THE ROLE OF THE GUT MICROBIOTA IN SUSTAINED WEIGHT LOSS FOLLOWING ROUX-EN-Y GASTRIC BYPASS SURGERY

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Roux-en-Y Gastric Bypass (RYGB) surgery is one of the most effective approaches for the treatment of severe obesity. Despite substantial weight loss following RYGB, a considerable proportion of patients experience weight regain or insufficient weight loss. The proposed research aimed to investigate the role of the gut microbiota in weight regain or suboptimal weight loss following RYGB. The gut microbiota composition in post-RYGB patients who experienced successful weight loss (SWL, n=6), post-RYGB patients who experienced poor weight loss (PWL, n=6), and non-surgical controls (NSC, n=6) who were age- and BMI-matched to the SWL group (NSC, n=6) were characterized through 16S rRNA gene sequencing. To further investigate the impact of the gut microbiota on weight profile, human fecal samples were transplanted into antibiotic-treated mice through oral gavage. Food intake and body weight were measured at weekly intervals for a month. At five weeks following colonization mice were randomly switched to a western diet or maintained on a normal diet. The results showed that *Lactobacillales*, *Enterobacteriales*, and *Verrucomicrobials* were enriched in both surgical groups compared to the NSC group. No significant difference was observed in the gut microbiota composition between PWL and SWL patients. However, transfer of the gut microbiota from human patients into antibiotic-treated mice resulted in significantly greater weight gain in PWL recipient mice compared to SWL recipient mice at four weeks following colonization (15.03±2.59% versus 7.88±1.28%, $F(2,41)=4.01$ $p=0.026$). We found that *Barnesiella*, *Gordonibacter*, *Parasutterella*, *Clostridium* cluster *XVIa* were effectively transferred from humans to mice and were associated with weight gain in recipient mice. Interestingly, *Barnesiella* that tended to be higher in PWL humans was also significantly higher in PWL recipient mice compared to SWL and NSC recipient mice. All three groups of recipient mice

gained weight when they were placed on the western diet regardless of human donor group. In summary, the results indicate that the gut microbiota are at least functionally different between PWL and SWL patients. Some taxa may contribute to weight gain after surgery. Future studies will need to determine the molecular mechanisms behind the effects of the gut bacteria on weight regain after RYGB.

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DEDICATION

Dedicated to my grandmother,

Esmat Sarjoughian

and my mother,

Mahnaz Pourvashi

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LIST OF ABBREVIATIONS

Angpt14.....Angiopoietin-4

ASA-24 .....Automated self-administered 24-hour recall

AUC .....Area under the curve

BH .....Benjamini Hochberg

BHS.....Bile salt hydrolase

BMI .....Body mass index

CA .....Cholic acid

CCK .....Cholecystokinin

CDCA .....Chenodeoxycholic acid

CYP7A1.....Cytochrome P450 7α-hydroxylase

DCA .....Deoxycholic acid

DDP4.....Dipeptidyl peptidase-4

ECC.....Enteroendocrine cell

ELISA .....Enzyme-linked immunoabsorbent assay

EWL.....Excess weight loss

FDR.....False discovery rate

FGF 15/19 .....Fibroblast growth factor 15/19

FIAF.....Fasting-induced adipose factor

FXR.....Farnesoid X nuclear receptor

GLP-1.....Glucagon-like peptide-1

GLP-2.....Glucagon-like peptide-2

GPR.....G-coupled protein receptor

LCA.....Lithocholic acid

LPS.....Lipopolysaccharide

MDS.....Multidimensional scaling

NSC.....Non-surgical control

OTU.....Operational Taxonomic Unit

PWL.....Poor weight loss

PYY.....Peptide YY

rRNA.....Ribosomal RNA

RYGB.....Roux-en-Y gastric bypass

SCFA.....Short chain fatty acid

SG.....Sleeve gastrectomy

SHP.....Small hepatic heterodimer binding protein

SWL.....Successful weight loss

TBA.....Total bile acid

TIU.....Trypsin inhibitory units

TLR.....Toll-like receptor

1. INTRODUCTION

Obesity has become a major public health concern in recent years. Obesity is defined as body mass index (BMI) of 30 kg/m^2 or above, while clinically severe or morbid obesity is defined as BMI of 40 kg/m^2 or above. It has been estimated that two-thirds of U.S. adults are obese or overweight (Flegal, Carroll, Kit, & Ogden, 2012). In 2015-2016, the prevalence of obesity reached 39.8% in adults and 18.5% in youth (Hales, Carroll, Fryar, & Ogden). Also, the rate of severe obesity has trended upward in recent years, and it has been found that the rise in morbid obesity was higher than the rise in obesity. Between 2000 and 2005, the prevalence of obesity increased by 24%, while the prevalence of severe obesity with BMI≥40 kg/m^2 increased by 50% and the prevalence of obesity with BM≥50 kg/m^2 increased by 75% (Sturm, 2007). The increased rate in morbid obesity has become a great concern as the most serious health problems are associated with morbid obesity (Sturm, 2007).

The most effective and sustainable treatment for severe obesity is bariatric surgery. Bariatric surgery is indicated for patients who have BMI≥40 kg/m^2 or patients who have a BMI≥35 kg/m^2 and at least one comorbidity. Due to the increased rate of severe obesity in recent years, the number of bariatric surgeries being performed has risen dramatically. Between 2011 and 2016, the number of bariatric surgeries performed in the U.S. increased by 36.7% (American Society for Metabolic and Bariatric Surgery, 2013). The most common bariatric surgery performed worldwide is Roux-en-YGastric Bypass (RYGB; Angrisani et al., 2015), which involves the creation of a small gastric pouch and bypass of a large portion of the stomach and proximal intestine.

RYGB results in a median of ~30% loss of baseline weight over the first year and resolves numerous comorbidities associated with obesity (Courcoulas et al., 2013; Puzziferri et

al., 2014). Despite the significant changes in weight and metabolic profile post-surgery, a significant portion of patients regain weight over time and experience reemergence of comorbidities after surgery (Karmali et al., 2013). The underlying mechanisms for weight regain are not fully understood and may involve several behavioral and biological factors. Since the failure of bariatric surgery is associated with considerable financial and emotional burden for patients, further research is needed to characterize the underlying mechanisms of weight regain and to improve the outcome of weight loss surgery. Given that there is compelling evidence on the link between the gut microbiota and metabolic abnormalities (Arora & Backhed, 2016), the purpose of this study is to explore the role of the gut microbiota and other microbiota-related factors in weight regain and unfavorable post-surgical outcomes.

1.1. Weight loss and weight regain following bariatric surgery

Bariatric surgery is the most effective treatment for severe obesity, resulting in considerable weight loss (15-30% of total body weight) and reduced all-cause mortality (Adams et al., 2007; Courcoulas et al., 2013; Sjostrom, 2013). Despite the significant weight loss, a proportion of patients experience weight regain or failure of sustained weight loss (Karmali et al., 2013). Studies have shown approximately 15-35% of patient experience insufficient weight loss (less than 50% excess weight loss) or experience significant weight regain (more than 15% of maximal weight loss) following RYGB (Jirapinyo, Abu Dayyeh, & Thompson, 2017). Weight regain may start as early as 1-2 years following surgery. However, patients are more likely to experience weight regain at a longer interval from their surgery (Shantavasinkul, Omotosho, Corsino, Portenier, & Torquati, 2016).

Data collected from 1,711 patients who had undergone RYGB in the Longitudinal Assessment of Bariatric Surgery study identified five distinct weight change trajectory groups

following RYGB. Patients in two trajectory groups (23.5%) discontinued losing weight at six months after surgery, and 2.1% of the patients started regaining weight at six months. Although patients grouped into other trajectories continued losing weight until two years, they started gaining weight after two years (Courcoulas et al., 2013). Weight regain is often associated with recurrence of comorbid conditions, which can lead to several health problems and associated financial burdens. Therefore, it is critical to understand the underlying mechanisms responsible for the failure of sustained weight loss to prevent weight regain and to improve the outcomes of surgery. A review by Karmali and colleagues (2013) identified factors that may contribute to weight regain post-surgery. These factors included non-compliance with dietary recommendations, hormonal/metabolic disturbances, psychological disorders, lack of physical activity, and surgical-related factors (Karmali et al., 2013). Given that the gut microbiota can affect host metabolism and can cause metabolic disorders, we explored the role of the post-operative gut microbiota in weight regain after surgery.

1.2. The gut microbiota as a contributor to obesity and metabolic syndrome

The complex microbial community in the intestine is composed of more than 100 trillion microbial cells, forming a genome which is more than 100 times greater than the human genome (Backhed, Ley, Sonnenburg, Peterson, & Gordon, 2005). The gut microbiota composition is dynamic and is influenced by diet, environment, and host genotype (Lozupone, Stombaugh, Gordon, Jansson, & Knight, 2012). Bacteroidetes and Firmicutes are the two major phyla that make up the gut microbiota in adults, while other phyla, including Actinobacteria, Proteobacteria, and Verrucomicrobia constitute a minor portion of the gut microbiota (Lozupone et al., 2012). Intestinal bacteria play several physiological roles, including energy harvest from indigestible carbohydrates, development of the immune system in the gut, regulation of the gut

barrier function, and protection against pathogenic bacteria (Gerard, 2016). Despite these beneficial effects, dysbiosis of the gut microbiota is implicated in the pathogenesis of several disorders, such as inflammatory bowel syndrome (Frank et al., 2007), obesity (Ruth E. Ley, Peter J. Turnbaugh, Samuel Klein, & Jeffrey I. Gordon, 2006; Turnbaugh, Backhed, Fulton, & Gordon, 2008), diabetes (Karlsson et al., 2013; Larsen et al., 2010; Qin et al., 2012), and cardiovascular diseases (Karlsson et al., 2012).

The first evidence of altered microbial composition in obesity came from a study showing that leptin-deficient ob/ob mice had higher Firmicutes and lower Bacteroidetes (Ley, 2005). This finding was further confirmed by human studies, which found a higher Firmicutes: Bacteroidetes ratio in obese patients compared to lean counterparts (R. E. Ley, P. J. Turnbaugh, S. Klein, & J. I. Gordon, 2006; Turnbaugh et al., 2009). However, the results regarding the Firmicutes: Bacteroidetes ratio are not consistent among studies as some studies found no change or lower Firmicutes: Bacteroidetes ratios in obese patients (Collado, Isolauri, Laitinen, & Salminen, 2008; Duncan et al., 2008; Fernandes, Su, Rahat-Rozenbloom, Wolever, & Comelli, 2014; Schwiertz, 2010). The conflicting results may be attributed to small sample sizes, different diets across studies, and different technical methods that were employed to identify the members of the microbial community in the gut. However, the general agreement among studies is that obesity is associated with reduced bacterial diversity and decreased microbial gene richness (Cotillard et al., 2013; Le Chatelier et al., 2013; Turnbaugh, 2009a).

In addition to the bacterial phyla of Firmicutes and Bacteroidetes, methanogenic Archaea were increased in obese patients compared to non-obese patients. Archaea are H_2 oxidizing microbes, and they accelerate the fermentation reaction in the gut by consuming the end product

of the fermentation (H_2) that is produced by Prevotellaceae. Increased fermentation provides more energy for the host and results in increased energy uptake (Patil et al., 2012; Zhang, 2009).

1.2.1. How do the gut microbiota contribute to obesity?

The gut microbiota can affect body weight and fat mass through different mechanisms, including regulation of energy extraction from diet, gut barrier function, appetite, food intake, and fat storage in adipose tissues. The following sections will explain the microbial-related pathways that could lead to the development of obesity.

1.2.1.1. Microbial processing of nutrients

The members of the gut microbial community play an essential role in the metabolism of carbohydrates, proteins, lipids, and nucleic acids. Metabolism of the dietary nutrients by the gut microbiota results in a wide range of bioactive molecules, such as vitamins, amino acids, fatty acids, and monosaccharides. These molecules are used as the substrates for gluconeogenesis and lipogenesis by the host (Kobyliak, Virchenko, & Falalyeyeva, 2016).

The gut microbiota produce short chain fatty acids (SCFA) through fermentation of indigestible complex carbohydrates. Short chain fatty acids produced by the gut microbiota mainly include acetate, propionate, and butyrate and provide 5-10% of the host's energy requirement (Puddu, Sanguineti, Montecucco, & Viviani, 2014). Butyrate is the major source of energy for intestinal epithelium cells, while propionate reaches the liver through the enterohepatic circulation and is used for gluconeogenesis. Acetate reaches the peripheral tissues and is used for lipogenesis (Puddu et al., 2014).

Short chain fatty acids are considered as signaling molecules that are involved in energy homeostasis. They bind to G-coupled protein receptors (GPR41 and GPR43) on enteroendocrine cells (EEC) and induce secretion of gut peptides, glucagon-like peptide-1 (GLP-1) and peptide

YY (PYY). Conventionally raised Gpr41-/- mice had lower body weight compared to wild-type mice despite similar food intake. However, germ-free Gpr41-/- mice and germ-free wild-type mice had similar body weight (Samuel et al., 2008). Another study also showed that Gpr43 knock-out mice are resistant to the development of obesity, insulin resistant, and dyslipidemia (Bjursell et al., 2011). All these findings suggest that SCFA and their receptors are involved in energy homeostasis, and this effect is dependent on the gut microbiota.

It has been suggested that the gut microbiota in an obese state are more efficient in extracting energy from diet through SCFA production (Fernandes et al., 2014; Turnbaugh, 2006). When feces from obese donors were fermented with galacto-oligosaccharides and lactulose, higher amounts of SFCA were produced compared to feces from lean donors (Aguirre, Jonkers, Troost, Roeselers, & Venema, 2014). Consistent with these results, studies in humans and mice have shown that an obesogenic microbiome is enriched with genes that encode enzymes for energy extraction from the diet (Turnbaugh, 2006, 2009a).

1.2.1.2. Microbial regulation of inflammation and intestinal permeability

Obesity is associated with low-grade inflammation and metabolic endotoxemia, a condition defined as an increase in plasma levels of lipopolysaccharide (LPS), which is a bacterial-derived molecule found in the outer membrane of gram-negative bacteria. It has been shown that a high-fat diet reduces the intestinal permeability through alterations of two major tight junction proteins, including Zonula Occluden-1 (ZO-1) and Occludin (Cani, Delzenne, Amar, & Burcelin, 2008). Increased intestinal permeability allows the translocation of LPS from the leaky gut into the systemic circulation. LPS triggers inflammatory response through activation of Toll-like receptor (TLR4/CD14) on the surface of innate immune cells (Cani & Delzenne, 2007). Therefore, increased circulating plasma LPS induces proinflammatory

responses at peripheral tissues and contributes to development of chronic inflammation, metabolic disorders, and obesity (de La Serre et al., 2010; Gnauck, Lentle, & Kruger, 2015; Patterson et al., 2016). The role of LPS in the pathogenesis of metabolic syndrome has been confirmed by a study in which chronic administration of LPS in mice induced glucose intolerance, weight gain, and insulinemia along with increased inflammatory markers and hepatic triglycerides. This effect was absent in transgenic Cd14-/- mice (Cani, 2007). Therefore, it appears that the gut microbiota-LPS-TLR signaling pathway could be an important contributor to the development of obesity.

1.2.1.3. Microbial regulation of bile acids

Bile acids are signaling molecules that are synthesized in the liver from cholesterol and are metabolized by the gut microbiota in the distal intestine. The gut microbiota deconjugate glycine and taurine-conjugated bile acids and transforms primary bile acids, including cholic acid (CA) and chenodeoxychlic acid (CDCA) into secondary bile acids, including deoxycholic acid (DCA) and lithocholic acid (LCA) (Ridlon, Kang, & Hylemon, 2006; Ridlon, Kang, Hylemon, & Bajaj, 2014). The intestinal bacteria that are involved in the biotransformation of bile acids mainly belong to the *Clostridium* species within the Firmicutes phylum (Ridlon et al., 2014).

Both primary and secondary bile acids have considerable effects on metabolism and energy homeostasis. Bile acids are potent activators of the Farnesoid X nuclear receptor (FXR) and G-protein coupled receptor (TGR5) (Thomas et al., 2009; Watanabe et al., 2006). FXR is expressed in multiple tissues, such as the intestine, liver, kidney, and adrenal glands and regulates bile acid synthesis and glucose and lipid metabolism. FXR in the intestine increases the expression of Fibroblast growth factor 15/19 (FGF15/19), which is transferred to the liver

through the enterohepatic circulation and reduces the activity of the first and rate-limiting enzyme cholesterol 7α-hydroxylase (CYP7A1) in the bile acid synthesis cascade (Stroeve et al., 2010). In addition, FXR directly inhibits bile acid synthesis in the hepatocytes through inducing the expression of small hepatic heterodimer binding protein (SHP), which subsequently reduces the CYP7A1 activity (Lu et al., 2000). On the other hand, TGR5 is mainly expressed in the intestine, brown adipose tissue, and muscle and plays an important role in the regulation of GLP-1 secretion from the gut and energy expenditure in peripheral tissues (Fouladi, Mitchell, Wonderlich, & Steffen, 2016; Thomas et al., 2009; Watanabe et al., 2006).

Alterations in the gut microbiota composition affect bile acid homeostasis and metabolism, which could further alter FXR/TGR5 signaling. Compared to conventionally-raised mice, germ-free mice and antibiotic-treated mice had higher levels of taurine-conjugated bile acids (taurocholic acid and tauro-β-muricholic acid) in different compartment tissues, including the liver, kidney, heart, and plasma (Swann, 2011). Increased tauro-β-muricholic acid in another study was shown to have a protective effect against nonalcoholic fatty liver disease (NAFLD) through inhibition of intestinal FXR signaling (Jiang et al., 2015). Degirolamo et al. showed that a 21-day VSL3 probiotic in mice resulted in a higher abundance of Streptococcaceae and Lactobacillaceae within Firmicutes and higher abundance of Bifidobacteriaceae within Actinobacteria compared to vehicle-treated mice. These alterations in the gut microbiota composition were associated with increased bile acid deconjugation, increased fecal bile acid excretion, decreased FXR-FGF15 signaling, and increased bile acid synthesis in the liver. These effects were absent in FXR knockout mice (Degirolamo, Rainaldi, Bovenga, Murzilli, & Moschetta, 2014).

The metabolic effects of the gut microbiota through regulation of bile acid metabolism were further confirmed by modulation of bacterial bile salt hydrolysis (BSH). Increased BSH levels in the intestine resulted in reduced body weight gain, decreased plasma cholesterol levels and decreased triglycerides content in the liver. Modulation of intestinal BSH was achieved through colonizing mice with *E. coli* MG1655 cloned with bsh genes (Joyce et al., 2014). Although the results from this study suggest that microbial BSH could serve as a new target to treat obesity, further research is warranted to determine the benefits of bacterial BSH modulation and bile acid profiles in humans.

1.2.1.4. Microbial regulation of gut-brain signaling

The gut-brain axis is a a bi-directional signaling axis and plays a major role in energy homeostasis. This axis involves neuronal pathways, e.g., brain, vagal and spinal nerves, and the enteric nervous system, as well as hormonal pathways, e.g., gut peptides, cytokines, and neuropeptides.

The gut-brain signaling axis communicates satiation and starvation signals from the gut to the brain, which is mainly initiated by the gut peptides, such as ghrelin, GLP-1, PYY, and others. These peptides are secreted from EEC at different sites of the gastrointestinal tract upon exposure to the influx of intestinal nutrients. Following release from the EEC, gut peptides relay satiation or starvation signals to the brain through local neuronal activation or endocrine effect. Upon receiving these signals by the central nervous system, nucleus tractus solitarius neurons in the brainstem and arcuate nucleus in the hypothalamus generate responses resulting in the regulation of energy intake and energy expenditure (Bauer, Hamr, & Duca, 2016).

The gut microbiota can influence signaling pathways in the gut-brain axis through SCFA. Short chain fatty acids generated through microbial fermentation of indigestible carbohydrates in

the gut activate GPRs (GPR43 and GPR41) expressed on the EEC, which have highest expressions in isolated distal L cells. Activation of these receptors releases gut peptides from EEC and initiates signaling pathways in the gut-brain axis. Administration of SCFA in mice induced GLP-1 secretion, reduced food intake, and protected against diet-induced obesity (Lin et al., 2012).

In addition to SCFA, the gut microbiota influence the gut-brain axis through altering the absorptive and secretory capacity of the intestinal epithelium, as well as changing the expression of intestinal cholecystokinin (CCK), GLP-1, and PYY peptides. Germ-free mice have increased intestinal taste receptors, increased intestinal sodium glucose transporters, decreased intestinal short and long fatty acid receptors (GPR41, GPR43, GPR40, and GPR120), and reduced expression of gut peptides (Duca, Swartz, Sakar, & Covasa, 2012; Swartz, Duca, de Wouters, Sakar, & Covasa, 2012). These intestinal epithelial changes induced by the absence of the gut microbiota could explain the reduced nutrient sensing and increased calorie intake in germ-free mice.

1.2.1.5. Microbial regulation of fasting-induced adipose factor

Fasting-induced adipose factor (FIAF), also known as angiopoietin-like 4 (Angptl4), is a lipoprotein lipase inhibitor and is produced by the intestine, liver, and adipose tissues. FIAF reduces lipoprotein lipase activity, which results in decreased liberation of fatty acids from lipoproteins and decreased fatty acid uptake by adipose tissues. Therefore, FIAF has an important role in regulating fat storage. Fasting increases plasma levels of FIAF, while chronic feeding leads to decreased levels of FIAF (Dijk & Kersten; Kersten et al., 2000).

Studies have shown that conventionalization of germ-free mice decreased expression of FIAF in intestinal epithelium, and this was associated with increased lipoprotein activity in white

adipose tissue and heart and increased body fat (Backhed, 2004). In addition, conventionalization of germ-free FIAF knockout mice resulted in significantly lower body fat gain compared to their wild-type counterparts, suggesting that the effect of the gut microbiota on fat accumulation is FIAF dependent (Backhed, 2004). Interestingly, unlike wild-type germ-free mice, germ-free FIAF knockout mice were not resistant to obesity under a high-fat diet, and this further supports the role of FIAF in the regulation of fat accumulation through the gut microbiota (Backhed, Manchester, Semenkovich, & Gordon, 2007). However, it is important to note that different bacterial species may have different regulatory effects on FIAF. Aronsson et al. (2010) have shown that *Lactobacillus paracasei* increased expression of FIAF from colonic cell lines *in vitro*. Furthermore, mice fed high-fat diet supplemented with *Lactobacillus paracasei* had lower body weight and higher levels of FIAF compared to mice fed high-fat diet without *Lactobacillus paracasei* (Aronsson et al., 2010). Therefore, dysbiosis of the gut microbiota may lead to changes in the expression of intestinal FIAF, which could be further involved in pathogenies of obesity.

1.3. The gut microbiota following bariatric surgery

A limited body of literature has explored the effect of bariatric surgery on the gut microbiota. Although there is some inconsistency in the observed alterations in the intestinal bacteria following RYGB, some common themes have emerged among studies. RYGB surgery is normally associated with an increase in bacterial richness (Kong et al., 2013; Palleja et al., 2016). Three prospective and one cross-sectional study have shown that the Proteobacteria phylum increased following RYGB (Furet, 2010; Graessler et al., 2013; Kong et al., 2013; Zhang, 2009). This finding has been further confirmed in rodent models of RYGB (Liou et al., 2013) (Li, 2011). One explanation for the increase in the Proteobacteria phylum could be

alterations in the bile acid flow following RYGB, which leads to increased bile-tolerant bacteria belonging to Proteobacteria (Sweeney & Morton, 2014). Within this phylum, *Escherichia coli (E. coli)* has been observed in higher abundance after surgery (Furet, 2010; Kong et al., 2013). Interestingly, Furet and colleagues reported that higher prevalence of *E. coli* was negatively correlated with fat mass and serum leptin, which was independent of food intake (Furet, 2010).

Another relatively consistent finding across studies has been the changes in the abundance of Firmicutes and Bacteroidetes. In a prospective study, the ratio of Firmicutes:Bacteroidetes decreased at three and six months post-RYGB, which was mostly due to an increase in *Bacteroides* within Bacteroidetes and a decrease in *Lactobacillus* and *Pediococcus* within Firmicutes (Furet, 2010). In addition, high-throughput sequencing of the gut microbiota of the same samples from the study by Furet revealed that several genera belonging to Firmicutes, including *Blautia*, *Dorea*, and *Lactobacillus,* were decreased post-RYGB (Kong et al., 2013). In a small cross-sectional study, Clostridia (a class belonging to Firmicutes) was lower in post-RYGB patients compared to their obese and lean counterparts (Zhang, 2009). Interestingly, higher Firmicutes:Bacteroidetes appears to be associated with the obese phenotype and this ratio decreased after weight-loss induced by dietary restriction (Ley et al., 2006). This suggests that the alterations in Firmicutes and Bacteroidetes that have been observed after surgery may contribute to weight loss post-surgery.

The changes in the gut microbiota composition are driven by the anatomical and physiological changes that occur in the gastrointestinal tract following RYGB surgery. Among these changes are increased gastric pH, changes in biliary flow, decreased intestinal transit time, increased dissolved oxygen, and increased delivery of undigested nutrients to the distal intestine.

Furthermore, changes in eating behavior, dietary regimen, and caloric restriction are expected to contribute to changes in the gut microbiota post-surgery.

In addition to RYGB, other bariatric surgery procedures are also associated with alterations in the gut microbiota. For example, sleeve gastrectomy (SG) has been associated with decreased abundance of Firmicutes and increased abundance of Bacteroidetes. Particularly, several bacteria within the clostridiales order, including *Clostridium*, *Dorea*, *Faecalibacterium*, *Eubacterium, Rominococcus*, and *Lachnospiracea* decreased following surgery (Damms-Machado et al., 2015). Changes in the gut microbiota following SG are moderate compared to those induced by RYGB surgery, and this may be attributed to the less invasive effect of the SG procedure on the gastrointestinal anatomy (Damms-Machado et al., 2015; Sanmiguel et al., 2017).

1.3.1. The role of the gut microbiota in weight and metabolic outcomes following bariatric surgery

Changes in the gut microbiota composition post-surgery influence host metabolism and adiposity. Colonization of germ-free mice with the post-bariatric surgery microbiota from human donors resulted in lower fat accumulation and reduced respiratory quotient compared to mice colonized with the microbiota of non-surgical obese controls (Tremaroli et al., 2015). In addition, microbiota transfer from a mouse model of RYGB into germ-free mice resulted in weight loss and decreased fat mass (Liou et al., 2013). These studies confirm the causal effects of the gut microbiota on weight loss and reduced adiposity following RYGB surgery.

In addition to weight profile, the altered composition of the gut microbiota after surgery may contribute to metabolic improvements following surgery. In fact, post-surgical improvements in metabolic markers correlated with several bacterial taxa. For example,

Propionibacterium was positively correlated with inflammatory markers, while, *Fecalibacterium prausnitzii* was negatively correlated with inflammatory markers (Furet, 2010; Murphy et al., 2017). Also, *Lactobacillus* was negatively correlated with fasting blood glucose (Patrone et al., 2016). *Akkerrmansia muciniphila* that was previously shown to be associated with decreased intestinal permeability and improved glucose metabolism (Dao et al., 2016) increased following RYGB (Graessler et al., 2013; Palleja et al., 2016). Furthermore, an increase in Proteobacteria following RYGB has been positively correlated with weight loss after surgery (Medina et al., 2017). Despite these associations, the causal relationship between the gut microbiota and metabolic improvements following bariatric surgery has not yet been determined, and further research is needed to determine the potential molecular mechanisms by which the gut microbiota can contribute to metabolic benefits of bariatric surgery.

1.3.2. Crosstalk between bile acids, gut peptides, and gut microbiota following bariatric surgery

Anatomical changes in the gastrointestinal tract following RYGB surgery result in substantial changes in the incretins secreted from the gut. GLP-1 and PYY are secreted from the EEC in the ileum and regulate gastrointestinal motility, satiety, and food intake (Holst, 2013). In addition, GLP-1 regulates glucose metabolism, insulin secretion, and improves β cell glucose sensitivity (Holst, 2013). Glucagon-like peptide-2 (GLP-2) is also secreted from the L cells and is believed to function primarily to enhance intestinal adaptation following RYGB (Holst, 2013). Following RYGB, bypass of the proximal intestine accelerates delivery of nutrients to the ileum, resulting in increased secretion of incretins. In fact, plasma GLP-1 and PYY levels are increased within days after surgery and contribute to improvements in glucose metabolism and insulin secretion in a weight-loss independent manner (Svane, Bojsen-Moller, Madsbad, & Holst, 2015).

GLP-1 and PYY response to a meal challenge was greater in a sample of post-RYGB patients who experienced successful weight loss (SWL) compared to post-RYGB patients who experienced poor weight loss (PWL), suggesting that these gut peptides could be long-term mediators of weight loss after surgery (de Hollanda et al., 2014; Dirksen et al., 2013; le Roux, 2007).

In addition to gut peptides, bile acid flow undergoes significant changes after surgery due to rearrangement of the enterohepatic circulation following RYGB. Plasma bile acid levels increase following RYGB surgery, and several studies have shown that the increase in bile acid levels was associated with improvements in glucose and lipid profiles (Fouladi et al., 2016). However, as discussed in a review by Fouladi and colleagues (2016), there are limited data on the effect of bile acids on weight outcome after surgery.

Although it has been assumed in prior literature that the increased levels of bile acids and gut peptides are the result of major changes in the gastrointestinal anatomy, the influence of the altered gut microbiota on changes in gut hormones after surgery has not yet been determined. Bile acid metabolism and secretion of gut peptides are both regulated by the gut microbiota as described in previous sections. However, to our knowledge, no study has determined the effect of post-surgical changes in the gut microbiota on bile acid composition and gut peptide profile after RYGB in humans. This is of clinical importance since bile acids and gut peptides are major contributors to post-surgical metabolic improvements and any potential changes in their profile may be associated with distinct metabolic benefits. In particular, the secondary bile acids produced by the gut microbiota are potent activators of TGR-5, which is expressed in multiple tissues and regulates GLP-1 secretion in the intestine (Thomas et al., 2009), regulates thermogenesis in adipose tissues (Watanabe et al., 2006), and ultimately and improves metabolic

disorders (Sato et al., 2007). Therefore, further elucidation of the post-surgical relationship between the gut microbiota and bile acids may introduce novel and non-invasive gut microbiota-targeted therapies for obesity.

1.4. Statement of the problem

In recent years, we have witnessed a drastic rise in the number of bariatric surgeries to treat morbid obesity despite the fact that they are invasive procedures which have both short and long-term risks to patients and have financial implications for the healthcare system. Bariatric surgery leads to more sustained and effective weight loss compared to other available treatments, such as lifestyle modification and pharmacological treatments which leads many healthcare professionals to preferentially recommend bariatric surgery to eligible patients. However, new studies have shown that the percentage of patients who fail to lose sufficient weight or who experience weight regain after an initial weight loss is significant. This leads to new research questions about what biological, psychological, and technical factors could influence the efficacy of bariatric surgery. Learning more about these factors could help health professionals to optimize weight outcomes following bariatric surgery. In addition, extending our knowledge of the biological and molecular mechanisms that are involved in the outcomes of bariatric surgery could lead to the development of less invasive and expensive interventions for the treatment of obesity.

Given that the role of the gut microbiota in the pathogenesis of obesity is well-established and that bariatric surgery induces major changes in body weight and shifts the microbial community of the gut, this study aimed to determine whether alternations in the gut microbiota could contribute to sustained weight loss after RYGB surgery. In addition, we further investigated the role of gut peptides and bile acids in sustained weight loss and their relationship

with the post-surgical gut microbiota. For this purpose, in a sample of participants who underwent RYGB 2-5 years prior, the gut microbiota, bile acids, gut peptides, and food intake were compared between individuals in the following groups: 1) those who experienced successful weight loss following RYGB (SWL), 2) those who experienced poor weight loss following RYGB (PWL), and 3) nonsurgical controls (NSC). By colonizing a murine model with the post-RYGB human gut microbiota, thereby creating a humanized mouse model, we explored the impact of the gut microbiota on weight status, food intake, and the gut peptides.

The specific aims and hypotheses for each aim of the present study were as follows:

Specific aim 1: To examine the gut microbiota composition and other biological variables (food intake, gut peptides, and bile acids) in participants who experienced SWL versus PWL 2-5 years post-RYGB and NSC.

Hypothesis 1a: Participants who experienced SWL post-surgery will exhibit different gut microbiota composition with higher bacterial diversity compared to patients who experienced PWL and NSC.

Hypothesis 1b: Participants who experienced SWL post-surgery will exhibit higher levels of pre- and post-prandial bile acids and gut peptides (GLP-1, GLP-2, PYY) compared to patients who experienced PWL and NSC.

Hypothesis 1c: Participants who experienced PWL post-surgery will report the least favorable dietary macronutrient intake (lowest percentage of protein, highest percentages of fat and carbohydrate) compared to patients who experienced SWL.

Specific aim 2: To examine the weight profile and gut peptides in mice colonized with human gut microbiota from SWL versus PWL post-RYGB participants and NSC.

Hypothesis 2a. Mice colonized with the gut microbiota of patients who have experienced PWL after RYGB will exhibit greater weight gain compared with mice colonized with the microbiota of patients who have experienced SWL post-surgery and NSC.

Hypothesis 2b: Mice colonized with the microbiota of patients who have experienced PWL after RYGB will show less favorable GLP-1 profile compared with mice colonized with the microbiota of patients who have experienced SWL post-surgery and NSC.

Hypothesis 2c: Mice colonized with the microbiota of patients who demonstrate PWL after RYGB will gain more weight on a western diet compared with mice colonized with the microbiota of patients who have experienced SWL post-surgery and NSC.

Exploratory aim: To investigate the relationship between the gut microbiota, bile acid and gut peptide profile using the data generated through Specific Aim 1 and 2 and the humanized mouse model, and to further clarify the mechanistic role these variables collectively play in mediating weight regulation following RYGB.

In summary, data generated through these specific aims provide novel information regarding the role of the gut microbiota in surgically-induced weight loss. Furthermore, the current research will lay the groundwork for future research on microbiota-targeted interventions as a novel approach to improve the outcome of weight loss surgery.

2. RESEARCH DESIGN AND METHODS

2.1. Study design

This study involved two distinct but interrelated phases: 1) A clinical study of post-RYGB participants and NSC participants; 2) A subsequent animal study using fecal samples obtained from human volunteers in the clinical phase of the study. This combined clinical/murine approach allowed us to examine the etiological role that the gut microbiota play in the regulation of weight following surgery.

2.1.1. Clinical study

This study was designed as a cross-sectional comparison between participants who experienced SWL and PWL post-RYGB and NSC participants who were age- and BMI-matched to the SWL post-RYGB group. Dietary intake, gut microbiota composition, serum bile acids, and plasma gut peptides were compared between the three groups of participants.

2.1.1.1. Participants

Three groups of participants who were female and 18-65 years old were recruited: 1) 2-5 years post-RYGB patients who experienced suboptimal or PWL defined as experiencing less than 50% of excess weight loss (EWL) at the time of recruitment (n=6); 2) 2-5 years post-RYGB patients who experienced SWL defined as 50% or more of EWL at the time of recruitment (n=6); 3) NSC matched to the SWL group on body mass index (BMI) and age (n=6). Pre-surgical body weight was assessed through self-report and current weight was obtained by weighing the participant barefoot and in light clothing on a Tanita scale. The following formula calculated ideal body weight (IBW) and %EWL:

$$IBW = 45.5\ kg + 2.3kg * (Height(inches) - 60)$$

$$\%EWL = \frac{Presurgical\ Weight\ (kg) - Current\ Weight\ (kg)}{Presurgical\ Weight\ (kg) - IBW(kg)} \times 100$$

Participants were excluded from participation if they met any of the following: 1)Taking antibiotics, commercially available prebiotics, or probiotics for the last three months, 2) Taking a medication which significantly alters gastrointestinal transit time (e.g., metoclopramide, erythromycin, drug with significant anticholinergic effects), 3) Diabetes at the time of study enrollment, 4) History of major gastrointestinal surgery or disorder (not including highly prevalent procedures such as cholecystectomy), 5) Smoking or any tobacco use in the past 3 months, 6) Currently pregnant or lactating, 7) History of bipolar or psychotic spectrum disorder.

This study was approved by the North Dakota State University Institutional Review Board (IRB Project Number: IRB-2015509-061). Written informed consent was obtained from all participants. The study was registered at www.clinicaltrials.gov (NCT02654496).

2.1.1.2. Study day

Participants underwent two study visits over the course of three days, including an Informed Consent/Screening Visit, during which they had a medical history, psychological interview, and underwent BMI measurement using bioelectrical impedance analysis (Tanita® scale) to determine inclusion/exclusion criteria. They then received training on a dietary recall software (Automated Self-Administered 24-hour Recall, ASA-24, National Cancer Institute) to record a journal of their dietary intake for three consecutive days. Participants completed the ASA-24 at home for two consecutive days preceeding Study Day 3. On Study Day 3, participants presented to the clinical research laboratory after 8 hours of fasting. Participants were asked to consume a liquid nutritional supplement (Ensure®) containing 220 calories, 9% fat, 11% carbohydrate, and 18% protein. Blood samples were collected through an indwelling

intravenous catheter at -5, 15, 30, 60, 90, and 120 minutes with the -5 minute timepoint occurring prior to ingestion of the supplement and the remaining times following supplement intake. For bile acid analysis, blood samples were collected in red top blood collection tubes and left at room temperature for 30 minutes and then centrifuged at 3000 rpm for 15 minutes at 4 °C. Serum was collected and stored in 2 ml vials at -80 °C. For gut peptide analysis, blood was collected in EDTA vacutainer tubes and stored on ice immediately after collection. The collected blood was aliquoted to three centrifuge tubes, and additives were added to each tube according to the downstream analysis. For active GLP-1 analysis, 40 µl of dipeptidyl peptidase-4 inhibitor (DPP4 inhibitor; EMD Millipore, Billerica, MA) was added to 4 ml blood to obtain a concentration of 10 µl/ml of DPP4 inhibitor. For PYY analysis, 300 µl of aprotinin (Phoenix, Burlingame, CA) was added to 3 ml of blood to obtain 0.6 trypsin inhibitory units (TIU) aprotinin per 1 ml of blood. No additive was added to blood for the analysis of GLP-2. Blood samples were centrifuged at 3000 rpm for 15 minutes at 4 °C. Plasma was collected and stored in 2 ml vials at -80 °C. Patients also provided fecal samples on Study Day 3 or within 1 day before or after the Study Day 3. Fecal samples were stored at -80°C upon collection.

2.1.1.3. Bile acid analysis

Serum bile acids were analyzed by Quest Diagnostics laboratory using Liquid-Chromatography/Tandem Mass Spectrometry. The lower limit of quantification for cholic acid (CA), chenodexoycholic acid (CDCA), deoxycholic acid (DCA), and total bile acids (TBA) was 0.5 µmol/L. The area under the curve (AUC) of serum bile acid concentrations versus time was calculated using a trapezoidal rule where 0 corresponds to the first blood draw and n corresponds to the last blood draw.

$$AUC_{0-n} = \sum \left\{ \frac{C_0 + C_1}{2} \times (t_0 - t_1) \right\} + \left\{ \frac{C_1 + C_2}{2} \times (t_1 - t_2) \right\} + \cdots$$

2.1.1.4. Gut peptide analysis

Plasma concentrations of active GLP-1, total PYY, and GLP-2 were measured by commercially available enzyme-linked immunosorbent assay (ELISA) kits (EMD Millipore, Billerica, MA). The limit of sensitivity of the assay for active GLP-1, total PYY, and GLP-2 was 0.14 pM, 6.5 pg/mL, and 0.3 ng/mL, respectively. The intra-assay and inter-assay variation of coefficients for all the assays were less than 15%. The AUC of plasma gut peptide concentrations versus time was calculated using the trapezoidal rule, as previously described.

2.1.1.5. DNA extraction from fecal samples

DNA was extracted from human fecal samples using phenol-chloroform extraction combined with physical disruption of bacterial cells and then followed by a DNA clean-up step, as previously described (Carroll et al., 2011; Carroll, Ringel-Kulka, Siddle, & Ringel, 2012).

100-200 mg frozen fecal samples were added to 0.2 ml tubes prefilled with Triple-Pure High Impact Zirconium Beads, 0.1 mm (Benchmark Scientific, Edison, NJ). Pre-warmed 750 µl lysis buffer containing 20 mg/ml BioUltra lysozyme (Sigma-Aldrich, St. Louis, MO) was added to each tube, and then the mixture was vortexed. After 30 minutes of incubation at 37 °C, 85 µl of 10% sodium dodecyl sulfate solution and 20 µl proteinase K were added to the mixture. Next, the mixture was vortexed and incubated for additional 30 minutes at 60 °C. Following incubation, 500 µl of Phenol:Chloroform:Isoamyl alcohol (25:24:1) was added, and the mixture was homogenized using a bead beater (Benchmark Scientific, Edison, NJ) at 4000 rpm for 90 seconds. Samples were then centrifuged at 13,000 rpm for 5 minutes. The supernatant was added to a new tube containing 500 µl Phenol:Chloroform:Isoamyl alcohol (25:24:1). Next, the mixture was centrifuged at 13,000 rpm for 5 minutes, and then the supernatant was added to a new tube

containing 500 µl Chloroform. After centrifuging at 13,000 rpm for 5 minutes, the supernatant was added to a new tube containing 1000 µL ethanol (100%) and 50 µL of 3 M sodium acetate (pH 5.2) for DNA precipitation. DNA then was stored at -80˚C for at least 1 hour. When samples were removed from the freezer, DNA pellets were formed by centrifuging the samples at 13,000 rpm for 5 min. Next, the top layer (ethanol) was discarded, and the DNA pellets were dissolved in 200 µl nuclease-free water. Next, 200 µL Buffer AL (QIAamp DNA Stool Mini Kit, Qiagen, Hilden, Germany) and 200 µl ethanol 100% were added to the mixture. The mixture was then passed through a spin column (QIAamp DNA Stool Mini Kit, Qiagen, Hilden, Germany). The spin column was washed with 500 µL Buffer AW1 (QIAamp DNA Stool Mini Kit, Qiagen, Hilden, Germany) and centrifuged at 8000 rpm for 1 min. The spin column was washed for the second time with 500 µL Buffer AW2 (QIAamp DNA Stool Mini Kit, Qiagen, Hilden, Germany) and centrifuged at 14,000 rpm for 3 min. Next, pre-warmed 100 µL nuclease-free water was added to spin columns and incubated for 5 minutes at room temperature. DNA was then eluted by centrifuging the spin columns at 8000 rpm for 1 min. DNA concentration was measured using Epoch™ Microplate Spectrophotometer (BioTek, Winooski, VT). All DNA samples were diluted to 40 ng /µL with nuclease-free water for downstream analysis.

2.1.1.6. Amplification of V4 region of 16S ribosomal RNA (rRNA) gene

To characterize the composition of the bacterial community, the V4 region of the 16S rRNA gene was amplified using forward primer 515 (5'-GAGTGCCAGCMGCCGCGGTAA-3') and reverse primer 806 (5'-ACGGACTACHVGGGTWTCTAAT-3') as previously described (Kleiman et al., 2017). In addition to the 515 and 806 sequences, the modified forward and reverse primers contained variable numbers (0-5) of padding nucleotides to increase downstream

sequence diveresity and quality. Sequencing of 16S rRNA included two subsequent polymerase chain reactions (PCR):

Step one: The 50 µl reaction contained 120 ng DNA (3 µl at 40 ng/µl) , 2 µl of the mixture of six forward primers at total concentration of 10 µM, 2 µl of the mixture of six reverse primers at total concentration of 10 µM, 10 µl buffer A (5X), 10 µl enhancer (5X), 1 µl dNTP Mix (10 Mm), 0.5 µl Robust DNA polymerase from KAPA2G Robust PCR kit (Kapa Biosystems, Wilmington, MA), and 21.5 µl nuclease-free water. For each batch of reactions, a no-template control containing only molecular-grade water was also used. The following thermal cycler conditions were used for amplification: initial denaturation at 95 ºC for 3 minutes, 10 cycles of 95 ºC for 30 seconds, 50 ºC for 30 seconds, 72 ºC for 30 seconds, and final extension at 72 ºC for 5 minutes.

Step two: A master mix containing 25 µl KAPA HiFi ReadyMix (2X) (Kapa Biosystems, Wilmington, MA), 6.25 µl adapter primer diluted to 10 µM (5'- AATGATACGGCGACCACC GAGATCTACA CGCCTCCCTCGCGCCATCAGAGATGTG-3'), and 7.5 µl molecular-grade, nuclease-free water for each reacion was prepared. Each reation then received 5 µl pure PCR product from step one and 6.25 µl of the sample's assigned 12-base error-correcting Golay barcode, modified from those described in Caporaso et al. (Caporaso et al., 2012). The barcode primer sequence had the following sequence: (5'-3') is CAAGCAGAAG ACGGCATACGAGAT [12-nucleotide index] GTGACTGGAGTTCAGACGTGTGCTC. For each batch of reactions, a no-template control containing only molecular-grade water was also used. The following thermal cycler conditions were used for amplification: initial denaturation at 95 ºC for 3 minutes, 22 cycles of 95 ºC for 30 seconds, 50 ºC of 30 seconds, 72 ºC for 30 seconds, and final extension at 72 ºC for 5 minutes.

Each PCR was followed by a clean-up step using a DynaMag-96 side magnet (Life Technologies, Carlsbad, CA). 90 µl HighPrep PCR reagent (MagBio Lausanne, Switzerland) was added to each PCR tube and was mixed thoroughly. After 5 minutes of incubation at room temperature, PCR tubes were placed on the magnet for 3 minutes and then the clear supernatant was discarded. While PCR tubes were still on the magnet, beads were washed for two times using 70% ethanol. Next, the beads were incubated at room temperature for 3-5 minutes. PCR tubes were removed from the magnet, and 40 µl nuclease-free water was added to each tube and was thoroughly mixed. PCR tubes were placed on the magnet for 1 minute until solution cleared. The clear supernatant was transferred to new PCR tubes.

Amplicons from step two were visualized using gel electrophoresis. For gel electrophoresis, 1.25% agarose in Tris-acetate-EDTA 1X buffer (TAE buffer) with 2 µl ethidium bromide was used. DNA samples, the no-template controls, and 1 Kb DNA ladder 1.0 µg/µl (Invitrogen, Carlsbad, CA) each were mixed with 6X Orange DNA Loading Dye (Thermo-Scientific, Waltham, MA) and were loaded on the gel. The gel ran for 45 minutes at 100 v. DNA fragments were visualized with UV light using FluorChem E System (ProteinSimple, San Jose, CA).

2.1.1.7. 16S rRNA gene sequencing

Following amplification of 16S rRNA genes, the libraries were quantified and combined into an equimolar pool for sequencing. Sequencing was performed on an Illumina MiSeq benchtop sequencer (Illumina, San Diego, CA) by the High-Throughput Sequencing Facility in the Carolina Center for Genome Sciences at the University of North Carolina at Chapel Hill School of Medicine. The 16S rRNA sequences were clustered into operational taxonomic units (OTU) using Ribosomal Database Project, which was run under an automated metagenomics

pipeline (https://github.com/mikesioda/BioLockJ_Dev). OTU tables at different taxonomic classifications were normalized using the following formula to account for differences in raw sequences between samples (Noble et al., 2017):

$$Log_{10}\left(\left[\frac{Raw\ count\ in\ sample\ (i)}{\#\ of\ sequences\ in\ sample\ (i)} \times Average\ \#\ of\ sequences\ per\ sample\right] + 1\right)$$

2.1.1.8. Statistical analysis

Nonparametric tests, including Kruskal-Wallis and Mann-Whitney U tests, were used to determine if there were group differences in age, BMI, years after surgery, %EWL, gut peptides, and bile acids. Pairwise comparison between PWL, SWL, and NSC were performed by Dunn's test with Hochberg adjustment using software R "dunn.test" package. An α level of less than 0.05 was considered significant.

A one-way ANOVA was used to identify bacterial taxa that had different abundance between PWL, SWL, and NSC participants. T-test comparisons were used for pairwise comparisons between groups using the "pairwise.t.test" function in R. Benjamini-Hochberg (BH) post hoc test with a false discovery rate (FDR) of P <0.10 was used for multiple testing corrections. The Spearman's rank-order correlation was used to measure the association between the gut microbiota and gut peptides and bile acids. For all analyses, rare taxa that were present in less 10% of samples were removed prior to analysis, and the cut-off for FDR was 10%.

Multidimensional scaling, a method for visualizing dissimilarities between samples in a dataset, was performed on the taxonomic tables at the genus level by using the "capscale" function of the R statistical package "vegan" with Bray-Curtis dissimilarity. The ADONIS test (permutational multivariate analysis of variance using Bray-Curtis distance matrices) was used to test whether the gut microbiota composition was significantly different among the groups. To

determine the species diversity within the gut microbial communities, Shannon Diversity Index was calculated for each group and then were compared using one-way ANOVA test.

2.1.2. Animal study

The animal phase of this work was designed to investigate the etiological role of the gut microbiota in weight regain or unfavorable weight outcomes. This study involved administration of the two-week antibiotic regimen to C57BL/6 mice to simulate an immunocompetent, but germ-free mouse model. Upon evidence of successful suppression of the murine intestinal microbiota (as indicated by 80-90% knockdown of colony counts when cultured), colonization with human fecal samples was completed through oral gavage. To colonize mice, each human fecal sample was split and provided to three mice. During the experiment, weight and food intake were measured at weekly intervals. Fecal samples were collected at pre-antibiotic, post-antibiotic, one day post-colonization, and four weeks post-colonization. Blood samples were collected at one week and four weeks post-colonization. Five weeks after colonization, mice in each group were randomly placed either on a western diet or normal chow diet. Weight and food intake were measured at weekly intervals, and fecal samples were collected four weeks after switching the diet.

2.1.2.1. Mice

Female wild-type C57BL/6 mice were used at the age of 12 weeks in this study. C57BL/6 mice were purchased from the Charles River Laboratories and were habituated to their environment for at least one week prior to experimentation. Mice were individually housed in sterile and disposable microisolator cages (Innovive, San Diego, CA) and were placed on ad libitum irradiated food and sterile water throughout the experiment. All the procedures were performed in a class II biosafety cabinet.

2.1.2.2. Antibiotic treatment

According to the protocol published by Reikvam and colleagues (2011), mice were orally gavaged with amphotericin-B 0.1 mg/ml twice a day, approximately 12 hours apart, for three days using sterile plastic feeding tubes (Instech Laboratories, Plymouth Meeting, PA). Afterward, mice were orally gavaged with a cocktail of antibiotics and antifungals containing amphotericin-B 0.1 mg/ml, vancomycin 5 mg/ml, neomycin 10 mg/ml, and metronidazole 10 mg/ml twice a day, approximately every 12 hours, for the following two weeks. Ampicillin, in a concentration of 1mg/ml, was added to drinking water. For each gavage, 10 ml/kg of the antibiotic cocktail was administered to mice.

2.1.2.3. Culturing of fecal samples

To monitor the depletion of the gut microbiota during the antibiotic phase, fecal pellets were collected five, ten, and 15 days following starting the oral gavage of the antibiotic cocktail. For each mouse, one fecal pellet was aseptically suspended in 1 ml sterile phosphate buffer saline and then cultured on a Trypticase Soy Agar using a sterile swab. Agar plates were incubated aerobically and anaerobically at 37 ºC for 24 and 72 hours, respectively.

2.1.2.4. Fecal transplant

Twelve hours following the conclusion of the antibiotic treatment protocol, mice were colonized with the gut microbiota from human donors (post-RYGB and NSC volunteers from the clinical phase of the study) each day for five days. Five fecal samples from each group of human donors were transferred to 15 antibiotic-treated mice. For each colonization, 0.5 g of the frozen human fecal sample was suspended and vortexed in 5 ml sterile phosphate-buffered saline (PBS), which had been purged with nitrogen gas to remove dissolved oxygen. Three mice were colonized from the same fecal suspension and a total of 0.2 ml of fecal suspension was

administered through oral gavage to each mouse once a day. To evaluate the efficiency of fecal transplant, fecal samples were collected at one day post-colonization and four weeks post-colonization. Fecal samples were stored at -80 ºC until downstream analysis.

2.1.2.5. Measurement of mouse plasma GLP-1

Blood samples were collected through facial bleeding at one week and four weeks after colonization. Blood was collected in EDTA coated tubes (Microvette CB300, Braintree Scientific, Braintree, MA) and immediately stored on ice. Blood was centrifuged at 3000 rpm for 15 minutes at refrigerated temperature. Plasma was collected and stored at -80 ºC for later analysis. Plasma GLP-1 was measured using a commercial mouse GLP-1 ELISA kit (Crystal Chem, Elk Grove Village, IL). The sensitivity of the assay was 1.24 pM, and the intra-assay variation of coefficients was less than 10%.

2.1.2.6. Western diet

Five weeks following colonization with human fecal samples, mice were randomly assigned into two groups using a table of random numbers. One group remained on the normal chow diet. This group included mice with NSC microbiota (n=7), mice with SWL microbiota (n=8), and mice with PWL microbiota (n=7). Another group was placed on a western diet (Harlan Teklad TD.88137, 15.2% Kcal from protein, 42.7% Kcal from carbohydrate, 42.0% Kcal from fat). This group included mice with NSC microbiota (n=7), mice with SWL microbiota (n=7), and mice with PWL microbiota (n=8). Mice remained on the western diet or normal chow diet for four weeks. Body weight was measured at weekly intervals. Fecal samples collected at four weeks following switching the diets were characterized with 16S rRNA genes sequencing.

2.1.2.7. Gut microbiota analysis of fecal samples

DNA extraction from fecal pellets, amplification of 16S rDNA gene, and sequencing of PCR products were performed as described in the methods section of the clinical study.

2.1.2.8. Statistical analysis

Kendall correlation analysis was performed to determine the correlation between human and mouse gut microbiota. Multidimensional scaling was performed on the taxonomic tables at the genus level by using the "capscale" function of the R statistical package "vegan" with Bray-Curtis dissimilarity. The ADONIS test (permutational multivariate analysis of variance using Bray-Curtis distance matrices) was further used to test whether the gut microbiota were significantly different among groups.

Two-way ANOVA was used to determine whether there were group differences in weight gain during the month following colonization. Weight gain at each time point was compared using pairwise-t-test with Hochberg adjustment. To control for the donors' BMI, a linear model was constructed regressing weight gain at each time point against the groups of recipient mice and the donors' BMI.

The following multiple regression model determined differences in the abundance of bacteria classified at the genus taxonomic level relative to weight gain at one week and four weeks post colonization:

$$Abundance = Weight\ gain\ (one\ and\ four\ weeks\ post\text{-}colonization) + Time + e$$

At each time point (one week and four weeks following colonization) differences in the abundance of bacteria relative to the weight gain were determined using simple linear regression:

$$Abundance = Weight\ gain + e$$

The following multiple regression model determined differences in the abundance of bacteria classified at a genus taxonomic level relative to the GLP-1 plasma at baseline, one week and four weeks post colonization:

$$Abundance = GLP\text{-}1(one\ and\ four\ weeks\ post\text{-}colonization) + time + e$$

Wilcoxon signed rank test was used to compare the plasma GLP-1 levels in a small subset of SWL and PWL recipient mic with an α level of 0.05. Simple linear regression was used to compare the abundance of bacteria at the genus level between the western and normal diets.

For all the statistical models, rare taxa that were present in less 10% of samples were removed prior to analysis. Benjamini-Hochberg method was used for multiple testing corrections. FDR less than 10% was considered significant. All the statistical tests were performed with R.

3. RESULTS

3.1. Clinical study

3.1.1. Patient characteristics

Six post-RYGB SWL patients, six post-RYGB PWL patients, and six NSC subjects participated in this study. All participants were female, and their age ranged between 38-45 years old. Pre-surgical BMI was not significantly different between PWL and SWL patients. However, PWL patients had the highest BMI following surgery compared to SWL patients and NSC subjects ($\chi^2(2)=8.57$, $p<0.05$). The average percentages of EWL in PWL and SWL patients were 43.01±1.42% and 61.41±3.51%, respectively (Table 1).

Table 1. Patient characteristics.

	PWL	SWL	NSC	p
N	6	6	6	
Age (years)	38.00±4.21	43.67±4.53	44.5±5.77	0.485[a]
Pre-surgical BMI (kg/m^2)	48.12±2.27	46.43±1.56	-----	0.093[b]
BMI on screening day (kg/m^2)	36.26±1.17	30.53±0.87	31.23±2.41	**0.007**[a]
Years after surgery	3.35±0.50	2.43±0.10	-----	0.589[b]
% Excess weight loss (% EWL)	43.01±1.42	61.41±3.51	-----	**0.002**[b]
Cholecystectomy (frequency)	50%	83.3%	16.7%	-----

a. p values from Kruskal-Wallis test; b. p values from Mann-Whitney U test. Data are presented as means ± standard errors.

3.1.2. Plasma gut peptides (active GLP-1, GLP-2, PYY)

No significant differences in plasma concentrations of fasting and postprandial gut peptides (active GLP-1, GLP-2, and PYY) were observed between SWL and PWL patients. However, both groups had greater plasma levels of gut peptides at different time points

following mixed macronutrient liquid meal ingestion compared to the NSC group. Both PWL and SWL patients had significantly greater active GLP-1-AUC, PYY-AUC, and GLP2-AUC compared to NSC participants (Figure 1).

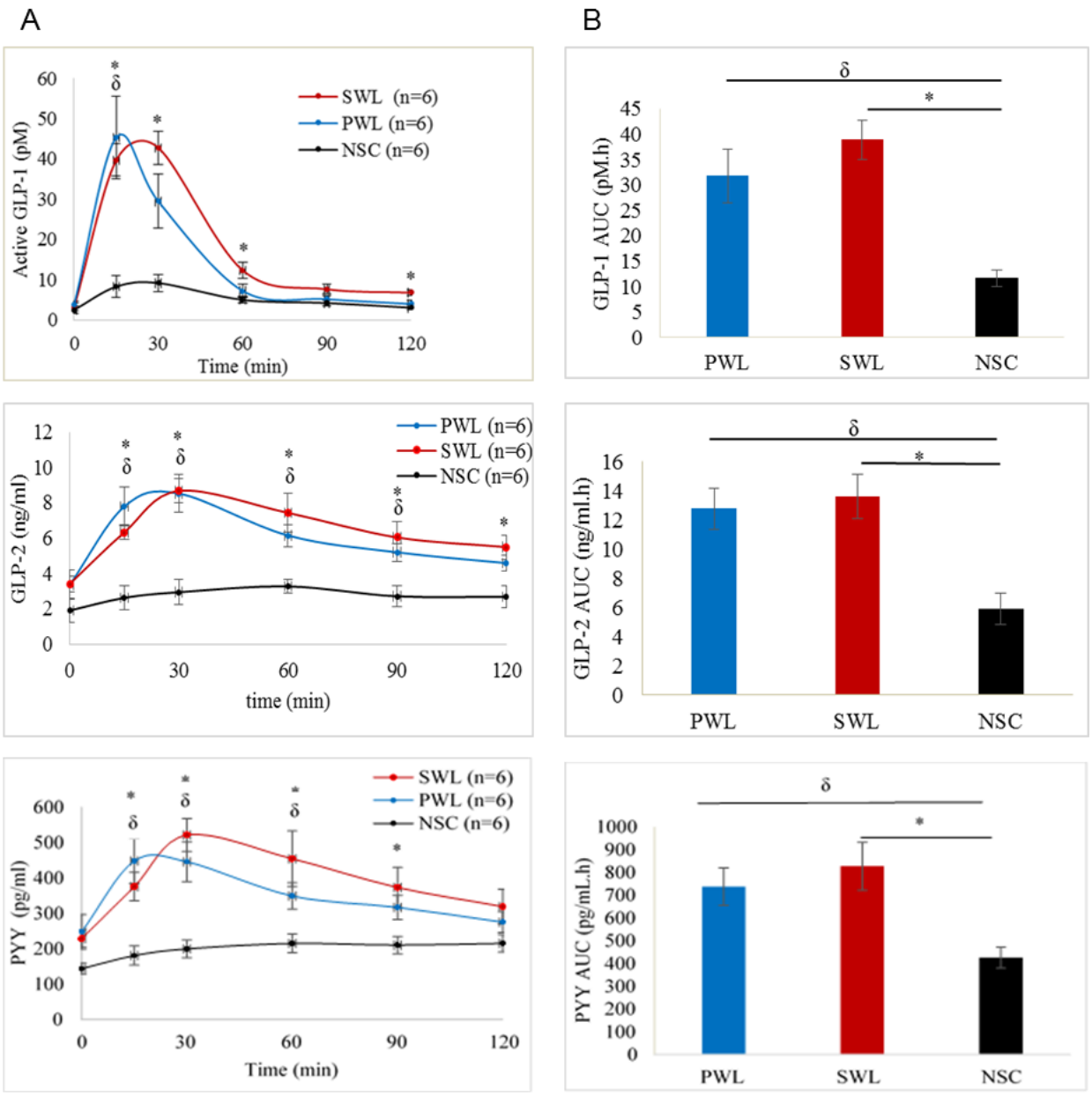

Figure 1. (A) Plasma concentrations of active GLP-1, GLP2, and PYY following liquid meal ingestion. (B) The AUC of plasma active GLP-1, GLP-2, and PYY concentrations versus time. Data are presented as means ± standard errors. Statistical tests are performed by Dunn's test with Hochberg adjustment. * SWL versus NSC $p<0.05$; δ PWL versus NSC $p<0.05$.

3.1.3. Serum bile acids

Serum levels of bile acids were associated with high variability among PWL, SWL, and NSC participants. PWL and SWL patients tended to have higher levels of serum bile acids compared to NSC participants following ingestion of a liquid meal (Figure 2). The AUC of serum total bile acids (TBA) concentration versus time was significantly greater for PWL patients compared to NSC participants (28.05±8.19 μM.h versus 10.47±2.56 μM.h, $p<0.05$). In addition, the AUC of serum CDCA concentrations versus time was significantly greater for SWL compared to NSC participants (15.15±4.60 μM.h versus 5.44±1.00 μM.h, $p <0.05$). No significant difference in serum bile acid concentrations was observed between PWL and SWL patients (Figure 2).

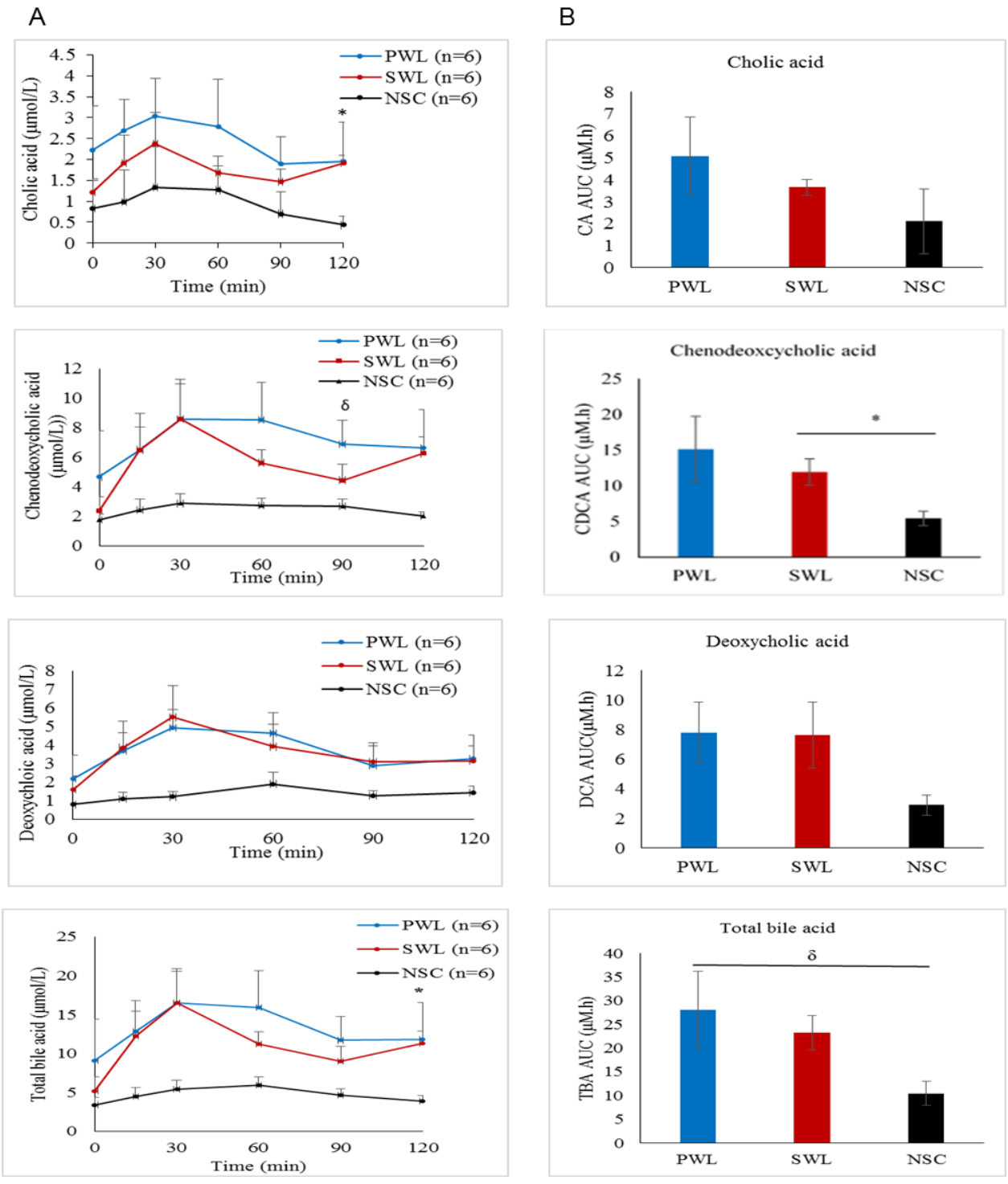

Figure 2. (A) Serum concentrations of CA, CDCA, DCA, and TBA following liquid meal ingestion. (B) The AUC of serum CA, CDCA, DCA, and TBA concentrations versus time. Data are presented as means ± standard errors. Statistical tests are performed by Dunn's test with Hochberg adjustment. * SWL versus NSC $p<0.05$; δ PWL versus NSC $p<0.05$.

3.1.4. Dietary intake

Patients recorded their dietary intake for three consecutive days through the ASA24 online dietary recall system. Daily total energy intake, total fat, protein, and carbohydrate did not differ between SWL, PWL, and NSC participants (Figure 3).

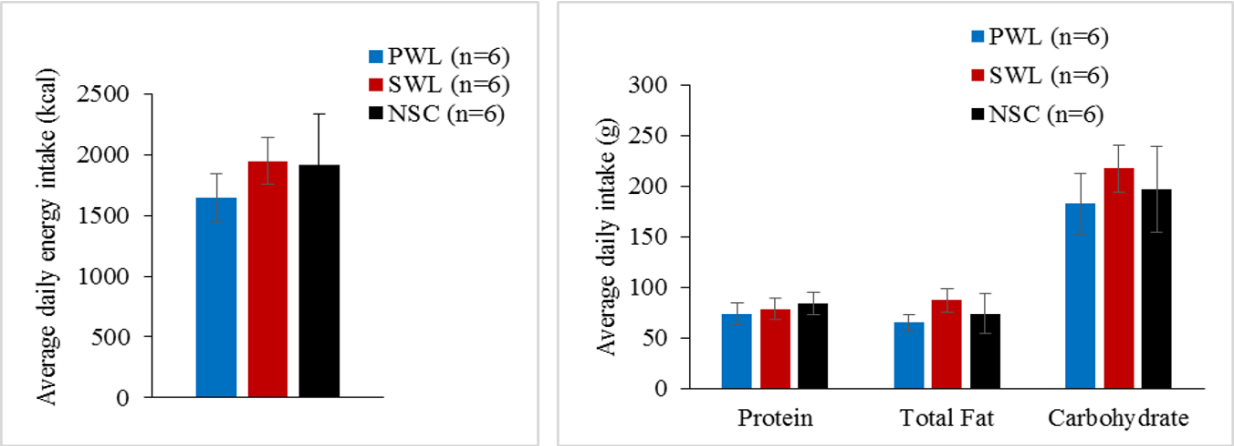

Figure 3. Food intake in SWL, PWL, and NSC participants. Data are presented as means ± standard errors. Statistical tests are performed by the Kruskal-Wallis test.

3.1.5. The gut microbiota

3.1.5.1. Gut microbiota composition

The composition of the fecal microbiota was characterized by high throughput sequencing of 16S rRNA genes. To determine how the gut microbiota composition differed between the three patient groups, we performed one-way ANOVA with log-normalized adjusted counts at the phylum, class, order, and genus levels.

At the phylum level, Verrucomicrobia had significantly higher abundance in PWL patients compared to NSC participants at a 10% FDR (Figure 4). At the class level, Verrucomicrobiae was similarly higher in PWL patients compared to NSC participants. In addition, Bacilli (Firmicutes) were significantly more abundant in PWL and SWL patients compared to NSC subjects at a 10% FDR (Figure 5). At the order level, Lactobacillales (Firmicutes) were enriched in SWL and PWL patients compared to NSC participants at a 10%

FDR (Figure 6). The abundance of Enterobacteriales within the Proteobacteria phylum tended to be higher in SWL and PWL groups compared to NSC group (one-way ANOVA, BH-corrected p=0.08).

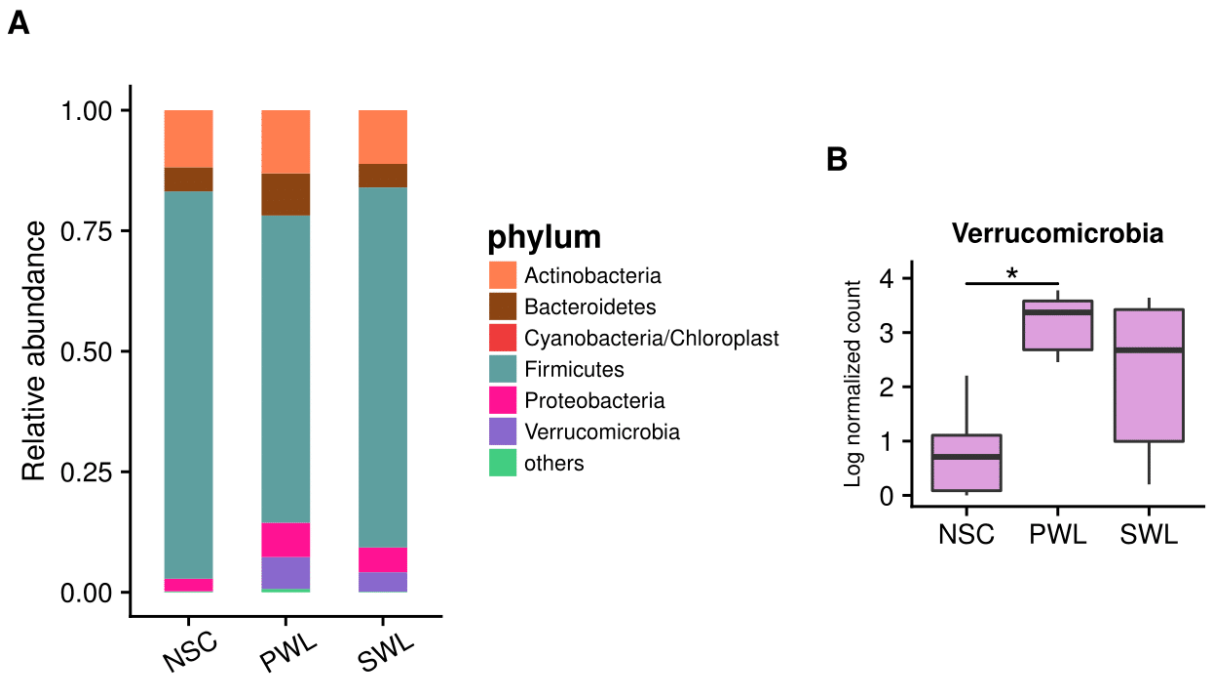

Figure 4. (A) Relative abundance of bacterial phyla in SWL, PWL, and NSC humans. (B) Box plot shows the abundance of Verrucomicrobia in SWL, PWL, and NSC humans. Statistical differences are analyzed by one-way ANOVA with Benjamini-Hochberg post hoc test: * BH-corrected $p<0.05$.

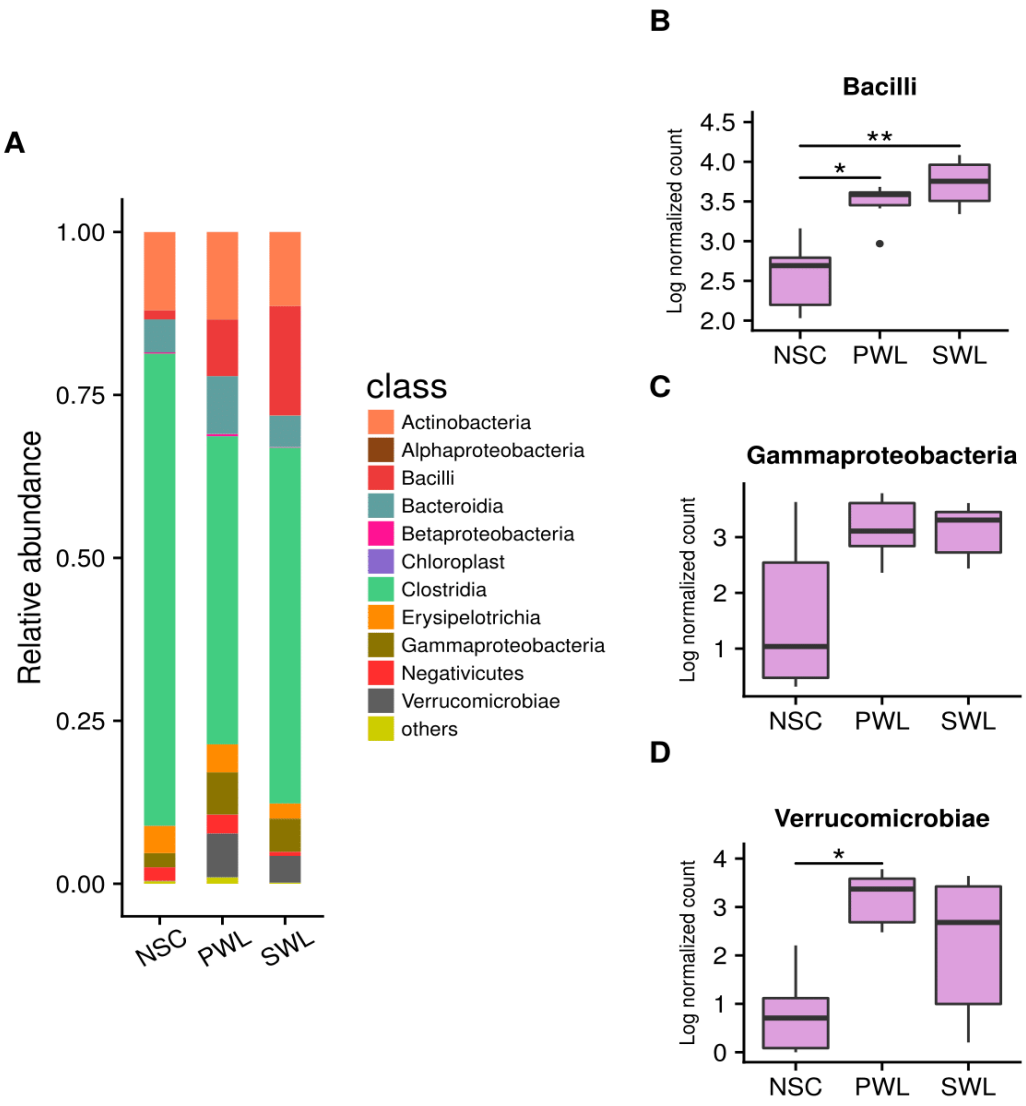

Figure 5. (A) Relative abundance of bacterial classes in SWL, PWL, and NSC humans. Box plots show the abundance of Bacilli (B), Gammaproteobacteria (C), and Verrucomicrobiae (D) in SWL, PWL, and NSC humans. Statistical differences are analyzed by one-way ANOVA with Benjamini-Hochberg post hoc test: * BH-corrected $p<0.05$; ** BH-corrected $p<0.01$.

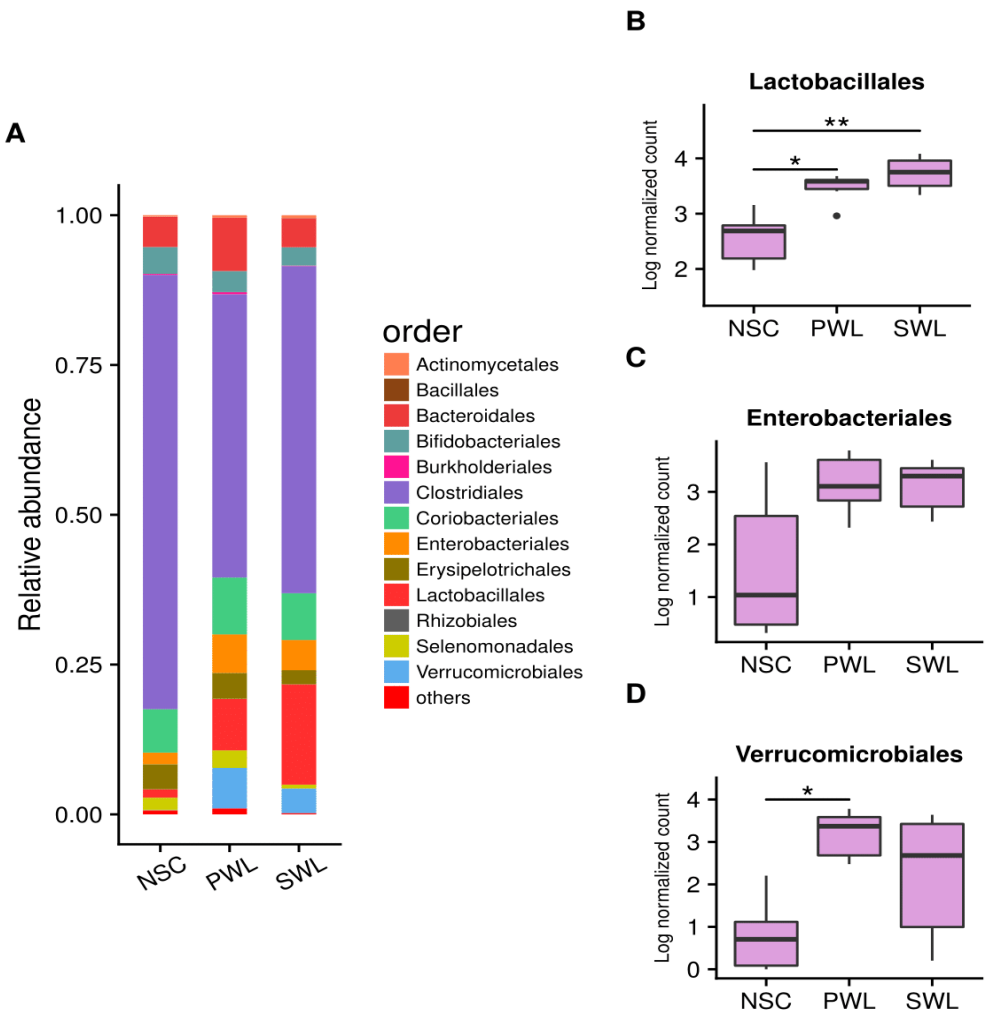

Figure 6. Relative abundance of bacterial orders in SWL, PWL, and NSC humans. Box plots show the abundance of Lactobacillales (B), Enterobacteriales (C), and Verrucomicrobiales (D) in SWL, PWL, and NSC humans. Statistical differences are analyzed by one-way ANOVA with Benjamini-Hochberg post hoc test: * BH-corrected $p<0.05$; ** BH-corrected $p<0.01$.

At the genus level, we observed a greater abundance of *Senegalimassilia* within the Actinobacteria phylum in PWL patients compared to SWL patients and NSC participants, and only the difference between PWL and NSC reached significance after multiple testing corrections. In addition, *Rothia* within the Actinobacteria phylum were significantly higher in PWL and SWL patients compared to NSC participants at a 10% FDR. *Streptococcus* was also significantly enriched in SWL patients compared to NSC participants (Figure 7).

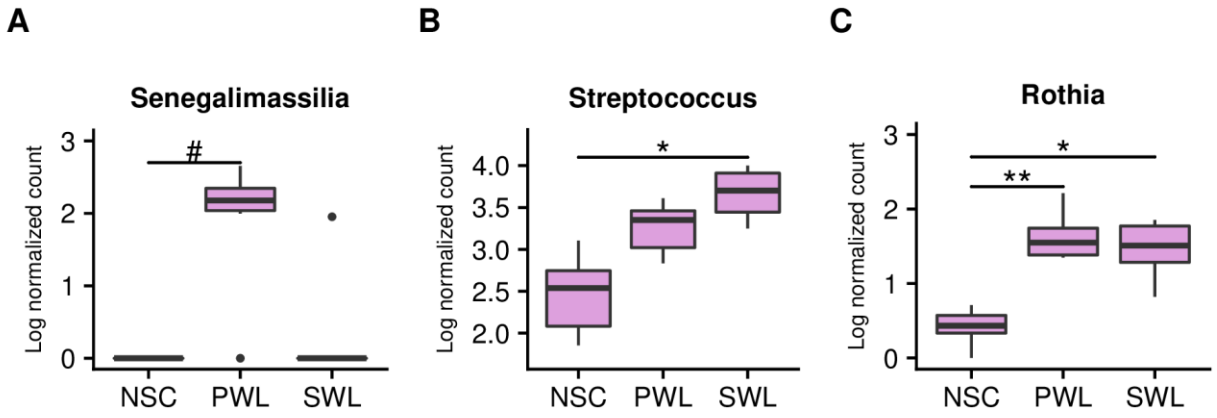

Figure 7. The abundance of bacterial genera that were significantly different between PWL, SWL, and NSC participants. Statistical differences are analyzed by one-way ANOVA with Benjamini-Hochberg post hoc test: # BH-corrected $p<0.1$; * BH-corrected $p<0.05$; ** BH-corrected $p<0.01$.

3.1.5.2. Dissimilarity between the PWL, SWL, and NSC microbial communities

To determine whether the gut microbiota composition was different among PWL, SWL, and NSC participants, multi-dimensional analysis on the taxonomic classification table at the genus level was performed (Figure 8). NSC participants (black symbols) and PWL patients (blue symbols) were separated on the first axis which explained 21% of the total variation in the gut microbial community. There was no clear separation between PWL and SWL microbial communities. Multivariate analysis of variance using the Bray Curtis distance matrix revealed that experimental groups (PWL, SWL, and NSC) influenced the gut microbial composition (ADONIS test, $F(2,15)=1.78$, $p= 0.002$).

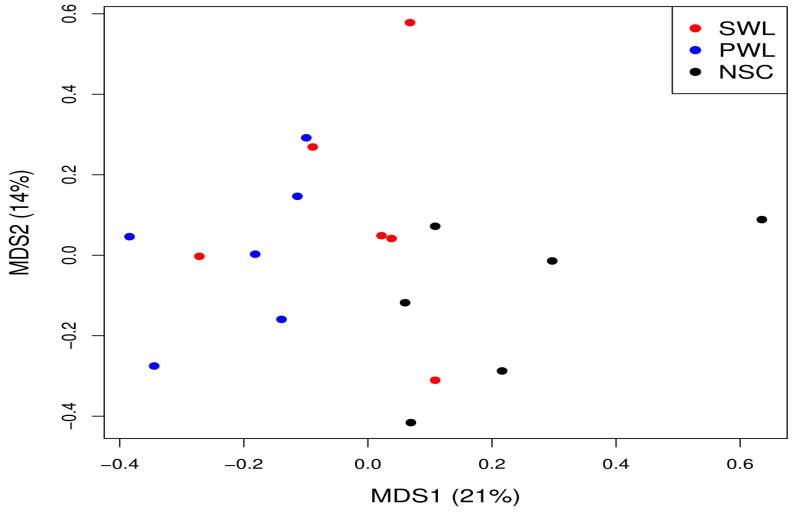

Figure 8. Summary of clustering patterns of microbial communities at the genus level from PWL, SWL, and NSC patients using multidimensional scaling analysis.

3.1.5.3. Within sample species diversity of the gut microbial community

Species diversity within the gut microbial community between PWL, SWL, and NSC participants was compared using Shannon Diversity Index (Figure 9). Shannon Diversity Index was significantly higher in PWL patients compared to NSC participants (BH-adjusted p= 0.014).

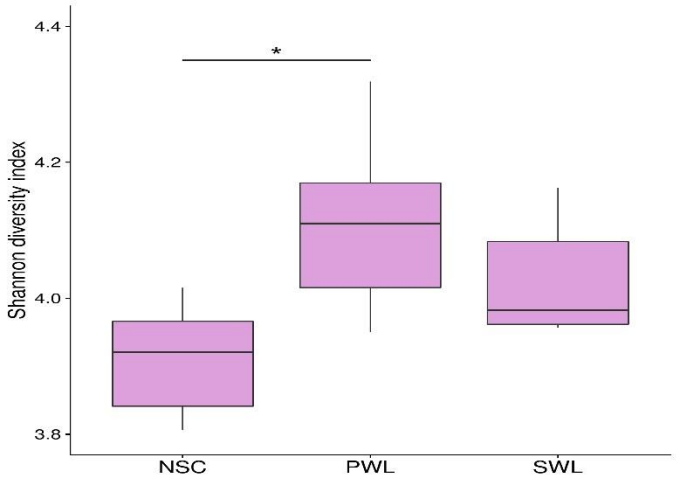

Figure 9. Shannon diversity indices in NSC, PWL, and SWL patients. Statistical differences are analyzed by one-way ANOVA with Benjamini-Hochberg post hoc test: * BH-corrected p<0.05.

3.1.5.4. Relationship between the gut microbiota and plasma gut peptides and serum bile acids

In a Spearman rank correlation analysis, the abundance of *Streptococcus* was positively correlated with postprandial plasma active GLP-1, GLP-2, and PYY (Table 2 and Figure 10). *Veillonella* and *Fusobacterium* were positively correlated with postprandial serum CA, CDCA, and TBA. *Rothia* was positively correlated with CDCA and TBA (Table 2). The abundance of *Rothia* was greater in PWL and SWL compared to NSC participants (BH-corrected p= 0.003). However, the abundance of *Veillonella* and *Fusobacterium* were not different between groups.

Table 2. Spearman correlation of gut microbiota at genus level with the AUC of plasma/serum active GLP-1, GLP-2, PYY, CA, CDCA, and TB concentrations versus time.

Variable	Bacterial genera	Rho Coefficient	p	BH-corrected *P*
GLP-1	*Streptococcus*	0.78	<0.001	**0.036**
GLP-2	*Streptococcus*	0.70	0.001	**0.095**
PYY	*Streptococcus*	0.71	0.001	**0.094**
CA	*Veillonella*	0.77	<0.001	**0.036**
CA	*Fusobacterium*	0.77	<0.001	**0.036**
CDCA	*Fusobacterium*	0.86	<0.001	**0.005**
CDCA	*Veillonella*	0.82	<0.001	**0.013**
CDCA	*Rothia*	0.75	<0.001	**0.038**
CDCA	*Anaerostipes*	-0.70	0.001	**0.095**
TB	*Fusobacterium*	0.75	<0.001	**0.038**
TB	*Rothia*	0.75	<0.001	**0.038**
TB	*Veillonella*	0.71	<0.001	**0.094**

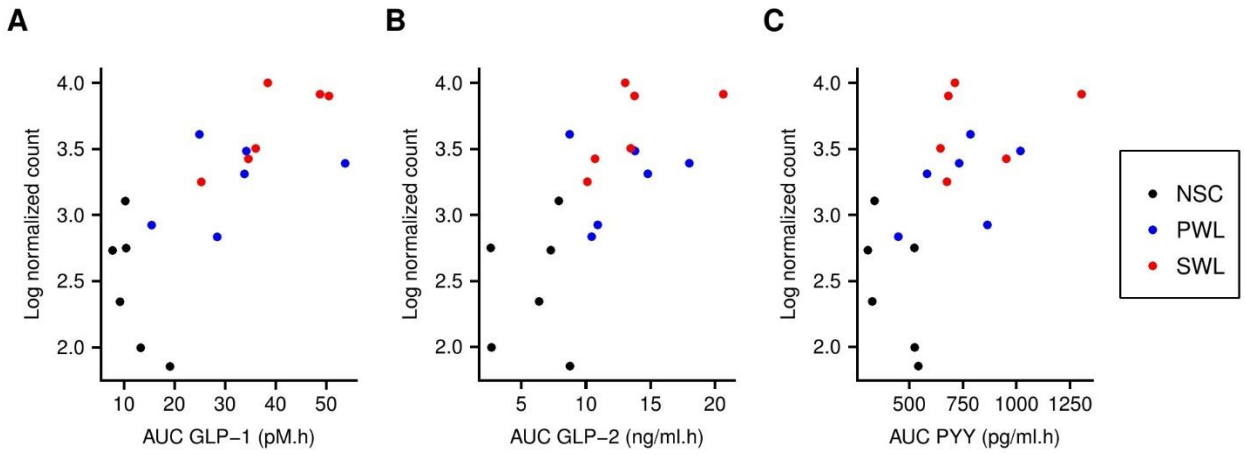

Figure 10. Correlation between *Streptococcus* and the AUC of plasma active GLP-1 (A), GLP-2 (B), and PYY (C) concentrations versus time.

3.2. Animal study

In the animal phase, C57BL/6 mice were treated with a broad spectrum of antibiotics for 17 days. Following antibiotic treatment, mice were colonized with human fecal samples for five days. Body weight and food intake were measured following colonization at weekly intervals. After five weeks, mice were randomly placed on a western diet or remained on a normal chow diet. Body weight was measured at weekly intervals for one month following randomization to the western diet.

3.2.1. Depletion of the gut microbiota by antibiotics

To validate the depletion of enteric microbes using antibiotics, we cultured fecal samples from each mouse during the phase of antibiotic treatment. Since a significant proportion of the enteric microbes are unculturable, we also sequenced the DNA samples obtained after the last dose of antibiotic treatment using 16S rRNA genes sequencing.

3.2.1.1. Culturing of fecal samples

Fecal samples were collected and cultured on the Trypticase Soy Agar on 5, 10, and 15 days following antibiotic treatment. We did not observe growth of bacteria or fungi on the agar-

filled Petri dishes under the aerobic and anaerobic conditions starting from the fifth day of antibiotic treatment. Lack of microbial growth via traditional microbial methods continued until mice were colonized with human gut microbiota (Figure 11).

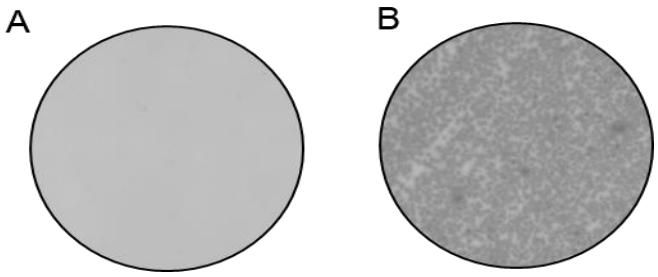

Figure 11. Microbial cultures of fecal samples on Trypticase Soy Agar. (A) Lack of microbial growth when fecal samples were collected at five days after antibiotic treatment and were cultured for 24 hours under aerobic condition. (B) Microbial growth when fecal samples were collected at five days after colonization and were cultured for 24 hours under aerobic condition.

3.2.1.2. 16S rRNA gene sequencing of fecal samples following antibiotic treatment

Fecal samples collected following the last dose of antibiotics were characterized with 16S rRNA gene sequencing. Compared with fecal samples that were collected at baseline (before antibiotic treatment) and five days after colonization with human fecal samples, post-antibiotic samples had the lowest number of sequence reads ($F(2,92)=38.99$, $p<0.001$; Figure 12).

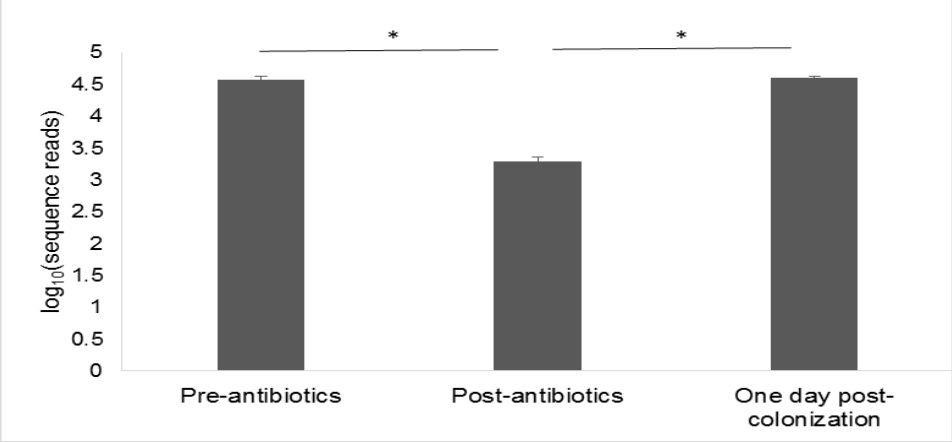

Figure 12. The average logarithm of number of sequence reads from fecal samples at pre-antibiotics, post-antibiotic, and one day post-colonization. Data are presented as means ± standard errors. Statistical differences are analyzed by one-way ANOVA with Hochberg post hoc test: * $p<0.001$.

3.2.2. Gut microbiota transfer from humans to mice

3.2.2.1. Correlation between human and mouse gut microbiota

To determine if the gut microbiota were transferred from human fecal samples into antibiotic-treated mice through oral gavage, the clustering patterns of the gut microbiota from humans and mice before and after colonization were plotted using multidimensional analysis. Figure 13 shows that the gut microbiota of mice at baseline clustered separately from the gut microbiota of humans and colonized mice on the second axis (MDS2), which described 13% of the total variation in the data. On the same axis, there was no distinct separation between the gut microbial communities of humans and colonized mice. However, they were clustered separately on the third axis (MDS3) that only explains 7.1% of the variation in the data (Figure 13). Antibiotic treatment resulted in a shift in the gut microbiota clustering on the first axis (MDS1=33%). Analysis of variance using Bray Curtis distance matrix revealed that the gut microbial composition was different between groups (ADONIS test, $F(5,193)=66.45$, $p= 0.001$).

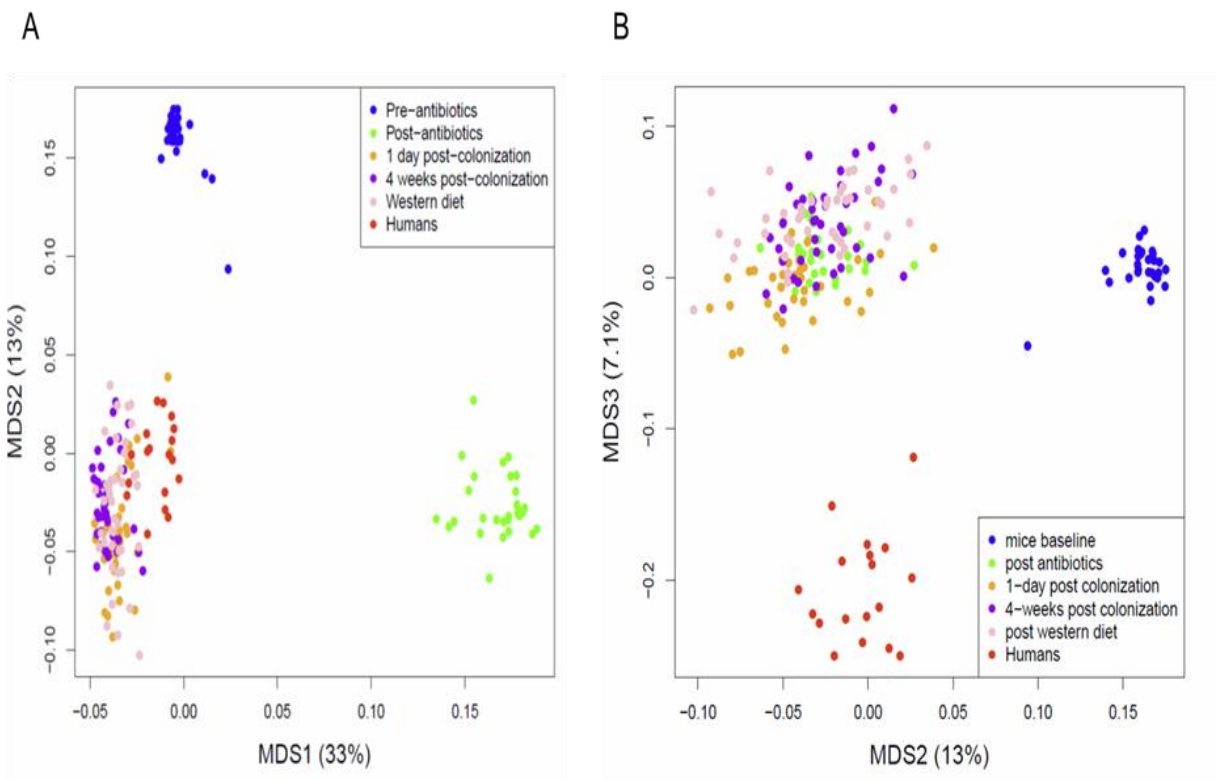

Figure 13. Summary of clustering patterns of microbial communities at the genus level for human patients, mice at pre-antibiotics, post-antibiotics, one day and four weeks post-colonization, and mice on western diet /normal diet using multidimensional scaling analysis. (A) MDS plot using the first two principle coordinates (MDS1 and MDS2). (B) MDS plot using the second and third principle coordinates (MDS2 and MDS3).

In addition, we performed Kendall correlation analysis on the genus level classifications and the first ten multidimensional scaling (MDS) axes to determine the correlation between human and mice microbiota after colonization. Results from Kendall analysis showed that mouse gut microbiota at one day and four weeks post-colonization were weakly correlated with human microbiota on MDS2 axis (tau=0.275, BH-corrected p= 0.002; Figure 14-A) and MDS3 axis (tau=0.253, BH-corrected p= 0.005; Figure 14-B). Out of 122 genera, 35 were significantly correlated between human and mouse microbiota (Table 3), suggesting that these genera were effectively were transferred from humans to antibiotic-treated mice.

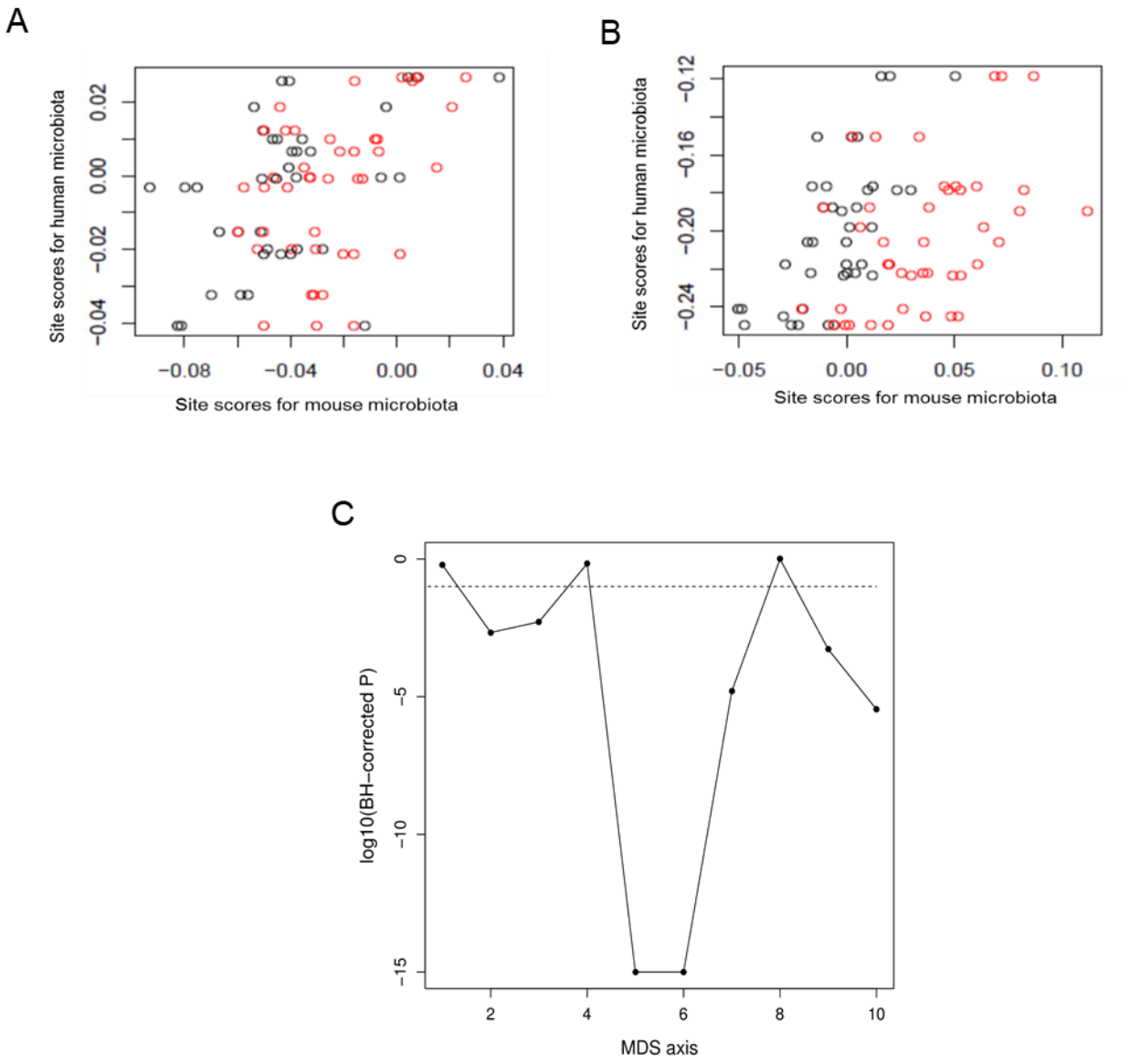

Figure 14. (A, B) Correlation between human gut microbiota and mouse gut microbiota at one day post-colonization (black circles) and four weeks post-colonization (red circles) on MDS2 (A) and MDS3 (B) axes. (C) BH-corrected *P*-values from Kendall test evaluating the correlation between human and mouse gut microbiota on the first ten MDS axes. Dotted line represents the significance level.

Table 3. Correlation between human and mouse gut microbiota.

Bacteria	Tau coefficient	*P*	BH-corrected *P*
Aestuariispira	0.593	<0.001	**<0.001**
Barnesiella	0.708	<0.001	**<0.001**
Butyricimonas	0.585	<0.001	**<0.001**
Paraprevotella	0.749	<0.001	**<0.001**
Slackia	0.635	<0.001	**<0.001**
Sutterella	0.514	<0.001	**<0.001**
Prevotella	0.416	<0.001	**<0.001**
Desulfovibrio	0.460	<0.001	**<0.001**
Mogibacterium	0.439	<0.001	**<0.001**
Coprobacillus	0.415	<0.001	**<0.001**
Holdemanella	0.428	<0.001	**<0.001**
Eggerthella	0.338	<0.001	**<0.001**
Eubacterium	0.371	<0.001	**<0.001**
Oxalobacter	0.407	<0.001	**<0.001**
Phascolarctobacterium	0.353	<0.001	**<0.001**
Odoribacter	0.320	<0.001	**<0.001**
Streptococcus	0.315	<0.001	**0.001**
Christensenella	0.320	<0.001	**0.001**
Butyricicoccus	-0.287	<0.001	**0.001**
Eisenbergiella	0.295	<0.001	**0.002**
Gemmiger	0.304	<0.001	**0.002**
Lactonifactor	0.279	<0.001	**0.003**
Clostridium XVIII	0.268	<0.001	**0.004**
Gordonibacter	0.266	<0.001	**0.004**
Romboutsia	0.284	0.001	**0.006**
Faecalibacterium	0.240	0.003	**0.012**
Ruminococcus	0.263	0.003	**0.012**
Akkermansia	0.218	0.006	**0.021**
Murimonas	0.226	0.010	**0.036**
Bifidobacterium	0.212	0.012	**0.043**
Parasutterella	0.199	0.013	**0.045**
Holdemania	0.199	0.014	**0.048**
Coprococcus	0.212	0.015	**0.049**
Clostridium XlVa	-0.190	0.017	**0.056**
Anaerofustis	0.204	0.018	**0.056**

3.2.2.2. Human and mouse gut microbiota diversity

Species diversity within the microbial communities in humans, mice before colonization, after antibiotics, one day and four weeks following colonization, and one month following western diet /normal diet were measured by Shannon Diversity Index (Figure 15). Human gut microbiota were significantly more diverse compared to mouse gut microbiota before and after colonization (BH-corrected p*<0.001).* Shannon Diversity Index for mouse gut microbiota following colonization tended to be higher compared to the mouse gut microbiota at baseline (before antibiotics) and this reached significance only in mice that were randomly placed on western diet or normal diet during the second phase of study (BH-corrected p=0.043). Shannon Diversity Index was higher in post-antibiotic samples compared to other groups (Figure 15).

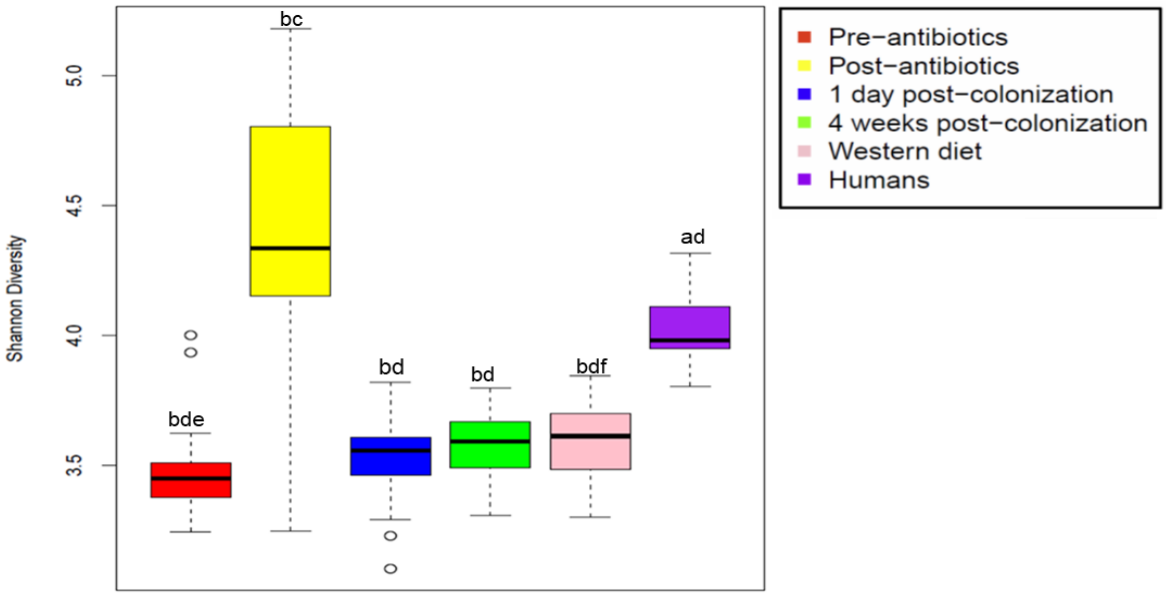

Figure 15. Shannon diversity indices for the gut microbiota in humans and mice at different time points. Statistical differences are analyzed by one-way ANOVA with Benjamini-Hochberg post hoc test. Significant pairwise comparisons (BH-corrected p<0.05) include a versus b, c versus d, e versus f.

Higher diversity in post-antibiotic group could be due to that rare taxa are more likely to be detected in samples with low microbial mass. Therefore, Shannon Diversity Index was

calculated after removal of taxa that present in less than 25% of samples. Following removal of rare taxa from the dataset, Shannon Diversity was significantly smaller in post-antibiotic samples compared to other groups (Figure 16).

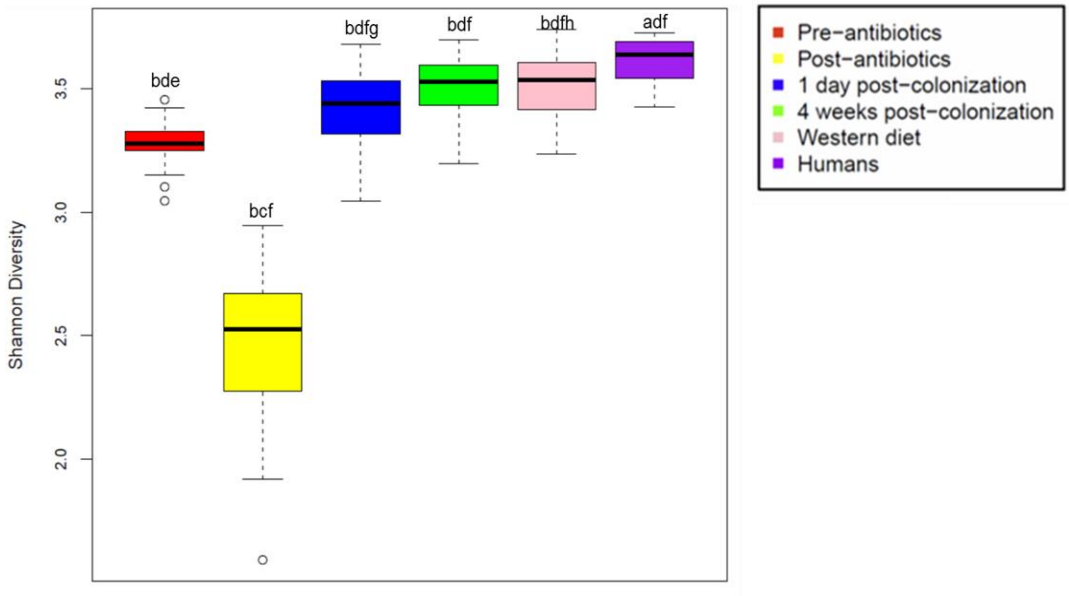

Figure 16. Shannon diversity indices for the gut microbiota in humans and mice at different time points after removal of rare-taxa. Statistical differences are analyzed by one-way ANOVA with Benjamini-Hochberg post hoc test. Significant pairwise comparisons (BH-corrected $p<0.05$) include a versus b, c versus d, e versus f, g versus h.

3.2.3. Weight profile in recipient mice

Mice colonized with human fecal suspensions were weighed weekly for one month following colonization. Baseline body weight prior to colonization was not significantly different between the groups of mice colonized with PWL, SWL, and NSC microbiota ($F(2,41)=1.53$, $p=0.229$).

Two-way ANOVA revealed that experimental group ($F(2,164)=13.26$, $p=0.02$) and time ($F(3,164)=25.25$, $p<0.0001$) significantly influenced body weight gain, while their interaction was not significant. Post hoc analysis showed that mice colonized with PWL microbiota gained

more weight than mice that were colonized with SWL microbiota at two weeks (p=0.009), three weeks (p=0.019), and four weeks (p=0.023) following colonization (Figure 17).

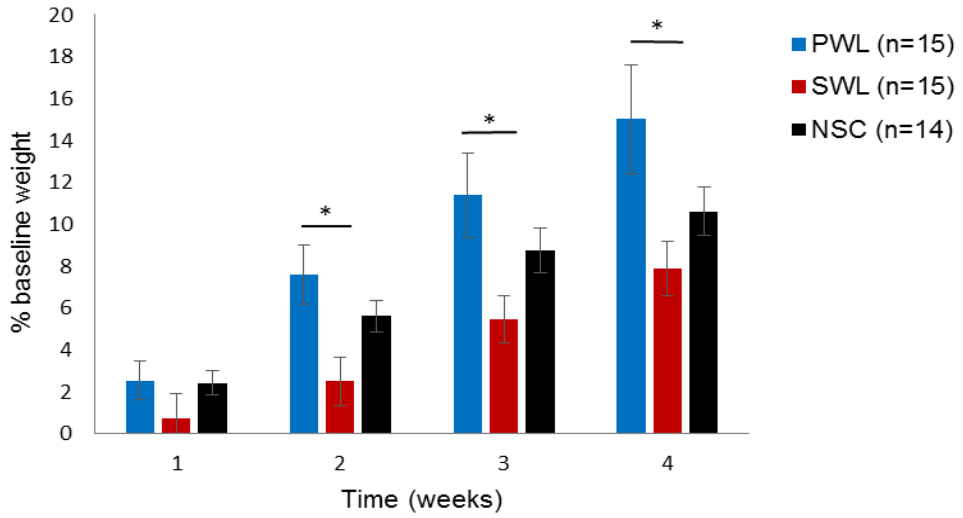

Figure 17. Weight gain in SWL, PWL, and NSC recipient mice at one, two, three, and four weeks following colonization. Data are presented as means ± standard errors. Statistical differences are analyzed by one-way ANOVA with Hochberg post hoc test: * $p<0.05$.

3.2.4. Food intake in recipient mice

Cumulative food intake was measured at weekly intervals following colonization. Cumulative food intake was not significantly different between mice that were colonized by PWL, SWL, and NSC microbiota at each time point (Figure 18).

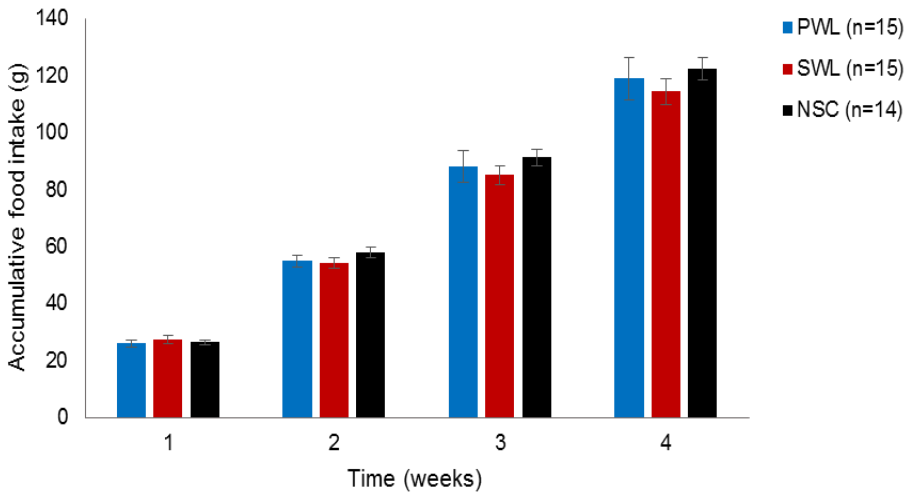

Figure 18. Food intake in recipient mice following one week, two weeks, three weeks, and four weeks following colonization. Data are presented as means ± standard errors.

3.2.5. Relationship between gut microbiota and body weight

To determine how the gut microbiota are associated with weight gain in recipient mice, we performed a series of linear regression models as described in Methods, Section 2.1.2.8. In our first linear regression model, we compared the continuous variable body weight and the categorical variable time (one week and four weeks post-colonization) with log-normalized adjusted counts of bacterial genera at one day and four weeks post-colonization. We observed that weight gain was associated with *Lachnospiracea_incertae_sedis* and *Barsnesiella* at a 10% FDR threshold. Several bacterial genera such as *Turicibacter, Gordonibacter, Pseudoflavonifractor, and Hungatella* increased over time following colorization, while *Bacteroides* and *Bilophila* decreased from one day to four weeks post-colonization (Table 4).

Table 4. Association between mouse weight gain and gut microbiota at the genus level using linear regression models.

Model	Bacteria	Weight		Time	
		p	Adjusted p	p	Adjusted p
A	*Turicibacter*	ns	ns	<0.0001	**<0.0001**
	Escherichia/Shigella	ns	ns	<0.0001	**<0.0001**
	Gordonibacter	0.004	ns	<0.0001	**<0.0001**
	Anaerotruncus	0.008	ns	<0.0001	**<0.0001**
	Pseudoflavonifractor	ns	ns	<0.001	**0.001**
	Bacteroides	ns	ns	<0.001	**0.002**
	Lachnospiracea_incertae_sedis	<0.001	**0.024**	0.001	**0.002**
	Hungatella	ns	ns	0.001	**0.003**
	Aestuariispira	0.011	ns	0.001	**0.004**
	Bilophila	0.074	ns	0.003	**0.010**
	Intestinimonas	ns	ns	0.005	**0.016**
	Acetatifactor	ns	ns	0.008	**0.024**
	Terrisporobacter	ns	ns	0.009	**0.024**
	Flavonifractor	ns	ns	0.013	**0.035**
	Murimonas	ns	ns	0.015	**0.040**
	Barnesiella	<0.001	**0.025**	0.016	**0.040**
	Parabacteroides	ns	ns	0.016	**0.040**
	Blautia	ns	ns	0.017	**0.041**
	Ruminococcus2	0.019	ns	0.020	**0.046**
	Streptococcus	ns	ns	0.021	**0.047**
	Faecalibacterium	ns	ns	0.021	**0.047**
	Prevotella	0.062	ns	0.026	**0.059**
	Clostridium IV	ns	ns	0.033	**0.071**
	Enterococcus	ns	ns	0.034	**0.072**
	Anaerostipes	0.074	ns	0.034	**0.072**
	Sutterella	0.043	ns	0.036	**0.073**
	Eubacterium	ns	ns	0.042	**0.083**
	Romboutsia	ns	ns	0.043	**0.084**
	Ruminococcus	ns	ns	0.044	**0.085**
	Collinsella	ns	ns	0.051	**0.094**
B	*Parasutterella*	0.002	**0.070**		
	Ruminococcus2	0.003	**0.076**		
	Clostridium XlVa	0.006	0.106		
C	*Gordonibacter*	0.001	**0.042**		
	Barnesiella	0.002	**0.070**		

A: Abundance = Weight gain (one week and four weeks post-colonization) + Time + e
B: Abundance = Weight gain (one week post-colonization) + e
C: Abundance = Weight gain (four weeks post-colonization) + e

Next, we executed simple linear regression models that included weight gain as a continuous variable to examine whether weight gain at one or four weeks post colonization was associated with any members of the microbial community at one day or four weeks following colonization, respectively. We found that weight gain at one week post-colonization was negatively associated with *Parasutterella* and positively associated with *Clostridium* cluster *XIVa* and *Ruminococcus 2*. Weight gain at four weeks post-colonization was positively associated with *Barnesiella* and negatively associated with *Gordonibacter* (Table 4).

Among bacterial genera that were associated with weight gain in recipient mice, the abundances of *Barnesiella* within the Bacteroidetes phylum (BH-corrected $p<0.001$), *Gordonibacter* within the Actinobacteria (BH-corrected $p=0.004$), *Parasutterella* within the Proteobacteria phylum (BH-corrected $p=0.045$), and *Clostridium* cluster *XIVa* within the Firmicutes phylum (BH-corrected $p= 0.056$) were significantly correlated with abundances in samples from human donors (Table 3). Figures 18-21 show the correlation of these bacteria with weight gain in recipient mice and also the abundance of corresponding bacterial genera in humans and recipient mice.

Barnesiella, *Parasutterella,* and *Clostridium* cluster *XIVa* were present in low or zero abundance in mice at baseline (before antibiotic treatment). Colonization with human fecal samples after antibiotic treatment transplanted these bacteria into recipient mice. *Barnesiella* tended to be higher in PWL patients compared to SWL and NSC participants ($p=0.032$, BH-corrected $p= 0.458$). Colonization with human fecal samples resulted in higher abundance of *Barnesiella* in PWL recipient mice compared to SWL and NSC recipient mice and this reached significance at four weeks post-colonization ($p<0.001$ and BH-corrected $p<0.001$; Figure 19).

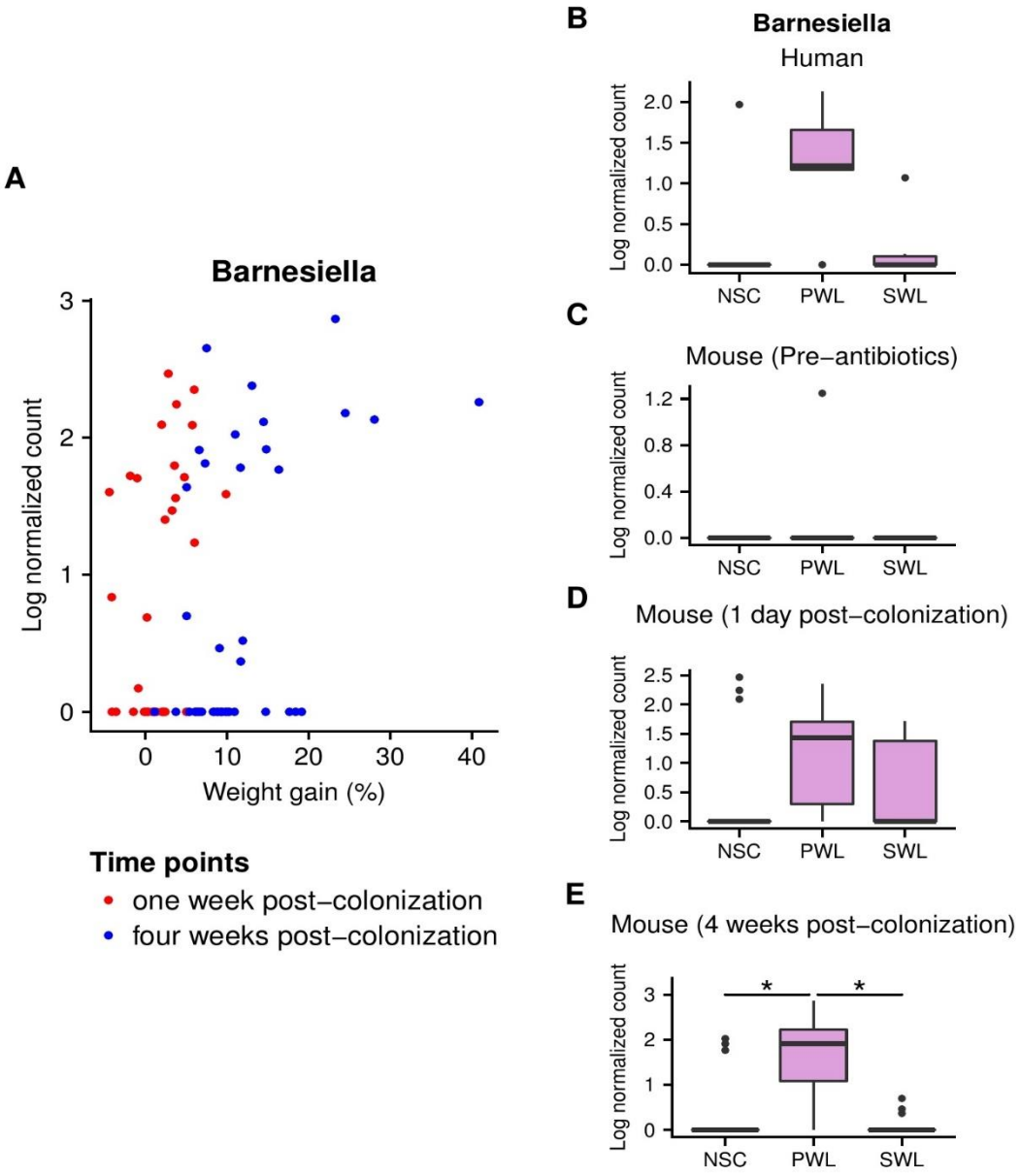

Figure 19. (A) Association between mouse weight gain at one and four weeks post-colonization and log normalized count of *Barnesiella* at one day and four weeks post-colonization. (B-E) Abundance of *Barnesiella* in humans and mice at pre-antibiotics, one day post-colonization, and four weeks post-colonization. * BH-corrected $p<0.05$.

The abundance of *Parasutterella* did not differ between PWL, SWL, and NSC patients. This genus was absent in mice at baseline (before antibiotics). Colonization with human fecal samples transplanted *Parasutterella* into recipient mice, which was negatively associated with weight gain at one-week following colonization. The abundance of *Parasutterella* did not differ

significantly in recipient mice although PWL recipient mice tended to have lower *Parasutterella* one day post colonization relative to SWL and NSC mice (p= 0.044, BH-corrected p= 0.130; Figure 20).

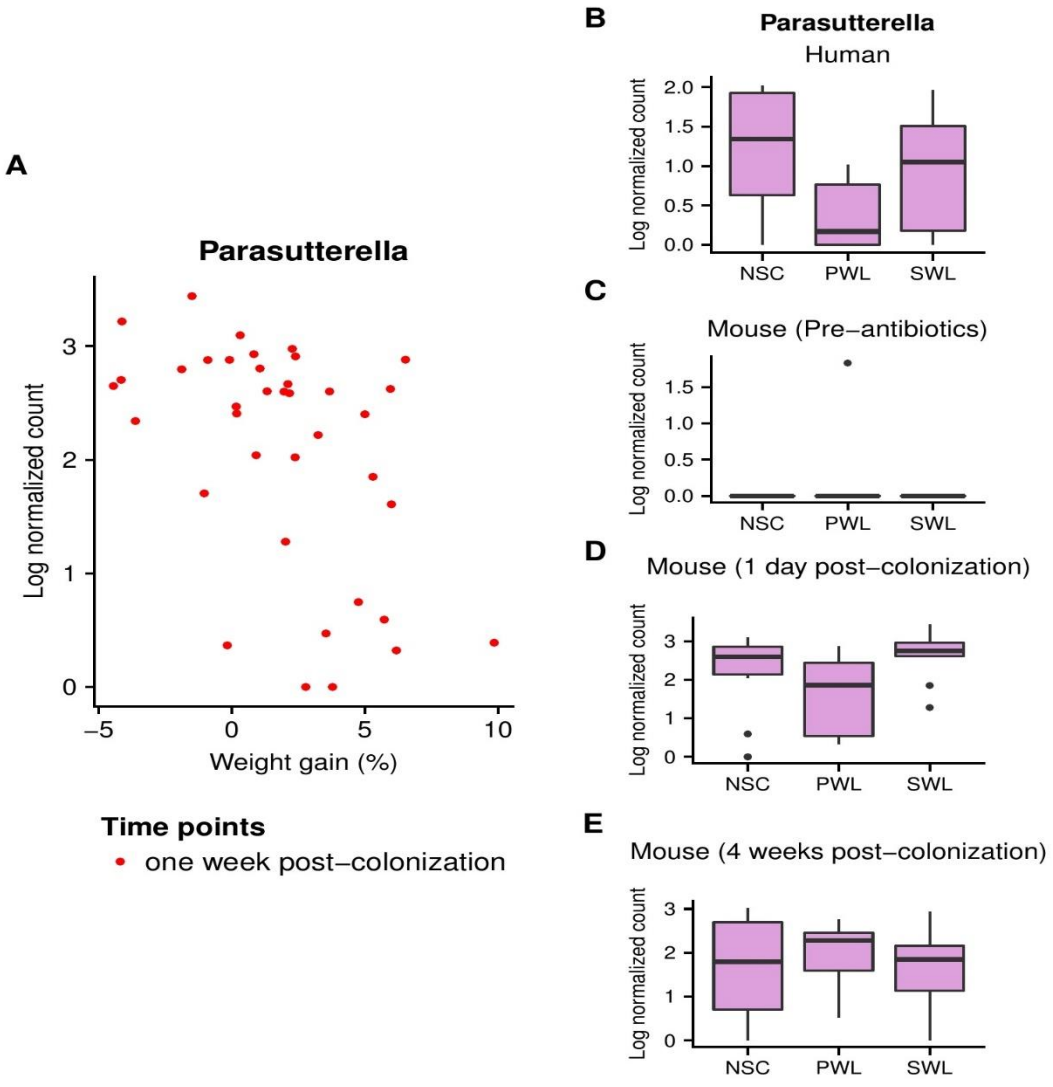

Figure 20. (A) Association between mouse weight gain at one week post-colonization and log normalized count of *Parasutterella* at one day post-colonization. (B-E) Abundance of *Parasutterella* in humans and mice at pre-antibiotics, one day post-colonization, and four weeks post-colonization.

The abundance of *Clostridium* cluster *XIVa* did not differ between PWL, SWL, and NSC participants. This genus was not present in PWL and SWL recipient mice but presented in NSC

recipient mice. Colonization with human fecal samples increased the abundance of *Clostridium* cluster *XIVa* in recipient mice, which was positively associated with weight gain at one week following colonization. NSC recipient mice had greater abundance of *Clostridium* cluster *XIVa* compared to SWL recipient (p<0.001, BH-corrected p= 0.013; Figure 21).

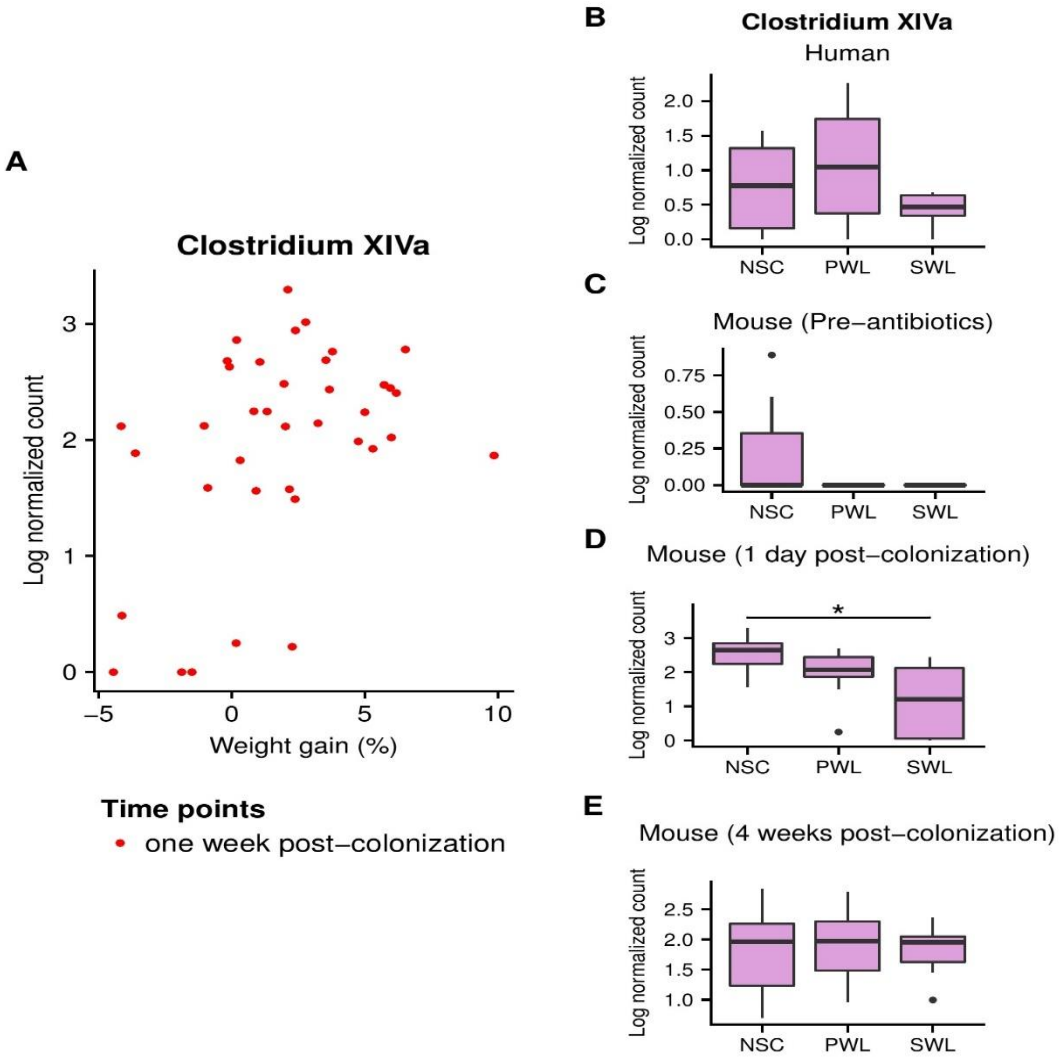

Figure 21. (A) Association between mouse weight gain at one week post-colonization and log normalized count of *Clostridium* cluster *XIVa* at one day post-colonization. (B-E) Abundance of *Clostridium* cluster *XIVa* in humans and mice at pre-antibiotics, one day post-colonization, and four weeks post-colonization. * BH-corrected p<0.05.

The abundance of *Gordonibacter* did not differ between PWL, SWL, and NSC patients. Gordonibacter was present in mice at baseline. Colonization with human fecal samples increased

the abundance of *Gordonibacter* in recipient mice from one day post-colonization to four weeks post-colonization (Table 4). At four weeks post-colonization, Gordonibacter was positively associated with weight gain although its abundance was not different significantly between the PWL, SWL, and NSC recipient mice (Figure 22).

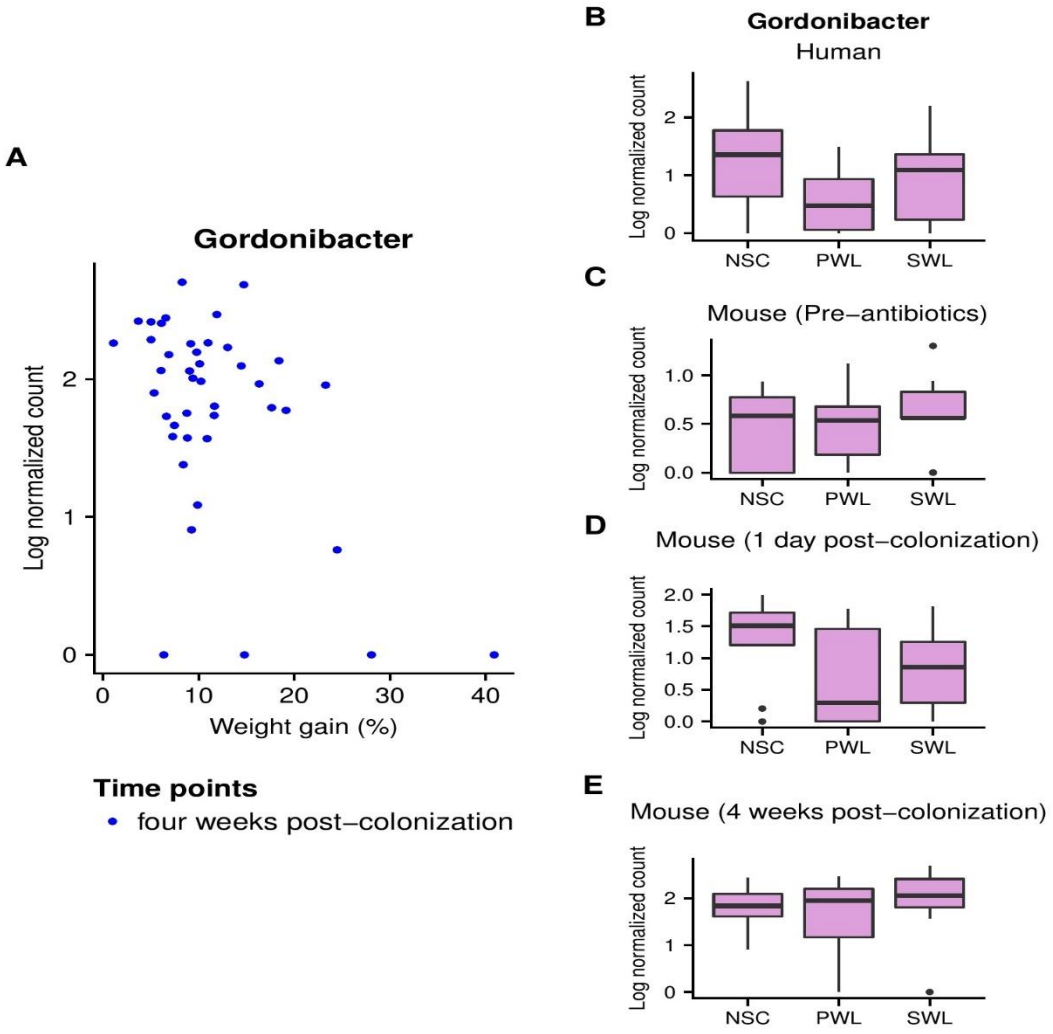

Figure 22. (A) Association between mouse weight gain at four weeks post-colonization and log normalized count of *Gordonibacter* at four weeks post-colonization. (B-E) Abundance of *Gordonibacter* in humans and mice at pre-antibiotics, one day post-colonization, four weeks post-colonization.

3.2.6. Plasma GLP-1 in recipient mice following colonization

Plasma GLP-1 levels one and four weeks following colonization were measured by ELISA. Plasma GLP-1 in SWL recipient mice was increased significantly from the baseline level 25.09±1.38 pM to 32.58±1.70 pM at one week following colonization and 31.97±1.69 pM at four weeks following colonization. Plasma GLP-1 in PWL recipient mice did not significantly increase compared to the baseline level (Figure 23).

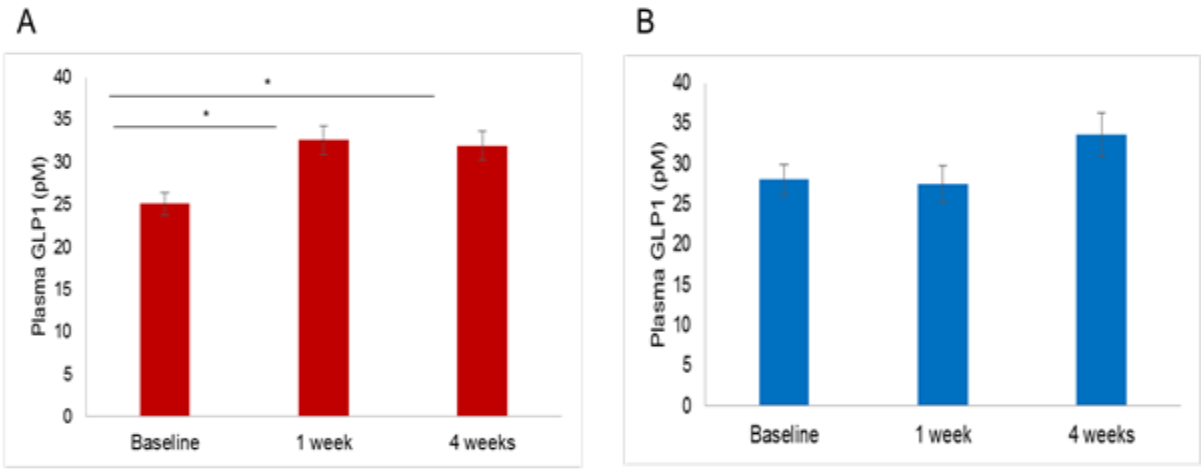

Figure 23. (A) Plasma GLP-1 in SWL recipient mice (n=8) at one week and four weeks following colonization. (B) Plasma GLP-1 in PWL recipient mice (n=6) at one week and four weeks following colonization. Data are presented as means ± standard errors. Statistical differences are analyzed by Wilcoxon signed rank test: * $p<0.05$.

3.2.6.1. Relationship between plasma GLP-1 and the gut microbiota

To determine whether plasma GLP-1 levels were associated with any members of the microbial community, we executed a model of linear regression that included GLP-1 concentrations and time (baseline, one and four weeks post-colonization) as independent variables and log-normalized counts of bacterial genera as a dependent variable. We observed that *Clostridium* cluster *XVIII* and *Clostridium* cluster *XIVa* were negatively associated with plasma GLP1 levels at the 10% FDR threshold. *Clostridium* cluster *XVIII* was present in mice at

baseline, and linear regression analysis showed that time (colonization) did not affect this genus. However, *Clostridium* cluster *XIVa* was present in low abundance at baseline, and colonization with human fecal samples increased the abundance of this bacterium in recipient mice (BH-corrected p<0.001; Table 5).

Table 5. Association between mouse plasma GLP-1 and gut microbiota at the genus level using linear regression.

Bacteria	GLP-1		Time	
	p	BH-corrected *P*	p	BH-corrected *P*
Clostridium XVIII	0.002	**0.084**	ns	ns
Clostridium XlVa	0.003	**0.084**	<0.001	**<0.001**

Linear regression model: Abundance= GLP-1+time+e
Time points included baseline, one week post-colonization, and four weeks post colonization

3.2.7. Effect of the western diet on body weight in recipient mice

Recipient mice in each group were randomly placed on a western diet or were remained on the normal chow diet at five weeks following colonization to determine how the PWL, SWL, and NSC gut microbiota could affect weight gain under a high caloric diet. Body weight was measured at weekly intervals for four weeks. The western diet induced significant weight gain in mice compared to the normal diet ($t(118.17)=17.28$, $p<0.001$; Figure 22). No significant difference was observed between mice grouped according to human donor group (SWL, PWL, or NSC) at any time point during either the western or normal diet phase of the study (Figure 24).

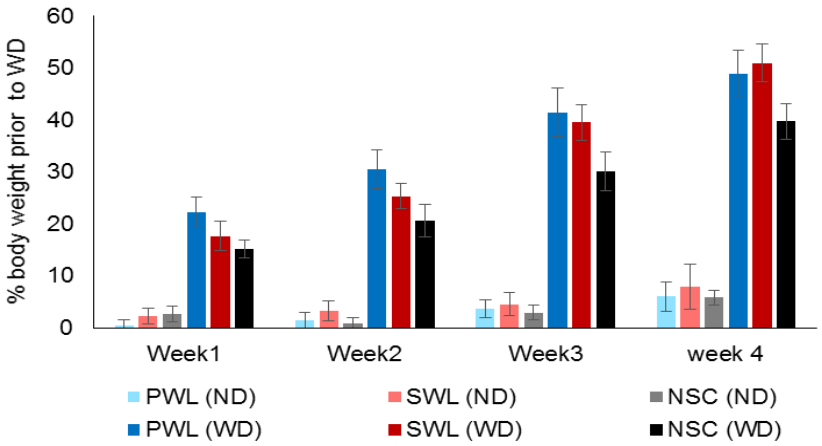

Figure 24. Weight gain (%) following randomization of mice to a normal diet and western diet.

3.2.7.1. Relationship between the gut microbiota and diet and weight gain

Western diet induced significant changes in the gut microbiota composition. Multidimensional analysis revealed distinct clustering patterns for the members of the gut microbial community at the genus level in mice that were on the western and normal diets (Figure 25). Multivariate analysis of variance using Bray Curtis distance matrix revealed that diet has a significant effect on the gut microbial composition (ADONIS test, $F(1,41)=4.94$, $p= 0.002$).

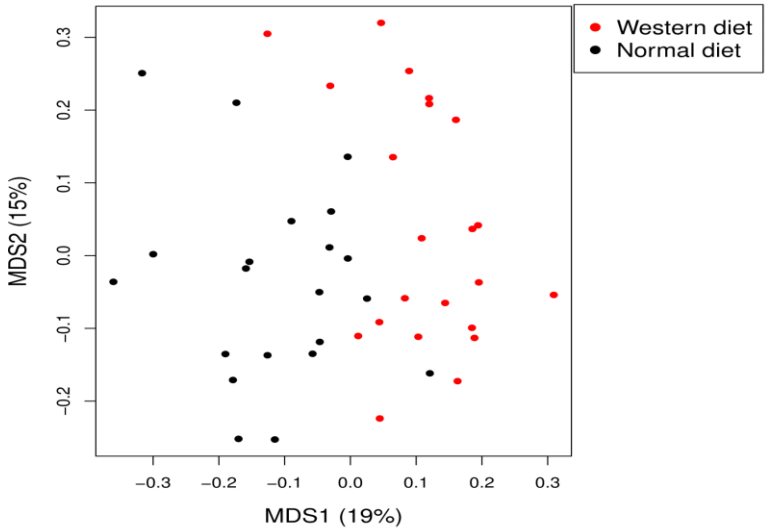

Figure 25. Summary of clustering patterns of microbial communities at the genus level in mice that were on the western and normal diets.

Abundances of several bacterial genera were different in mice on the western diet compared to the normal diet (Table 6). *Lactococcus* within the Firmicutes phylum was present in low abundance in mice on the normal diet, but was significantly enriched in mice that were placed on the western diet. In addition, western diet increased *Hungatella* and decreased *Turicibacter* and *Anaerosporobacter* within the Firmicutes phylum (Figure 26). Among the genera in table 6, *Gordonibacter* had been transplanted from human fecal samples in recipient mice and was decreased significantly in mice that were placed on the western diet compared to the normal diet.

Table 6. Bacterial genera that had significantly different abundances in mice on the western diet compared to mice on the normal diet.

Bacteria	p	BH-corrected p
Lactococcus	<0.001	<0.001
Streptophyta	<0.001	<0.001
Turicibacter	<0.001	<0.001
Hungatella	<0.001	0.001
Gordonibacter	<0.001	0.005
Flavonifractor	<0.001	0.005
Anaerosporobacter	0.005	0.054

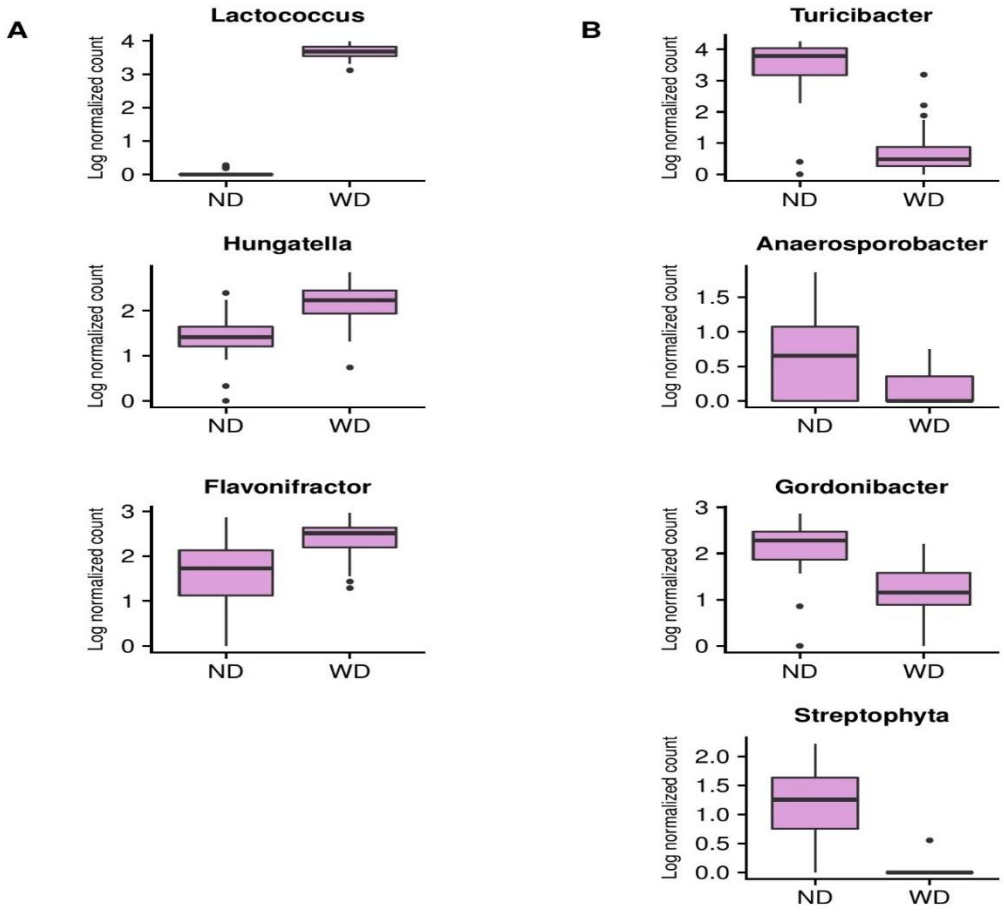

Figure 26. (A) Bacterial genera that were significantly in greater abundance in mice on the western diet compared to mice on the normal diet. (B) Bacterial genera that were significantly in lower abundance in mice on the western diet compared to mice on the normal diet.

The bacteria that were positively or negatively associated with the western diet did not differ between PWL, SWL, and NSC recipient mice. In addition, weight gain in mice on the western diet was not associated with any members of the microbial community at the genus level.

4. DISCUSSION

4.1. Clinical study

The clinical portion of this study was mainly designed to test the hypothesis that the gut microbiota composition would be different between post-RYGB patients who experienced insufficient weight loss or weight regain and post-RYGB patients who experienced successful weight loss. It was further hypothesized that SWL patients would have higher plasma levels of gut peptides and bile acids compared to PWL patients and that the gut microbiota would contribute to the alterations in bile acid and gut peptide profiles.

The hypotheses of this study have been generated based on other studies which have found that bariatric surgery, particularly the RYGB procedure, induces major shifts in the gut microbiota composition and that the altered gut microbiota contribute to weight loss and metabolic benefits after surgery (Liou et al., 2013; Tremaroli et al., 2015). However, no studies have yet determined if the altered gut microbiota would contribute to different weight outcomes after surgery.

4.1.1. The gut microbiota

Our study demonstrated that the gut microbiota were more diverse in post-RYGB patients compared to NSC participants. This was consistent with studies showing increased richness and bacterial diversity following RYGB (Ilhan et al., 2017; Kong et al., 2013; Palleja et al., 2016). Increased microbial diversity and gene richness have been shown to be associated with a healthier metabolic phenotype (Cotillard et al., 2013; Le Chatelier et al., 2013). Anatomical changes induced by RYGB are likely the major driving force responsible for the increase in bacterial diversity since changes in the gut environment allow the overgrowth of acid-sensitive, facultative anaerobic, and bile-tolerant microbes.

We found a higher abundance of Lactobacillales, Enterobacteriales, and Verrucomicrobiales in post-RYGB patients compared to NSC participants. Within the Lacobacillales order, *Streptococcus* was the only genus that was found in higher abundance in SWL patients compared to NSC participants. In addition, *Rothia* was the only member of Actinobacteria that was present in greater abundance in post-surgical patients compared to NSC participants. Several studies using different methods, such as real-time quantitative polymerase chain reaction, shotgun metagenomics sequencing, pyrosequencing, and Illumina sequencing have shown that Proteobacteria were greater in post-RYGB patients. Within this phylum, *Escherichia coli* was enriched both short-term and long-term following surgery (Furet, 2010; Graessler et al., 2013; Kong et al., 2013; Palleja et al., 2016; Tremaroli et al., 2015; Zhang et al., 2009). Proteobacteria following RYGB positively correlated with weight loss (Medina et al., 2017) and *Escherichia* was positively correlated with %EWL (Ilhan et al., 2017), suggesting that increased Proteobacteria may contribute to weight loss following surgery. Increased availability of oxygen to the more distal part of the intestine following RYGB may explain the overgrowth of the aero-tolerant members of Proteobacteria. Furthermore, starvation during the first few months of surgery due to food restriction could promote the growth of bacteria with higher efficiency for energy extraction, such as *E. coli* (Kong et al., 2013). Although we did not observe similar changes in Proteobacteria and *E.coli* in our study, probably due to the low power of our study, we still found Enterobacteriales, an order within Proteobacteria, tended to be higher in both surgical groups compared to the NSC group.

Verrucomicrobia was present in a low abundance in NSC individuals but were higher in post-surgical patients. *Akkermansia muciniphila,* a mucin degrading bacterium belonging to the Verrucomicrobia phylum, is associated with several metabolic benefits, such as improvements in

glucose homeostasis, intestinal integrity, inflammation, and body weight (Dao et al., 2016; Everard, 2011). Using shotgun sequencing, Palleja et al. showed that the relative abundance of *A. muciniphila* increased at three months after surgery, but returned to the baseline level at 12 months after surgery (Palleja et al., 2016). Despite the higher abundance of Verrucomicrobia in the surgical group in our study, *A. muciniphila* was not different among the surgical and non-surgical groups. This could be attributed to the low power of our study or to the participants included in this study being more distal to the time of surgery.

Creation of a small gastric pouch and bypass of the distal part of the stomach increases the pH of the gastric pouch following RYGB. In addition, reduced gastric acid contact with nutrients in the distal part of stomach reduces acid secretion and increases the distal excluded stomach pH to ~ 4 (Liou et al., 2013). Increased gastric pH weakens the defense barrier against the oral microbiome and therefore allows the oral bacteria to colonize in the gastrointestinal tract. This explains the higher abundance of *Streptococcus* in post-RYGB patients in ours and other studies (Ilhan et al., 2017; Medina et al., 2017; Palleja et al., 2016). In addition to *Streptococcus,* Ilhan et al. demonstrated that other bacteria associated with oral cavity and periodontitis, such as *Prevotella, Veillonella, Enterococcus*, and *Haemophilus* were increased in post-RYGB patients (Ilhan et al., 2017). In line with these findings, oral microbiota, such as *Rothia*, *Enterococcus, Streptococcus, Staphylococcus* were enriched in the fecal microbiota of proton pump inhibitor users. This supports the hypothesis that increased gastric pH plays a role in shifting the structure of the gut microbiota toward the oral microbiota (Imhann et al., 2016). Some of the oral bacteria that can be transferred into the distal part of the gastrointestinal tract, such as *Streptococcus, Prevotella,* and *Veillonella* have the capacity to ferment proteins and carbohydrates (Dai, Wu, & Zhu, 2011). Increased fermentation of carbohydrates and proteins

leads to generation of SCFA and branched chain fatty acids (BCFA), which regulate the hunger/satiety response, improve insulin sensitivity, glucose metabolism, and lipid metabolism (Chambers et al., 2015; Heimann, Nyman, Pålbrink, Lindkvist-Petersson, & Degerman, 2016; Ilhan et al., 2017; Lin et al., 2012).

The comparison of the PWL and SWL gut microbiota composition in our clinical study failed to support the hypothesis that SWL patients would have a more diverse gut microbiota profile than PWL patients. We did not observe any differences in the abundance of bacteria at the phylum, class, and order levels. This finding was surprising as the PWL patients had significantly greater BMI at the time of study enrollment than SWL patients. Lack of differences between PWL and SWL microbiota could be due to the fact that the average BMIs for both PWL and SWL patients were in the range of obesity, and therefore BMI per se might not have any further effect on the gut microbiota composition. In other words, it is possible that there is a ceiling effect after which BMI no longer influences the composition of the gut microbiota. This assumption is supported by Tremaroli's study that found two groups of obese patients with average BMIs of 31.90±2.84 kg/m^2 and 44.32±4.71 kg/m^2, serving as controls for post-RYGB patients, had similar microbiomes (Tremaroli et al., 2015).

Nevertheless, lack of significant differences in the gut microbiota composition between PWL and SWL patients in our study could be due to our small sample size. Given that significant differences were detected between the surgical and non-surgical patients with our small sample size, we can conclude that the effect of surgery may be more overwhelming to the gut microbiota composition compared to the effect of BMI. It is also worth noting that similar gut microbiota composition may not translate into similar functionality. Therefore, studies with larger sample

size which use metagenomic and transcriptomic tools are needed to determine if the gut microbiota in PWL and SWL patients have different compositions and/or functions.

4.1.2. Plasma gut peptides

Consistent with previous studies, we found that postprandial plasma levels of gut peptides (active GLP-1, GLP-2, and PYY) were higher in post-surgical patients compared to non-surgical patients. Increased levels of the gut peptides following RYGB have been widely reported in previous literature and they contribute to remission of diabetes and suppression of appetite (Svane et al., 2015). GLP-1 levels, in response to an oral glucose tolerance test or a meal, increased in post-RYGB patients with or without a history of diabetes at one week after RYGB and remained elevated at one year post-surgery. This was associated with reduced fasting glucose and insulin levels along with improved insulin sensitivity in both groups and increased insulin secretion only in patients with diabetes (Bojsen-Moller et al., 2014; Jorgensen et al., 2012). Administration of GLP-1 receptor antagonists to post-RYGB patients with diabetic remission decreased insulin secretion and glucose tolerance, while administration did not affect insulin secretion before surgery (Jorgensen et al., 2013). This suggests that exaggerated GLP-1 response after RYGB surgery is an important mediator of glucose control and improved β-cell function. GLP-2 and PYY levels are similarly increased after RYGB (Jacobsen et al., 2012; Nannipieri et al., 2013; Peterli, 2012). Gastrointestinal anatomical changes and rapid transit of nutrients to the distal part of the intestine following RYGB explain the early and long-term rise in the L cell-derived gut hormones.

Postprandial gut peptides did not differ between SWL and PWL groups, suggesting that gut peptides are not likely to be the major mediator of sustained weight loss after bariatric surgery in our sample. This finding is in contrast with a study that showed that patients with

favorable weight loss outcomes (i.e., more than 30% weight loss) had greater GLP-1 and PYY responses than patients with less favorable weight outcomes (patients with less than 25% weight loss; le Roux, 2007). Dirksen et al. also found greater GLP-1 release during a multiple-meal test in patients who experienced successful weight loss versus those who experienced poor weight loss 12 months after surgery. In the same study, plasma PYY levels were not different between good weight loss and poor weight responders (Dirksen et al., 2013). Furthermore, Hollanda et al. demonstrated that GLP-1 response to a meal was greater in SWL versus PWL in patients studied at ≥ 24 months after surgery. In this study, PYY and GLP-2 response did not differ between the two groups of patients (de Hollanda et al., 2014). These two latter studies along with our study do not support a role for PYY and GLP-2 in weight regulation following RYGB. PYY response to a meal was attenuated in obese patients compared to normal weight subjects in an earlier study (le Roux et al., 2006). Therefore, the lower BMI of SWL patients (25.1 kg/m^2) compared to PWL patients (37.8 kg/m^2) in le Roux's study may be the reason for the higher PYY response observed in SWL patients versus PWL patients. On the other hand, in Dirksen and Hollandas' studies and our study, the SWL patients were overweight or obese, and their average BMI for SWL patients ranged between 27-30 kg/m^2 and for PWL patients ranged between 36-40 kg/m^2.

Previous studies have found an increased GLP-1 response in SWL patients compared to PWL patients (de Hollanda et al., 2014; Dirksen et al., 2013; le Roux, 2007). Lack of difference in GLP-1 response to a meal between PWL and SWL patients in our study could be attributed to the small samples size. However, mechanistic and interventional studies in animals suggested that GLP-1 did not play a role in weight reduction after RYGB. In one such study, GLP-1 receptor knockout mice lost a similar amount of weight and fat mass and similarly sustained the weight loss following RYGB compared to wild-type mice (Ye et al., 2014). In addition, mice

with GLP-1 secretion deficiency showed comparable weight loss and improvements in glucose homeostasis compared to wild-type mice in another study (Mokadem, Zechner, Margolskee, Drucker, & Aguirre, 2014). These studies call into question whether GLP-1 moderates the effects of RYGB on body weight and glucose homeostasis after surgery and more research is needed concerning this relationship.

Interestingly, we observed that *Streptococcus* which was present in higher abundance in post-surgical patients compared to non-surgical participants was positively associated with active GLP-1, indicating that *Streptococcus* may contribute to the increased level of gut peptides after surgery. *Streptococcus* is a member of a group of lactic acid producing bacteria, and Streptococcus *thermophilus* has been widely used in probiotics. Yadav et al. have shown that administration of VSL#3 probiotics containing *Lactobacillus*, *Streptococcus*, and *Bifidobacterium* strains to high-fat diet fed mice prevents weight gain and improves glucose tolerance. This effect was associated with increased butyrate and increased GLP-1, suggesting that these bacteria regulate GLP-1 release through short chain fatty acid production (Yadav, Lee, Lloyd, Walter, & Rane, 2013). Furthermore, it has been shown that *Lactobacillus* and *Streptococcus* were enriched in patients who underwent biliointestinal bypass and this was associated with higher plasma levels of GLP-1, supporting that lactic acid producing bacteria, including *Streptococcus* may contribute to increased GLP-1 levels after surgery (Federico et al., 2016).

4.1.3. Serum bile acids

Similar to gut peptides profiles, post-prandial bile acids tended to be higher in post-surgical patients but not different between SWL and PWL groups. Numerous studies have shown that serum bile acid levels are increased after gastric bypass surgery (Ahmad, Pfalzer, & Kaplan,

2013; Albaugh et al., 2015; De Giorgi et al., 2014; Nakatani et al., 2009; Pournaras et al., 2012; Simonen et al., 2012; Steinert et al., 2013), and one recent study reported that the increase in bile acid levels is independent of calorie restriction (Jahansouz et al., 2016). The increase in bile acids occurs as early as a few weeks after surgery (Jahansouz et al., 2016; Pournaras et al., 2012), and one study showed increased bile acid levels could be sustained for 2-4 years after surgery (Patti et al., 2009). In accordance, post-RYGB participants in our study were investigated on average 3.2 years following surgery, and they showed higher levels of bile acids compared with the NSC group. Postprandial bile acids were elevated in RYGB participants compared to postprandial bile acids in NSC participants, although some other studies have also found higher levels of fasting bile acids following RYGB compared to pre-surgery or controls (Kohli et al., 2013; Steinert et al., 2013; Werling et al., 2013).

Studies have shown that increased bile acids are associated with improved glucose and lipid metabolism (Patti, 2009); however, studies on the role of increased bile acids in weight loss after surgery are still relatively lacking. In agreement with Dirksen's study (Dirksen et al., 2013), our study did not show any difference in bile acid profiles between PWL and SWL patients. Previously, it was shown that Fibroblast Growth Factor 19 (FGF19), which is stimulated by bile acids in the intestine, was not different between patients who experienced failed weight loss and successful weight loss (de Hollanda et al., 2014). This may indicate that bile acid levels and their targets are mainly affected by surgery and are not altered by weight changes after surgery.

Increased bile acids after RYGB surgery could be due to the bypass of the proximal intestine and the accelerated delivery of undiluted bile acids to the lower part of the intestine (Fouladi et al., 2016). In addition, changes in intestinal bile acid absorption, bile acid synthesis, and bile acid metabolism could further contribute to increased bile acid levels after bariatric

surgery (Bhutta et al., 2015; Ionut, Burch, Youdim, & Bergman, 2013; Myronovych et al., 2014). Of note, changes in the gut microbiota following surgery might affect the bile acid profile since the gut microbiota regulate bile acid composition under normal conditions (Sayin et al., 2013). One study showed that patients who were nine years post- RYGB had greater primary and secondary bile acids and this was associated with enrichment of 7α-dehydroxylation genes in their gut microbiota compared to obese patients. This indicates that the gut microbiota after surgery may be more efficient in metabolizing bile acids compared to the gut microbiota in obese patients (Tremaroli et al., 2015). Enhanced microbial metabolism of bile acids was associated with increased bile acid synthesis, reductions in weight gain, plasma cholesterol and hepatic triglycerides (Degirolamo et al., 2014; Joyce et al., 2014). In our study, we observed that total bile acids were higher in post-surgical patients compared to NSC participants and this was mainly due to increased primary bile acids (CDCA). In contrast to Tremaroli's study, we did not observe any significant differences in secondary bile acids between post-RYGB and NSC groups. Our small sample size does now allow us to make a clear conclusion on the microbial metabolism after RYGB.

Nevertheless, we found that some genera, including *Viellonella* and *Anaerostipes* within the Firmicutes phylum, *Fusobacterium* within the Fusobacteria phylum, and *Rothia* within the Actinobacteria phylum were positively correlated with primary bile acids (CA and CDCA) and TBA. The gut microbiota hydrolyze conjugated bile acids and biotransform primary bile acids into secondary bile acids. Previously, it has been shown that anaerobic bacteria (*Bacteroides*, *Bifidobacteria*, *Clostridia*, and *Veillonella*) can deconjugate bile salts (Aries, Crowther, Drasar, & Hill, 1969). Bile salt hydrolases (BSH) are the major enzymes responsible for deconjugation of bile salts and are widely distributed among the members of the gut community. Functional

metagenomics analysis revealed that BSH activity is mostly present in Firmicutes (30%), Bacteroidetes (14.4%), and Actinobacteria (8.9%) (Jones, Begley, Hill, Gahan, & Marchesi, 2008). Association some of the genera with primary bile acids in our study may imply the involvement of the bacteria in the deconjugation process. Particularly, the role of *Rothia* in bile acid metabolism may be pronounced after RYGB as the abundance of this genus was higher in post-RYGB patients. *Rothia* consist of aerobic and facultative anaerobic species. They are abundant in the distal ileum and also present in the oral microbiota (Segata et al., 2012; Villmones et al., 2018). Increased gastric pH and oxygen concentration in the distal GI might increase the abundance of this genus in the fecal microbiota following RYGB. Such changes in the gut microbial community after RYGB can change bile acid composition and therefore affect host metabolism.

4.1.4. Summary

In summary, we found that surgery had a sustainable effect on the gut microbiota composition, gut peptides, and bile acids, with few differences between the two surgical groups. *Streptococcus* and *Rothia* were correlated with gut peptides and bile acids, respectively, suggesting that these bacteria may play a role in the post-surgical changes in gut peptides and bile acids. With the limitation of our small sample size, we were not able to detect any significant differences in the gut microbiota between SWL and PWL patients. A humanized mouse model was, therefore, used to further investigate any potential difference in the composition and function of the gut microbiota between PWL, SWL, and NSC participants.

4.2. Animal study

The animal part of this study was mainly designed to test the hypothesis that the gut microbiota in PWL patients would contribute to weight regain following surgery and the gut

microbiota from SWL patients would contribute to sustained weight loss. It was further hypothesized that the gut microbiota from PWL patients would promote greater weight gain under high caloric intake compared to the gut microbiota from SWL patients.

4.2.1. Transplantation of human gut microbiota into mice

Our animal study involved a 17-day period of antibiotic administration to C57BL/6 mice to deplete the gut microbiota and to mimic the germ-free mouse model. Although germ-free mice are widely used in the microbiota research, antibiotic-treated mice are an alternative approach when using germ-free mice is not feasible due to lack of infrastructure and special facilities. Antibiotic-treated mice also offer other advantages over germ-free mice. Germ-free mice have underdeveloped lymphatic and immune systems due to lack of exposure to microbial components and therefore do not mimic normal physiological conditions. Furthermore, germ-free mice are not available for all strains, which could be used in different disease models. Therefore, protocols have been established to deplete the enteric microbes in mice by using broad-spectrum antibiotics (Reikvam et al., 2011). Hintze et al. (2014) showed that mice treated with antibiotics and then gavaged with human fecal samples adopted a distinct microbiota profile, which resembled their human donors. Metabolomic analysis of cecal metabolites revealed that the metabolic profiles of humanized mice were different from control and antibiotic-treated mice (Hintze et al., 2014), suggesting that the gut microbiota composition and function are transmissible from human donors to antibiotic-treated mice.

Our results showed that oral gavage of fecal samples to mice transplanted a number of bacteria from humans into recipient mice. In fact, colonization with human fecal samples resulted in a new configuration of the gut microbiota in the recipient mice, which was different from the gut microbiota at baseline before antibiotic administration. Consistent with other studies

of this type, not all bacterial taxa from humans survived in the recipient mice, which is likely a result of the different environments and host and/or loss of viability during the pre-colonization process of the human stool samples. Accordingly, in Hintze's study, 57% of sequence mass in humanized mice was shared exclusively with their human donor (Hintze et al., 2014).

In our study, some of the bacteria that were transplanted in mice from humans were stable or increased in abundance throughout the experiment, while others, including *Streptococcus* and *Bifidobacterium* were decreased from one-day post-colonization to four weeks post-colonization. Similarly, one study demonstrated that the gut microbiota in mice that had been inoculated with the gut microbiota of obese or lean mice were not stable and underwent some changes with time within each group (obese or lean recipient mice). Despite the changes in the gut microbiota over time, the difference in the gut microbiota of obese and lean recipient mice persisted until six weeks following colonization (Ellekilde et al., 2014).

4.2.2. Body weight, food intake, and the gut microbiota

We observed that mice which received the gut microbiota from PWL patients gained more weight compared to the mice that received the gut microbiota from SWL patients, supporting our hypothesis that the gut microbiota from PWL patients could contribute to weight regain after surgery. Studies have shown that transfer of gut microbiota from obese donors resulted in greater adiposity gain in recipient mice compared to the gut microbiota of lean donors (Ridaura et al., 2013; Turnbaugh et al., 2008; Vijay-Kumar et al., 2010). These findings may raise the question of whether the greater weight gain that we observed in PWL recipient mice would be simply the result of the higher BMI of their donors. To test this, we controlled for the effect of the donors' BMI when comparing the weight gain between PWL, SWL, and NSC recipient mice. Weight gain in PWL mice remained significantly higher compared to SWL mice

after controlling the effect of donors' BMI. In addition, despite a significant difference between BMIs of PWL and NSC donors, the difference in weight gain of their counterpart recipient mice did not reach significance, supporting that BMI did not drive the difference in weight gain between SWL and PWL recipient mice.

Similar food intake among the groups of recipient mice indicates that the PWL microbiota would contribute to weight gain through mechanisms independent of food intake. Previously, Liou et al. (2013) showed that transfer of the gut microbiota from RYGB mice into germ-free mice resulted in decreased body weight compared to the gut microbiota from SHAM mice. Similar to our result, food intake between the RYGB and SHAM recipient mice was comparable (Liou et al., 2013). Increased energy production from diet, increased fat accumulation in adipose tissues, and inducing low grade-inflammation are potential mechanisms by which the gut microbiota could affect the host metabolism and body weight and composition (Seganfredo et al., 2017).

We observed that *Barnesiella, Clostridium* cluster *XIVa, Gordonibacter,* and *Parasutterella* were transplanted from human donors into mice and were also associated with weight gain in recipient mice. *Barnesiella* and *Clostridium* cluster *XIVa* were positively associated with weight gain, while *Gordonibacter* and *Parasutterella* were negatively associated with weight gain. *Gordonibacter* is one of the members of the Coriobacteriaceae family within the Actinobacteria phylum. Coriobacteriaceae has been reported to play a role in the regulation of glucose metabolism. In germ-free mice colonized with the gut microbiota, Actinobacteriaceae was strongly associated with low levels of hepatic glucose and glycogen (Claus et al., 2011). Increased Actinobacteria after RYGB was associated with a decrease in gamma-glutamyl transpeptidase and alkaline phosphatase (Medina et al., 2017). In addition, some studies found

that the family Coriobacteriaceae was decreased in diabetic patients compared to non-diabetic controls (Karlsson et al., 2013; Liu et al., 2018). Coriobacteriaceae in the cecum was also higher in mice that were resistant to the onset and maintenance of obesity and high fat liver (Clavel et al., 2014; Le Roy et al., 2013). Consistent with these results, our study showed that lower abundance of *Gordonibacter* was associated with greater weight gain in recipient mice.

Interestingly, we observed that *Barnesiella*, which was positively associated with weight gain, presented in the higher abundance in PWL recipient compared to SWL and NSC recipient mice, which mirrored the trend observed in their human donors. This suggests that *Barnesiella* may be one of the members of the microbial community that could promote weight regain after surgery. Le Roy et al. (2013) demonstrated that transfer of the gut microbiota from mice that responded to a high-fat diet to germ-free mice resulted in hyperglycemia, insulin resistance, and increased hepatic lipogenesis, while these metabolic abnormalities were not present in germ-free mice that received the gut microbiota from non-responders to a high-fat diet. Furthermore, recipient mice colonized with the gut microbiota of responders to high-fat diet had higher *Barnesiella* compared to mice colonized with the gut microbiota of non-responders, suggesting that *Barnesiella* may contribute to the propensity to development of metabolic disorders (Le Roy et al., 2013). Another study showed that higher abundance of *Barnesiella* in inflammasome-deficient mice was associated with hepatic steatosis and inflammation (Henao-Mejia, 2012).

We demonstrated that *Parasutterella*, a member of the Proteobacteria phylum, was associated with lower weight in recipient mice during the first week. Studies on this genus are rare, and its effect on health and disease is not evident. *Parasutterella* has been recently negatively correlated with hypothalamic inflammation in obese patients, and its abundance is influenced by food intake. Patient with lower fat intake had higher abundance of *Parasutterella*

and decreased mediobasal hypothalamus inflammation and gliosis (Kreutzer et al., 2017). Administration of galacto-oligosaccharides to mice increased the abundance of *Parasutterella*, which was positively correlated with SCFA and amino acids (Cheng et al., 2018). On the other hand, this genus was enriched in the submucosa of patients with Crohn's disease (Chiodini et al., 2015) and was more abundant in individuals who were carriers of methicillin-resistant *Staphylococcus aureus* compared to non-carriers (Dong et al., 2018). The microbial symbiosis associated with hypertriglyceridemia-induced acute necrotizing pancreatitis included a higher abundance of *Parasutterella* compared to acute necrotizing pancreatitis without hypertriglyceridemia (Huang et al., 2017). The clinical relevance of these findings is yet to be determined.

Measurements of fasting plasma GLP-1 showed that GLP-1 increased from the baseline level to the post-colonization level in SWL recipient mice. However, GLP-1 did not increase in PWL mice following colonization. We observed that *Clostridium* cluster *XIVa* was negatively associated with plasma GLP-1. *Clostridium* cluster *XIVa* presented in a very low abundance in mice at baseline and increased following colonization with human fecal samples. Interestingly, *Clostridium* cluster *XIVa* was positively associated with weight gain, and it seems that lower abundance of *Clostridium* cluster *XIVa* in SWL recipient mice might have contributed to lower weight gain and higher plasma GLP-1 levels at one week following colonization. The *Clostridium* cluster *XIVa* within the Firmicutes phylum includes species belonging to the *Clostridium*, *Eubacterium*, *Ruminococcus*, *Coprococcus*, *Dorea*, *Lachnospira*, *Roseburia,* and *Butyrivibrio* genera (Lopetuso, Scaldaferri, Petito, & Gasbarrini, 2013). In obese patients compared to non-obese patients the Firmicutes:Bacteriodetes ratio was higher. Increased abundance of Firmicutes was attributed to an increase in *Clostridium* cluster *XIVa* (Verdam et

al., 2013). A reduction in clostridial species was also observed in post-RYGB patients compared to their BMI-matched non-surgical controls (Tremaroli et al., 2015). Many species belonging to *Clostridium* cluster *XIVa* have butyrate producing properties, and therefore they can increase energy harvest from diet and contribute to obesity (Ruth E. Ley et al., 2006; Schwiertz, 2010). Several species belonging to this cluster, such as *Coprococcus catus*, *Eubacterium ventriosum*, *Ruminococcus obeum*, *Butyrivibrio fibrisolvens*, and *Dorea longicatena* were associated with obese phenotype (Federico et al., 2016; Kasai et al., 2015). However, some members of the *Clostridium* cluster *XIVa* may have metabolic benefits. For example, studies have shown that diabetes is associated with decreased abundance of *Roseburia* (Karlsson et al., 2013; Qin et al., 2012). Furthermore, restoration of *Roseburia* in high-fat-fed mice through chitin-glucan (CG) supplement decreased body weight gain and improved glucose and lipid metabolism (Neyrinck et al., 2012).

4.2.3. Effect of the western diet on humanized mice

To determine if the PWL microbiota would contribute to greater weight gain under a high caloric diet, mice were switched to a western diet or remained on a normal diet five week following colonization. All mice that were kept on the western diet significantly gained weight, and there was no significant difference in weight gain among groups of mice with different microbiota. This finding rejects our hypothesis that PWL microbiota would promote greater weight gain compared to SWL microbiota when mice are placed on a western diet. Lack of a difference in weight gain between mice with different gut microbiota could be attributed to the fact that we placed mice on the western diet five weeks following colonization. We have shown that several bacterial genera following colonization changed over time after colonization, and therefore the gut microbiota might not have represented the PWL and SWL microbiota at the

time of dietary change. In addition, we showed that the western diet induced significant changes in the gut microbiota in all the three groups of mice. Therefore, it is possible that the effect of the western diet is overwhelming compared to the effect of the baseline microbiota. In support of this theory, Turnbaugh et al. (2009) demonstrated that the gut microbiota colonized from a human donor into germ-free mice reached a stable configuration by 28 days after colonization. However, placing mice on a western diet induced a significant shift in the microbial community that was evident from one day following the western diet (Turnbaugh, 2009b). This result indicates that diet has a significant impact on the gut microbiome composition over a short time interval.

We observed that the western diet was associated with a bloom in the class of Bacilli (mainly *Lactococcus*). At the genus level, *Hungatella* and *Flavonifractor* within the Clostriadales class were increased. Some other genera, including *Turicibacter*, *Anaerosporobacter*, *Gordonibacter*, and *Streptophyta* in all groups of microbiota groups of mice decreased. In Turnbaugh's study, the western diet induced an increase in the relative abundance of the class of bacilli (mainly *Enterococcus*) and Erysipelotrichia and a decrease in the members of Bacteroidetes (Turnbaugh, 2009b). The different effects of the western diet on the microbial community between our study and Turnbugh's study may be explained by the use of antibiotic-treated mice instead of germ-free mice in our study and also different baseline microbial community before switching to the western diet.

4.2.4. Summary

In summary, by using a humanized mouse model we showed that the gut microbiota in PWL patients were more obesogenic and can contribute to weight regain after surgery compared to the SWL microbiota. Our study revealed that a number of members of the gut microbial

community might be accountable for the greater weight gain in recipient mice. Of note, *Barnesiella* was associated with the PWL microbiota and therefore may be implicated in weight regain after RYGB. Future studies are warranted to confirm these data and determine the molecular mechanisms by which the gut microbiota induce weight regain. Furthermore, metagenomics and transcriptomic studies are needed to determine the functional impact of the PWL and SWL gut microbiome on body weight and metabolism.

4.3. Limitations

Our study has a number of limitations. First, the sample size in our clinical study was small, and therefore the study might have been low powered to detect any difference in the gut microbiota composition, gut peptides, and bile acids between PWL, SWL, and NSC subjects. However, by transplanting the human microbiome into mice, we were able to detect the bacteria that might have contributed to weight gain in the human donors and overcome some of the limitations inherent to the clinical study.

Second, due to the cross-sectional nature of the clinical study, data on the percentage of weight regain after surgery could not be obtained. Therefore, it was not possible to accurately determine whether participants initially experienced successful weight loss followed by weight regain, or whether they never achieved 50% EWL following surgery. This could be of importance as patients with different weight gain profiles (initially failed weight loss versus weight loss followed by regain) may exhibit different metabolic and microbial profiles. Additionally, pre-surgery weight was collected through self-report and may have been subject to retrospective recall bias.

Third, cholecystectomy may affect serum bile acid synthesis and metabolism (Berr, Stellaard, Pratschke, & Paumgartner, 1989) and the gut microbiota (Keren et al., 2015). In our

study, 83% of SWL patients and 50% of PWL underwent cholecystectomy. The difference in the rate of cholecystectomy between groups may act as a confounding variable, and this will need to be more carefully examined in larger studies on this topic.

Fourth, the use of humanized mouse model has limitations. Transplantation of the gut microbiota from a human donor with a specific metabolic phenotype into germ-free mice or antibiotic-treated mice has been the primary approach used to investigate the relationships between the specific members of the gut microbiota and a disease phenotype. However, determining the cause and effect relationship from such studies should be interpreted with caution as the observed association between an individual bacterial taxon and a metabolic phenotype may be dependent on the reconfigured microbial community in the recipient mice or on the communication within members of the microbial community (Ussar, Fujisaka, & Kahn, 2016). For example, Goodrich et al. demonstrated that the family Christensenellaceae was negatively correlated with BMI in a large data set including 977 individuals. They also showed that this family was associated with reduced weight gain in recipient mice colonized with obese and lean donors, suggesting that Christensenellaceae is associated with the the lean phenotype. This was further supported by the finding that addition of *Christensenella minute* to the microbiota inoculum from an obese donor and its transfer to germ-free mice resulted in lower weight gain compared to microbiota inoculum without *Christensenella minute*. However, the addition of *Christensenella minute* reshaped the microbial community and enriched other taxa, such as Oscillospira and Rikenellaceae (Goodrich et al., 2014). This suggests that the reshaped microbiota may be functionally active in reducing weight gain rather than specific species per se.

Finally, genetic host and environment closely interact with the gut microbiome, and therefore the association between specific members of the gut microbiota and a disease

phenotype observed in one study may not be replicated with a different genetic background host or a different environment.

4.4. Future directions

Longitudinal studies and the use of complex metagenomic analyses will help us to improve our understanding of how the surgically induced alterations in the structure and function of the gut microbiome would affect or predict weight outcomes following bariatric surgery.

In addition, findings from our study support the need for additional studies exploring interventions targeting the gut microbiota as a potential tool to improve weight outcomes after surgery. Using the humanized mouse model, we expect to be able to identify interventions for reducing microbiota-related unfavorable weight outcome that are worthy of subsequent exploration in clinical trials. One potential intervention would be the administration of probiotics in the humanized mouse model to examine the potential for this intervention to initiate reversal of weight regain.

Our study also supports future studies to investigate the relationship between alterations in the gut microbiota and other surgically related complications, such as mood disorders or changes in eating behavior.

5. CONCLUSION

The results of this study led to the conclusion that RYGB surgery induces major shifts in the gut microbial community and these changes are present in both SWL and PWL patients. However, the humanized mouse model suggested that the PWL microbiota is either functionally or compositionally different enough from SWL patients to contribute to weight gain after RYGB. At the genus level, *Barnesiella, Clostridium* cluster *XIVa, Gordonicater,* and *Parasutterella* were found to be correlated with weight gain in the humanized mouse model. Particularly, *Barnesiella* was associated with the PWL microbiota and therefore might have contributed to weight regain after surgery. Future studies should be pursued to confirm the potential contributions of these bacteria to weight regain and to unravel the molecular and genomic mechanisms behind their effects on weight outcomes after RYGB surgery.

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