius, Knight, & Koren, 2015). With recent developments in 16S rRNA gene fingerprinting technologies, metabolomics, cultivation of previously "uncultivable" microorganisms, and bioinformatic tools, microbiome researchers are able to deeply

characterize microbe-host related health conditions. With the initiation of the Human Microbiome Project, the gut microbiomes of healthy (Peterson *et al.*, 2009) and unhealthy humans have been characterized, including disorders such as diabetes (Cani *et al.*, 2007), obesity (Ley, *et al.*, 2005), autism (Kang *et al.*, 2013, Hsiao *et al.*, 2013), and irritable bowel syndrome (Wu, Bushmanc, & Lewis, 2013).

Host-microbe energy balance studies have demonstrated several important findings: 1) Microbiome composition differs between obese and lean hosts (Ley, *et al.*, 2005). 2) Microbes can regulate host fat storage (Backhed *et al.*, 2004) and are involved in weight loss (Turnbaugh, *et al.*, 2006). 3) Microbes can also transmit host phenotypes (Tremaroli, *et al.*, 2015). More recently, microbiome research has evolved to identify causative interactions between the microbiome and host metabolism. With these new findings, the microbiome's role on host energy regulation has been expanded from energy extraction to hormonal and neurological regulation as well as changes in circadian rhythms and mood. Knowing that bariatric surgeries induce hormonal, neurological, and behavioral changes on hosts, it is crucial to understand the relationship between microbiome after RYGB and weight loss, remission of metabolic syndrome, and quality of life improvements.

1.3 Dissertation framework

Recent studies interrogating the human gut microbiome suggest that the gut microbiota play a significant role in the regulation of host metabolism. Many of the findings have been attained from animal studies and do not completely translate to the human metabolism. Bariatric surgery serves as an invaluable model for gut microbiota and host energy metabolism studies as it imposes drastic temporary or permanent

environmental changes on microbiota. Diet and other environmental factors change considerably after bariatric surgery, and this needs to be considered for better understanding of the factors that lead to successful and sustained weight loss post surgery. Therefore, the underlying hypothesis for my research is that bariatric surgery imposes changes on the environmental conditions in the gut, altering the host-microbiome and contributes to energy metabolism. I pursued four major research goals to address my hypothesis: 1) determine differences in microbiome and metabolism after Roux-en-Y gastric bypass and Laparoscopic Adjustable Gastric Banding (LAGB); 2) identify microbial signatures of successful RYGB surgery; 3) evaluate spatial and temporal changes in the microbiome and metabolism after RYGB surgery; and 4) characterize microbial response to environmental changes due to alterations in gut pH similar to those that happen after RYGB.

To accomplish these goals, I applied integrative molecular, microbial, and ecological approaches. Below, I provide a brief summary of the organization of each chapter.

Chapter 2. Before addressing the research questions, I present a detailed background on microbiome research in the context of obesity, weight loss, and bariatric surgery. This chapter provides a comprehensive literature review on the microbiome in obese and post-bariatric surgery patients, the role of microbiome on host energy regulation, methods to study microbiome structure and function, and factors that influence microbiome structure and function.

Chapter 3. RYGB surgery alters gastrointestinal anatomy and imposes environmental changes on gut microorganisms. One environmental change is increased

gastrointestinal pH due to a reduction in gastric acid secretions. An increased pH can change microbial community dynamics, enabling the survival of acid-sensitive bacteria. An altered microbial community can perform modified microbial reactions, especially fermentation. Hence, pH is crucial for the understanding of microbial outcomes after RYGB surgery. Another variable is the delivery of different carbon sources to the colon. To tackle this research goal, I developed enrichment cultures from a fecal sample collected from a healthy individual. I exposed the cultures to different pH conditions. For each pH condition, I tested the secondary effects of the carbon source on the microbial community structure and fermentation pathways by tracking microbiome composition using16S rRNA gene and fermentation end products in each enrichment culture. I assessed how small changes in initial pH and carbon type altered microbial community dynamics and interpreted those changes as a possible outcome of RYGB surgery.

Chapter 4. As shown in chapter 3, pH is an important environmental factor that controls the metabolic interactions among the gut microbial species. pH inhibition or aid of certain gut species can have fundamental impact on the metabolic outcome of the microbial processes. Based on the observations from chapter 3, I investigated the impact of pH on microbial interactions using pure cultures of *Veillonella dispar*, *Bacteroides thetaiotaomicron*, and *Streptococcus salivarius*. By tracking down growth and metabolism of these monocultures and under various co-culture scenarios, I investigated the impact of pH-driven metabolic partnerships, especially as they relate to lactate metabolism.

Chapter 5. RYGB and LAGB are two commonly performed bariatric surgeries for treatment of morbid obesity. LAGB surgery is relatively unsuccessful compared to RYGB. While changes in gut microbiota structure and metabolism after RYGB surgery have been established in small cohorts for humans and animals, and altered microbiota after RYGB has been shown to enhance weight-loss in mice studies. On the other hand, gut microbiota changes post-LAGB surgery has not been reported. I hypothesized that RYGB imposes greater changes on the microbiome than LAGB, and this is one of the reasons why RYGB is more successful than LAGB. I provide a holistic approach to addressing this hypothesis by integrating microbiota diversity, metabolites, and clinical data into an analysis of variations between normal weight, obese, post-RYGB, and post-LAGB subjects. I analyzed fecal microbiota composition and diversity to establish differences between LAGB and RYGB microbiota. I correlate the abundance of several fecal metabolites with the microbiota in surgical weight loss groups. Finally, I discuss the microbiome and fecal fatty acid data in the scope of clinical data that we collected including diet, exercise and weight loss of the subjects. I show that changes in the microbiome after RYGB are due to the surgical intervention rather than changes in the lifestyle of the subjects.

Chapter 6. RYGB surgery patients experience varying success rates after surgery. RYGB patients harbor different gut microbiota than obese and lean people; however, the structure of the microbial community and how it affects long-term surgical success are unknown. Some microbial metabolites have long been associated with host energy metabolism. Therefore, the RYGB-altered gut microbiota might enhance weight loss via production of specific metabolites. Here, I hypothesize that some microbiota-

specific metabolites are responsible for successful and sustained weight loss after RYGB surgery. For this research goal, I divided the RYGB population into successful and unsuccessful groups using three approaches: 1) greater or less than 20% weight regain, 2) 2-means clustering based on their excess weight loss percentages and, 3) greater or less than 85% maximum excess weight loss percentage. I investigated the gut microbial ecology in successful and unsuccessful subjects and analyzed global metabolomics data to identify possible biomarkers specific to successful RYGB surgery.

Chapter 7. RYGB is a non-reversible surgery, which can have long-term impacts on patients' life styles and metabolisms. Patients often experience drastic weight loss in the first few months following surgery but regain some weight over the long-term. The microbiota's roles in long-term, successful weight loss and weight management following bariatric surgery are unknown. Moreover, microbiota studies rely heavily on luminal samples, with changes in the microbiota at mucosal surfaces after bariatric surgeries being unknown. In this chapter, I presented the results of longitudinal changes in the microbiota after RYGB surgery in mucosal and luminal surfaces. Moreover, my comprehensive analysis on fecal metabolome that includes soluble and non-soluble metabolites in addition to fecal bile acids provide a deeper understanding to sustained changes in gut microbiome and metabolome after RYGB surgery in connection to weight loss.

Chapter 8. This chapter summarizes the key findings of the research chapters 3-7 and provides recommendations for future studies to enhance the understanding of microbe-host interactions and microbiome mediated host metabolism regulation.

CHAPTER 2

BACKGROUND ON OBESITY, MICROBIOME, AND BARIATRIC SURGERY

2.1 Obesity

Obesity is a worldwide problem that affects millions of individuals (Roth *et al.*, 2009), and is of a great concern because of it is associated with multiple comorbidities that lead to metabolic syndrome (Ali *et al.*, 2006). Obesity develops as a result of an imbalance between energy intake and energy expenditure (Roth *et al.*, 2009). Even though the origins of obesity are unknown, factors such as host genetics, dietary habits, host psychology and sedentary lifestyle are known to contribute to the development of obesity (Nieuwdorp *et al.*, 2014). Additionally, microorganisms that reside in the human gut, "the gut microbiota", have been proposed as one of the factors that control obesity (Turnbaugh *et al.*, 2006). This chapter summarizes current findings related to: 1) microbiota implicated in host energy balance, and development of obesity and 2) approaches to study the gut microbiota, 3) factors affecting gut microbiome, and 4) microbiota related weight loss and weight gain mechanisms.

2.2 Discovery of microbiome as a potential regulator of human energy balance

2.2.1 Increasing recognition of host - microbiota symbiosis in the development of obesity

The interactions between the host and gut microbiota are often mutualistic: the host provides nutrients and space to live to the microbiota (Macpherson & Harris, 2004), and, in return, microbiota support the host health with various functional interactions (Tremaroli & Backhed, 2012). For instance, the microbiota prompt a healthy immune

response (Round & Mazmanian, 2009), metabolize drugs (Xu *et al.*, 2007), provide energy sources to colonocytes (Pryde *et al.*, 2002), produce bioactive molecules such as vitamins (Sekirov *et al.*, 2010), hydrolyze indigestible dietary components (Sekirov *et al.*, 2010), and contribute to the host energy metabolism (Tremaroli & Backhed, 2012). Additionally, the gut microbiota preserve the healthy function of the gut by competing for food and space with intruder microorganisms (Macpherson & Harris, 2004).

In the context of obesity and its comorbidities such as diabetes, disruption of symbiosis between the host and gut microbiota could be an important contributor to energy expenditure. The gut microbiota's role on energy balance has been studied extensively in the past 10 years, and microbially enhanced energy harvest has been proposed to occur by multiple mechanisms summarized here: 1) microbiota hydrolyze undigested polysaccharides and increase bioavailable energy (Wolf, 2006), 2) microbiota produce short chain fatty acids, mainly acetate, propionate and butyrate; these can be readily absorbed by the host (Turnbaugh *et al.*, 2006) and serve as signaling molecules to receptors in the gut or brain (Lin *et al.*, 2012), and 3) microbiota are involved in hormone regulation pathways (Lin *et al.*, 2012, Yano *et al.*, 2015).

2.2.2 Animal models show causality for body weight regulation

Human studies exhibit two important challenges that can hinder isolation of cause and effect relationships between the microbiota and body weight regulation. The first challenge is the inter-individual variations in host genetics and metabolism, and the second one is the difficulty to control environmental variables such as diet and exercise. Animal models, especially gnotobiotic mice models provide an opportunity to test mechanistic hypotheses (Thi Loan Anh *et al.*, 2015); they provide a platform for gut

microbiota study designs with less inter individual variability and strict control of environmental and dietary variables such as identical food intake, and homogeneous genetic background (Spor *et al.*, 2011). Moreover fecal transplants to gnotobiotic animals have facilitated the translation of microbiota's potential impact on host energy metabolism (Turnbaugh *et al.*, 2006). Furthermore, gnotobiotic animal studies allow researchers to study the effect of specific microbes on host body weight one at a time or in specific important combinations.

Microbiota's role on energy metabolism can occur in two directions: weight gain and weight loss. Some studies that focus on weight gain include co-habitation of the gnotobiotic mice gut with specific microorganisms and fecal transplantations to prove specific hypothesis. For example, cohabitation of a fermenter (*Bacteroides thetaiotaomicron)* with a methanogen (*Methanobrevibacter smithii*) in the mice gut increased dietary energy extraction (Samuel & Gordon, 2006). In another case, absence of one specific type of microorganism, *Christensenella minuta,* in the mice gut showed increased weight gain due to altered microbial community dynamics (Goodrich *et al.*, 2014). Fecal transplants to mice provided scientific proof that microorganisms can increase the host's energy intake. For instance, fecal transplantation from conventionally raised mice to gnotobiotic mice increased body weight and liver adiposity (Backhed *et al.*, 2004). Moreover, in a later study, when the transplantation was performed from obese humans to lean mice, an increase in mice body weight and adiposity was observed due to increased dietary energy harvest (Turnbaugh *et al.*, 2006). Later research also showed that increased adiposity after fecal transplantation to mice correlated with the

abundance of endotoxin producing bacteria in the colon (Everard *et al.*, 2013, Fei & Zhao, 2013), and this finding further strengthen the microbiota.

On the other side of the energy balance equation, fecal transplantation studies also showed the microbiota's potential to initiate weight loss in mice. For instance, transfer of microbiota from mice that had bariatric surgery (Roux-en-Y Gastric Bypass) to gnotobiotic mice resulted with decreased body weight and adiposity (Liou *et al.*, 2013). As a result, the RYGB-recipient mice produced higher propionate in comparison to acetate compared to the controls, these metabolic changes as a result of the fecal transplantation indicated causality (Liou *et al.*, 2013). The uses of gnotobiotic animals and their colonization with microbiota from humans or animals with different metabolic types have strengthened the acceptance of the microbiota's role in host weight regulation.

2.2.3 Human studies on the regulation of the microbiome

Animal studies provide mechanistic insights into the microbial regulation of host energy balance. However, they provide limited representation of the human metabolism due to physiological differences including the anatomy of the gastrointestinal tract (Thi Loan Anh *et al.*, 2015). Human gut microbiota studies grant indispensable medical translational power. Initial studies done on human microbiota structure based on 16S rRNA gene and metagenome sequencing analyses showed that obese and lean individuals have different proportions of the two most abundant phyla of the human gut: Firmicutes and Bacteroidetes, and their associated genes (Ley *et al.*, 2006, Turnbaugh *et al.*, 2009). However, soon after Duncan *et al.* (2008) described how environmental factors contributed to the abundance of these phylotypes more than the body type (Duncan *et al.*, 2008), and many studies reported contradictory *Bacteroidetes*/*Firmicutes* ratios in obese

and lean populations (Armougom *et al.*, 2009, Schwiertz *et al.*, 2009, Walters *et al.*, 2014).

Environmental factors that affect microbiota in humans include, but are not limited to: diet, exercise, and host genetics. Diet has been shown to influence the gut microbiota more than the metabolic type or body mass index (Ley *et al.*, 2006). Weight loss diets that have higher protein to carbohydrate ratio decrease the abundance of *Firmicutes* in the obese individuals (Duncan *et al.*, 2008). Additionally, Jumpertz *et al.* showed that a decrease in nutrient load led to 20% increase in *Bacteroidetes* and decrease in *Firmicutes* relative abundance in lean and obese individuals (Jumpertz *et al.*, 2011). On the other hand, a meta-analysis of the gut microbiota in an obesity study revealed that *Bacteroidetes* species are linked to weight loss independent of diet and body type (Angelakis *et al.*, 2012).

Besides diet, an exercise based weight-loss strategy in adolescents correlated with higher *Bacteroides* and lower *Clostridium* levels (Santacruz *et al.*, 2009). Human genetics and genetic variations observed in obese populations also have been linked to changes in the gut microbiota (Ley, 2015). A gene polymorphism, peroxisome proliferator-activated receptor γ2 (PPAR-γ2) g*ene,* observed in obese individuals was negatively correlated with *Bacteroides* abundance (Zuo *et al.*, 2011).

Human microbiota studies often report contradictory results; however studies with proper designs that include dietary control and obesity-related host genotypes would strengthen our understanding on the role of microbiota on host energy regulation.

2.3 Genetic and environmental factors dictate microbiota structure and function in obesity

As seen in Figure 2.1, genetic and environmental factors have effects on the structure of gut microbial communities. Diets that contain high amounts of fats, and antibiotic treatments are known to disturb microbiota structure (Krajmalnik-Brown *et al.*, 2012). A healthy microbiota structure can be reestablished by fecal transplantation, customized synbiotics (pro- and pre-biotics), and balanced diet that contains high amount of fiber (Sonnenburg & Fischbach, 2011).

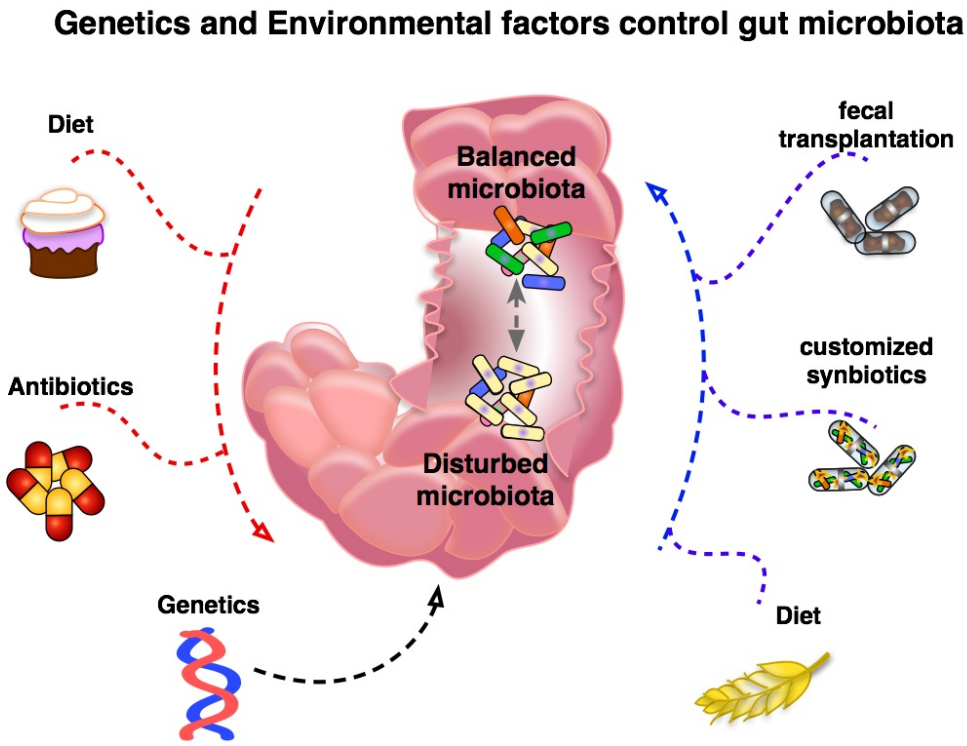

Figure 2.1 Genetic and environmental factors control gut microbiota structure.

2.3.1 Diet is one of the major factors that shape microbiota structure

Soon after birth, our gastrointestinal tracts receive food and food-associated microorganisms. The dietary ingredients are not only fermentation substrates for the

colonic microbiota, but also form an inoculum for the gastrointestinal tract (David *et al.*, 2014). Diet accompanies the development of microbiota during infancy; microbiota structure greatly varies among formula-fed and breast-fed infants (Harmsen *et al.*, 2000).

Introduction of solid food to the diet alters the microbiota and initiates the first step to adulthood microbiota (Fallani *et al.*, 2011). Even though microbiota reach a stable state at some point in time (Yatsunenko *et al.*, 2012), short-term and long-term dietary habits have shown to alter the microbiome (Wu *et al.*, 2011, David *et al.*, 2014). David *et al.* (2014) described how short-term shifts in the diet composition from high carbohydrate to high fat changed the microbiota by increasing the abundance of bile tolerant *Alistipes*, *Bilophila,* and *Bacteroides*, and reduced the abundance of plant polysaccharide degraders such as *Roseburia* sp., *Eubacterium rectale,* and *Ruminococcus bromii* (David *et al.*, 2014). High fat diets change the microbiota by increasing the amount bile acids delivered to the colon (Fava *et al.*, 2013). Besides dietary fat, proteins also rapidly change the microbiota (David *et al.*, 2014). Apart from the composition of the diet, short-term changes in caloric intake also showed rapid and drastic changes in microbiota composition (Jumpertz *et al.*, 2011). An increase of 1000 kcal/day in the diet was associated with 20% increase in Firmicutes and increased energy harvest from the diet (Jumpertz *et al.*, 2011).

Besides short-term dietary changes, long-term dietary patterns also shape the gut microbiota. Duncan *et al.* (2008) showed that a four-week consumption of high-protein low-carbohydrate diet decreased the abundance of butyrogenic microorganisms as well as *Bifidobacterium* (Duncan *et al.*, 2008). Drastic differences in the diet of children in

Western and African societies showed that gut microbiota composition varies based on diet (De Filippo *et al.*, 2010).

A diet with high fiber composition correlated with the abundance of cellulose degrading *Prevotella* and *Xylanibacter* (De Filippo *et al.*, 2010). Dietary fiber fosters abundance of microorganisms, microbial interactions including synthrophies, and metabolic outcomes such as butyrate production for healthy colons (Fischbach & Sonnenburg, 2011).

Changes in the diet not only change the microbial structure, but they also affect microbial functions, such as amounts and types of short chain fatty acids that are produced (Fava *et al.*, 2013). For instance, David *et al.* (2014) reported that isovalerate and isobutyrate were more abundant in the feces of a group of humans consuming an animal based diet whereas acetate and butyrate were more abundant in a group of humans consuming a plant based diet.

2.3.2 Bariatric surgery alters the microbiome in an irreversible way

Diet, exercise, and weight loss drugs often fail to provide sufficient weight loss for morbidly obese individuals (Body mass index > 40) (Blackburn, 2005). Bariatric surgery procedures are currently the most efficient management strategy of obesity and its comorbidities (Aron-Wisnewsky *et al.*, 2012). The bariatric surgeries mechanism of action can be restrictive, hormonal, behavioral, and malabsorptive (Blackburn, 2005). As shown in **Figure 2.2**, laparoscopic gastric banding (LAGB) and vertical sleeve gastrectomy (VSG) are restrictive surgeries; biliopancreatic diversion (BPD) is a malabsorptive surgery, whereas Roux-en-Y gastric bypass (RYGB) is restrictive and malabsorptive (Aron-Wisnewsky *et al.*, 2012).

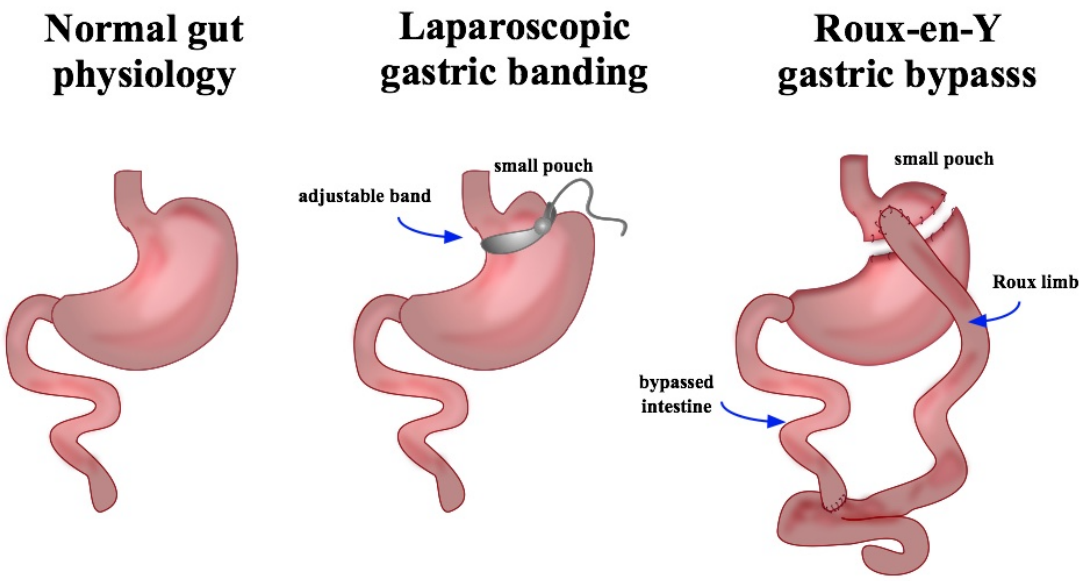

Figure 2.2. Gut physiology after two commonly performed bariatric surgeries: LAGB and RYGB.

Physiological and anatomical changes due to bariatric surgery procedures result in changes in gut microbiota (Zhang *et al.*, 2009, Furet *et al.*, 2010, Aron-Wisnewsky *et al.*, 2012). Those changes include, but are not limited to: bile flow, colonic pH, vagal modulation, enteric and adipose tissue hormones, flow of nutrients and quantities of nutrients delivered to the colon (Li *et al.*, 2011). To date, alterations in the microbiota after RYGB surgery in animals (Li *et al.*, 2011, Liou *et al.*, 2013, Osto *et al.*, 2013) and in humans (Zhang *et al.*, 2009, Furet *et al.*, 2010, Graessler *et al.*, 2013, Kong *et al.*, 2013) have been documented. Changes in the microbiota of rats were also documented for the VSG surgery (Ryan *et al.*, 2014). For both types of surgeries, increases in *Gammaproteobacteria* and *Verrucomicrobia* species in the gut were observed (Zhang *et al.*, 2009, Liou *et al.*, 2013, Ryan *et al.*, 2014). Those differences in the microbiota structure did not bring the microbiota from obese to normal weight states (Liou *et al.*,

2013), in fact resulted in microbiomes that were distinct from the microbiomes of metabolic types (Zhang *et al.*, 2009).

Fecal transplants from post-RYGB surgery animals to germ-free animals reduce body weight and adiposity (Liou *et al.*, 2013). The study published by Liou *et al.*, (2013) provided the first line of evidence that transmissible post-RYGB microbiota can lead to weight loss and to reduction of body fat. Even though this finding is promising, more studies are needed to identify which microorganisms or microbial consortia that develops after bariatric surgery lead to weight loss or remission of metabolic syndrome. Identifying microbiota that are responsive to the successful surgery can enhance the understanding of microbiota related weight loss. Moreover, microbiota of other bariatric surgery procedures that initiate weight loss as well as resolution of the metabolic syndrome requires further evaluation. Identification of microorganisms or microbial consortia, and their mechanisms of action could eliminate the need of bariatric surgery for morbid obesity.

2.3.4 Host genetics might have a role on regulations of the microbiome

Variations in diet, lifestyle, and genetics make it difficult to distinguish microbiota's contribution to the host energy balance. Among these factors, host genetics have been shown to influence gut microbiota composition (Spor *et al.*, 2011). When gut microbiota structure of more than 500 individuals were investigated using beta diversity metrics, host genetics were an important clustering factor of the microbiota: twins had the most similar microbiota (Peterson *et al.*, 2009, Yatsunenko *et al.*, 2012), and individuals from the same families had closer microbiota structure to each other than to the unrelated individuals (Yatsunenko *et al.*, 2012).

The effects of host genome on gut microbiota structure were previously speculated. One hypothesis is that twins or siblings are often subjected to similar environmental exposures/diets; therefore, they develop similar microbiota structures (Turnbaugh *et al.*, 2009). Another view is that expression of host genes can change the environment for microorganisms, and therefore, the microbiota structure (Spor *et al.*, 2011). Host genes can control a single species or a group of taxa and can have putative pleiotropic effect on groups of microorganisms (Benson *et al.*, 2010). Animal models have successfully shown that single-nucleotide polymorphisms (SNPs) on the host genome results with different microbiota structure compared to the wild type animals (Spor *et al.*, 2011). For instance, mice deficient in Toll like receptor 5 developed metabolic syndrome and experienced reduced levels of *Bacteroidetes* in the colon (Vijay-Kumar *et al.*, 2010).

2.3.5 Antibiotics can shape microbiome in early age and adulthood

Widespread use of antibiotics has been associated with the development of many health conditions such as asthma (Russell *et al.*, 2012) and diarrhea (Young & Schmidt, 2004). Short-term or long-term alterations in the gut microbiota structure due to the use of antibiotics have been previously reported (Jernberg *et al.*, 2007, Antonopoulos *et al.*, 2009, Dethlefsen & Relman, 2011, Elena Perez-Cobas *et al.*, 2013). Even though the dominant members of the microbiota recovered from the antibiotic treatment within weeks, some minor members often disappeared (Dethlefsen *et al.*, 2008).

Antibiotics are considered as one of the environmental factors that contribute to human energy metabolism due to the use of antibiotics as growth promoters in the animal husbandry (Angelakis *et al.*, 2012). Due to variations in the mode of actions of

antibiotics and differences in the target microorganisms, the effects of antibiotics on the microbiota associated host metabolism have showed results. While some studies reported increased body weight (Thuny *et al.*, 2010, Angelakis *et al.*, 2012), some others reported a decrease in body fat (Cani *et al.*, 2008).

Exposure to antibiotics such as amoxicillin, clarithromycin, vancomycin, and erythromycin was associated with childhood (Azad *et al.*, 2014, Bailey *et al.*, 2014) and adulthood (Thuny *et al.*, 2010, Angelakis *et al.*, 2012) obesity, and even though the mechanisms of action are unknown, these antibiotics are known to be more efficient in the treatment of infections due to Gram-positive bacteria. On the other hand, mice models have shown that antibiotic treatment can protect against obesity and adiposity (Cani *et al.*, 2008). Antibiotics that are efficient against Gram-negative bacteria have shown that, lipopolysaccharides produced by Gram-negative bacteria initiate metabolic endotoxemia, which leads to increased inflammation and adiposity (Cani *et al.*, 2008, Everard *et al.*, 2013).

2.4 Insights into microbial mechanisms that control host energy balance

Microorganisms that reside in the human intestines contribute to host energy balance in multiple ways: they increase the energy harvest (Turnbaugh *et al.*, 2006), regulate the energy intake via production of hormones and neurotransmitters that are relevant to the appetite (Clarke *et al.*, 2014), alter bile acid metabolism (Nieuwdorp *et al.*, 2014), and interact with the host at the epithelial surfaces (Nieuwdorp *et al.*, 2014). These processes are illustrated in Figure 2.3.

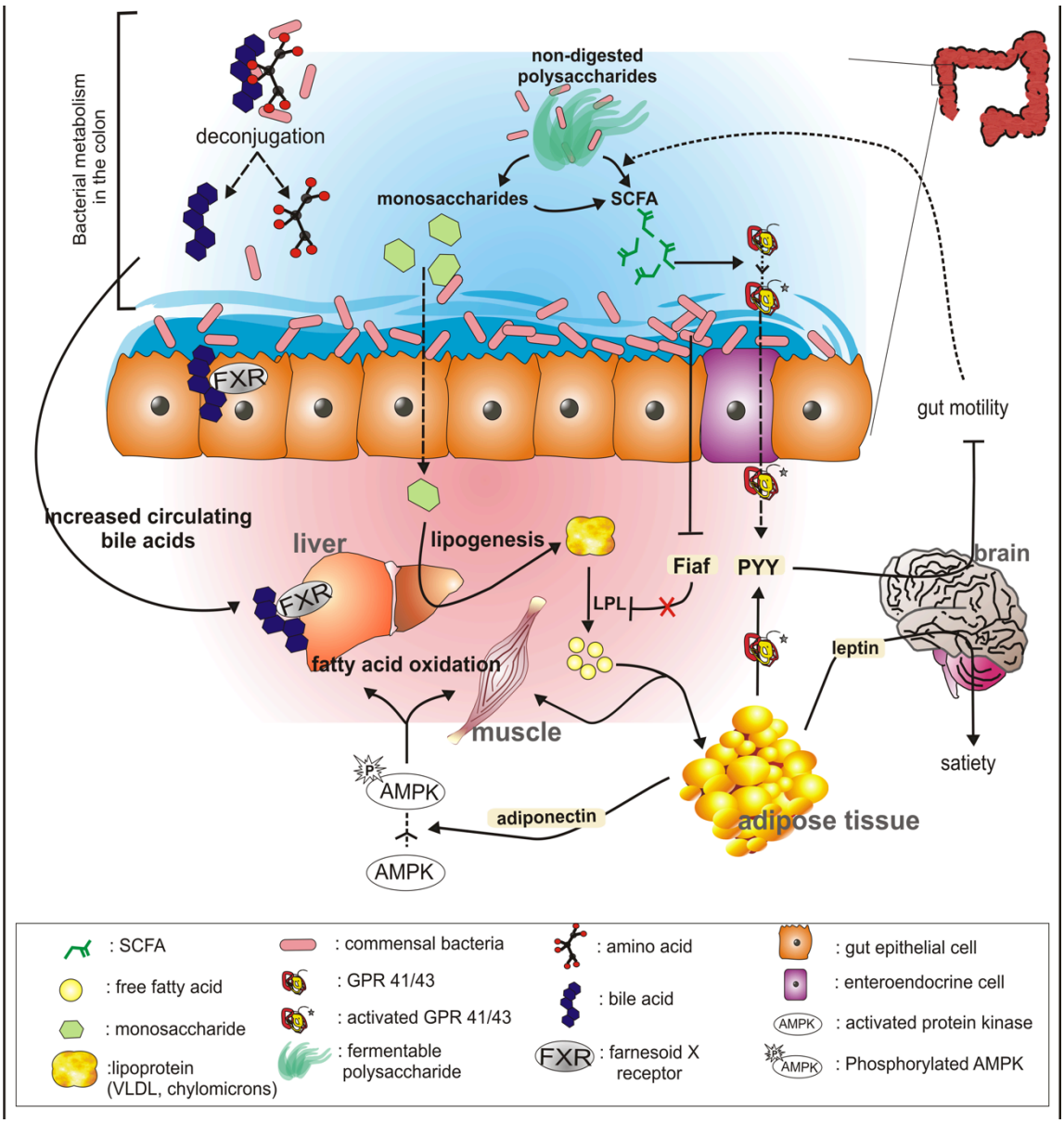

Figure 2.3 Gut microbiota interacts with the host at multiple levels. Interactions between the host and gut microbiota regulate host metabolism. Modified from Krajmalnik-Brown *et al.,* 2012.

Genomes of many gut microbes contain genes such as glycoside hydrolases that are essential to break down many plant polysaccharides such as arabinose, xylan, and pectin (Gill *et al.*, 2006). By converting otherwise energy-neutral complex nutrients into short chain fatty acids, the microbiome can increase energy extraction from the diet (Krajmalnik-Brown *et al.*, 2012). Besides increased energy harvest from the nutrients, gut microbes have shown to interfere with the absorption of digested nutrients (Jumpertz *et al.*, 2011).

Acetate, butyrate, and propionate are the major microbially produced short chain fatty acids (SCFAs) (Macfarlane & Macfarlane, 1997). Gut bacteria also produce minor SCFAs: isobutyrate and isovalerate, gases (H_2, CO_2, and CH_4 etc.), and vitamins (Macfarlane & Macfarlane, 1997). Figure 2.4 demonstrates the stoichiometry of these reactions. Hydrogen generation during fermentation, especially during acetate generation reactions, might have implication on overall SCFAs balance (Krajmalnik-Brown *et al.*, 2012). For instance gnotobiotic mice experiment has shown that co-culturing mice gut with fermenter *Bacteroides thetaiotaomicron* and hydrogen-consumer *Methanobrevibacter smithii* increased total acetate production by removing hydrogen, which limits acetate production (Samuel & Gordon, 2006).

Once SCFAs are absorbed and reach to the blood stream, they can be stored in the liver or adipose tissue; therefore, they can contribute to energy harvest. SCFAs can also signal many receptors that are involved in energy regulation such as G protein coupled receptors (GPRs): GPR41 and GPR43 (Tazoe *et al.*, 2008). Propionate and, to a lesser extent, butyrate can regulate weight-loss hormones, such as glucagon-like peptide-1 (GLP-1), and both were shown to protect against obesity (Lin *et al.*, 2012). Besides

GLP-1, propionate can induce secretion of appetite-reducing hormone peptide YY (PYY) (Chambers *et al.*, 2015).

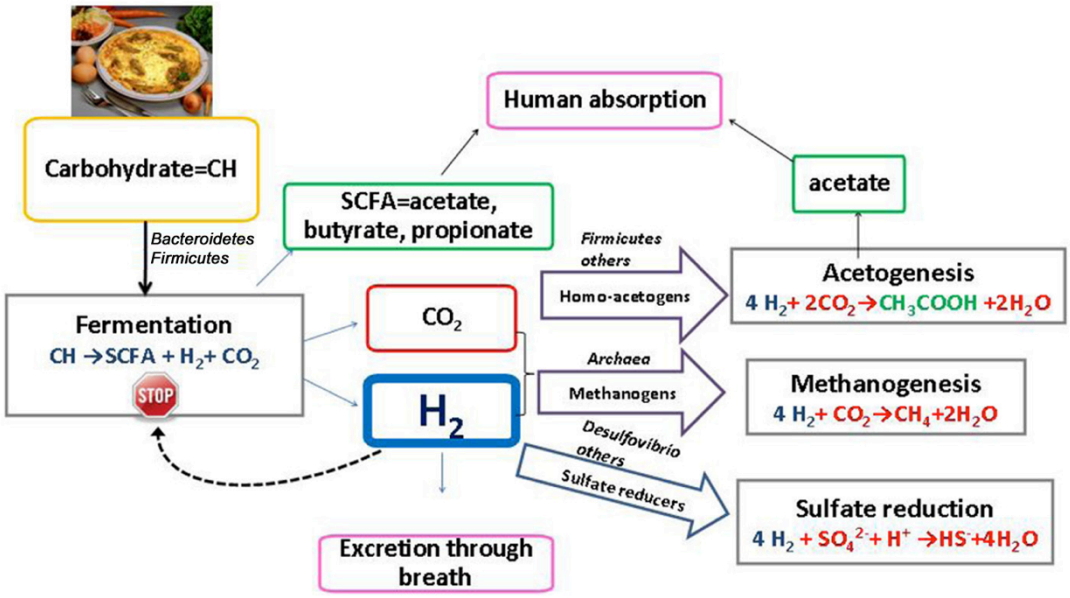

Figure 2.4. Regulation of microbial reactions by hydrogen gas. (Krajmalnik-Brown *et al.,* 2012)

Most recently, it has been shown that microbiota can modulate the bile acid receptors: Farnesoid X receptor (FXR) and G-protein coupled receptor TGR5 by signaling via transformation of the bile acids (Fiorucci & Distrutti, 2015). Those receptors are involved in regulation of insulin and glucagon secretions (Fiorucci & Distrutti, 2015), therefore contribute to host energy metabolism. Increase in activation of FXR receptor has been shown to reduction in body weight despite high-fat diet (Watanabe *et al.*, 2006). After vertical sleeve gastrectomy surgery, an increased activation of FXR receptor has shown to increase weight loss in mice that were fed high-fat diet (Ryan *et al.*, 2014).

Gut microorganisms can also alter amino acids. It has been shown that gut microorganisms can transform amino acids into neuro-transmitting molecules such as

serotonin (Clarke *et al.*, 2014). Serotonin can regulate appetite, stress and depression (Halford *et al.*, 2005). Spore-forming microorganisms have shown to increase serotonin production in mice models by signaling enterochromaffin cells that secrete serotonin (Yano *et al.*, 2015).

2.5 Multi-omic approaches to study human microbiome in obesity

To understand the role of microbiota on host body weight regulation, we need to answer two essential questions: who are they and what do they do? Modern technology allows us to characterize the microbial community structure and their functions that affect the host metabolism by offering novel techniques including metagenomics, transcriptomics, and metabolomics (Figure 2.5). These techniques have advantages and limitations and I discuss them below as well as the advancements in microbiome era due to the initiation of Human Microbiome Project.

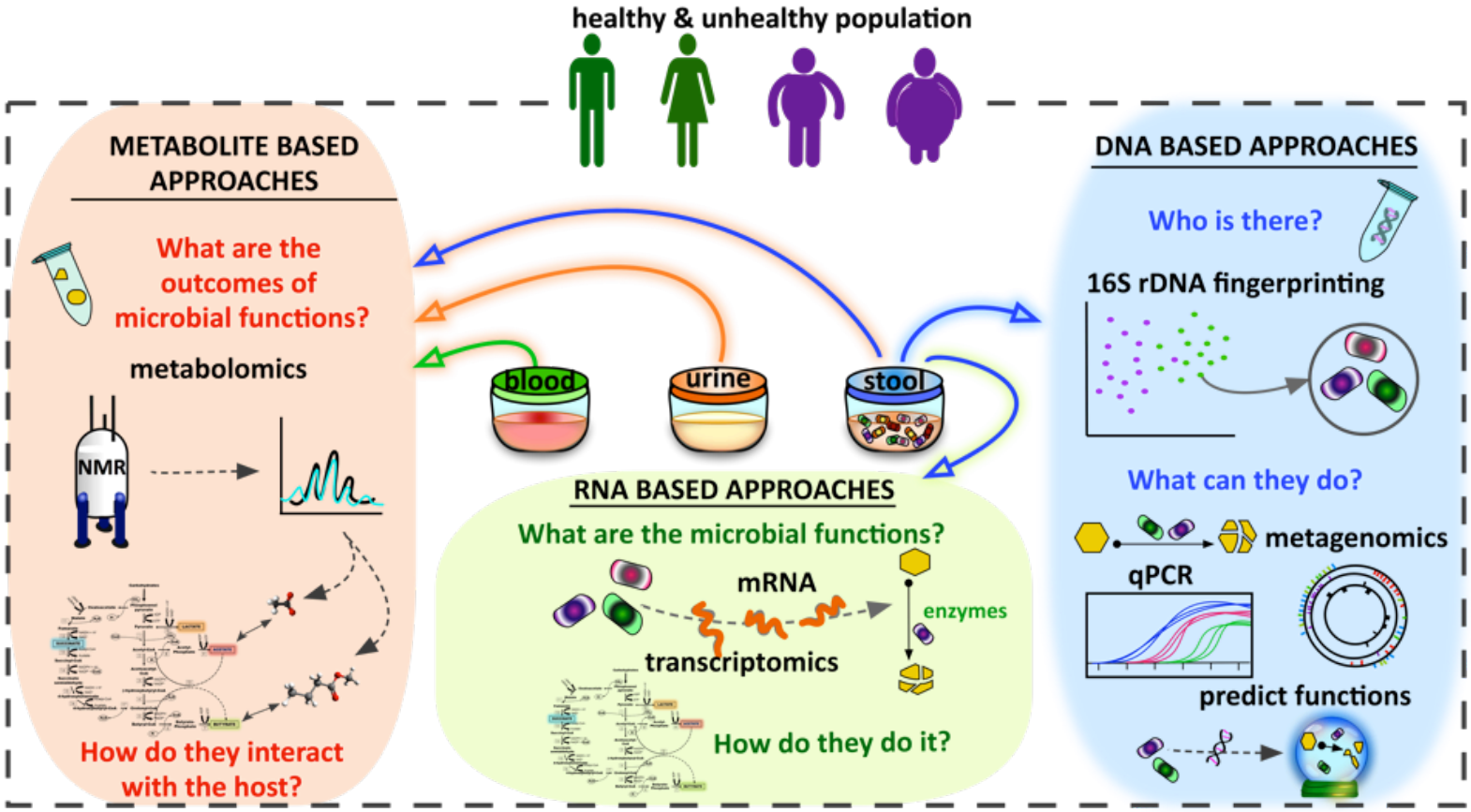

Figure 2.5. Illustration of DNA-, RNA-, and metabolite-based approaches to study microbiome host interactions in the context of obesity **.**

2.5.1 Human microbiome project – goals and aims

The human genome project (HGP), aimed to sequence and map all the genes of *Homo sapiens,* brought profound changes to the understanding of the genetic basis for some diseases. Despite its breakthrough, the HGP focused on the human host, and excluded our second genome: "the microbiome". The human microbiome is composed of the genes of the trillions of microorganisms that reside on the surfaces of the human body (Relman & Falkow, 2001, Lederberg, 2002). With the recognition of the host-associated microbiota's importance to human health, the National Institute of Health (NIH) launched the Human Microbiome Project (HMP). Two important forces that impelled this project were: the lack of studies on microbial genomic compositions of a baseline healthy population, and the rapid development of faster and cheaper sequencing technologies (Peterson *et al.*, 2009).

The overarching goals of the HMP were to provide a reliable database of microorganisms living in symbiosis with the human host and standard protocols of sample preparation, sequencing and data analysis to a broad scientific community (Proctor, 2011, Gevers *et al.*, 2012). In order to achieve its goals, the HMP employed a multiple tier approach. The first stage aimed to sequence 500 new reference bacterial whole genomes and 16S rRNA gene of 250 healthy individuals at five anatomical regions including the gut (Peterson *et al.*, 2009). During this phase, metagenomes of the same individuals were sequenced and then analyzed with bioinformatic tools to resolve the functional potential of the host-associated microbial communities (Peterson *et al.*, 2009, Gevers *et al.*, 2012). The second stage began in 2012, after the completion of the first

one, and its main goal is to expand the scope of microbiome sequencing from healthy to different disease conditions (Peterson *et al.*, 2009).

Initial findings of the HMP surprised the scientific community because the human microbiome turned out to be unexpectedly diverse in contrast to the human genome (Turnbaugh *et al.*, 2007). Microbiota composition greatly varied among healthy individuals, but interestingly enough the microbial metagenomes (based on cataloged functions) were similar within populations (Huttenhower *et al.*, 2012). The HMP accomplishments have provided great milestones for molecular, ecological, evolutionary and microbiological sciences, in addition to the potential to identify causality of diseases that were not traditionally considered of microbial origin. These milestones can potentially lead to opportunities to develop personal medicine strategies to modify the microbiome for therapeutic purposes. Because of its high plasticity and potential to be modified, the microbiome might have higher therapeutic value than the human genome.

2.5.2 Who is there: 16S rRNA gene profiling

Every bacteria or archaea cell contains at least one copy of the 16S rRNA gene in their genomes. The conserved and hypervariable regions of the 16S rRNA gene make it as a target for phylogenetic resolution of microbial communities. Microorganisms can be identified and abundances can be estimated by targeting one or a couple of the nine-hypervariable regions of the 16S rRNA gene (Hamady & Knight, 2009). There are a variety of pre-genomic methods such as denaturing gradient gel electrophoresis, fluorescent in situ hybridization (FISH), and sequencing to study microbial communities using the16S rRNA gene as a marker (Rittmann *et al.*, 2008), although sequencing is currently by far the most feasible technique some of the other mentioned techniques have

other advantages such as price and time frames for data generation and analysis. Different sequencing platforms such as 454 pyrosequencing, Ion Torrent, and Illumina (HiSeq and MiSeq) can be used for sequencing. Illumina is currently the leading sequencing technology due to its short turn-around time, high accuracy, low cost, and comparable read lengths to pyrosequencing (Nelson *et al.*, 2014).

16S rRNA gene profiling of microbial communities in the human gut provides a snapshot of the community, taxonomic resolution of uncultivable microorganisms, and phylotypes at low abundance including keystone species. Keystone species are organisms that are low in abundance, but have essential functions in their habitats, once they are removed from the ecosystem, the ecosystem function gets disrupted (Mills *et al.*, 1993).

16S rRNA gene profiling usually allows identification and a certain degree of estimation of the abundance of microorganisms; it can provide comparison of multiple microcosms at a time and helps to identify which taxa differentiates microcosms from each other (Hamady & Knight, 2009). In comparison to clone libraries, higher throughput 16S rRNA gene sequencing enables researchers to calculate within sample diversity (alpha diversity) and between-sample diversity (beta diversity), which provide an understanding of differences in microbiota structure and communities based on host-associated conditions (Lozupone *et al.*, 2012). Another advantage of high throughput 16S rRNA gene sequencing is the possibility to predict the microbiota's potential function of using bioinformatic tools that generate metagenomes based on the available 16S rRNA gene data (Langille *et al.*, 2013).

Like every other technology, methods based on the16S rRNA gene have limitations; I discuss here four important limitations. First, although 16S rRNA gene databases have been growing exponentially in the last 10 years, they are still incomplete and are not sufficient to cover all the human gut associated microorganisms; this leads to abundance of many unidentified sequences in most datasets. Therefore, microorganisms with biological importance for the host health can be neglected unintentionally. Second, methods based on the 16S rRNA gene rely on genomic content; this captures the presence of microorganisms, but does not capture the activity of microorganisms. Third, genomes of microorganisms vary in their 16S rRNA gene numbers, which makes it difficult to estimate absolute number of species. Fourth, different sample-preparation methods, such as DNA extraction; PCR-amplification errors, and biases due to primer selection, can yield different results (Ercolini, 2004, Goodrich *et al.*, 2014), which is a common limitation for all genomic methods.

2.5.3 Which activities are taking place? Transcriptomics with RNASeq and gene expression assays with quantitative PCR

DNA based techniques can identify and estimate the relative abundance of microorganisms and their genes; however they cannot measure microbial activity. Microbial activities can be determined with transcriptomics, which catalogues RNA molecules including mRNA, tRNA, and rRNA (Wang *et al.*, 2009) or quantitative PCR assays on reverse transcribed mRNA (Rittmann *et al.*, 2008). In this section, I focused on RNA-seq methods rather, than qPCR, since RNA-seq provides a more global approach rather than targeted approach. Among the RNA molecules, mRNA (transcripts) is most helpful to microbial ecologist to uncover microbial functions. Previous studies have

shown that RNA-sequencing is currently the most accurate metatranscript quantification method, even more than the microarrays (Fu *et al.*, 2009, Wang *et al.*, 2009).

RNA-seq can reveal some of the activity of the gut microbiota. Based on a study that was performed on fecal samples collected from 10 healthy individuals; carbohydrate metabolism and energy production were enriched functions, whereas amino acid and lipid metabolisms were underrepresented functions of the microbiota (Jose Gosalbes *et al.*, 2011). Fecal transplantation from obese and lean humans to mice revealed that the obese and lean associated gut microbiome were enriched in different functions; obese microbiota expressed more of amino acid biosynthesis genes and lean microbiota expressed more of polysaccharide degradation genes (Ridaura *et al.*, 2013).

Even though transcriptomics is invaluable for human microbiota studies, the cost and challenges of working with RNA have delayed a widespread adaptation of this powerful technique to microbial communities that are associated with the human gut. These challenges include but are not limited to: 1) the difficulty of microbial mRNA isolation from fecal and biopsy samples, 2) fast degradation of microbial mRNAs, 3) the differences in sample preservation and extraction methods which greatly influence integrity and quantity of the RNA recovered (Xiong *et al.*, 2012), and 4) only small fraction (~5%) of the extracted RNA is mRNA; therefore, cumbersome methods are required to remove 16S rRNA gene, which hinders mRNA sequencing (Giannoukos *et al.*, 2012). Yet another challenge with RNAseq comes from data analysis. Most of the current bioinformatic tools are designed to handle eukaryotic RNA sequencing data, although new bioinformatic tools for microbial RNA are under development (McClure *et al.*, 2013). Microbial functions are important for host health and the development of

easier RNA-sequencing methods for sure will aid the identification of novel microbiota associated weight loss and weight gain mechanisms.

2.5.4 What chemicals are being produced? Biochemical views including metabolomics

Microbially derived metabolites in the human gut serve as communication signals between microorganisms and other microorganisms and the host. Identification and quantification of these metabolites, metabolomics or fingerprinting microbial chemical outcomes (Marcobal *et al.*, 2013), can contribute to the understanding of microorganisms' importance to the human health (Beckonert *et al.*, 2007), especially in the pathophysiology of obesity and diabetes (Calvani *et al.*, 2014). The most common analytical platforms to detect and quantify microbial metabolites in the urine, blood, or feces are nuclear magnetic resonance (NMR) spectroscopy and gas/liquid chromatography mass spectrometry (LC or GC-MS) (Beckonert *et al.*, 2007, Wikoff *et al.*, 2009, Calvani *et al.*, 2014).

Depending on the hypothesis being tested, global or targeted metabolomics approaches can be employed to study gut metabolism with these two platforms. Global approaches capture a broader metabolites range; however they require more bioinformatics power (Oresic, 2009). On the other hand, targeted approaches limit the number of metabolites detected, but they provide higher metabolic resolution of a certain function, for example metabolites of the bile metabolism (Oresic, 2009).

Use of metabolomics techniques has brought deeper understanding to the microbial regulation of host metabolism. Comparison of plasma metabolites of conventionally raised mice to germ-free mice revealed differences in many microbially produced/associated metabolites, especially amino acid metabolism products that are

absent in the plasma of germ free mice (Wikoff *et al.*, 2009). Metabolomics approaches also revealed in mice studies that microbial fermentation end products such as propionate have the potential to regulate satiety via activation of G protein – coupled receptors (Samuel *et al.*, 2008). Similar results have been reported in humans (Calvani *et al.*, 2010). Use of NMR on urine samples collected from obese and lean individuals showed many metabolites derived from gut microbiota, including hippuric acid, 2-hydroxyisobutyrate, and xanthine were able to distinguish obese metabolic type from lean metabolic type (Calvani *et al.*, 2010), although a contribution of these metabolites to the obesity pathophysiology requires further investigation.

Microbially derived metabolites have been associated with obesity-linked comorbidities. For instance, identification in humans of microbially produced deoxycholic acid (DCA) suggested that the gut microbiota are involved in the development of obesity associated liver carcinoma (Yoshimoto *et al.*, 2013). Production of volatile compounds by the gut microbiota was recently associated with the development of obesity associated non-alcoholic fatty liver disease (Raman *et al.*, 2013). Metabolomics in conjunction with 16S rRNA gene profiling and transcriptomics hold great promise to provide the missing link between the gut microbiota and development of obesity with its comorbidities. Optimization of metabolomics techniques as well as a reduction in the cost of analysis will greatly enhance microbiome studies by making metabolomics more widely available to the scientific community.

2.6 Limitations of the current approaches to study microbiota in the context of obesity

2.6.1 Fecal microbiota are not equal to gut microbiota

Studies of the human gut microbiota frequently are performed on fecal samples and less commonly on tissues that are collected during colonoscopy. Sampling bias is of major concern when considering if fecal microbiota is representative of: 1) the microbiota of the different parts of the colon (Macfarlane *et al.*, 1992) and 2) the microbiota of luminal and mucosal surfaces (Zoetendal *et al.*, 2002). Other challenges with study design include lack of control on fecal-sample preservation and flaws in experiment designs that exclude age, diet, and gender matched controls (Goodrich *et al.*, 2014). Here, I summarize and discuss biases associated with interpretation of human gut microbiota from fecal samples.

2.6.2 Proximal and rectal colons are different ecosystems

The limited accessibility of the distal and proximal sections of the human colon has forced human gut microbiota research to rely on fecal microbiota analyses. In humans, microbiota associated with different parts of the colon can be studied post-mortem (Macfarlane *et al.*, 1992) or during colonoscopies (Zoetendal *et al.*, 2002). Gastrointestinal samples collected during an autopsy showed that luminal microbiota varies along the different colon sections (Hayashi *et al.*, 2005). The differences are possibly due to gradients of substrate concentrations and variations in the environmental conditions such as pH and moisture across the colon (Macfarlane & Macfarlane, 1997).

On the other hand, mucosal microbiota of different colon sections didn't show variation (Zoetendal *et al.*, 2002, Wang *et al.*, 2003). From a practical standpoint, animal

studies or bioreactors that simulate human colon offer the study of both microbiota structure and metabolism of different parts of the colon. For instance, weaned pigs that were fed oligosaccharides had different abundance of fermentation end products and higher abundance of *Bifidobacterium* in the proximal than the distal colon (Tzortzis *et al.*, 2005). Reactors that simulate different parts of the colon such as Simulator of Human Intestine Microbial Ecology (SHIME), also confirmed that microbiota develop differently due to gradient of conditions in the colon (Molly *et al.*, 1994).

In vitro and *in vivo* studies have shown that fecal samples are not representatives of the proximal colon (Mai & Draganov, 2009). However, fecal samples are still valuable since they collect and carry the microbiota along the GI tract, and they reflect changes in the gut microbiota during health or disease states. Experimental, bioinformatic, and mathematical approaches are necessary to better evaluate how fecal samples are representative and predict the microbiota of the proximal colon.

2.6.3 Luminal space and mucosal space are different

The human colon offers a number of habitats to microorganisms. A vertical cross section of the colon shows gradient concentrations of oxygen and substrate from mucosal to luminal surfaces that may lead to localization of the microbiota (Zoetendal *et al.*, 2002). Due to the decreasing oxygen gradient from mucosal to the luminal surfaces (Rabus & Widdel, 1995), mucosal microbiota consist of more oxygen tolerant and facultative anaerobe species, while luminal microbiota composes of more strictly anaerobic species (Van den Abbeele *et al.*, 2011).

Besides oxygen, nutrients have defining role on the microbiota structure. Host glycans and dietary polysaccharides are the substrates for mucosal and luminal

microbiota, respectively (Van den Abbeele *et al.*, 2011), as shown in Figure 2.6. Luminal communities are more susceptible to environmental variables such as pH and retention time than the mucosal communities, and these variables can change luminal communities rapidly in comparison to mucosal communities (Van den Abbeele *et al.*, 2011).

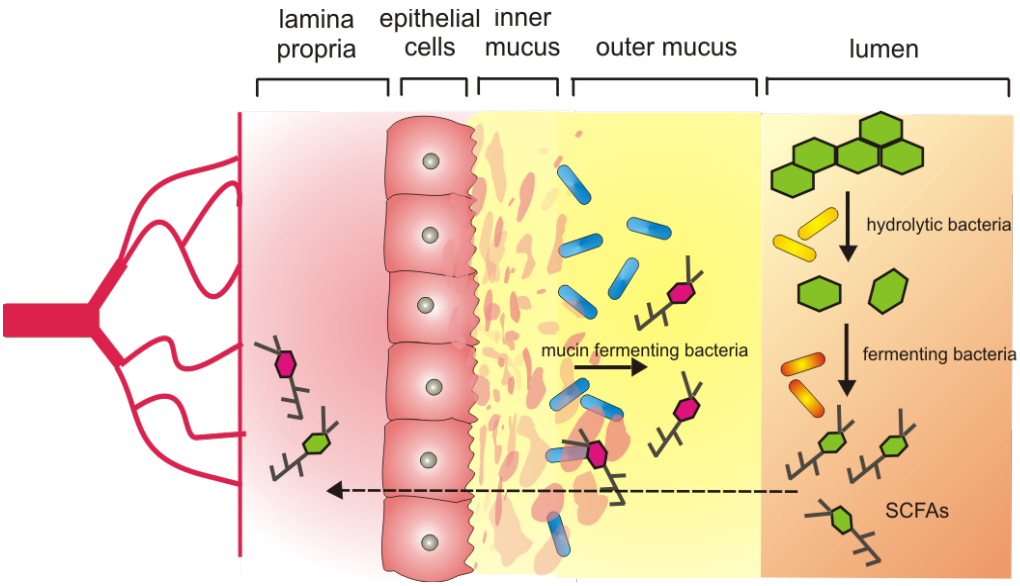

Figure 2.6. An illustration of a cross section of a healthy colon with its microbiota. The outer mucus layer and the colon lumen are colonized by different microbiota and therefore, produce different metabolic end products.

Contrasts in mucosal and luminal communities facilitate differential roles of microbiota. Mucosal and luminal microbiota have differential roles on host-microbe interactions and body homeostasis, as mucosal communities prime the immune system and maintain the health of the mucosal layer, whereas luminal microbiota provide additional nutrients to the host by fermenting undigested polysaccharides (Van den Abbeele *et al.*, 2011).

The decision to use luminal or mucosal samples for microbiota analysis should be done based on the goals of the study. For instance, mucosal microbiota are particularly important in cases where direct interaction with the microbiota and the host epithelial cells or immune system are essential, such as conditions like leaky gut, irritable bowel syndrome, and diabetes (Walker *et al.*, 2011). For dietary modulation studies luminal samples might be more representative, since these microbiota are often associated with the food particles (Walker *et al.*, 2008). Due to differences in mucosal and luminal microbiota, scientist should be aware that luminal samples do not always represent the mucosal microbiota.

CHAPTER 3

PH MEDIATES MICROBIAL AND METABOLIC INTERACTIONS IN FECAL ENRICHED COMMUNITIES

3.1 Introduction

Gut microorganisms use a variety of fermentative pathways to harvest energy, and the pathway utilized depends on many factors, including pH and available fermentation substrate (Chung *et al.*, 2016; Walker, Duncan, Leitch, Child, & Flint, 2005). The pH varies along the human colon (approximately from 5 to 7) (Evans *et al.*, 1988), with the type and abundance of fermentation products, bicarbonate secretion by colonic epithelial cells, and nutrients establishing the pH (Fallingborg, 1999; Novak *et al.*, 2011; Nugent, Kumar, Rampton, & Evans, 2001). Many of the dietary nutrients are substrates for microbial metabolism, such as oligosaccharides or simple sugars, and they have an impact on pH as they promote acid production via fermentation (Campbell, Fahey, & Wolf, 1997). Colonic pH along with gut microorganisms deviate from normal during gastrointestinal diseases or conditions such as colorectal cancer (Ohigashi *et al.*, 2013), inflammatory bowel disease (Nugent *et al.*, 2001), and constipation (Camilleri *et al.*, 2010). Exogenous factors such as use of a proton pump inhibitor (William & Dangizer, 2016) and bariatric surgery (Li *et al.*, 2011) also alter gut pH. It is likely that an abnormal pH in the gut can alter microbiota composition and its metabolism (Kassinen *et al.*, 2007; Li *et al.*, 2011).

pH imposes selective pressure on microbial growth and metabolism. While some bacteria, such as in *Bacteroides*, can grow over a wide range of pH values (Sylvia H. Duncan, Louis, Thomson, & Flint, 2009), others, such as in *Veillonella* and

Streptococcus, are inhibited by acidic pH (Bradshaw & Marsh, 1998), although some species from these genera can thrive in acidic pH (Bradshaw & Marsh, 1998). Furthermore, pH is an important determinant of the distribution of major fermentation end products. For example, butyrogenic reactions occur at mildly acidic pH (Walker *et al.*, 2005), propionate production often occurs at neutral pHs (Belenguer *et al.*, 2007), and acetogenic reactions occur at various pHs depending on the microbial species producing it (Belenguer *et al.*, 2007). These microorganisms form metabolic networks in biofilms (Al-Ahmad *et al.*, 2007), hence differences in their tolerance to acid production in mixed community settings can alter metabolic processes. However, to date, the majority of the *in-vitro* studies (Chung *et al.*, 2016; Walker *et al.*, 2005) that characterize microbial growth and function have neglected the impact of pH on microbial and metabolic interactions.

Dietary carbohydrates have a major effect on the structure and function of microbial communities in the human gut (Chung *et al.*, 2016; De Filippo *et al.*, 2010). Prebiotic polysaccharides such as inulin and pectin can stimulate the growth of certain microbial species, and the community structures depend on starting pH (Chung *et al.*, 2016). Monosaccharides and disaccharides can be found in the diet in their monomeric forms (Tappy & Le, 2010) and as building blocks of common polysaccharides such as cellulose, inulin, and oligofructose (Tappy & Le, 2010). Among the components of dietary carbohydrates, glucose fermentation at different pH conditions has been well documented (Carlsson & Griffith, 1974; Egert *et al.*, 2007; Miller & Wolin, 1981; Weaver, Krause, Miller, & Wolin, 1989). In contrast to glucose, fermentation of other saccharides such as fructose and cellobiose by human gut microbiota has had minimal

attention. Fructose is more accessible to the colonic microbiota than glucose because it is absorbed less efficiently than glucose in the small intestine (Tappy & Le, 2010). Cellobiose is a disaccharide whose bioavailability in the colon is limited by its hydrolysis by the gut microbiota (Nakamura, Oku, & Ichinose, 2004). Therefore, the relative roles of these organic substrates and pH on the microbial community and its function remain unclear and demand more investigation.

Here, we address the effects of pH and carbohydrates on the structure and function of anaerobic microbial communities derived from fecal slurry obtained from a healthy human. We carried out batch *in vitro* experiments using glucose, fructose, or cellobiose as the organic substrate and initial pH values of 6.0, 6.5, or 6.9; these values range from normal to unusually low values associated with gastrointestinal abnormalities in the distal colon. We evaluate impacts on the microbial communities' structure and metabolic end products. We apply electron-equivalent mass balances to quantify the effects of pH and substrate on community function.

3.2 Materials and Methods

3.2.1 Experimental Design

A fecal specimen was collected from a female healthy subject and transported to the laboratory on ice packs. After homogenizing one gram of the specimen in 50 mL sterile anaerobic 1X phosphate buffer saline at pH 7.2, we produced the fecal slurry used in the experiments. The inoculum was diluted to a final concentration of 0.04 g/L solids, and all inoculations were carried out in anaerobic glove box.

The culturing medium was an anaerobic fermentation medium (Lee, Salerno, & Rittmann, 2008) that contained 30 mM sodium bicarbonate ($NaHCO_3$), 2% cysteine-

sulfide solution, and 10 mM of one fermentable substrate (glucose, fructose, or cellobiose). Glucose and fructose are monosaccharaides that have the same electron equivalence (24 electrons per mole), although they have different chemical properties and metabolism by bacteria (Andreese.Jr, Schaupp, Neuraute.C, Brown, & Ljungdah.Lg, 1973). By comparing glucose and fructose fermentation, we aimed to identify microbial development and function based on availability of different types of monosaccharides without varying the amounts of electrons provided to the bacteria for metabolism. Because the cultures had the same mmoles of substrate in the batch bottles, cellobiose cultures received twice the amount of the electrons that fructose or glucose cultures received, since cellobiose is a disaccharide. By comparing cellobiose fermentation to glucose fermentation, we aimed to understand the impact of electron availability on microbial metabolism and community structure at different pH values.

After preparing the medium anaerobically under a stream of 20/80 % CO_2/N_2 gas, we distributed 50 mL of medium into triplicate 125-mL serum bottles and then adjusted the pH to 6.0, 6.5, or 6.9 with 10% hydrochloric acid. Before inoculation, we had flushed the headspace of the bottles with 20/80% CO_2/N_2 gas and equilibrated the bottle contents to atmospheric pressure (1 atm). We labeled the cultures based on their initial pH and substrate: Glu6.0, Glu6.5, Glu6.9, Fru6.0, Fru6.5, Fru6.9, Cello6.0, Cello6.5, and Cello6.9.

All inoculated bottles were incubated at 37^{o}C in a shaking incubator (New Brunswick Scientific, Enfield, CT) at 150 rpm. The duration of the experiments was 72 hours: the first 24-48 hours to reach stationary phase and establish biomass, and another 24 hours to ferment substrate. We sampled the liquid and gas phases at the beginning

and end of each experiment. All experiments were performed in triplicate, and the means and standard deviations of the triplicates are reported.

3.2.2 Growth and Fermentation End-Product Measurements

We documented growth by measuring optical density at 600 nm (Varian Cary 50 Bio UV) and pH using a pH meter (ThermoScientific Orion). We sampled the liquid phase at the time of inoculation and at the end of 72 hours using sterile syringes equipped with sterile 20-gauge needles and filtered the supernatant through 0.2-µm PVDF membranes (Acrodisc, LC 13 mm syringe filter).

We analyzed substrates and metabolites using High-Pressure Liquid Chromatography (HPLC) (LC-20AT, Shimadzu) equipped with a carbohydrate column (Aminex HPX-87H column, Biorad) as previously described (Lee *et al.*, 2008). Short-chain fatty acids (acetate, formate, butyrate, isobutyrate, isovalerate, valerate, propionate, lactate), alcohols (ethanol and methanol) were analyzed using 5 mM H_2SO_4 as the eluent, a 0.6-ml/min-flow rate, a column temperature of 50^{o}C, and a 50-min run time. The carbohydrates (glucose, fructose, and cellobiose) were analyzed using 18-ohm water as eluent, a 0.6-ml/min-flow rate, a column temperature of 30^{o}C, and 30 minutes of run time. The SCFAs and alcohols were detected with a photodiode array detector (PDA, Shimadzu), and the sugars and alcohols were detected with a refractive index detector (RID 10A, Shimadzu). We normalized the mmole of SCFAs produced to mmole of hexose consumed.

In order to perform electron-equivalent mass balances, we measured the total chemical oxygen demand (COD) of the samples before filtering and soluble COD after 0.2-µm filtration using HACH COD analysis kit (HACH Co, Loveland, CO, U.S.A). We

calculated the electron equivalents of sugars, fermentation end products, and biomass using the stoichiometric equations as specified in Rittmann and McCarty (Rittmann & McCarty, 2001). We also calculated theoretical alkalinity based on initial pH, partial pressure of CO_2, and pKa of the $HCO3^-$ using the equation specified in Rittmann and McCarty (Rittmann & McCarty, 2001). The calculated pKa of $HCO3^-$ was 6.16 when the ionic strength of the media was 0.03.

3.2.3 DNA Extraction and Sequencing

We extracted DNA from the inoculum and the resulting mixed fermentative consortia using QIAamp Mini Stool kit (Qiagen, CA) and followed the manufacturer's recommendation for pathogens with minimal modification. Briefly, we incubated the lysis solution and bacteria mix at 95^{o}C to enhance the lysis of Gram (+) bacteria. We verified the quantity and quality of DNA samples by NanoDrop measuring the absorption at 260 and 280 nm and stored the extracts at -80^{o}C until sequencing.

We amplified genomic DNA with a bar-coded primer set targeting the V2-V3 regions of 16S rDNA (Kang *et al.*, 2013). Sequencing libraries were prepared according to Claesson *et al.*(Claesson *et al.*, 2010) and purified PCR products were sent to the DNASU Genomics Core Facility at the Virginia G. Piper Center for Personalized Diagnostics in the Biodesign Institute at Arizona State University (Tempe, AZ), which provided pair-end reads (2x100 bp) using the HiSeq2000 platform (Illumina Inc., San Diego, CA). We received fastq files and deposited the sequences to Sequence Read Archive under SAMNO3120391-400 accession numbers.

3.2.4 Sequence Analysis

We analyzed data using the QIIME 1.8 suite (Caporaso, Kuczynski, *et al.*, 2010). We filtered the sequences using default values and setting the minimum quality score to 21 and minimum length to 192. We clustered sequences into operational taxonomic units (OTUs) at the 97% level of sequence similarity using Uclust (Edgar, 2010), picked the most abundant sequence as representatives of each cluster, and then assigned taxonomy to the sequences using RDP algorithm at 50% threshold (Wang, Garrity, Tiedje, & Cole, 2007) and Greengenes Database 2013 release (DeSantis *et al.*, 2006). We aligned representative sequences using Pynast (Caporaso, Bittinger, *et al.*, 2010) and identified chimeric sequences with ChimeraSlayer (Haas *et al.*, 2011). We calculated within sample (*alpha*) diversity indexes: Phylogenetic Distance Whole Tree (Faith, 1992) for diversity and Chao1 (Chao, 1987) for richness. The weighted Unifrac metric (Lozupone *et al.*, 2006) was used to calculate inter-sample diversity (*beta* diversity).

3.2.5 Statistics

Since our data size is small (n=3), non-parametric tests were more suitable for our data sets. We used Mann Whitney U test for significance and accepted P values less than 0.05 as significant. To find relationships between pH, microbial phylotypes, and metabolic end products, we performed Pearson and Spearman correlation tests, and accepted correlation coefficients with P values < 0.05 as significant associations. All the statistical procedures were carried out with Statistical Package for Social Sciences version 22.

3.3 Results and Discussion

3.3.1 Mixed Community Structure Depended more on pH than Carbon Type

In order to identify whether pH or the substrate exerted greater selective pressure on the microbial communities, we compared Unifrac distances (Lozupone, Hamady, & Knight, 2006) of the cultures to each other and visualized the result on principal coordinates, as shown in Figure 3.1A. We performed unweighted and weighted Unifrac analysis. Unweighted analysis, which provides information on changes in taxa that are not abundant, did not yield clusters based on pH or carbon source (data not shown). Weighted Unifrac, which relies on abundance of phylotypes in addition to their presence or absence, provides information regarding most abundant taxa. Since we had enrichment cultures from the same inoculum, weighed Unifrac was more suitable for our data set and revealed important changes.

Weighted Unifrac analysis shows that enrichment samples were distant from the inoculum and clustered according to pH on Principal Coordinate 1 (PC1), which explained 67% of the variation in the whole dataset, and PC2, which explained 22%. The pH-6.0 cultures formed a cluster distant to the right on PC1 and lower on PC2. The pH-6.5 and -6.9 cultures overlapped on PC1, although the pH-6.9 cultures were higher on PC2.

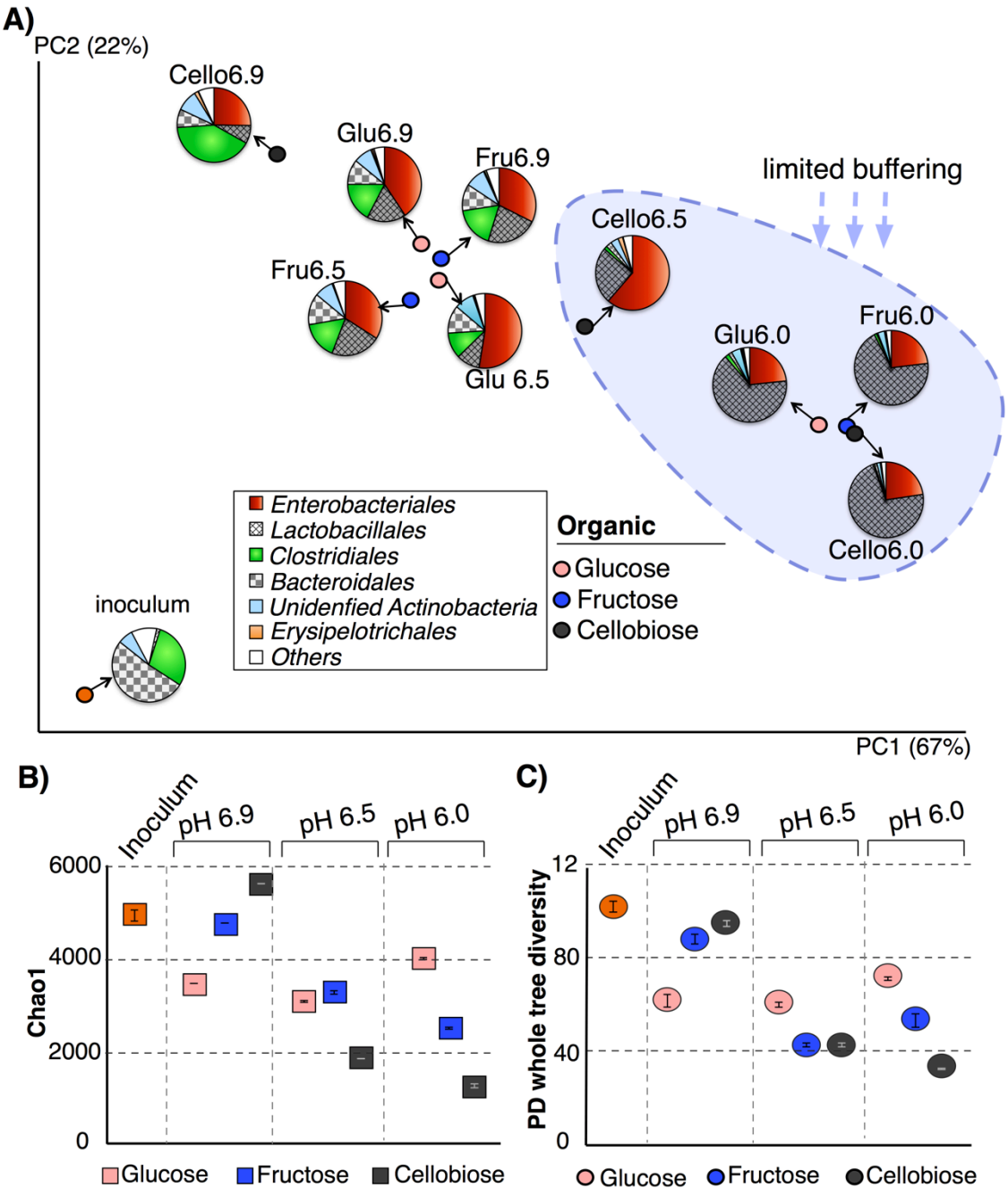

Figure 3.1. Beta and alpha diversity analysis of pH enrichments. (A) Weighted Unifrac analysis visualized on Principal Coordinates shows that mainly the initial pH along with buffering determined the main phylotypes that drove the community structures in the system. (B) Phylogenetic diversity tree and (C) Simpson reciprocal indexes calculated from 16S rRNA gene sequences for inoculum, pH 6.0, 6.5, and 6.9 cultures.

The medium's buffering capacity played a substantial role in the development of microbial communities. As shown in Table 3.1, 30 mM HCO_3^- provided 3.2-3.5 mM alkalinity to pH-6.0 cultures, whereas it provided 10.7-11.9 and 26.8-31.7 mM alkalinity to the pH-6.5 and -6.9 cultures, respectively. Greater bicarbonate buffering at pH 6.5 and 6.9 led to much smaller drops in pH in 72 hours, and this correlated with the development of similar microbial communities. The concentration of acids produced in pH-6.0 cultures and Cello6.5 cultures by 72 hours far exceeded the concentration of alkalinity, as summarized in Table 3.1. Therefore, rapid fermentation led to insufficient buffering, and the pH dropped dramatically. The substantial drop in the pH appeared to promote the survival of only acid-tolerant species, a trend that drove microbial communities away from pH-6.5 and -6.9 cultures.

Among the pH 6.5 cultures, Cello6.5 produced higher concentration of acid due to the double amount of electrons provided; hence, it did not have as much pH buffering as glucose and fructose cultures. The drop in the pH of Cello6.5 culture moved its community structure towards to pH-6.0 cultures. pH-6.9 cultures provided sufficient buffering for all cultures, and all three cultures developed similarly. The experimental design also probed the impact of different organic substrate (glucose or fructose) on community structure. Substrate type led to no clustering pattern.

Table 3.1. Amount of biomass produced, initial pH, final pH, and initial theoretical alkalinity values of the experiments.

Sample type		Biomass (OD)	Initial pH	Final pH	Initial Alkalinity (mmoles/L)	Total acids produced[a] (mmoles/L)
pH-6.0	Glu	0.34± 0.03	5.99±0.04	4.50±0.22	3.54±0.28	16.16±2.13
	Fru	0.27±0.03	5.95±0.31	4.27±1.19	3.30±0.52	17.71±0.69
	Cello	0.29±0.03	5.95±0.05	4.33±0.03	3.23±0.35	18.13±0.61
pH-6.5	Glu	0.35±0.05	6.45±0.13	6.13±0.18	10.66±2.99	12.54±1.39
	Fru	0.39±0.03	6.53±0.04	6.39±0.09	12.37±1.04	13.83±0.70
	Cello	0.49±0.04	6.51±0.02	4.57±0.12	11.88±0.64	16.19±2.17
pH-6.9	Glu	0.32±0.02	6.86±0.07	6.77±0.01	26.82±4.23	13.71±1.20
	Fru	0.40±0.02	6.92±0.02	6.75±0.06	30.08±1.45	14.05±0.51
	Cello	0.55±0.01	6.94±0.03	6.45±0.05	31.75±1.96	23.07±0.23

Glu, Fru, and Cello indicate that the initial substrate was glucose, fructose, or cellobiose, respectively. [a]measured after 72 hours

Figure 3.1A also shows the relative distributions of order-level phylotypes on the Principal Coordinates that clustered communities based on alkalinity and buffering. *Bacteroidales*, the most abundant order in the inoculum, was reduced in all cultures, while *Clostridiales*, *Lactobacillales* and *Enterobacteriales* increased in all the cultures. The main factors that separated the pH 6.0 cultures from the other cultures were the greater abundance of *Lactobacillales* (>65%) and lower abundances of *Enterobacteriales* and *Bacteroidales*.

Besides beta diversity (the Unifrac metric), we calculated alpha diversity parameters to understand within-community diversity based on pH and substrate. Figures 3.1B and 3.1C demonstrate Chao1 and PD whole tree indexes for richness and diversity, respectively. The Chao1 index shows that lower starting pH and alkalinity led to lower diversity overall. This trend was accentuated for cellobiose, a disaccharide composed of

two glucose molecules, except when buffering was strongest at pH 6.9. The PD whole tree index (Figure 3.1C), a phylogeny-based diversity index, showed similar patterns to Chao1. In summary, alpha diversity reinforces that pH had the primary effect on community structure and the substrate type had the secondary effect. Sugars like fructose and cellobiose, which likely reach the human colon (Nakamura *et al.*, 2004; Tappy & Le, 2010), should be important for maintaining microbial diversity in the human gut, as long as the pH is not substantially decreased.

3.3.2 Acetate, Lactate, and Propionate Accumulation Depended on pH

Figure 3.2 shows the major short chain fatty acids (SCFAs) in mmoles produced per mmole hexose consumed. Acetate and propionate were present in every culture, and they were the dominant end products of the pH-6.5 and pH-6.9 cultures. Acetate and propionate concentrations were 2- to 5-fold higher in pH-6.5 cultures and 7- to 27-fold higher in pH-6.9 cultures than pH-6.0 cultures. Lactate was the main end product of the pH-6.0 cultures, but was undetectable or very low in all the pH-6.5 and -6.9 cultures. Lactate, which can be fermented to acetate and propionate (Seeliger, Janssen, & Schink, 2002), probably accumulated in the pH-6.0 cultures due to the acid stress on lactate-utilizing bacteria (Giraud, Lelong, & Raimbault, 1991).

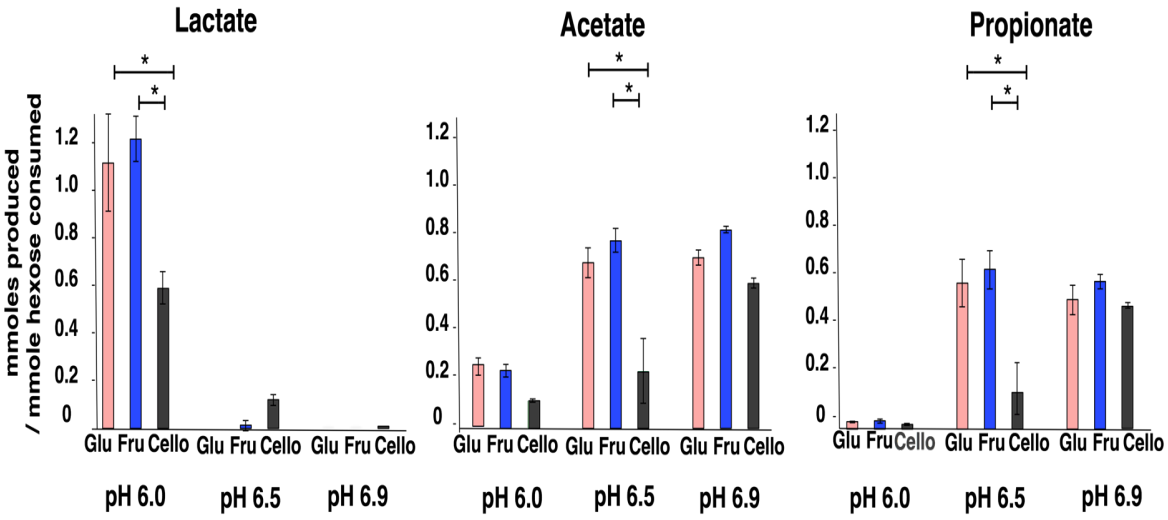

Figure 3.2. Major fermentation end products -- lactate, acetate, and propionate -- in mixed cultures fed glucose, fructose, or cellobiose at initial pH values of 6.0, 6.5, or 6.9. The mmol of each acid produced was normalized per mmole of hexose consumed. Error bars represent the standard deviations of triplicates for each condition. * indicates Mann Whitney U test $P < 0.05$

Minor fermentation products were butyrate, ethanol, and citrate (Table 3.2). Butyrate accumulation was minimal at all pH values. Similar to lactate production, butyrate production is favored at acidic conditions, such as pH ≤ 5.5 (Walker *et al.*, 2005), but butyrate producers require longer acclimation and incubation periods (Le Blay, Michel, Blottiere, & Cherbut, 1999). Thus, low butyrate generation might be explained by short experimental time of 72 hours. Citrate, an intermediate in propionate and acetate fermentation (Newbold *et al.*, 2005), was only detectable in pH-6.0 cultures, as well as in the Cello6.5 culture. Our results are consistent with those of Ramos et al, who showed that pH regulates citrate fermentation to acetate, with citrate fermentation to acetate becoming greater at a higher pH (6.2 in comparison to pH 5) (Ramos, Lolkema, Konings, & Santos, 1995). Citrate accumulation in these cultures indicated incomplete

acetate and propionate fermentation, and the presence of citrate and lactate explained low acetate and propionate concentrations in the pH-6.0 and Cello6.5 cultures. We did not observe this trend in the Glu6.5, Fru6.5 and pH-6.9 cultures, because the initial substrate was completely depleted, indicating that fermentation intermediates were converted into final fermentation products.

Table 3.2. Electron balances based on each metabolite's electron equivalence. % Recovery was calculated based on how much of the initial electron equivalents could be tracked by measurements at the end of the experiment. Values represent means with standard deviation.

	pH 6.0			pH 6.5			pH 6.9		
	Glu	**Fru**	**Cello**	**Glu**	**Fru**	**Cello**	**Glu**	**Fru**	**Cello**
Substrate	13.29±1	13.59±1	26.18±3	13.102	11.94±1	24.31±1	12.21±0	11.83±0	19.95±1
Products									
Lactate	7.37±1	8.24±1	7.46±1	0.00±0	0.08±0	1.61±0	0.00±0	0.00±0	0.01±0
Acetate	1.14±0	1.10±0	0.98±0	2.80±0	2.94±0	2.14±1	3.09±0	3.27±0	5.08±0
Propionate	0.24±0	0.28±0	0.29±0	4.32±1	4.39±0	2.40±4	4.10±0	4.26±0	7.60±0
Butyrate	0.48±0	0.50±0	0.43±0	0.53±0	0.60±0	0.52±0	0.61±0	0.61±0	0.75±0
Formate	0.02±0	0.10±0	0.18±0	0.03±0	0.01±0	0.10±0	0.06±0	0.01±0	0.00±0
Isobutyrate	0.03±0	0.05±0	0.03±0	0.61±1	0.18±0	0.98±0	0.10±0	0.08±0	0.12±0
Valerate	0.08±0	0.16±0	0.22±0	0.10±0	0.18±0	0.16±0	0.20±0	0.20±0	0.16±0
Isovalerate	0.04±0	0.09±0	0.25±0	0.00±0	0.06±0	0.43±0	0.00±0	0.12±0	0.09±0
Citrate	0.40±0	0.64±0	1.42±0	0.00±0	0.02±0	2.97±2	0.00±0	0.00±0	0.01±0
Hydrogen	0.08±0	0.15±0	0.02±0	0.29±0	0.15±0	0.28±0	0.13±0	0.04±0	0.04±0
Ethanol	0.21±0	0.21±0	0.29±0	0.68±0	0.62±0	0.67±0	0.77±0	0.69±0	1.34±0
Biomass	0.89±0	0.76±0	1.15±0	1.22±0	1.09±0	1.86±1	0.74±0	1.07±0	1.55±1
RS[a]	0.00±0	0.00±0	13.21±4	0.00±0	0.00±0	5.36±5	0.00±0.0	0.00±0	0.00±0
Total	10.76±1	12.06±1	25.63±3	9.91±1	9.69±0	18.82±1	8.65±1	9.67±0	15.42±1
% Recovery	80.90±4	88.78±5	97.96±1	75.85±3	81.22±2	77.41±1	73.12±6	81.69±3	77.38±4

RS[a] stands for remaining substrate at the end of experiment

The electron mass balances in Table 3.2 show that 73 to 98% of the electrons of the initial substrate were captured by our SCFAs and biomass measurements. The unaccounted electron equivalents likely were present in amino acids and other metabolic products that were not measured. Electron balances verified that the majority of the electrons were accumulated in intermediate molecules such as lactate and citrate when HCO_3^- alkalinity was limiting, and electrons were accumulated in mainly acetate and propionate when buffering with HCO_3^- was sufficient.

3.3.3. The Interactions Between Lactate-producing and -Consuming Microbial Communities were Driven by pH

Figure 3.3 shows that the inoculum consisted of diverse butyrate-producing species, including *Faecalibacterium* (6.8%), *Roseburia* (2.4%), and *Lachnospira* (4.7%). However, these butyrate producers were almost eradicated by batch culturing, and the low abundances of these phylotypes in the resulting cultures (<1%) can explain the observed low butyrate yields. In contrast, genus-level phylotypes including *Citrobacter*, *Escherichia*, *Streptococcus*, and *Veillonella* were present in low abundances in the inoculum, but became the majority of the phylotypes in the cultures.

Due to substantial differences in lactate, propionate, and acetate concentrations with the different pH conditions, we focused the role of pH on lactate-producing and -utilizing communities. These genus-level phylotypes were mainly from *Lactobacillales*, *Enterobacteriales*, *Clostridiales*, and *Baceroidales* orders that drove the cultures with limited buffering away from sufficient buffering (Figure 3.1). We examined phylotypes similar to dominant genera of lactate-producing *Streptococcus* (Bender, Sutton, & Marquis, 1986) and *Citrobacter* (Oh, Kim, Park, Kim, & Ryu, 2008), lactate-utilizing

Veillonella (Kolenbrander, 2006), and lactate-producing and lactate-utilizing *Bacteroides* (Schultz & Breznak, 1979) and *Escherichia (Bunch, MatJan, Lee, & Clark, 1997; Clark, 1989)*. The initial pH and the buffering capacity (affecting the final pH) had substantial impact on the relative abundance of these phylotypes. Only when buffering was sufficient (at pH 6.5 and 6.9), we observed that the substrate type had the secondary effects on abundances of phylotypes (similar to alpha diversity indices).

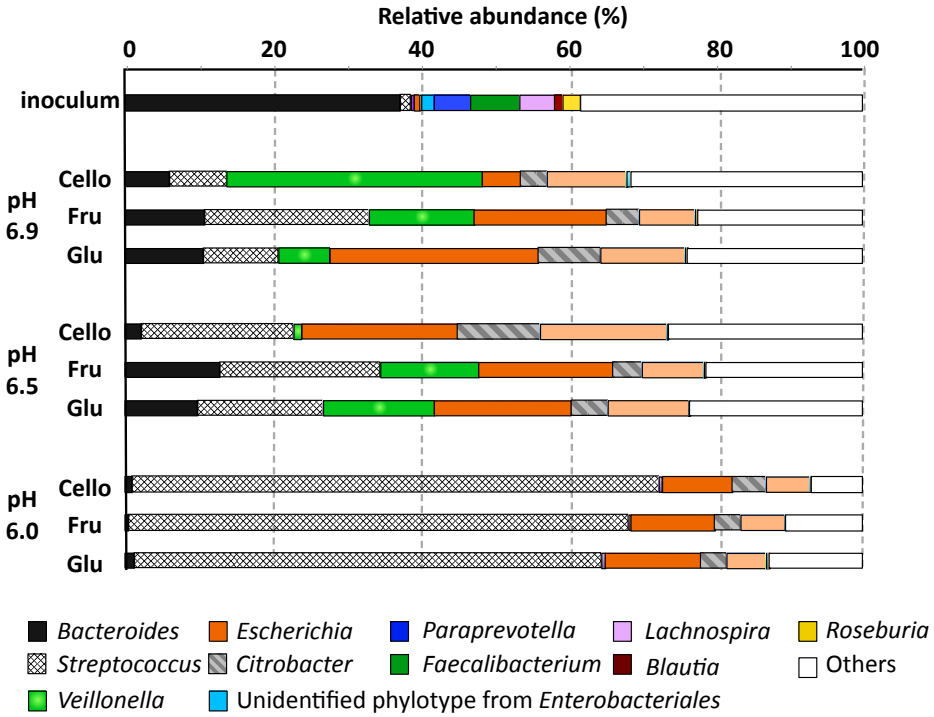

Figure 3.3. Relative abundance of phylotypes at the genera level in inoculum and fermentation cultures with initial pH values of 6.0, 6.5, or 6.9, and with glucose, fructose, or cellobiose as initial substrate. Green bars represent genera that belong to *Bacteroidetes*, blue bars represent genera that belong to *Firmicutes*, and orange bars represent genera that belong to *Proteobacteria.*

Lactate-producing *Streptococcus* phylotypes comprised only 1.5% of the inoculum, and they increased to 61-73%, 17-20%, and 7-22% in the pH-6.0, -6.5 and -6.9

cultures, respectively (Figure 3.3). Abundances of *Citrobacter,* another lactate-producer, did not mimic the trend of *Streptococcus* abundance, and it was at the greatest abundance in Cello6.5 cultures. Figure 3.4 shows parametric correlation coefficients for the associations between fermentation end products, pH, and major microbial phylotypes. The abundance of *Streptococcus* phylotypes strongly and positively correlated with lactate concentration (Pearson $r = 0.91$, $P < 0.05$), and the dominance of *Streptococcus* phylotypes at pH 6.0 can explain lactate accumulation in these cultures. *Citrobacter* did not significantly correlate with the abundance of citrate or lactate (Pearson correlation coefficient $r = 0.56$, $P > 0.05$), possibly because citrate is an intermediate of fermentation by *Citrobacter,* which gains energy from fermenting glucose to citrate and lactate and subsequently fermenting citrate and lactate to acetate (Gyaneshwar, Kumar, & Parekh, 1998).

Veillonella, reported lactate utilizers (Kolenbrander, 2006), were less than 1% of the inoculum and in the pH-6.0 cultures; their low abundance in the pH-6.0 cultures could be due to poor acid tolerance (Kolenbrander, 2006). *Veillonella* phylotypes varied from 1 to 35% in the pH 6.5 and 6.9 cultures, with the highest in the Cello6.9 (35%) and the lowest in the Cello6.5 culture (~1%). Unlike *Bacteroides*, *Veillonella* lacks the ability to ferment sugars, but it ferments organic acids, such as lactate and pyruvate, to propionate and acetate (Kolenbrander, 2006). The abundance of *Veillonella* phylotypes had negative correlation with lactate accumulation (Pearson correlation coefficient $r = -0.61$, $P < 0.01$) and positive correlation with propionate accumulation (Pearson $r = 0.70$, $P < 0.05$).

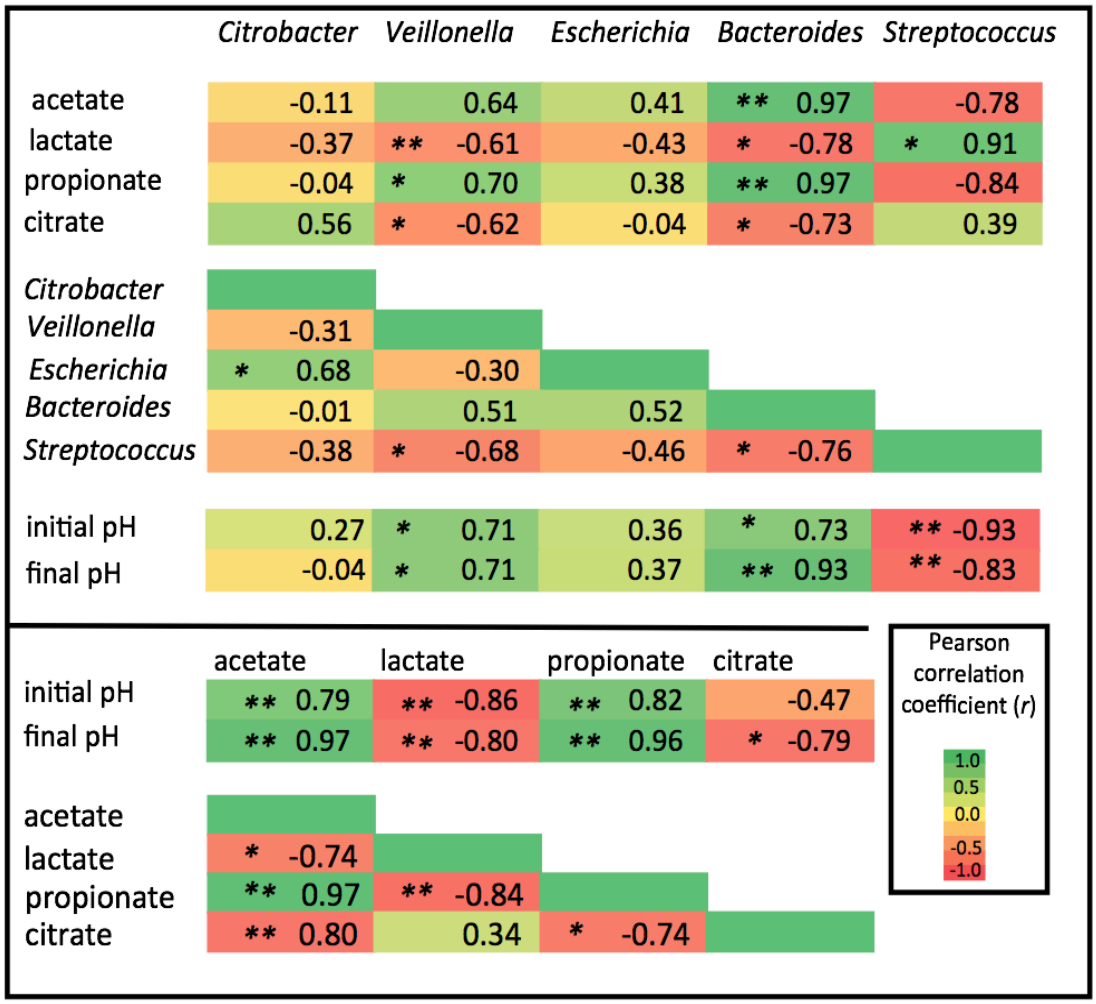

Figure 3.4. Parametric correlation coefficients (Pearson's) between combinations of taxa, initial pH, and fermentation end products. * Indicates correlation coefficient significant when $P < 0.05$, and ** indicates correlation coefficient significant when $P < 0.01$.

Bacteroides phylotypes were 37% of the inoculum, but they declined to less than 2% in the pH-6.0 cultures and 2-12 % of the pH-6.5 and -6.9 cultures. This observation is consistent with *Bacteroides* having weak acid tolerance (Walker *et al.*, 2005). The abundance of *Bacteroides* phylotypes correlated negatively with lactate concentration (Pearson correlation coefficient $r = -0.78$, $P < 0.05$), but positively with acetate and propionate concentrations (Pearson $r = 0.97$, $P < 0.01$). Along the same lines, high

lactate accumulation and low accumulations of acetate and propionate in the Cello6.5 culture can be explained by the low relative abundances of the lactate consumer and propionate producers *Veillonella* and *Bacteroides*. The lower abundances of *Bacteroides* and *Veillonella* phylotypes in pH 6.0 cultures happened in concert with lactate accumulation.

Escherichia were relatively abundant in all cultures (5-28%), especially for pH 6.5 and 6.9. The high abundance of *Escherichia* at pH 6.5 and 6.9 suggests that they replaced *Streptococcus* for the lactate-production *niche*. *Escherichia* are able to either produce or consume lactate, and this metabolic versatility may explain why it was a key member of all culture communities. Lactate-utilizing and acetate- and propionate-producing bacteria such as *Bacteroides, Escherichia* and *Veillonella*, and butyrate-producing microorganisms such as *Faecalibacterium* and *Roseburia* are essential for removing accumulated lactate in the colon (S. H. Duncan, Louis, & Flint, 2004). An absence of lactate consumers in the colon can lead to reduced pH of the colon via d-lactic acidogenesis, and the consequence of lower pH is a deterioration of the host's health (Fiddiangreen, 1993).

Our results on the microbial composition have implications for microbiota consuming simple carbohydrates post-bariatric surgery. The microbiota composition at pH 6.5 and 6.9 were similar to the reported colonic microbiota of post-Roux-en-Y gastric bypass (RYGB) surgery patients (Zhang *et al.*, 2009). RYGB surgery enriched *Gammaproteobacteria* phylotypes (Zhang *et al.*, 2009), more specifically *Escherichia* (Furet *et al.*, 2010; Kong *et al.*, 2010) and *Citrobacter* (Graessler *et al.*, 2013), as well as phylotypes most closely related to *Veillonella,* a lactate-consuming propionate-producing

in the *Firmicutes* phylum (Graessler *et al.*, 2013). Because this weight-loss surgery reduces gastric acid secretions, it might select for less acid-sensitive microorganisms (Palleja *et al.*, 2016) and increase fecal propionate concentration (Liou *et al.*, 2013). A limitation of this study is that we utilized batch bottles and enriched for microbial species with single carbon source, which cannot represent the complexity of the human gut. Nevertheless, findings of our study can be useful for the interpretation instances in which the pH of the intestines changes due to scenarios in which buffering is low compared to acid production in or input to the human intestine.

We studied how pH, alkalinity, and carbohydrate substrate affect the microbial community structure and function of a mixed-culture inoculum taken from the stool of a healthy human. Low pH, caused by limited bicarbonate alkalinity, had by far the strongest impact on community structure and metabolism. Impacts of substrate type on microbial community structure were secondary and evident only when alkalinity was not sufficient. Thus, a transient shift in pH from 6 to ~4 led to a less-diverse microbial community that formed less of acetate and propionate, but more lactate. As a consequence of limited buffering, a drop in the pH disrupted the growth of some community members, hence restrained microbial and metabolic interactions between lactate-producing and lactate-utilizing communities.

CHAPTER 4

PH CONTROLS THE LACTATE METABOLISM IN CO-CULTURES OF *VEILLONELLA*, *STREPTOCOCCUS*, AND *BACTEROIDES* SPECIES

4.1 Introduction

As explained in chapter 3, pH and alkalinity are important for microbial community structure and function. Microorganisms have different levels of tolerance to their surrounding pH; different environments such as host gastrointestinal (GI) tracts, host oral cavity, and soils expose microorganisms to fluctuating pH conditions (Lund *et al.*, 2014). Fluctuations in proton conditions influence the growth and metabolism of microorganisms, especially neutralophilic bacteria, depending on their ability to overcome acid stress (Lund *et al.*, 2014).

Chapter 3 demonstrated the impact of pH on mixed communities and metabolism. Particularly, lactate metabolism and microorganisms that are involved in lactate production and fermentation were affected by the initial pH imposed on them. Lactate producing *Streptococcus* (Russell & Hino, 1985) and *Bacteroides* (Falony *et al.*, 2009), and lactate-consuming *Veillonella (Al-Ahmad et al., 2007)* responded to the changes in the initial pH and alkalinity among the other members of the microbiota. Therefore, it is important to study microbial interactions between these lactate-producing and -consuming microorganisms at the species level using mono and co-cultures.

Streptococcus, *Bacteroides,* and *Veillonella* are important members of the human gut and oral microbiota (Suau *et al.*, 1999, Al-Ahmad *et al.*, 2007). Symbiosis between lactate-producing *Streptococcus,* and lactate-consuming *Veillonella* (Egland *et al.*, 2004) can overcome gut acidosis due to removal of accumulated lactic acid (Russell & Hino,

1985). Unlike these two species *Bacteroides* species are more versatile and can do both lactate production and consumption (Falony *et al.*, 2009), although its efficiency to produce lactate compared to *Streptococcus* and to consume lactate compared to *Veillonella* are unknown.

Lactate can be fermented into acetate and propionate via methylmalonyl-CoA or acrylyl-CoA pathways (Seeliger, Janssen, & Schink, 2002). The active lactate fermentation pathway depends on microbial composition (Bourriaud *et al.*, 2005) and environmental conditions (Ushida & Sakata, 1998) and due to the acid stress imposed on microorganisms at pH 6.0, lactate fermentation was inhibited which lead to lactate accumulation.

The objectives of this chapter were: (i), to reveal the impact of pH on interactions between lactate producing and consuming species and (ii) to demonstrate the impact of co-existence of metabolically-bond species on each other's growth in the context of pH. We selected *Streptococcus salivarius*, a probiotic that enhances the immune function (Cosseau *et al.*, 2008), *Veillonella dispar,* a species that can aggregate on *Streptococcus* cells (Palmer *et al.*, 2007), and *Bacteroides thetaiotaomicron,* a versatile species that can form many metabolic reactions and can enhance growth of specific groups of microorganisms (Samuel & Gordon, 2006).

Using the same initial pHs from chapter 3, we grew *S. salivarius*, *B. thetaitomicron*, and *V. dispar* as mono-cultures and as co-cultures at different combinations and we characterized their growth and metabolism in response to co-culturing and initial pH. Our results showed that pH could impact the lactate metabolism due to different acid tolerance of microbial species.

4.2 Materials and Methods

4.2.1 Batch Experimental Conditions for Co-Culture Experiments

Streptococcus salivarius, *Veillonella dispar*, and *Bacteroides thetaiotaomicron* (*B. theta)* were purchased from American Type Culture Collection. I grew all the strains using reinforced clostridial medium (7714) (attc.org) with 10 mM glucose for *S. salivarius* and *B. theta* and 10 mM lactate for *V. dispar*. I adjusted the strains to 10-fold-diluted medium in order to reduce yeast extract and peptone fermentation by the cultures. I prepared 10-fold diluted reinforced clostridial medium with addition of 10 mM glucose and 30 mM of HCO_3^- anaerobically. I distributed 15 mL of medium into serum bottles and autoclave-sterilized them. Then, I adjusted the pH to 6.0, 6.5, and 6.9 with 10% HCl, and equilibrated the bottles over-night.

For inoculation, I prepared over-night seed cultures by inoculating sterile media with frozen stocks of the cultures. After measuring protein content with the BCA protein assay (Thermo-Scientific Inc.) of the over-night cultures, I inoculated 0.3 mg/L of each culture to bottles set to the different initial pHs. I grew *S. salivarius*, *V. dispar*, and *B. theta* in monocultures, *S. salivarius* and *B. theta* with *V. dispar*, and finally I grew all three cultures together at three different initial pH settings. I mixed the cultures equally based on their protein content.

Both mixed culture, monoculture, and co-culture bottles were exposed to same environmental conditions. Briefly, I incubated the inoculated bottles at 37^{o}C and shaken at 150 rpm to achieve mixing. The duration of the experiments was 72 hours; first 24-48 hours to reach stationary phase and establish biomass, and another 24 hours to ferment substrate. I collected liquid and gas samples at time 0, 16, 36, and 72 hours for biomass

and metabolic analysis. All experiments were performed in triplicates, and the means and standard deviations of the triplicates were calculated for reporting.

4.2.2 Growth and Fermentation Product Measurements

The samples collected at time 0, 16, 36, and 72 hours were subjected to biomass, pH, and fermentation end product measurements. Using a spectrophotometer (Varian, AA20, Australia), I measured the biomass at 660 nm. pH was measured with a pH meter (Orion, U.S.A). For fermentation end product analysis, I filtered the supernatant through 0.2 μm PVDF membranes (Acrodisc, LC 13 mm syringe filter).

I analyzed substrates and metabolites using High-Pressure Liquid Chromatography (HPLC) (LC-20AT, Shimadzu) equipped with a carbohydrate column (Aminex HPX-87H column, Biorad) as previously described (Lee *et al.,* 2008). Short-chain fatty acids (acetate, formate, butyrate, isobutyrate, isovalerate, valerate, propionate, lactate), alcohols (ethanol and methanol) were analyzed using 5 mM H_2SO_4 as the eluent, 0.6 ml/min flow rate, the column temperature of 50^{o}C, and 50 minutes of run time. The carbohydrates (glucose, fructose and cellobiose) were analyzed using 18-ohm water as eluent, 0.6 ml/min flow rate, the column temperature of 30^{o}C, and 30 minutes of run time. The SCFAs and alcohols were detected with a photodiode array detector (PDA, Shimadzu) and the sugars and alcohols were detected with a refractive index detector (RID 10A, Shimadzu). I normalized the amount of SCFAs produced per mmole of hexose consumed. Additionally, I calculated electron equivalence of each product, substrate and the biomass produced, hence provided electron balance.

4.2.3 Extraction of DNA, Plasmid Construction, and Quantitative PCR Analysis of 16S rRNA Genes from *V. dispar*, *S. salivarius*, and *B. theta.*

I extracted DNA from the pure cultures and co-cultures using QIAamp Mini Stool kit (Qiagen, CA). I followed the manufacturer's recommendation for pathogens with few modifications. Briefly, I incubated the lysis solution and bacteria mix at 95^{o}C to enhance the lysis of Gram (+) bacteria. I verified the quantity and quality of DNA samples by NanoDrop measuring the absorption at 260 and 280 nm. We stored the extracts at -80^{o}C until sequencing.

I performed PCR using 8F and 1525R primers that target 16S rRNA gene for each of the strains as described (Zhao *et al.,* 2013). Using TOPO TA Cloning kit (Invitrogen), I constructed plasmid for qPCR standards. I used the primers and protocols for *B. theta* from Liu *et al.* (Liu *et al.*, 2003), *V. dispar* and *S. salivarius* from van den Bogert *et al.* (van den Bogert *et al.*, 2011). I generated the standard curves by running serial dilutions of the standards from $1x10^{-1}$ to $1x10^{-7}$ ng/uL.

The qPCR reactions were performed in 20 ul reactions: 10 uL SYBR green Ex Taq I mastermix, 8.6 ul water, and 0.2 uL of each primers with a concentration of 10 mM, and 1 uL 10 times diluted DNA. Once I obtained gene copies for each strain, I normalized them based on how many 16S rRNA genes exists in each strain: *V. dispar* contains four, *B. theta* contains five, and *S. salivarius* contains six copies of 16S rRNA gene per cell.

4.3 Results and Discussion

4.3.1 Co-cultures of *Streptococcus*, *Bacteroides* and *Veillonella* produced different amounts of acetate, lactate, and propionate

Lactate, acetate, and propionate were the main fermentation end products of the cultures similarly to the results from mixed communities reported in chapter 3, as shown in Figure 4.1, *V. dispar* mono-cultures have very low concentration of all products and did not consume the glucose in the media since they cannot utilize glucose (Kolenbrander, 2006). Accumulations of SCFAs at minor quantities in these cultures are probably a result of fermentation of yeast extract.

All monocultures had similar levels of acetate accumulation and the concentrations were less than 4 mM. Propionate was at minor quantities in *S. salivarius* and *V. dispar* cultures, while it was at greater abundance in *B. theta* cultures. *S. salivarius* produced mainly lactate and minor quantities of acetate and propionate. *S. salivarius* showed higher lactate accumulation at pH 6.5 and 6.9 compared to 6.0. *B. theta* cultures produced mainly lactate and propionate and small quantities of acetate, regardless of the initial pH. *B. theta* produced more lactate at pH 6.9 than pH 6.0 and 6.5, whereas it produced more propionate at pH 6.0 than 6.5 and 6.9. This finding suggests that *B. theta* is either more efficient producing lactate at pH6.0 than at pH6.5 and pH6.9, or it can convert lactate to acetate and propionate more efficiently at pH6.0. Compared to *B. theta*, *S salivarius* was more efficient producing lactate at pH6.5, and at pH6.9 their lactate production levels were comparable.

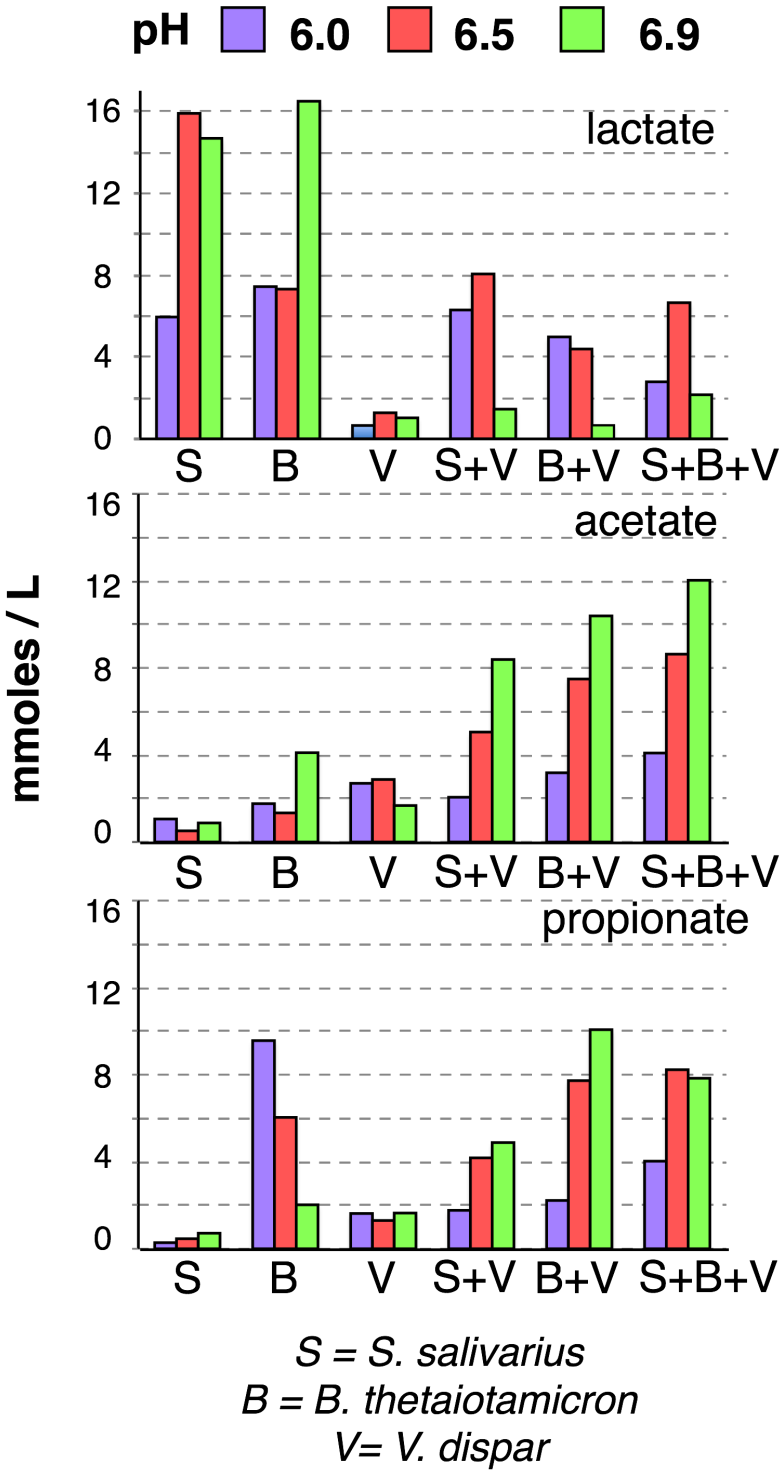

Figure 4.1. Main fermentation product distribution of *V. dispar*, *S. salivarius*, *B. theta* in monocultures and co-cultures at pH 6.0, 6.5 and 6.9.

Co-culture experiments depicted different fermentation results. When *B. theta* and *S. salivarius* were co-cultured with *V. dispar*, lactate levels dropped in the cultures and propionate and acetate concentrations increased at pH 6.5 and 6.9. We did not observe this trend in pH 6.0 cultures because growth of *V. dispar* is limited at low pH. *B. theta* co-cultures produced more propionate than *S. salivarius* co-cultures because unlike *S. salivarius*, *B theta* has been shown to produce propionate (Liu, *et al.*, 2003). Co-culturing *S. salivarius* and *B. theta* with *V. dispar* produced comparable levels of acetate. When all three strains were cultured together, the trends were similar to two strain co-cultures; however, acetate production was greater than propionate production.

Starting pH had an impact on co-cultures metabolism. Both *S. salivarius* and *B. theta* co-cultures with *V. dispar* produced more lactate at lower starting pH and more acetate and propionate at higher starting pH. This could be due to the limited growth of V. dispar at low pH.

Our co-culturing strategy under different initial pH scenarios showed that microbial partnerships are controlled by pH and they have an important role in lactate metabolism and fermentation end-product distribution. Figure 4.2 illustrates these microbial interactions. *Veillonella*, a genus that lacks carbohydrate fermentation genes rely on lactate-producing species for survival (Kolenbrander, 2006). *Veillonella* and *Streptococcus* are found together in oral biofilms or dental cavities (Egland *et al.*, 2004) due to the metabolic interactions regards to carbohydrate metabolism. Our results showed that starting pH could control the metabolic interactions between *S. salivarius* and *V. dispar*, therefore can alter the fermentation end product distribution. When we co-cultured *V. dispar* with *B. theta*, we observed similar fermentation end-product trends to

co-culture with *S. salivarius* although the results were less drastic due to *B. theta*'s ability to produce acetate and propionate from glucose fermentation intermediate lactate (Liu, *et al.*, 2003).

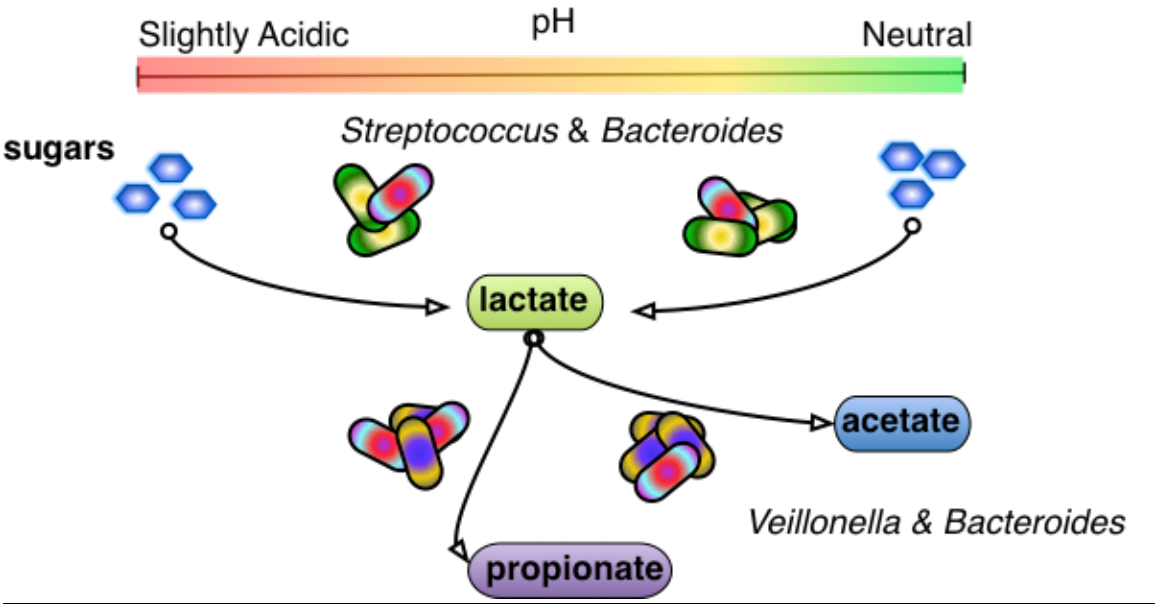

Figure 4.2. Illustration of microbial interactions that yield acetate, lactate, and propionate fermentation regards to initial pH.

4.3.2 Co-Culturing *Streptococcus*, *Bacteroides* and *Veillonella* Had Different Effects on Each Other's Growth

As seen in Figure 4.3, by the end of 72 hours pH dropped in *B. theta* and *S. salivarius* monocultures, and the drop was greater when the initial pH was 6.0. This result was consistent with mixed-culture results that were presented in chapter 3. The amounts of acids produced at pH 6.5 or 6.9 was not concentrated enough to drop the pH below the pKA of HCO_3^-, therefore these cultures remained well-buffered until the end of the experiment.

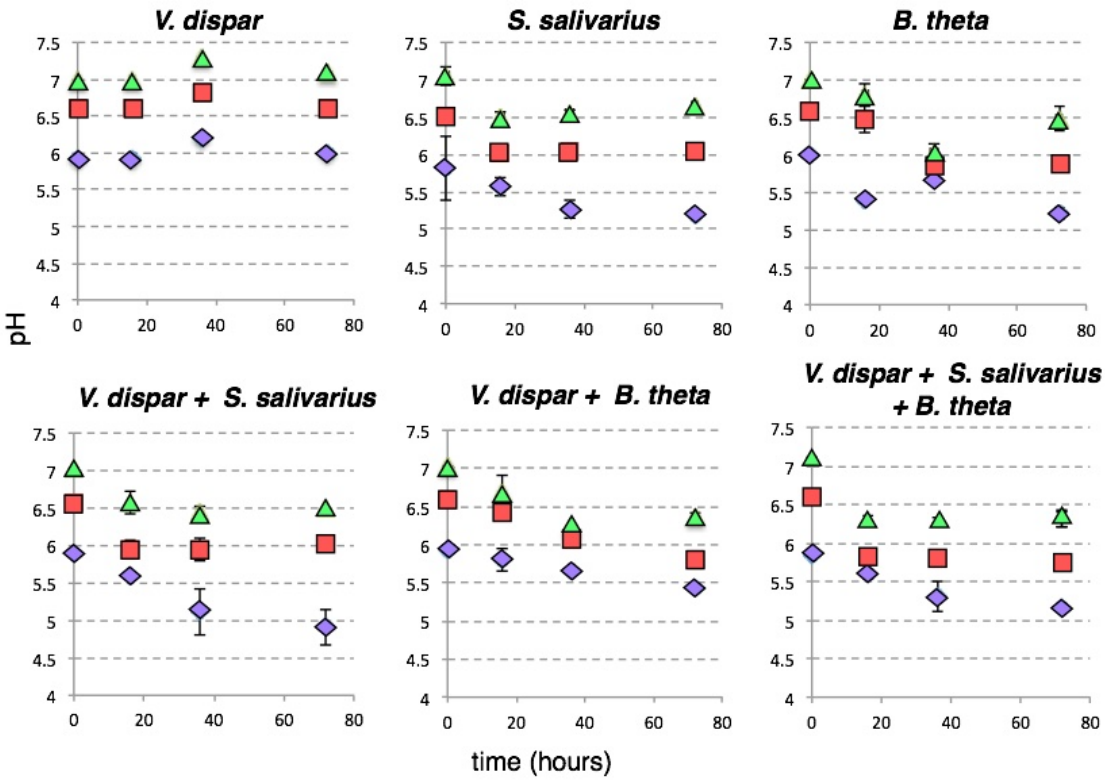

Figure 4.3. Changes in pH over time (72 hours) in monocultures of *V. dispa*r, S. *salivarius*, *B. theta*, and their co-cultures. Green triangles represent pH 6.9 cultures, red squares represent pH 6.5 cultures, and purple diamonds represent pH 6.0 cultures.

V. dispar monocultures did not change the pH; this was mainly because *V. dispar* cannot utilize glucose as energy source (Kolenbrander, 2006), so its growth was limited by the nutrients from the yeast extract. Co-culturing *S. salivarius*, and *B. theta* with *V. dispar* showed similar pH drop patterns to *B. theta* or *S. salivarius*. When we co-cultured the three strains together, the drop in the pH was more prominent compared to other co-cultures.

Changes in the pH were consistent with the amount of 16S rRNA gene copies at the end of the experiment. Figure 4.4 shows number of cells per culture detected for each of the strains: *V. dispar*, *S. salivarius*, and *B. theta* in monocultures and co-cultures at initial pHs of 6.0, 6.5, and 6.9. Based on these, the growth for *V. dispar* was undetectable at pH 6.0 or 6.5, and it was the lower than *S. salivarius* and *B. theta* for pH 6.9 cultures. *S. salivarius* had limited growth at pH 6.0 and cells per culture were higher at pH 6.0 and 6.5. *B. theta* had the highest amount of cells per culture, even though the number of copies at pH 6.0 was lower compared to pH 6.5 and 6.9, the difference was less than 10 fold.

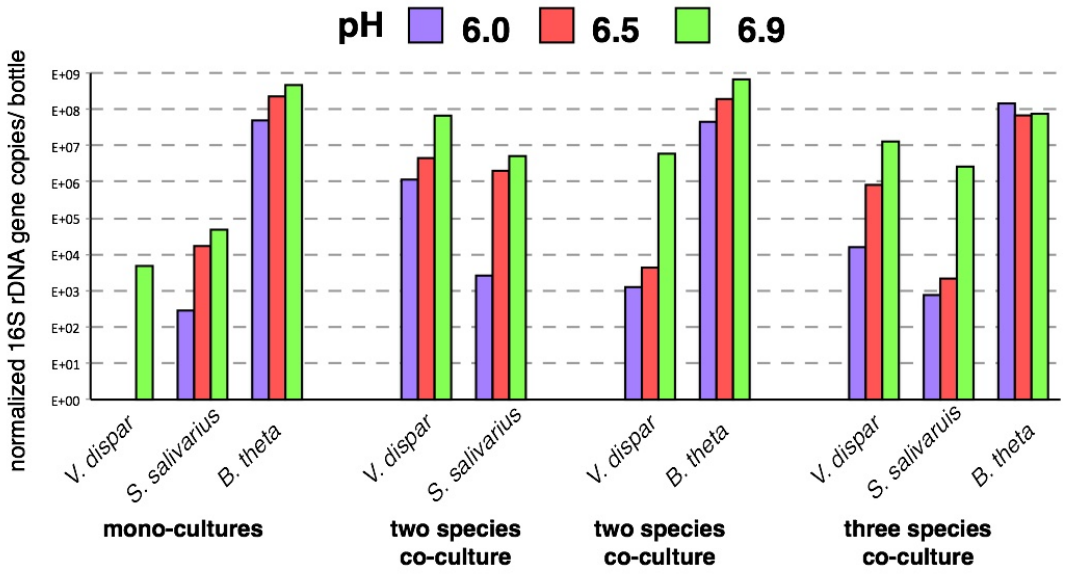

Figure 4.4. Normalized 16S rDNA copy numbers of *V. dispar*, *S. salivarius*, and *B. theta* at pH 6.0, 6.5, and 6.9 in monocultures and co-cultures

When *V. dispar* was co-cultured with *S. salivarius*, *B. theta*, or with both, cells per culture was above the detection limit. 16S rRNA gene copy numbers were the greatest at pH 6.9 and comparable at pH 6.0 and 6.5. When co-cultured, *V. dispar* generated more 16S rRNA gene copies with *S. salivarius* than with *B. theta*. The difference in the growth

of V. dispar was even more prominent at pH 6.0, co-culturing with *S. salivarius* helped *V. dispar* to grow better at pH 6.0.

Co-culturing *V. dispar* with *S. salivarius* had a positive impact on *S. salivarius* growth as well. More cells of *S. salivarius* were detected when it was co-cultured compared to grown on monocultures. Presence of *V. dispar* did not affect the amount of *B. theta* cells. When all three strains were together, *V. dispar* and *S. salivarius* showed similar trends of 16S rDNA copy numbers to their two species co-culture. In three species co-cultures, their copy numbers were lower in general, possibly due to competition with *B. theta* for resources.

Our results from this co-culture experiment showed that pH is important for microbial interactions in between species. Metabolic interactions among *Streptococcus* and *Veillonella* species rely on both of the species tolerance on pH. *Streptococcus*-*Veillonella* co-occurrence in dental biofilms has shown previously (Al-Ahmad *et al.*, 2007), however, *B. theta* supporting the growth of *V. dispar* has not been shown before.

The human GI tract is a complex ecosystem with multiple elements affecting its function; therefore, it is difficult to determine causality from *in-vivo* studies. Small differences in pH shift SCFA producing pathways, which could lead to important changes to host metabolism by altering production of lactate, acetate, and propionate.

Co-culture experiments verified the major findings of the mixed culture experiments from chapter 3 that pH is important for the distribution of fermentation end products and the growth of neutralophilic microorganisms. For some strains, it can influence the end products just because pH limits the growth, i.e., *S. salivarius*. For some, i.e., *B. theta,* pH doesn't affect its growth but have a substantial role in the

fermentative pathways. In case of lactate utilizing, *V. dispar,* the proton conditions limited its growth at pH 6.0. Co-culture experiments help us identify those physiological differences and metabolic interactions at strain level.

We can expand the implications of pH on these model microorganisms on host health. For instance, lactate accumulation can lead to acidosis (Russell & Hino, 1985) in the gut. Absence of lactate consumers in the colon due to perturbations of microbiota or diseases can lead to reduced pH of the colon via d-lactic acidogenesis, and consequently deteriorate the host's health (Fiddiangreen, 1993). Here we showed that even though *B. theta* can consume some lactate, co-culturing *B. theta* and *S. salivarius* with *V. dispar* significantly reduced the total amount of lactate being accumulated. Introducing *V. dispar* to the co-cultures also increased the total amount of propionate being produced, which exerts beneficial effects on host metabolism. Propionate is a short chain fatty acid that can reduce liver fattiness by signaling free fatty acid receptors (Nilsson *et al.*, 2003) and increase intestinal gluconeogenesis (De Vadder *et al.*, 2014). By impacting the survival of lactate consuming species, gastrointestinal pH can indirectly affect gut metabolism. A slight increase in gut pH can increase propionate production (Walker *et al.*, 2005). Conditions or treatments such as proton pump inhibitor use that increase gut pH demonstrated an increase in propionate production and abundance in lactate utilizers (Imhann *et al.*, 2016).

In summary, we demonstrated that slight differences in initial pH have an impact on growth or metabolism of lactate utilizing and producing communities. pH has primary effect on metabolic interactions between microorganisms that establish partnerships based on their metabolic requirements. Greater propionate production at higher pH due

to survival of lactate consuming microorganisms such as Veillonella holds promise for a probiotic to enhance liver and gut health.

CHAPTER 5

WEIGHT LOSS-ASSOCIATED GUT MICROBIOMES AND METABOLITES IN GASTRIC BYPASS AND BANDING PATIENTS

5.1 Introduction

Rising obesity rates and ineffective weight management strategies have increased the prevalence of bariatric surgery approaches to treat morbid obesity (Korner *et al.*, 2009). At present, laparoscopic adjustable gastric banding (LAGB) and Roux-en-Y gastric bypass (RYGB) are the most commonly performed surgeries (Yurcisin, Gaddor, & DeMaria, 2009); however, RYGB achieves greater and more sustained weight loss, as well as better resolution of obesity-related comorbidities such as diabetes and hyperlipidemia (O'Brien, 2010).

Both bariatric operations limit food intake. While LAGB surgery places an adjustable band around the proximal stomach to restrict the flow of ingested food, RYGB surgery creates a small gastric pouch by partitioning the stomach into proximal and distal parts (Korner *et al.*, 2009). Additionally, with the RYGB, a section of jejunum (i.e., the Roux limb) is then connected to the proximal gastric pouch, thereby excluding the majority of the stomach, duodenum, and proximal jejunum from exposure to ingested food (Korner *et al.*, 2009). The digestive secretions produced by the bypassed stomach, duodenum, and pancreas reconstitute with the flow of chyme through a surgical connection with the Roux limb (i.e., the biliopancreatic limb) at a certain length distal to the connection of the Roux limb with the gastric pouch (Wittgrove & Clark, 2000). In addition to limiting food intake, and unlike the LAGB, RYGB may induce malabsorption of digested food depending upon the length of the Roux limb, change hormonal response

for appetite (Korner *et al.*, 2009), increase intestinal transit, and alter bile-acid metabolism (le Roux *et al.*, 2006). These factors have been shown to contribute to weight loss after RYGB surgery (Korner *et al.*, 2009). Although an altered response of the orexigenic hormone ghrelin has also been observed after LAGB, the clinical significance of this finding remains uncertain (Shak *et al.*, 2008).

A role of gut microbiota on host energy regulation and metabolism has been postulated. For example, the structure of the gut microbial community has been shown to differ between lean and obese hosts (Ley *et al.*, 2005), and altered microbial metabolism in obese hosts appears to contribute to weight gain (Turnbaugh *et al.*, 2006). Humans (Furet *et al.*, 2010; Zhang *et al.*, 2009) and animals (Li *et al.*, 2011; Liou *et al.*, 2013) that had undergone RYGB were shown to exhibit significantly different microbial-community structures than obese or lean comparators. To our knowledge, changes in gut microbiota after LAGB surgery have not been studied.

Recently, the transplantation of fecal from post-RYGB mice to gnotobiotic mice altered gut microbiota and induced weight loss (Liou *et al.*, 2013) and the deposition of adipose tissue (Tremaroli *et al.*, 2015) independently from diet. Although the microbiota-associated mechanism of weight loss remains unclear, microbial metabolites, such as a short-chain fatty acid (SCFA), propionate, which was found to increased after RYGB surgery in mice (Liou *et al.*, 2013), have been implicated as signaling molecules that interact with the free-fatty-acid receptor in a way that protects against diet-induced obesity (Lin *et al.*, 2012). Besides propionate, two branched-chain-fatty-acids (BCFAs) -- isobutyrate and isovalerate -- also serve as signaling molecules that show potent activity

against the G protein-coupled receptor GPR41 (Le Poul *et al.*, 2003) and free-fatty-acid receptors 2 and 3 (Schmidt *et al.*, 2011).

A comparative analysis of the transformations in gut microbiota after successful and unsuccessful bariatric operations in humans, along with changes in microbial metabolites, may provide further insights into the mechanism of weight loss following these operations. Moreover, microbiota and microbial metabolites after LAGB and RYGB, in connection to diet composition, body mass index, and percent excess weight loss warrant further investigation. The objectives of our study were to: i) determine differences in microbial community structure after RYGB and LAGB surgeries, ii) identify microbial signatures of surgical weight loss, and iii) identify microbe-produced metabolites that contribute to successful bariatric surgery outcomes. To accomplish these objectives, we studied the microbial community structure and metabolic products in fecal samples from individuals who had previously undergone either RYGB or LAGB.

5.2 Materials and Methods

5.2.1 Experimental Design and Subject Characteristics

The Institutional Review Boards of Mayo Clinic and Arizona State University (IRB# 10-008725 for both) approved our study and all subjects provided signed informed consent. Our study compared gut microbial ecology in four groups: subjects who had previously undergone RYGB or LAGB surgery, healthy normal weight (NW) individuals, and morbidly obese controls who were scheduled to undergo bariatric surgery. The subjects were recruited at Mayo Clinic, Scottsdale between 2011 and 2014. Importantly, the morbidly obese subjects were on a recommended weight loss diet in preparation for their upcoming surgeries and they were in negative energy balance. We

use abbreviations “PreB-Ob” and NW for pre-bariatric surgery morbidly obese and normal-weight subjects, respectively.

We excluded subjects from the study who had undergone other types of gastrointestinal surgery or had uncontrolled gastrointestinal, endocrine, or other chronic diseases. Prebiotic/probiotic agents were avoided two weeks prior to feces collection and antibiotic use was not allowed within two months of feces collection. Participants were asked to refrain from the use of medications that can alter gastrointestinal secretory or motor function (e.g., proton pump inhibitors or prokinetic agents) for at least 2 weeks prior to feces collection; however, if they were unable to do so, this did not disqualify them from participation.

In order to avoid short-term disturbances in the microbiota, we included only subjects who had their surgeries more than 9 months before the sample collection. Fecal samples were collected at the Mayo Clinic, Scottsdale, shipped to Arizona State University on dry ice, and stored at -80^{o}C until the time of analysis.

Around the time of feces collection, we obtained 4-day food diaries and food frequency questionnaires from the subjects, and the subjects were educated by a dietitian on how to accurately record their food intake. Dietary information was analyzed using DietOrganizer software (dietorganizer.com).

5.2.2 DNA extraction, 16S rRNA gene sequencing, and genomic predictions

We extracted genomic DNA using the QIAamp Mini Feces kit (Qiagen, CA) following the manufacturer’s recommendations, but with an additional lysis step at 95^{o}C to enhance the DNA extraction from Gram-positive bacteria. DNA extracts were sent to University of Minnesota Genomic Center. Sequencing was performed with an Illumina

Miseq Instrument using V4-V6 primers (F 5′-GTGCCAGCMGCCGCGGTAA-3′ and R 5′-ACAGCCATGCANCACCT-3′). Sequences were submitted to NCBI Sequence Read Archive under accession numbers from SAMN05001554 to SAMN05001616. Forward and reverse reads were paired using PANDAseq (Masella, Bartram, Truszkowski, Brown, & Neufeld, 2012) and analyzed using the QIIME 1.8 suite (Caporaso *et al.*, 2010). After quality filtering, we attained a total of 3 369 231 sequences and median number of sequences per sample was 50 035. Details of the analysis were previously reported (Kang *et al.*, 2015) with the following modification: clustering was performed at 99% sequence similarity. We calculated within-sample (alpha) diversity metric: Phylogenetic Distance Whole Tree (Faith, 1992). We used the Unifrac metric (Lozupone, Hamady, & Knight, 2006) to calculate inter-sample (beta) diversity and Linear Discriminant Analysis Effect Size scores to identify microbial discriminants of bariatric surgeries (Segata *et al.*, 2011). In order to understand the role of gut microbiota in metabolite production, we predicted functional-gene content in the samples from 16S rRNA gene data using Phylogenetic Investigation of the Communities by Reconstruction of Unobserved States (PICRUSt) (Langille *et al.*, 2013). We analyzed wide-scale properties of the microbiota after bariatric surgeries using the BugBase tool (Caporaso *et al.*, 2010; Langille *et al.*, 2013).

5.2.3 Biochemical Characterization of Feces

For each fecal specimen, approximately one gram of wet weight (precise weight was recorded and used for calculations) was diluted with 20 mL of milliQ water (18 ohms). The homogenate was vortexed for 10 minutes and centrifuged at 13,000 rpm for 15 minutes before supernatants were filtered through 0.2-um PVDF membranes (PALL

Corporation). SCFAs were analyzed using High-Performance Liquid Chromatography (HPLC) (LC-20AT, Shimadzu) equipped with a carbohydrate column (Aminex HPX-87H column, Biorad) and photodiode array detector (PDA, Shimadzu) (Lee, Salerno, & Rittmann, 2008). The SCFAs analyzed included acetate, formate, butyrate, isobutyrate, isovalerate, valerate, propionate, and lactate. We normalized the SCFA concentrations to grams of fecal dry weight.

A global metabolomics approach was employed to obtain assignment and quantitation of small metabolites via ^{1}H-NMR. The 1D ^{1}H NMR spectra of all samples were collected following standard Chenomx (Edmonton, Alberta) sample preparation and data collection guidelines (Weljie, Newton, Mercier, Carlson, & Slupsky, 2006). For our study, we prepared and analyzed a subset of samples (only RYGB, NW, and PreB-Ob groups) in triplicates for extraction and duplicates for NMR. NMR samples were prepared as fecal extracts described above but then but diluted with a 10% (v/v) spike of a NIST calibrated reference solution (100% D_2O, 5 mM 2,2-dimethyl-2-silapentane-5-sulfonate-d6 (DSS) and 0.1% sodium azide). The resulting 500 uM DSS internal reference standard yields quantitation compared to spectral features and intensities for a wide-range of metabolites found in a Chenomx NMR reference library (DeAngelis *et al.*, 2013).

5.2.4 Statistics

Statistical analyses were conducted using the IBM SPSS Statistics for Macintosh, Version 23.0 (Armonk, NY: IBM Corp). Group medians were compared using the Mann-Whitney U test and the P-values corrected using Monte Carlo simulations. Median values were reported with median absolute deviation values. Spearman's rank-order

correlation coefficients that pass the critical values were used to assess associations between microbiome and weight loss. Corrected P_{adj} values < 0.05 using Monte Carlo simulations were considered statistically significant.

5.3 Results and Discussion

5.3.1 RYGB is More Effective than LAGB

Fecal samples were collected from 63 subjects: 24 post-RYGB, 14 post- LAGB, 10 NW, and 15 PreB-Ob subjects. Table 5.1 shows subject characteristics in each group. The median age among the groups was not statistically significant.

Table 5.1. Subject characteristics of each experimental group: NW, RYGB, LAGB and PreB-Ob. For age and time after surgery categories, the numbers represent median values with standard deviation.

	NW	RYGB	LAGB	PreB-Ob
Age	41±15	50±9	47±11	54±9
Gender (F/M)	7/3	16/8	14/2	8/7
Time after surgery (months)	NA	34.5 ± 12.8	36.0 ± 14.0	NA

Figure 5.1A shows the calculated body mass index (BMI) of all subjects. NW and RYGB subjects had lower BMI than the LAGB and PreB-Ob subjects. The median BMI (kg/m^2) of the populations were: NW: 22.32, RYGB: 30.8, LAGB: 36.6, and PreB-Ob: 43.48. Despite substantial weight loss, the majority of the subjects from RYGB and LAGB groups remained in the overweight or obese categories based upon BMIs being > 25 kg/m^2. The RYGB group was composed of mainly over-weight and obese subjects, whereas the LAGB group was mainly composed of obese and severely obese subjects (BMI > 30). The median pre- or post-surgical BMIs of the groups did not differ significantly (P>0.05).

In order to assess surgical success, we calculated percent excess weight loss (% EWL), a commonly used clinical outcome that defines the weight loss normalized to the excess-weight loss needed to reach an ideal body weight defined by a BMI of 25 (Boza *et al.*, 2010). Figure 5.1B shows that %EWL was significantly higher for the RYGB group than the LAGB group ($P<0.05$), an observation consistent with previous long-term (Boza *et al.*, 2010) and short-term findings (Tice, Karliner, Walsh, Petersen, & Feldman, 2008). The higher success rate in the RYGB group compared to the LAGB group was not related to a difference in the time of data collection with respect to how long ago was the surgery: The median times of data collection after the surgery were 35 and 34 months for RYGB and LAGB, respectively. Additionally, Figure 5.1C shows that %EWL was independent of the average calories consumed based on 4-day food diaries, as LAGB and

RYGB groups consumed similar amounts of calories that were less than the NW and PreB-Ob. Importantly, even though LAGB and RYGB subjects consumed similar amounts of calories per day, %EWL was significantly lower for LAGB group than RYGB group. Based on food frequency questionnaires as shown in Table A.1, the RYGB group consumed fewer calories than the other groups, and PreB-Ob consumed more than the others, although this finding was not statistically significant.

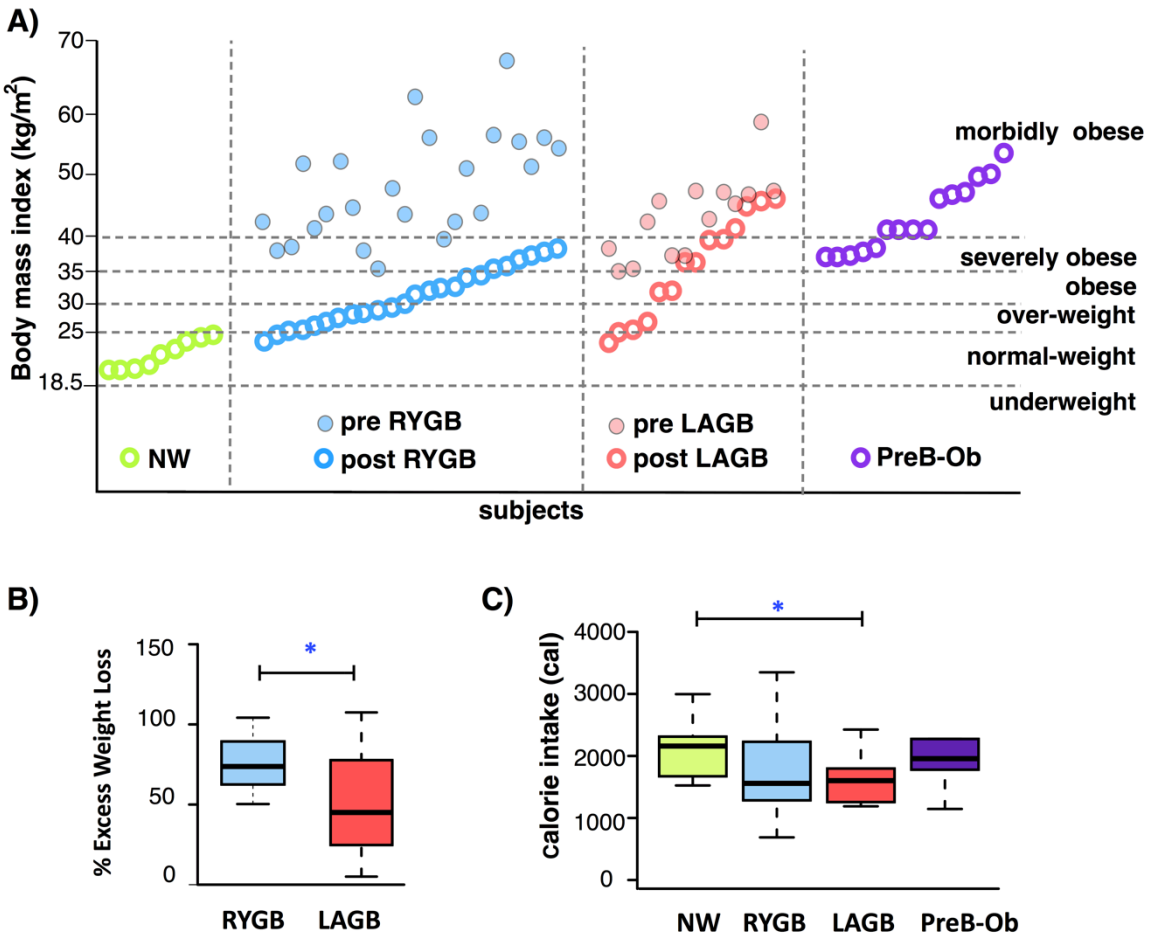

Figure 5.1. Subject's body mass index (BMI), % excess weight loss (%EWL), and average daily calorie intake. (A) BMI distribution of the subjects that participated in this study. Empty circles represent BMI at the time of sampling and full circles represent recorded BMI before surgeries retrospectively. The horizontal dashed lines indicate the BMI-based weight categories according to National Institutes of Health guidelines (nhlbi.nih.gov). (B) Percent excess weight loss (%EWL) for the RYGB and LAGB groups. The RYGB group had significantly higher % EWL than the LAGB subjects. (C) Median calorie intake per experimental group. * Denotes Mann-Whitney U test $P < 0.05$.

5.3.2 Post-RYGB and PreB-Ob subjects had distinctive fecal microbiomes and metabolomes

We evaluated community-level differences in the fecal microbiota of NW, RYGB, LAGB, and PreB-Ob populations using unweighted and weighted Unifrac metrics (Lozupone *et al.*, 2006). Weighted Unifrac analysis, which takes into account abundances of operational taxonomic units (OTU) and reflects differences in the most dominant OTUs, did not show any specific clustering pattern (Figure A.1A), although NW group was significantly distant from the other groups on PC1 (Figure A.1B). Due to the dominance of *Firmicutes* and *Bacteriodetes* phylotypes, as shown in Table A.2, the impact of the surgeries on microbiome was not obvious. Unweighted Unifrac analysis accounts for the presence or absence of OTUs; hence, it is a metric that reflects changes in less dominant phyla. Figure 5.2A depicts the unweighted Unifrac distances on principal coordinates (PCoA). In PC1 the microbiome of RYGB and PreB-Ob subjects formed clusters and there were some degree of separation from the other groups. The microbiomes of NW and LAGB groups did not show any clustering pattern. As shown in Figure A.2, microbiomes did not cluster based on other patients data such as: BMI, diet composition, gender, or age. Therefore, RYGB surgery was the principal factor responsible for the alteration of gut microbial community structure.

As seen in Figure 5.2B, the unweighted Unifrac distance between the RYGB and PreB-Ob groups was greater than the distance between the RYGB and NW groups, evidence that RYGB community profiles were more similar to NW than PreB-Ob profiles. This trend was also valid for LAGB subjects; the distance between LAGB and

Pre-Ob was greater than the distance between LAGB and NW. Overall, both surgeries change the microbiomes more towards that observed in NW subjects.

Since we observed the greatest difference between RYGB and PreB-Ob groups on PCoA-based unweighted Unifrac distances, we visualized these groups with the class-level phylotypes that promoted their separation. Figure 5.2C illustrates that the presences of unique phylotypes from *Bacilli*, *Gammaproteobacteria*, *Fusobacteriia*, and *Mollicutes* caused the microbiome of RYGB subjects to separate from the microbiome of PreB-Ob subjects. These phylotypes were the most abundant in the RYGB group ($P<0.05$), as shown in Figure 5.2D. They also were in greater abundance in the LAGB group compared to non-surgical groups, although not statistically different ($P > 0.05$). Based on linear discriminant analysis (LDA) effect-size algorithm (Segata *et al.*, 2011), as shown in Figure A.3**,** *Bacilli*, *Gammaproteobacteria*, *Fusobacteriia,* and *Synergistales* were the microbial signatures discriminating RYGB microbiota from nonsurgical microbiome (NW and PreB-Ob). *Fusobacteriia* and *Bacilli* also discriminated RYGB from the LAGB group. Only two genus-level phylotypes, *Prevotella* and an unidentified *Parabacteroides,* discriminated LAGB from the nonsurgical subjects.

Our findings reinforce previous studies showing that *Gammaproteobacteria* and *Fusobacteriia* increased in relative abundance after RYGB (Furet *et al.*, 2010; Graessler *et al.*, 2013; Zhang *et al.*, 2009). High levels of *Gammaproteobacteria* phylotypes have been associated with weight loss in post RYGB subjects (Furet *et al.*, 2010), but *Fusobacteriia* have not been evaluated in the context of weight loss or adiposity. In addition to *Gammaproteobacteria* and *Fusobacteriia*, *Bacilli* and *Mollicutes* were enriched after RYGB surgery, a trend not previously reported. Interestingly, *Bacilli*

includes many species that are known to protect against diet-induced obesity, such as *Enterococcus sp.* (Nadal *et al.*, 2009).

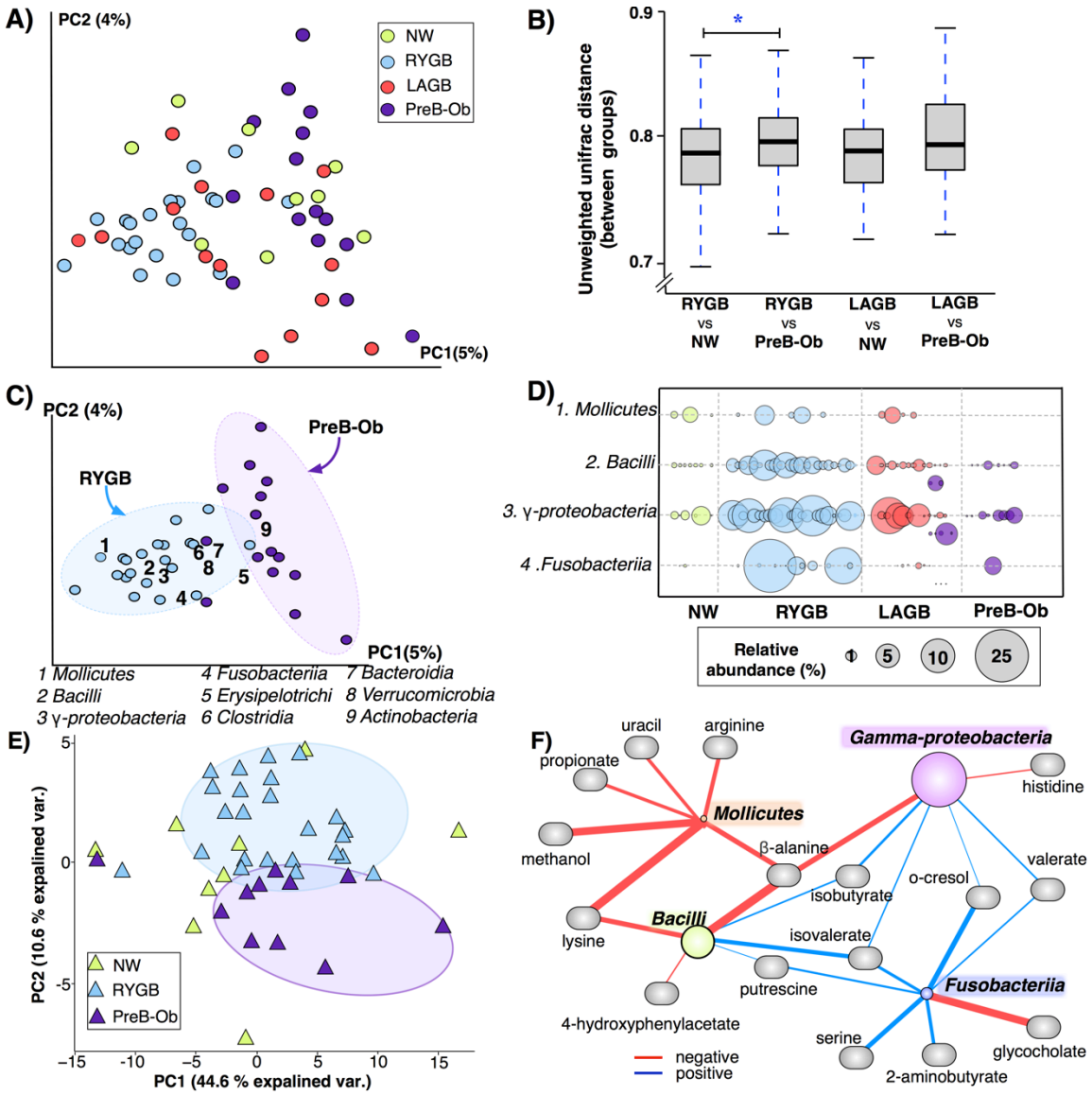

Figure 5.2. Microbial community structure after RYGB and LAGB surgeries compared to NW and PreB-Ob controls. A) Unweighted Unifrac distances between the samples visualized using principal coordinate analysis showed two distinctive clusters: RYGB and PreB-Ob. B) Pairwise comparison of median unweighted Unifrac distances between the groups suggested that both surgeries result in microbial profiles that resemble normal more than obese microbiota. C) Class-level phylotypes on principal coordinates show the taxa that are responsible for clustering of RYGB subjects.

D) Relative abundance of the four main class level phylotypes that separated the RYGB group from normal, LAGB, and PreB-Ob groups. E) Principal component analysis (PCA) of fecal metabolites from NW, RYGB, and PreB-Ob subjects show that RYGB and PreB-Ob had different metabolomes. F) Spearman's rho correlations between *Gammaproteobacteria*, *Fusobacteriia*, *Mollicutes*, and *Bacilli* and fecal metabolites. The line thickness indicates the strength of the correlation.

We evaluated whether distinctive microbiomes of RYGB and PreB-Ob groups also resulted in distinctive fecal metabolomes by using principal component analysis (PCA). Figure 5.2E shows the distribution of fecal metabolomes of NW, RYGB and PreB-Ob groups based on 64 metabolites that were detected with NMR. Even though subjects didn't separate on PCA based on their metabolomes, similar to the observations that are derived from 16S rRNA gene, RYGB and PreB-Ob groups showed the greatest separation. NW metabolomes did not separate from the other groups, although they showed narrow distribution. These findings suggest that obesity and RYGB surgery not only change the microbial community structure (microbiome) but also change the microbial community functions, reflected by the metabolome.

To probe the associations between fecal microbiome and metabolome, we calculated Spearman's rho coefficients between the four phylotypes (*Gammaproteobacteria*, *Mollicutes*, *Bacilli*, and *Fusobacteriia*) that separated RYGB from PreB-Ob and fecal metabolites. *Gammaproteobacteria*, *Fusobacteriia*, and *Bacilli* positively correlated with amino acid degradation products: isobutyrate, isovalerate, and o-cresol, as shown in Figure 5.2F. Putresine concentration positively correlated with the relative abundances of *Bacilli* and *Fusobacteriia.* *Fusobacteriia* also positively correlated with 2-aminobutyrate, a catabolic product of branched-chain-amino-acids. On the other hand, *Mollicutes* relative abundance negatively correlated with many

metabolites including lysine, methanol, propionate, uracil and arginine. β-alanine, an amino-acid derivative, negatively correlated with the following order level phylotypes: *Mollicutes*, *Bacilli*, and *Gammaproteobacteria*. Metabolites that positively correlated with *Gammaproteobacteria*, *Fusobacteriia,* and *Bacilli* were at greatest abundance in the RYGB group compared to other groups. We show the differential connection between fecal microbiome and metabolome after two bariatric surgeries. Our findings indicated that the impact of bariatric surgery on the microbiome is reflected at the functional level by gut metabolism; hence those differences can contribute to the weight loss.

5.3.3 Bariatric surgery reduces microbial diversity in the gut

We also evaluated the variation within the microbial communities. As seen in Figure 5.3A, within-community unweighted Unifrac distance was the smallest in the RYGB group compared to the other groups ($P < 0.05$). Thus, we postulated that RYGB induced a more uniform microbiome in subjects and that this might be related to lower community diversity. According to the alpha diversity indexes for the communities, RYGB and LAGB groups had lower diversity than the controls, as seen in Figure 5.3B.

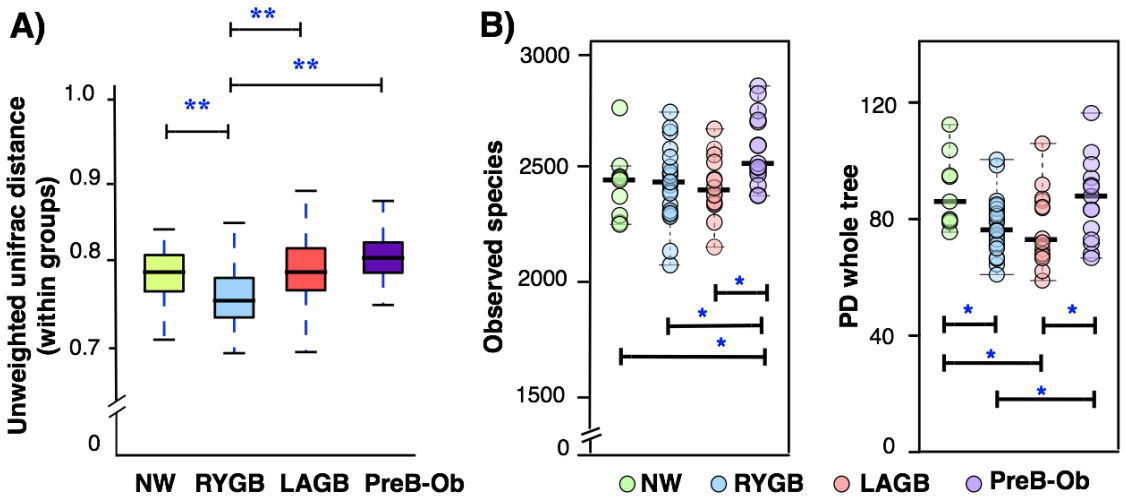

Figure 5.3. Within-community variation and alpha diversity indexes reveal differences in the community structure. A) Within-group Unweighted Unifrac distance was the smallest for the RYGB group. B) Alpha diversity parameters: observed species and PD whole tree showed greater diversity in nonsurgical groups compared to RYGB and LAGB.

The PreB-Ob group had the highest diversity and, ranked highest in terms of within-community Unifrac distances. This result contrasts with previous studies that have associated obesity with low alpha diversity (Qin *et al.*, 2010; Turnbaugh *et al.*, 2006), possibly because our PreB-Ob group followed a pre-bariatric surgery weight loss diet. We can speculate that this diet, as seen in Figure A.4, could have increased microbial diversity and established a healthier microbiota (David *et al.*, 2014). The fiber intake trends, as seen in Figure A.4, align with the alpha-diversity trends, supporting previous observations that fiber intake is important for microbial diversity (De Filippo *et al.*, 2010). Similarly, the lower diversity in the RYGB and LAGB groups may be explained by the restrictive diets imposed after surgery. Patients often develop food intolerances after bariatric surgery, further limiting their dietary choices (Schweiger, Weiss, & Keidar, 2010). Moreover, surgery-induced alterations in environmental

conditions, such as higher pH imposed on gut microbiota after RYGB surgery, might further contribute to a reduction in gut microbial diversity, thereby reducing the variation within the RYGB group.

Overall, RYGB and LAGB had different long-term effects on gut microbiomes. RYGB had a stringent clustering effect on microbial phylotypes, whereas LAGB did not impose any condition that distinguished its microbiome from non-surgical groups. Community-level differences between the RYGB and other groups may be explained by changes to the gastrointestinal environmental created by the surgery. The rearrangement of the gastrointestinal tract after RYGB alters gut pH, oxygen content, and bile-acid concentrations, and nutrient exposure that are delivered to the colon (Li *et al.*, 2011). Similar changes would not be anticipated after LAGB surgery, as anatomical rearrangement is not performed. Therefore, RYGB may create environmental changes in the gut critical for new colonization and succession of microbial species in the colon.

5.3.4 Weight-loss associated oral microorganisms were enriched in the fecal microbiomes after RYGB

Table A.4 documents that the RYGB group had more facultative anaerobes and fewer anaerobes than the other three groups, which supports a previous hypothesis that RYGB surgery increases the oxygen content of the gut (Zhang *et al.*, 2009). Figure 5.4 shows phenotypes that were most prevalent in the RYGB group similar to anaerobic and facultative anaerobic genera. In addition to previously reported *Escherichia* (Liou *et al.*, 2013), *Klebsiella* (Graessler *et al.*, 2013), and *Fusobacterium* (Furet *et al.*, 2010), we observed a higher abundance of phylotypes from *Firmicutes* such as *Streptococcus* and *Veillonella.* Some of these phylotypes are from the most abundant phylotypes of the

gastric mucosa, such as *Veillonella*, *Enterococcus*, *Haemophilus*, *Fusobacterium*, *Prevotella,* and *Streptococcus*, and are commonly found together in individuals with dental cavities or periodontitis (Liljemark & Bloomquist, 1996). Proton pump inhibitors, which reduce gastric acid secretions, increased the abundance of oral cavity associated microorganisms including *Enterococcus*, *Streptococcus*, and *Veillonella* (Imhann *et al.*, 2016). The great abundance of these phylotypes after RYGB suggests that the conditions in the gut after this surgery allowed higher transport and survival of oral microorganisms and promoted growth of the upper-gastrointestinal tract species in the more distal bowel by a similar aggregation mechanism as oral biofilm formation.

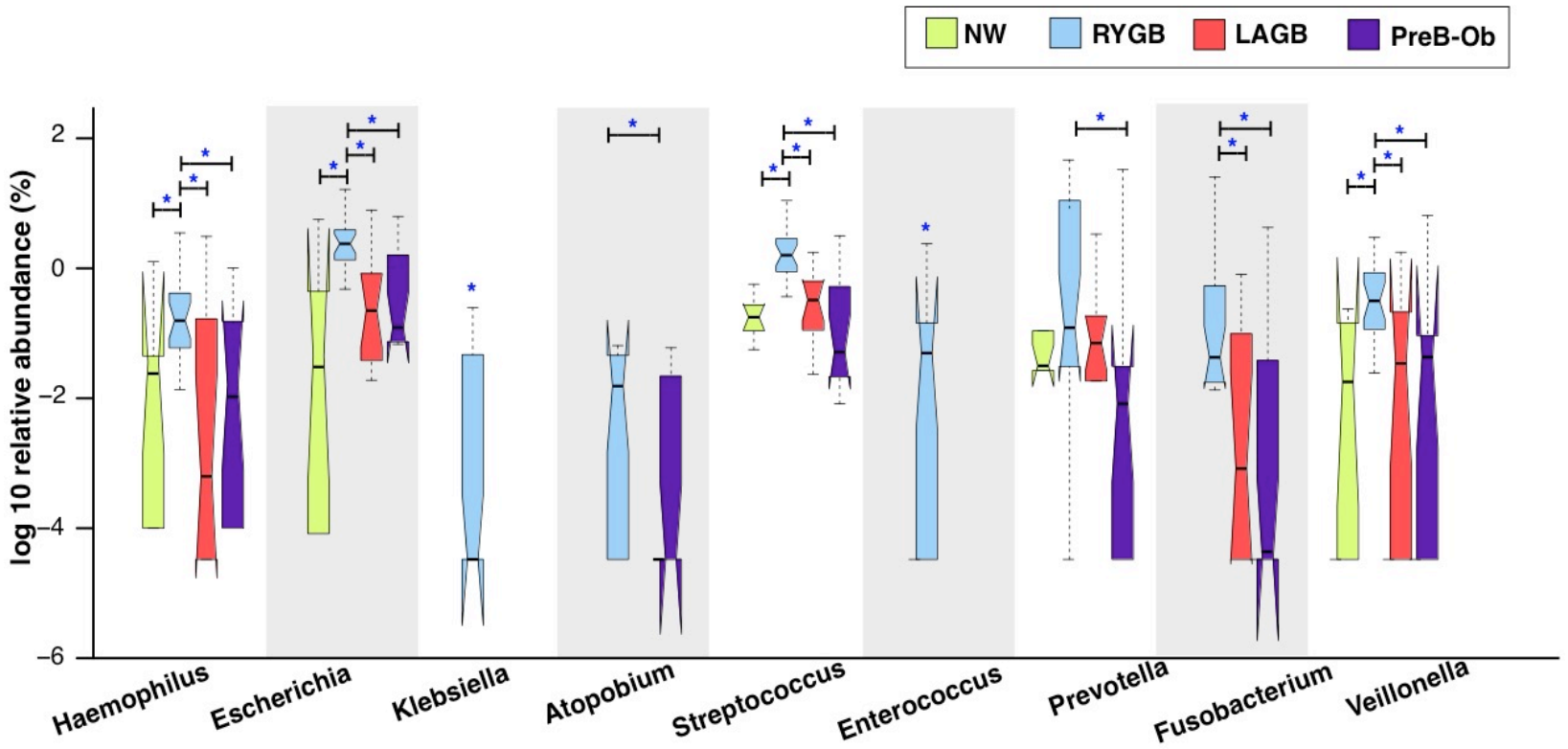

Figure 5.4. Significantly abundant genera in the RYGB subjects compared to the other groups. * Denotes $P < 0.05$ of Mann Whitney U-test adjusted results. The box-plots were not presented if the phylotypes were under detection limit for certain groups.

Gut pH could have been another important confounding factor for enrichment of these phylotypes. *Fusobacteria*, *Prevotella*, *Atopobium*, and *Escherichia* (Ling *et al.*, 2010) are typically found at high relative abundance in patients with bacterial vaginosis, a condition that results in an increased vaginal pH (from 4 to 6) (Ling *et al.*, 2010), and they also proliferated in the large bowel of mice when gastric acid secretion was inhibited (Kanno *et al.*, 2009). Since RYGB reduces gastric acid contact with the nutrient stream (Rubin, Nguyen, & Schwentker, 2004) and increases the distal excluded stomach pH to ~ 4 (Liou *et al.*, 2013), these facultative anaerobes from the oral and gastric mucosa, which thrive at more neutral pH levels, can bypass harsh stomach acid in transit to the colon. In contrast, the decreases seen in these species after LAGB, where normal digestive physiology is retained, further supports that the observed microbiome changes were due to rearrangement of the gastrointestinal tract after RYGB surgery. We did not see a significant difference in fecal pH (NW = 7.4, RYGB = 7.2, LAGB= 7.8, and PreB-Ob = 7.45), probably because biochemical selection of species happens within the stomach and small intestine, before microorganisms reach the colon, where production of fatty acids through fermentation results in a decline in the pH.

A majority of the genus-level phylotypes that were enriched in the RYGB group positively correlated with %EWL, since the RYGB group had greater excess weight loss than the LAGB. Table 5.2 shows Spearman correlation coefficients of the relationships between genera and EWL% and BMI. When we performed Spearman's correlation on the combined data set (n= 63), we observed that *Prevotella*, *Escherichia*, *Veillonella*, *Enterococcus*, and *Carnobacterium* with unidentified phylotypes from *Gemellaceae* and *Enterococcaceae,* positively correlated with the %EWL. Nadal *et al.*, reported an

increase in *Prevotella* and a decrease in *Enterococcus* associated with weight loss in adolescents (Nadal *et al.*, 2009). In humans and animals, an increase in the relative abundance of *Escherichia* after RYGB surgery has been associated with weight reduction and adiposity independently from diet (Furet *et al.*, 2010; Liou *et al.*, 2013). *Bacteroides*, *Oscillospira, Sutterella*, and an unknown phylotype from *Ruminococcaceae* negatively correlated with %EWL.

As seen in Table 5.2, *Methanobrevibacter*, *Ruminococcus*, and *Coprococcus* moderately and negatively correlated with BMI, whereas *Sutterella*, *Bacteroides, Oscillospira,* and *Holdemania* positively correlated with the BMI. *Methanobrevibacter* (Zhang *et al.*, 2009), *Ruminococcus,* and *Coprococcus* (Kasai *et al.*, 2015) have been associated with an obese metabolic type, but we speculate that these changes may reflect diverse diets and host physiology of the subjects studied and, therefore, the relationship is ambiguous.

Table 5.2. Spearman correlation coefficients between genus-level phylotypes in fecal samples and excess weight loss and body mass index (BMI) distributions of subjects.

	EWL %			BMI	
	Spearman's rho	P value		Spearman's rho	P value
Unidentified *Carnobacteriaceae*	**0.531**	0	*Methanobrevibacter*	**-0.305**	0.018
Unidentified *Enterococcaceae*	**0.467**	0	*Ruminococcus*	**-0.322**	0.012
Unidentified *Gemellaceae*	**0.464**	0	Unidentified *RF39*	**-0.328**	0.01
Enterococcus	**0.454**	0	Unidentified *SHA*	**-0.331**	0.01
Escherichia	**0.448**	0	*Coprococcus*	**-0.373**	0
Veillonella	**0.424**	0.001	Unidentified *Coriobacteriaceae*	**-0.374**	0.003
Prevotella	**0.388**	0.002	*Oscillospira*	**0.209**	0.109
Klebsiella	**0.312**	0.013	*Bacteroides*	**0.307**	0.017
Bacteroides	**-0.314**	0.014	*Sutterella*	**0.308**	0
Oscillospira	**-0.348**	0.006			
Sutterella	**-0.358**	0			
Unidentified *Ruminococcaceae*	**-0.437**	0			

5.3.5 Butyrate, Propionate, and Branched-Chain-Fatty-Acids were at Great Concentrations only in Post-RYGB Group

As seen in Figure 5.5A, the concentration of acetate, a major carbohydrate-fermentation product, was higher in the PreB-Ob group, followed by the RYGB and LAGB groups. NW group had the lowest acetate concentration. The RYGB group had the highest concentrations of butyrate and propionate. LAGB and NW groups had comparable levels of butyrate and propionate, and they were lower than the RYGB and PreB-Ob groups. Figure 5.5B shows the fecal butyrate-to-acetate and propionate-to-acetate ratios in the groups, which indicates preferred fermentative

pathways by gut microbes. Butyrate-to-acetate ratio was significantly highest in the RYGB group, and propionate-to-acetate ratio was significantly higher in RYGB compared to LAGB and PreB-Ob groups. Figure 5.5C shows that the RYGB samples had the highest concentrations of branched-chain-fatty-acids (BCFA) isobutyrate and isovalerate.

In addition to carbohydrate-fermentation products, we measured branched-chain-amino-acids (BCAA) fermentation products: branched-chain-fatty-acids (BCFAs). When we looked at the abundance of leucine, isoleucine, and valine in post-RYGB in comparison to NW and PreB-Ob groups, we observed that the concentrations were not statistically different among the groups, although NW group had slightly lower concentrations of these BCAAs (Figure 5.5D).

Using PICRUSt to analyze predicted genes involved in the BCFA production and consumption pathways, we found that BCFA production associated genes such as leucine 2,3-aminomutase and butyryl-CoA dehydrogenase, were significantly more abundant in the NW group compared to the other groups, as shown in Figure 5.5E. The predicted genes involved in BCFA production and consumption pathways were in greater and lower abundances in the RYGB group, respectively compared to the other groups.

High propionate-to-acetate and butyrate-to-acetate ratios in RYGB subjects can be explained by the higher relative abundance of propionate- or butyrate-producing species such as *Escherichia*, *Veillonella*, *Klebsiella, Fusobacterium,* and *Prevotella* (Strobel, 1992). These phylotype**s** form a metabolic network that involves production of vitamins and fermentation of carbohydrates and proteins.

For example, *Streptococcus, Escherichia, Klebsiella, Prevotella,* and *Enterococcus* ferment sugars and produce lactate and pyruvate for microorganisms that cannot utilize sugars, such as *Veillonella* (Hojo, Nagaoka, Ohshima, & Maeda, 2009). *Veillonella* produces vitamin K, which stimulates the growth of *Prevotella* (Hojo *et al.*, 2009). As illustrated in Figure 5.5F, *Fusobacterium* degrade proteins and peptides into amino acids (Bachrach, Rosen, Bellalou, Naor, & Sela, 2004). *Prevotella* and *Enterococcus* ferment BCAAs (valine and leucine) into BCFAs (isovalerate and isobutyrate) (Takahashi, Saito, Schachtele, & Yamada, 1997). Finally, *Prevotella* and *Fusobacterium* produce alkali to neutralize the pH due to acid production during amino acid fermentation to BCFA (Takahashi *et al.*, 1997).

SCFAs and BCFAs can have many effects on host energy metabolism. Acetate has been associated with fat depositions in the liver (Siddiqui *et al.*, 2015), whereas butyrate and propionate have been associated with weight loss (Lin *et al.*, 2012). Higher fecal propionate concentrations also were observed in mice that received fecal transplants from lean or post-RYGB mice, and they correlated with diet-independent weight loss (Liou *et al.*, 2013; Ridaura *et al.*, 2013). Propionate and, to a lesser extent, butyrate can regulate weight loss hormones, such as glucagon-like peptide-1 (GLP-1), and both were previously shown to protect against obesity (Lin *et al.*, 2012). Besides GLP-1, propionate can induce secretion of the satiety-inducing, appetite-reducing hormone peptide YY (PYY) (Chambers *et al.*, 2015). Therefore, propionate-to-acetate ratio is an important factor for the evaluation of microbiota's contribution to host energy balance.

Conversion of BCAA to BCFA is optimal at pHs above neutral, such as pH 8 (Thierry, Maillard, & Yvon, 2002). The reduction in gastric acid secretion after RYGB

(Rubin *et al.*, 2004) may stimulate the production of BCFA by increasing the BCFA-producing bacteria or favor thermodynamics of these reactions. A previous study showed that leptin-deficient obese mice had lower isobutyrate levels than controls, indicating that the obese mice had lower leucine and valine fermentation (Won *et al.*, 2013). Moreover, increased isobutyrate, isovalerate, and other SCFA have been associated with increased satiety via increases in PYY (Haenen *et al.*, 2013). An increase in satiety hormones, including PYY, was previously observed in individuals who have had RYGB (Karra & Batterham, 2010). Our results of higher concentrations of BCFAs in the RYGB group, independent from diet, suggest that RYGB surgery enhanced the abundance of bacteria and pathways producing BCFAs. In summary, our analysis showed that RYGB surgery increased fecal butyrate, propionate, isovalerate, and isobutyrate. RYGB had higher concentration of SCFAs and BCFAs that can interact with free fatty acid receptors and regulate the hunger/satiety response, suggesting a microbiota-associated role in sustained weight loss. These important changes were not observed after LAGB.

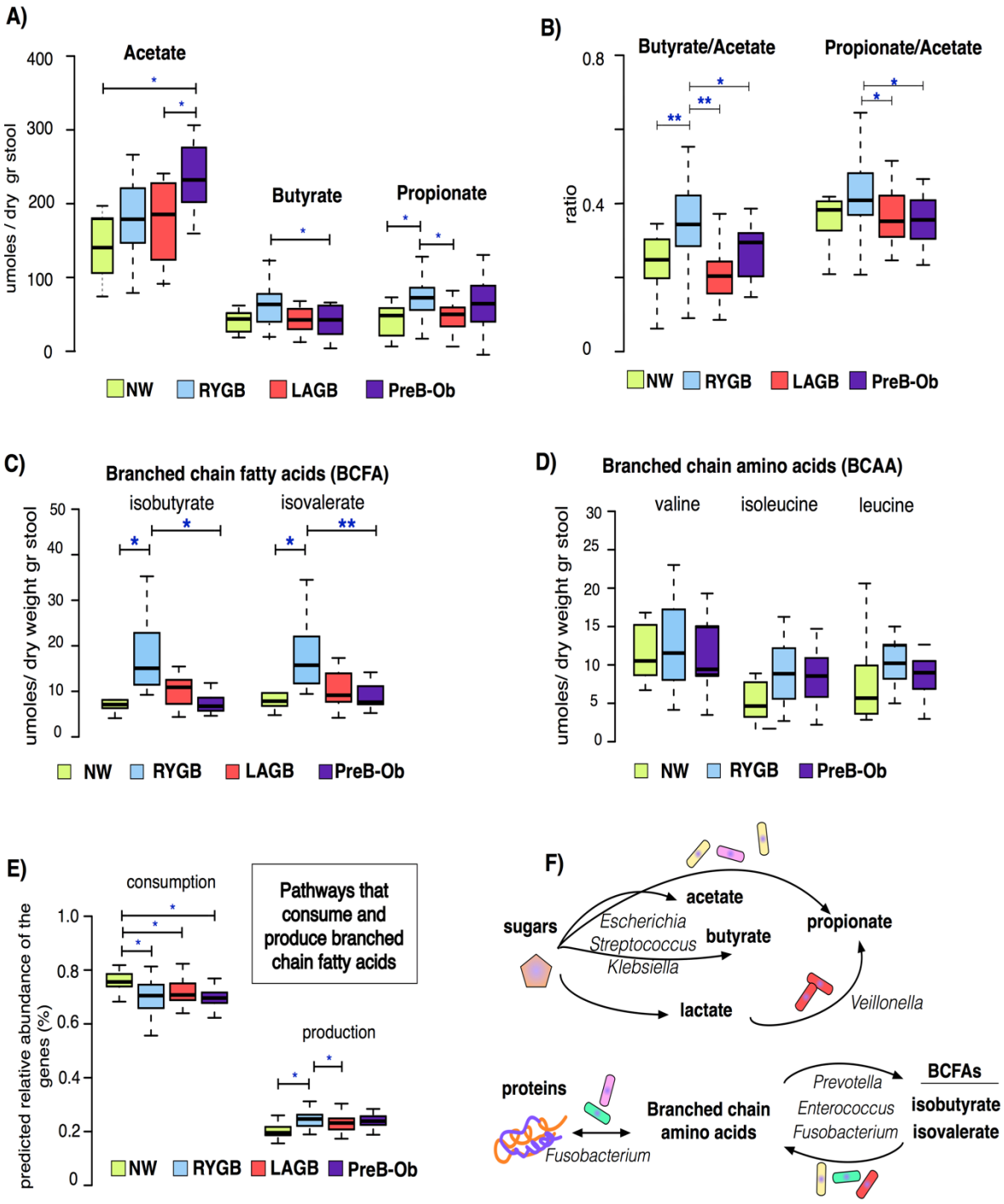

Figure 5.5. Fecal fermentation products and substrates. A) The most abundant fermentation products: acetate, propionate, and butyrate showed different distribution among the groups. B) Ratios of butyrate to acetate and propionate to acetate were greatest in the RYGB group compared to NW, LAGB, and PreB-Ob groups.

C) Isobutyrate and isovalerate, which are fermentation products of branched chain amino acids (isoleucine and valine) were at greater abundance in the RYGB group compared to the other groups. D) Branched-chain-amino-acids: valine, leucine, and isoleucine concentrations did not significantly vary among the groups. E) PICRUSt predicted relative abundance of the genes involved in the consumption and production of BCFAs. F) Illustration of conversion of carbohydrates and proteins into short chain and branched chain fatty acids by significantly abundant taxa in RYGB subjects. *denotes Mann-Whitney U-test Padj value < 0.05 and **denotes Padj<0.01

We observed major differences in the structure of the fecal microbiome and its metabolites in post-RYGB and post-LAGB subjects, compared to PreB-Ob and NW subjects. The changes were more substantial after RYGB than after LAGB, and can be related to the very different environmental conditions caused by the radically altered gastrointestinal anatomy following RYGB. Greater abundances of metabolites associated with weight loss, such as short chain fatty acids and branched chain fatty acids, were observed in post-RYGB subjects compared to post-LAGB subjects. These new data on microbial community structure and function significantly expands knowledge on how the microbiome is associated with weight loss following bariatric surgery.

CHAPTER 6

MICROBIAL SIGNATURES OF SUCCESSFUL AND UNSUCCESSFUL ROUX-EN-Y GASTRIC BYPASS SURGERY

6.1 Introduction

In Chapter 5, I summarized the major differences in the gut microbiome of post-RYGB individuals. I detected higher abundances of *Streptococcus*, *Veillonella*, *Enterococcus*, and many phylotypes from *Gammaproteobacteria* such as *Escherichia* and *Klebsiella* compared to nonsurgical controls. Higher abundance of phylotypes from *Gammaproteobacteria*, especially *Escherichia,* compared to individuals without RYGB surgery had been reported in many other studies (Zhang*, et al.*, 2009, Furet*, et al.*, 2010, Tremaroli*, et al.*, 2015). Besides *Gammaproteobacteria*, an increase in the relative abundance of *Verrucomicrobia* such as *Akkermansia* (Zhang *et al.*, 2009) and a decrease in the relative abundances of *Lactobacillus* and *Bifidobacterium* (Furet *et al.*, 2010) were also previously reported. Similar changes in the gut microbiota have been observed in rats (Li *et al.*, 2011) and mice (Liou *et al.*, 2013) post-RYGB surgery. To date, these observations are only valid for post-RYGB subjects or animals that successfully lost weight, but not for the unsuccessful ones that regain some of the lost excess weight in the long-term. Even though RYGB is a relatively successful surgery with high percent excess weight loss (%EWL), up to 40% of the patients fail to lose weight or regain some of the lost excess weight (Prachand *et al.*, 2006). In cases of unsuccessful outcomes, revisional surgeries are performed to enhance weight loss after primary surgeries, which might increase post-surgery complications (Shimizu*, et al.*, 2013).

The success of RYGB relies on many factors; changes in dietary habits, the psychology, neurology, and hormonal response of the patients (Sarwer*, et al.*, 2011). Changes in hormonal response after RYGB, for example an increase in Glucagon like peptide (GLP1) and peptide tyrosine tyrosine (PYY) after RYGB (Berthoud*, et al.*, 2011) was associated with reduction in appetite, and weight loss (le Roux*, et al.*, 2007). Since the gut microbiome can regulate the production of neurotransmitters or hormones such as PYY (Cani & Delzenne, 2009), the microbiome might contribute to weight loss via interfering with host satiety signaling pathways.

A mechanistic link to microbiota associated weight loss after RYGB was shown in mice (Liou *et al.*, 2013). Fecal transplantation from sham-operated, sham-operated weight-management, and RYGB-operated mice to gnotobiotic mice resulted in reduced body weight and adiposity independently from the diet only in the RYGB-microbiota recipient mice (Liou *et al.*, 2013). Even though, the microbiota's role in post-RYGB weight loss is unknown, some hypothesized mechanisms include increased abundance of microbial products such as short chain fatty acids (SCFAs) (Lin *et al.*, 2012) that mediate cross talk between the host and the gut microbiota. SCFAs can reduce food intake by altering gut hormonal response towards satiety (Fruebis *et al.*, 2001, Lin *et al.*, 2012). On the other hand, microbially produced protein degradation products such as putrescine and cadaverine can act as leptin (hunger hormone) signaling molecules due to their toxicities (Hyman *et al.*, 2007). Microbiome composition and metabolism in unsuccessful RYGB patients has not been reported, and differentiation of microbiome and metabolites in successful and unsuccessful patients can enhance the understanding of microbial contribution to successful and sustainable weight loss.

Among different microbiota associated weight loss mechanisms post-RYGB, we focus on microbial products that are involved in the stimulation of weight-loss and weight-gain associated hormones or neurotransmitters. The objectives of this research were: (i) to define what conditions define a successful RYGB surgery, (ii) to characterize fecal microbiota structure at least 13 months after successful and unsuccessful RYGB surgery, and (iii) to identify microbial metabolites that might contribute to the success of RYGB surgery. In order to achieve these objectives, we employed 16S rRNA gene sequencing to analyze microbial structure and H-Nuclear Magnetic Resonance to analyze microbial metabolites/functions.

6.2 Materials and Methods

6.2.1 Subjects and Fecal Sampling

The Institutional Review Board of Mayo Clinic Scottsdale and Arizona State University approved our study (IRB # 10-008725). We recruited: 9 normal-weight (NW), 12 pre-operative morbidly obese individuals (Ob-), and 24 post-RYGB participants for this study.

The RYGB subjects that qualified for our study had their surgery at least 13 months (median = 35 months) before they donated their samples. Each subject donated a fecal specimen, and shipped it to Mayo Clinic in Scottsdale, Az. The samples were shipped to Arizona State University from Mayo Clinic on dry ice. Upon arrival I stored them at -80^{o}C until further analysis. 16S rRNA gene sequencing and metabolomics were performed on each sample. Participants filled out diet diaries, food frequency questionnaires, and symptom checklist 90.

6.2.2 DNA Extraction, Sequencing and 16S rDNA Data Analysis

DNA extraction, sequencing and 16S rDNA data analysis were described in detail in Chapter 4's materials and method section. Briefly, I extracted DNA using the QIAamp Mini Stool kit with enhanced lysis for Gr (+) bacteria. The sequencing was performed with an Illumina Miseq Instrument using V4-V6 primers at University of Minnesota, IL Sequencing facility. We paired the forward and reverse reads using PANDAseq (Masella *et al.*, 2012) and analyzed data using QIIME 1.8 suite (Caporaso *et al.*, 2010).

6.2.3 Metabolite Extraction and H-NMR Analysis of Metabolites

For each fecal specimen, approximately one gram of wet weight (precise weight was recorded and used for calculations) was diluted with 20 mL of milliQ water (18 ohms). This step was performed in triplicates for process control. The homogenate was vortexed at the highest speed for three minutes to achieve complete mixing. Then the homogenates were centrifuged at 13,000 rpm for 15 minutes and the supernatants were filtered through 0.2-um PVDF membranes (PALL Coorparation). 30 uL of reference solution was added to 270 uL of the filtrate. The reference solution was composed of 100% D_2O, 5 mM DSS and 0.1% sodium azide. The resulting mixture was loaded into 3mm H-NMR tubes (Bruker Inc) (duplicates for analytical controls) and shipped to Pacific Northwest National Laboratories (PNNL) on ice blocks for NMR analysis. There, the metabolite analysis was performed using a Bruker 500 MhZ NMR as previously described (Kajimoto *et al.*, 2014). Chenomx suite 8.1 was used to collect NMR spectrums (chenomx.com). From the metabolome data, we calculated the mean of the six data points for each compound and normalized the concentrations to per gram dry weight of feces.

6.2.4 Statistical Analysis

We performed statistical analyses with Statistical Package for Social Sciences (SPSS v22) (Chicago, IL) and R using Bioconductor package (www.bioconductor.org). For the subject metadata, which includes, age, BMI, diet information, and symptom checklist 90, we used Mann-Whitney's U test since this data had non-normal distribution. We considered P values smaller than 0.05 as significant. We normalized the H-NMR and 16S rRNA gene data using the following methods: dividing the metabolite quantities for each subject by the respective dry weight of the stool sample normalized data. Any zero quantity for a metabolite was considered a missing value. For NMR data, we conducted one-way analysis of variance (ANOVA) with post-hoc tests of all pairwise comparisons using Tukey-Kramer p-value adjustments (Tukey, 1949) for multiple comparisons. We also conducted Mann-Whitney's U test and performed Bonferroni correction.

For 16S rRNA gene data, we used a similar approach to NMR normalization. After assuming the zeros were missing values, we normalized the data using the 75th quartile method. We conducted pairwise differential abundance tests using edgeR which assumes a negative binomial distribution for OTU count data and conducts a likelihood ratio test (McCarthy *et al.,* 2010). Then, we performed DeSeq2 test, which is more conservative compared to edgeR for significance to provide a secondary check.

6.2.5 Refining the Definition of Successful RYGB

We used three methods to sub-group RYGB subjects into successful and unsuccessful categories: For the first method, we classified the individual who lost less than 50% of the %EWL or regained ≥ 20% of the % nadir EWL as unsuccessful. All RYGB patients in our study lost more than 50% EWL; however, seven of these patients

regained at least 20% EWL and were classified as URYGB. The remaining 14 were classified as SRYGB. Throughout this chapter, I will refer to this classification as 20% regain method. This method has been commonly used in clinical studies to assess the success of bariatric surgery procedures (Dayyeh *et al.*, 2011). Since the definition of successful bariatric surgery is subjective and unclear (Puzziferri*, et al.*, 2008), we utilized machine-learning algorithms to understand which parameters are important in defining success.

For the second method, we used k-means clustering (Hartigan *et al.*, 1979) and picked 2 as seed size: one for successful and one for unsuccessful, I refer to this method as 2-means clustering. For the third method, we evaluated the distribution of %EWL, max % EWL, %EWL rate and changes in BMI, decided to use max 85%-maxEWL as a cut-off as it formed two clusters. In order to understand how these three methods define success, we calculated the following weight loss associated metrics: Delta BMI, weight loss percent, lowest weight loss percent, weight loss percent rate, excess weight loss percent at follow-up, nadir excess weight loss percent, and regained excess weight loss. The formulas used to calculate these metrics were summarized in Appendix 5. Then we evaluated how good the methods separate successful ones from the unsuccessful ones:

6.3 Results and Discussion

6.3.1 Subject Characteristics and Categorizing RYGB Subjects into Successful and Unsuccessful Groups

RYGB group contained 24 individuals with different degrees of %EWL; the median was 73.75%, the minimum was 51%, and the maximum was 104%. We used three different classification methods --- 20% regain, 2-means clustering, and 85%-

maxEWL --- to evaluate the success of surgery. As shown in Figure 5.1A, with 20% regain method we observed relatively good separation of successful and unsuccessful groups based on delta BMI and weight loss percent metrics but poor separation based on other metrics. 2-means clustering method had the best separation of successful and unsuccessful subjects based on majority of the metrics as seen in Figure 5.1B; those metrics were excess weight loss at follow-up, nadir excess weight loss percent, and weight loss percent rate. Figure 5.1C shows the metrics based on 85%-EWL; similar to 2-means clustering method, 85%-maxEWL method resulted in good separation based on excess weight loss at follow-up, nadir excess weight loss percent, and weight loss percent rate.

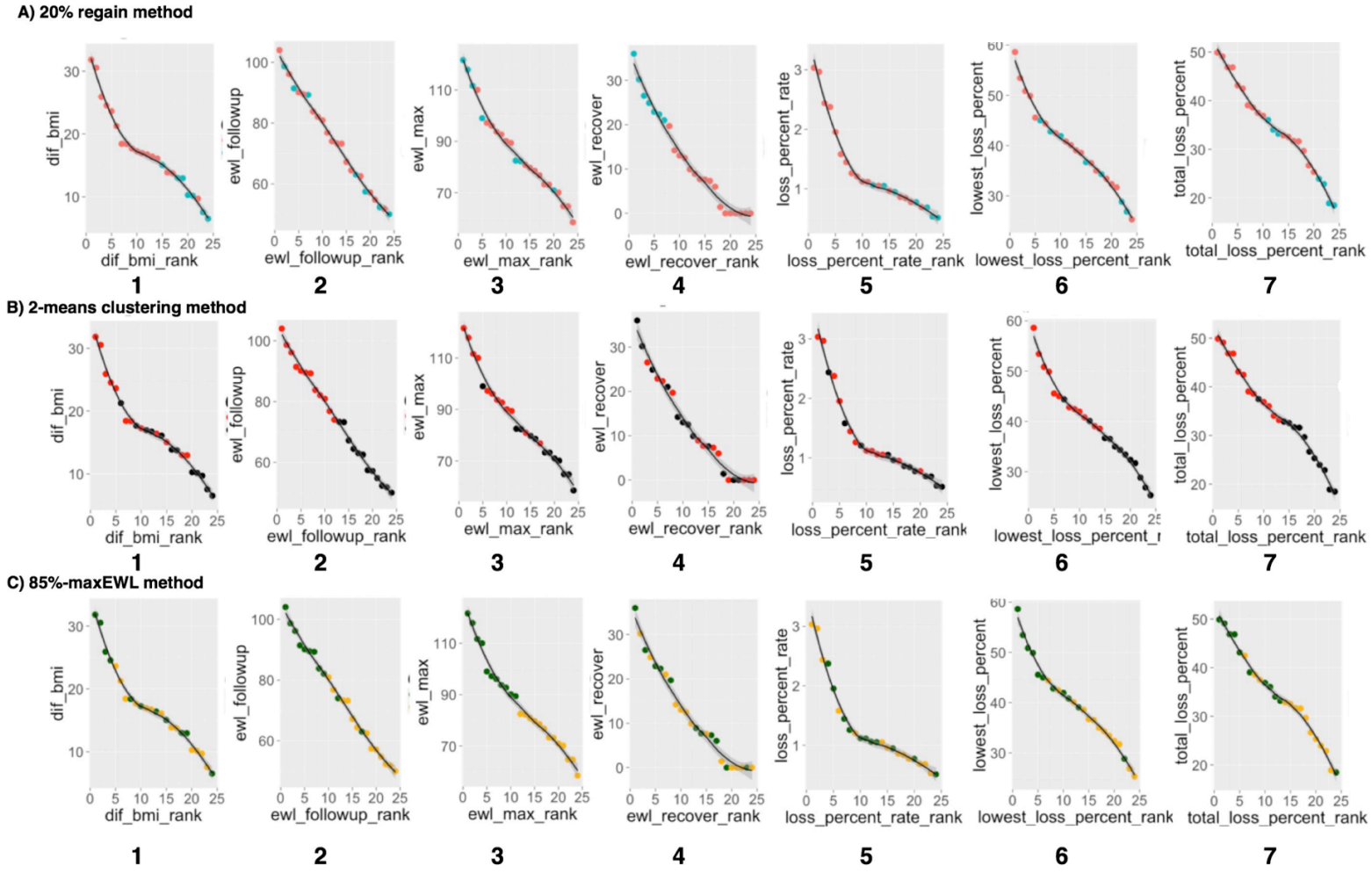

Figure 6.1 Weight loss metrics based on A) 20% regain, B) 2-means clustering, and C)85%-maxEWL classification methods. Numbers represent: 1-Delta BMI, 2-EWL percent, 3-Nadir EWL, 4-Regained EWL, 5-Weight loss percent rate, 6-Lowest weight loss percent, 7- Total weight loss

Figure 6.2 shows %EWL, % nadir EWL, and % regain of EWL of successful and unsuccessful RYGB groups based on three different types of classification: 20% regain, 2-means clustering, and 85%-maxEWL methods. As seen in Figure 6.2A, 20% regain method formed SRYGB and URYGB groups with significantly different %EWL and %regain, but not nadir %EWL. Grouping based on 2-means clustering and 85%-maxEWL yielded similar results for %EWL and % nadir EWL but opposite trends for %regain. With 2-means clustering method, %EWL and nadir %EWL were statistically different between SRYGB and URYGB groups, whereas %regain was not different. Based on 85%-maxEWL method, only nadir %EWL was statistically different between SRYGB and URYGB groups. Among the three classification methods we used, 20% regain was the only method that separated the subjects that regained weight. SRYGB and URYGB groups had significantly different %EWL with 20% regain and 2-means clustering methods. Neither of the classification methods we used in this chapter showed statistical difference on diet composition between successful and unsuccessful individuals.

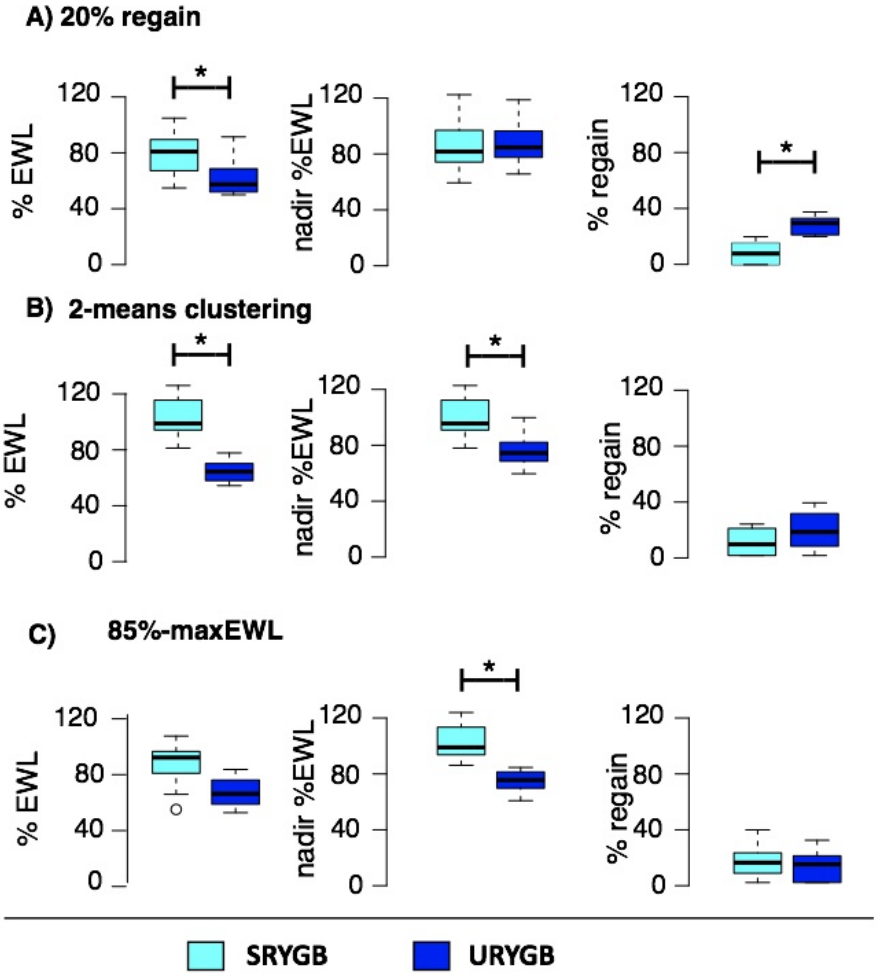

Figure 6.2 %EWL, nadir %EWL, and %regain of successful and unsuccessful RYGB groups classified by **A)** 20% regain method, **B)** 2-means clustering method, **C)** 85%-maxEWL method.

In order to better understand whether we can predict the success of RYGB using the datasets we collected (clinical metadata, diet, 16S rRNA gene data, and NMR metabolomics data), we used Naïve Bayes classifier (Russel and Norvig 2002) and evaluated the predictions based on the classification methods (20% regain, 2-means clustering, and 85% maxEWL) we used. Figure 6.3 shows the results of how selected

features from 16S rRNA gene and NMR dataset can accurately classify subjects into their true categories. As seen in Figure 6.3A, based on genus-level phylotype presences/abundances, all three classification methods did poorly on predicting NW and Ob(-) subjects into their true groups. Among the three methods, 85%-maxEWL method performed better placing SRYGB and URYGB subjects into their true groups than 20% regain and 2-means clustering methods. 85%-maxEWL method can place successful subjects into successful group with 71.8% accuracy and unsuccessful ones to unsuccessful group with 68% accuracy.

When we tried to classify subjects based on NMR metabolomics data, we did not achieve accuracies as high as we got from 16S rRNA gene data. As seen in Figure 6.3B, metabolomics data failed to predict the success of surgery. Among the three classification methods, 20% regain performed better placing SRYGB subjects into SRYGB group but failed to place URYGB subjects into URYGB group. All classification methods did poorly placing URYGB into URYGB group. The majority of the URYGB subjects were placed into SRYGB group, which indicated metabolomics data is good enough to distinguish the effect of surgery on metabolome, however not good enough to assess the success of the surgery based on the classification methods provided.

Overall these results indicate that 16S rRNA gene data is a better predictor of the success of RYGB than NMR based fecal metabolomics data. Additionally, the ways we define success have an impact on the prediction of success. 85%-maxEWL method provides better separation and classification of post RYGB subjects into successful and unsuccessful groups, compared to 20% regain and 2-means clustering methods.

A) Predictions based on 16S rDNA genus-level phylotype data

	Predicted			
NW	NW	Ob(-)	SRYGB	URYGB
20% regain	**44.62**	19.31	13.65	22.42
2-means clustering	**32.63**	41.76	20.26	5.46
85%-maxEWL	**19.27**	34.63	32.63	13.47
Ob(-)	NW	Ob(-)	SRYGB	URYGB
20% regain	34.74	**35.47**	10.17	19.62
2-means clustering	63.1	**27.58**	0.28	9.04
85%-maxEWL	33.43	**37.22**	10.74	18.61
SRYGB	NW	Ob(-)	SRYGB	URYGB
20% regain	4.27	2.47	**64.7**	28.63
2-means clustering	6.86	15.56	**35.9**	41.67
85%-maxEWL	6.73	9.31	**71.8**	12.17
URYGB	NW	Ob(-)	SRYGB	URYGB
20% regain	13.29	17.14	40.86	**28.71**
2-means clustering	2.04	16.5	33.84	**47.62**
85%-maxEWL	2.6	13.8	15.62	**67.98**

B) Predictions based on H-NMR metabolomics data

	Predicted			
NW	NW	Ob(-)	SRYGB	URYGB
20% regain	**49.39**	17.87	32.52	0.22
2-means clustering	**9.69**	39.31	35.19	15.81
85%-maxEWL	**4.45**	41.43	21.71	32.41
Ob(-)	NW	Ob(-)	SRYGB	URYGB
20% regain	21.72	**64.53**	8.16	5.59
2-means clustering	19.93	**68.63**	2.49	8.95
85%-maxEWL	7.96	**70.74**	4.26	17.04
SRYGB	NW	Ob(-)	SRYGB	URYGB
20% regain	8.03	15.35	**64.4**	12.22
2-means clustering	2.34	16.23	**45.49**	36.23
85%-maxEWL	2.86	8.76	**38.71**	49.68
URYGB	NW	Ob(-)	SRYGB	URYGB
20% regain	13.86	2.43	52.86	**30.86**
2-means clustering	14.88	12.59	24.43	**47.11**
85%-maxEWL	12.93	9.7	24.21	**53.15**

Figure 6.3. Machine-learning results on predictions of success of RYGB surgery under three scenarios: 20% regain, 2-means clustering, and 85%-maxEWL. Panel A summarizes the results of predictions based on 16S rDNA results and Panel B summarizes the results based on H-NMR metabolomics data. The true class predictions are bolded and the matrix of accuracy percentages are colored as a heatmap, with higher accuracies tending toward darker blue and lower accuracies tending toward white.

6.3.2 Gut Microbiota Post SRYGB was Peculiar and Different than Post URYGB

As I demonstrated in Chapter 5, post-RYGB subjects had different microbiome structure than the non-surgical subjects from NW and Ob(-) groups. After demonstrating RYGB had an impact on microbial community structure, I investigated how different classification approaches (20% regain, 2-means clustering, and 85%-maxEWL) cluster RYGB subjects based on their success, and I present the results in Figure 6.4. When I used the weighted Unifrac approach, the methods: 20% regain, 2-means clustering and 85% -maxEWL failed to form clusters based on success, even though the 20% regain method showed slight separation of unsuccessful subjects from the successful subjects. This finding indicated that the most abundant OTUs do not contribute to the success of the surgery.

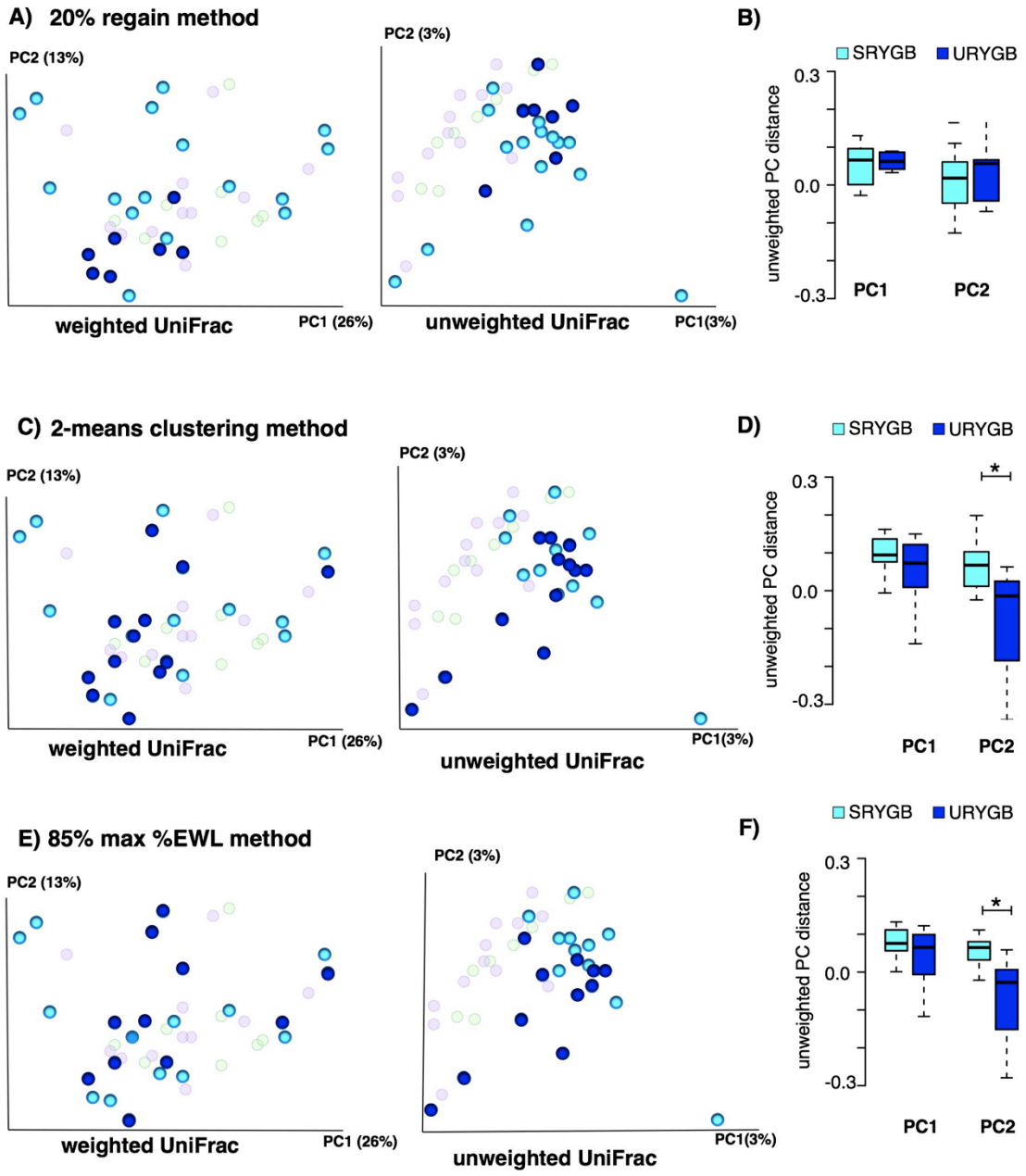

Figure 6.4. Beta diversity analyses on post-RYGB samples. Subject microbiomes are color-coded based on success of the surgery: light blue as SRYGB and dark blue as URYGB. Figure A, C, and E show weighted and unweighted Unifrac distances among the subjects based 20% regain, 2-means clustering, and 85% max-%EWL methods. Figures B, D, and F show the difference among SRYGB and UYGB groups on the PC1 and PC2 axes.

When I employed unweighted Unifrac approach, which relies on presence or absence of OTUs in a phylogenetic context, giving more weight to minor taxa, 2-means clustering and 85%-maxEWL methods formed gradient clusters on PC2. As shown in Figure 6.4D and 6.4F, the PC2 distances were statistically different among successful and unsuccessful groups for two of the success definitions used.

Based on these findings, I concluded that the definition of regain of more than 20% of %EWL does not separate the microbiome structure significantly but might be important for the distribution of more abundant OTUs. 2-means clustering method and 85% max EWL methods generated similar results: They did not contribute to clustering based on weighted Unifrac metric but showed some level of separation based on unweighted Unifrac metric. These findings indicate that presence of unique and possibly low abundant phylotypes are important for success in terms of excess weight loss or nadir excess weight loss rather than abundance of highly abundant phylotypes.

6.3.3 Abundance of Genus-Level Phylotypes in Successful and Unsuccessful RYGB Groups Relied on Definition of Success

Classification results have shown that 16S rDNA data at the genus level is better predictor of the success of the surgery than patient metadata, psychology of the patients or fecal metabolome data. As discussed in detail in Chapter 5, many genus-level phylotypes such as *Veillonella*, *Streptococcus*, *Enterococcus*, *Klebsiella*, *Megaspheara*, *Haemophilus* and *Prevotella* were at greater abundance in post-RYGB subjects than in non-surgical subjects (NW and Ob(-)).

In order to better understand whether microbial phylotypes that were enriched after RYGB have any connection to successful and sustainable weight loss, we compared

their normalized abundances in successful and unsuccessful groups that were defined based on three different methods (20% regain, 2-means clustering, and 85%-maxEWL), as shown in Figure 6.5.

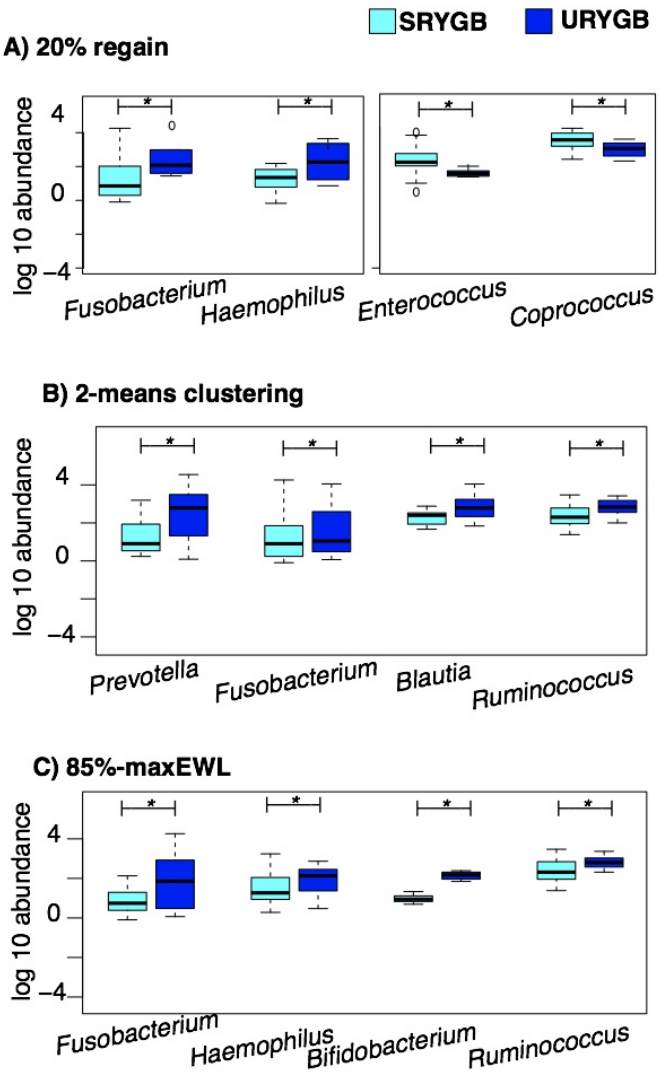

Figure 6.5. Normalized log10 abundance of genus-level phylotypes that were significantly more abundant in the SRYGB compared to URYGB based on all three methods used to define success: 20% regain, 2-means clustering, and 85%-maxEWL.

Based on 20% regain method, *Fusobacterium* and *Haemophilus* were more abundant; *Enterococcu*s and *Coprococcus* were less abundant in the URYGB compared to SRYGB. Interestingly, those phylotypes were significantly more abundant in post-RYGB subjects than the nonsurgical subjects. Based on 2-means clustering and 85%-maxEWL methods, four different genus-level phylotypes were statistically more abundant in the URYGB than the SRYGB, and we did not observe any phylotype that was significantly more in the SRYGB than URYGB based on these methods. For 2-means clustering, these phylotypes were: *Prevotella*, *Fusobacterium*, *Blautia* and *Ruminococcus*, whereas for 85%-maxEWL method, they were: *Fusobacterium*, *Haemophilus*, *Bifidobacterium*, and *Ruminococcus*. Interestingly, *Fusobacterium* was the common signature of unsuccessful RYGB based on all three classification methods, *Haemophilus* was common for 20% regain and 85%-maxEWL methods, and *Ruminococcus* was common for 2-means clustering and 5% maxEWL methods.

Fusobacterium was reported to be more abundant in post-RYGB subjects (Furet*, et al.*, 2010) and our findings reported in Chapter 5 confirm that. Higher abundance of *Fusobacterium* species in oral microbiome (Goodson*, et al.*, 2009) and fecal microbiome (Angelakis*, et al.*, 2012) were previously associated with obese metabolic type in humans. Increased abundance of *Fusobacterium* was also observed in many health conditions such as colorectal cancer (Kostic*, et al.*, 2012), and it is known to produce lipopolysaccharides that can induce inflammation (Sveen *et al.,* 1977). Since obesity is associated with low-grade inflammation (Everard*, et al.*, 2013), *Fusobacterium* might be involved in the weight gain processes after RYGB.

Haemophilus has not been associated with weight gain or weight loss before. *Ruminoccoccus* was found to decrease in individuals that lose weight via dieting and exercise (Nadal*, et al.*, 2009, Santacruz*, et al.*, 2009). Interestingly, *Bifidobacterium* species were found more abundant in not obese compared to obese populations (Santacruz*, et al.*, 2010). *Prevotella* was found more abundant in obese population than normal weight or post-RYGB populations (Zhang*, et al.*, 2009), here we observed higher *Prevotella* in post-RYGB population compared to nonsurgical, however significantly more in URYGB than SRYGB.

Our results have shown that the method used to define success can yield different outcomes in terms of associating microbial phylotypes with success of the surgery. Although some phylotypes such as *Fusobacterium* in this case, appear to be signature of unsuccessful RYGB regardless of the method used to define success. Understanding *Fusobacterium* metabolism and its interactions with the host might explain weight gain or inability to loose excess weight after RYGB.

6.3.4 Fecal Metabolites Mainly from Amino Acid Degradation Pathways form Signatures of Successful and Unsuccessful Bariatric Surgery

Fecal metabolome profiles of post-RYGB, NW, and Ob(-) groups were different. Figure 6.6 shows principal component analysis based on fecal metabolomes. Principal component analysis demonstrated that post-RYGB metabolomes are different than NW and Ob(-) metabolomes. Additionally, NW and Ob(-) metabolomes were different from each other as well. Ob(-) subjects had the highest fecal concentrations of many of the metabolites detected including short chain fatty acids (SCFAs), and amino acids (AAs),

whereas NW subjects had relatively lower concentration of the majority of the metabolites compared to RYGB and Ob(-) groups.

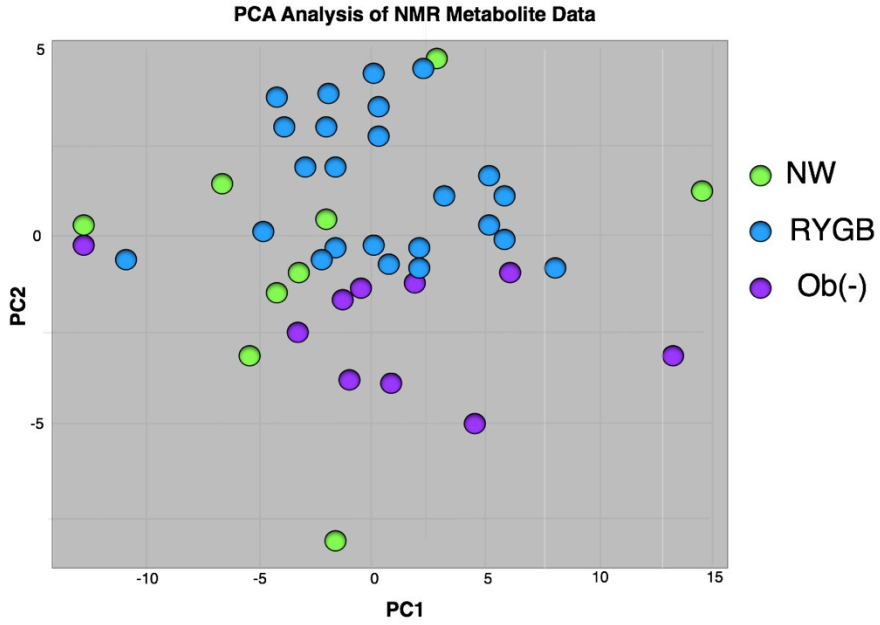

Figure 6.6. Principal component analysis performed on fecal metabolomes of individuals from NW, post-RYGB, and Ob(-) groups.

Branched chain fatty acids: isovaleric and isobutyric acids and phenlyacetic acid were significantly more abundant in the RYGB group compared to NW and Ob(-) groups as seen in Table 6.1. Lactic acid was also significantly more abundant in the RYGB group. Molecules that form conjugates with bile acids (taurine and glycocholic acid) and choline, which is the precursor of neurotransmitter acetylcholine, were significantly more abundant in the Ob(-) group compared to RYGB group**.** Additionally, amino acid arginine was significantly lower in the RYGB group compared to NW and Ob(-) groups.

Table 6.1. Log 2 transformed concentrations of metabolites that were significantly more or less abundant in the RYGB group compared to NW and Ob(-).

	NW	RYGB	Ob(-)
Isobutyric acid*	2.55±0.91	3.31± 0.78	2.54±0.82
Isovaleric acid*	2.31±0.82	3.24±0.75	2.21±0.76
Phenylacetic acid**	1.98±1.08	2.72±0.86	1.72±0.79
Choline**	-4.43±1.24	-3.97±1.37	-2.85±1.36
L-arginine**	-0.60±0.54	-1.82±0.83	-0.95±0.8
L-lactic acid*	-2.48±0.54	-1.46±1.36	-2.34±0.93
Taurine**	0.50±0.93	-1.13±1.72	2.07±2,38

* indicates significantly greater abundance and ** indicates significantly less abundance.

Isobutyric, isovaleric, phenylacetic, and lactic acids were at greater abundance in the RYGB group, slightly higher in SRYGB groups however there was no statistical difference between SRYGB and URYGB regardless of how we define the success. Next, we evaluated how defining success of the RYGB surgery with three different methods can identify signatures of successful or unsuccessful RYGB surgery. When we compared the abundances of fecal metabolites in successful and unsuccessful groups using ANOVA, we did not observe any significant difference between the groups regardless of the classification method used. With Mann-Whitney's U test, we detected a few metabolites that distinguished successful groups from unsuccessful ones. I also performed Receiver Operator Characteristics (ROC) analysis based on three different definitions of success. As seen in Figure 6.7A, 4-hydroxyphenylacetate, xanthine, ribose, choline, and taurine emanated as signatures of unsuccessful RYGB with Area Under the Curve (AUC) values of greater than 0.6. 2-means clustering and 85%-maxEWL methods yielded comparable results. As shown in Figures 6.7B and 6.7C, putrescine, malonate, ethanol, and hydroxyisobutyrate were signatures of unsuccessful surgery with both methods. In summary, ROC analysis failed to identify signatures of successful surgery but showed some indications of unsuccessful surgery.

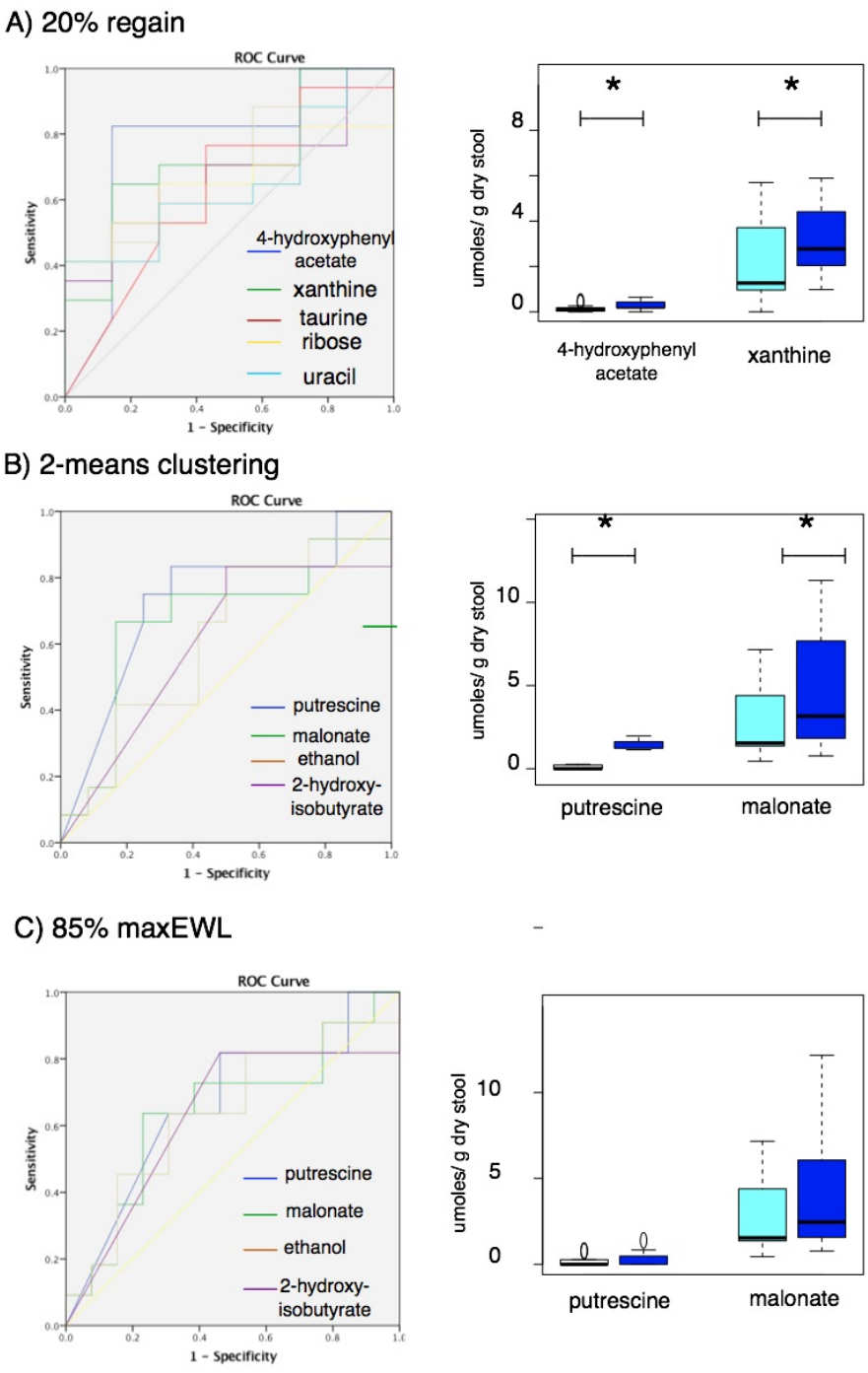

Figure 6.7 Receiver operator characteristics (ROC) analysis of fecal metabolites shows signs of successful and unsuccessful surgeries. A) 4-hydroxy-phenylacetate and xanthine were signatures of unsuccessful surgery based on 20% regain method. Putrescine and malonate were signatures of unsuccessful surgery based on B) 2-means clustering method and C) 85% maxEWL method

Based on Mann-Whitney's U test results, with the 20% regain method xanthine and 4-hydroxyisobutyrate were significantly more abundant in the URYGB than SRYGB. Putrescine and malonate were significantly more abundant in the URYGB than SRYGB based on 2-means clustering method.

In summary we fatty acids did not distinguish successful and unsuccessful subjects, even though isobutyric and isovaleric acids were at greater abundance in the SRYGB group based on 20% regain method. However, we should not neglect the fact that the unsuccessful subjects in this study had some degree of weight loss (at least 50% of the EWL); therefore these branched chain fatty acids are possibly contributing to weight loss. The branched-chain fatty acids isobutyrate and isovalerate serve as signaling molecules that show potent activity against the G-protein-coupled receptor GPR41 (Le Poul*, et al.*, 2003) and free-fatty-acid receptors 2 and 3, which are involved in the regulation of appetite (Schmidt*, et al.*, 2011). Higher abundance of these branched chain fatty acids in post-RYGB groups can give a slight edge and contribute to host weight loss via signaling hormonal and neural networks of the host.

Based on ROC analysis, 4-hydroxyphenylacetate and xanthine were signatures of URYGB based on 20% regain method, and significantly more abundant in the URYGB subjects. This finding indicates that these two metabolites might contribute to weight gain in the long-term. Many microbial species, especially the ones that degrade nucleic acids such as *Enterococcus* species are known to produce xanthine from purine degradation (Huycke*, et al.*, 2002), and xanthine triggers lipolysis in the adipose tissues and then lead to release of hunger hormone ghrelin (Beavo*, et al.*, 1970). Xanthine has been observed at higher concentrations in obese patients compared to normal-weight

patients (Calvani*, et al.*, 2010). On the other hand, 4-hydroxyphenylacetate is the precursor of 4-cresol, and mainly produced from *Clostridia* species (Holmes*, et al.*, 2011). 4-hydroxyphenylacetate has not been associated with host metabolism yet although it has been observed to be at greater concentrations in aged mice compared to young mice (Calvani*, et al.*, 2014). Putrescine and malonate discriminated URYGB subjects from SRYGB subjects based on 2-means clustering and 85%-maxEWL methods, and URYGB subjects had significantly higher fecal concentrations of these metabolites compared to SRYGB subjects based on these two methods.

Fusobacterium, *Bacteroides* and *Pseudomonas* can degrade ornithine and produce putrescine (Noack*, et al.*, 2000). *Fusobacterium* was at greater abundance in the URYGB group than the SRYGB one regardless of the classification method used. Li *et al.* detected an increase in urinary putrescine in rats after RYGB surgery (Li*, et al.*, 2011) but did not associate putrescine with weight loss or weight gain. In a different study putrescine has been associated with the accumulation of fat and it has also been suggested as urinary biomarker for diabetes (Kim*, et al.*, 2010).

Besides putrescine, *Fusobacterium* species can produce malonate (Dzink & Socransky, 1990). Malonate can inhibit oxidation of glutamate because it is an inhibitor of succinate dehydrogenase enzyme (Greene & Greenamyre, 1995). Malonate has been reported in many toxicity studies but it hasn't been associated with weight gain or weight loss.

In conclusion, success of bariatric surgery depends on many factors, and the description of successful RYGB to date is variable and subjective. Our results show that gut microbiome can be one of the contributors of successful and sustainable weight loss

after RYGB. Fecal metabolome was not a good indicator of the successful RYGB surgery, although we identified a few promising metabolites as signatures of unsuccessful RYGB. Pathways involved in weight loss and weight gain are redundant, so identification of multiple microbial signatures would be important for the estimation of success of RYGB. Sample size was our main limitation to validate some of the findings with robust statistics; future studies with greater number of subjects will help validate these findings.

CHAPTER 7

LONGITUDINAL CHANGES IN LUMINAL AND MUCOSAL MICROBIOME, AMINO ACID, AND BILE ACID METABOLISM AFTER RYGB SURGERY

7.1 Introduction

Roux-en-Y gastric bypass (RYGB) is an efficient treatment strategy for morbid obesity and its comorbidities such as diabetes mellitus (Ryan *et al.*, 2014). RYGB changes hormonal response (Korner *et al.*, 2009), energy metabolism (Korner *et al.*, 2009), and bile-acid circulation in the body (Ryan *et al.*, 2014). A number of studies have shown that RYGB transforms gut microbiota (Furet *et al.*, 2010; Graessler *et al.*, 2013; Palleja *et al.*, 2016; Tremaroli *et al.*, 2015; Zhang *et al.*, 2009), although only a few of them (Furet *et al.*, 2010; Graessler *et al.*, 2013; Palleja *et al.*, 2016) have monitored gut microbiota before and after the surgery in the same individuals. Studies with human subjects have shown that gut microbiota composition shifts promptly, as soon as three months after the surgery (Furet *et al.*, 2010) and those shifts have been reported prominent even 12 months after the surgery (Palleja *et al.*, 2016). Post-RYGB patients have unique diets; they begin consuming small portions of solid food only eight-nine months post-operatively (Brolin, Robertson, Kenler, & Cody, 1994), therefore diet can impact the results of cohort studies, and it is essential to validate their results with cross-sectional studies.

Changes in gut microbiota after RYGB have been associated with remission of diabetes in humans (Graessler *et al.*, 2013) and weight loss in rats (Liou *et al.*, 2013) and in humans (see Chapter 5). These associations possibly rely on production of microbial

metabolites because (as discussed below) these metabolites are key for host-microbial interactions. Metabolic products of the gut microbiota after RYGB exert beneficial effects on host metabolism (Liou *et al.*, 2013). Microorganisms produce a variety of metabolites that enhance communication between the host and the microbes; these molecules vary from fermentation end-products such as short chain fatty acids (SCFAs) to secondary bile acids (Nicholson *et al.*, 2012). SCFAs butyrate and propionate, which are known to induce satiety in animals due to their affinity to free fatty acid receptors (Nilsson, Kotarsky, Owman, & Olde, 2003), increase in concentrations after RYGB surgery. On the other hand, acetate can induce fattiness in the liver (Hanson & Ballard, 1967), and microbial hydrogen metabolism can control acetate production (Nie, Liu, Du, & Chen, 2008). Bile acids are another group of metabolites that are microbially transformed, and their concentrations change in the plasma after RYGB surgery (Kohli *et al.*, 2013; Ryan *et al.*, 2014), some researchers consider bile acids and their transformation one of the possible factors that leads to successful weight loss after bariatric surgery (Li *et al.*, 2011). RYGB surgery also increases the abundance of amino acid degradation genes (Palleja *et al.*, 2016), although the metabolic products have not been identified or quantified in humans yet.

Additionally, human gut microbiome studies after RYGB to date have only relied on fecal samples (Furet *et al.*, 2010; Graessler *et al.*, 2013; Palleja *et al.*, 2016; Tremaroli *et al.*, 2015; Zhang *et al.*, 2009). Microbiota of mucosal surfaces in individuals with morbid obesity and after RYGB are unknown.

Microorganisms inhabit luminal and mucosal surfaces of our gastrointestinal tracts. Due to invasive nature of mucosal sample collection, a vast majority of the gut

microbiota studies rely on luminal sample collection (Durban *et al.*, 2011). It has been previously shown that mucosal and luminal samples from healthy individuals vary in microbiota composition, and luminal microbiota do not represent the mucosal microbiota (Durban *et al.*, 2011). Many factors including nutrients, physical and chemical conditions in the gut, and immune system interactions diverge mucosal microbiota from luminal microbiota (S. Macfarlane & Dillon, 2007). For instance, a decreasing vertical oxygen gradient exists from mucus layer to the lumen, which creates a facultative anaerobic to obligate anaerobic zone (Espey, 2013). Another important difference between mucosal and luminal microbiota is the organic substrate: luminal communities often utilize undigested diet components such as plant polysaccharides, whereas mucosal communities utilize host-derived glycans (Hooper, Midtvedt, & Gordon, 2002). The composition of the mucosal microbiota can change drastically during gastrointestinal diseases (S. Macfarlane & Dillon, 2007). Inflammatory diseases such as ulcerative colitis (UC) affect the colonic mucosa or sub-mucosa, and UC patients had different microbiota than healthy individuals (S. Macfarlane & Dillon, 2007). Similarly, luminal and mucosal microbiota composition change in colorectal cancer (Chen, Liu, Ling, Tong, & Xiang, 2012). Variations in the mucosal and luminal microbiota were also observed in diabetic mice (Amar *et al.*, 2008; Cani *et al.*, 2008).

The objectives of this study were: (i) to investigate microbial and metabolic changes after the surgery and compare findings with cross-sectional studies, (ii) to explore if changes in microbiota after surgery increase weight loss and reduce weight gain associated metabolic products, (iii) to reveal differences between mucosal and luminal communities in morbidly obese individuals before and after RYGB surgery.

Here, we characterized microbiome structure and metabolism after RYGB using multi-omic techniques including, 16S rRNA gene fingerprinting, gas chromatography mass spectrometry, liquid chromatography mass spectrometry, and nuclear magnetic resonance. We also provided a comprehensive analysis on changes to microbiome and metabolome after RYGB by comparing cohort and case-study results.

7.2 Materials and Methods

7.2.1 Study Design and Ethical Consideration

The Institutional Review Board of Mayo Clinic Scottsdale and Arizona State University (IRB# for both = 10-008725) approved our study. We recruited 10 morbidly obese subjects that were scheduled to undergone RYGB surgery and 10 normal weight controls. One of the subjects dropped the study after baseline sampling and two of them missed the 6-months sampling. The pre-surgical population consisted of 5 female and 5 male subjects whereas normal group consisted of 7 females and 3 males. Median age of the subject groups was not statistically different among the groups. In order to confirm results of case-control studies with this cohort study, we expanded our study to include subjects that had RYGB surgery 9 months to 60 months before the sample collection. We collected samples from pre-bariatric morbidly obese subjects (RYGB baseline) before the surgery, 6-months and 12-months after the surgery in addition to 36 retrospective (retro-RYGB) and 10 normal-weight (NW) subjects. Fecal samples were collected from all the individuals at each time point. Subjects donated the fecal samples 216±41 days and 455±124 days after the surgery. Rectal biopsies were collected during non-sedated sigmoidoscopy from 10 NW subjects and 9 pre-bariatric surgery subjects before and 12-months after the surgery at Mayo Clinic, Scottsdale U.S.A. Mayo Clinic

personnel immediately transferred the samples into sterile RNAse/DNAse free cryogenic tubes with sterile forceps. The tubes were instantly submerged in liquid nitrogen for one minute and then transferred to a -80 ^{o}C freezer on dry ice. Samples were shipped to ASU on dry ice and placed into a -80 ^{o}C freezer immediately upon receipt. The samples were shipped to ASU from Mayo Clinic on dry ice and were kept at -80^{o}C until analysis.

7.2.2 DNA and RNA Extraction Protocols

We extracted microbial DNA and RNA from fecal and biopsy samples to represent luminal and mucosal microbiota. For DNA and RNA extractions, we used MOBIO PowerSoil DNA extraction (MOBIO Laboratories, Carlsbald, CA, USA) and MOBIO PowerSoil RNA extraction kits (MOBIO Laboratories, Carlsbald, CA, USA), respectively. RNA extracts were treated with MOBIO DNase Max kit (MOBIO Laboratories, Carlsbald, CA, USA), and cDNA synthesis was performed using Omniscript Reverse Transcription kit (Qiagen, Santa Clarita, CA, USA) according to manufacturer's instructions.

7.2.3 16S rRNA Gene Sequencing and Analysis

We prepared sequencing libraries using the protocols from Earth Microbiome project using V4 forward and reverse primers with Illumina Miseq Instrument (Gilbert, Jansson, & Knight, 2014). PANDAseq (Masella, Bartram, Truszkowski, Brown, & Neufeld, 2012) paired reads were analyzed using QIIME 1.9 suite (Caporaso *et al.*, 2010). The analysis was followed by Kang *et al.* with the following modification: clusters were formed at 99% sequence similarity in addition to 97% sequence similarity (Kang *et al.*, 2015).

We utilized alpha and beta diversity metrics of Phylogenetic Diversity Whole Tree (Faith, 1992), and Unifrac (Lozupone, Hamady, & Knight, 2006) to better understand microbial community structures.

7.2.4 Quantitative-PCR on Hydrogen Consuming Microorganisms

We performed quantitative polymerase chain reaction (q-PCR) on targeted microbial genes (*fthfs*, *mcrA*, and *dsrA*) to detect homoacetogenesis, methanogenesis, and sulfate reduction using Eppendorf thermocyclers according to previously described methods (Ontiveros-Valencia *et al.*, 2012; Parameswaran, Torres, Lee, Krajmalnik-Brown, & Rittmann, 2009). We carried out quantitative PCR assays in 20 uL reactions consisting of 1X SYBR Premix Ex Taq (Tli Rnase H Plus) (Takara), 10 ng/ ul genomic DNA or cDNA, 10 mM forward and reverse primers, and DNase free water. We quantified formyltetrahydrofolate synthetase (*fthfs*) gene for homoacetogens(Leaphart & Lovell, 2001), dissimilatory sulfide reductase (*dsrA*) gene (Kondo, Nedwell, Purdy, & Silva, 2004) for sulfate reducers, and methyl coenzyme-M reductase (mcrA) gene for methanogens (Luton, Wayne, Sharp, & Riley, 2002).

7.2.5 H-NMR Analysis of Water-Soluble Fecal Metabolites

For each fecal specimen, approximately one gram of wet weight (precise weight was recorded and used for calculations) was diluted with 20 mL of milliQ water (18 ohms). This step was performed in triplicates for process control. The homogenate was vortexed at the highest speed for three minutes to achieve complete mixing. Then the homogenates were centrifuged at 13,000 rpm for 15 minutes and the supernatants were filtered through 0.2-um PVDF membranes (PALL Coorparation). 30 uL of reference solution was added to 270 uL of the filtrate. The reference solution was composed of

100% D_2O, 5 mM DSS and 0.1% sodium azide. The resulting mixture was loaded into 3mm H-NMR tubes (Bruker Inc) and shipped to Pacific Northwest National Laboratories (PNNL) on ice blocks for NMR analysis. There, the metabolite analysis was performed using a Bruker 500 MhZ NMR as previously described (Kajimoto *et al.*, 2014).

7.2.6 Gas Chromatography-Mass Spectrometry Analysis of Fecal Metabolites

Fecal samples were lyophilized before extraction. Fecal metabolite derivatization and GC-MS analysis were based on the protocols described by Kaiser *et al.* (Kaiser *et al.*, 2013).

7.2.7 Liquid Chromatography-Mass Spectrometry Analysis of Bile Acids

Bile acids were extracted from fecal samples at PNNL following the protocol from Humbert *et al.* (Humbert *et al.*, 2012). Briefly, fecal matter was lyophilized and a protocol using NaOH extraction was performed. Once the bile acids were extracted, they were identified and quantified with High Pressure Liquid Chromatography (HPLC-MS/MS) coupled with tandem mass spectrometry as described by Hubert *et al.* (Humbert *et al.*, 2012).

7.2.8 Statistical Analyses of Microbiome and Metabolome Data Sets

We used Statistical Package for Social Sciences (SPSS) for all the statistical analysis and R packages. The NMR, GC-MS, and GC-LS generated datasets were log2 transformed. The 16S rRNA gene sequence data was normalized based on 75^{th} quartile method described by Paulson *et al.* (Paulson, Stine, Bravo, & Pop, 2013). For the longitudinal data analysis, we performed Wilcoxon signed-rank test and accepted P values less than 0.05 as significant. For group comparisons, we used Mann-Whitney's U

test and accepted P values that were False Discovery Rate corrected and less than 0.05 as significant.

7.3 Results and Discussion

7.3.1 Subjects Achieved the Greatest Weight Loss Six Months after the RYGB Surgery

Figure 7.1 shows short-term and longer-term effects of RYGB surgery on weight loss. The median body mass index (BMI) before surgery was 45 kg/m^2, and in 6 months it dropped to 35 kg/m^2. The median BMI dropped minimally from 6 months to 12 months and remained at an average of 33kg/m^2 as shown in Figure 7.1A. Even though subjects lost substantial amount of weight, and the drop was statistically significant at 6 and 12 months, the majority of the subjects remained clinically obese with BMIs higher than 30 kg/m^2 even one year after RYGB. Percent excess weight loss (%EWL) calculations, as seen in Figure 7.1B, confirmed that the subjects achieved the greatest weight loss during the initial 6 months and the subjects maintained the weight loss benefits a year after the surgery.

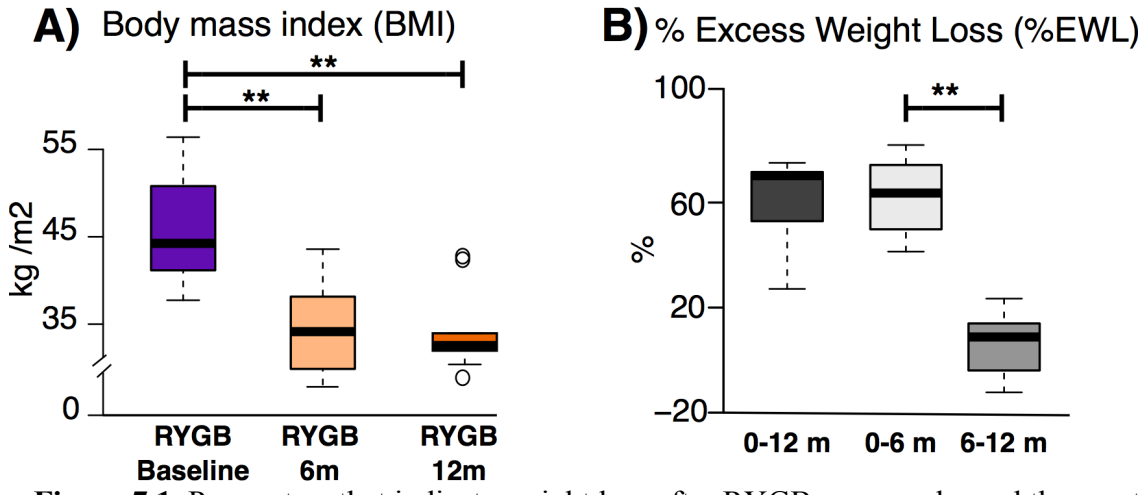

Figure 7.1. Parameters that indicate weight loss after RYGB surgery showed the greatest weight loss period being 6-months after the surgery. A) Body mass index (BMI) index of subjects before the surgery (baseline), 6-months and 12-months after the surgery.

B) % Excess Weight loss 12 months after the surgery, 6 months after the surgery and 6 to 12 months after the surgery.

Weight loss after the surgery was correlated to the diet and exercise regimens of the subjects. Table 7.1 summarizes total dietary calories, dietary composition and calories burned as a result of exercise of the subjects. Based on total calories, morbidly obese subjects (RYGB baseline) were consuming fewer calories than the NW subjects. The subjects reduced the amount of calories they consume by 30% during the first 6 months. Compared to their baseline, subjects were consuming 22% less calories at 12 months, yet the weight loss benefits were sustained. The subjects also increased the amount of calories consumed due to exercise after the surgery, which possibly contributed to their weight-loss and maintenance of body weight. The dietary composition of the morbidly obese subjects did not significantly change after the surgery, although compared to NW individuals, carbohydrates formed smaller fraction of the diets of post RYGB subjects.

Table 7.1. Dietary composition of the normal-weight (NW), pre-surgical morbidly obese baseline (RYGB-base), 6 months (RYGB-6m), and 12 months (RYGB-12m) after the surgery samples.

	NW	RYGB-base	RYGB-6m	RYGB-12m
Calorie intake (cal)	2159±681	1820±706	1311±513	1419±465
Carbohydrate %	51±7.4	42.5±7.6	40.0±5.8	37.0±6.5
Fat%	33±5.7	36.5±6.3	36±5.4	38.0±5.6
Protein %	14±2.9	19.5±4.2	20.0±8.0	21±8.3
Fiber intake (g)	21±9.1	14±12.5	18±6.5	14±4.6
Exercise (cal)	2076±3074	532±1446	1845±1908	952±1012

7.2 RYGB Changed Microbial Community Structure Longitudinally and the RYGB-retro Cohort Validated Cross-Sectional Study Results.

Figure 7.2A underscores the longitudinal changes in luminal microbiome after RYGB surgery. We performed unweighted (Figure 7.2A) and weighted Unifrac (Figure B.1) analyses to understand changes in microbiome structure after the surgery. We observed greater differences in community structure after the surgery with unweighted Unifrac, which enhances the differences in the microbiome structure of minor phylotypes of the community because the analysis is based on changes on presence or absence and not on abundances. Based on unweighted Unifrac distances, changes in the luminal microbiome were apparent as short as 6 months after the surgery. This difference could be due to changes in the gut environment (Zhang *et al.*, 2009) and changes in the diet (Brolin *et al.*, 1994) after RYGB.

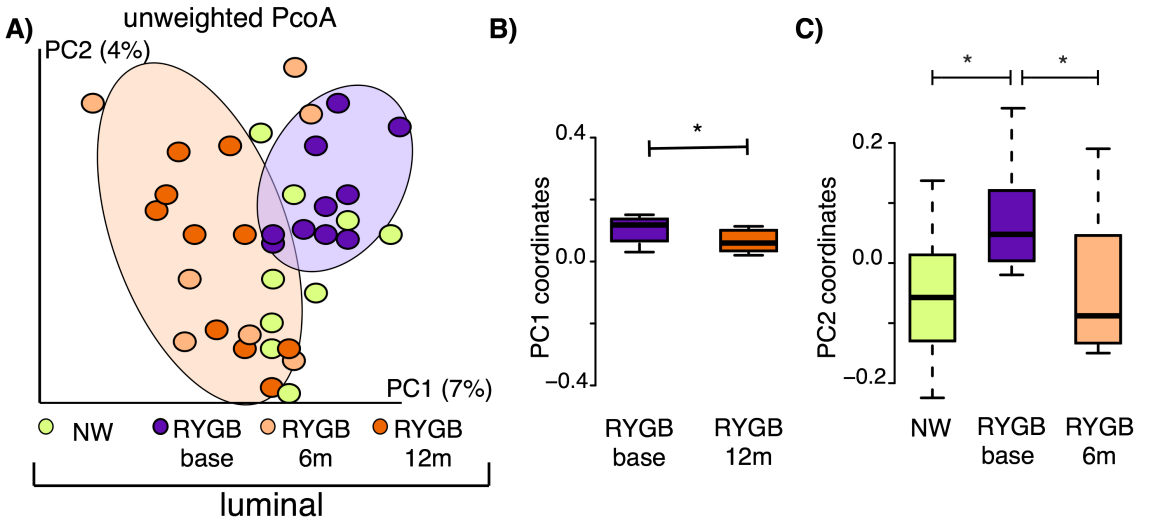

Figure 7.2. Longitudinal changes on luminal microbiome structure after RYGB surgery. A) Unweighted Unifrac analysis shows that minor taxa in the lumina changes after RYGB. B) The location of RYGB-base and RYGB-12m subjects on principal coordinate 1 (PC1) based on luminal samples. C) The location on PC2 of RYGB-base subjects NW and RYGB-6m subjects based on luminal samples

On PC1, baseline and 12-months samples form two distinct clusters. Additionally, RYGB-12m samples were distant from NW group samples, which indicated that after RYGB surgery microbiome does not become similar to NW microbiome. As shown in Figure 7.2B, the location of RYGB-base and RYGB-12m samples were significantly different on PC1 axis. Also, the PC2 axis, which explains less variation of the dataset than PC1, showed that RYGB-base samples were located distantly from NW samples. Additionally, impact of the surgery after 6 months was more apparent on PC2. Our results on luminal microbiome were consistent with the previous reports (Furet *et al.*, 2010; Palleja *et al.*, 2016) showing that microbiome structure changes a few months after RYGB and the changes persist in the long-term such as one year after the surgery.

Figure 7.3 shows the relative abundance of significantly enriched or depleted genus-level phylotypes in the luminal space one year after RYGB. The surgery altered relative abundances of 21 genus level phylotypes. The majority of enrichments or depletions of genus-level phylotypes occurred within the first 6 months after the surgery and were sustained at least one year after the surgery. Interestingly, the abundances of these phylotypes were significantly different in RYGB-6m and RYGB-12m groups compared to NW group. Even though RYGB surgery is known to enrich phylotypes from *Gammaproteobacteria* (Furet *et al.*, 2010; Palleja *et al.*, 2016; Zhang *et al.*, 2009), our analysis showed that RYGB impacts the abundance of many genus-level phylotypes from other phyla such as *Firmicutes*, *Actionobacteria*, *Fusobacteria*, and *Bacteroides*.

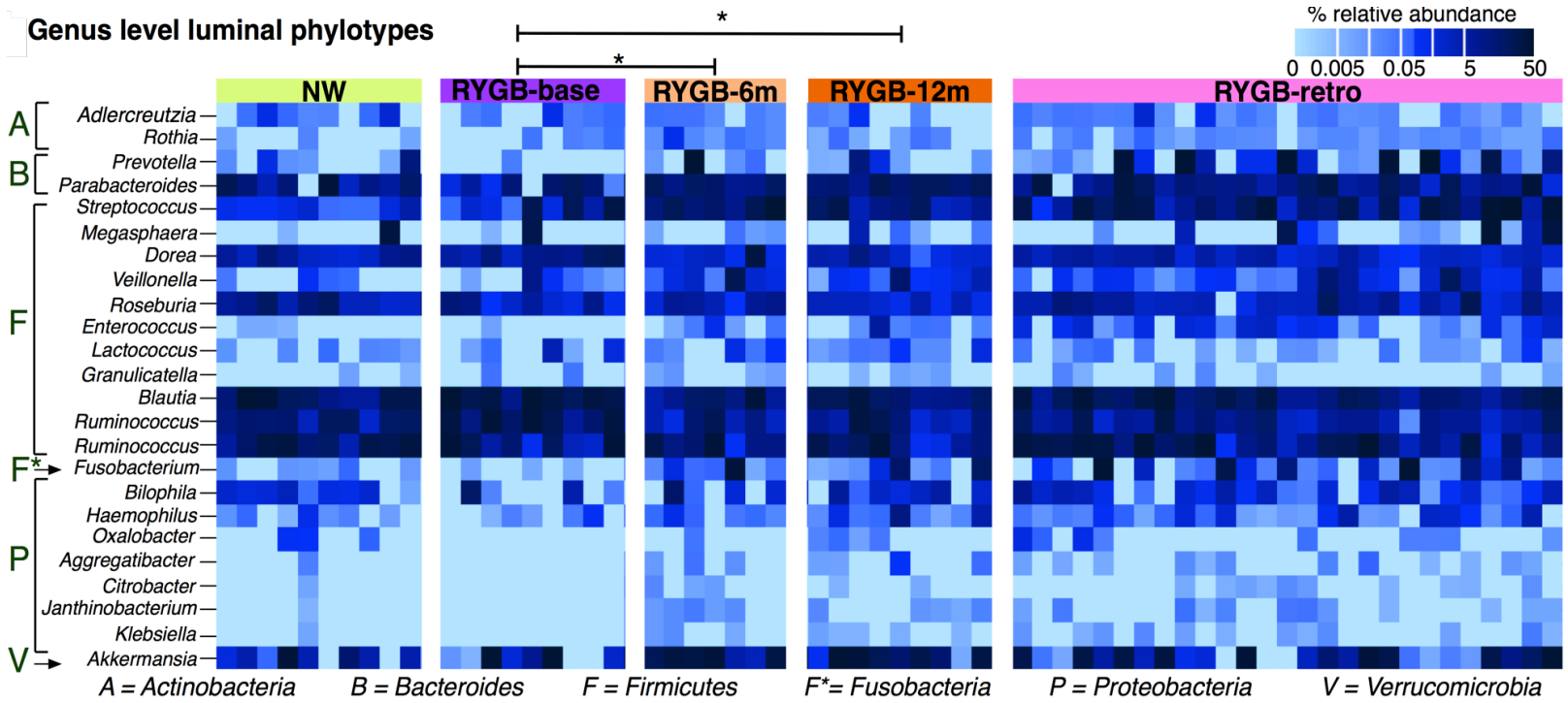

Figure 7.3. Heat-map visualization of genus-level phylotypes significantly enriched or depleted prospectively 6 and 12months after RYGB surgery, and RYGB-retro (cross sectional RYGB subjects 34 months (median) after the surgery). Luminal genus-level phylotypes in NW, RYGB-base, RYGB-6m, RYGB-12m, and RYGB-retro groups

We observed an increase in the abundance of *Proteobacteria* phylotypes: *Rothia*, *Aggregatibacter*, *Granulicatella, Citrobacter*, *Janthinobacterium*, and *Klebsiella*. *Firmicutes* was another phylum that had many phylotypes whose relative abundances were affected by the surgery; while most of the phylotypes such as *Streptococcus*, *Enterococcus*, *Lactococcus*, *Veillonella*, and *Granulicatella* got enriched, other phylotypes such as *Ruminococcus*, *Blautia*, and *Roseburia* got depleted after the surgery. *Akkermansia* from *Verrucomicrobia* and *Adlercruetzia* and *Rothia* from *Actinobacteria* were also at greater abundance after RYGB.

Figure 7.3 also shows the relative abundance of the aforementioned phylotypes in a cross-sectional retrospective RYGB group (RYGB-retro). RYGB-retro group consisted of post-RYGB subjects that were unrelated and donated a sample 13 months to 60 months after the surgery; therefore it was a heterogeneous group. The trends we observed in the retro-RYGB group paralleled the RYGB-6m and RYGB-12m groups. This sustained changes in the microbiome after RYGB indicates that surgery imposed permanent environmental changes that affected gut microbiota, and the data presented here shows that those changes occurred soon after the surgery. This observation was mainly derived from comparison of unrelated individuals; therefore it was unknown whether if it was the surgery or any other interpersonal variation affecting their abundance and leading to different profiles compared to non-surgical controls (NW and RYGB-baseline). To our knowledge, this is the first study that characterized post-RYGB microbiome by employing both longitudinal (changes in the same individual) and cross-sectional (changes comparing post surgery to other controls) studies.

Our analysis demonstrated that after RYGB surgery there is a wide spectrum of changes in the luminal space. The abundances of phylotypes such as *Klebsiella*, *Akkermansia*, *Citrobacter* etc. in post-RYGB subjects were not only statistically different than their baseline counterparts but also normal weight controls. Our analysis also demonstrated that in the short-term and long-term, abundances of several genus-level phylotypes from diverse group of microorganisms changed after RYGB surgery, and those sustained changes might possibly change the metabolic outcome of the gut microbiome, and contribute to weight loss.

7.3 Post-RYGB Surgery Microbiota Changes Fecal Metabolome, Reduces Acetate Production and Enhances the Fermentation of Amino Acids.

Changes in the gut microbiome structure after RYGB surgery were also reflected on the microbiome functione by analizing the luminal metabolome. Figures 7.4A and 7.4B show fecal metabolomes after RYGB on principal components detected by NMR and GC-MS based methods, respectively. Principal component analysis for metabolites detected by NMR showed that RYGB-6m and RYGB-12m fecal metabolomes clustered away from their baseline (RYGB-base). GC-MS metabolome data showed comparable patterns to NMR metabolome data. GC-MS data, which includes dietary components in addition to microbial metabolites in the fecal samples revealed greater differences after the surgery. In Figure 7.4C, we overlayed metabolomes of the RYGB-retro subjects to the metabolomes illustrated in Figure 7.4B metabolomes of the RYGB-retro group clustered together with RYGB-6m and RYGB-12m subjects. This observation indicates that RYGB surgery not only changes microbiome in the short-term and in the longer-term, but also that the impact of the surgery on metabolome is greater than any other

inter-personal variation that might influence metabolome such as diet and lifestyle. Moreover, observing similar clustering patterns with metabolome (Figures 7.4A and 7.4B) and microbiome (Figures 7.2A), strengthens the connection between microbiome and metabolome after RYGB surgery and shows that these changes are severe and one directional.

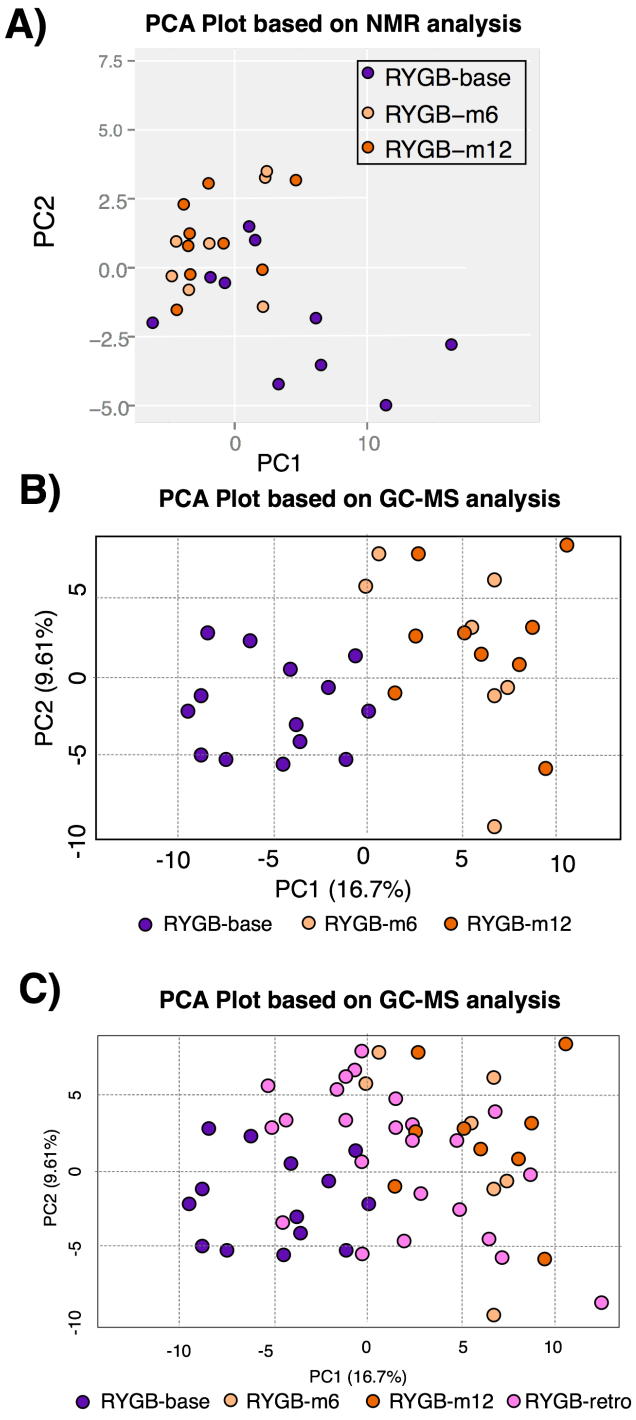

Figure 7.4. Fecal metabolites detected with H-NMR and GC-MS. Principal component analysis based on metabolites detected by (A) NMR and (B) GC-MS based metabolomes before and after the surgery. C) Principal component analysis based on GC-MS metabolome that includes RYGB-retro group samples.

The major SCFAs of the human gut; acetate, butyrate, and propionate did not show any significant difference before or after the surgery, the results are summarized in Table 7.2. The concentrations of these fatty acids in RYGB-retro and RYGB-12m groups were comparable. Even though, concentrations did not show any significant difference, propionate to acetate and butyrate to acetate ratios increased 6 and 12 months after the surgery, and the difference between baseline and 12 months samples was statistically significant. Higher butyrate and propionate to acetate ratios after the surgery compared to baseline indicates that there is a shift in microbial metabolism from acetate production to butyrate and propionate production. Butyrate and propionate are molecules that can signal free fatty acid receptors, and induce satiety response in the brain (Lin *et al.*, 2012), and shifts in microbial metabolism to produce more of them can be potential mechanisms that microorganisms contribute to weight loss.

Table 7.2. Dry weight normalized concentrations of acetate, butyrate, propionate, propionate to acetate and butyrate to acetate ratios in NW, RYGB-base, RYGB-6m, and RYGB-12m, and RYGB-retro groups. The numbers represent median values of the groups.

	Propionate / Acetate	**Butyrate / Acetate**	**Acetate**	**Butyrate**	**Propionate**	**sCOD /tCOD***
			µmoles/ g stool	µmoles/g stool	µmoles/g stool	%
NW	0.396	0.248	114.1	31.3	45.2	20
RYGB-base	0.366	0.244	226.7	47.0	75.4	25
RYGB-6m	0.375	0.267	240.8	61.1	103.4	22
RYGB-12m	0.407	0.315	162.8	41.8	69.6	18
RYGB-retro	0.408	0.343	182.7	68.6	81.4	21

* %Soluble Chemical oxygen demand (sCOD) of total chemical oxygen demand (tCOD).

In order to evaluate fermentation efficiency within the groups, we measured total and soluble chemical oxygen demand (COD) of fecal samples collected from subjects and calculated % soluble COD (SCOD) of total COD (TCOD), SCOD is the soluble fraction of TCOD that does not include solids and is a good proxy of fermentation efficiency or soluble calories remaining in feces. As seen in Table 7.2, %SCOD was greatest in the RYGB-base group and after the surgery the percentage dropped significantly. Percentage of soluble COD was similar in RYGB-12m and RYGB-retro groups. This finding indicated that due to differences in microbiome, a reduction in dietary calories (see Table 7.1), and microbial processes, the fermentation capacity of microbiome drops after the surgery.

Accumlation of hydrogen gas, a fermentation end product, can mediate fermentation pathways (Samuel & Gordon, 2006) the type and abundance of hydrogen consuming microorganisms will affect fermentation end products and their ammounts. Figure 7.5 shows abundances of *fthfs, mcrA,* and *dsrA* genes for homoacetogenesis. methanogenesis, and sulfate reduction, respectively. The copy numbers of the genes for methanogenesis and sulfate reduction were relatively lower than the genes for homoacetogenesis (Figure 7.5A). We observed an increase in *fthfs* copy numbers after RYGB surgery although these results were not similar to what we observed on the RYGB-retro group. *mcrA* copy numbers were significantly lower in the obese(-) group compared to the others. *dsrA* copy numbers did not significantly vary among the groups. Figure 7.5B demonstrates the expression levels of these genes before and after the RYGB surgery. RYGB-base group had the highest expression of *fthfs* gene and the lowest expression of the *mcrA* gene.

Previously, higher levels of methanogens were observed in obese populations in comparison to normal weight populations (Mathur *et al.*, 2013). High levels of methanogens were also observed in anorexic individuals compared to normal-weight individuals (Armougom, Henry, Vialettes, Raccah, & Raoult, 2009). In our study, methanogens were almost non-detectable in many of the subjects from RYGB-base group hence this is different to the previous reports where methanogens are associated with obese phenotype (Mathur *et al.*, 2013). Our presurgical morbidly obese population was on negative energy balance, this probably changed their gut microbial ecology and its interpretation based on host phenoytype. Breath hydrogen concentrations were not significantly different among the groups, even though there was an increase in breath hydrogen concentration after the surgery. This finding indicated that subjects in this study either did not generate significantly different amounts of hydrogen or had similar levels of hydrogen consumption activity. The expression of *mcrA* gene was relatively higher after the surgery and this finding was consistent with breath methane concentrations observed among the groups (Figure 7.5C). Similar to *mcrA* gene expression, *dsrA* expression was greater after the surgery. After the surgery, the subjects increased their protein consumption (Table 7.1), which might explain higher *dsrA* expression, an indication of sulfate reduction. Previously, animal based diets, which contain high levels of protein and fat, were shown to increase sulfate-reducing microorganisms (David *et al.*, 2014).

The expression results of hydrogen consumer genes were similar between RYGB-12m and RYGB-retro groups, which indicated RYGB surgery affects microbial hydrogen metabolism and have an impact on fermentation efficiency.

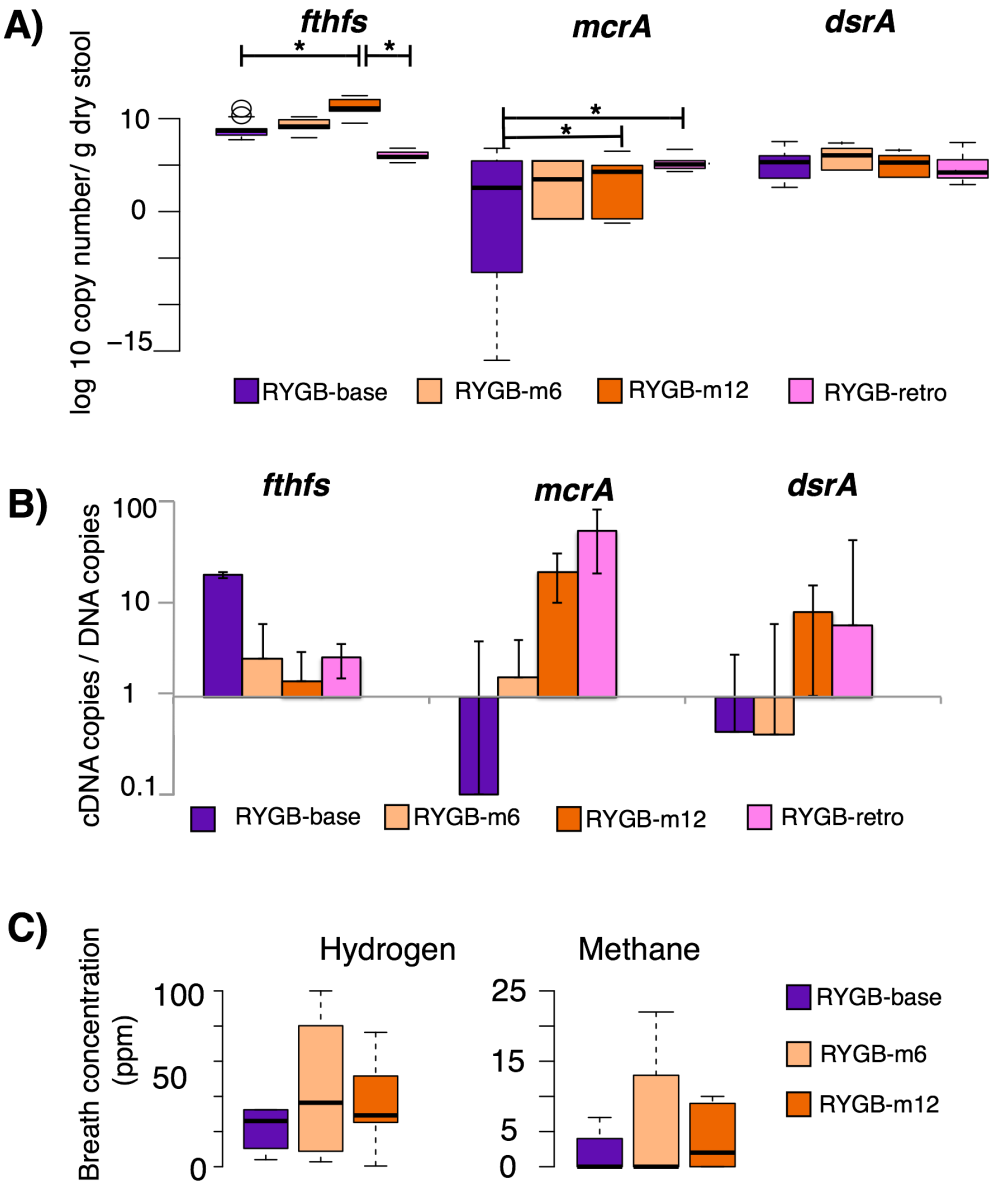

Figure 7.5. Hydrogen consumers in RYGB-base, RYGB-m6, RYGB-m12, and RYGB-retro (retrospective RYGB subjects) subjects. A) Q-PCR analysis of genes that are involved in hydrogen consumption: *fthfs* for homoacetogens, *mcrA* for methanogenesis, and *dsrA* for sulfate reducers. B) Expression of *fthfs*, *mcrA*, and *dsrA* genes. C) Breath hydrogen and methane concentrations before and after the RYGB surgery.

We looked also at abundance of branched-chain-amino-acids, as these were important findings in chapter 5. Fermentation of branched-chain-amino-acids increased after RYGB surgery. As seen in Figure 7.6A, the fecal concentrations of branched-chain-fatty-acids: isobutyrate and isovalerate increased after RYGB. The RYGB-retro and RYGB-12m groups had similar concentrations of these BCFAs; therefore we can deduce that an increase in the abundance of these BCFAs is a result of changes induced by bariatric surgery. Branched chain amino acids (BCAA): leucine, isoleucine, and valine were at significantly lower abundance 12 months after the surgery and their concentrations were comparable among RYGB-retro and RYGB-base groups, despite a higher protein consumption by this group (Figure 7.6C). Even though BCAA concentrations were variable, their fermentation products were always greater post-RYGB. Figure 7.6B shows that microorganisms in the human gut can ferment branched-chain amino acids such as leucine and isoleucine to branched chain fatty acids: isobutyrate and isovalerate. These species among others got enriched after RYGB. In summary, BCFA usually produced microbially from BCAA were at higher concentrations after surgery, BCAA were at lower concentrations despite higher protein consumption suggesting that higher BCFA concentrations were due to after RYGB induced higher microbial conversion rates from BCAA to BCFA. Overall, changes in microbiome due to RYGB surgery potentially alter luminal amino acid metabolism, and can contribute to weight loss by producing branched chain fatty acids that are capable of signaling free fatty acid receptors (Newgard *et al.*, 2009).

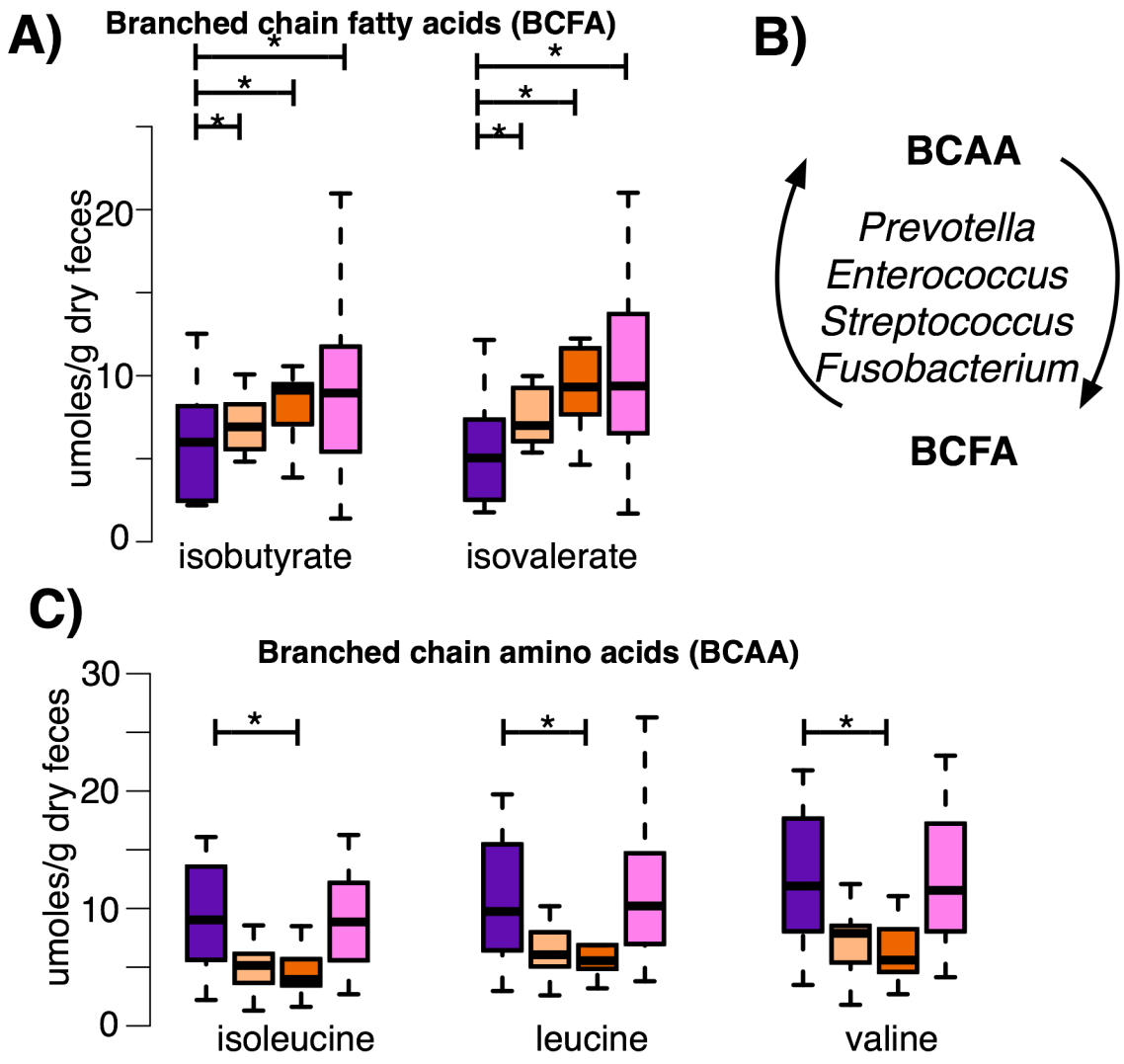

Figure 7.6. Branched chain amino acid and fatty acid metabolism. A) Branched chain fatty acids: isobutyrate and isovalerate measure with NMR after RYGB surgery prospectively and retrospectively. B) Concentrations of isoleucine, leucine, and valine, branched-chain-amino-acids (BCAA) measured with NMR after RYGB surgery prospectively and retrospectively. C) Microorganisms enriched post surgery that convert BCAA into branched chain fatty acids (BCFA). The color pattern in figure 7.2 was followed and colors: purple, light orange, orange, and pink represent RYGB-base, RYGB-6m, RYGB-12m, and RYGB-retro groups, respectively.

Besides SCFAs and BCFAs, we analyzed widespectrum of metabolites. Most of the metabolites detected with H-NMR and GC-MS were at greater abundance in the RYGB-baseline group and after the sugery their concentrations dropped (Tables 7.3 and B.1). Considering that fecal water was analyzed with NMR and lyophilized fecal matter was analyzed with GC-MS, we observed variations in the metabolites detected with these techniques. NMR technique provided mainly volatile and water-soluble compounds, whereas GC-MS identified many metabolites of the undigested nutrients and components of microbial cells. The majority of the metabolites detected were significantly more abundant in the baseline samples compared to 12-months after the surgery samples, however as shown in Table 7.3 besides isovalerate and isobutyrate, concentrations of xylose and isopropanol increased after RYGB surgery**.** Greater abundance of fecal xylose after the surgery indicates whether the subjects adapted more plant based diets, or they lost some microbial hydrolytic capabilities to breakdown xylose. Fiber intake of the subjects did not increase or decrease significantly after the surgery, although it was statistically lower in post RYGB subjects compared to NW subjects. Our results on fiber intake along with fecal xylose lead us to speculate that a reduction of microbial pathways that are involved in xylose degradation occurred after RYGB surgery. An increase in the concentration of isopropanol after the surgery is possibly due to enrichment of *Clostridia* species in the lumen, as many *Clostridia* species are known to produce isopropanol (Lee, Chou, Ham, Lee, & Keasling, 2008). Although health effects or weight loss effects of isopropanol on host physiology are unknown, increased concentrations of isopropanol accompanied with weight loss suggest that isopropanol might be a future target for microbiome associated weight loss detection and enhancement strategies.

Table 7.3. Dry weight concentrations of luminal metabolites that were statistically different between RYGB-baseline and RYGB-12months samples**.**

	NW	RYGB baseline	RYGB-6months	RYGB-12months
Alanine	10.05	16.49	9.72	6.89
Cadaverine	0.22	0.77	0.58	0.73
Glucose	5.84	26.14	6.08	3.94
Glutamine	3.54	5.83	2.61	2.54
Isopropanol	0.17	0.08	0.20	0.20
Methanol	0.92	1.70	1.10	0.67
Succinate	2.89	4.56	2.51	2.06
Taurine	0.66	1.31	1.00	0.85
Threonine	1.01	1.94	0.70	0.68
Thymidine	0.00	8.00	0.00	0.00
Tyrosine	0.24	0.82	0.00	0.00
Uracil	0.66	1.52	0.76	0.52
Uridine	4.99	7.80	4.84	3.40
Valerate	2.47	3.38	1.85	2.05
Valine	0.00	1.31	0.00	0.00
Xylose	7.92	9.57	13.92	16.36
Leucine	6.65	9.74	6.06	5.55
Lysine	4.44	7.38	3.76	2.54
Isoleucine	5.40	9.03	5.14	3.96
Isovalerate	4.97	5.04	7.00	9.32
Isobutyrate	4.83	5.99	6.92	9.12

Based on GC-MS results, amino acids and sugars were mainly at greater abundance at baseline compared to 6-months and 12-months (Table B.1). Concentrations in the RYGB-retro group were similar to RYGB-12m group. This could be due to unvarying post-RYGB diets in combination with the altered GI tract due to surgery. Interestingly, the concentrations of many saturated fats increased after the surgery. This could be due to a reduction of fatty acid absorption and reduced bile acid secretions (see below) due to the altered GI after the surgery.

7.4 RYGB Surgery Drops Fecal Bile Acid Concentrations

Figure 7.7 shows the fecal bile acids measured at baseline, 6 months and 12 months after the RYGB surgery. Fecal concentrations of primary bile acids: CA and CDCA and their glycine and taurine conjugated forms (TCA, GCA, TCDCA, and GCDCA) significantly dropped 6 months after the surgery, and remained at similar concentrations even at 12 months after the surgery, as seen in Figure 7.7A.

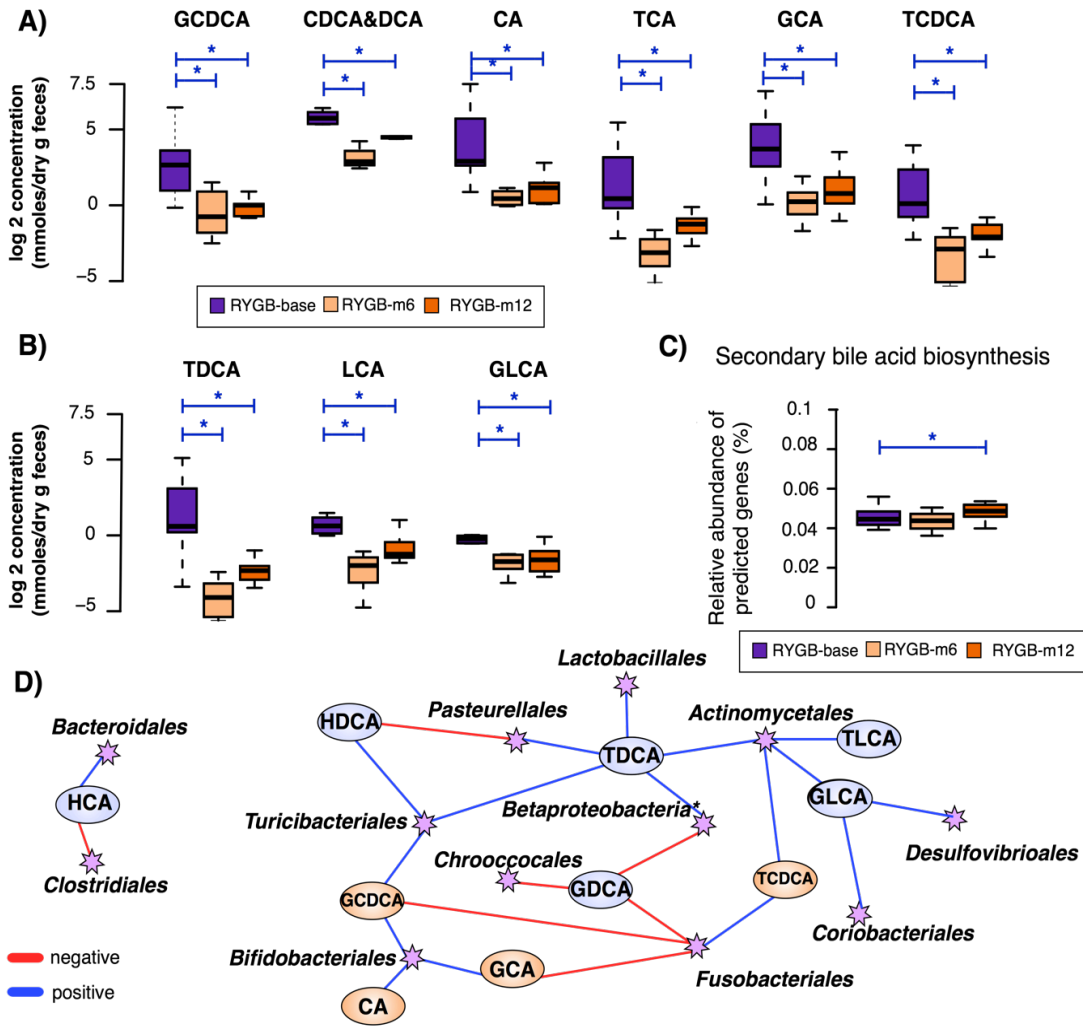

Figure 7.7. Fecal bile acids measured before and after RYGB surgery. A) Fecal primary bile acids. B) Fecal secondary bile acids that are produced by gut microbiota. C) Bile acid biosynthesis genes predicted via PICRUSt. D) Fecal bile acid and microbiome co-occurrence network based on Spearman's rho correlation coefficient.

Figure B.2 illustrates the conjugation and transformation reactions of CA and CDCA and secondary bile acids produced from them by gut microbiota. Our findings show that not only primary bile acids, but also microbially produced secondary bile acids significantly diminished after RYGB surgery, possibly due to less amounts of primary bile acids. Interestingly, abundances of PICRUSt (Langille *et al.*, 2013) predicted genes (Figure 7.6C) of secondary bile acid biosynthesis were greater after the surgery even though the secondary bile acid concentrations dropped after the surgery.

The concentrations of secondary bile acids, solely produced by the gut microbiota (Jason M. Ridlon, Kang, Hylemon, & Bajaj, 2014), were also greater at the baseline in comparison to 6 months or 12 months after the surgery. As seen in Figure 7.7B, the concentrations of the following secondary bile acids: LCA, its glycine conjugated form GLCA and taurine conjugated DCA (TDCA) significantly dropped after surgery.

Table B.2 summarizes the median concentrations of primary and secondary bile acids observed in NW and retro-RYGB groups in comparison to RYGB-12m and RYGB-base groups. The concentrations observed in RYGB-retro group were similar to the RYGB-12m group, which indicates that the response of surgical modification on bile acid metabolism is very strong and can be predicted even if the baseline time-points before the surgery are missing. Interestingly, bile acid levels of post-RYGB groups were similar to NW. Overall, our findings indicate that fecal concentrations of primary and secondary bile acid drop after RYGB surgery, and lower levels that are similar to normal-weight individuals were maintained even years after the surgery.

Considering that gut microbiota can produce secondary bile acids (J. M. Ridlon, Kang, & Hylemon, 2006) and deconjugate primary bile acids (Jason M. Ridlon *et al.*,

2014), and concentrations of bile acids can affect gut microbiota composition (Jason M. Ridlon *et al.*, 2014), we performed co-occurrence network analysis between order-level microbial phylotypes and bile acids. As shown in Figure 7.7D, many secondary bile acids such as TDCA, HDCA, GDCA, TLCA, and GLCA positively correlated with *Actinomycetales* and *Turicibacteriales* phylotypes, *Actinomycetales* contain primary bile acid hydrolyzing microorganisms (Islam *et al.*, 2011), although microorganisms from the *Turicibacteriales* order have not been previously described as secondary bile acid producers. *Bifidobacteriales* order positively correlated with primary bile acid concentrations: CA, GCA, and GCDCA. Strikingly, *Fusobacteriales*, which gets enriched after RYGB negatively correlated with the concentrations of those primary bile acids.

Our findings are different to previous studies (Pournaras *et al.*, 2012; Sayin *et al.*, 2013), which reported increased bile acids, especially secondary bile acids after RYGB surgery in blood samples. One difference among those studies and ours is that we measured fecal samples whereas the others measured serum ones; hence these findings are not comparable. Fat and cholesterol intake play a big role in the production and secretion of bile acids (Jason M. Ridlon *et al.*, 2014). As seen in Table 7.1, the subjects did not reduce the fat % of their diets although they consumed lower calories after RYGB, which overall leads to lower amounts of fat being consumed. Lower delivery of fat to the GI tract might have played a role in the lower concentrations of primary and secondary bile acids measured in this study in feces.

Given that bile acids have been reported to modify the gut microbiome (Jason M. Ridlon *et al.*, 2014), lower delivery of bile acids to the colon might have played a role on some of the microbiome compositional changes observed.

7.5. Mucosal-microbiome structure and composition changed after RYGB.

We expand our analysis on gut microbiome and also investigated whether changes in the luminal microbiome were prominent in the mucosal microbiome as well. Using weighted and un-weighted Unifrac metrics (Lozupone *et al.*, 2006), we investigated changes in mucosal and luminal communities after RYGB. As seen in Figure 7.8A, the location microbes occupy in the colon (luminal or mucosal) had the greatest effect on clustering based on unweighted Unifrac metric, which enhances the differences in microbiome based on presence of unique phylotypes allowing to quantify changes in minor taxa. This change was statistically different, this is illustrated in Figure 7.8B.

A key difference between mucosal and luminal surfaces is the substrates that are available to microorganisms; in luminal surfaces, substrates are usually dietary molecules whereas in mucosal surfaces they are host-derived glycans (Hooper *et al.*, 2002). Another difference is the electron acceptor type on mucosal and luminal surfaces (Espey, 2013). Oxygen derived from the eukaryotic tissues is gradually depleted in the mucosal layer by facultative anaerobes and therefore, luminal surfaces become anaerobic (Zoetendal *et al.*, 2002). Microorganisms that live on luminal surfaces are also affected by other host-associated factors such as transit time, frequency of dietary intake and bile acids (G. T. Macfarlane & Macfarlane, 1993).

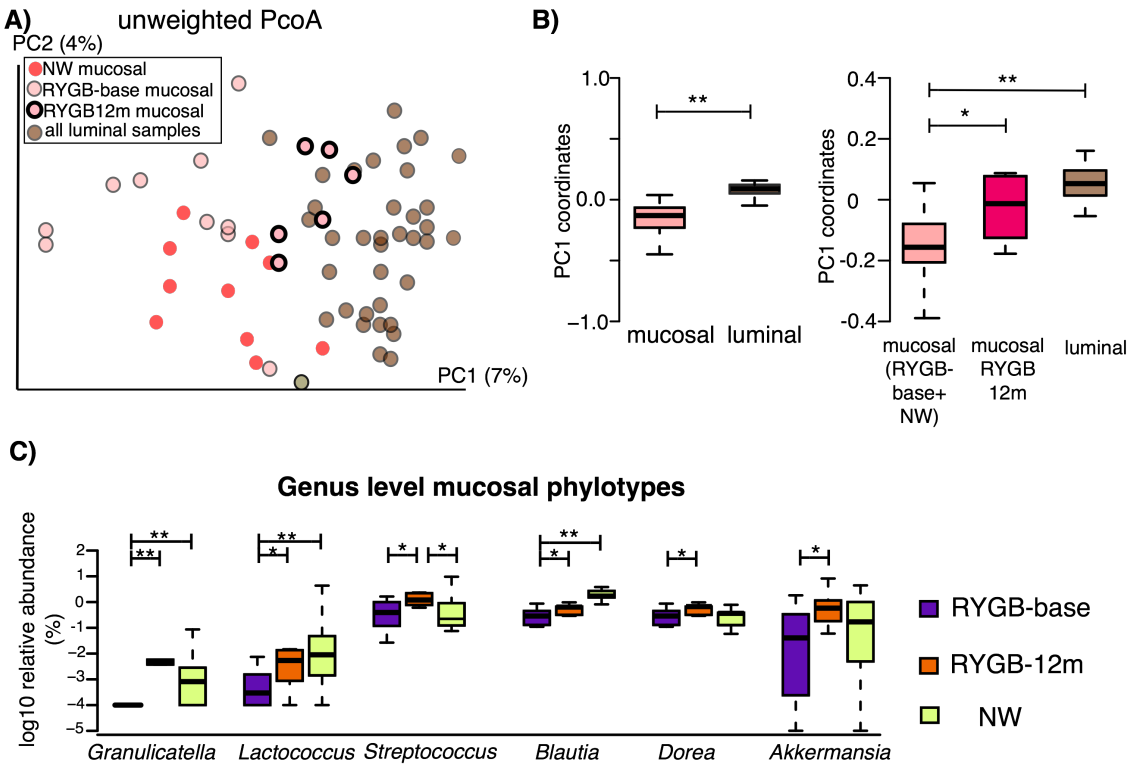

Figure 7.8. Mucosal and luminal microbiome after RYGB surgery. A) Unweighted Unifrac analysis of luminal and mucosal samples after RYGB. B) Location of mucosal and luminal samples on PC1 coordinate. The location of mucosal RYGB-12m samples was significantly different than other mucosal and closer to luminal samples. C) Genus-level phylotypes that were significantly higher after RYGB surgery in the mucosa.

The effect of surgery on mucosal microbiome is reflected on PC1; before and after surgery samples formed two separate clusters (Figure 7.8A). Additionally, mucosal baseline RYGB and RYGB-12m samples were distant from the mucosal NW samples. Interestingly, mucosal microbiomes after RYGB were more similar to luminal communities than the mucosal microbiome of presurgical morbidly obese and normal weight individuals. Based on unweighted Unifrac distances, RYGB-12m mucosal samples appeared closer to luminal samples than mucosal samples on PC1, which indicated that possible outward movement of mucosal microbiome towards lumina or

inward movement of luminal microbiome towards mucosa after RYGB surgery. This finding indicated that possibly due to a reduction in the nutrient stream, luminal microbiota moved towards to mucosa for resources and colonized the mucosal surfaces, which resulted in the observation of luminal phylotypes in the mucosal space.

Mucosal microbial communities are important in obesity and diabetes research because inflammation has been suggested as one of the factors that contribute to development of these conditions (Devaraj, Hemarajata, & Versalovic, 2013). Previously disrupted gut barrier has been associated with the development of obesity and inflammation (Brun *et al.*, 2007) and microbiome has been associated with obesity and inflammation in animal studies (Everard *et al.*, 2013). Mucosal microbiome characterization in the context of obesity has been limited to only a few animal studies (Cani *et al.*, 2007; Everard *et al.*, 2013; Kellermayer *et al.*, 2011) and changes in mucosal microbiome has not been characterized for post-RYGB subjects. Our findings contribute to the understanding of microbiome changes in the context of weight loss both in the luminal and the mucosal space. Additionally, both short-term and long-term changes in the luminal microbiome structure have not been evaluated until our study. Our data showed that the luminal microbiome changes as early as 6 months after surgery and that a unique RYGB luminal microbiome gets established and is sustained in the longer-term or at least until one year post surgery.

Six genus-level phylotypes got significantly enriched in the mucosa after RYGB surgery; these phylotypes were *Granulicatella*, *Lactococcus*, *Streptococcus*, *Blautia*, *Dorea*, and *Akkermansia*, as shown in Figure 7.8C. Relative abundances of these phylotypes were also greater in the NW mucosa compared to the RYGB-baseline

mucosa. After the surgery, abundances of these phylotypes in the RYGB mucosa became similar to the abundance observed in the NW mucosa. Besides *Akkermansia*, the remainder microorganisms are from *Firmicutes* phylum and have capabilities to form biofilms and play a role in lactate metabolism (Antharam *et al.*, 2013). *Lactococcus*, *Streptococc*us, and *Granulicatella* are lactate-producing microorganisms, whereas *Dorea* and *Blautia* are lactate oxidizers (Jost, Lacroix, Braegger, & Chassard, 2013). *Streptococcus* and *Lactococcus* species have been used as probiotics to enhance gut barrier and integrity (Radha & Goethe, 2013); weak gut barrier has been associated with the development of fattiness (Everard *et al.*, 2013). *Akkermansia* is a known mucin degrader, and in animal models, its presence has been shown to improve gut barrier, reduce fattiness of the host organs, and protect against insulin resistance and obesity (Derrien, Vaughan, Plugge, & de Vos, 2004; Everard *et al.*, 2013). An increase in the relative abundance of *Akkermansia* in mice after RYGB has been reported (Liou *et al.*, 2013) and previously by our group in human fecal samples (Zhang *et al.*, 2009), our results validate these findings.

Conclusion

We demonstrated the impact of RYGB surgery on gut microbiome, metabolome, and bile acid concentrations. Changes in fecal metabolome mirrored the changes in luminal microbiome, which showed that composition and quantities of dietary compounds reaching the colon were altered after the surgery, hence substrate availability to the gut microorganisms was altered too. As a result microbial metabolite profiles changed after the surgery. Bile acid delivery to the colon diminished after the surgery and as a result microbiome and metabolome profiles were affected. For the first time, we

showed that changes in gut microbiome after RYGB are not limited to luminal space; due to surgical alterations and nutritional limitations; microorganisms of the mucosal space were permanently transformed too. The changes in microbiome were more prominent in the luminal space; possibly due to greater environmental changes occur in lumina than mucosa after RYGB.

CHAPTER 8

KEY FINDINGS AND RECOMMENDATIONS FOR FUTURE WORK

8.1. Key Findings

The gut microbiome has been associated with development of obesity, but microbiome structure and function in weight-gain and weight-loss have not been fully explored due to the complexity of the problem. Bariatric surgery provides a unique aspect to obesity-microbiome research, because it alters the conditions in the gut and in return changes the microbiome and it makes it possible to study changes in microbiome in the context of sustainable weight loss. My research advances the knowledge on microbiome in obesity, surgical weight loss and host energy metabolism. My work details and provides comprehensive analysis on; (i) how environmental factors such as gut pH after bariatric surgery affect the microbiome, (ii) differences in microbiome structure of different post-bariatric surgery patients, (iii) the contribution of microbiome to sustainable weight loss, and (iv) spatial and temporal changes in microbiome after Roux-en-Y gastric bypass surgery.

After Roux-en-Y gastric bypass, reduction in gastric acid secretions and rearrangement of the gastrointestinal tract alter colonic environment (Aron-Wisnewsky *et al.*, 2012). Reduction in gastric acid secretion due to proton pump inhibitors has been shown to affect gut microbial communities (Seto *et al.*, 2016). A possible explanation of the altered microbiome after RYGB could be increased pH along the gastrointestinal tract. Typically, microorganisms of the oral cavity and ingested microorganisms through our diets are eliminated in the stomach or small intestine due to harsh acidic conditions (Lang *et al.*, 2014). Lower gastric acid exposure after RYGB might enhance the survival

of oral microorganisms in the colon. In Chapter 3, I evaluated whether small differences in colonic pH with varying levels of buffering has an impact on development of microbial communities from fecal inoculum and community function. My fecal enrichment cultures at three different initial pH (6.0, 6.5, and 6.9) showed that even small differences in pH could set off great differences in microbiome structure and function. Abundances of microbial phylotypes from *Streptococcus* and *Veillonella*, *Bacteroides* and *Escherichia*; phylotypes commonly found in dental plaques (Al-Ahmad *et al.*, 2007) responded differently to the varied pH conditions..

Besides their growth, these microorganisms' functions and microbial interactions were mediated by pH. In Chapter 4, I investigated the impact of small variations in starting pH on metabolic interactions of lactate-producing and -consuming communities. After enriching these microorganisms at different initial pH, I characterized their metabolisms and functions in mono- and co-cultures. I demonstrated that *Veillonella* and *Streptococcus* enhance each other's growth and metabolism, whereas versatile microorganisms that contribute to redundancy of gut function such as *Bacteroides* do not rely on other microorganisms to provide resources. Due to sensitivity of *Veillonella* to acidic environments, lactate produced by *Streptococcus* accumulated at pH 6.0, and *Veillonella* fermented lactate into acetate and propionate at pH 6.5 and 6.9. Our results from this chapter provide insights into pH-derived changes in gut microbiota and metabolism after RYGB.

In Chapter 5, I demonstrated differences in microbial community structure and function in patients that underwent two different types of bariatric surgeries: Roux-en-Y gastric bypass (RYGB) and laparoscopic adjustable gastric banding (LAGB).

Differences in microbiome in post-RYGB and nonsurgical controls have been demonstrated before (Zhang *et al.*, 2009, Tremaroli *et al.*, 2015) although findings were summarized for bariatric surgeries in general. Since LAGB surgery is relatively unsuccessful compared to RYGB, I tested the hypothesis that microbiome evolves differently after different types of bariatric surgeries, and have varying effects on weight loss. The greater changes imposed on microbiome after RYGB due to anatomical rearrangement compared to LAGB resulted with different microbiomes and different weight loss achievements. Different microbiomes as a result of different surgeries affected the distribution of fermentation end products. Branched chain fatty acids that are fermentation products of branched chain amino acids were greater in fecal metabolomes after RYGB compared to LAGB or nonsurgical subjects. Furthermore, our work also showed that microbial products that are associated with appetite regulation such as butyrate and propionate were significantly higher in post-RYGB subjects compared to post-LAGB subjects.

After identifying key differences in microbiome structure between LAGB and RYGB surgeries, I focused on microbial components that made RYGB surgery more successful. Even though RYGB microbiome structure has been known, which gut microbes or microbial metabolites contribute to the success of the surgery is unknown. In Chapter 6, I first addressed the problem of defining successful surgery, as the definition can be relatively subjective. In the scope of this chapter, I found that *Fusobacterium* consistently shows up in unsuccessful subjects regardless of the definition of success. In this chapter, I also showed that putrescine, a microbial degradation product of ornithine, which was produced by mainly *Fusobacterium*, was at greater concentration

in unsuccessful subjects. Putrescine has been associated with weight gain in animals, therefore my results from this chapter indicate that putrescine along with *Fusobacterium* could be important targets to manage the success of RYGB surgery.

Human microbiome research, especially in the context of obesity, has relied heavily on cross-sectional studies based on single time-point stool collections. Therefore, it was essential to investigate longitudinally luminal and mucosal changes in the gut microbiome after RYGB surgery. In Chapter 7, I documented those microbial and metabolic changes in the luminal space 6 months and 12 months after RYGB as well as previously undocumented changes in mucosal microbiome after RYGB surgery. My findings reveal that changes in microbiome occur in the luminal and the mucosal space and the scale of changes in the luminal space are much greater. I also demonstrated that wide-spectrum changes in gut metabolism, from microbially produced fatty acids, amino acid degradation products to altered bile acids, occur as short as 6 months after RYGB and permanent. Additionally, by comparing the results of this cohort study to the findings of Chapter 5, which is a case control study, I demonstrated the microbial and metabolic changes after RYGB are due to surgery and not due to diet or other interpersonal variations.

Finally, I present research ideas that can enhance microbiome research in the field of obesity, and for continuation of this dissertation.

8.2 Recommendations for future work

8.2.1 Develop Continuous Flow Stirred Reactors to Understand Early Colonization of the Gastrointestinal Tract and Factors that Can Affect Colonization

The pH and substrate gradients along the gastrointestinal tract (GI) initiate colonization of microorganisms to different sections of the colon. Moreover, many oral and food associated microorganisms that are acid-sensitive and cannot colonize the gut because the gastric acid in the GI tract eliminates them (Lang *et al.*, 2014). Small changes in colonic pH after RYGB surgery due to reduced gastric acid secretions could be a major factor affecting the development of post-RYGB microbiome. In Chapter 3, I demonstrated that small differences in initial pH have tremendous effects on the development of fecal communities, and especially microorganisms involved in lactate and propionate metabolism.

Findings from Chapter 3, Chapter 4, and reports from the literature suggest that RYGB surgery interferes with the colonization of oral or diet-associated microbiome in the downstream GI tract. To better understand colonization of the gut under the influence of gastric pH and after RYGB, I propose to develop continuous stirred flow reactors (CSTRs) in sequence similar to the human gut reactor Simulator of Human Intestinal Microbial Ecology (SHIME) (Molly *et al.*, 1994) but with more components. One CSTR will generate the oral microbiome and be fed twice daily with a media representing the average Western diet (McDonald *et al.*, 2013). The "oral" CSTR will be connected to two stomach CSTRS in parallel. Both stomachs will have same amount of acid, the pH will set between 1-2. The first stomach reactor will retain contents of oral CSTR for one hour, i.e. a normal GI tract, whereas the second one for 15 minutes, i.e. the RYGB GI

tract. Both stomach CSTRs will deliver their contents to two separate and additional CSTRs that represent colons. The first colon CSTRs for both RYGB and normal GI reactors will have fecal seeds; the second CSTRs will be sterile from the beginning to understand microbial community function.

This design will allow the researcher to test two kinds of hypotheses: First, reduction in gastric acid secretions or lower exposure time to gastric acid after RYGB allows survival of pH sensitive microorganisms from the oral cavity or food. Second, changes in gastric acid production after RYGB changes the microbial interactions and synthrophies in the colon.

8.2.2 Role of Branched Chain Amino Acids and Fatty Acids on Host Energy Regulation

Gut microbiota ferments dietary carbohydrates and produce mainly short chain fatty acids (SCFAs) (acetate, propionate, and butyrate), and some minor products such as hydrogen gas (Macfarlane & Macfarlane, 2003). Fermentation of proteins yields SCFAs and many phenoloic and indolic compounds. Fermentation of branched chain amino acids (leucine, valine and isoleucine) produces branched chain fatty acids (isobutyrate, isovalerate, 2-methylbutyrate (Windey *et al.*, 2012).

Protein fermentation in the gut is limited due to efficient digestion and absorption of dietary proteins in the duodenum and small intestine (Borgstrom *et al.*, 1957); therefore protein fermentation products are often neglected in gut microbiome studies. Protein fermentation products were previously observed at greater concentrations in the subjects that were on animal-based diet compared to plant-based diet (David *et al.*, 2014) and in subjects with colorectal cancer (Wang *et al.*, 2012).

SCFAs produced by gut microbiome can interact with the host epithelial cells; for instance, butyrate is the energy source of colonyctes, and a reduction in its production was associated with the weakening of the integrity of the colonic epithelial layer (Wang *et al.*, 2012). SCFA can also serve as signaling molecules for endogenous free fatty acid receptors and protect against diet-induced obesity (Lin *et al.*, 2012). Furthermore, isobutyrate and isovalerate have been shown to signal those receptors (Di Paola & Lorusso, 2006), but did not receive much attention due to their lower concentrations in the gut.

In Chapter 5, 6, and 7, I demonstrated BCFAs (isobutyrate and isovalerate) were at greater concentrations in post-RYGB subjects, mainly in the highly successful ones. Subjects that undergo RYGB surgery often develop malabsorption against some nutrients and proteins are speculated to be one of those (Odstrcil *et al.*, 2010).

I propose to study the effect of greater production of these BCFAs on host metabolism and energy regulation. Whether these products are more or less potent against these receptors are unknown. For this study, I propose a colonic epithelial tissue culture study. Main BCFA producers such as monocultures of *Enterococcus* (Hume *et al.*, 2004) can be grown on BCAA media and the filtered supernatant of these media can be exposed on single layer of epithelial cells. Using qPCR techniques, activities of these receptors can be quantified. The proposed study will help the understanding of branched chain amino acid fermentation on host metabolism and, in particular, on signaling pathways. It will also provide an understanding on how changes in the gut microbiome post-RYGB can alter host metabolism.

8.2.3 Understand the Role of Hydrogen on Energy Regulation

The role that gut microorganisms play in host weight gain or loss is unknown. However, the products of fermentation, like short chain fatty acids (SCFAs) and gases (CO_2, H_2, CH_4), are known to contribute to host energy balance (Boden & Shulman, 2002). Therefore, it stands to reason that microbial fermentation products regulate weight gain and loss. The main SCFAs of the human gut microbiota are acetate, propionate, and butyrate (Macfarlane & Macfarlane, 1993) and an increase in the fecal acetate concentration is associated with weight gain (Jumpertz *et al.*, 2011), because acetate can be converted into fat by the liver (Yamashita *et al.*, 2001). In contrast, fecal propionate is associated with weight loss via a mechanism that involves appetite regulation (Sleeth *et al.*, 2010, Arora *et al.*, 2011).

Acetate-producing fermentative reactions evolve H_2 gas, while propionate-producing reactions consume H_2 gas (Mitsumori & Sun, 2008). Hydrogen accumulation inhibits acetate-producing fermentation (Shock & Helgeson, 1990) and stimulates propionate-producing reactions. Besides propionate producers, H_2 produced in the gut can be consumed by hydrogen-consuming microorganisms: methanogens, sulfate reducers, and homoacetogens (Gibson *et al.*, 1990). Methane and hydrogen have also been associated with host metabolism: breath methane and hydrogen concentrations were higher in obese subjects with greater body mass index than lower body mass index (Mathur *et al.*, 2011, Mathur *et al.*, 2013). Moreover, methanogens in the gut were at higher abundance in the obese subjects compared to normal-weight or post-RYGB surgery subject (Zhang *et al.*, 2009). Other studies found methanogens detected at greater abundance in anorexic individuals (Armougom *et al.*, 2009) and depleted in obese

individuals (Million *et al.*, 2011). Co-habiting the most abundant methanogen of the human gut: *Methanobrevibacter smithii* with a polysaccharide degrading bacteria: *Bacteroides thetaiotaomicron* in gnotobiotic mice showed that *M. smithii* enhances polysaccharide degradation and acetate production of *B. thetaiotaomicron* by removing the accumulated H_2 (Samuel *et al.*, 2007).

In Chapter 7, I summarized the results for hydrogen and hydrogen consuming microorganisms. It is necessary to study, *in-vitro,* how H_2 partial pressure influences acetate and propionate production with a reactor that simulates the conditions of the human gut. I propose to study this concept using a reactor that was developed in our laboratory, the Membrane Biofilm Reactor (MBfR) (Lee & Rittmann, 2000), which delivers a constant H_2 partial pressure through hollow fibers. Hydrogen-consuming microorganisms form biofilms by attaching to the membrane surface and consuming hydrogen. The unique design of MBfR provides a steady H_2 partial pressure while retaining microorganisms and mimicking the conditions in the gut, such as pH, temperature, and retention time.

I propose two hypotheses to be tested with this design: (1) H_2 gas accumulation due to fermentative reactions in the gut promotes the production of the weight loss-associated microbial metabolite propionate and inhibits the production of weight gain-associated metabolite acetate. (2) The type and abundance of H_2-consuming microorganisms (homoacetogens, methanogens, and sulfate reducers) in the gut contribute to weight balance via acetate, methane, or H_2S production. By applying different constant H_2 partial pressures to fermentative communities, and comparing the resulting microbiota and metabolites (especially acetate and propionate ratios), it will be

possible to identify how different H_2 partial pressures shape gut microbiota and contribute to host energy balance.

Identification of H_2 gas-regulated gut microbial dynamics can lead to weight loss therapies by supplementing the gastrointestinal tract with right type of microorganisms (probiotics). Obesity, a disease that affects millions of people and costs billions of dollars annually, often cannot be treated with exercise and diet regimens. Gut microbiota-based approaches can provide cost-effective solutions.

Findings from this study will enable subsequent studies that involve the transplant of laboratory-derived microbial cultures with desired properties to mouse gastrointestinal tracts and finally lead to clinical trials.

8.2.4 Understand Gut-Brain Connection: Develop Microcosms and Test What Makes Post-RYGB Microbiome Induce Weight-Loss

RYGB is considered as a metabolic surgery due to its beneficial effects on host health (Li *et al.*, 2011). It induces healthier dietary habits and improves physiology and psychology of the patients (Holsen *et al.*, 2015). Changes in hormonal response and metabolic improvement have been observed in a number of different studies (Vincent & le Roux, 2008, Berthoud *et al.*, 2011, Nannipieri *et al.*, 2013). Additionally, neurobiological changes after RYGB has been reported (Romanova *et al.*, 2004) and altered serotonin signaling has been shown in mice that underwent RYGB surgery (Carmody *et al.*, 2015).

Most recently, gut microbiome have been demonstrated to play a role in hormonal and neurobiological regulation (Lin *et al.*, 2012, Sayin *et al.*, 2013, Ryan *et al.*, 2014). Given the evidence that gut microbiome can alter hormonal and neurobiological

responses and alterations in those responses after RYGB were associated with weight loss, I propose to study changes in hormonal response and microbiome in humans and in animals after RYGB surgery. The first phase of the study will be focusing on analysis of human microbiome before and after surgery along with fecal, urinary, and blood metabolomes and screening for hormonal and neurological changes in host body. This study will be the extension of Chapter 7, which includes an extra component of understanding of metabolic changes in the host physiology.

I propose a more targeted approach for the second phase of this study. After characterizing which microorganisms are associated with the induction of hormonal and neurological response, I propose the development of multiple-species microcosms that are involved in neurotransmitter and hormone production. In particular, I propose the study of microorganisms that alter weight loss or behavior associated metabolites such as serotonin, peptide YY, and ghrelin which were associated with the regulation of host metabolism.

Third phase of this study will include transplant the fecal microbiome into germ-free mice from successful and unsuccessful RYGB subjects that did or not demonstrate altered hormonal response, weight loss and behavioral improvements. Microbiome transplants from the laboratory-developed microcosms to germ-free mice should be performed to demonstrate which microorganisms or microbial interactions are responsible of hormonal, neurological, and metabolic changes after RYGB.

The outcomes of this study will strengthen the tie between microbiome and host energy balance, and also will show that RYGB is also a microbial surgery. The findings

will also aid the development of microbiome-associated therapies on individuals with obesity, metabolic condition and psychological problems.

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APPENDIX A

MICROBIAL COMMUNITY STRUCTURE AND SUBJECT METADATA RESULTS THAT SUPPORT FINDINGS OF CHAPTER 5

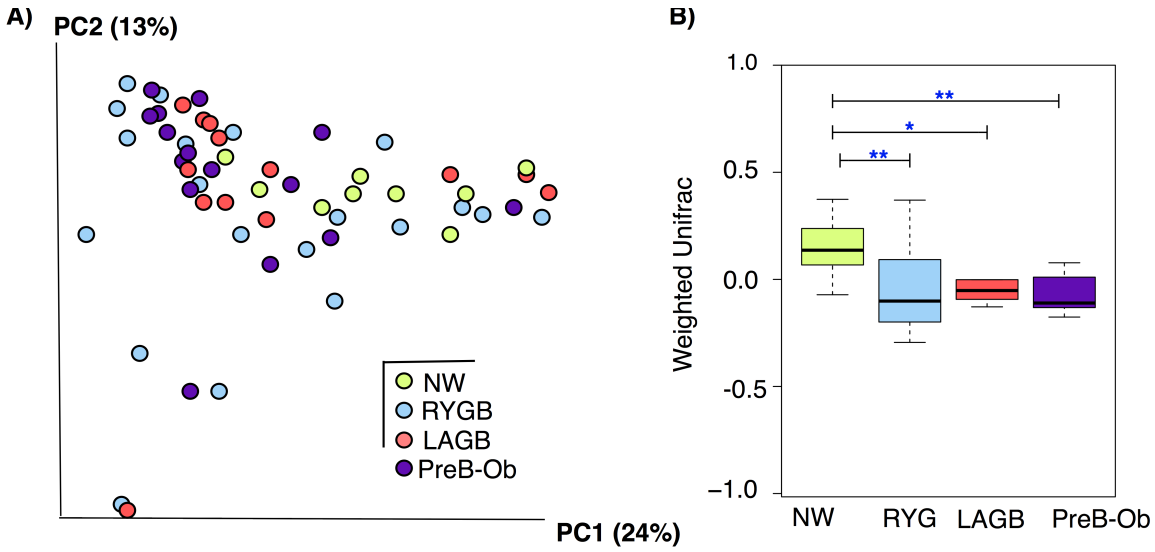

Figure A.1. Microbial community analysis of dominant OTUs after RYGB and LAGB surgeries compared to NW and PreB-Ob controls. A) Weighted Unifrac analysis illustrated on Principal coordinates. B) Median PC1 coordinates illustrated for each group. * Mann Whitney U test $P < 0.05$ and **Mann Whitney U test $P < 0.01$.

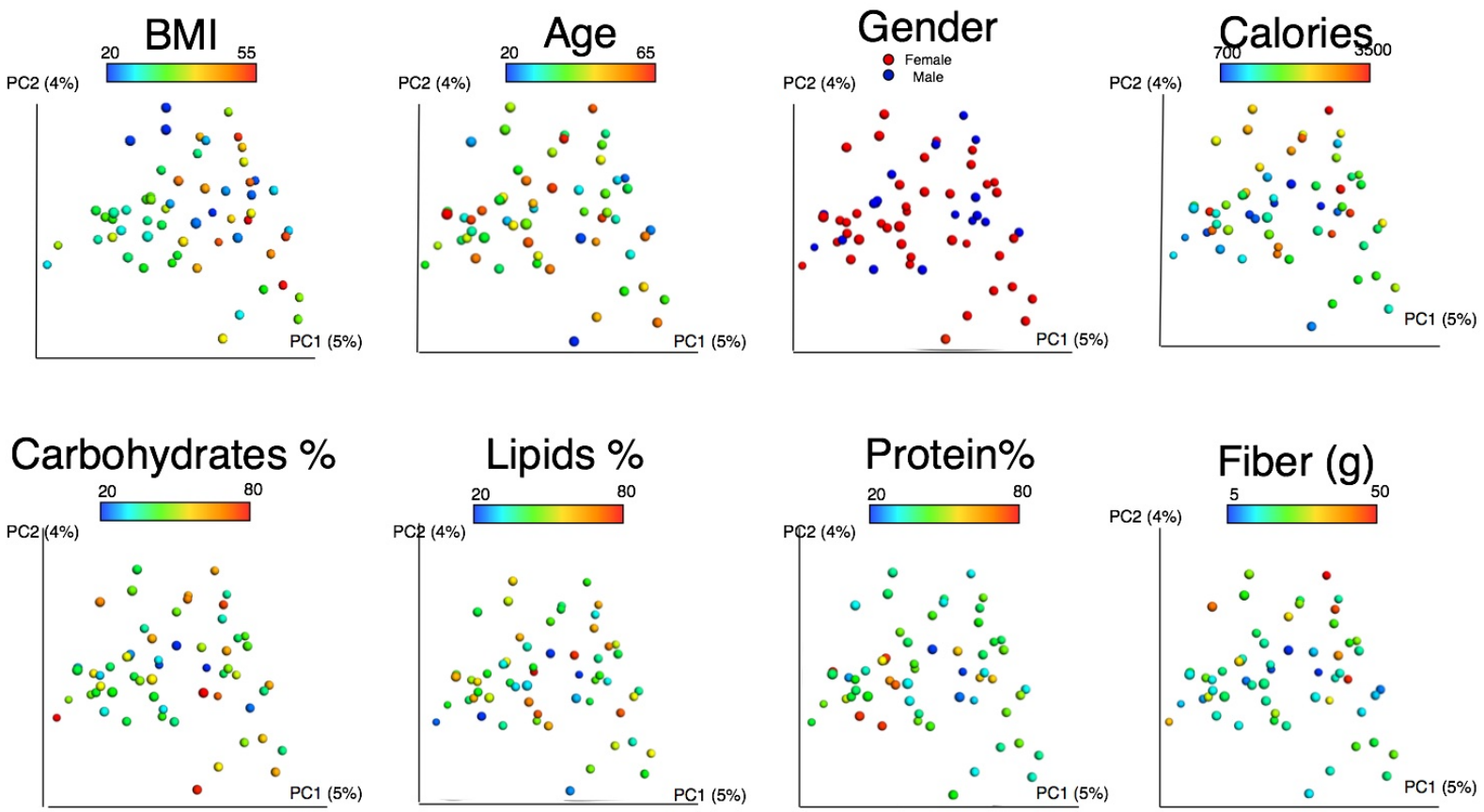

Figure A.2. Unweighted Unifrac distance visualized on principal coordinates. Color changes are based on body mass index (BMI), age, gender, total calories consumed, carbohydrate, lipid and protein fractions of the diet, and fiber consumption.

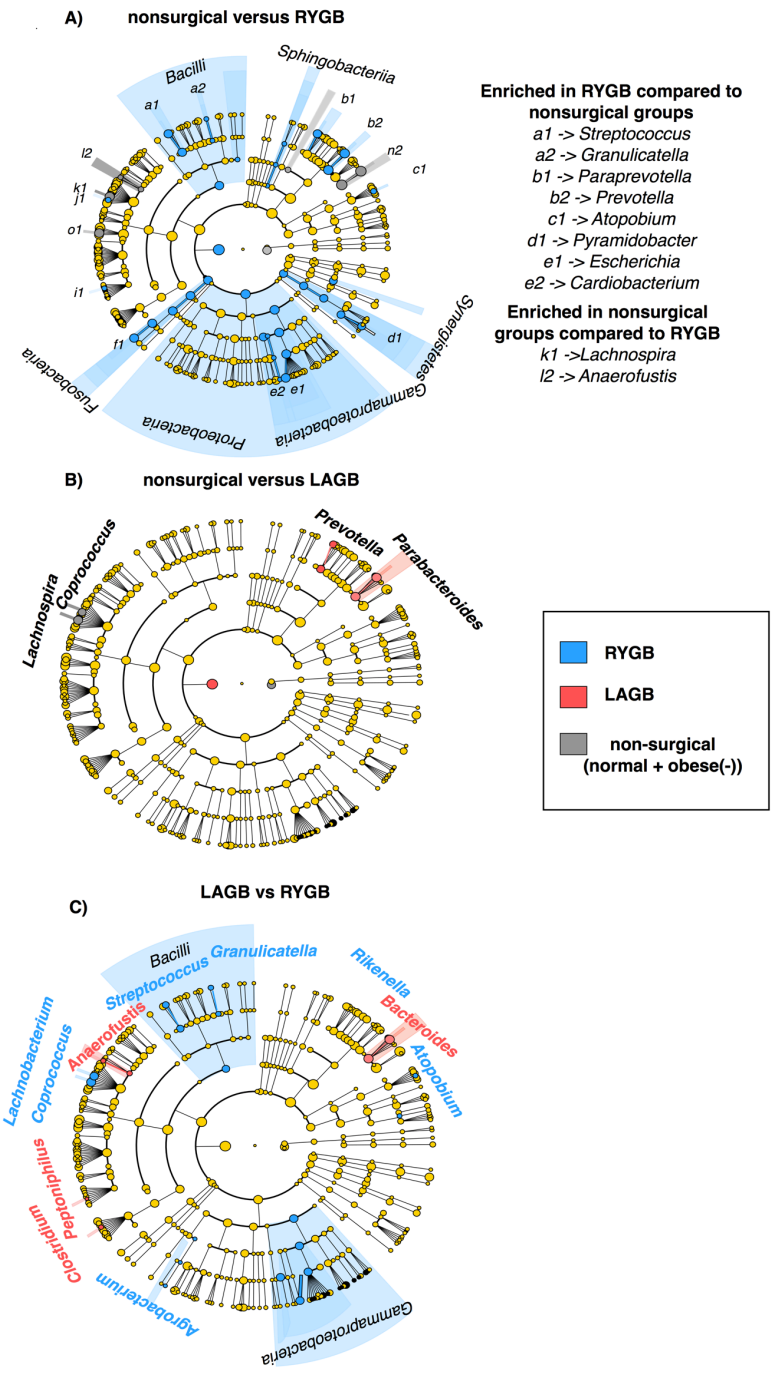

Figure A.3. Linear discriminant-effect score (LEFSE) analysis shown on a cladogram. A) Discriminant analysis comparing RYGB group to non-surgical group. B) LAGB group vs nonsurgical group. C) RYGB group versus LAGB group. The blue shading represents possible microbial signatures of RYGB surgery, whereas red shading represents possible microbial signatures of LAGB surgery. The grey shading represent microbial signatures of non-surgical subjects.

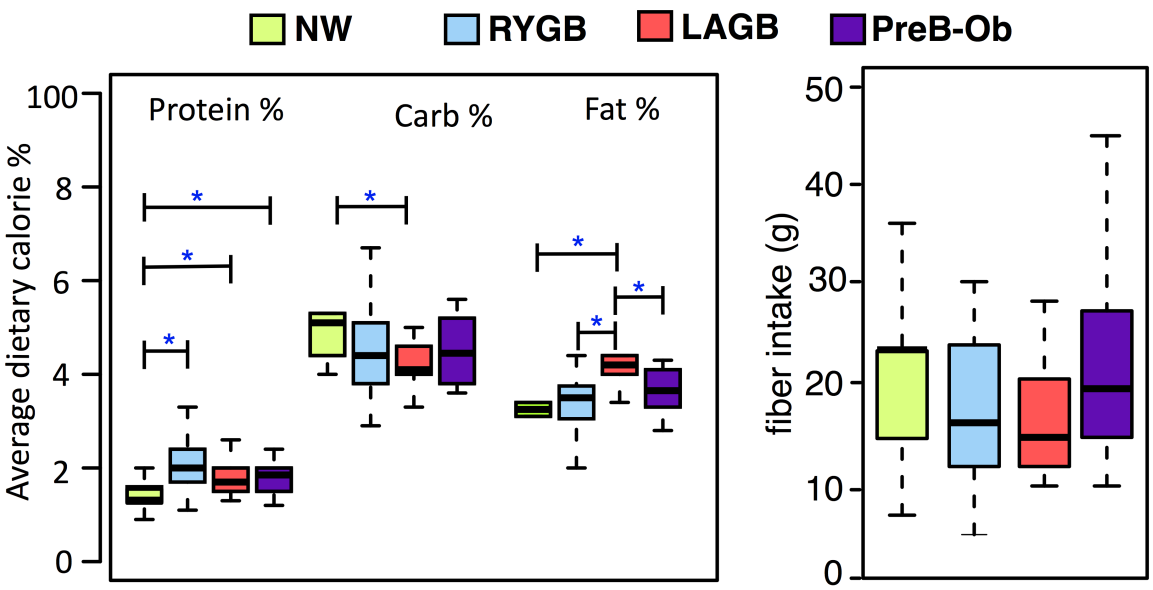

Figure A.4. Box-plots representing median values for dietary composition of NW, RYGB, LAGB, and Ob(-) subjects.

Table A.1. Median values of the items from the food frequency questionnaires that summarizes annual dietary habits of the subjects from NW, RYGB, LAGB, and PreB-Ob groups.

	NW	RYGB	LAGB	PreB-Ob
Calories consumed	1371±313	1101±286	1332±473	1719±639
Fat (gr)	51±9	51±16	56±12	82±31
Fat %	36±6	39±5	43±7	40±4
Saturated fat (gr)	17±4	15±5	20±4	25±9
Saturated fat %	11±2	12±3	14±4	12±2
Monounsaturated fat (gr)	20±5	21±6	26±7	32±13
Polyunsaturated fat (gr)	10±4	11±3	13±4	18±8
Protein (gr)	52±9	49±11	48±10	78±26
Protein %	15±1	16±3	16±3	17±3
Carbohydrates (gr)	198±41	116±94	168±52	222±72
Carbohydrates %	47±5	45±6	41±7	44±5
Cholesterol (gr)	142±22	151±63	254±112	240±98
Fiber (gr)	15±7	13±4	13±7	19±6
Alcohol %	2±3	0±2	0±2	0±2
Sweets %	12±4	12±9	10±6	8±4
Vitamin A	638±147	487±247	567±121	688±142
Beta Carotene	5129±2708	2898±2403	2985±2647	4034±1385
Vitamin C	85±31	69±36	48±46	71±32
Vitamin E	7±3	7±3	6±2	9±3
Vitamins B1 and B2	1±0	1±	1±0	1±1
Niacin	15±1	13±	14±6	21±8
Folate	494±54	315±	335±190	501±183
Vitamin B6	2±0	1±0	1±1	2±1
Calcium	688±136	568±196	627±189	813±293
Zinc	9±2	8±1	7±2	11±4
Iron	10±3	8±4	8±3	13±4
Potassium	1955±450	1780±557	2003±714	2944±999
Sodium	2188±486	1795±702	2381±683	3758±1569
Magnesium	261±84	230±106	225±89	329±102

All values are median ±median absolute deviation.

Table A.2. Relative abundances of phylotypes at the phyla-level in NW, RYGB, LAGB, and PreB-Ob groups.

	Actinobacteria	*Bacteroidetes*	*Firmicutes*	*Proteobacteria*	*Verrucomicrobia*
NW	1.0 ± .04	40.1±9.6	48.5±7.7	2.5±1.1	0.4±0.3
RYGB	0.3±0.4	55.8±14.5	34.4±15.3	4.3±2.1	0.3±0.3
LAGB	0.5±0.4	53.8±16.2	36.3±16.8	3.3±1.4	0.2±0.4
PreB-Ob	0.2±0.1	52.2±12.6	42.2±7.5	3.2±1.6	0.1±0.4

All values are median ±median absolute deviation.

Table A.3. NMR detection of metabolites that were statistically different in the RYGB group compared to nonsurgical groups**.**

Compounds	Median			FDR Padj		
	NW	PreB-Ob	RYGB	NW vs PreB-Ob	NW vs RYGB	RYGB vs PreB-Ob
4-Hydroxyphenyl-acetate	0.24±0.17	0.26±0.12	0.15±0.08	0.766	0.094	0.016
Arginine	0.25±0.25	0.33±0.20	0.19±0.20	.095	0.827	0.013
Glycocholate	0.27±0.20	0.11±0.11	0.00±2.62	0.552	0.006	0.047
Histidine	0.58±0.35	1.05±1.12	0.69±0.26	0.370	0.736	0.451
Isobutyrate	5.07±1.50	6.23±1.66	11.10±3.67	0.882	0.020	0.013
Isovalerate	4.39±1.09	4.29±1.40	10.05±2.92	1.000	0.007	0.002
Lysine	6.04±2.27	9.86±3.90	5.69±1.97	0.038	0.677	0.033
Methanol	1.17±0.42	2.92±1.82	1.49±0.91	0.056	0.392	0.072
Propionate	45.68±22.17	93.48±45.21	92.55±28.30	0.412	0.414	0.051
Putrescine	0.00±0.00	0.00±0.00	0.19±0.01	0.766	0.222	0.587
Serine	6.16±2.93	5.95±1.08	7.61±1.76	0.882	0.207	0.283
Uracil	2.22±0.85	3.36±1.32	3.09±1.33	0.295	0.254	1.000
Valerate	8.30±3.07	12.77±7.15	17.08±6.41	0.941	0.040	0.099
o-Cresol	0.58±0.19	0.45±0.14	0.92±0.38	0.656	0.079	0.016
β-Alanine	0.27±0.12	0.23±0.19	0.00±0.85	0.824	0.142	0.107

Padj values represent False Discovery Rate corrected MannWhitney U test P values.

Table A.4. Physiological characteristics of microbiota composition among NW, RYGB, LAGB, and PreB-Ob groups. The values represent median values. Pairwise group comparisons with the Kruskal Wallis test showed no significant differences of the functional microbial fractions among the groups (FDR corrected P value > 0.05).

	Gram (-) %	Aerobes %	Facultative anaerobes %	Biofilm formers %	Stress tolerant %
NW	27.6	1.6	0.8	4.0	9.9
RYGB	41.9	3.6	1.9	5.5	3.7
LAGB	47.6	2.5	0.9	6.6	9.9
PreB-Ob	46.8	5.0	0.0	5.2	1.1

APPENDIX B

16S RRNA GENE SEQUENCING, METABOLITE, AND BILE-ACID RESULTS THAT SUPPORT FINDINGS OF CHAPTER 7

Table B.1. GC-MS analysis of fecal metabolites in NW, RYGB-base, RYGB-6m, and RYGB-12m subject groups. Log2 transformed concentrations were reported.

		RYGB-base	RYGB-6m	RYGB-12m	RYGB-retro	NW
Amino acid	**l-leucine**	3.68	1.62	1.51	2.85	3.69
	l-methionine	0.39	-1.49	-1.59	-0.19	0.55
	l-threonine	-1.04	-2.23	-2.29	-1.33	-1.14
	l-valine	1.68	0.44	0.58	1.25	1.92
	l-alanine	3.80	3.32	3.09	3.73	4.10
	l-isoleucine	3.18	-0.34	-2.33	-1.24	-0.05
Microbially produced amino acid	**dehydroalanine**	-2.43	-2.20	-2.00	-2.26	-2.27
Nucleic acid	**uracil**	2.12	1.46	1.16	2.04	2.51
Saturated fatty acid	**heptadecanoic acid**	2.83	4.04	4.65	3.99	3.27
	octadecenoic acid	6.05	7.27	7.78	7.21	7.21
	oleanitrile	0.63	1.36	1.54	0.93	0.77
	palmitic acid	7.33	8.26	8.63	8.11	7.72
	stearic acid	7.01	7.88	8.14	7.88	7.77
	pentadecanoic acid	0.58	1.94	2.26	1.83	1.89
	arachidic acid	2.61	4.48	4.80	4.23	3.41
	linoleic acid	2.35	1.11	1.53	1.60	2.90
	heneicosanoic acid	-0.93	0.50	0.31	-0.02	-0.37
	dodecanoic acid	0.93	2.68	3.49	2.89	2.90
	dodecanoic acid	0.93	2.68	3.49	2.89	2.90
	oleamide	2.20	2.93	3.03	3.31	3.23
Short chain fatty acid	**l-(+)lacticacid**	0.45	1.47	1.22	0.83	0.74
Sugars	**pectin**	1.69	-0.69	-1.50	0.33	1.36
	d-xylose	3.38	1.10	-0.79	2.21	2.97
	d-ribose	4.14	3.07	2.06	3.90	4.22
	d-fructose	2.10	0.07	-0.23	1.50	2.41
	d-glucose	4.01	1.82	1.92	3.18	3.65

Table B.2. Primary and secondary bile acids measured in fecal matter from the subjects that formed NW, RYGB-retro, RYGB-12m, and RYGB-base groups. P stands for primary bile acids and S stands for secondary bile acids.

Bile acid type	Acronym	Type	NW	RYGB-Retro	RYGB -12m	RYGB-base
Chenodeoxycholic acid and deoxycholic acid	CDCA and DCA	P+S	5.17	4.38	4.50	5.61
Cholic acid	CA	P	1.82	1.16	1.15	2.87
Gylco-chenodeoxy-cholic acid	GCDCA	P	0.56	0.63	0.01	2.28
Taurocholic acid	TCA	P	-0.02	-1.52	-1.25	-0.10
Glycocholic acid	GCA	P	1.99	1.45	0.78	2.90
Tauro-chenodeoxy-cholic acid	TCDCA	P	-0.47	-1.94	-2.09	0.00
tauro-αmuricholic acid	TalphaMCA	P	-6.24	-7.57	-3.44	-7.88
Taurohydroxy-cholic acid	THDCA	S	-5.78	-5.92	-5.28	-3.63
Glycocholic acid	GDCA	S	0.39	0.13	-0.48	1.60
Taurodeoxycholic acid	TDCA	S	-0.88	-2.97	-2.64	0.15
Lithocholic acid	LCA	S	-0.55	-1.86	-1.56	0.38
Glycolithocholic acid	GLCA	S	-1.93	-1.18	-1.93	-0.50
Taurolithocholic acid	TLCA	S	-0.77	-1.09	-1.30	-0.13
Hyacholic aicd	HCA	S	-1.03	-1.89	-1.84	-1.59
Ursodeoxycholic acid	UDCA	S	-1.10	-1.08	-1.05	0.87
Glycoursodeoxycholic acid	GUDCA	S	-3.87	-3.66	-4.11	-1.64
Tauroursodeoxycholic acid	TUDCA	S	-7.80	-7.34	-6.69	-6.46

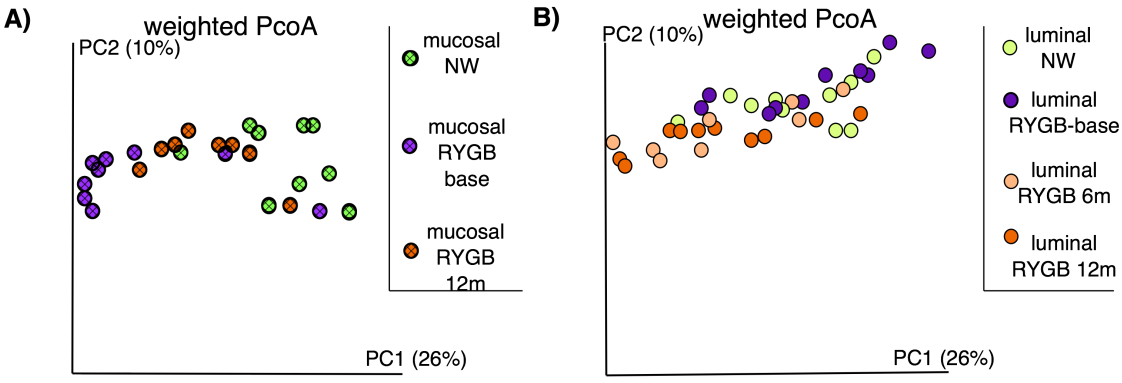

Figure B.1. Weighted Unifrac analysis of mucosal and luminal communities. A) Mucosal communities of NW, RYGB-base, and RYGB-12m groups. B) Luminal communities of NW, RYGB-base, and RYGB-12m groups.

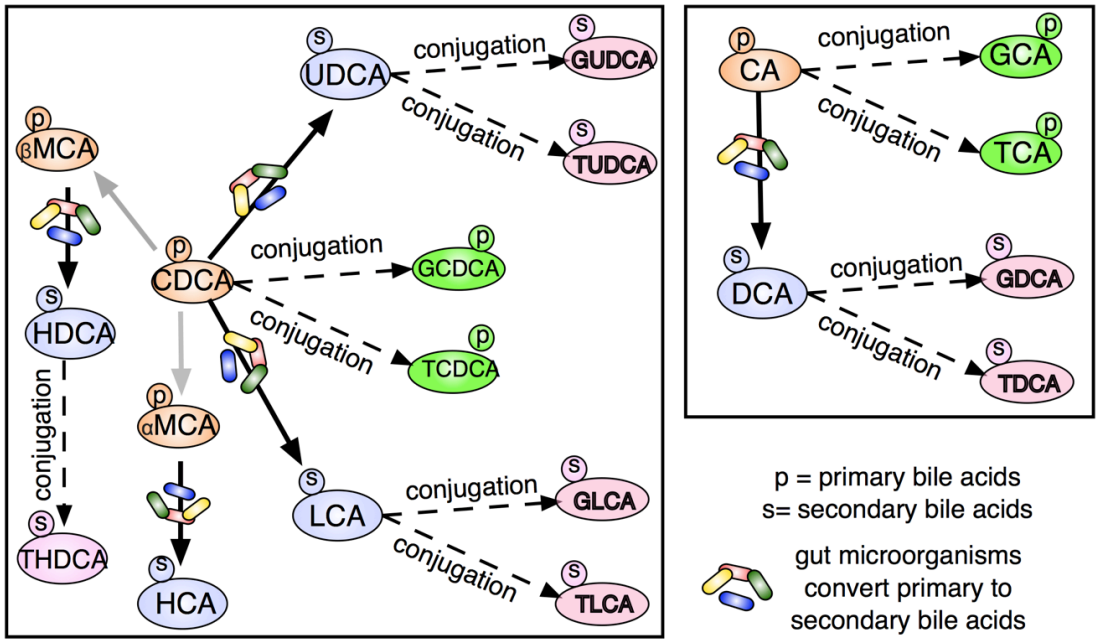

Figure B.2. Transformation reactions of primary bile acids to their conjugated forms or to secondary bile acids. CDCA and CA are two primary bile acids that are produced in the liver from cholesterol. They can be conjugated with glycine or taurine by the host. UDCA, HDCA, HCA, and LCA are all secondary bile acids derived from CDCA by gut bacteria. The gut bacteria can produce DCA from CA and its glycine and taurine conjugates. CA=Cholic acid, DCA=deoxycholic acid, GDCA = glycodeoxycholic acid, TDCA= taurodeoxycholic acid, GCA= glycocholic acid, TCA=taurocholic aci. CDCA = chenodeoxycholic acid, LCA= lithocholic acid, HCA= Hyacholic acid, HDCA=Hyodeoxycholic acid, UDCA=Ursodeoxycholic acid, GUDCA=glycoursodeoxycholic acid, TUDCA= tauroursodeoxycholic acid, THDCA= taurohydroxydeoxycholic acid, GLCA= glycolithocholic acid, TLCA=taurolithocholic acid, GCDCA= glycochenodeoxycholic acid, TCDCA=taurochenodeoxycholic acid, αMCA=α-muricholic acid, βMCA = β-muricholic acid.