Profiling the Bacterial and Fungal Gut Microbiome of Children with and without Prader-Willi Syndrome (PWS)

by

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Abstract

Obesity is a complex multifactorial disease that has a large array of direct (e.g. altered metabolic and inflammatory profile) and indirect (e.g. mental health concerns etc.) consequences on health and is associated with increased morbidity and mortality. Prader-Willi Syndrome (PWS) is the most common syndromic form of childhood obesity, characterized by abnormally increased and insatiable appetite (hyperphagia). The pathogenesis of hyperphagia and weight gain in PWS is poorly understood and management strategies have been met with limited success.

The gut microbiome has been implicated in several metabolic disorders such as obesity and diabetes. Interest in the role of the gut microbiome in genetic forms of obesity has emerged, however the specific role of the gut microbiome in PWS and childhood obesity is not fully understood.

This thesis work features a cross-sectional case-control study with two major objectives. The first objective was to characterise and compare the gut microbiome composition in children with and without PWS. The second objective was to determine if collected metadata could help to explain the compositional differences between groups.

A total of fifty children (n=25 PWS and 25 Controls) aged 3-17 (male and female) were recruited for this study. For each participant, a stool sample, a 3-day dietary record, a hyperphagia questionnaire, and anthropometric measures were collected. For this work, both bacterial (16S rRNA via Illumina) and fungal (ITS2) sequences were considered. This is a novel

result as the fungal component of the gut microbiome in PWS has not been previously explored.

The gut microbiome was compared both between PWS and control (CON) groups and between weight-classified subgroups ("overweight/obese" (OWOB) and "normal weight" (NW) subgroups). Assessments of alpha-diversity (Chao1, Shannon and Simpson indices), beta-diversity (Bray-Curtis) and differential abundance (DESeq2) were performed. Spearman correlations and canonical correspondence analysis (CCA) were used to assess associations between the microbial community (bacterial and fungal) and collected hyperphagia and dietary intake information.

Significant differences in fungal community structure (beta-diversity) were observed between the PWS and CON groups. These differences were observed when comparing PWS and CON individuals ($P_{PWS\ vs\ CON} < 0.001$), as well as when comparing the PWS and CON groups in both the NW and in the OWOB weigh-class subgroupings ($P_{NW\ PWS\ vs\ CON} = 0.02$ and $P_{OWOB\ PWS\ vs\ CON} < 0.001$ respectively). Comparing within the PWS and CON groups for differences in NW and OWOB individuals did not yield any significant differences in beta-diversity. Unlike the fungal community, no differences were observed in the bacterial community structure between groups.

Higher bacterial Shannon diversity was observed in the CON group compared to the PWS group (p=0.04). For the fungal alpha-diversity assessment, the OWOB PWS group was found to have increased Chao1 richness compared to the OWOB CON group (p=0.04). No other significant differences were observed in alpha-diversity metrics.

The PWS group was found to be characterized by an increased relative abundance of the genus *Candida* and relative decreased abundance of *Saccharomyces* relative to that of the CON group. No differentially abundant taxa were found in the bacterial profile of the PWS and CON groups (2 group analysis), however differences were observed in pairwise analyses.

Group type (PWS vs CON) was found to have the greatest potential to explain variation in fungal communities in the CCA model. No statistically significant findings were observed between taxa and the hyperphagia and dietary intake data using correlations, however dietary components were a significant contributor to variation explained by the CCA model of fungal communities.

The results of this thesis work provide a rationale for future exploration of the fungal component of the gut microbiome in PWS to further elucidate the clinical implications of these findings. Additionally, an understanding of the unique gut microbial profile of children with PWS has the potential to unveil more personalized approaches for effective treatment of excessive weight gain and hyperphagia, ultimately leading to improvements in overall health and quality of life.

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List of Abbreviations

BMI: body mass index

CCA: Canonical correspondence analysis

CCK: cholecystokinin

CDC: Centers for Disease Control and Prevention

CON: Control group

DEL: deletion

DESeq2: Differential Expression analysis for Sequence count data version 2

FDR: false discovery rate

FMT: Fecal microbiome transplant

GH: growth hormone

GI: gastrointestinal

GLP-1: glucagon-like peptide 1

HOMA-IR: homeostatic model assessment insulin resistance

ITS2: Internal Transcribed Spacer 2

kcal: kilocalorie

LDA LEfSe: Linear Discriminant Analysis Effect Size

lfcSE: log fold change Standard Error

log2FC: log2FoldChange

LPS: lipopolysaccharide

MACs: microbiota-accessible carbohydrates

NW: Normal weight

OTU: Operational taxonomic Unit

OWOB: Overweight/obese

PCoA: Principle Coordinate Analysis

PERMANOVA: Permutational analysis of variance

PWS: Prader-Willi syndrome

PYY: peptide tyrosine tyrosine

QoL: quality of life (QoL)

T2D: type 2 diabetes

TLR: Toll-Like Receptors (TLR)

UPD: uniparental disomy

WHO: World Health Organization

Chapter 1: Introduction

1.0 Background

1.0.1 Obesity overview

Obesity has been described as an accumulation of excess adiposity that often results in impaired health. The National Institutes of Health and the World Health Organization defines obesity as a Body Mass Index (BMI) of ≥30 kg/m^2 (Harrison, 1985; Ulijaszek, 2003). BMI calculation measures an individual's body weight adjusted for height squared [weight (kg)/height (m^2)].

Weight status for adults is determined using BMI, with 'healthy' body weight classified as a BMI between 18.5 and 24.9 kg/m^2, 'overweight' ranging between 25.0 and 29.9 kg/m^2, and obesity as ≥30 kg/m^2 ("Clinical guidelines on the identification, evaluation, and treatment of overweight and obesity in adults: executive summary. Expert Panel on the Identification, Evaluation, and Treatment of Overweight in Adults," 1998; Jensen et al., 2014). For children over 2 years of age, weight status is determined using BMI percentiles, based on the Centers for Disease Control and Prevention (CDC) 'BMI-for-age growth charts.' In the pediatric population, 'overweight' is defined as a BMI between the 85th -95th percentile for an individual of the same age and sex, and pediatric 'obesity' is defined as a BMI at or above the 95th percentile for an individual of the same age and sex. Current guidelines assess weight status by calculating and plotting weight for-length in children under 2 years old.

Obesity is one of the largest global health problems of our time and is associated with significant detrimental health outcomes (including increased risk of developing type 2 diabetes mellitus, hypertension, non-alcoholic fatty liver disease, obstructive sleep apnea, cardiovascular complications, dyslipidemia and a variety of metabolic dysfunctions), as well as increased healthcare and economic costs. In Canada, approximately one in every three children meets the criteria of being overweight or obese, which greatly increases their risk for developing multiple comorbidities and their likelihood of maintaining obesity into adulthood. Insight into the

pathogenesis and treatment of childhood obesity is urgently needed. Obesity is undoubtedly multifactorial, and major contributing factors include genetic predisposition, diet, physical activity, environmental factors, as well as economic, psycho-social, and cultural factors. More recently, the role of the gut microbiome in the pathogenesis and etiology of obesity has emerged. Alterations of composition and subsequent functionality of the gut microbiome has been shown to play a role in the risk and pathogenesis of obesity and metabolic dysfunction (Singer-Englar, Barlow, & Mathur, 2019). The mechanisms of action through which the microbiota may influence obesity are still being elucidated, however links have been made between the gut microbiota and changes in GI tract physiology and function (including barrier-function), influences in triglyceride production and deposition in adipocytes, increases in lipogenesis, decreases in fatty-acid oxidation, alterations in immune function and regulation, changes in insulin sensitivity, and regulation of bile acid synthesis to name a few (Boulangé, Neves, Chilloux, Nicholson, & Dumas, 2016). More detailed mechanisms will be discussed further in this thesis work.

Research into genetic or syndromic forms of obesity can provide unique insight into the pathophysiology of obesity and potential treatment. One of the most well-known obesogenic genetic syndromes is Prader-Willi Syndrome (PWS).

1.0.2 Prader-Willi Syndrome overview

Prader-Willi Syndrome (PWS) is the most common syndromic form of obesity. This rare genetic disorder occurs 1 in 10,000 to 1 in 15,000 live births and has no prevalence-bias to a particular sex or ethnicity (Irizarry, Miller, Freemark, & Haqq, 2016b). PWS results from an imprinting defect of the paternal genes on chromosome 15q11-q13; however the genetics of PWS is complex and the exact genes responsible for the PWS phenotype is not known (Irizarry et al., 2016b). Approximately 70% of cases of PWS attribute their origin to deletions of the paternal 15q11-q13. Maternal uniparental disomy (UPD) accounts for 20–30% of cases, and is caused by maternal meiotic nondisjunction followed postzygotic mitotic loss of a single paternal chromosome 15 (Cassidy, 1995). A minority of cases are due to imprinting defects (which can occur with no detectable mutation) and through microdeletions in the imprinting center of the

15th chromosome (Nicholls, Saitoh, & Horsthemke, 1998). Depending on the exact genetic origin, the symptoms and deficits in PWS may differ in severity (Nicholls & Knepper, 2001). Imprinting defects of the same region on chromosome 15 has been implicated for both Prader-Willi and Angelman syndromes. It is the loss of the paternal contribution that is linked to PWS and the loss of the maternal contribution that is linked to Angelman syndrome (Gurrieri & Sangiorgi, 2011). In these syndromes, the presence of a second non-affected copy of the gene(s) on chromosome 15 does not correct the defect.

PWS is characterized by a myriad of clinical features including distinct physical features, failure to thrive and low muscle tone during infancy, deficiencies in growth hormone (GH) secretion, delayed motor and cognitive development, behavioral difficulties, disrupted metabolic and endocrine function and sleep disturbances (Irizarry et al., 2016b). Of most relevance to this thesis work is the extreme food-seeking and insatiable hyperphagia (abnormally increased appetite for food) phenotype which begins to develop in childhood and carries through to adulthood (Irizarry et al., 2016b). This abnormal feeding behavior driven by insatiable appetite can lead to progressive weight gain (if the environment is not strictly controlled) and greatly increases the risk for developing severe obesity, metabolic dysfunction, cardiorespiratory difficulties, and other co-morbidities associated with excessive weight gain such as type 2 diabetes and negative psychosocial consequences at an early age (Butler, Manzardo, Heinemann, Loker, & Loker, 2017; McAllister, Whittington, & Holland, 2011).

The need for strict control of access to food (Kayadjanian, Schwartz, Farrar, Comtois, & Strong, 2018) to control the food seeking characteristic of PWS is a source of stress for both individuals with PWS and caregivers. Physical and mental aspects of quality of life (QoL) have been shown to be impaired in individuals with PWS compared to individuals in a healthy population (Caliandro et al., 2007; Caliandro, Grugni, Taruscio, Kodra, & Padua, 2011). Of the factors studied, weight was the major component that negatively influenced the reported physical aspects of quality of life (Caliandro et al., 2007). Children and adolescents with obesity have been shown to have drastically lower QoL scores than the general population. (Schwimmer, Burwinkle, & Varni, 2003; Varni, Limbers, & Burwinkle, 2007). There is no doubt

that obesity greatly depreciates QoL for children and their caregivers. Parents of children with rare diseases tend to have more stress and report lower QoL scores (Dellve, Samuelsson, Tallborn, Fasth, & Hallberg, 2006). With a syndrome like PWS where obesity is usually observed, the QoL in both parents and children have been shown to be even lower than typically developing children with obesity (Wilson, Wiersma, & Rubin, 2016). In addition to the effects on QoL, there are also reported increased economic burden associated with PWS. For example, in Europe the estimated annual cost per PWS patient in 2012 ranged from € 3937 to € 67,484 (depending on the country) (López-Bastida et al., 2016). This estimate included both direct healthcare and non-healthcare costs as well as loss of labour productivity.

The combination of decreased QoL, higher economic burden and the complex clinical picture presented by PWS makes it clear that families affected by PWS would require more support. As the literature has identified behavioural issues (such as hyperphagia) and weight problems as a major source of decreased quality of life for patients with PWS, research aimed towards improving quality of life measures through inexpensive and non-invasive treatment and prevention programs are needed.

Currently, treatment of the hyperphagia that is characteristic of PWS has been largely unsuccessful and attempts to control weight and prevent metabolic decompensation through dietary interventions have had limited success (Crinò, Fintini, Bocchini, & Grugni, 2018). In addition, the pathogenesis of hyperphagia and weight gain in PWS is poorly understood (Heymsfield et al., 2014). Because of this, a need to expand our focus and look for new treatment angles is paramount, especially for pediatric populations so we may intervene early and prevent future negative health outcomes.

Early intervention in nutritional management has been shown to have potential benefits in impeding excessive weight gain (Pipes & Holm, 1973). In terms of nutritional management, the most success has been found by enforcing an energy restricted diet of 7kcal/cm per day to induce weight loss or 8-11 kcal/cm per day to maintain weight (Holm & Pipes, 1976; Irizarry, Miller, Freemark, & Haqq). These measures convert to daily intakes of 600-1300 kcal/day depending on the age of the patient. When limited to this level of intake, which is much lower

than that of healthy children, it is difficult to ensure proper nutrition is being maintained (Crinò et al., 2018), with previous studies indicating deficiencies in variety of essential nutrients including vitamin D, tocopherol, calcium, and iron (Lindmark, Trygg, Giltvedt, & Kolset, 2010; Mackenzie et al., 2018). In addition, the optimal nutritional balance for individuals with PWS is still a matter of debate and results from dietary interventions remain inconclusive (Tan et al., 2019). Other interventions aimed at attempting to reduce hyperphagia include drug treatments and bariatric surgery. Several medical agents have been researched with varying levels of success ; however there is no currently approved drug treatment to treat hyperphagia in PWS (Crinò et al., 2018). Pharmaceutical approaches have included medications used to 1) target carbohydrate metabolism and insulin regulation (e.g. metformin), 2) improve lipid digestion/absorption (e.g. orlistat), 3) to increase energy expenditure (e.g. sibutramine, lorcaserin, bupropion, naltrexone), 4) to address hormonal disturbances in PWS such as hyperghrelinemia (e.g. ghrelin O-acyltransferase (GOAT) inhibitors such as RM-853 by rhythm pharmaceuticals) and 5) reduce appetite/food seeking behavior (topiramate, rimonabant, somatostatin analog, exenatide, liraglutide) (Crinò et al., 2018). Bariatric surgery has been increasingly used as treatment of morbid obesity in the general population and has also been used in the PWS population (Crinò et al., 2018; Irizarry et al., 2015). Along with the limitation of being an invasive procedure, Obesity Canada reports that access to bariatric surgeries in Canada is extremely limited and has very long wait times throughout the process; a process that is even more arduous for the PWS population as there are very few bariatric surgeons in Canada who are trained to do surgery in children or adolescents (Harwick, Iuppa, & Fekety, 1969).

As discussed, there are currently no effective treatment options for individuals with PWS that allow them to live independently. Since quality of life is drastically decreased with pediatric obesity and is even lower in PWS, research into new avenues for treatment is an important endeavor.

Recent research suggest that the gut microbial community may play an important role in the etiology of obesity and maintenance of weight gain. Specifically, intervention trials have

been conducted to provide appetite regulating effects harnessing the potential of the gut commensals through several mechanisms including endocrine regulation and altered gastric motility (Boulangé et al., 2016). Gaining a better understanding of the gut microbiome could lead to potential new approaches for effective, non-invasive, and relatively inexpensive treatment of PWS and childhood obesity.

1.0.3 Microbiome overview

The human body is host to over 10–100 trillion microbes that colonize numerous body habitats including the skin, oral cavity, vagina and gastrointestinal (GI) tract. Specific microbial ecosystems have reliably been shown to differ between body sites, and each body site has been shown to have different potential implications for host health. There is a wealth of evidence in support of the theory that our ancestors have co-evolved with the microbial communities that inhabit our bodies and that these microbial communities play an important role in several key physiological processes of their hosts, including influencing immune development and regulating host metabolism (Singer-Englar et al., 2019). This thesis work focused on the gut microbiome, which encompasses the host's largest and most diverse microbial network. The human GI tract hosts a robust ecosystem composed of a dynamic microbial community of an estimated 10^{14} microbes, including bacterial, archaea, eukaryotes (such as fungi) and viral members (Hillman, Lu, Yao, & Nakatsu, 2017).

The development of an individual's gut microbial community is influenced by several factors. Among these factors, colonization history has been shown to have a major influence on the establishment of the gut microbiome. The GI tract is colonized at birth, and community development is affected by several factors such as mode of delivery (vaginal vs caesarean), diet (example: breastfed VS formula/bottle-fed), and antibiotic exposure (Mathur & Barlow, 2015). The environmental exposures in the first 3 years of life are crucial to the acquisition of an "adult-like" microbiome, which has been shown to remain relatively stable throughout life. The human gut microbiome generally reaches the characteristics of what is considered to be a stable adult microbiome after the age of 3 (Boulangé et al., 2016) .

As the industrialization of the world continues, there are certain changes in our environment and lifestyle that may impede the formation and sustainment of a mutualistic microbial community. While improvements in sanitation and hygiene (clean water, antimicrobial products, antibiotics, etc.) have significantly reduced microbial associated morbidity and mortality, it is worth noting that there has been a coinciding dramatic increase in the incidence of chronic non-communicable diseases such as inflammatory bowel diseases, cancers, autoimmune diseases, asthma, type 2 diabetes and obesity (Logan, Jacka, & Prescott, 2016). This observed inverse relationship has led to the hypothesis that modern sanitization standards have had adverse effects on commensal microbes (Mathur & Barlow, 2015). In addition to the environmental triggers, there have also been notable changes in traditional dietary patterns that have had ill effects on our commensal microbes. Compared to the traditional ancestral diet, the "Western" diet of much of the industrialized world features a distinct reduction in fiber intake, amongst many other changes (E. C. Deehan & Walter, 2016). Diets low in fiber and other indigestible dietary carbohydrates, more generally categorized as microbiome-accessible carbohydrates or MACs, provides insufficient nutrients for the gut microbes. This may lead to several consequences, including a depletion of species reliant on these substrates and a subsequent reduction in important fermentation end products involved in physiological and immunological host functions (Sonnenburg & Sonnenburg, 2014). This "fiber gap" is a key factor that differentiates the ancestral GI environment, where the symbiotic partnership was co-evolved, to the present day(E. C. Deehan & Walter, 2016). This is suggested to be a major driving factor in the reduction of the beneficial gut microbiome(Gentile & Weir, 2018).

Disruptions of the microbial ecosystem such as shifts in the diversity and/or composition is often described using the term "dysbiosis", particularly when characterizing a specific pathological condition (Singer-Englar et al., 2019; Sze & Schloss, 2016; Tseng & Wu, 2019). Dysbiosis in the gut microbiome has been linked to a variety of diseases and conditions, including inflammatory bowel disease, non-alcoholic fatty liver disease, as well as diabetes and obesity (Mathur & Barlow, 2015). While several studies have reported disease-associated changes in the gut microbiome when compared to healthy populations, further studies are

warranted to elucidate the importance of these observed associations. Caution must be executed when attempting to determine causality, and if these findings are directly or clinically relevant for the development or progression of a given disease (Haller, 2018). While much of the mechanisms have yet to be fully elucidated, it is clear that gut microbes contribute to human health through roles in host metabolism and energy homeostasis, including breaking down nondigestible foods, production of Short Chain Fatty Acids (SCFA), vitamin synthesis and energy harvest. In addition, the gut microbiome also contributes to the development and modulation of the host immune system and provides protection against enteric pathogens (Mathur & Barlow, 2015). Links between the gut microbiome and the central nervous system (CNS) have also begun to emerge and may be indirectly involved in appetite regulation (Niccolai, Boem, Russo, & Amedei, 2019).

1.0.4 The gut microbiome, the immune system and metabolic dysfunction

The gut microbiome associated with obesity is generally characterized by a combination of factors including: 1) a low degree of biodiversity, 2) an over-abundance in pathobiont bacteria, such as members of the family *Enterobacteriaceae*, the sulphate reducer species *Bilophila wadsworthia (Turnbaugh et al., 2008)* and other species associated with an inflammatory phenotype and 3) an underrepresentation of species associated with metabolic health such as *Akkermansia muciniphila* (Depommier et al., 2019).

One major contribution to the obesogenic profile from the gut microbiome is the role of the microbiome on the host immune function. The gut has been well established as an important immune organ and the gut microbiome plays a key role in the development of the host innate immune system (Matam Vijay-Kumar, Chassaing, Kumar, Baker, & Singh, 2014). Disturbances in the gut microbial community may lead to increased intestinal permeability via weakened mucous layer and/or changes in tight-junction topography and functioning. This compromised intestinal barrier may lead to bacterial/bacterial product infiltration and a subsequent inflammatory response mediated in large part by Toll-Like Receptors (TLR), especially TLR4 and TLR5. For example, mice lacking the toll-like receptor TLR5 had an altered gut microbiome and developed obesity, hyperphagia, insulin resistance, and exhibited pro-

inflammatory gene expression (M. Vijay-Kumar et al., 2010). Chronic low-grade inflammation is one hallmark of metabolic disorders such as obesity and Type 2 Diabetes (T2D).

Individuals with PWS have been shown to have increased systemic low-grade inflammation, with some studies suggesting this inflammation is independent of adiposity levels and insulin resistance status (Viardot et al., 2010). However, other studies have found that compared to individuals with non-genetic forms of obesity, individuals with PWS have a paradocically increased insulin sensitivity, increased adiponectin, as well as decreased levels of pro-inflammatory adipokines and visceral fat (Haqq et al., 2011). As gut dysbiosis has been linked with low-grade inflammation, the possible connection and implications in the PWS population may be of even more clinical significance. The gut microbiota may be playing a role in the observed improved inflammatory profile in PWS, or might be able to provide insight on why there are mixed findings in the literature. The possibilities of using the microbiome as a biomarker for impaired metabolic health continues to be a future direction in the field.
The bacterial product lipopolysaccharide (LPS) has been implicated in many models of how the microbiome interacts with the host in inflammatory disease states. Increased LPS levels and increased inflammation has been observed in patients with obesity and T2D (Creely et al., 2007; Dasu, Devaraj, Park, & Jialal, 2010), with LPS being largely implicated to contribute to the pathophysiology of weight gain and insulin resistance through LPS-mediated TLR4 signaling. In addition, healthy subjects given LPS exhibited changes in insulin sensitivity (van der Crabben et al., 2009).

1.0.5 Overview of appetite regulation

The regulation of food intake and appetite modulation is complex and involves communication between the CNS and circulating hormones produced by peripheral organs. Endocrine regulators of appetite include both orexigenic (appetite promoting) hormones such as ghrelin, and anorexigenic (satiety promoting) gut hormones, including cholecystokinin (CCK), peptide YY (PYY) glucagon-like peptide (GLP)-1, pancreatic polypeptide (PP), obstatin and oxyntomodulin (OXM). Leptin, another anorexigenic hormone, is expressed and secreted by

white adipose tissue adipocytes and impairements in leptin have been shown to directly influence obesity (Montague et al., 1997). Many studies have looked into the role of gut microbial-derived chemical signals in the activation and modulation of intestinal satiety pathways. For example, activation of enteroendocrine cells (EECs) by bacterial signals may directly or indirectly (via the enterocytes) trigger local and systemic release of PYY and GLP1, thereby inducing satiety (Fetissov, 2017).

1.0.6 Hyperphagia and TLR4 signalling

Hyperphagia is a major concern and a key characteristic in PWS. Disturbances in the intestinal feedback mechanisms involving the action of CCK and leptin on vagal afferent neurons have been linked to a hyperphagic phenotype (Covasa, 2010). LPS can trigger the activation of TLR4 on vagal afferent neurons, reducing leptin sensitivity and ultimately influencing the ability of CCK action, which contributes to increased hyperphagia (De La Serre, De Lartigue, & Raybould, 2015; de Lartigue, Barbier de la Serre, Espero, Lee, & Raybould, 2011).

More recently, it has been shown that this LPS mediated TLR4 signalling can mediate the metabolic benefits of caloric restriction (Fabbiano et al., 2018; S. Wang et al., 2018); something that could prove to be of great clinical significance in the PWS population as the current most common and most effective management/treatment strategy for these individuals involve careful monitoring of dietary intake and caloric restriction (Crinò et al., 2018).

The microbiome may also regulate host metabolism through the actions of SCFAs. SCFAs play a role as energy substrates for the host and for the microbial community and are in large part introduced to the lower intestinal tract through microbial fermentation of dietary fibers and starch (Ríos-Covián et al., 2016). They have several immunomodulatory effects including improving intestinal barrier function by promoting the production of antimicrobial peptides (pathogen exclusion)(Zhao et al., 2018), increasing mucin production (Willemsen, Koetsier, van Deventer, & van Tol, 2003) and upregulating tight-junction proteins(H.-B. Wang, Wang, Wang, Wan, & Liu, 2012). They also act as immune cell regulators by activating regulatory T cells and assisting in the attenuation of immune response (Smith et al., 2013). The

secretion of gut hormones, especially hormones involved with appetite regulation such as glucagon-like peptides (GLP-1, GLP-2) and PYY are affected by SCFAs through several mechanisms of action (Gill, van Zelm, Muir, & Gibson, 2018; Lin et al., 2012; Gwen Tolhurst et al., 2012). SCFAs have protective effects against the development of obesity and insulin resistance, with direct effects observed through dietary supplementation as noted by association studies (Makki, Deehan, Walter, & Bäckhed, 2018). Butyrate specifically has been shown to have many beneficial effects on the host including enhancing thermogenesis in brown fat and limiting the oxygen availability in the intestinal tract through β-oxidation by luminal microbes, which limits the proliferation of pathogenic microbial species, and ultimately protects against dysbiosis (Byndloss et al., 2017). Diets rich in MACs have been shown to improve metabolic outcomes, decrease risk of developing obesity and assist in the regulation of appetite (Ríos-Covián et al., 2016).

In summary, gut microbes and their human hosts share an important mutualistic relationship that has evolved over time. The host affects the microbes through diet, environment factors and the use of antibiotics, and they in turn affect energy harvest, metabolism, gut permeability and nutrient uptake, as well as immune responses and inflammation, all of which may play roles in contributing to the development of obesity (Mathur & Barlow, 2015). As such, the gut microbiome has emerged as a novel target responsive to dietary manipulation that can be modulated rapidly to promote weight loss and satiety.

1.0.7 Microbiome in PWS

Few studies have examined the gut microbiome in children with obesity (Ajslev, Andersen, Gamborg, Sørensen, & Jess, 2011; Bergstrom et al., 2014; Bervoets et al., 2013; Kalliomaki, Collado, Salminen, & Isolauri, 2008; Karlsson et al., 2012; Nadal et al., 2008; Payne, Chassard, Banz, & Lacroix, 2012; Riva et al., 2017; Scheepers et al., 2014; Trasande et al., 2012; Vael, Verhulst, Nelen, Goossens, & Desager, 2011), and fewer still have looked to the gut microbiome as a potential therapeutic target for obesity in the pediatric population.

Attempts to characterize the dysbiosis present in the gut microbiome of pediatric individuals with obesity have had some varied results. For example, an increase in the Firmicutes-to-Bacteroides ratio has been suggested in several studies as a marker for dysbiosis in obesity, including in the pediatric literature (Bervoets et al., 2013); however this theory is highly contentious as results from studies vary greatly. In the studies by Bergström et al. (Bergstrom et al., 2014) and Ignacio et al. (Ignacio et al., 2016) obesity/overweight was found to be associated with an increase in taxa from the Firmicute phylum (*Clostridium leptum*, *Eubacterium hallii*, and *Lactobactobacillus* spp.) However other studies such as those conducted by Scheepers et al. (Scheepers et al., 2014) and Vael et al.(Vael et al., 2011) observed decreased levels of bacteria belonging to Firmicutes such as *Clostridium difficile* and the genus *Staphylococcus* in the higher BMI groups.

Species/families such as *Akkermansia muciniphila*, *Bifidobacteriaceae, Methanobrevibacter smithii*, and *Desulfovibrionaceae*, were repeatedly associated with a lower BMI (Bergstrom et al., 2014; Borgo et al., 2017; Ignacio et al., 2016; Karlsson et al., 2012). *Bacteroides fragilis* was overrepresented in overweight groups (Ignacio et al., 2016; Scheepers et al., 2014; Vael et al., 2011) and findings for the associations between *Enterobacteriaceae* and BMI showed contradictory results (Bergstrom et al., 2014; Karlsson et al., 2012).

Only two studies to date have examined the microbiome of children with PWS. A hospitalized intervention trial conducted in China studied a group of patients with PWS (n=17) and simple obesity (n=21) using a diet rich in non-digestible carbohydrates (with significant increase in dietary fiber from 6 to 49g per day) over 90 days to induce significant weight loss and concomitant favorable structural changes of the gut microbiome. These changes were associated with reduced degree of hyperphagia , a 7.6 percent weight loss, reduced inflammation and improved metabolic measures (C. Zhang et al., 2015). Baseline dysbiosis was apparent in both the obese children with and without PWS, however the bacterial composition between the PWS and non-PWS groups was not statistically significant. When the pre-intervention gut microbiome from a participant was transplanted into germ-free mice, it

induced higher inflammation and larger adipocytes compared with the post-intervention microbiome from the same individual (C. Zhang et al., 2015).

An additional published abstract compared children and young adults with "simple" obesity (n = 17), hypothalamic obesity (n = 12 with Prader-Willi syndrome), hypothalamic lean (n = 10 with Prader-Willi) and healthy lean controls (n = 20). Obesity had significant effects on microbial community structure. Operational taxonomic unit (OTU) richness and diversity was lower in both obese groups compared to both lean groups. According to study authors, 5% of total variance was explained by PWS pathology (M. J. Khan et al., 2015).

Finally, after the commencement of this thesis work, another study looking at the gut microbiome in PWS was published (Olsson et al., 2019). This work analyzed the gut microbiome of 17 PWS patients (average age 29.4) and 17 obese individuals with similar BMI, gender, and age. As their "non-obese control group" researchers in this study also analyzed samples from parents of PWS patients. In this work, individuals with PWS had higher diversity than individuals with common obesity, and community structure in the PWS group more closely resembled that of the PWS parents than the simple obesity group (Olsson et al., 2019). Finally, several differentially abundant taxa were identified between groups, including increases in abundance of the genus *Akkermansia* and *Desulfovibrio* and decreases in the genus *Dorea* in the PWS group compared to the simple obesity group (Olsson et al., 2019). Results from germ-free mice experiments as part of this study revealed improved insulin tolerance independent of fat-mass or body weight in the mice transplanted with fecal content of PWS patients compared to those transplanted with non-syndromic obesity (Olsson et al., 2019). Overall, study authors proposed that the gut microbiome in PWS may be metabolically protective compared to individuals with non-syndromic obesity.

Together, these studies indicate an etiological contribution of dysbiotic gut microbiome in the metabolic derangements and obesity associated with PWS; however, the composition and the specific role of the gut microbiome in weight and metabolic control in PWS and childhood obesity is not fully understood.

1.1 Study Justification

The previously described hospitalized intervention study with patients with PWS (C. Zhang et al., 2015) provides an excellent starting point for future research, however it is not without its limitations.

First, this study accounts only for the bacterial component of the gut microbial ecosystem. The role of the fungal (mycobiome) component of the gut microbiome on human health remains poorly explored (Mar Rodríguez et al., 2015). Some links to specific over/under abundance of fungal species have been observed in studies comparing individuals with and without obesity, though these patterns are not as well established in the literature as the bacterial component of the microbiome. Study authors were able to discern between patients with and without obesity by their specific fungal composition and were able to further distinguish metabolically "healthy" from "unhealthy" obesity. Specific fungi were also associated with increases in metabolic markers (Mar Rodríguez et al., 2015).

In addition, it has been suggested that attempting to infer possible cross-kingdom interactions between the bacterial and fungal components could also provide a more holistic and comprehensive understanding of how the robust ecological community of the gut microbiome can influence host physiology (Suhr & Hallen-Adams, 2015). Bacteria and fungi can interact in many ways, and an overrepresentation of some fungi may suppress the growth of certain beneficial commensal bacteria and vice versa. For example, an inverse association has been previously described between Candida (fungus) and Bacteroides (bacteria) (Hoffmann et al., 2013). Several bacterial species are capable producing antifungal molecules that affect morphology and virulence of Candida (Hogan, Vik, & Kolter, 2004). The bacterial microbiota reduces Candida albicans colonization by excluding and out-competing Candida for adhesion sites and producing inhibitory molecules (Kennedy & Volz, 1985). Short-chain fatty acids (specifically butyric acid) produced by lactic acid bacteria may also prevent pathogenic C. albicans from causing disease in the gut through mechanisms that inhibit filamentation of the fungus (Noverr & Huffnagle, 2004). The fungal community present in the gut of individuals with

PWS has yet to be characterized. This information could provide a foundation for novel treatment and prevention strategies to the excessive weight gain seen in this population.

Another limitation of the study by Zhao et. al. (C. Zhang et al., 2015) was that it only assessed children with obesity. This paper did not characterize the gut microbiome of normal-weight individuals with PWS, nor did it aim to determine whether the microbial profile differs between normal-weight and obese children with PWS. This is also a limitation of the study by Olsson et al. (Olsson et al., 2019). Additionally, the study population in the Chinese cohort was hospitalized for the entirety of the study period, making the findings less generalizable compared to a free-living population (C. Zhang et al., 2015).

In addition to the lack of research on the mycobiome in PWS, previous reports of the gut microbiome in PWS have also not fully assessed potential associations between the gut microbiome, hyperphagia and dietary intake. Looking into these possible associations could provide further insight on how to most effectively modulate the gut microbiome to confer health benefits.

Finally, participants in Zhao et. al underwent an additional 30% caloric restriction which could confound the results of the study. In addition, the intervention consisting of traditional Chinese medicine not necessarily easily accessible and is difficult to replicate in other populations. Future work described in the final chapter of this thesis work addresses these concerns and suggests substituting this traditional Chinese medicine dietary component with items more typical to the Canadian diet.

1.2 Study objectives and hypotheses

The prevailing goal of this thesis research is to identify interventions which favorably modify gut microbiome for dietary management of PWS and childhood obesity. Specifically, this work has the **overall objective** to explore the gut microbiome in children with Prader-Willi Syndrome (PWS) compared to controls. A novel aspect of this work is the analysis of the fungal component of the gut microbiome which has previously not been explored in the PWS population. This thesis work features a population that was under free-living conditions (not in

hospital). Subjects were not altering their core diets, restricting calories, or changing physical activity; therefore, results from this work are more generalizable.

We **hypothesized** the gut microbiome would differ in composition, diversity, and community structure between PWS (normal weight and overweight/obese) and typically developing children (normal weight and overweight/obese). Differences were also expected within and between obese/nonobese subgroups. In other words, we hypothesized that there would be differences between the dysbiosis present in the overweight/obese PWS and the overweight/obese control groups. More specifically, differences in abundance of specific members of the bacterial and fungal community could contribute to phenotypic differences observed between the 4 subgroups. We also aimed to assess associations between hyperphagia phenotype and dietary intake and the gut microbiome composition.

Identifying and characterizing the microbial composition present in children with PWS and obesity is an important first step to guide the design of effective therapies to achieve weight control and management of hyperphagia based on manipulation of the gut microbiome. This is especially pertinent as there is currently no established effective therapy for PWS-related hyperphagia and obesity.

1.3 Thesis Outline

This thesis work is part of an overarching research project which aims to gather and apply knowledge of the microbiome in PWS in order to provide a prebiotic intervention to favourably modify gut microbiome composition and subsequent metabolic health.

Chapter two describes a cross-sectional study which sought to characterize and compare the gut microbiome profile of North American Children with and without PWS. This chapter contains our rationale, study design, results, and discussion of this research. Chapter two will conclude with a summary of the study results, as well as limitations and a brief discussion of impacts and considerations for future research.

Our goals for a fiber intervention in PWS will be further discussed in chapter three. Chapter three will also discuss methods to modulate the gut microbiome and will also briefly describe the creation of a pilot trial to assess tolerance to the fiber intervention trial.

Chapter 2: Profiling the gut microbiome in children with and without PWS

2.1 Preface

Research from that study was approved by the University of Alberta's Health Research Ethics Board: 'Profiling of the gut microbiome in children with PWS: a fiber intervention to target hyperphagia (AIM 1)' (ID: RES0033157).

This work was funded in part by the Women Children Health Research Institute (WCHRI) and through a grant from the Prader-Willi Syndrome Association (USA).

The analysis of data contained in this thesis has employed one set of statistical methods. Additional analyses, using different methods, are underway in the Haqq research group.

2.2 Background

Prader-Willi Syndrome (PWS) is the most common syndromic form of childhood obesity, affecting approximately one in 15,000 live births (Irizarry et al., 2016b). PWS is characterized by failure to thrive and low muscle tone during infancy, followed by food-seeking and hyperphagia in childhood (Irizarry et al., 2016b). This abnormal feeding behaviour translates into increased risk for developing severe obesity, metabolic dysfunction, cardiorespiratory difficulties, and death (Butler et al., 2017; McAllister et al., 2011). The food seeking characteristic of PWS is a source of stress for both individuals with PWS and caregivers, especially related to the need for strict control of access to food (Kayadjanian et al., 2018). The pathogenesis of hyperphagia and weight gain in PWS is poorly understood (Heymsfield et al., 2014), and current treatment options have had limited success (Crinò et al., 2018; Tan et al., 2019). Imbalances in gut microbial composition have been linked to health and disease, including obesity and metabolic dysfunction. However, exactly how changes in gut microbial composition and function influence childhood obesity remains under debate (Collado, Isolauri, Laitinen, & Salminen, 2010; Galley, Bailey, Kamp Dush, Schoppe-Sullivan, & Christian, 2014; John & Mullin, 2016; Muhammad Jaffar Khan, Gerasimidis, Edwards, & Shaikh, 2016; Tun et al., 2018).

To date, a handful of studies on the gut microbiome composition of PWS have been published (M. J. Khan et al., 2015; Nicholls et al., 1998; Olsson et al., 2019; Wu et al., 2017; C. Zhang et al., 2015). Together these studies suggest that the gut microbiome may play a role in the metabolic derangements and obesity associated with PWS.

Findings that specific fungi can modulate both local and systemic immunological responses in the host have led to increased interest in exploring the gut mycobiome (Wheeler et al., 2016). Furthermore, bacteria and fungi interact within the gut, influencing each other in several ways such as through mutualism, amensalism, commensalism, parasitism or competition (Richard & Sokol, 2019). These interactions additionally occur through a variety of mechanisms and could potentially result in a wide array of consequences on the host physiology and health. For example, by consuming oxygen present in the gut (through

mitochondrial activity), *Candida spp.* have been found to support the growth of and create a favourable environment for *Clostridium difficile*, a pathogenic anaerobic bacterium, in aerobic conditions (Lambooij, Hoogenkamp, Brandt, Janus, & Krom, 2017; van Leeuwen et al., 2016). The studies looking at the gut microbiome in PWS thus far have neglected to consider the fungal component of the microbiome and have not attempted to look at the differences between NW and OWOB members of this population.

The current study aims to further contribute to this growing body of literature and provides novel findings on the gut mycobiome composition in PWS and how it differs from that of typically developing individuals. Additionally, this work will attempt to link information on hyperphagia and dietary intake with the microbiome data in this population.

2.3 Materials and Methods:

2.3.1 Study population and Study Design

Children with confirmed diagnosis of PWS (ages 3-17 years, male and female, n=25) were recruited from endocrine clinics at the Stollery Children's Hospital (Edmonton, Alberta, Canada) as well as remotely through collaboration with the Foundation for Prader-Willi Research (Canada/USA) and the USA-PWS association between February 2017 to July 2018. Age-, sex- and BMI-z scores matched controls (n=25) were recruited from advertisements through the University of Alberta bulletin boards and e-mail distribution lists. Participants were excluded from the study for the following reasons: (a) a pre-existing condition that could influence body weight; (b) prior antibiotic exposure within 30 days, or (c) administration of medications or supplements known to influence gut microbiota composition (e.g. probiotic supplements and antidepressants). Exceptions were made for children with PWS who were taking growth hormone, as this treatment is commonly used to counteract an endogenous deficiency of growth hormone in PWS (Irizarry, Miller, Freemark, & Haqq, 2016a). All patients were free-living and not in hospital.

A stool sample, a 3-day dietary record, a hyperphagia questionnaire validated in PWS (Dykens, Maxwell, Pantino, Kossler, & Roof, 2007), and anthropometric measurements (height,

weight and waist circumference) were collected from each participant. This metadata will be discussed in more depth later in the methods.

This was a cross-sectional case-control study with two major objectives. The first objective was to characterise and compare the gut microbiome composition in children with and without PWS. The second objective was to determine if collected metadata could help to explain the compositional differences between groups.

In order to characterize and compare the microbiome of the PWS and CON groups, participants were subcategorized into 4 subgroups based on weight classification. Participants with a BMI percentile at or above 85% were placed in the "overweight/obese" (OWOB) subgroup and participants with a BMI % below this threshold were placed in the "normal weight" (NW) subgroup. This distinction is based on the Centers for Disease Control and Prevention (CDC) definitions of pediatric weight classification (Lau et al., 2007; Nihiser et al., 2007). By subcategorizing the groups, we aimed to 1) assess differences in microbiome composition both between and within the groups 2) specifically assess whether patterns in the microbiome composition differ with obesity phenotype comparing genetic and non-genetic derivations of OWOB. In addition, as weight status has been demonstrated as a factor that influences microbiome composition, we wanted to determine the role of weight status in differences observed in the microbiome between groups.

Statistical assessments were performed for both the bacterial and the fungal communities. Comparisons were performed systematically, and alpha diversity, beta diversity and differential abundance tests were all assessed in A) 2 group (PWS vs CON), B) 4 subgroup (NW PWS, OWOB PWS, NW CON, OWOB CON), and C) pairwise subgroup analyses (NW CON vs NW PWS; NW OWOB CON vs OWOB PWS; NW PWS vs OWOB PWS; NW CON vs OWOB CON). Pairwise analyses were used to gain insight between the effects of weight status (NW vs OWOB) and the effects of group type (PWS vs CON) (Appendix B).

2.3.2 Anthropometric measurements

Height was measured to the nearest 0.1cm using a wall-mounted stadiometer for children. Weight was measured to the nearest 0.1kg using an electronic weighing scale. Waist circumference was recorded to the nearest 0.1cm with a non-stretch measuring tape between the bottom of the lower rib and the iliac crest. BMI percentile and BMI z-scores were calculated using the standardized procedure based on the Centers for Disease Control and Prevention Growth charts (Lau et al., 2007; Nihiser et al., 2007).

2.3.3 Dietary intake and hyperphagia assessment

A 3-day dietary intake record validated in children (Day, 2001) was completed by participants/parents to assess macro-and-micronutrient intake. Particular attention was given to macronutrient and dietary fiber intake in the analysis. The dietary record was analyzed using Processor SQL (version 11.4, ESHA Research, Salem, OR, 2006). Diet data was adjusted using an energy-adjusted residual model to control for variation caused by total energy intake (Willett, Howe, & Kushi, 1997). A PWS-specific hyperphagia questionnaire containing questions about hyperphagic behavior (5 questions), drive (4 questions) and severity (2 questions) was also administered (Dykens et al., 2007). The "Dykens Hyperphagia Questionnaire" can be found in the Appendix under Appendix F. Differences in indices of dietary intake and hyperphagia scores between the PWS and the control groups, as well as between subgroups were tested using two-sided Wilcoxon test and Dunn test, respectively.

2.3.4 Molecular and statistical analyses of fecal microbiota

Stool samples were collected by participants using the OMNIgene 200 stool collection kit (DNAGenotek, Ottawa, ON, CAN). DNA was extracted from the fecal homogenates combining enzymatic and mechanical cell lysis with the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA, USA). In OMNIgene·GUT kits, samples were diluted in a proprietary solution, which has been previously shown to keep microbial DNA stable (Anderson et al., 2016). Bacterial 16S rRNA V4 sequences and fungal ITS2 sequences were PCR-amplified with dual-

barcoded primers, as per the protocol of Kozich et al. (2013) (Kozich, Westcott, Baxter, Highlander, & Schloss, 2013). Amplicons were sequenced with an Illumina MiSeq using the 300-bp paired-end kit (v.3) by Microbiome Insights (Vancouver, BC, Canada). The potential for contamination was addressed by co-sequencing DNA amplified from specimens and from four each of template-free controls and extraction kit reagents processed the same way as the specimens. Two positive controls, consisting of cloned SUP05 DNA, were also included (number of copies = 2*10^6). Datasets containing less than 1,000 reads were excluded from all analyses.

2.3.5 Processing of sequencing data

Quality-controlled bacterial and fungal reads were taxonomically classified using Greengenes (v. 13_8) and UNITE as the reference databases, and then clustered into 97%-similarity operational taxonomic units (OTUs) with the Mothur software package (v.1.39.5) (Schloss et al., 2009), following the recommended procedure (https://www.mothur.org/wiki/MiSeq_SOP).

2.3.6 Data Filtering

The purpose of the data filtering is to identify and remove features that are unlikely to be of use when modeling the data. Features having very few counts were filtered based on their abundance levels (minimum counts) across samples (prevalence). Details of the filtered data can be found in table 2.

For the bacterial and fungal microbiome analyses, OTUs were retained if at least 15% of the samples had at least 4 counts. If an OTU did not meet this threshold, it was considered noise and filtered out from further analysis. OTUs were also removed based on inter-quartile range (IQR) set at 10% for comparative analyses to minimize the influence of low variance OTUs to bias results.

2.3.7 Data Normalization

Currently, there are no consensus guideline with regard to which normalization techniques should be used. The literature provides suggestions for researchers to explore different approaches based on specific characteristics in a given data set. Then it is suggested to visually examine the separation patterns (i.e. PCoA plot) to assess the effects of different normalization procedures with regard to experimental conditions of interest and biological probability (McMurdie & Holmes, 2013b; Thorsen et al., 2016).

A recent publication assessing methods of normalizing microbiome data from an ecological perspective found that rarefied data outperformed all the other normalization methods (scaling and transformation based methods) for producing accurate Bray-Curtis dissimilarities, including subsequent PCoA visualization and PERMANOVA analysis (McKnight et al., 2019). Sequence data was normalized following rarefication due to differences in sequencing depth and the uneven skew of reads (Appendix C), as is the recommendation for samples that differ significantly (i.e. >10X) (Weiss et al., 2015). To stabilize the variance of the data, the centered log ratio (CLR), which is commonly used and recommended because of the compositionality of microbiome data was also applied to the fungal data (Gloor, Macklaim, Pawlowsky-Glahn, & Egozcue, 2017). Previous literature has also applied CLR normalization to fungal sequences before analysis (Tipton et al., 2018).

2.3.8 Alpha diversity analysis

This method is used to measure the diversity present within a sample or community. Alpha diversity can be characterized via the total number of species (richness), the abundances of the species (evenness) or measures that considered both richness and evenness. For our analysis, a combination of different indices was used to assess alpha diversity in order to get a more holistic view of this measure and because each measure emphasizes different factors in their respective assessments. The Chao1 index estimates richness, while also inferring the number of rare organisms that may have been lost due to under-sampling. Indices such as the Shannon and Simpson indices consider the number (richness) and the abundance of organisms (evenness) to describe the diversity of a community.

Alpha diversity analysis was performed using the phyloseq package (McMurdie & Holmes, 2013a) through the '*MicrobiomeAnalyst*' web-based software program (Chong, Liu, Zhou, & Xia, 2020; Dhariwal et al., 2017). The results were plotted across samples and summarized as box plots for each group or experimental factor. Further, the statistical significance of grouping based on experimental factor was also estimated using both parametric (t-test/Anova) and nonparametric tests (Mann-Whitney/ Kruskal-Wallis) as appropriate based on the skew of the data. Significance was set at $p<0.05$. Both raw and normalized data was assessed. Alpha diversity was assessed at several taxonomic levels including OTU and genus level.

2.3.9 Beta diversity:

Beta-diversity measures provide a way to compare the diversity or composition between two microbial communities. These methods compare the changes in the presence/absence or abundance of all the taxa present in a dataset and summarize these into how 'similar' or 'dissimilar' the samples are. Each sample gets compared to every other sample generating a distance or dissimilarity matrix. Our Beta-diversity analysis involved two steps. First, the similarity or distance between sample was measured using the non-phylogenetic Bray-Curtis distance index. Next, the results from the matrix developed were visualized using the ordination-based method, Principle Coordinate Analysis (PCoA) in a 2-dimensional plot where each point represents the entire microbiome of a single sample. Each axis of the plot reflects the percent of the variation between the samples. The X-axis and Y-axis represent the highest and second highest dimension of variation, respectively.

Beta diversity analysis was performed both at a feature and genus level using the Phyloseq package in R (McMurdie & Holmes, 2013a) through the '*MicrobiomeAnalyst*' web-based software program (Chong et al., 2020; Dhariwal et al., 2017). Figure 2 shows the Principle Coordinate Analysis (PCoA) derived ordination plot represented in 2 dimensions. Each point or sample displayed on PCoA plots was colored based on sample group (e.g. PWS vs CON). For the feature level or genus level analysis, statistical significance was tested using Permutational

ANOVA (PERMANOVA).

2.3.10 Differential abundance testing

Several differential analysis methods exist to identify features that are significantly different between conditions under study. These methods include classical parametric (T-test/ANOVA) and non-parametric (Mann-Whitney/Kruskal-Wallis) univariate analyses, biomarker discovery tools such as Linear Discriminant Analysis (LDA) Effect Size (LEfSe) and more computationally demanding methods such as DESeq2. Each method considers and treats data differently, and considerations for each method must be applied. For example, when the number of samples is high (>50 samples), rarefying or proportion normalized data paired with non-parametric tests have been shown to yield as high sensitivity as other more robust methods for identifying differential features.

Differential abundance results were assessed at a genus level using DESeq2. DESeq2 is considered to be a robust method to estimate differential features, with low false positive rates (Weiss et al., 2015). This method is considered to have the highest power to compare groups, especially for cases where there are less than 20 samples per group, as is the case with our data.

The genus level was selected as the sequencing and taxonomical labeling methodology has an acceptable level of resolution at this level of classification. All analyses were adjusted for False Discovery Rate (FDR) using the '*MicrobiomeAnalyst*' web-based software program (Chong et al., 2020; Dhariwal et al., 2017).

2.3.11 Integrating metadata with microbiome sequence data

To assess what collected factors could be contributing to the differences we observed between the microbial communities in PWS and CON groups, several methods were applied to integrate the collected metadata (see Appendix A) with the microbiome data.

I. *2.3.11.1 Univariate Analysis*

Spearman correlation coefficients were calculated to assess the correlation of dietary intake and hyperphagia scores with bacterial and fungal genera. Analysis was separated by group (PWS vs CON). Statistical significance of the correlations was determined after adjustment with the Bonferroni method for FDR, and correlations between genus level taxa and metadata variables were visualized using a heatmap. Significance was set at $p<0.25$ for q values (p values after FDR).

II. *2.3.11.2 Multivariate analysis*

Any observed differences in overall gut microbiome composition for PWS and CON groups that is identified by the ordination analysis (PCoA) will be further evaluated to determine the percentage of variability that can explained using multivariate modeling. To further expand upon ordination results, the next step in our analysis was to perform a canonical correspondance analysis (CCA) to determine the contribution of collected metadata factors in shaping the microbiome differences between groups. First, CCA was used to determine how much the metadata variables collectively explain the variation observed between groups using the “cca” function in the vegan package in R. From there, the most strongly associated factors were determined using permutation tests.

10,000 permutations were performed to evaluate the significance of each CCA model using “anova.cca” function in “vegan”. For all CCA models, the *P* values from the permutation tests were said to be significant if P was less than 0.05. This would suggest that the CCA model explained more variance of the gut microbiome than expected by chance.

CCA can be used to identify and measure associations among two sets of variables. The outputted information from the CCA includes measures of variance or “inertia”. The quotient of the “constrained inertia” over the total inertia indicates how good the overall 'fit' was /how much variation could be explained by the model. Further, the amount of the constrained inertia expressed by each CCA axis can be determined.

CCA can therefore provide insight on how much variation the collected metadata can explain, and statistical testing can give an indication of which factors exert the most influence on shaping the microbiome.

2.4 Results:

The first step of our analysis was to evaluate if any significant differences were observed in the collected metadata between groups. This metadata included participant characteristics, hyperphagia assessment and dietary intake assessment (see Appendix A). Any differences observed were taken into further consideration when interpreting higher level of statistical analyses.

2.4.1 Participants' characteristics

This study included a group of 25 children with PWS (14F:11M; median age = 6.3 (3-17y); median body mass index (BMI) percentile = 79.3%; Genetic PWS subtype (15 deletion: 10 uniparental disomy) and a group of 25 children without PWS (9F:16M; median age = 8.8 (3 - 17y); median BMI percentile = 76.6%). Of the 25 PWS participants, 15 were classified as "normal-weight (NW)", with the remaining 10 being classified in the over-weight/obese category (OWOB), as per the pediatric association guidelines (above the 85th BMI percentile) (Lau et al., 2007). For the 25 control (CON) participants, 17 were classified as NW and 8 OWOB. The details of the collected participants' characteristics can be seen in Table 1.

No significant differences were observed in the participant characteristics between the PWS and CON groups.

2.4.2 Hyperphagia assessment

The median total hyperphagia score of individuals with PWS was 19/55 (Range: 12-39). A breakdown of the hyperphagia score subcategories can be found in Table 1. Hyperphagia total score ($p=0.014$) and the subcategorized 'drive' score ($p=0.039$) and 'severity' score ($p<0.001$) were significantly higher in PWS than in controls. No statistical significance between

groups was observed for the 'behaviour' subscore.

2.4.3 Dietary intake

After adjusting data for total energy intake (Willett et al., 1997), carbohydrate intake and total energy intake differed significantly between the PWS and CON groups ($p<0.001$ and $p = 0.002$ respectively), with the Control group having the higher intake for both measures. Full dietary intake information (adjusted and non-adjusted) can be found in Appendix A.

2.4.4 Microbiome (bacteria and fungi) read data generated from amplicon sequencing

An average of 57,133 and 5,732 quality-filtered reads were generated from the V4 region of bacterial 16S rRNA and fungal ITS2. 13 samples from the ITS2 sequencing contained less than 1,000 sequences; these samples were excluded from downstream analyses, (6 samples from PWS group and 7 from controls removed leaving total of 37 samples assessed). A total of 2,498 bacterial OTUs and 255 fungal OTUs were identified for the microbial communities.

2.4.5 Fungal Microbiome Compositional Analysis Results

Compositional assessments of the fungal component of the gut microbiome were first assessed between the PWS and CON groups. Further pairwise subgroup assessments were also performed and interpreted.

2.4.5.1 PWS vs CON Group Fungal Analysis

From the 37 samples used in this analysis, a total of 73 low abundance features were removed based on prevalence. 40 features remained after the data filtering step was performed. Full filtering results for all pairwise test can be found in table 2. An overview of genus level taxonomic composition can be seen in Appendix D.

I. Alpha diversity results:

No differences were observed in alpha diversity measures (Chao1, Shannon, Simpson) between PWS and CON groups, using either the original and filtered/normalized data and testing with parametric and non-parametric tests (figures 1-3).

II. Beta diversity results:

Beta diversity analysis was performed at a feature level using the phyloseq package in R (McMurdie & Holmes, 2013b). A summary of the beta diversity results can be found in figure 4. Figure 5 displays the Principle Coordinate Analysis (PCoA) ordination plot represented in 2 dimensions for the 2-group analysis comparing the entire PWS and CON groups. For the feature level analysis, statistical significance was found using Permutational ANOVA (PERMANOVA) with an F-value: 4.2049; R-squared: 0.10725; p-value<0.001. The first PCoA dimension (PCoA Axis 1) explained 21.2% of variation in the data. The second PCoA dimension (PCoA Axis 2) explained 13.8% of variation in the data.

III. Differential abundance testing:

The genus level was selected as the sequencing and taxonomical labeling methodology has an acceptable level of resolution at this level of classification. Appropriate false discovery tests were applied to ensure identified features have the highest likelihood of being genuinely different between groups rather than an artifact of statistical testing procedures.

After adjusting for FDR, three taxa were identified as differentially abundant between the PWS and CON groups (Figure 6). Saccharomyces, Candida, and unclassified Basidiomycota were identified as differential features (P<0.001 for all three taxa), with larger abundances of Saccharomyces in the Control group and higher abundances of Candida and unclassified Basidiomycota in the PWS group.

The next step in our analysis was to compare subgroups in a pairwise fashion to look at the influence and group type and weight status on these overall differences.

2.4.5.2 Pairwise comparisons of fungal microbiome

I. NW CON vs NW PWS Fungal analysis:

No differences were observed in alpha diversity measures (Chao1, Shannon, Simpson) between NW PWS and NW CON subgroups at any taxonomic level, using either the original and filtered/normalized data and testing with parametric and non-parametric tests (figures 1-3 indicate observed p-values). For the feature level analysis of Beta-diversity, statistical significance was found, with an F-value: 2.267; R-squared: 0.1066; p-value < 0.022. The first PCoA dimension (PCoA Axis 1) explained 26% of variation in the data. The second PCoA dimension (PCoA Axis 2) explained 23.6% of variation in the data (figure 7). No significant differences were found in differential abundance between the NW CON and NW PWS groups (Table 3).

II. OWOB CON vs OWOB PWS Fungal analysis:

The Chao1 richness measure of alpha diversity calculated from the OWOB PWS group was found to be higher than that of the OWOB CON group (figure 8) when using parametric test (t-test/ANOVA) on the filtered and normalized data at OTU level (p-value: 0.048733; [T-test] statistic: -2.1777). For the feature level analysis of Beta-diversity, statistical significance was found, with F-value: 4.0703; R-squared: 0.22525; p-value < 0.001. The first PCoA dimension explained 30.6% of variation in the data. The second PCoA dimension explained 20.9% of variation in the data (figure 9).

A relative increase in Saccharomyces was observed in the OWOB CON group (p=0.003) and whereas Candida was more abundant in the OWOB PWS group (p=0.003) (figure 10).

III. NW PWS vs OWOB PWS Fungal analysis:

No statistically significance was observed between groups in any measure of alpha or beta-diversity (p>0.05) (figure1-4 and 11). Unclassified Basidiomycota was observed to be

differentially abundant with a greater abundance observed in the OWOB PWS group (p=0.03) (figure 12).

IV. NW CON vs OWOB CON Fungal analysis:

No significance was observed in measures of alpha-diversity, beta-diversity or differential abundance was observed (p>0.05) (figure1-4, 11 and table 4).

In conclusion, only the NW: PWS vs CON and the OWOB: PWS vs CON pairwise analyses had significant differences in Beta-diversity. This finding may suggest that genotype rather than weight status has more of an impact on the observed fungal community differences. The differences in unclassified Basidiomycota are most strongly due to the contribution from the OWOB PWS group. There were no differences in differential abundance for the NW subgroup analysis, however there were differences with the OWOB subgroups. A graphical summary of differential features in the fungal communities can be found in figure 13. Additionally, there was higher Chao1 richness in the OWOB PWS group compared to the OWOB CON group.

2.4.6 Bacterial Microbiome Compositional Analysis Results

2.4.6.1 PWS vs CON Group Bacterial Analysis

From the 50 samples used in this analysis, a total of 1719 low abundance features were removed based on prevalence and 39 low variance features based on IQR. 348 features remained after the data filtering step was performed. Full filtering results for all pairwise test can be found in table 2. An overview of genus level taxonomic composition can be seen in Appendix E.

I. Alpha diversity results:

No differences were observed in Chao1 richness or Simpson diversity between the two groups (figure14 and 17), however Shannon alpha diversity measures were higher in the CON group compared to the PWS groups using parametric tests (p=0.04) (figure15 and 16).

II. Beta diversity results:

No statistical significance was found in beta-diversity between groups (Bray-Curtis PCoA, PERMANOVA with an F-value: 1.1449; R-squared: 0.023296; p-value = 0.309) (figure 18).

III. Differential abundance testing

After adjusting for FDR, there were no differentially abundant bacteria between the PWS and CON groups (table 5).

2.4.6.2 Pairwise comparisons of bacterial microbiome

No differences were observed in alpha diversity measures (Chao1, Shannon, Simpson) between the subgroups at any taxonomic level, using either the original and filtered/normalized data and testing with appropriate parametric and non-parametric tests ($p>0.05$, figures 14-17). No statistical significance was found in beta-diversity between subgroups (figure 18).

Further pairwise subgroup analysis followed to see if group type or weight status affected the differential abundance. (figures 19-22).

I. NW CON vs NW PWS Bacterial analysis:

Staphylococcus (p=0.004), *Lactobacillus* (p=0.01), *Escherichia* (p=0.04) and unclassified RF39 (p=0.04) were all found to be more abundant in the NW PWS groups (figures 19), whereas an increase in unclassified Bacteroidales (p=0.04) was observed in the NW CON subgroup.

II. OWOB CON vs OWOB PWS Bacterial analysis:

SMB53 ($P<0.001$) was more abundant in the OWOB CON group, whereas *Lachnospira* (p=0.04) was elevated in the OWOB PWS group (figure 20).

III. NW PWS vs OWOB PWS Bacterial analysis:

Within the PWS group, a larger abundance of *Escherichia* (p=0.001) was observed in the NW subgroup compared to the OWOB subgroup (figure 21).

IV. NW CON vs OWOB CON Bacterial analysis:

5 taxa were identified as differentially abundant within the different weight classifications of the CON group. *Bifidobacterium* was found to be more abundant in the OWOB CON group (p<0.001) whereas *Phascolarctobacterium*, unclassified Bacteria, *Alistipes* and *Haemophilus* were more abundant in the NW CON group (figure 22).

While no significance was seen in differential abundance between the two groups (PWS vs CON), there were significant features that were identified in the pairwise subgroup analysis. Other than higher abundance of *Escherichia* in the NW PWS compared to the NW CON group and the OWOB PWS group, there were no overlapping taxa that were differentially abundant between pairwise tests.

2.4.7 Results of integrating metadata with microbiome sequence data

2.4.7.1 Univariate Analysis

2.4.7.1.1 Correlation between gut microbiota and hyperphagia

After adjusting for FDR using the Bonferroni methodology, there were no significant correlations between either bacterial or fungal genus and hyperphagia questionnaire scores (total & subcategories) (figure 24).

2.4.7.1.2 Correlation between gut microbiota and dietary intake

The bacterial genus SMB53 was slightly positively correlated with polyunsaturated fat intake in the PWS group (p=0.23, Spearman correlation=0.65) (figure 24A) and the fungal genus

Alternaria was slightly positively correlated with saturated fat intake in the PWS group (p=0.16, Spearman correlation=0.73) (figure24C). No other genus was found to be significant after FDR adjustments (figure 24).

2.4.7.2 Multivariate analysis

Several CCA analyses were performed on fungal relative abundance count data and the collected metadata.

Firstly, a CCA model incorporating all collected metadata (with the adjusted values for dietary intake) was performed using the "cca" function in the vegan package of R.

Looking at the proportion of constrained inertia expresses how good the overall 'fit' of this model was. In other words, we can find that 58% of variance in the fungal abundance matrix can be explained by the model containing all collected metadata. Variables were then plotted onto a biplot of the first two most influential constrained axes. The amount of the constrained inertia expressed by each constrained axis (i.e. those that are linear combinations of the explanatory variables) were added to the plot and can be seen in the brackets of the CCA. The first CCA dimension (CCA Axis 1) explained 7.4% of variation in the data. The second CCA dimension (CCA Axis 2) explained 7.1% of variation in the data (figure 25).

Next, statistical significance of the model was tested using ANOVA. The overall model using all collected metadata was not statistically significant (p=0.09). Testing the model by each metadata variable by term rather than the overall model using ANOVA found that "Group Type" (PWS vs CON, p=0.001), fat intake (p=0.003) and sugar intake (p=0.002) were all significant in describing the constructed CCA model.

Specific hypothesis driven CCA models were then tested. Models testing the effects of hyperphagia scores (total score and subscores) were not found to be statistically significant after permutation analyses (ANOVA). Models testing the effects of dietary intake variables were significant using ANOVA (p=0.029). The model looking at dietary factors explained 36% of total variance, with the first CCA dimension explaining 7.2% of variation in the data and the second

CCA dimension explaining 6.7% of variation in the data. Energy intake (p=0.025), protein intake (p=0.019), fat intake (p=0.006) and cholesterol intake (p=0.005) being the factors having the most influence on the model.

Models testing metadata factors that differed between the two groups were applied. This model consisted of testing "GroupType + Drive + Severity + Total + ENERGY_KCAL + Carbs + Fat + SatFat + Sugar" specifically with the abundance matrix. This model was found to be overall statistically significant (p=0.024), with Grouptype (p<0.001) Energy intake (p=0.045) and Fat Intake (p=0.013) being the factors that have the most influence on the model. The first CCA dimension (CCA Axis 1) explained 8.3% of variation in the data. The second CCA dimension (CCA Axis 2) explained 8.0% of variation in the data. These variables collectively explained 29% of the gut mycobiome variation.

2.5 Discussion:

In this study we demonstrate that the faecal microbiome of children with PWS differs significantly to typically developing children and explore the differences between NW and OWOB subcategories of these two populations. Overall, our analyses on the PWS and CON microbiome emphasize the importance of sequencing the fungal component of the microbiome alongside the more commonly explored bacterial component of the gut ecosystem.

Significant differences in fungal community structure were observed between the PWS and CON groups. These differences were observed when comparing PWS vs CON in both the NW and in the OWOB weigh-class groupings. However, results of beta-diversity in the fungal community within different weight classifications of the PWS or CON groups did not reveal significant differences in overall community structure. The results of the pairwise beta-diversity tests suggest that genotype (PWS vs CON) rather than weight status (NW vs OWOB) is a more influential factor in the differences in mycobiome profiles. As the gut mycobiome of PWS has not previously been explored, this novel finding may initiate interest in further work exploring the mycobiome in PWS, including the potential impact on health and possible therapeutic manipulations.

The PWS group was found to be characterized by an increased relative abundance of the genus *Candida* and relative decreased abundance of *Saccharomyces* relative to that of the CON group. No significant differences were observed between the NW PWS and NW CON groups, conversely, increased relative abundance of *Candida* and relative decreased abundance of *Saccharomyces* was found in the OWOB PWS and OWOB CON groups. This may imply that the OWOB groups have a more significant impact on the overall observed differences in the relative abundance of *Candida* and *Saccharomyces* in the PWS vs CON comparison. In other words, it appears that within an obese profile, there are differences between fungal taxa abundance depending on genetic profile.

While there is still much unknown as to what classifies as a clinically significant difference in taxa abundance, we can first begin by observing which taxa are over or underrepresented in disease populations and from there develop more insight on the pathophysiology of the organism-host interaction within the context of the greater microbial community. Interestingly, previous literature has found that *Candida* and *Saccharomyces* exhibit strong negative abundance correlations to each other. *Candida* are normally harmless commensals on many human body sites. However, when the host immunity or the normal gut microbial community is compromised, this fungus can become pathogenic (Enaud et al 2017). It has been suggested that a higher relative abundance of *Candida*, and a concurrent lower abundance of *Saccharomyces* may play a role in the dysbiosis of inflammatory diseases such as IBD (Hoarau et al., 2016; Sokol et al., 2016). *Candida* has been previously reported as the most abundant genera detected in obese patients (Borgo et al., 2017; Mar Rodríguez et al., 2015) and some studies have suggested a link between expansion in *Candida* (especially *Candida albicans*) with diabetes. Conversely, some fungal species such as *Saccharomyces boulardii* have been suggested to have potential gut health benefits or probiotic effects (Ward et al., 2017). Previous work with other rare genetic diseases has also noted differences in the gut mycobiome communities between affected individuals and typically developing controls. Children with Rett syndrome (a neurological disorder mainly caused by mutations in the *MeCP2* gene) were found to have differences in fungal beta-diversity as well as an increased abundance of the genus *Candida* (Strati et al., 2016). This once again matches the pattern of findings from this work.

Twin research has demonstrated a heritable component exists for the gut microbiota composition (Julia et al., 2016), however additional research has suggested that when compared to host genetics, priority effects (ecology), diet and lifestyle may be the most influential factors that shape the human gut microbiome (Rothschild et al., 2018). While it may not be as strong of a factor, host genotype does factor into the composition of gut microbiota (Goodrich, Davenport, Clark, & Ley, 2017; Kreznar et al., 2017; Spor, Koren, & Ley, 2011; Tabrett & Horton, 2020).

Saccharomyces, Basidiomycota, and *Candida* are three taxa that were observed to differ in abundance levels between groups. These species are known to dominate the GI tract of humans and are likely true members of the mycobiome (Nash et al., 2017). While evidence for a "core mycobiome" is lacking, a few species are more well characterized. Relatively commonly detected gut fungi such as *Debaryomyces hansenii* and multiple *Penicillium* species are not likely to be true members of the human gut as they do not grow at 37 C. These species are likely allochthonous and pass through the GI system from environmental or dietary exposure without colonizing the gut. It is also unclear if these taxa exert any influence on the host or the host's gut microbiota (Suhr & Hallen-Adams, 2015). While these potentially allochthonous taxa were observed in the overall composition, they did not differ significantly in abundance between groups. We found the genus *Alternaria* was slightly positively correlated with saturated fat intake in the PWS group, however this genus is known to be a plant pathogen and it is likely that it may be found in the GI tract due to dietary consumption or environmental exposure rather than being a true member of the gut community (Suhr & Hallen-Adams, 2015).

As was observed in previous reports looking at the gut mycobiome (Mar Rodríguez et al., 2015), no significant differences were detected in the alpha diversity between NW and OWOB subjects of this study. Although the results of Chao1 richness measure between overall PWS and CON was not statistically significant, there was a trend towards increased richness in the PWS group, with differences in richness being particularly relevant for individuals with OWOB. Within the OWOB classification, the PWS group was found to have increased Chao1

richness compared to the CON group (p=0.04). This may further suggest that genotype plays a more important role than weight status in differentiating mycobiome profiles.

As discussed in the meta-analysis by Sze et. al 2016 (Sze & Schloss, 2016), alpha diversity metrics are able to distill complex data into a single value, however the potential applicable clinical significance of these metrics is not currently understood. High diversity of the gut microbiota is usually considered to be an indication of community stability and is more generally associated with a healthy gut ecosystem. While 'differences in diversity' was noted to be a significant discriminatory marker between health and disease in their meta-analysis, Sze. et al (2016) point out that caution must still be exercised when discussing these metrics. Additional context is needed to interpret the results of diversity metrics in a given population comparison.

While our results found higher bacterial Shannon diversity in the CON group compared to the PWS group (p=0.04), it may be worth noting that the range of alpha-diversity scores on the PWS group were broader than that of the control group. No differences were observed in the pairwise comparisons of bacterial alpha diversity or in any other alpha diversity measure in the bacterial component of the gut microbiome. In their recent publication, study authors suggested that certain features of the microbiome profile observed in their PWS population such as increased phylogenetic diversity may be contributing to the more beneficial inflammatory profile seen in PWS (Olsson et al., 2019). However, it's important to note here that this research only studied the bacterial component of the gut microbiome. Conversely, in the study on a Chinese pediatric cohort, no differences in alpha diversity metrics were found in the baseline microbiome of PWS and control groups (C. Zhang et al., 2015).

In line with the results of Zhang et al. (C. Zhang et al., 2015), we found no significant differences in bacterial community structure (Beta-diversity) between PWS and CON groups. While there were no overall differences in bacterial differential abundance between PWS and CON groups, differences were observed in the pairwise analyses. Within the NW subgroups, the PWS group tended to have higher abundances of bacterial taxa often associated with inflammation and metabolic disorders. These include higher abundances of *Staphylococcus* and

Lactobacillus. *Lactobacillus* has been found to be more abundant in obese and overweight children (Bervoets et al., 2013), with study authors finding a positive-correlation between this species and BMI (Ignacio et al., 2016) and plasma hs-CRP (Bervoets et al., 2013). Positive associations between BMI and *Staphylococcus* have also been previously reported (Befus et al., 2015). Children and adolescents with a high daily energy intake showed high faecal concentrations of *Staphylococcus*, and *Staphylococcus aureus* in feces was a marker for obesity risk during development (Bervoets et al., 2013; DiBaise, Frank, & Mathur, 2012). The bacterial genus *SMB53* was slightly positively correlated with polyunsaturated fat intake in the PWS group and this taxon was found to be more abundant in the OWOB CON group compared to the OWOB PWS group. *SMB53* may be important for the abnormal metabolism of type 2 diabetes (Horie et al., 2017), and is often reported in the obese microbiome profile. Conversely, the OWOB PWS group had higher abundance of *Lachnospira* compared to the OWOB CON group. *Lachnospira* has been shown to negatively correlate with BMI and diabetes incidence in adult populations (Del Chierico et al., 2018). Whether the observed differences in abundance between PWS and controls in our research are of clinical significance remains to be seen.

Interestingly, bacterial genera that were increased in PWS were generally facultative anaerobes. There has been recent literature that has linked presence of oxygen in the adult gut with unfavourable health consequences and a "dysbiotic" microbial ecosystem composition that is overrepresented by more oxygen-tolerant microbes (Henson & Phalak, 2017; Litvak, Byndloss, Tsolis, & Bäumler, 2017). The SCFA butyrate plays an important role in intestinal permeability by both the upregulation of tight junction proteins and the promotion of mucin production (H.-B. Wang et al., 2012; Willemsen et al., 2003). β-oxidation of butyrate has also been recognized as an important regulator of oxygen availability (limiting the expansion of facultative anaerobic bacteria) and is known to drive energy metabolism in colonic epithelial cells (Vacca, 2017). The "oxygen hypothesis" posits that chronic inflammation of the GI tract may results in increased release of reactive oxygen species into the intestinal lumen (Zhu & Li, 2012), which in turn creates an environment that favors facultative anaerobes (Albenberg et al., 2014; Hartman et al., 2009). This environment promotes both a decrease in obligate anaerobes such as *F. prausnitzii* that have been shown to produce anti-inflammatory compounds (Miquel

et al., 2015) and causes increased inflammation through disruption of barrier function (Kelly et al., 2015). This combination is thought to establish a positive feedback loop that accelerates the inflammation and subsequent disease process (Miquel et al., 2015). As obesity is associated with chronic low-grade inflammation and disruption in intestinal barrier function, these microbial patterns, especially when seen in the NW subgroup may imply that the gut environment in PWS is potentially in a state of oxygen imbalance. While this theory of oxygen imbalance cannot be confirmed in the present study, this avenue of research could potentially lead to new biomarker discovery to provide insight on the health of individuals with PWS. It is also possible that designing interventions that promote butyrate production could assist in reversing the effects of the overly oxygenated gut. In previous research, results of fecal transplantation to germ-free mice resulted in improved insulin tolerance in mice transplanted with the PWS microbiome compared to those mice transplanted with the microbiome of obese individuals, with no differences in their fat mass or body weight (Olsson et al., 2019). This result lead study authors to suggest that the gut microbiome in PWS may be contributing to the protective factors that are seen clinically in this population. Similarly, the differences in taxa between the OWOB PWS and OWOB CON group described above also hint at a more protective microbiome profile present in the PWS group. As the fungal component was not studied in the work by Olsson et al, it is less clear what contribution the mycobiome could be playing to these findings and future research should aim to see a more holistic view of the microbiome to further pinpoint what are driving these differences.

Some literature has looked at interactions between bacteria and fungi in the gut microbiome. For example, *Lactobacillus* spp. has been shown to have a protective effect against *C. albicans* mucosal and systemic infections (Wagner et. al., 1997), and short-chain fatty acids (specifically butyric acid) produced by lactic acid bacteria may also prevent pathogenic *C. albicans* from causing disease in the gut through mechanisms that inhibit filamentation of the fungus (Noverr & Huffnagle, 2004). Whether other butyrate producing bacteria could have the same effect on *Candida* virulence remains to be seen at this time. It could be theorized that while *Lactobacillus* and *Candida* may both individually be associated with disease states, their interactions within the microbial community could play an important role in maintaining host

health and preventing disease. While this is hypothetical, further research into microbial interactions are warranted to help pull together the findings of abundance results.

A previously published work describing the microbiome in PWS noted the lack of dietary assessment as a limitation of their work (Olsson et al., 2019). The current study collected dietary intake information using a 3-day dietary intake record to assess macro-and-micronutrient intake. This tool is validated to assess dietary intake in children (Day, 2001). Consistent with previous findings (Mackenzie et al., 2018), the PWS group had a lower carbohydrate and total energy intake compared to the control group. Also, in congruence with previous findings, both total fat and saturated fat intake was lower in the PWS group, however these differences no longer remained after adjusting values for energy intake. Although Mackenzie et al. noted a greater fiber intake in PWS compared to controls (Mackenzie et al., 2018), no differences were observed in fiber intake between the PWS and CON groups in this study. As fiber intake is strongly associated with microbiome composition, this could possibly be another factor partially explaining the lack of significantly different bacterial profiles between groups in this work. It is important to note that the population in our study may be biased in that participants were largely children of high socio-economic status and usually from a household with at least one parent with a high degree of education. The caregivers of children with PWS were often extremely diligent with the control of food intake and diet quality, thus the results of dietary intake from this population may not fully represent the general PWS population. Additionally, as the control group was recruited in large part through university channels, this population may also not be fully generalizable.

The current study utilized canonical correspondence analysis (CCA) to assess the influence of multiple factors on the fungal microbial communities. While not statistically significant using correlations, dietary components were a significant contributor to variation explained by the CCA model with 36% of variation in fungal communities being explained by the dietary measures. The effect of specific dietary components in the model was secondary only to group type (PWS vs CON), which had the most potential to explain variation in samples. The literature has noted that the fungal community can also be largely influenced by diet (Richard &

Sokol, 2019) and additional research on how the mycobiome can be modulated with diet could lead to new therapeutics.

How the gut microbiota interacts with the host may differ between individuals and therefore the same microbial profile in individuals with this altered genetic background may result in different effects. This may be especially pertinent in individuals with an altered immune profile such as individuals with PWS since the microbiome is known to play several immunomodulatory roles. Further studies are needed to elucidate potential effects of the microbiome in PWS individuals. While mouse models of PWS are still not ideal (as no single mouse model is able to encapsulate all the characteristics of the human syndrome) (Carias & Wevrick, 2019), one avenue for exploration could include fecal transplantation in germ-free PWS mouse models compared to mouse models for other forms of obesity and control mice. Having a better understanding of how the microbiome can affect clinically relevant parameters could ultimately help in the design of novel therapeutics for PWS.

Some methodological strengths and limitations that can be observed include the stringent data filtering and normalization techniques applied. The rationale for the chosen methods is outlined in the earlier text, however it is worth noting that a consensus has not been reached on which methods are best for analysis of microbiome data. This is especially the case for the fungal component of the gut microbiome as research of the mycobiome's role in human health is still in its infancy. Every method comes with its own series of pros and cons that must be evaluated and the specific nuances of the data must be considered as there is much variability in the features of microbiome sequence data. For example, rarefying count data helps to reduce false positive (type 1 error) results that could occur due to large differences in reads between samples, however as rarefied data represent only a portion of the original data, decreased sensitivity (type 2 error) could be more prominent. As our interests are strongly aligned with finding factors that are most likely to have clinical significance, this work focused on findings with effect sizes large enough to be identified despite the stringent protocol. This is not to say that small changes or more rare taxa are less likely/unlikely to contribute significantly to the functional potential of the overall microbial community, however

by being more conservative in this initial observational study, we can focus on the few key findings and their possible clinical implications.

Our samples were collected from a wide age range (3-17 years old), which could be considered another limitation. While the microbiome is considered to be “adult like” and stable after the age of 3, age can still be a factor shaping the gut microbiota profile. Additionally, because hyperphagic behavior in PWS varies in age of onset (Irizarry et al., 2016b), the results and associations between the microbiome and hyperphagic behavior may have been less apparent than if we had used a population that all exhibited this clinical feature. Despite this limitation, there was still a significant difference in hyperphagia scores between the PWS and control groups, with higher scores in the PWS group as would be expected.

Another limitation of this work can be attributed to sample size, which is challenging as PWS is a rare disease. Due to the current sample size, the confidence of findings is somewhat limited. Additional work with a greater sample size would allow for further confidence in findings. Additionally, we conducted our differential abundance analyses at a genus level as this level of resolution can be analyzed with more confidence using the given sequencing methods. Emerging research has suggested that functional differences in the gut microbiome and its relationship with diseases might be strain-specific (Chenhong Zhang & Zhao, 2016), therefore future research using methodologies with higher resolution (e.g. shotgun metagenomic sequencing etc.) could be important in further elucidating findings and may also be an important step in moving towards clinical applications.

Finally, a discussion of dysbiosis, causality and what claims can be made from this research is warranted. The term “dysbiosis”, while widely used in the microbiome field is ambiguously defined and often misused. Dysbiosis is typically used to describe whatever microbiome features are different between the group of interest (disease) and the control group which a) reduces the complexity of the microbiome to a binary categorization of “healthy” and “dysbiotic” b) makes the assumption that the microbiome of the control individuals is “healthy” or that there is a universally established “healthy” configuration of microbes c) implies something is imbalanced or dysfunctional with the microbiome and d)

implies that the observed differences in microbiome features are related and possibly causal to the disease state (Brüssow, 2020; Hooks & O'Malley, 2017; Olesen & Alm, 2016).

Currently, it is unclear whether dysbioses may be a cause or consequence, or just a bystander of a disease state (Lynch, Parke, & O'Malley, 2019). The possible mechanisms by which "dysbiosis" could cause disease are still under investigation and most findings that attempt to ascertain the relevance of microbiome composition to disease remain speculative (Fischbach, 2018). Additionally, the term dysbiosis may erroneously imply that a universally accepted definition of a "healthy human gut microbiome" has been determined (McBurney et al., 2019). Because of this, some experts in the field caution against the use of the term and suggest that if this term is to be used that context is needed for interpretation of findings.

There are several reasons why establishing what constitutes a "healthy human gut microbiome" in both composition and features (diversity, genetic potential, metabolic function, etc.) is extremely difficult, if not impossible. The healthy microbiome cannot be defined by a single ideal community composition and the effects of specific microbiome features on host health are often context dependent (McBurney et al., 2019). The presence/relative abundance of a given microbe may be associated with both a healthy or disease profile depending on the disease in question. For example, *Akkermansia* is associated with a metabolically healthy profile when looking into the microbiome literature on obesity (Dao et al., 2015; Schneeberger et al., 2015). Contrarily, *Akkermansia* has been inversely associated with prevalence of multiple Sclerosis (Cantarel et al., 2015; Jangi et al., 2016).

The differing microbial features discovered between the PWS and CON populations and weight-based subgroups in this research cannot establish causality or directionality. Caution must be exuded when attempting to interpret these results and the implications they may have for health and disease. As discussed earlier, the assessment of beta-diversity determined that there were significant differences in fungal community structure between PWS and CON groups regardless of weight-status, and that specific taxa differed in relative abundance in both two-group and subgroup fungal analyses. Specific taxa also differed in relative abundance in the

subgroup bacterial analyses, however no differences were observed in overall bacterial community structure.

There are numerous factors that could begin to explain why differences in microbial features were observed. It is known that human gut microbiome communities are highly individualized and show a high degree of interindividual variation. In the adult-like (after ~3 years of age) microbiome, genetic and other host factors are said to account for ~10% of the microbiome variation, while specific environmental factors (e.g. diet, antibiotic/medication use, etc.) account for ~20% (Rothschild et al., 2018; J. Wang et al., 2016). This leaves a vast majority of the microbiome variation unexplained. These unaccounted-for differences are largely considered to be driven by stochastic elements and ecological processes, such as priority effects, that establish and shape the gut ecosystems (Martínez et al., 2018).

When considering the potential implications of our results, context is important. For example, it was observed that the genus Saccharomyces (a commonly consumed yeast in food production) had a higher relative abundance in the CON group compared to the PWS group. Interestingly, the CON group also reported higher intake of carbohydrates. The fungal microbiome is affected by dietary intake (Chin et al., 2020; David et al., 2014) and it is possible that the relative increase in Saccharomyces found in the CON group is driven at least in part by the difference in diet between groups and the relative decrease of Saccharomyces in the PWS group may simply be the influence of diet (less carbohydrate intake) rather than a marker of disease or “dysbiosis”.

As discussed in McBurney et al. 2019, ecosystem functions may also be an important avenue for consideration and the overall ecosystem may potentially be more influential to the host than the abundance/presence/absence of specific individual members of the gut microbiome (McBurney et al., 2019). Several factors highlight the likely importance of overall ecosystem health to the host well-being. First, there is a notable functional redundancy present in the gut microbiome and an established high degree of interindividual variation in community structure between individuals (Moya & Ferrer, 2016). The large range of what the microbiome in individuals without disease can encompass further emphasizes that it is likely not possible to

identify a specific microbiome feature that is universally healthy. Additionally, consideration should be made for the complexity of the potential interactions of the bacteria, archaea, fungi, viruses, and protozoa in the gut microbiome (within the ecosystem itself and with the host) and the potential effects these microbes and their metabolites could play on host health (alone or through community interactions). The inclusion of the fungal component of the gut microbiome in this thesis research may provide further insight to how the overall ecosystem interacts and its relationship with health host and disease, though this research is still in its infancy and no conclusions regarding causality or recommendations can be determined by the results of this work alone.

Establishing causal relationships between microbiome features is exceedingly difficult and only a few cases such as Helicobacter pylori-associated peptic ulceration and gastric cancer (Parsonnet et al., 1991) and Clostridium difficile infection-associated diarrhea (Gupta, Allen-Vercoe, & Petrof, 2015) have been extensively documented (Lynch et al., 2019). While the microbiome has been causally linked to obesity in mouse models (Turnbaugh et al., 2006) multiple meta-analyses have found little to no consistent difference in the gut microbiomes of obese and lean patients (Duvallet, Gibbons, Gurry, Irizarry, & Alm, 2017; Finucane, Sharpton, Laurent, & Pollard, 2014; Walters, Xu, & Knight, 2014).

The use of animal models to gain insight on mechanisms of actions and possible causality in the microbiome field has been greatly improved with the introduction of germ-free and gnotobiotic organisms. Causal relationships between microbiomes and phenotypes such as obesity in germ-free mice are not however necessarily indicative of a causal relationship between microbiomes and these phenotypes in humans, or even within healthy colonized mice. It has been established that the lack of a microbiome during development has significant consequences for the maturation and function of the GI tract and the immune system (Tlaskalová-Hogenová et al., 2011). The current understanding of microbiome-mediated contributions to obesity are heavily intertwined with immune function (Singer-Englar et al., 2019) and results obtained from germ-free mice should be interpreted with care.

In the previous studies of the PWS microbiome, germ-free mice models were used to suggest causality and/or to try and elucidate effects of the microbiome on health outcomes (Olsson et al., 2019; C. Zhang et al., 2015). There are several reasons why translating results from animal models and applying them to human disease can be challenging and must be done with caution. In addition to concerns about experimental design, control and rigor, there are a few elements that are often overlooked from consideration before conclusions are made. Historically in the microbiome field, there is sometimes an issue of pseudoreplication, where the ‘‘N’’ for statistical analyses and inferences is reported as the number of recipient animals instead of donor numbers. The low “N” for donors used in most studies is unlikely to capture the interindividual variability of the human gut microbiome, which is typically more prominent than the effect sizes caused by disease states (Lloyd-Price et al., 2019). Thus, even if differences in microbiome features did play a causal role in disease, a small donor group would likely not powered to reflect these differences, nor would the small donor pool be sufficiently representative of the larger group (Jens Walter, Armet, Finlay, & Shanahan, 2020). Appropriate power to account for real biological variation must be considered carefully before conclusions are drawn. Pooling donor samples is also of concern as it removes the component of interindividual variation and changes the true N for statistical interpretations. Finally, there is still a lack of mechanistic insight for the large majority of animal model research, with most studies in the microbiome field not attempting to identify causal components of disease states (Jens Walter et al., 2020).

In the germ-free mouse experiment conducted by Olsson, the donors were not randomly selected but rather were chosen based on the abundance of what study authors called “key microbial taxa” that characterised the PWS gut microbiome (determined based on the differential abundance results of the same publication) (Olsson et al., 2019). Study authors reported that characteristics that differentiated the PWS and control patients in the study were reflected in the germ-free recipient mice and repeated the germ-free experiment with another specifically chosen donor-pair (total experiment n=2 PWS donors and n=2 control donors). In the germ-free mouse experiment from Zhang and colleagues, the gut microbiota from the same PWS volunteer (who was chosen as they had they most drastic improvement in tested health

parameters) was transferred to mice to compare the microbiome effects pre- and post high-fiber intervention (C. Zhang et al., 2015). While Olsson and colleagues did repeat their experiment with a new donor-pair to try and confirm their finding on improved insulin tolerance, in the work by Zhang only a single donor was used to represent the entire population. In both studies, the donors were not randomly assigned but rather specifically selected based on their microbiome composition and health biomarkers. Interpretation of these results and what they could mean for the microbiome of the larger PWS population must be done with care, especially as results from a single donor is unlikely to fully represent the larger group and would likely not have the statistical power to make causal conclusions. PWS is a phenotypically heterogenous group with varying degrees of symptom severity which makes extrapolating findings to all individuals even more of a challenge. Individuals with PWS are known to have altered inflammatory, endocrine, and metabolic profiles (Irizarry et al., 2016b), thus the gut environment may already differ than that of the control group and these differences may influence the composition and function of the microbial ecosystem and microbe-host interactions.

Some microbial features were identified as differing between the PWS and CON groups, as well as the NW and OWOB subgroups of this thesis work. The current case-control study does not have the capacity to determine if any of these microbial features may have a functional or causal role in pathology and health of our population. Animal models could be utilized as one potential avenue to try and elucidate the potential impact of our findings. Comprehensive recommendations for human microbiota-associated murine models to establish causal relationships between altered (dysbiotic) gut microbiomes and human disease have recently been suggested (Jens Walter et al., 2020). In addition to ensuring experimental rigor and standards are upheld and being cautious to avoid common misleading statistical inferences as described previously, future research to determine causality should aim to gain mechanistic insight whenever possible through the identification and functional testing of candidate “causal components”. We would also want to ensure that microbiome engraftment was successful (and not overlook the fungal component of the gut) and see whether ‘‘dysbiotic’’ patterns were transferred from our human cohort to the recipient animals before making any conclusions.

As suggested by Fischbach, research establishing causation and molecular mechanisms in the microbiome should emphasize findings that are practical and easy to measure for clinical translation, have a large effect size and have been shown to be unambiguously driven by the microbiota (Fischbach, 2018). To minimize technical artifacts, there is a need for more robust sampling protocols and standards in the microbiome field and consensus for experimental methodology to employ. In doing so, the aggregation of the data from many studies can become more feasible. Future hypothesis-driven clinical research is needed to determine causality between microbiome features (diversity/structure/functional changes) and validated markers of host health (McBurney et al., 2019). This could include large comprehensive, multidisciplinary multi-center prospective cohort studies that measure and record a variety of known factors of interest that may influence the microbiome including lifestyle and environmental factors (e.g. diet, antibiotic use, socioeconomic status, geography, education etc.) along with repeated biological samples (blood and fecal samples) over time. The repeated measures within subjects and the large sample size in this design may help to overcome intraindividual variability. The biological samples, additionally assessed with multiple "omic" platforms such as functional metabolomics and metagenomics, could then be combined with the collected metadata and the indicators of health that correlate with "microbiome features" can be identified and their relationship validated. This multiomic characterization of microbiome-related biomarkers can be then further explored with mechanistic studies the potential to devise microbiome-targeted strategies using the validated biomarkers can be applied. Ideally such trials would be run from an ecological perspective (Jens Walter, Maldonado-Gómez, & Martínez, 2018) using a multidisciplinary approach and attempts at personalizing the intervention approach could be made. Finally, once the metabolomic markers in biological samples (blood, feces, and urine) and host-specific indicators of health are systematically identified, validated, and agreed on, consensus statements can be devised and used to guide regulatory agencies.

2.6 Conclusions:

Despite the limitations of the present work, several novel findings have expanded our knowledge and understanding of the potential role of the microbiome in PWS. Here we have

shown a unique bacterial and fungal signature differentiates PWS from control individuals, as well as weight status within groups. Based on the results of this work, differences between the gut microbiome community in PWS and CON seem to be more strongly associated with differences in genotype (PWS vs CON) than weight status. Additionally, within NW and OWOB groups, different taxa are differentially abundant. These taxa may be contributing to the altered metabolic and inflammatory profile in PWS. Specifically, differences in microbiome profiles for OWOB individuals with or without PWS seem to differ the most. The significant differences in taxa abundance and community structure of the mycobiome in PWS compared to controls is a novel finding and has not been investigated previously.

The knowledge that a unique microbial profile may be contributing to the specific pathogenesis of hyperphagia and obesity in PWS is an indication that research into a specific and more personalized intervention for this population may be efficacious. Therapeutic manipulation of the gut microbiome has strong potential for treatment of diseases. The specifics of how and if the mycobiome might contribute remains to be determined. Additionally, these findings bring up the question of whether a combination of host factors (such as GI motility, inflammation, and changes in endocrine and exocrine function) creates an environment that favours a particular microbial community, or if a microbial community confers a specific gut environment or a combination of the two.

Our understanding of how the genetic mutations that characterize PWS may influence the microbe-host interactions and their subsequent effects on obesity and metabolic complications is limited. The current literature currently lacks adequate validated biomarkers that can predict the development of obesity and in high-risk subjects. The gut microbiome has the potential to provide new insights not only for biomarker discovery but also as a potential target for intervention to safely and effectively modulate weight-gain and improve metabolic function. This is especially important in high risk pediatric populations as early intervention can greatly improve health outcomes.

The study of the mycobiome in human health and disease is still in its infancy and there is limited knowledge on how various microbial communities interact within an ecosystem and

how these interactions may affect host health. Further gut mycobiome studies are needed to better understand and interpret the findings of this study, and more broadly to better understand what factors influence the composition, interactions, and functionality of the microbiome in different populations.

Chapter 3: Summary and future directions

Prader-Willi Syndrome (PWS) is the most common syndromic form of childhood obesity. The development and maintenance of PWS-associated obesity may involve several possible pathophysiological mechanisms. Previous research has implicated the gut microbiome in the development and pathology of obesity and metabolic dysfunction (Singer-Englar et al., 2019). PWS is a genetic disease, thus modulating the gut microbiome of these individuals is unlikely to completely reverse the pathophysiology associated with this disorder. Instead, the goal is to attempt to develop beneficial treatment and management strategies to try and minimize symptoms such as hyperphagia, improve metabolic health markers and prevent the development of obesity and subsequent detrimental health outcomes.

The results from the present study provides evidence that there are notable differences in the gut microbiome between children with and without PWS. Specifically, this work unveiled that the gut mycobiome community could potentially be an important avenue for future exploration as differences in the fungal community between groups were more notable than those of the bacterial microbiome community. While differences in diet and hyperphagia scores were not significantly correlated with microbial community structure using Spearman's correlations, dietary factors were found to be influential in CCA models of the mycobiome. Rather than being correlated with individual taxa, measures like hyperphagia and dietary intake may be more associated with overall community structure.

This thesis work is the first to investigate the fungal community present in the gut microbiome in individuals with PWS. Future work can expand upon these findings for a better understanding of how this information could be applied towards new therapeutic options in this population. Additionally, more recent research may even suggest a role of the gut

microbiome in the interplay between energy metabolism, mood and cognitive function (Agustí et al., 2018). Behavioural concerns are also frequently cited as a concern for patients and caregivers of individuals with PWS (Wilson et al., 2016), thus modulating the gut microbiome may have the potential to impact both physical and mental health parameters.

Several avenues to favourably modify the gut microbiome have been proposed and studied. This includes more direct methods such as probiotic supplementation and fecal microbiome transplants (FMT) as well as more indirect methods such as prebiotic supplementation and other dietary interventions.

The use of probiotics is one popular option to influence the gut microbiome that has been studied extensively. The gut microbial population established in adults tends to be stable, resilient, and resistant to outside colonizers. Probiotic use in healthy adult populations does not generally alter the gut microbiome composition (Kristensen et al., 2016) and when it does, it has been noted that the microbes are usually transient/do not persist very long following cessation of probiotic use (Alander et al., 2001; Charbonneau, Gibb, & Quigley, 2013; Firmesse, Mogenet, Bresson, Corthier, & Furet, 2007). These observations may be in part explained through ecological theory. Firstly, an organism must be able to overcome the specific challenges faced in an environment in order to be established into that community. Many probiotics are not adapted to overcome the many challenges put in place by the host (immune system, other defenses) and/or may not be able to compete with the established microbiota (J. Walter, 2008; Jens Walter & Ley, 2011). Longer colonization has been observed when the species/strains in question are more core members (autochthonous) of the host microbiome (Maldonado-Gómez et al.). Autochthonous organisms are generally adapted for the host environment, and are more likely to fill an existing niche and therefore may persist for longer (J. Walter, 2008; Jens Walter & Ley, 2011). Additionally, previous colonization history affects the availability and types of niches (De Meester, Vanoverbeke, Kilsdonk, & Urban, 2016; Fukami, 2015). Niche modification may also play a role in explaining the large inter-individuality in responses to probiotics in the literature, even if they contain autochthonous species. Ecological theory also suggests the presence of phylogenetically or functionally related organisms within

the resident microbiome could impede colonization through competitive exclusion mechanisms (Mayfield & Levine, 2010). This being said, there have been demonstrated benefits of consuming these probiotics even if the bacteria are only present transiently (Bermudez-Brito, Plaza-Díaz, Muñoz-Quezada, Gómez-Llorente, & Gil, 2012). While in the gut, these probiotic bacteria may use the resources to produce short chain fatty acids and other substrates that the resident microbiota may utilize. This cross-feeding ability can be beneficial to the metabolic function of the established microbiome (Ceapa et al., 2013; McNulty et al., 2011; Scott, Antoine, Midtvedt, & van Hemert, 2015). They may also act to prevent the invasion of potentially pathogenic bacteria from establishing (through their ability to improve the host barrier function, influence on host immune system and their use of available resources (Bermudez-Brito et al., 2012)) thus aiding the resident microbiota fend off competition. Finally, these bacteria can alter enzymatic activity of the resident microbiota (Ouwehand, Lagström, Suomalainen, & Salminen, 2002), both if they are established into the community or if they are transiently present. Several avenues for development of "next-generation probiotics" are being explored including the creation of genetically modified microorganisms (Chang, Ruan, & Kao, 2019) and selection of taxa that may have a stronger ecological potential to survive and provide benefits in the host.

Fecal microbiota transplantation (FMT) is an even more direct procedure to manipulate the gut microbiota and is achieved by transferring fecal microbiota from donors into recipients with the end goal of ameliorating a "dysbiosis" present in the recipient. Clinically FMT is currently used as an effective approach to treatment of *Clostridium difficile*- associated diseases (Chang et al., 2019). Recently there have been a few trials assessing the use of FMT for therapy in other diseases including obesity and metabolic disorders that have had mixed results (Z. Zhang et al., 2019). Several concerns arise when discussing FMT for clinical application, especially uncertainty surrounding the complex and undefined microbiota composition. Although the short-term adverse effects of FMT are generally reported to be relatively minor, the long-term effects of these treatments will still need to be carefully evaluated. There are still many unknowns surrounding the most effective implementation of FMT as a potential therapeutic for obesity and metabolic dysfunction. Future mechanistic studies of FMT will

hopefully reveal more on which changes in composition and function of gut microbiome affect metabolic outcomes for patients. Additional insights on optimal fecal microbial preparation, effective dosage, duration of intervention and method of delivery are also needed to best implement this technique as a reliable therapeutic tool.

Finally, dietary supplementation is another more indirect way of modulating the gut microbiome. Microbiome-accessible carbohydrates (MACs) are important in the diet to provide nutrients for gut microbes (Edward C. Deehan et al., 2017). Insufficient intake of MACs may lead to a depletion of species reliant on these substrates and a subsequent reduction in important fermentation end products involved in physiological and immunological host functions (Sonnenburg & Sonnenburg, 2014). Intake of MACs have been implicated in the reduction of chronic diseases (Dahl & Stewart, 2015; Delcour, Aman, Courtin, Hamaker, & Verbeke, 2016), low-grade systemic inflammation (Buyken et al., 2014), obesity (Armet, Deehan, Thöne, Hewko, & Walter, 2019; Du et al., 2009), metabolic syndrome (Chen, Chen, Wang, Qin, & Bai, 2017; Wei et al., 2018), type 2 diabetes (Yao et al., 2014), and CVD (Pereira et al., 2004; Veronese et al., 2018), as well as reduction in mortality rates (Yang, Zhao, Wu, Ma, & Xiang, 2015). Some mechanisms of action for these health-promoting effects of MACs have been studied more extensively. These include promotion of bile acids and cholesterol sequestration and excretion (Gunness & Gidley, 2010), reduction of glucose and lipid absorption (McRorie & McKeown, 2017) and production of SCFAs (Ríos-Covián et al., 2016). SCFAs have a wide variety of immunological, metabolic, and endocrine effects including satiety promotion, improved inflammatory profiles, and ameliorating glucose and lipid metabolism (Koh, De Vadder, Kovatcheva-Datchary, & Bäckhed, 2016; Makki et al., 2018).

Finally, combinations of these methods have been proposed to modify the microbiome. This includes “Synbiotics”; a combination of pre and probiotics with the end goal of providing a synergistic health benefit. A recent review concluded that dietary supplementation in populations with obesity and metabolic syndrome with synbiotics prepared using selected combinations of strains and prebiotics may provide anti-inflammatory effects and promote weight loss (Ferrarese, Ceresola, Preti, & Canducci, 2018). These observed effects were thought

to be in large part conferred due to SCFA production and modulation of the microbial community.

Another important consideration for how to modulate the gut microbiome to confer health benefits is addressing specific considerations for the population of interest. Individuals with PWS are suggested to have some impaired gastrointestinal function; Gastric emptying in individuals with PWS has been reported to be relatively normal (Choe et al., 2005; Hoybye, Barkeling, Naslund, Thorén, & Hellstrom, 2007) or slightly slower than nonaffected individuals (Arenz, Schwarzer, Pfluger, Koletzko, & Schmidt, 2010). Individuals with PWS have increased frequency of constipation (Kuhlmann, Joensson, Froekjaer, Krogh, & Farholt, 2014) compared to the general population. Additionally, PWS patients often have difficulty interpreting body signals (such as mechanistic feedback responses to inhibit eating (Stevenson et al., 2007)) as well as expressing symptoms (Hurren & Flack, 2016). One mechanism by which dietary fibers can modulate appetite and promote satiety is dependant on the viscosity of the fiber and the subsequent slowing of gastric motility. However as gastric function is somewhat impaired in PWS, this mechanism for reducing hyperphagia may not be effective in this population.

Based off the results from this thesis work, our research group led by Dr Andrea Haqq at the University of Alberta is currently working on a dietary fiber intervention in children with PWS (NCT04150991)(Haqq, 2020) that aims to take advantage of other mechanisms to achieve appetite reduction, such as the promotion of SCFA production. As described previously, SCFAs have a large range of beneficial effects and have been shown to modulate appetite in several ways. SCFAs may initiate stimulation of PYY and GLP-1 through SCFA mediated G-protein coupled receptors (GPCRs). Increased SCFA circulation is correlated with increases of anorectic gut hormones such as GLP-1 & PYY (G. Tolhurst et al., 2011; Zhou et al., 2006; Zhou et al., 2008) and increased leptin expression in adipocytes. MRI imaging shows SCFAs target appetite centers in the hypothalamus (Anastasovska et al., 2012; So et al., 2007). SCFAs have also been shown to improve glucose homeostasis, lipid metabolism and reduce insulin resistance (Delzenne & Kok, 2001; Gao et al., 2009; Vadder et al., 2014). Together, the metabolic effects resulting from SCFAs point to a protective state against obesity.

To date, only one study specifically aiming to modulate the gut microbiome composition of children with PWS has been published. In a 90-day hospitalized intervention trial with PWS (n=17) and obese (n=21) children, a diet rich in non-digestible carbohydrates induced significant weight loss and resulted in structural changes of the gut microbiota that were associated with reduced inflammation (C. Zhang et al., 2015). The study authors also saw a statistically significant improvements in hyperphagia scores in the PWS children (assessed by the Dykens Hyperphagia Questionnaire). The planned study aims to replicate and improve upon the mixture of prebiotics and whole grains used in the Zhang (2015) study which included combination of whole grains, traditional Chinese medicinal foods and prebiotics (C. Zhang et al., 2015). More specifically, this study will be using a mixture of fructo-oligosaccharides, digestion-resistant maltodextrin, and resistant starch (RS4) along with adding whole food sources of insoluble fiber. A more in-depth review of the literature that provided rationale for the selected fibers and dosages and a summary can be found in Appendix G.

An important factor for consideration of dosage and fiber selection for this intervention trial was tolerance to the intervention. Dietary fibers are often associated with undesirable gastrointestinal (GI) symptoms such as flatulence and abdominal pain, however the tolerability of a given fiber can vary greatly between individuals. The presence, frequency and severity of GI symptoms varies based on several factors, including properties of the fiber itself, the medium by which the fiber is consumed, and a combination of host factors including the resident gut microbiota (Grabitske & Slavin, 2009). A critical review of tolerance to dietary fiber intervention in pediatric populations was written and used as additional background for dosage rational. This work was written in 2017 and can be found in in Appendix H. Finally, a pilot study was developed to assess tolerability and the ease of the incorporation of the fiber intervention. A tolerance study journal (Appendix I) was completed by parents. In addition to daily assessments of tolerance, participants could indicate the amount of supplement consumed and write any additional comments they may have had in this journal. Results from this pilot study were taken into consideration for determining dosage and method of incorporation in the full clinical trial.

There are several challenges and hopes for this proposed intervention trial. High inter-individual variability in responses to dietary intervention has been a longstanding issue in the development of effective treatment strategies for obesity. Several factors influence the response of the host to interventions and thus the ability to combine several measures to predict responses to interventions may lead to the development of personalized medicine. The gut microbiome could also be an important factor to consider in the development of personalized medical intervention. Previously, blood parameters, dietary habits, anthropometrics, physical activity, and gut microbiome features measured in a sample cohort were combined using machine-learning to create an algorithm that was able to accurately predict personalized postprandial glycemic response to meals (Zeevi et al., 2015). The algorithm was then further validated and applied in a blinded randomized controlled dietary intervention which resulted in significantly lower postprandial glycemic responses and consistent alterations to the observed gut microbiome composition. Together, these results led to study authors to suggest that future personalized diets may be able to successfully modify elevated postprandial blood glucose and subsequent metabolic outcomes.

While there is still much work to be done, similar strategies could be developed for other factors that influence the development and progression of obesity (Bray et al., 2018). The PWS high fiber intervention study being conducted by our research group will be observing the relationship between changes in gut microbial composition and function with changes in hyperphagia, hormones (acylated-ghrelin, PYY, insulin, GLP-1, adiponectin, leptin), insulin sensitivity (HOMA-IR), inflammatory biomarkers (high sensitive C-reactive protein (hs-CRP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), lipids) and metabolites (amino acids, branched chain ketoacids, acylcarnitines, ceramides, Trimethylamine N-oxide (TMAO), choline and betaine). The large amount of information gathered in this trial may provide valuable information for the development of even more personalized nutrition for the PWS population.

Overall, this future research aims to expand upon this thesis work to provide potential new approaches for effective and practical non-pharmacologic treatment of excessive weight gain and hyperphagia associated with PWS to improve overall health and quality of life.

There is still much to be understood about the microbiome's role in health and disease and additional research specifically considering the microbiome in PWS is warranted. There are many avenues for future interventions to harness the potential of the gut microbial ecosystem as a potential therapeutic target to ameliorate the lives of individuals with PWS.

Tables and Figures

Table 1. Participant Characteristics

		PWS (n=25)	Control (n=25)	P-values
Background Characteristics	**Sex (F/M)**	14/11	9/16	0.162
	Age (Years)	6.2 (5.2, 12.9)	8.8 (6.4, 10.5)	0.800
	BMI %ile	79.3 (65.5, 94.1)	76.6 (51.2, 91.5)	0.507
	BMI z-score	0.8 (0.4, 1.6)	0.73 (0.02, 1.4)	0.588
	Weight Status (OWOB/NW)	10/15	8/17	0.565
	Genetic subtype	15-DEL 10-UPD	-	-
Hyperphagia Data	**Hyperphagia Score Behaviour (/25)**	7 (5, 10)	6 (6, 8)	0.172
	Hyperphagia Score Drive (/20)	8 (7, 11)	7 (6, 8)	**0.019***
	Hyperphagia Score Severity (/10)	4 (2, 4)	2 (2, 2)	**0.000***
	Hyperphagia Score Total (/55)	19 (16, 26)	15 (14, 18)	**0.005***
Dietary intake Data	**ENERGY (Kcal)**	1360.69 (1175.7, 1499.8)	1911.5 (1540.4, 2064.4)	**0.000***
	Fib (g)	18.50 (14.9, 21.78)	18.38 (15.3, 22.2)	0.945
	Prot (g)	61.41 (51.9, 71.3)	65.95 (58.6, 84.5)	0.058
	Carb (g)	149.24 (102.1,171.9)	254.25 (211. 7, 287.0)	**0.000***
	Fat (g)	47.23 (28.4, 53.9)	63.21 (47.3, 77.4)	**0.016***
	SatFat (g)	14.39 (9.6, 20.1)	25.2 (19.5, 31.1)	**0.004***
	MonoFat (g)	15.31 (9.8, 23.4)	22.31 (13.7, 31.9)	0.155
	PolyFat (g)	6.6 (4.8, 10.0)	8.65 (7.3, 16.1)	0.486
	TransFat (g)	0.83 (0.2, 4.0)	1.24 (0.7, 2.8)	0.443
	Chol (mg)	160.34 (72.0, 273.0)	151.79 (129.8, 282.2)	0.334
	Sugar (g)	52.63 (26.8, 69.4)	81.53 (51.1, 108.8)	**0.022***

Table 1. Participant Characteristics

Data presented as Median (25th and 75th percentiles). Hyperphagia scores obtained from validated questionnaire containing questions about hyperphagic behavior (5 questions), drive

(4 questions) and severity (2 questions). Total Hyperphagia assessment scores ranged from 12 to 39 out of a total of 55 for the PWS group and from 12 to 25 for the control group. Minimum possible score for the hyperphagia questionnaire is 11/55. *P-values for comparison between total PWS group (n = 25) and total control group (n = 25) determined using independent Student's t test (* indicates where $p<0.05$).

Abbreviations: Deletion (DEL), Uniparental disomy (UPD), Overweight/Obese (OWOB), Normal weight (NW)

Table 2. Data filtering summary

Table: Data filtering summary				
Data used:	# of samples	# of low abundance features removed based on prevalence	# of low abundance features removed based on variance (IQR)	# of features remaining
Fungal:				
PWS vs CON	37	73	N/A	40
NW: CON vs PWS	21	57	N/A	19
OWOB: CON vs PWS	16	41	N/A	21
PWS: NW vs OWOB	19	49	N/A	25
CON: NW vs OWOB	18	45	N/A	21
Bacterial:				
PWS vs CON	50	1719	39	348
NW: CON vs PWS	32	1060	41	364
OWOB: CON vs PWS	18	1480	43	378
PWS: NW vs OWOB	25	1351	45	398
CON: NW vs OWOB	25	1230	39	346

Table 2. Data filtering summary

Columns represent the number of samples in a given analysis, the numbers of features removed based on prevalence (minimum count of 4, 15% of samples), variance (IQR) and finally the number of features remaining for analysis. Rows indicate the group analysis performed and is separated by fungal and bacterial analysis.

Abbreviations: Interquartile range (IQR), Overweight/Obese (OWOB), Normal weight (NW), Prader-Willi Syndrome (PWS), Control group (CON)

Figure 1. Fungal Chao1 alpha diversity

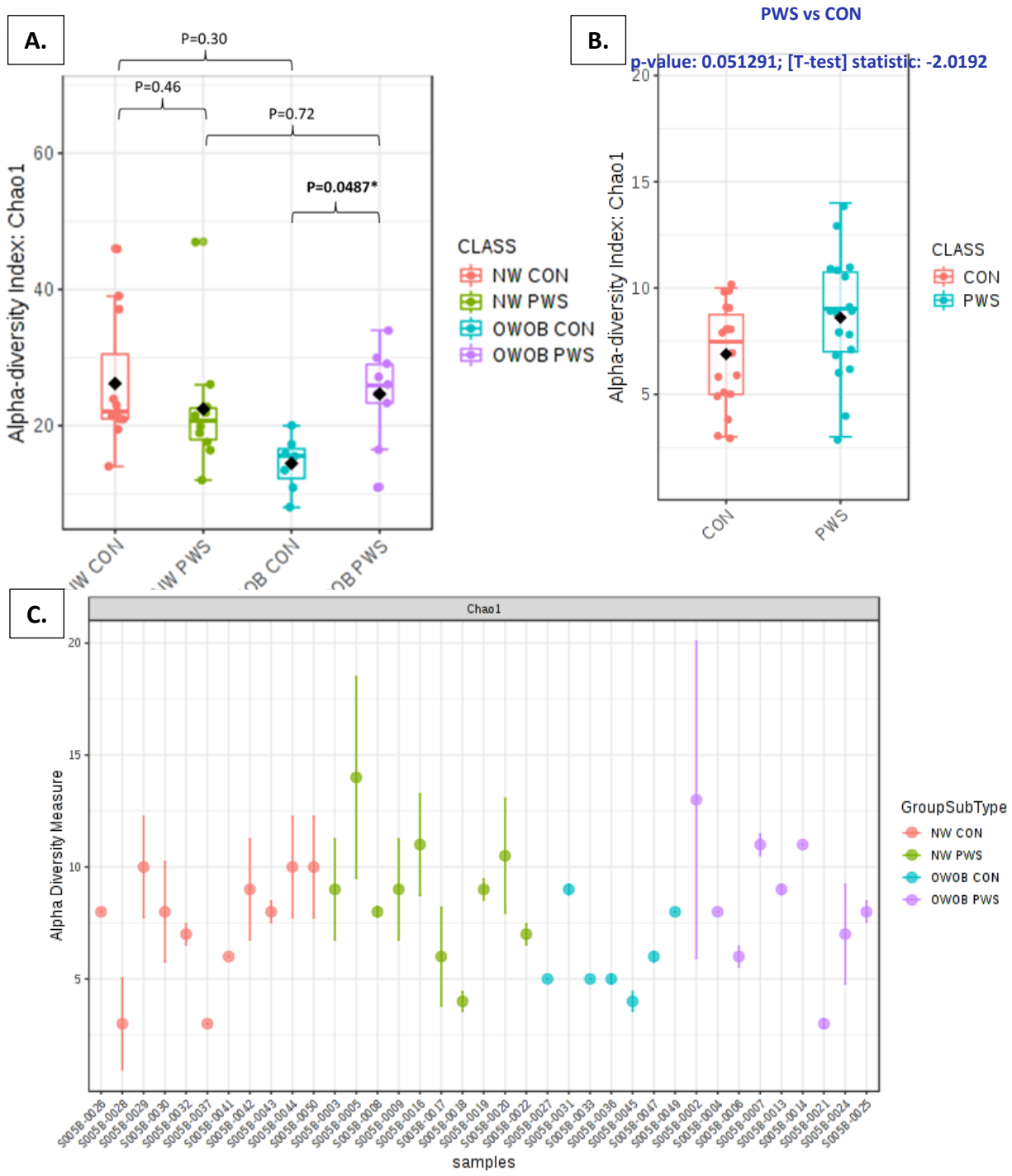


Figure 1: Fungal Chao1 alpha diversity

4-way ANOVA of fungal Chao1 alpha diversity index. Significance of pairwise tests indicated with p-values.

No overall significance between the two groups (PWS vs CON, p>0.05).

The Chao1 richness was higher in the OWOB PWS group than the OWOB CON group (p-value: 0.048733; [T-test] statistic: -2.1777). No other significant differences in chao1 richness was observed. A) Chao1 alpha diversity of 4 subgroups. B) Chao1 alpha diversity of 2 groups. C) Chao1 alpha diversity of individual samples.

Abbreviations: Overweight/Obese (OWOB), Normal weight (NW), Prader-Willi Syndrome (PWS), Control group (CON)

Figure 2. Fungal Shannon alpha diversity

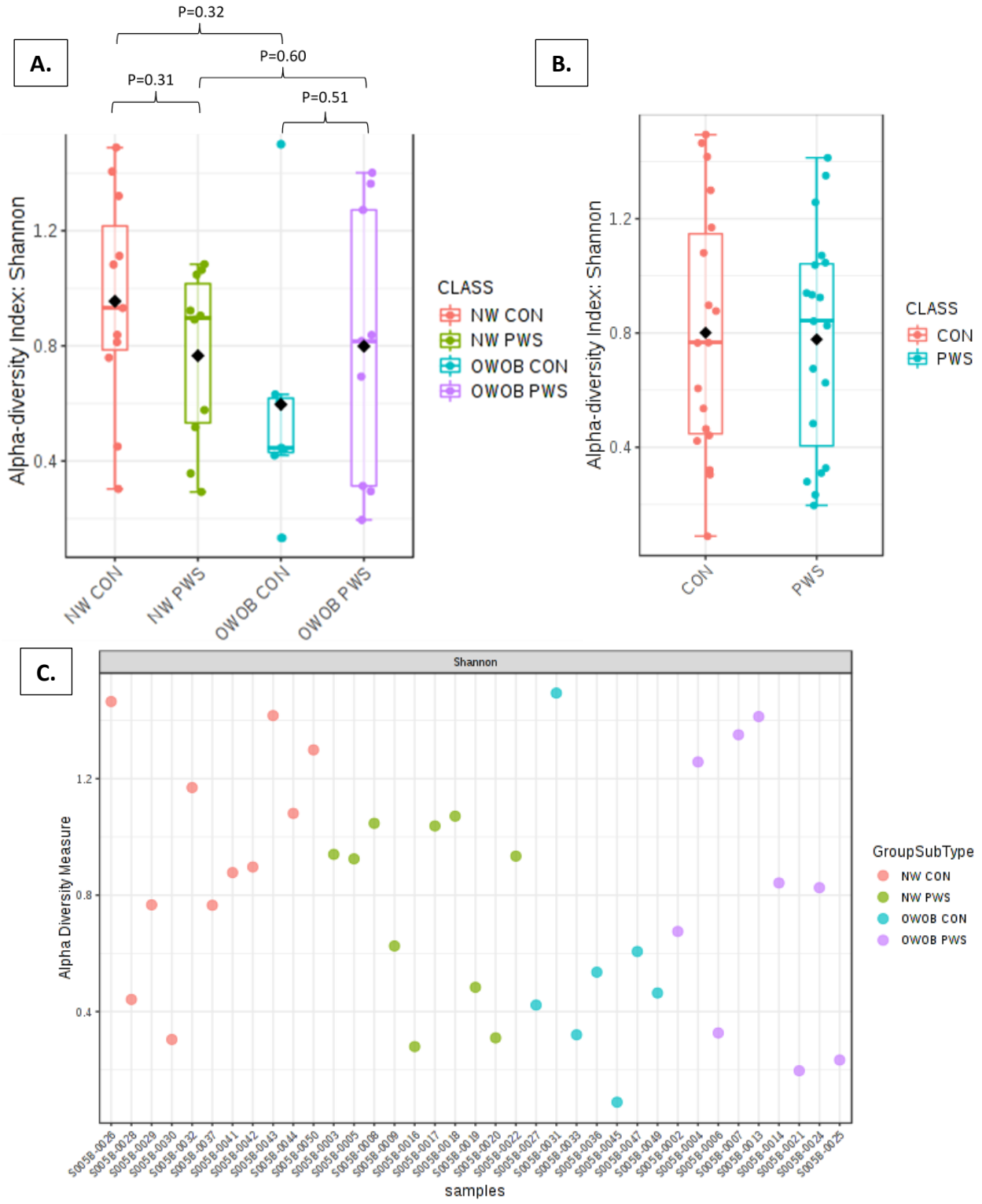


Figure 2: Fungal Shannon alpha diversity
4-way ANOVA of fungal Shannon alpha diversity index. Significance of pairwise tests indicated with p-values.

No significant differences in fungal Shannon alpha diversity index were observed. A) Shannon alpha diversity of 4 subgroups. B) Shannon alpha diversity of 2 groups. C) Shannon alpha diversity of individual samples.

Abbreviations: Overweight/Obese (OWOB), Normal weight (NW), Prader-Willi Syndrome (PWS), Control group (CON)

Figure 3. Fungal Simpson alpha diversity

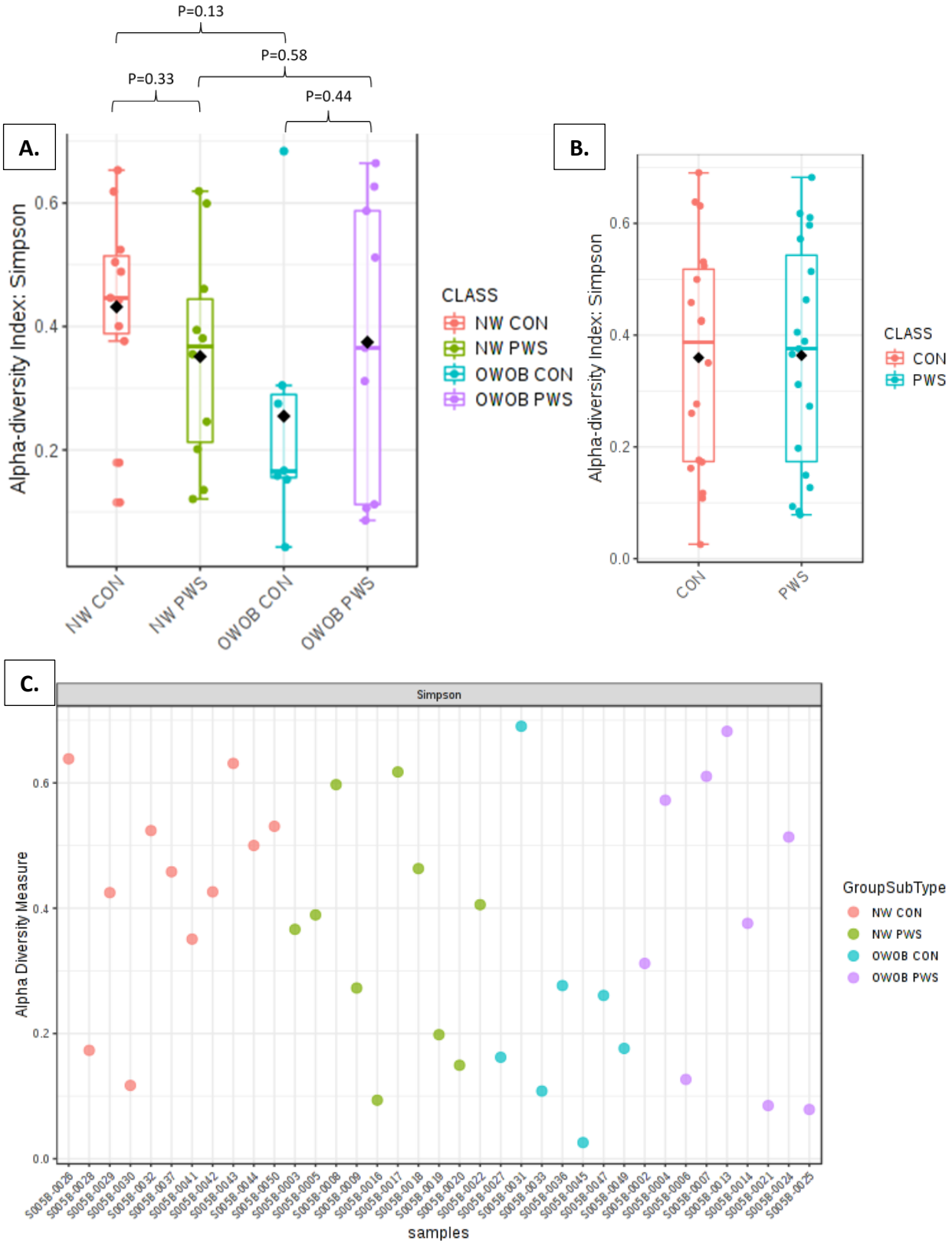
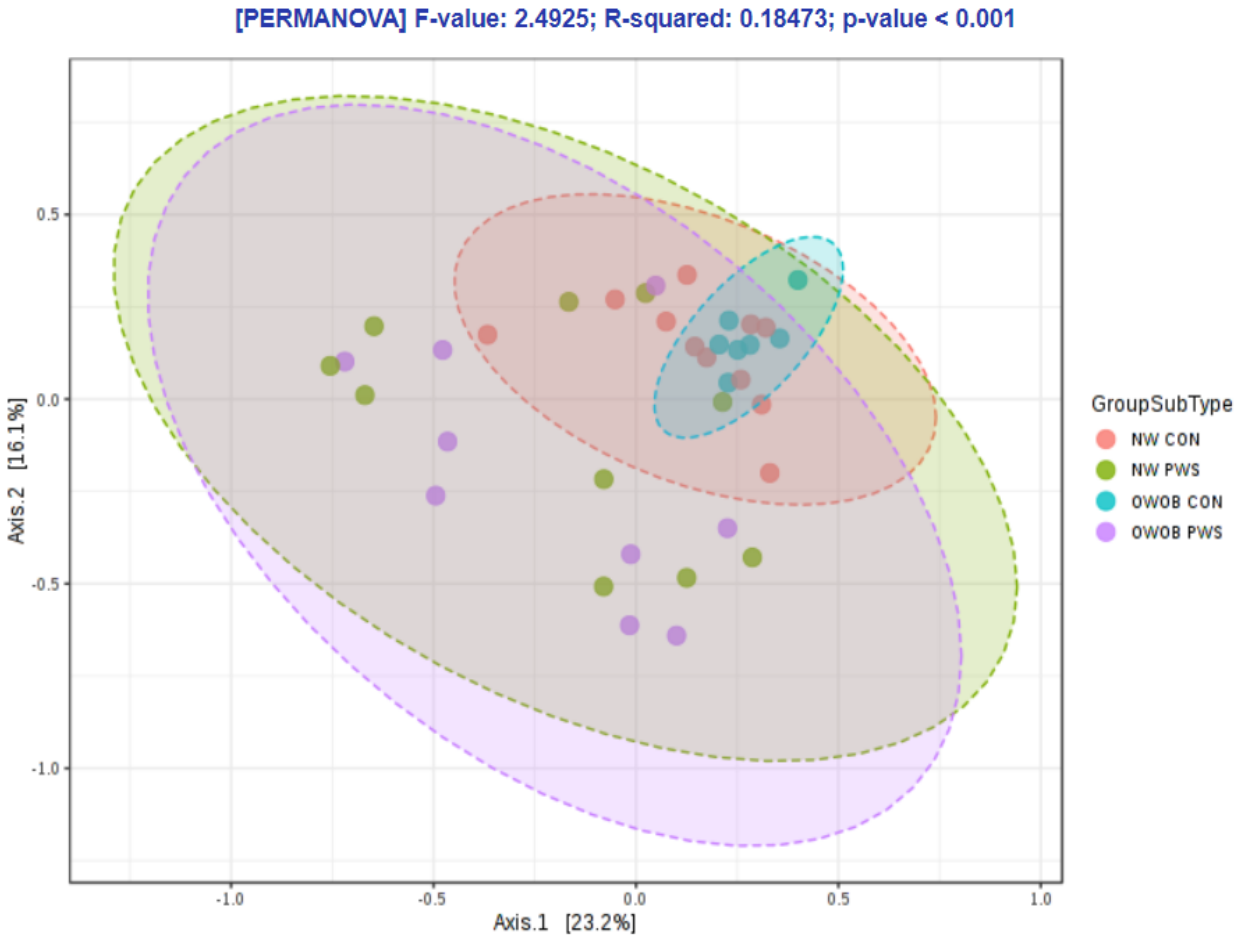


Figure 3: Fungal Simpson alpha diversity
4-way ANOVA of fungal Shannon alpha diversity index. Significance of pairwise tests indicated with p-values.

No significant differences in fungal Simpson alpha diversity index were observed. A) Simpson alpha diversity of 4 subgroups. B) Simpson alpha diversity of 2 groups. C) Simpson alpha diversity of individual samples.

Abbreviations: Overweight/Obese (OWOB), Normal weight (NW), Prader-Willi Syndrome (PWS), Control group (CON)

Figure 4. Summary of Fungal beta diversity:



PWS vs CON:	**[PERMANOVA] F-value: 4.2049; R-squared: 0.10725; p-value<0.001 ***
NW PWS vs NW CON:	**[PERMANOVA] F-value: 2.267; R-squared: 0.1066; p-value < 0.022 ***
OWOB PWS vs OWOB CON:	**[PERMANOVA] F-value: 4.0703; R-squared: 0.22525; p-value < 0.001 ***
NW PWS vs OWOB PWS:	[PERMANOVA] F-value: 0.7229; R-squared: 0.04789; p-value < 0.649
NW CON vs OWOB CON:	[PERMANOVA] F-value: 0.70381; R-squared: 0.042135; p-value < 0.671

Figure 4. Summary of Fungal beta diversity:

2D PCoA plot using bray-curtis distance (4 subgroups)

Each point displayed on PCoA plots was colour coded based on sample subgroup and the explained variances are shown in brackets. Significance of each pairwise subgroup analysis is listed in the table under the PCoA. Differences were found between the two-group analysis (PWS vs CON) and the two-weight adjusted group analysis (NW PWS vs NW CON and OWOB PWS vs OWOB CON). Overall statistical significance of the 4 subgroups at the feature level was found out using Permutational ANOVA (PERMANOVA), with an F-value: 2.4925; R-squared: 0.18473; p-value < 0.001. The first PCoA dimension (PCoA Axis 1) explained 23.2% of variation in the data. The second PCoA dimension (PCoA Axis 2) explained 16.1% of variation in the data.

Abbreviations: Overweight/Obese (OWOB), Normal weight (NW), Prader-Willi Syndrome (PWS), Control group (CON), PCoA- Principle Coordinate Analysis, PERMANOVA- Permutational analysis of variance

Figure 5. 2D PCoA plot using bray-curtis distance for PWS vs CON groups.

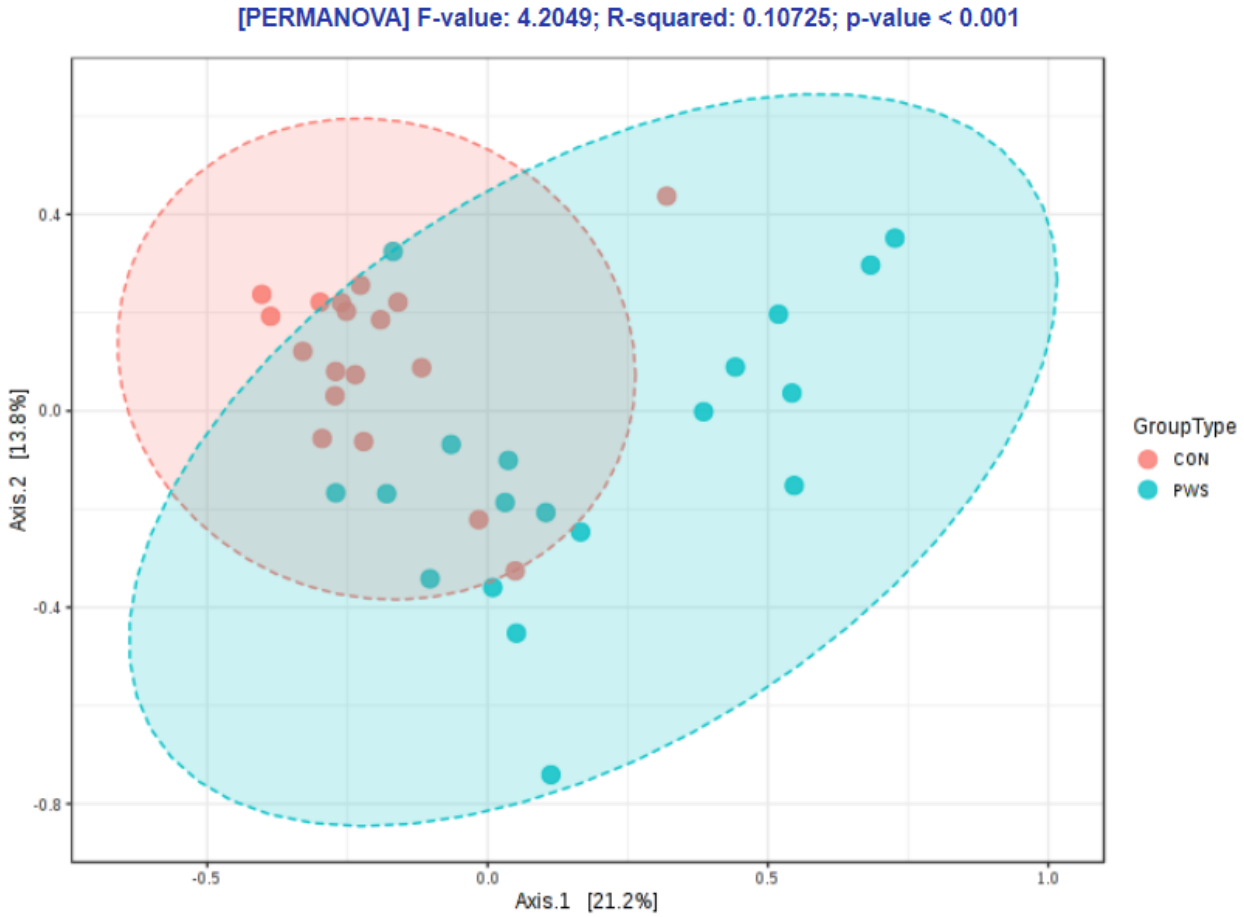
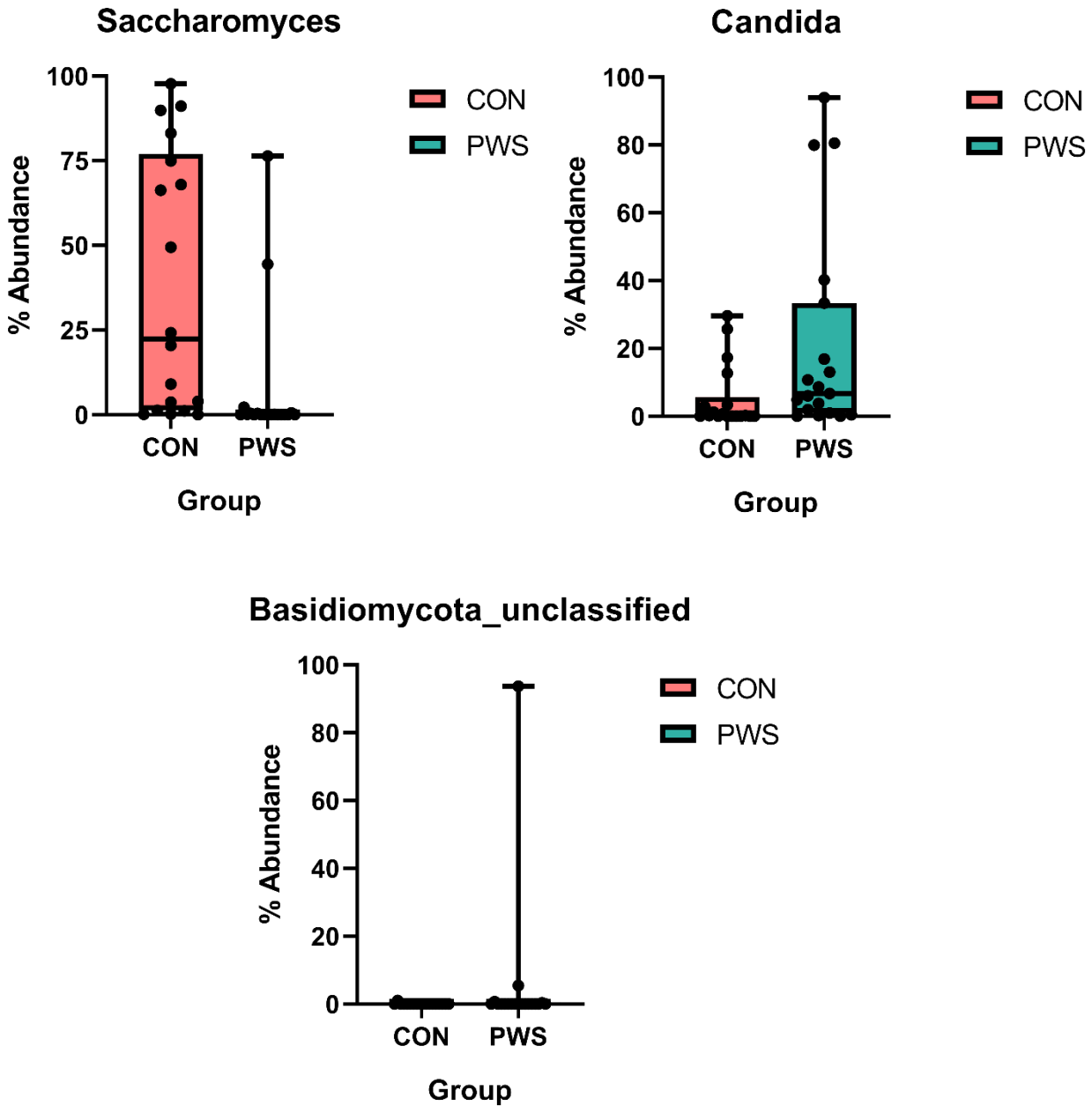


Figure 5: 2D PCoA plot using bray-curtis distance for PWS vs CON groups.

Each point displayed on PCoA plots was colour coded based on sample group (PWS vs CON) and the explained variances are shown in brackets. Statistical significance at the feature level was found out using Permutational ANOVA PERMANOVA, with an F-value: 4.2049; R-squared: 0.10725; p-value<0. 001. The first PCoA dimension (PCoA Axis 1) explained 21.2% of variation in the data. The second PCoA dimension (PCoA Axis 2) explained 13.8% of variation in the data.

Abbreviations: Prader-Willi Syndrome (PWS), Control group (CON), PCoA- Principle Coordinate Analysis, PERMANOVA- Permutational analysis of variance

Figure 6. Differential features identified by DESeq2 analysis at Genus level based on PWS samples vs CON samples as the grouping variable/experimental factor.



Genus	log2FC	lfcSE	Pvalues	FDR
Saccharomyces	**-5.1935**	**0.96588**	**7.58E-08**	**6.06E-07 ***
Candida	**6.0249**	**1.169**	**2.55E-07**	**1.02E-06 ***
Basidiomycota_unclassified	**6.6992**	**1.8785**	**0.000362**	**0.000966 ***
Cyberlindnera	-2.216	1.6009	0.16628	0.32242
Ascomycota_unclassified	-1.633	1.2785	0.20151	0.32242
Alternaria	0.55258	2.9326	0.85054	0.95672
Cladosporium	0.34449	1.8851	0.855	0.95672
Fungi_unclassified	0.042957	0.79153	0.95672	0.95672

Figure 6: Differential features identified by DESeq2 analysis at Genus level based on PWS samples vs CON samples as the grouping variable/experimental factor.

Features are considered to be significant based on their adjusted p-value (adj. p-value cutoff = 0.05, significance indicated with *). Saccharomyces, Candida, and unclassified Basidiomycota were identified as differential features (P<0.001), with larger abundances of Saccharomyces in the Control group and higher abundances of Candida and unclassified Basidiomycota in the PWS group.

Abbreviations: Prader-Willi Syndrome (PWS), Control group (CON), False discovery rate (FDR), log2FoldChange (log2FC), log fold change Standard Error (lfcSE)

Figure 7. 2D PCoA plot using bray-curtis distance (NW CON vs NW PWS).

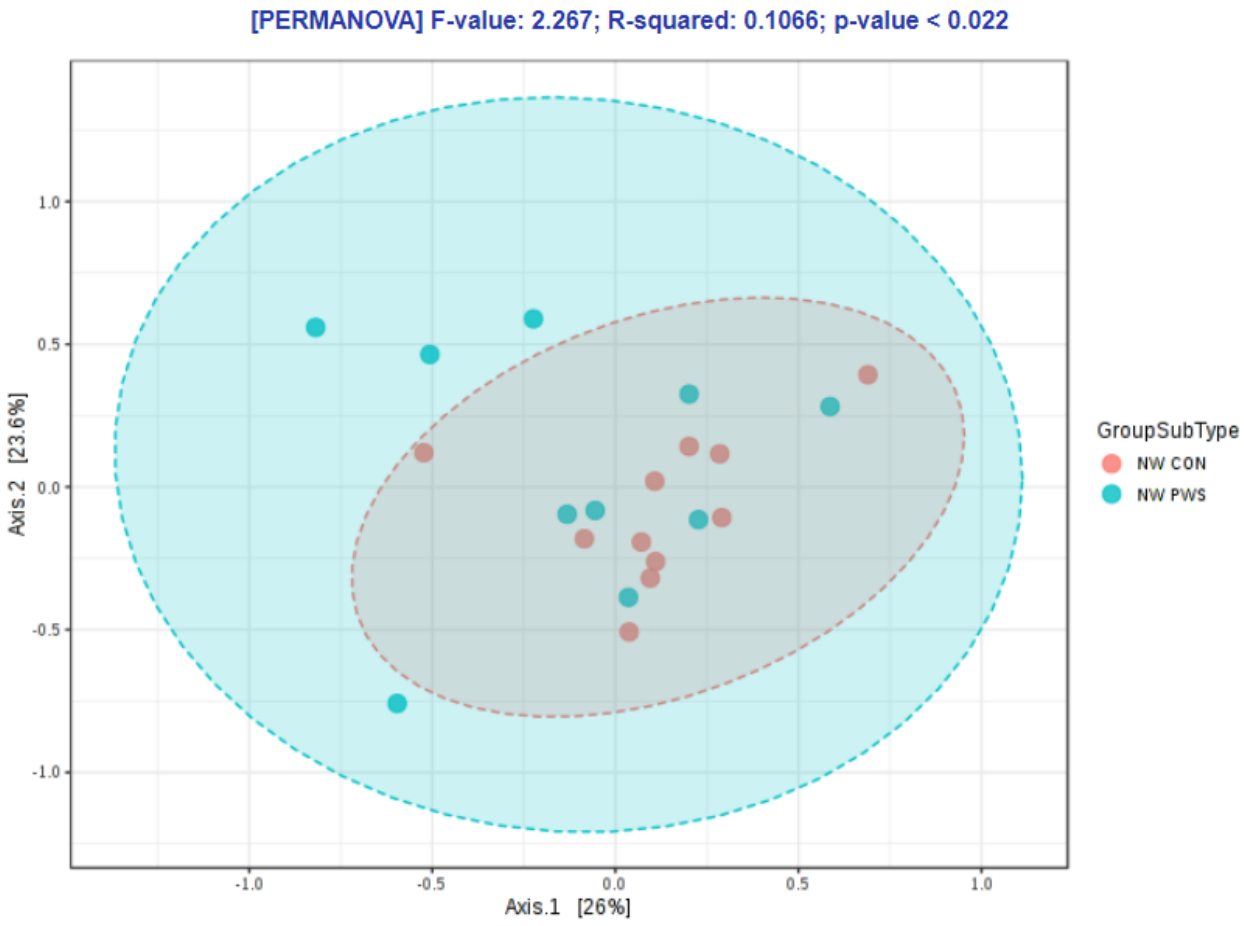


Figure 7: 2D PCoA plot using bray-curtis distance (NW CON vs NW PWS).

Each point displayed on PCoA plots was colored based on sample group and the explained variances are shown in brackets. Statistical significance at the feature level was found out using Permutational ANOVA (PERMANOVA), with an F-value: 2.267; R-squared: 0.1066; p-value < 0.022. The first PCoA dimension (PCoA Axis 1) explained 26% of variation in the data. The second PCoA dimension (PCoA Axis 2) explained 23.6% of variation in the data.

Abbreviations: Prader-Willi Syndrome (PWS), Control group (CON), Normal weight (NW), PCoA-Principle Coordinate Analysis, PERMANOVA- Permutational analysis of variance

Table 3. Differential features identified by DESeq2 analysis at Genus level based on NW PWS samples vs NW CON samples as the grouping variable/experimental factor.

Genus	log2FC	lfcSE	Pvalues	FDR
Candida	4.5516	1.8313	0.012942	0.10353
Ascomycota_unclassified	3.262	2.226	0.14281	0.55219
Cyberlindnera	-3.3304	2.6397	0.20707	0.55219
Saccharomyces	-1.1851	1.4699	0.42012	0.77146
Rhodotorula	-1.7011	2.9271	0.56113	0.77146
Fungi_unclassified	-0.53205	1.1067	0.63068	0.77146
Cladosporium	-1.0533	2.9217	0.71845	0.77146
Alternaria	-0.85501	2.9435	0.77146	0.77146

Table 3: Differential features identified by DESeq2 analysis at Genus level based on NW PWS samples vs NW CON samples as the grouping variable/experimental factor.

Features are considered to be significant based on their adjusted p-value (adj. p-value cutoff = 0.05, significance indicated with *). No significantly differential fungal genus identified between groups.

Abbreviations: Prader-Willi Syndrome (PWS), Control group (CON), Normal weight (NW), False discovery rate (FDR), log2FoldChange (log2FC), log fold change Standard Error (lfcSE)

Figure 8. Fungal alpha diversity index Chao1 between OWOB PWS and OWOB CON groups.

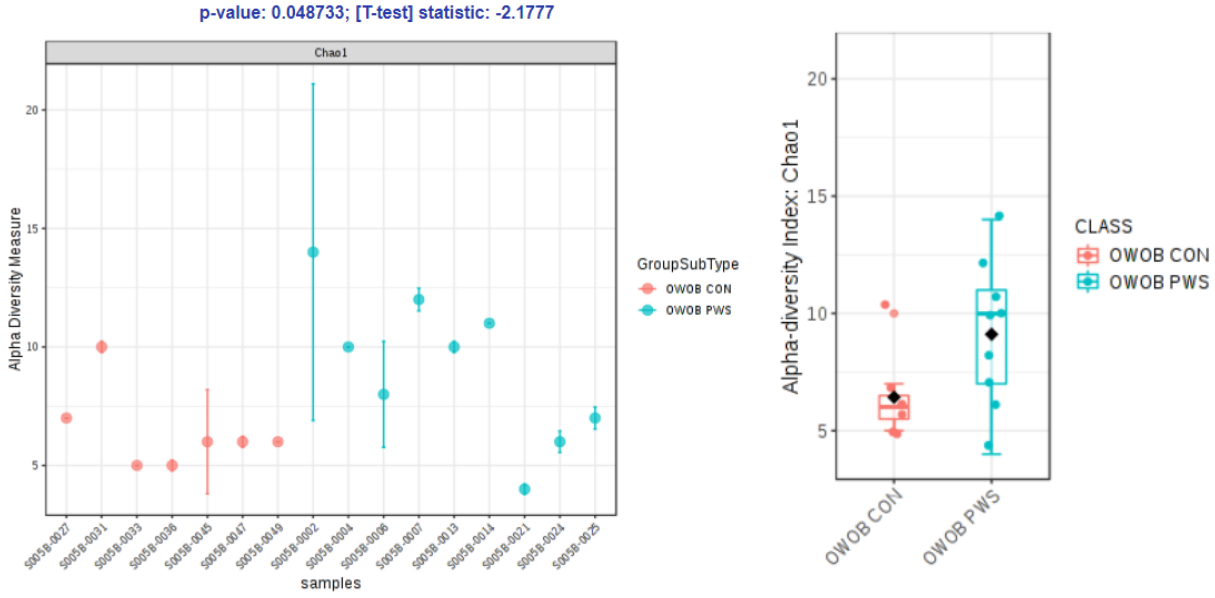


Figure 8: Fungal alpha diversity index Chao1 between OWOB PWS and OWOB CON groups.

A) Sample level chao1 alpha diversity scores and B) group level comparisons between the PWS and CON groups. Higher chao1 diversity was observed in the PWS group compared to the CON group (p=0.04) using parametric tests (t-test/ANOVA) on the filtered and normalized data at OTU level (p-value: 0.048733; [T-test] statistic: -2.1777).

Abbreviations: Prader-Willi Syndrome (PWS), Control group (CON), Overweight/Obese (OWOB)

Figure 9. 2D PCoA plot using bray-curtis distance (OWOB CON vs OWOB PWS).

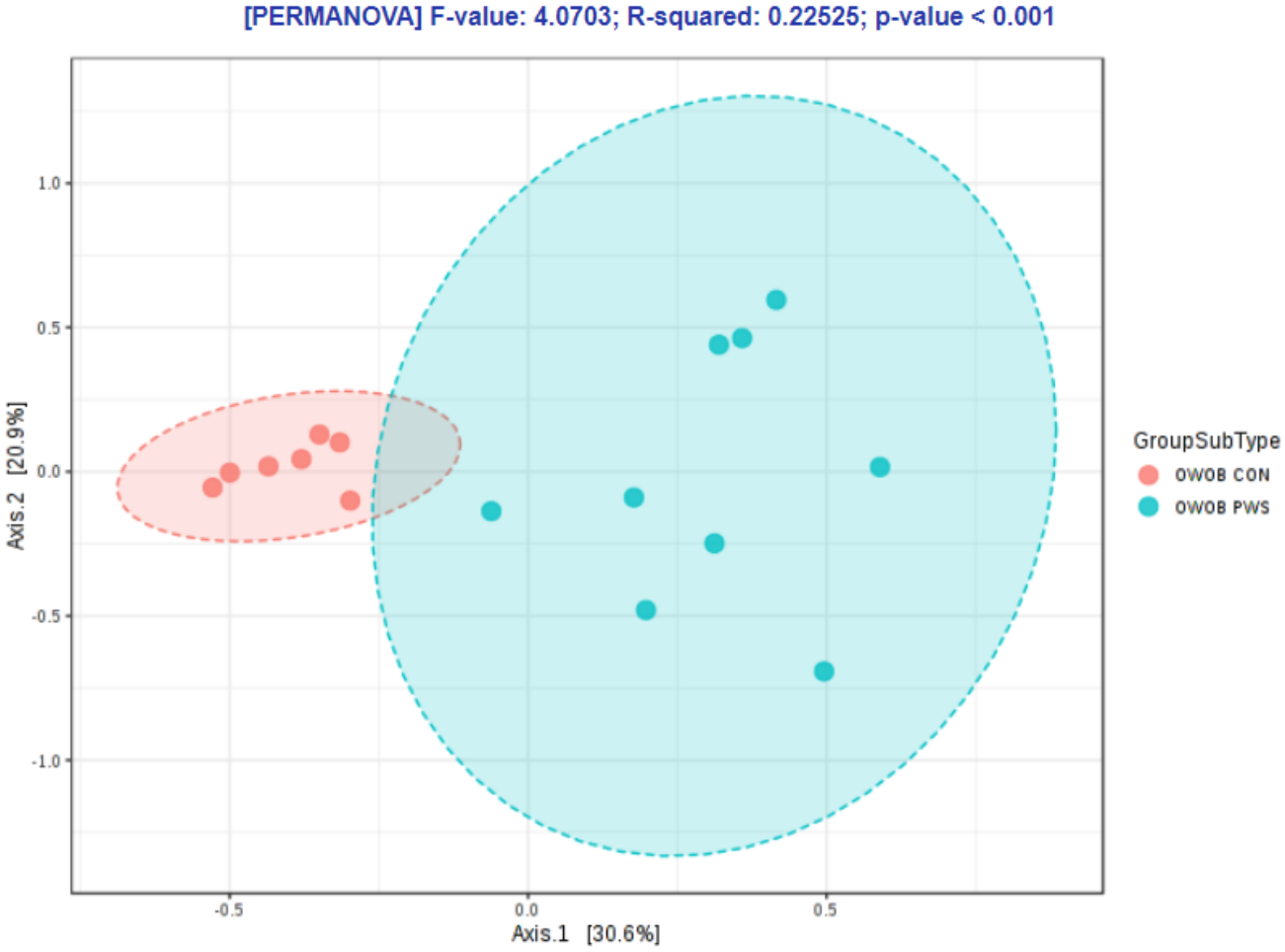
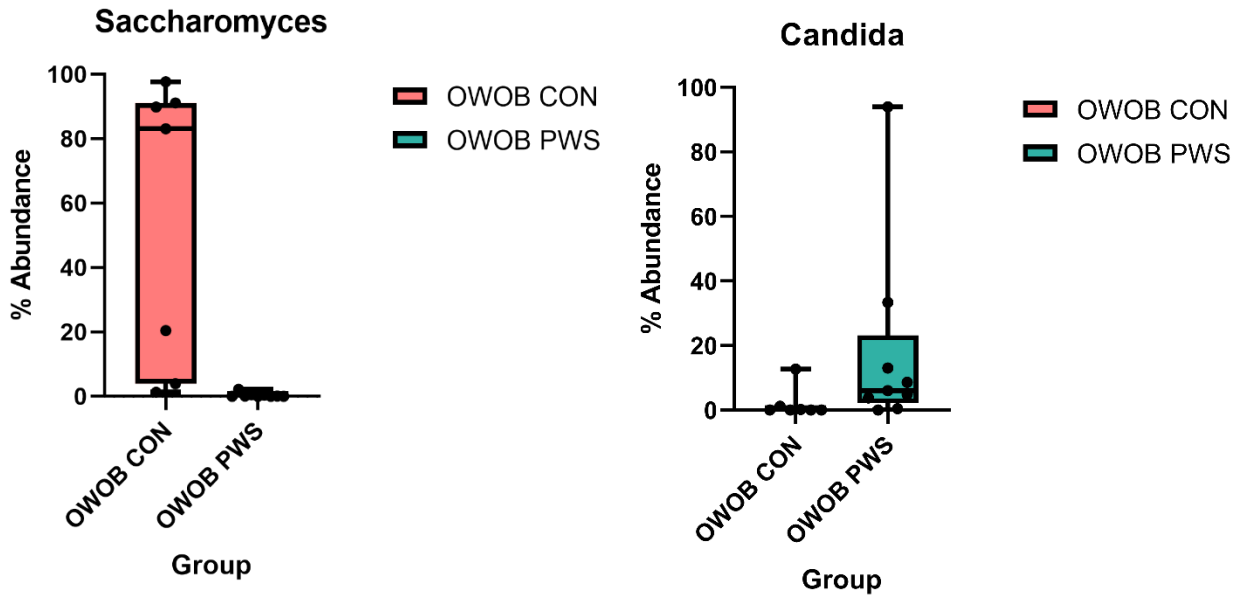


Figure 9: 2D PCoA plot using bray-curtis distance (OWOB CON vs OWOB PWS).

Each point displayed on PCoA plots was colored based on sample group and the explained variances are shown in brackets. Statistical significance at the feature level was found out using Permutational ANOVA (PERMANOVA), with an F-value: 4.0703; R-squared: 0.22525; p-value < 0.001. A) Feature level: The first PCoA dimension (PCoA Axis 1) explained 30.6% of variation in the data. The second PCoA dimension (PCoA Axis 2) explained 20.9% of variation

Abbreviations: Overweight/Obese (OWOB), Prader-Willi Syndrome (PWS), Control group (CON), PCoA- Principle Coordinate Analysis, PERMANOVA- Permutational analysis of variance

Figure 10. Differential features identified by DESeq2 analysis at Genus level based on OWOB PWS samples vs OWOB CON samples as the grouping variable/experimental factor.



Genus	log2FC	lfcSE	Pvalues	FDR
Saccharomyces	**-4.4034**	**1.3096**	**0.000772**	**0.00309 ***
Candida	**4.9947**	**1.5872**	**0.001651**	**0.003302 ***
Fungi_unclassified	1.7538	1.5359	0.2535	0.338
Basidiomycota_unclassified	2.4069	2.5252	0.34052	0.34052

Figure 10: Differential features identified by DESeq2 analysis at Genus level based on OWOB PWS samples vs OWOB CON samples as the grouping variable/experimental factor.

Features are considered to be significant based on their adjusted p-value (adj. p-value cutoff = 0.05, significance indicated with *). Saccharomyces and Candida were identified as differential features (P=0.003), with larger abundances of Saccharomyces in the OWOB CON group and higher abundances of Candida in the OWOB PWS group.

Abbreviations: Prader-Willi Syndrome (PWS), Control group (CON), Overweight/Obese (OWOB), False discovery rate (FDR), log2FoldChange (log2FC), log fold change Standard Error (lfcSE)

Figure 11. 2D PCoA plot using bray-curtis distance for within grouptype analysis

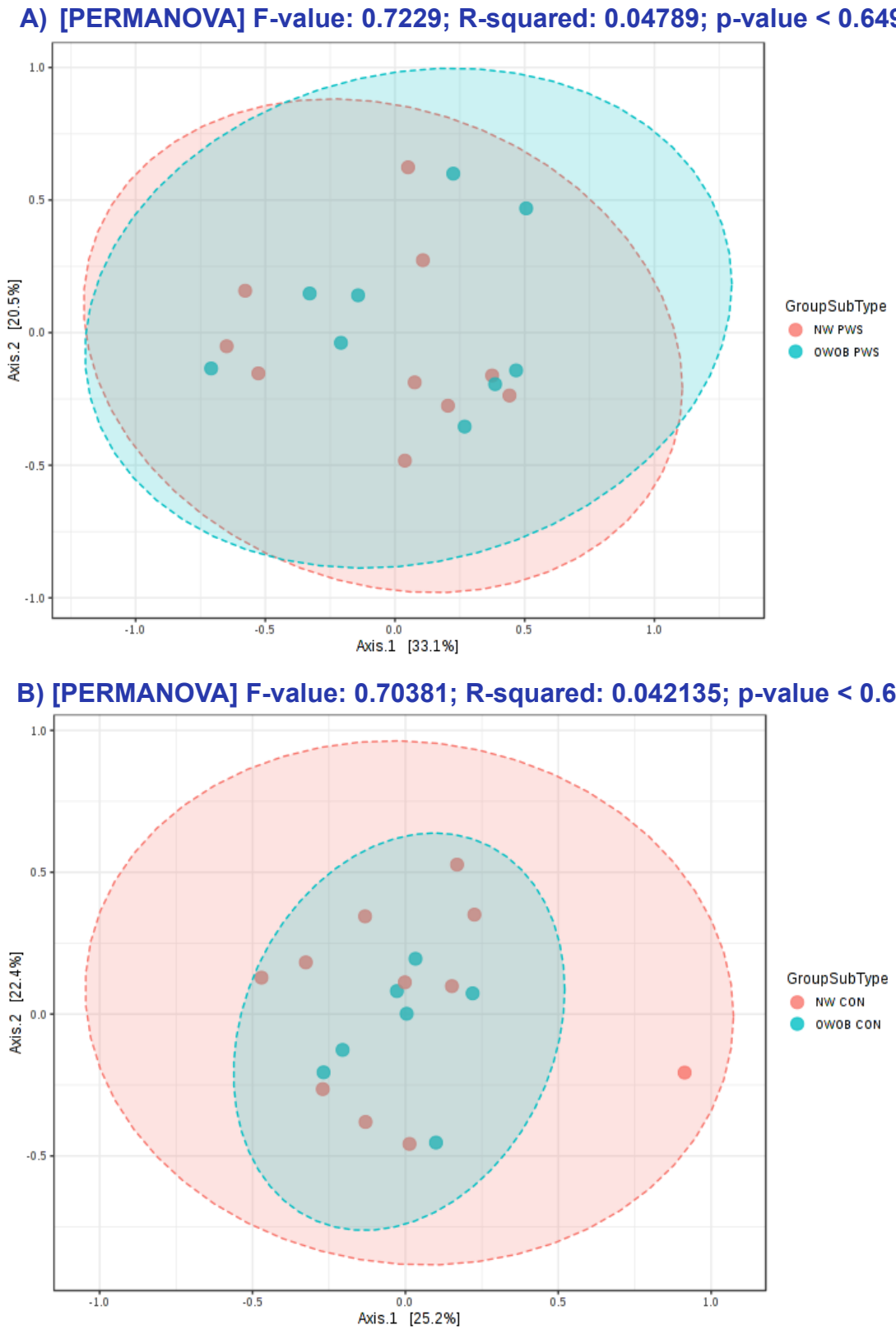
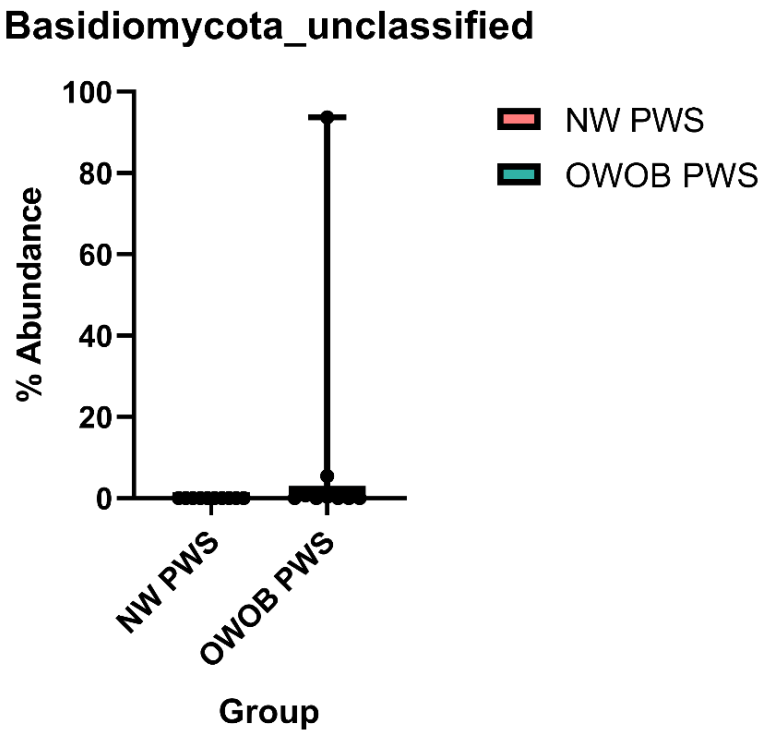


Figure 11. 2D PCoA plot using bray-curtis distance for within grouptype analysis

Each point displayed on PCoA plots was colored based on sample group and the explained variances are shown in brackets. No significant differences were found for the bacterial communities for either the A) NW and OWOB PWS subgroups or B) NW and OWOB CON subgroups.

Abbreviations: Overweight/Obese (OWOB), Normal weight (NW), Prader-Willi Syndrome (PWS), Control group (CON), PCoA- Principle Coordinate Analysis, PERMANOVA- Permutational analysis of variance

Figure 12. Differential features identified by DESeq2 analysis at Genus level based on NW PWS vs OWOB PWS samples as the grouping variable/experimental factor.



Genus	log2FC	lfcSE	Pvalues	FDR
Basidiomycota_unclassified	**6.3788**	**2.1518**	**0.003032**	**0.03032 ***
Ascomycota_unclassified	-5.433	2.4315	0.025457	0.12728
Clavispora	2.2341	2.8068	0.42607	0.84667
Saccharomyces	-1.4405	1.9469	0.45937	0.84667
Cyberlindnera	-1.1816	2.2398	0.59782	0.84667
Fungi_unclassified	0.6436	1.2699	0.61228	0.84667
Mrakia	-1.1781	2.3749	0.61986	0.84667
Alternaria	-1.1782	2.8315	0.67733	0.84667
Cladosporium	-0.66642	2.5983	0.79758	0.8862
Candida	-0.07855	1.4791	0.95764	0.95764

Figure 12: Differential features identified by DESeq2 analysis at Genus level based on NW PWS vs OWOB PWS samples as the grouping variable/experimental factor.

Features are considered to be significant based on their adjusted p-value (adj. p-value cutoff = 0.05, significance indicated with *). Higher abundances of unclassified Basidiomycota were seen in the OWOB PWS group (P=0.03).

Abbreviations: Prader-Willi Syndrome (PWS), Control group (CON), Overweight/Obese (OWOB), Normal weight (NW), False discovery rate (FDR), log2FoldChange (log2FC), log fold change Standard Error (lfcSE)

Table 4. Differential features identified by DESeq2 analysis at Genus level based on NW CON samples vs OWOB CON samples as the grouping variable/experimental factor.

Name	log2FC	lfcSE	Pvalues	FDR
Rhodotorula	-5.1067	3.0691	0.096125	0.5722
Candida	-2.6171	2.1506	0.22364	0.5722
Cyberlindnera	-2.5867	2.6174	0.32302	0.5722
Fungi_unclassified	0.91695	1.1289	0.41666	0.5722
Ascomycota_unclassified	-1.2419	1.7456	0.47683	0.5722
Saccharomyces	0.087262	1.0319	0.93261	0.93261

Table 4: Differential features identified by DESeq2 analysis at Genus level based on NW CON samples vs OWOB CON samples as the grouping variable/experimental factor.

Features are considered to be significant based on their adjusted p-value (adj. p-value cutoff = 0.05, significance indicated with *). No significantly differential fungal genus identified between groups.

Abbreviations: Prader-Willi Syndrome (PWS), Control group (CON), Overweight/Obese (OWOB), Normal weight (NW), False discovery rate (FDR), log2FoldChange (log2FC), log fold change Standard Error (lfcSE)

Figure 13.

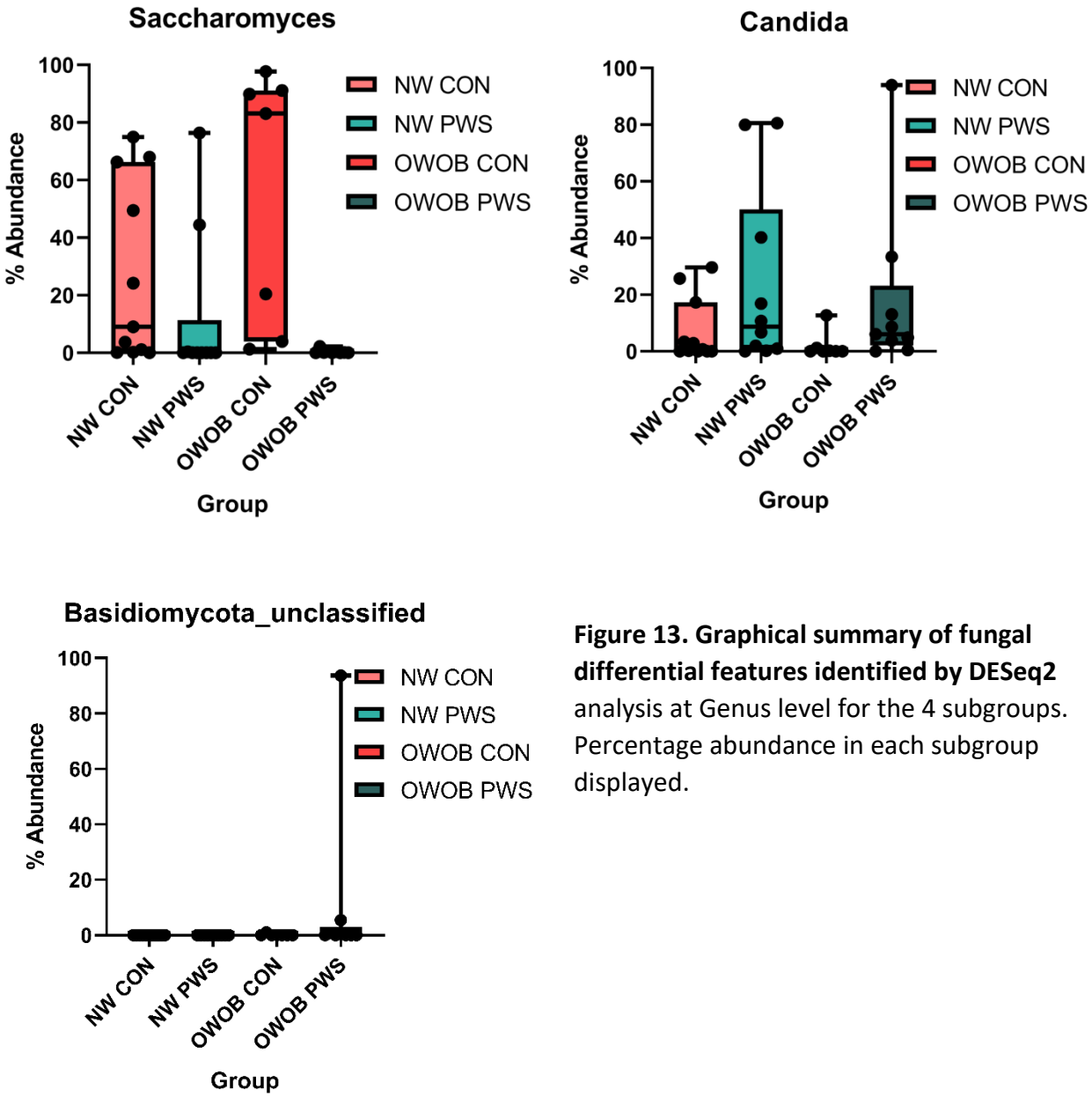


Figure 13. Graphical summary of fungal differential features identified by DESeq2 analysis at Genus level for the 4 subgroups. Percentage abundance in each subgroup displayed.

Figure 14. Bacterial Chao1 alpha diversity

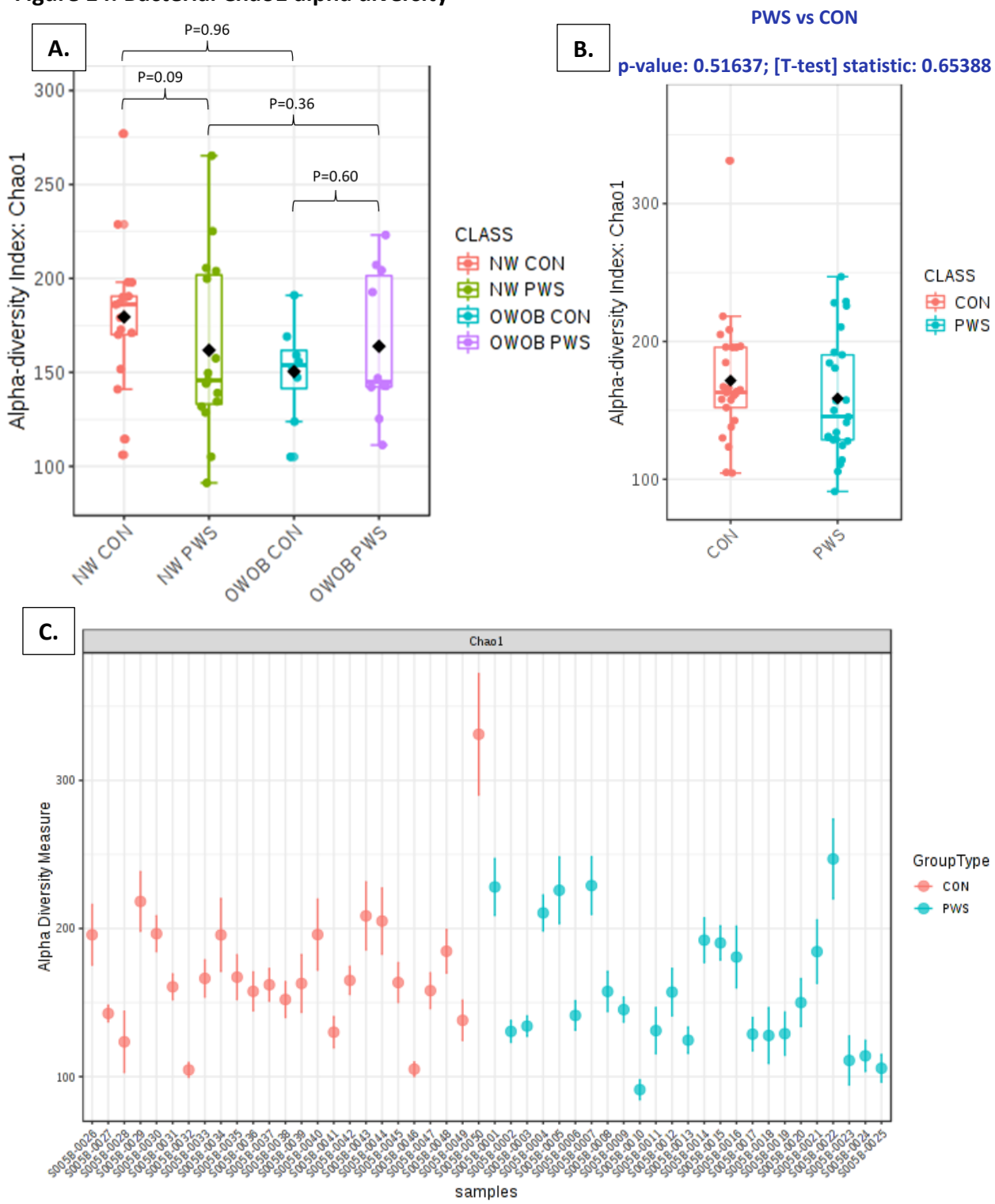


Figure 14: Bacterial Chao1 alpha diversity
4-way ANOVA of bacterial Chao1 alpha diversity index. Significance of pairwise tests indicated with p-values.

No significant differences in chao1 richness were observed. A) Chao1 alpha diversity of 4 subgroups. B) Chao1 alpha diversity of 2 groups. C) Chao1 alpha diversity of individual samples.

Abbreviations: Overweight/Obese (OWOB), Normal weight (NW), Prader-Willi Syndrome (PWS), Control group (CON)

Figure 15. Pairwise Bacterial Shannon alpha diversity

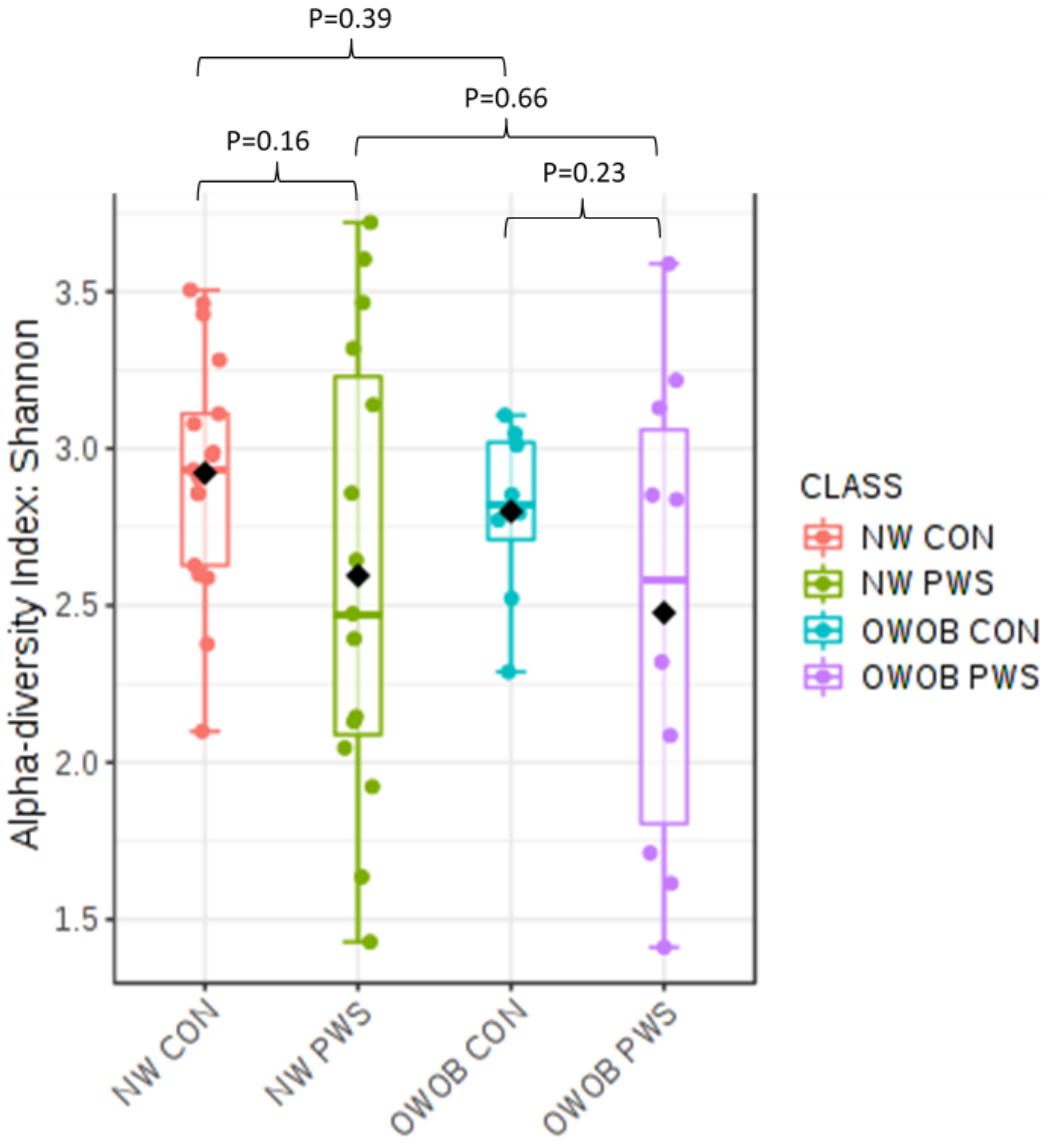


Figure 15: Bacterial Shannon alpha diversity
4-way ANOVA of bacterial Shannon alpha diversity index. Significance of pairwise tests indicated with p-values.

No significant differences in Bacterial Shannon alpha diversity index in pairwise subgroup tests.

Abbreviations: Overweight/Obese (OWOB), Normal weight (NW), Prader-Willi Syndrome (PWS), Control group (CON)

Figure 16. Bacterial Shannon alpha diversity index for PWS vs CON

A.

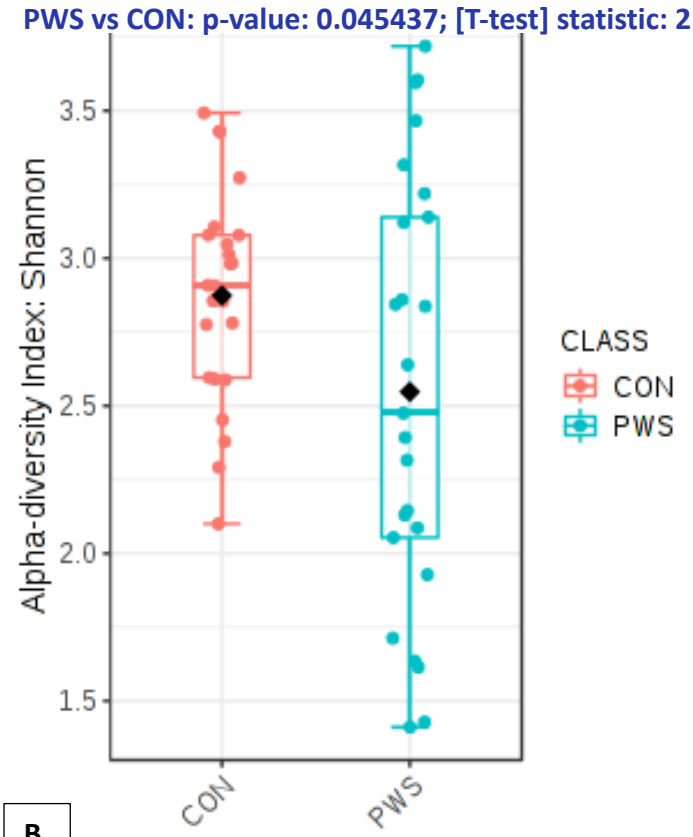


Figure 16: Bacterial Shannon alpha diversity index for PWS vs CON

A) Sample level shannon alpha diversity scores and B) group level comparisons between the PWS and CON groups. Higher Shannon diversity was observed in the CON group compared to the PWS group (p=0.04) using parametric tests.

Abbreviations: Prader-Willi Syndrome (PWS), Control group (CON)

B.

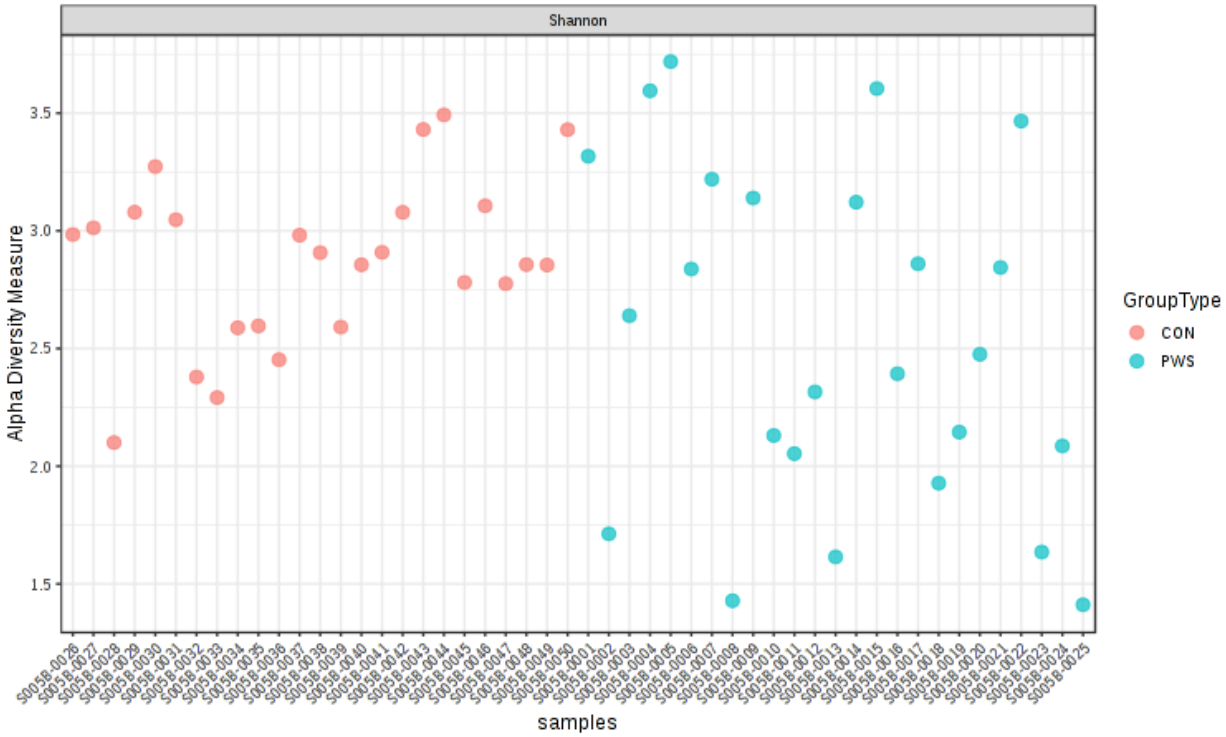


Figure 17. Bacterial Simpson alpha diversity

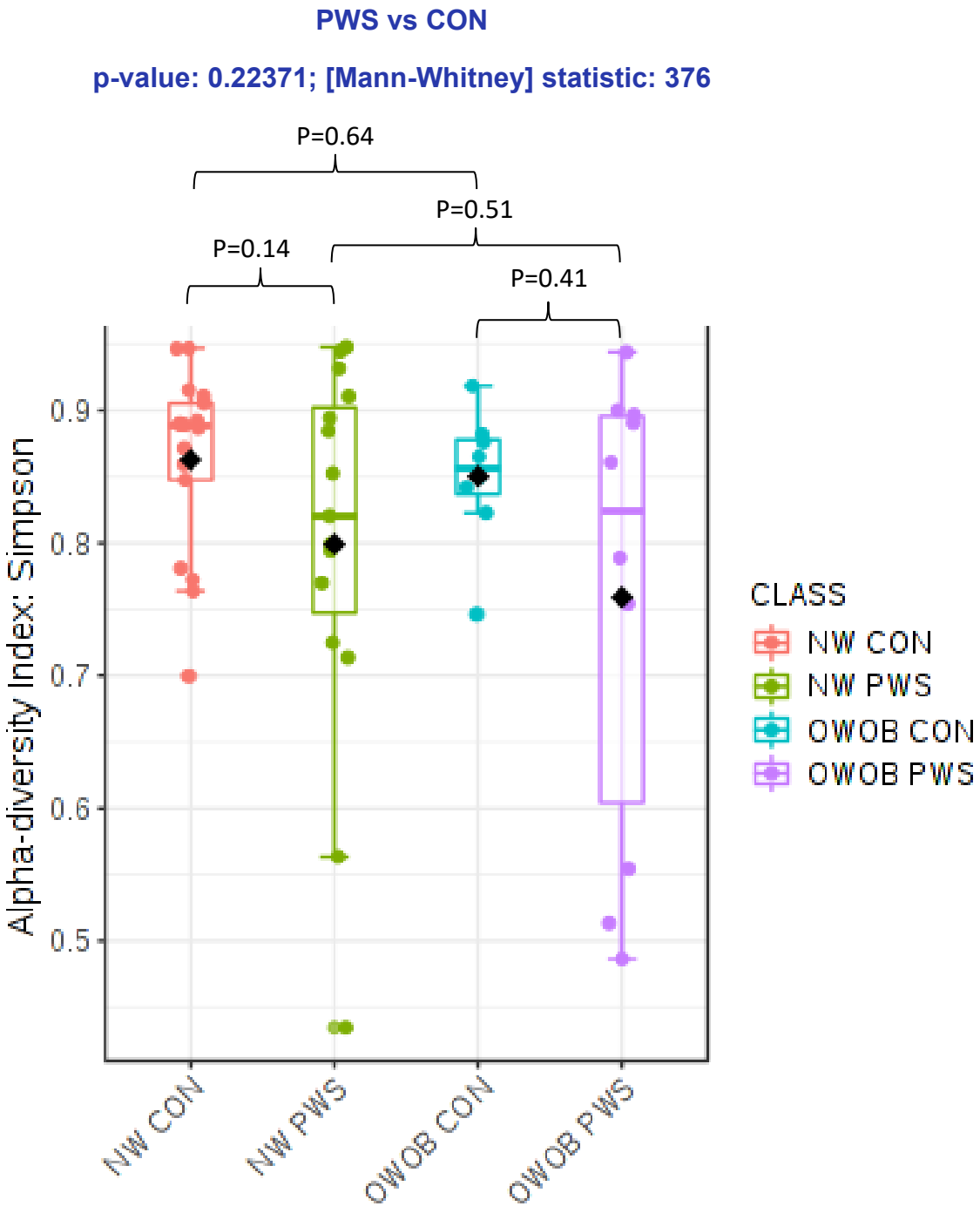
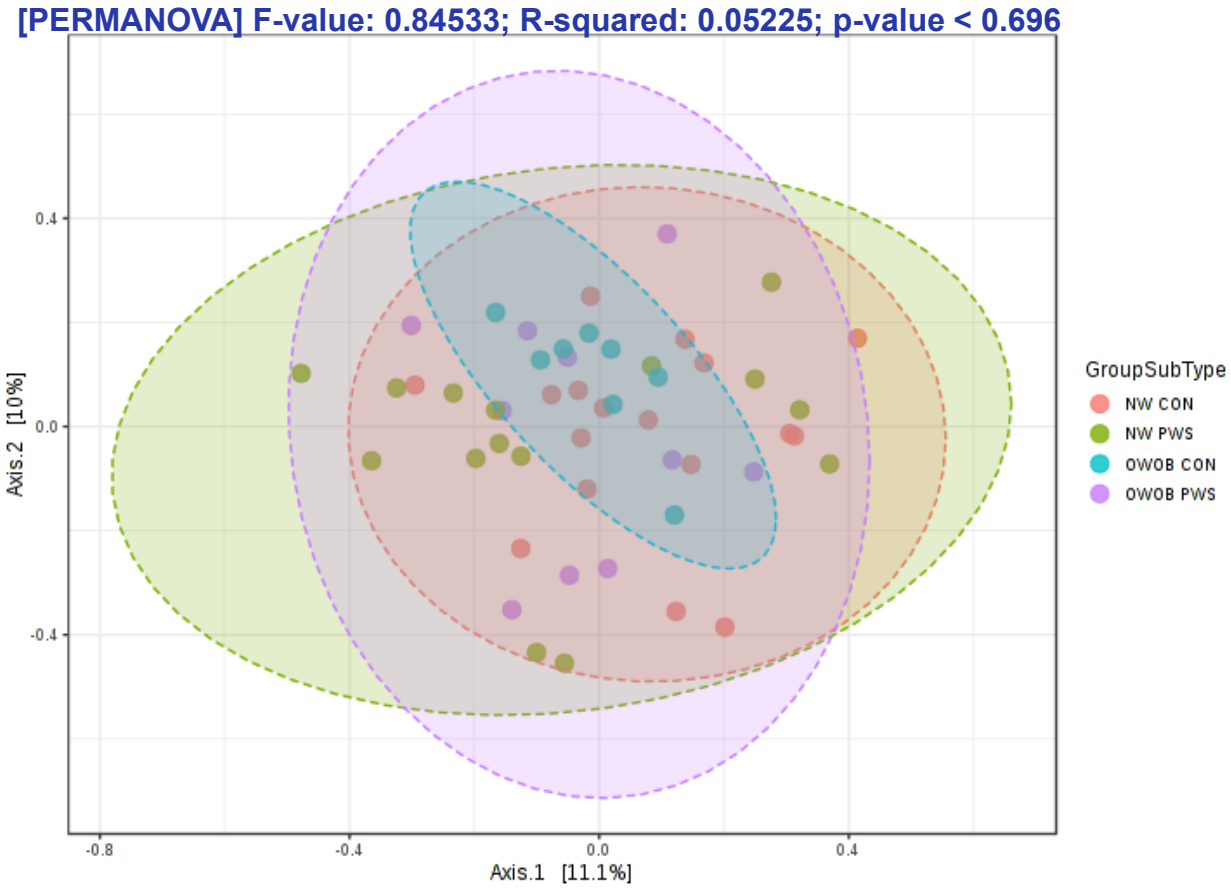


Figure 17: Bacterial Simpson alpha diversity
4-way ANOVA of bacterial Shannon alpha diversity index. Significance of pairwise tests indicated with p-values.

No significant differences in bacterial Simpson alpha diversity index were observed.

Abbreviations: Overweight/Obese (OWOB), Normal weight (NW), Prader-Willi Syndrome (PWS), Control group (CON)

Figure 18. Summary of Bacterial beta diversity:



PWS vs CON:	[PERMANOVA] F-value: 1.0771; R-squared: 0.021947; p-value < 0.359
NW PWS vs NW CON:	[PERMANOVA] F-value: 0.93137; R-squared: 0.030111; p-value < 0.469
OWOB PWS vs OWOB CON:	[PERMANOVA] F-value: 0.83274; R-squared: 0.049471; p-value < 0.516
NW PWS vs OWOB PWS:	[PERMANOVA] F-value: 0.52002; R-squared: 0.02211; p-value < 0.884
NW CON vs OWOB CON:	[PERMANOVA] F-value: 0.92354; R-squared: 0.038604; p-value < 0.533

Figure 18. Summary of Bacterial beta diversity: 2D PCoA plot using bray-curtis distance (4 subgroups)

Each point displayed on PCoA plots was colour coded based on sample subgroup and the explained variances are shown in brackets. Significance of each pairwise subgroup analysis is listed in the table under the PCoA. No statistical significance was found for any measures of beta diversity (P>0.05).

Abbreviations: Overweight/Obese (OWOB), Normal weight (NW), Prader-Willi Syndrome (PWS), Control group (CON), PCoA- Principle Coordinate Analysis, PERMANOVA- Permutational analysis of variance

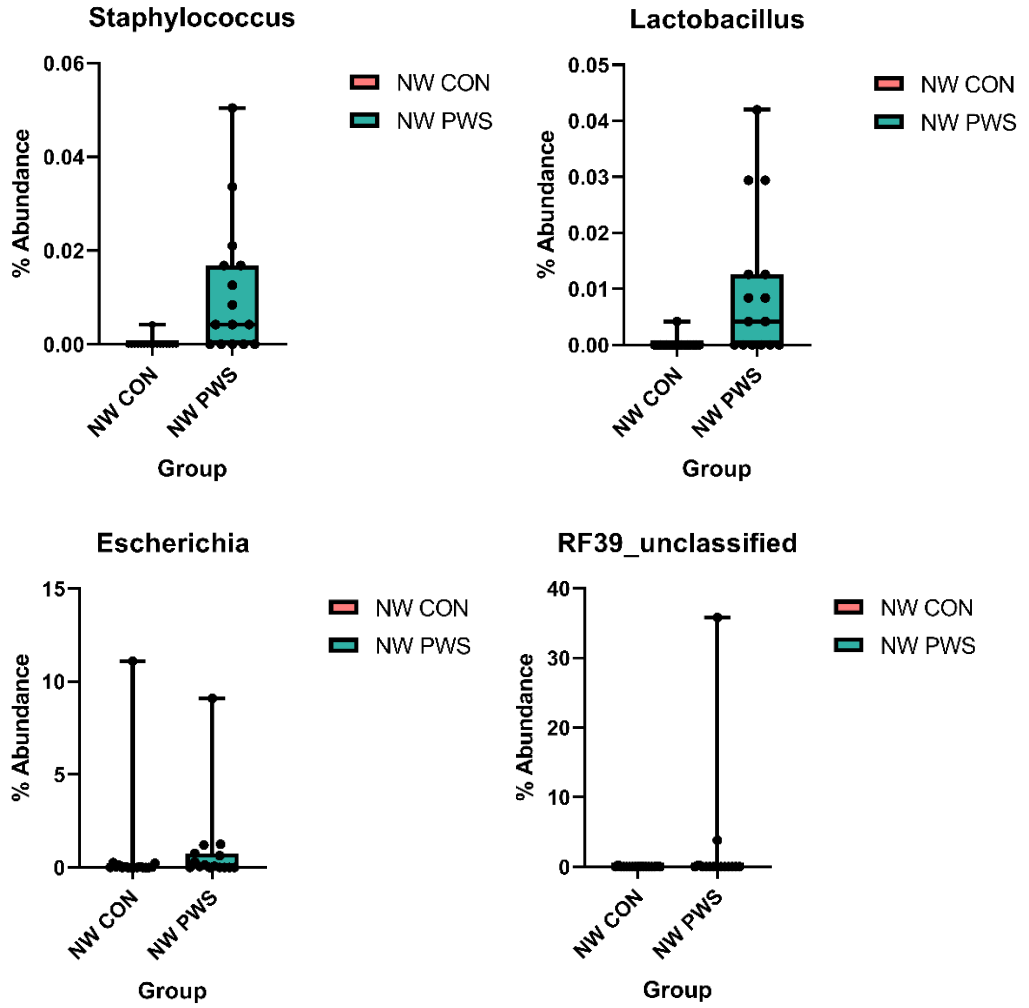
Table 5.

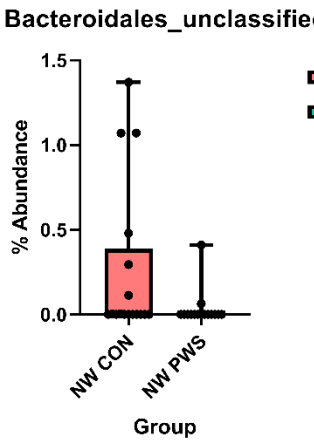
Genus	log2FC	lfcSE	Pvalues	FDR
SMB53	-1.5271	0.5833	0.008844	0.4422
Anaerostipes	-1.6409	0.78553	0.03671	0.5756
Escherichia	1.8146	0.89268	0.042079	0.5756
Oscillospira	-0.61325	0.32035	0.055577	0.5756
Bacteria_unclassified	-1.2547	0.68696	0.067786	0.5756
Akkermansia	2.1464	1.2628	0.089179	0.5756
Prevotella	2.1919	1.331	0.099609	0.5756

Table 5. Differential abundance assessment with DESeq2 at bacterial genus level for 2 group (PWS vs CON) analysis.
Features are considered to be significant based on their adjusted p-value (adj. p-value cutoff = 0.05, significance indicated with *). No significantly differential fungal genus identified between groups.

Abbreviations: Prader-Willi Syndrome (PWS), Control group (CON), False discovery rate (FDR), log2FoldChange (log2FC), log fold change Standard Error (lfcSE)

Figure 19. Bacterial DESeq2 Differential Abundance Analysis NW CON vs NW PWS





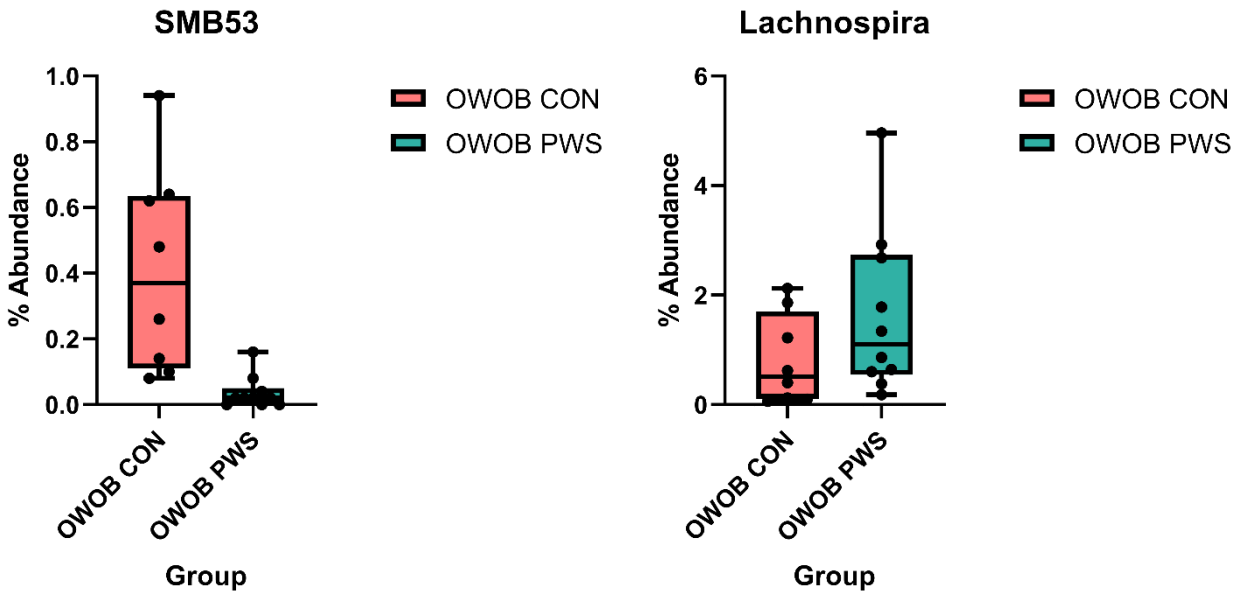
Name	log2FC	lfcSE	Pvalues	FDR
Staphylococcus	**3.6372**	**0.91725**	**7.3296E-5**	**0.0040313**
Lactobacillus	**3.6462**	**1.0425**	**4.6956E-4**	**0.012913**
Escherichia	**2.9845**	**1.0019**	**0.0028943**	**0.042875**
RF39_unclassified	**4.9039**	**1.6753**	**0.0034216**	**0.042875**
Bacteroidales_unclassified	**-5.231**	**1.8123**	**0.0038977**	**0.042875**
Holdemania	1.7646	0.75925	0.020117	0.18441
Anaerostipes	-1.5881	0.72301	0.028057	0.19943
Adlercreutzia	1.7381	0.79604	0.029008	0.19943
Bacteria_unclassified	-1.6674	0.78125	0.032823	0.20059
Lachnobacterium	-3.1867	1.5439	0.039012	0.20367
Oscillospira	-0.82303	0.40222	0.040734	0.20367

Figure 19: Bacterial DESeq2 Differential Abundance Analysis NW CON vs NW PWS

Features are considered to be significant based on their adjusted p-value (adj. p-value cutoff = 0.05, significance indicated with *). Staphylococcus (p=0.004), Lactobacillus (p=0.01), Escherichia (p=0.04) and unclassified RF39 (p=0.04) were all found to be more abundant in the NW PWS groups, whereas an increase in unclassified Bacteroidales (p=0.04) was observed in the NW CON subgroup.

Abbreviations: Prader-Willi Syndrome (PWS), Control group (CON), Normal weight (NW), False discovery rate (FDR), log2FoldChange (log2FC), log fold change Standard Error (lfcSE)

Figure 20. Bacterial DESeq2 Differential Abundance Analysis OWOB CON vs OWOB PWS



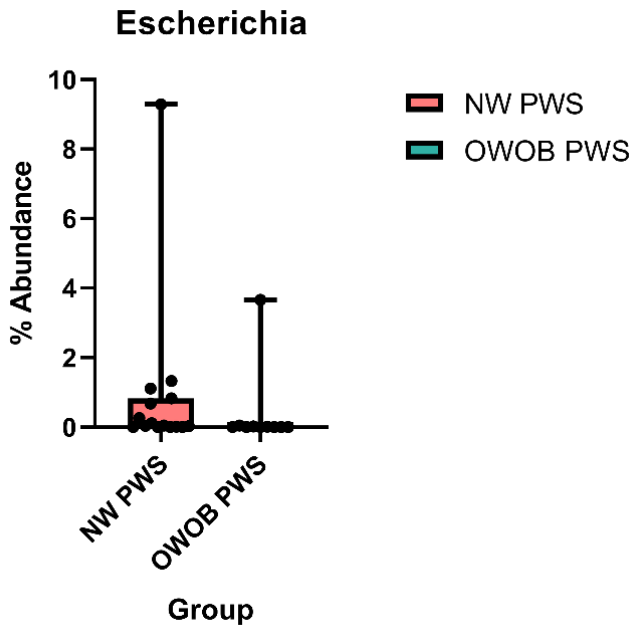
Name	log2FC	lfcSE	Pvalues	FDR
SMB53	**-2.8255**	**0.64105**	**1.0453E-5**	**4.8086E-4** *
Lachnospira	**1.8269**	**0.59452**	**0.0021197**	**0.048754** *
Turicibacter	-3.3086	1.2251	0.0069185	0.10387
Enterobacteriaceae_unclassified	2.3378	0.89541	0.0090326	0.10387
Alistipes	1.577	0.71129	0.026613	0.19483
Bacteria_unclassified	2.2345	1.0189	0.0283	0.19483
Lachnospiraceae_unclassified	0.71645	0.32944	0.029647	0.19483

Figure 20: Bacterial DESeq2 Differential Abundance Analysis OWOB CON vs OWOB PWS

Features are considered to be significant based on their adjusted p-value (adj. p-value cutoff = 0.05, significance indicated with *). SMB53 (P<0.001) was more abundant in the OWOB CON group, whereas Lachnospira (p=0.04) was elevated in the OWOB PWS group.

Abbreviations: Prader-Willi Syndrome (PWS), Control group (CON), Overweight/Obese (OWOB), False discovery rate (FDR), log2FoldChange (log2FC), log fold change Standard Error (lfcSE)

Figure 21. Bacterial DESeq2 Differential Abundance Analysis NW PWS VS OWOB PWS



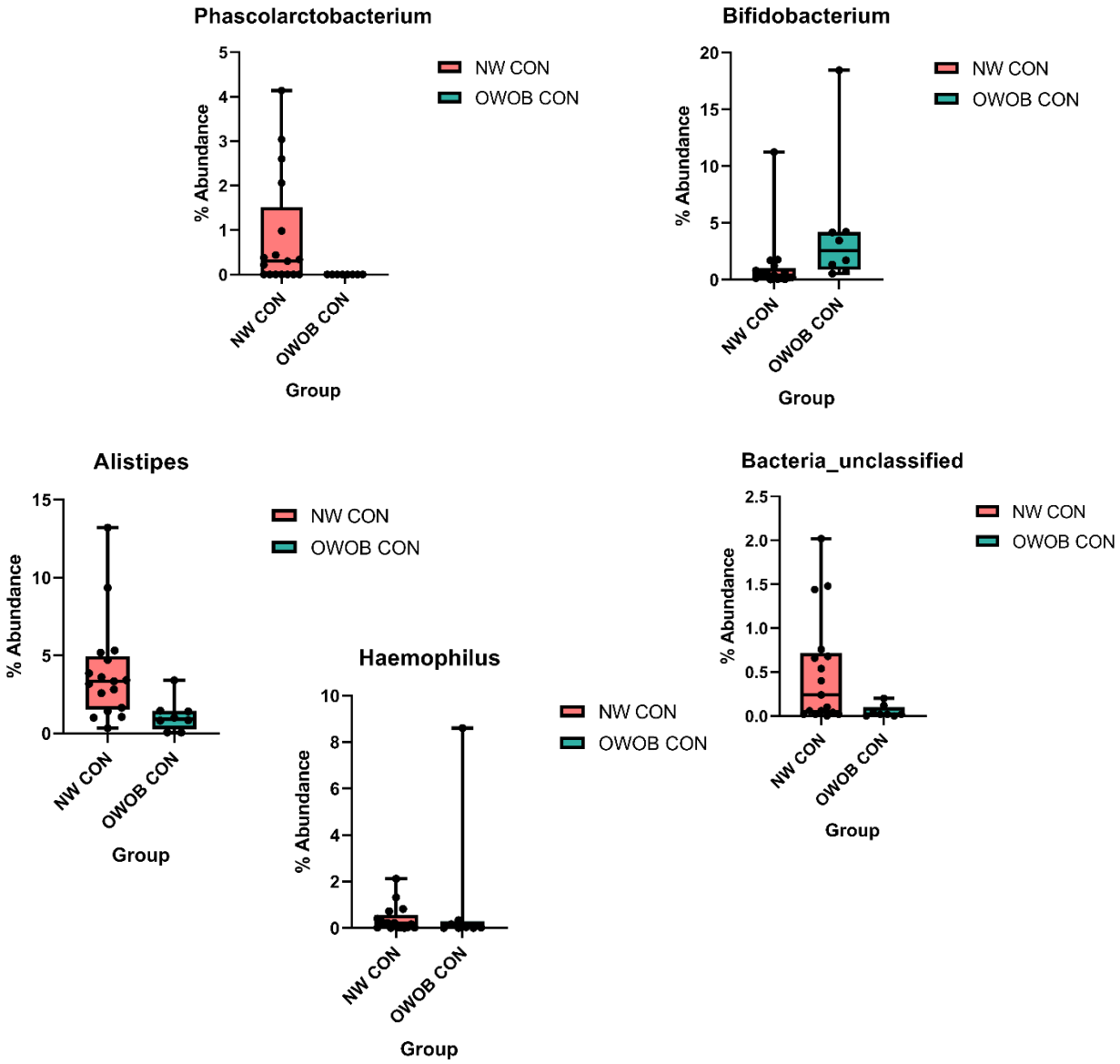
Name	log2FC	lfcSE	Pvalues	FDR
Escherichia	**-4.3764**	**1.0551**	**3.3582E-5**	**0.0017799**
RF39_unclassified	-5.6725	2.0655	0.0060261	0.11476
Turicibacter	-3.258	1.1971	0.0064958	0.11476
SMB53	-1.7398	0.75318	0.020888	0.27677
Dialister	2.7637	1.2492	0.026941	0.28558
Eubacterium	-2.7212	1.3234	0.03977	0.33145

Figure 21: Bacterial DESeq2 Differential Abundance Analysis NW PWS VS OWOB PWS

Features are considered to be significant based on their adjusted p-value (adj. p-value cutoff = 0.05, significance indicated with *). Within the PWS group, a larger abundance of Escherichia (p=0.001) was observed in the NW subgroup compared to the OWOB subgroup.

Abbreviations: Prader-Willi Syndrome (PWS), Control group (CON), Overweight/Obese (OWOB), Normal weight (NW), False discovery rate (FDR), log2FoldChange (log2FC), log fold change Standard Error (lfcSE)

Figure 22. Bacterial DESeq2 Differential Abundance Analysis NW CON VS OWOB CON



Name	log2FC	lfcSE	Pvalues	FDR
Phascolarctobacterium	**-8.1775**	**1.7305**	**2.2965E-6**	**1.1023E-4**
Bifidobacterium	**2.7405**	**0.64198**	**1.9647E-5**	**4.7153E-4**
Alistipes	**-1.7547**	**0.58048**	**0.0025038**	**0.034881**
Bacteria_unclassified	**-2.7547**	**0.92519**	**0.0029068**	**0.034881**
Haemophilus	**-2.581**	**0.98257**	**0.0086199**	**0.082751**
Collinsella	3.3516	1.369	0.014356	0.11485
Lachnospira	-1.403	0.662	0.034062	0.2044
Christensenellaceae_unclassified	-2.261	1.0669	0.034067	0.2044
Lachnospiraceae_unclassified	-0.90611	0.44811	0.043168	0.23023

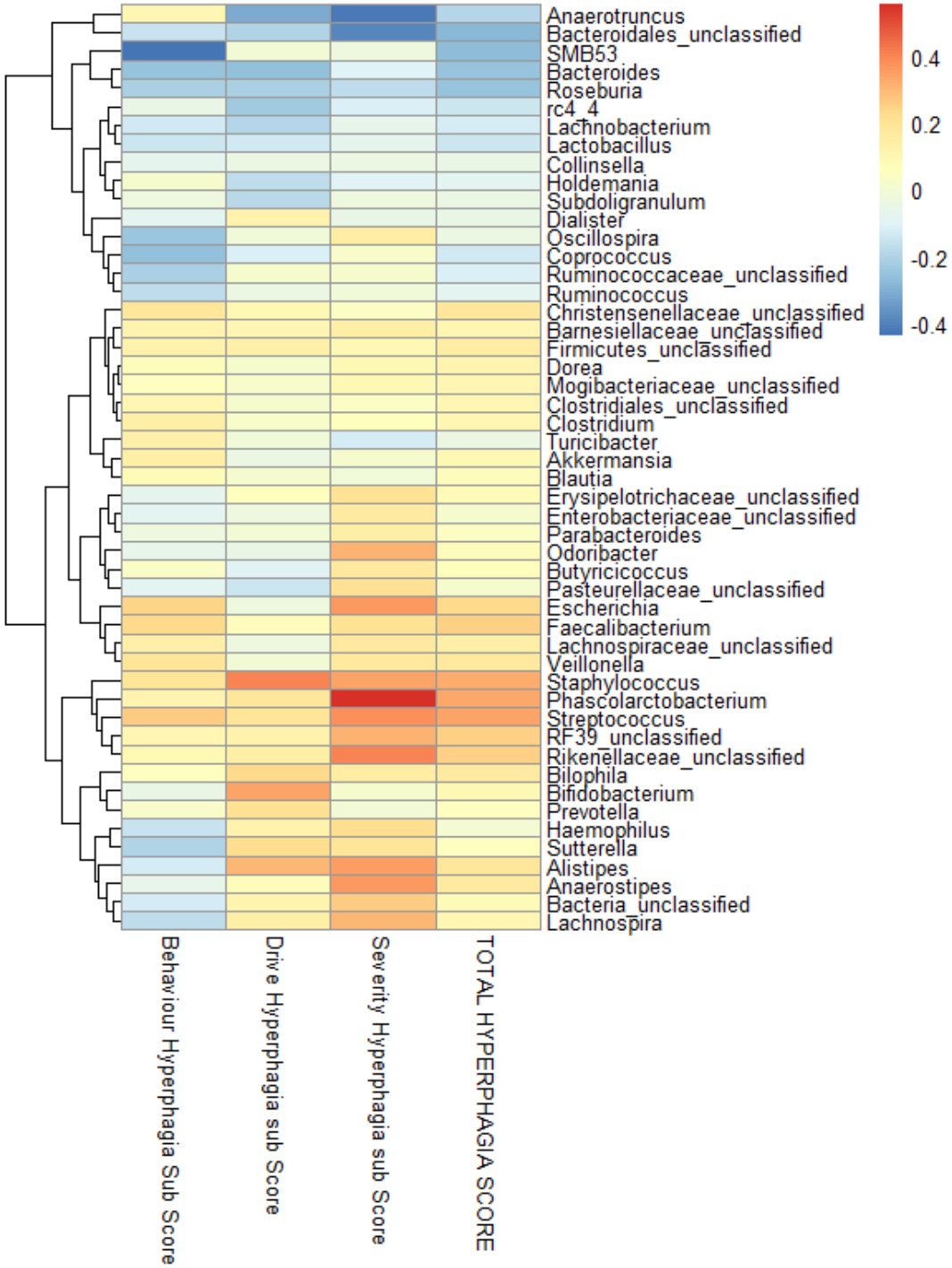
Figure 22: Bacterial DESeq2 Differential Abundance Analysis NW CON VS OWOB CON

Features are considered to be significant based on their adjusted p-value (adj. p-value cutoff = 0.05, significance indicated with *). Bifidobacterium was found to be more abundant in the OWOB CON group ($p<0.001$) whereas Phascolarctobacterium, unclassified Bacteria, Alistipes and Haemophilus were more abundant in the NW CON group

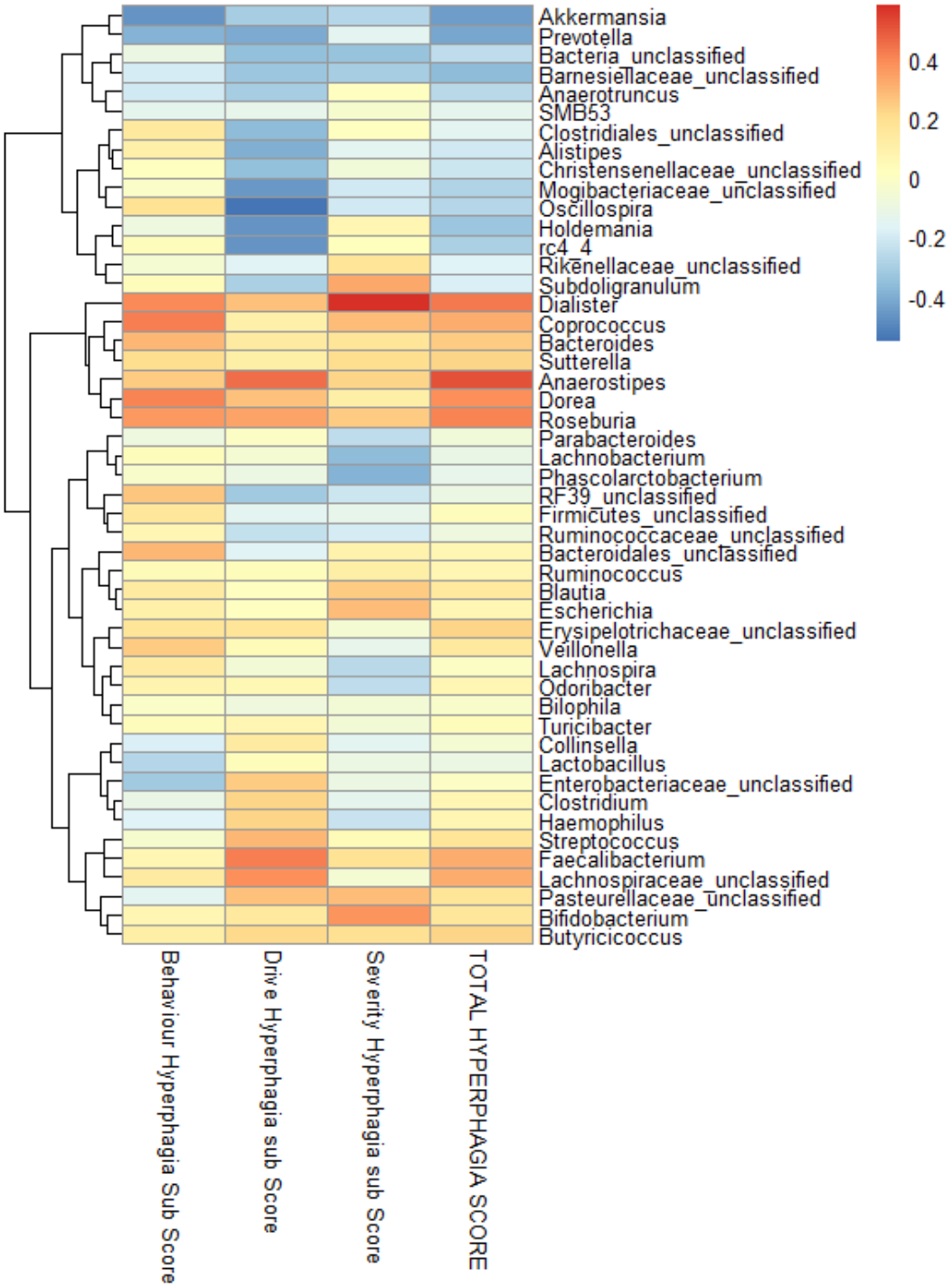
Abbreviations: Prader-Willi Syndrome (PWS), Control group (CON), Overweight/Obese (OWOB), Normal weight (NW), False discovery rate (FDR), log2FoldChange (log2FC), log fold change Standard Error (lfcSE)

Figure 23. Correlation between gut microbiota and hyperphagia scores.

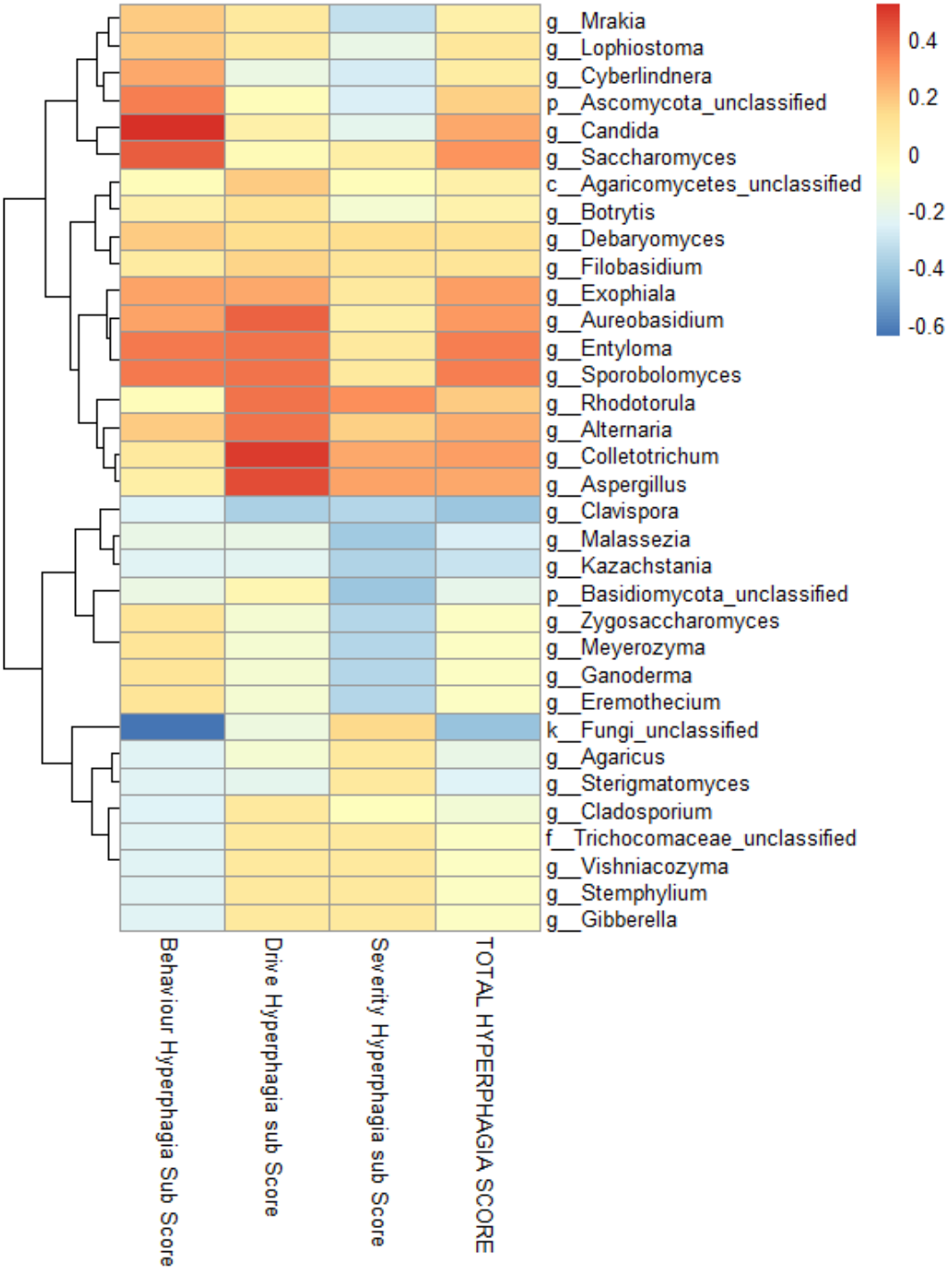
A)



B)


Akkermansia
Prevotella
Bacteria_unclassified
Barnesiellaceae_unclassified
Anaerotruncus
SMB53
Clostridiales_unclassified
Alistipes
Christensenellaceae_unclassified
Mogibacteriaceae_unclassified
Oscillospira
Holdemania
rc4_4
Rikenellaceae_unclassified
Subdoligranulum
Dialister
Coprococcus
Bacteroides
Sutterella
Anaerostipes
Dorea
Roseburia
Parabacteroides
Lachnobacterium
Phascolarctobacterium
RF39_unclassified
Firmicutes_unclassified
Ruminococcaceae_unclassified
Bacteroidales_unclassified
Ruminococcus
Blautia
Escherichia
Erysipelotrichaceae_unclassified
Veillonella
Lachnospira
Odoribacter
Bilophila
Turicibacter
Collinsella
Lactobacillus
Enterobacteriaceae_unclassified
Clostridium
Haemophilus
Streptococcus
Faecalibacterium
Lachnospiraceae_unclassified
Pasteurellaceae_unclassified
Bifidobacterium
Butyricicoccus
0.4
0.2
0
-0.2
-0.4
Behaviour Hyperphagia Sub Score
Drive Hyperphagia sub Score
Severity Hyperphagia sub Score
TOTAL HYPERPHAGIA SCORE

C)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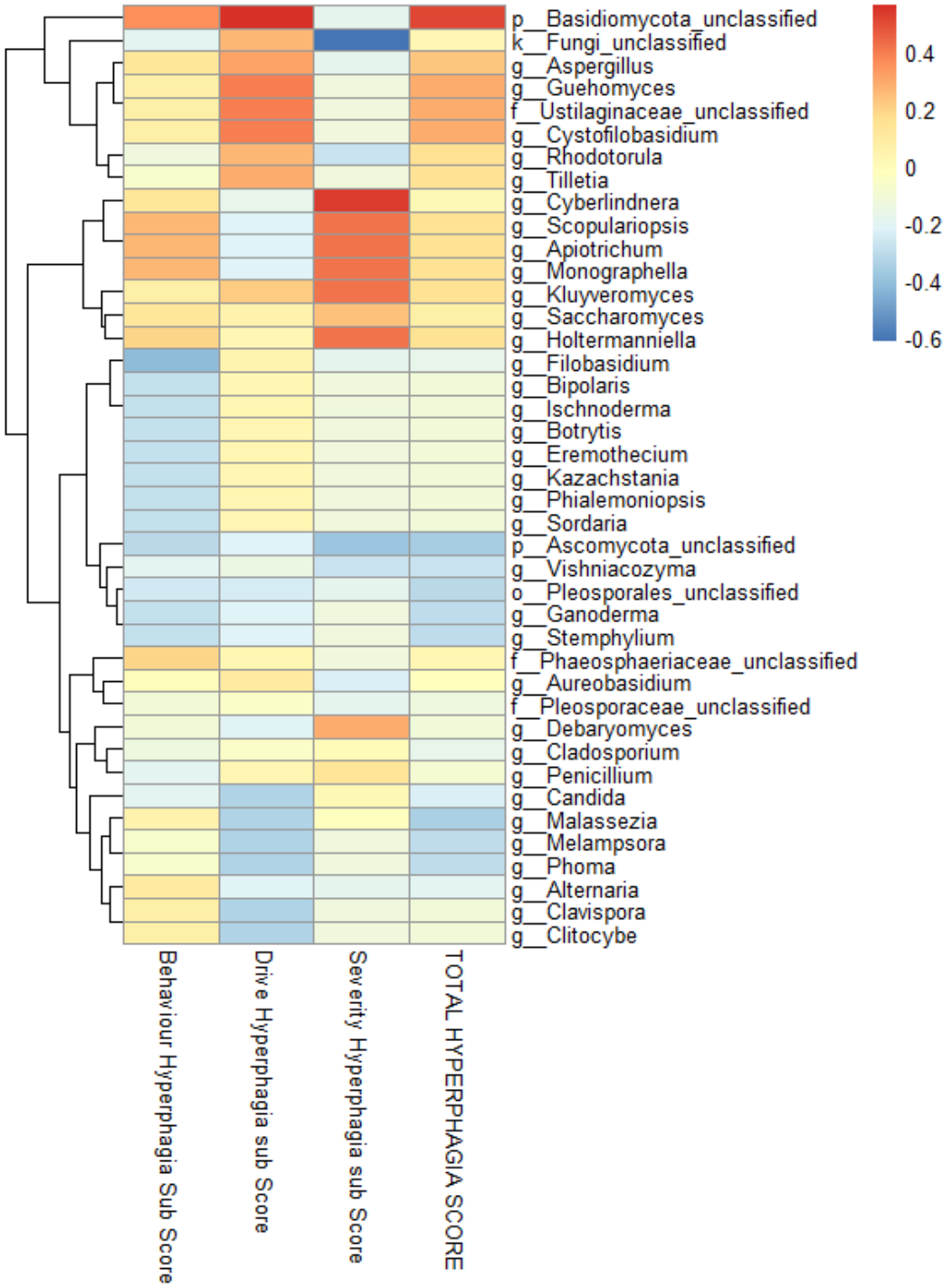
g__Cyberlindnera
p__Ascomycota_unclassified
g__Candida
g__Saccharomyces
c__Agaricomycetes_unclassified
g__Botrytis
g__Debaryomyces
g__Filobasidium
g__Exophiala
g__Aureobasidium
g__Entyloma
g__Sporobolomyces
g__Rhodotorula
g__Alternaria
g__Colletotrichum
g__Aspergillus
g__Clavispora
g__Malassezia
g__Kazachstania
p__Basidiomycota_unclassified
g__Zygosaccharomyces
g__Meyerozyma
g__Ganoderma
g__Eremothecium
k__Fungi_unclassified
g__Agaricus
g__Sterigmatomyces
g__Cladosporium
f__Trichocomaceae_unclassified
g__Vishniacozyma
g__Stemphylium
g__Gibberella
0.4
0.2
0
-0.2
-0.4
-0.6
Behaviour Hyperphagia Sub Score
Drive Hyperphagia sub Score
Severity Hyperphagia sub Score
TOTAL HYPERPHAGIA SCORE

D)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k__Fungi_unclassified
g__Aspergillus
g__Guehomyces
f__Ustilaginaceae_unclassified
g__Cystofilobasidium
g__Rhodotorula
g__Tilletia
g__Cyberlindnera
g__Scopulariopsis
g__Apiotrichum
g__Monographella
g__Kluyveromyces
g__Saccharomyces
g__Holtermanniella
g__Filobasidium
g__Bipolaris
g__Ischnoderma
g__Botrytis
g__Eremothecium
g__Kazachstania
g__Phialemoniopsis
g__Sordaria
p__Ascomycota_unclassified
g__Vishniacozyma
o__Pleosporales_unclassified
g__Ganoderma
g__Stemphylium
f__Phaeosphaeriaceae_unclassified
g__Aureobasidium
f__Pleosporaceae_unclassified
g__Debaryomyces
g__Cladosporium
g__Penicillium
g__Candida
g__Malassezia
g__Melampsora
g__Phoma
g__Alternaria
g__Clavispora
g__Clitocybe
0.4
0.2
0
-0.2
-0.4
-0.6
Behaviour Hyperphagia Sub Score
Drive Hyperphagia sub Score
Severity Hyperphagia sub Score
TOTAL HYPERPHAGIA SCORE

Figure 23: Correlation between gut microbiota and hyperphagia scores.

Spearman's correlations were calculated and tested for significance, then adjusted for FDR using the Bonferroni methodology. Results were then plotted onto a heatmap. The heatmap legend represents spearman correlation scores.

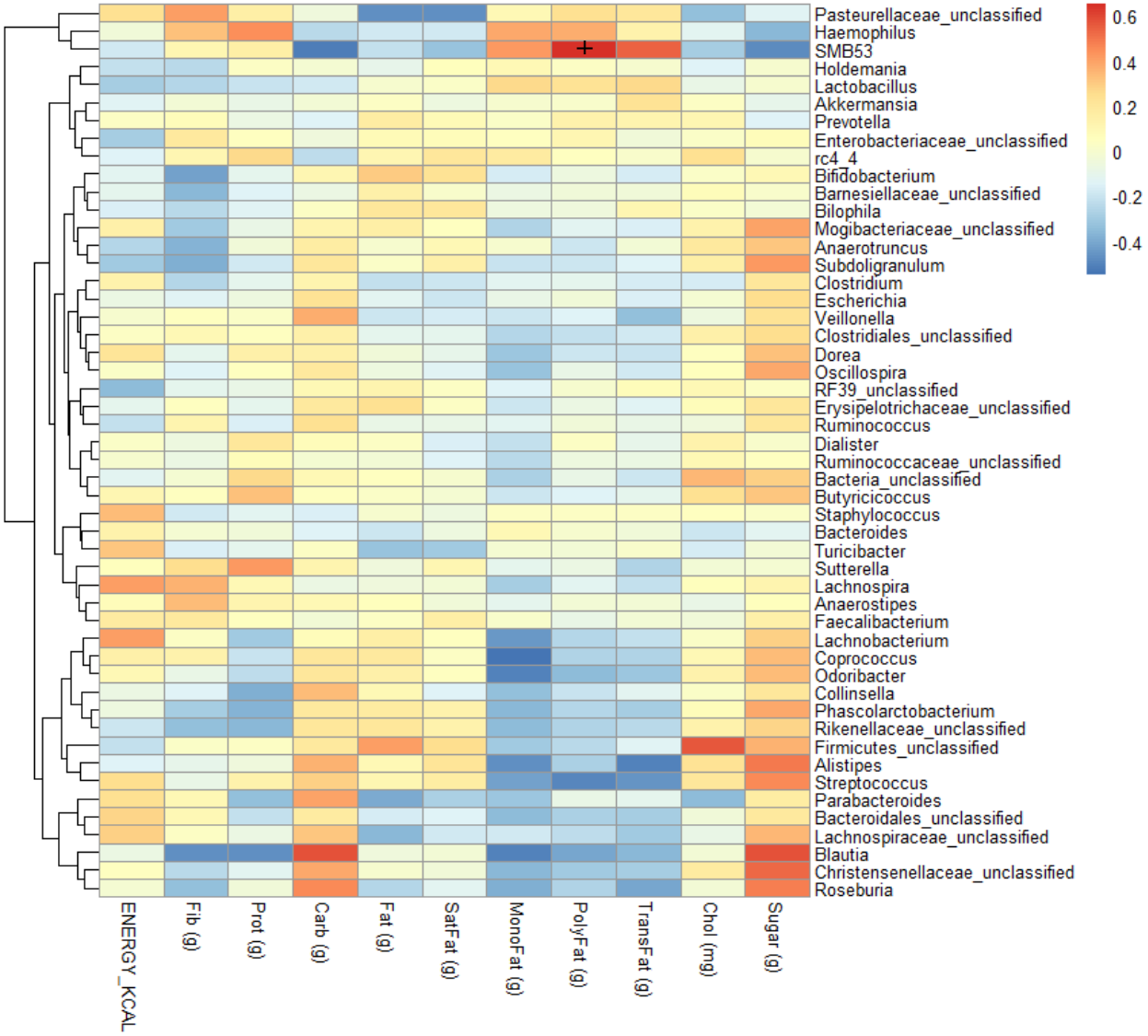
(A) Correlation of hyperphagia scores with bacterial genus-level abundance in the PWS group. (B) Correlation of hyperphagia scores with bacterial genus-level abundance in the CON group. (C) Correlation of hyperphagia scores with fungal genus-level abundance in the PWS group. (D) Correlation of hyperphagia scores with fungal genus-level abundance in the CON group. No significant differences found after FDR adjustments.

* indicates the p values less than 0.05. + indicated a p-value less than 0.25.

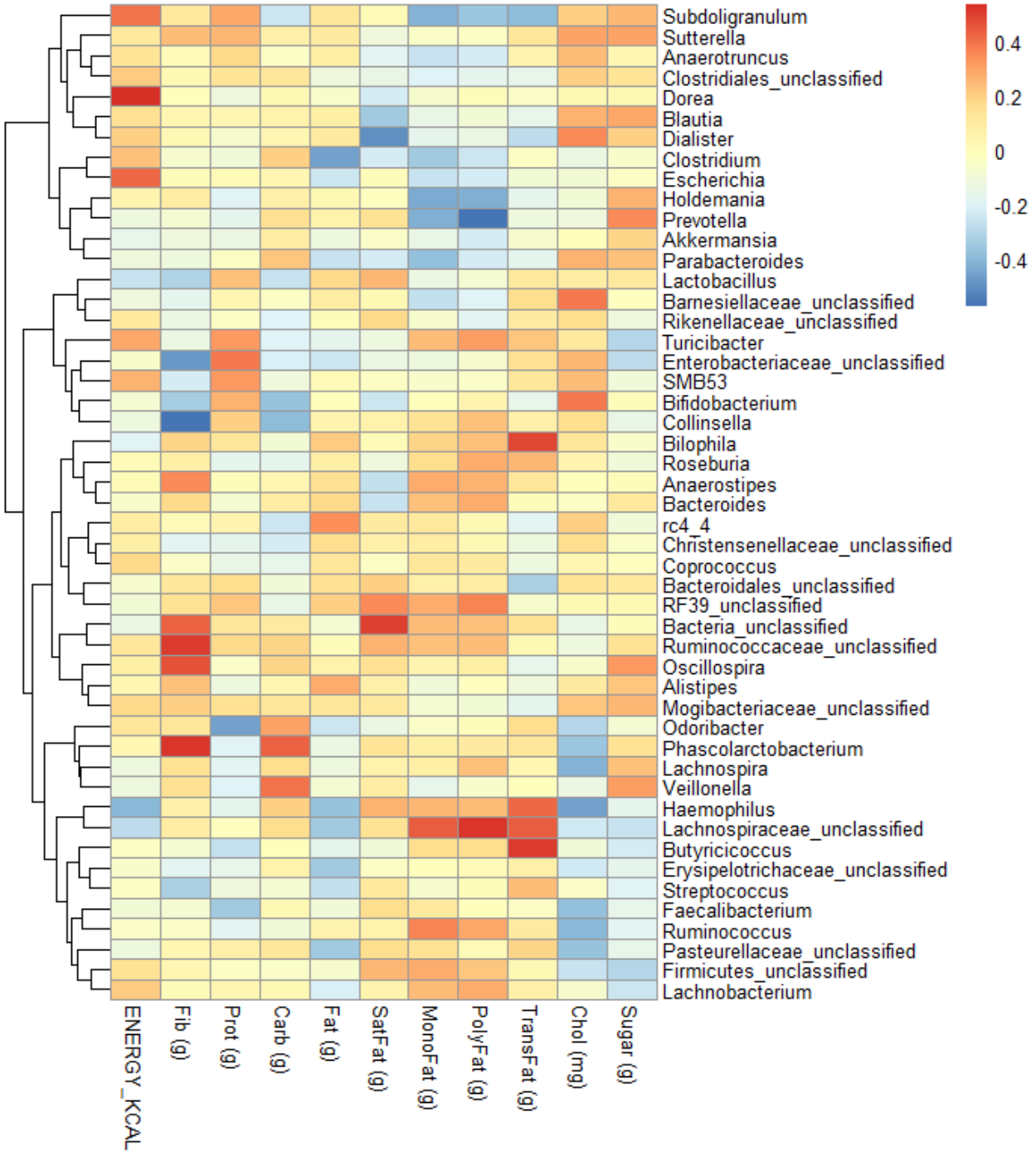
Abbreviations: Prader-Willi Syndrome (PWS), Control group (CON), False discovery rate (FDR)

Figure 24. Correlation between gut microbiota and dietary intakes.

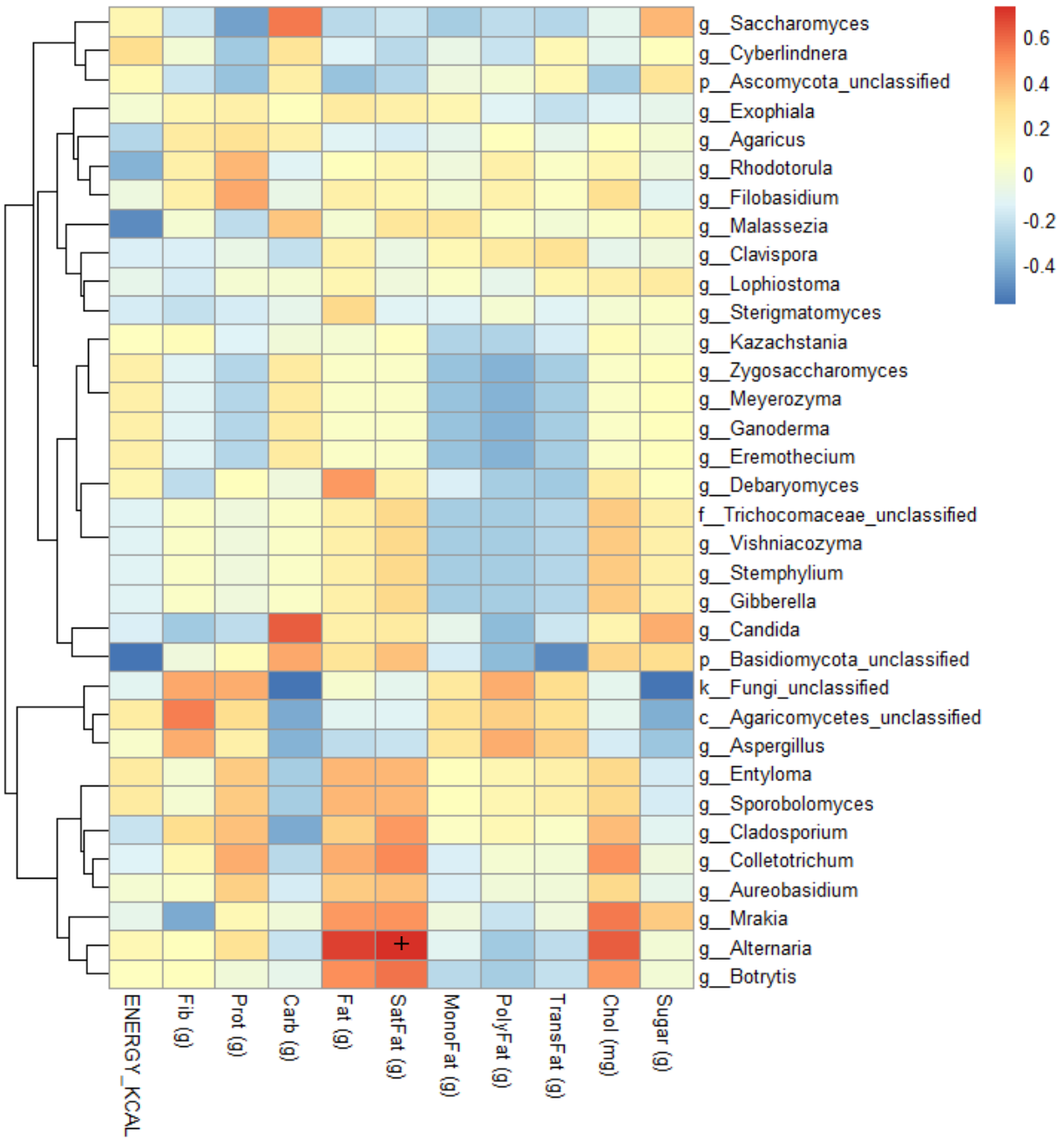
A)



B)


Subdoligranulum
Sutterella
Anaerotruncus
Clostridiales_unclassified
Dorea
Blautia
Dialister
Clostridium
Escherichia
Holdemania
Prevotella
Akkermansia
Parabacteroides
Lactobacillus
Barnesiellaceae_unclassified
Rikenellaceae_unclassified
Turicibacter
Enterobacteriaceae_unclassified
SMB53
Bifidobacterium
Collinsella
Bilophila
Roseburia
Anaerostipes
Bacteroides
rc4_4
Christensenellaceae_unclassified
Coprococcus
Bacteroidales_unclassified
RF39_unclassified
Bacteria_unclassified
Ruminococcaceae_unclassified
Oscillospira
Alistipes
Mogibacteriaceae_unclassified
Odoribacter
Phascolarctobacterium
Lachnospira
Veillonella
Haemophilus
Lachnospiraceae_unclassified
Butyricicoccus
Erysipelotrichaceae_unclassified
Streptococcus
Faecalibacterium
Ruminococcus
Pasteurellaceae_unclassified
Firmicutes_unclassified
Lachnobacterium
0.4
0.2
0
-0.2
-0.4
ENERGY_KCAL
Fib (g)
Prot (g)
Carb (g)
Fat (g)
SatFat (g)
MonoFat (g)
PolyFat (g)
TransFat (g)
Chol (mg)
Sugar (g)

C)


g__Saccharomyces
g__Cyberlindnera
p__Ascomycota_unclassified
g__Exophiala
g__Agaricus
g__Rhodotorula
g__Filobasidium
g__Malassezia
g__Clavispora
g__Lophiostoma
g__Sterigmatomyces
g__Kazachstania
g__Zygosaccharomyces
g__Meyerozyma
g__Ganoderma
g__Eremothecium
g__Debaryomyces
f__Trichocomaceae_unclassified
g__Vishniacozyma
g__Stemphylium
g__Gibberella
g__Candida
p__Basidiomycota_unclassified
k__Fungi_unclassified
c__Agaricomycetes_unclassified
g__Aspergillus
g__Entyloma
g__Sporobolomyces
g__Cladosporium
g__Colletotrichum
g__Aureobasidium
g__Mrakia
g__Alternaria
g__Botrytis
ENERGY_KCAL
Fib (g)
Prot (g)
Carb (g)
Fat (g)
SatFat (g)
MonoFat (g)
PolyFat (g)
TransFat (g)
Chol (mg)
Sugar (g)
0.6
0.4
0.2
0
-0.2
-0.4

D)

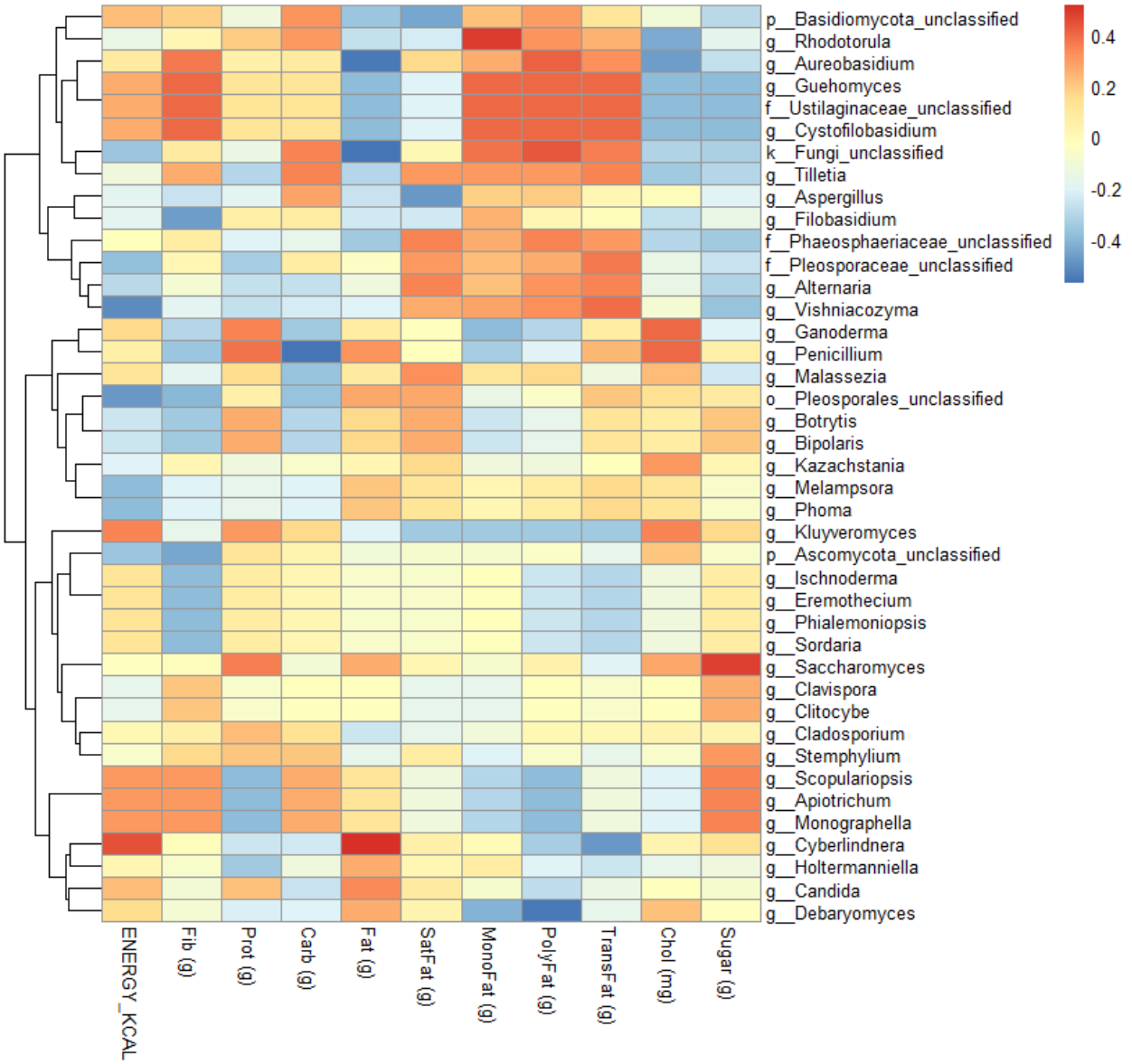


Figure 24: Correlation between gut microbiota and dietary intakes.

Spearman's correlations were calculated and tested for significance, then adjusted for FDR using the Bonferroni methodology. Results were then plotted onto a heatmap. The heatmap legend represents spearman correlation scores.

(A) Correlation of dietary intakes with bacterial genera in the PWS group. (B) Correlation of dietary intakes with bacterial genera in the CON group. (C) Correlation of dietary intakes with fungal genera in the PWS group. (D) Correlation of dietary intakes with fungal genera in the CON group.

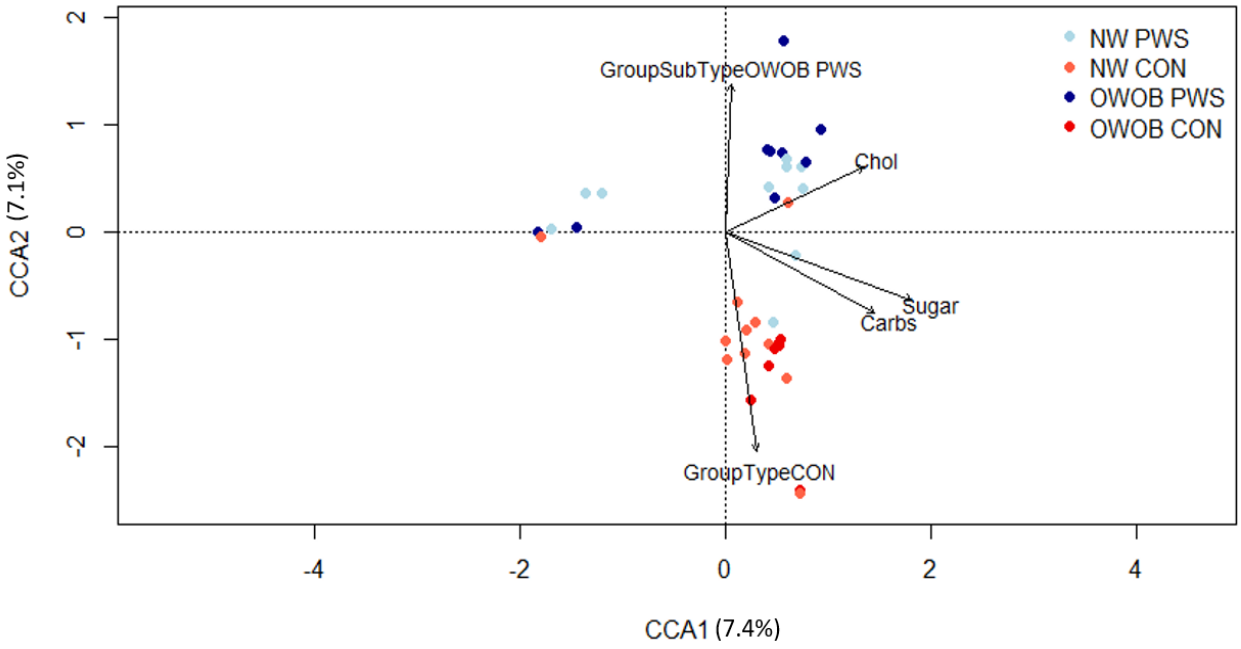
* indicates the p values less than 0.05. + indicated a p-value less than 0.25.

The bacterial genus SMB53 was slightly positively correlated with polyunsaturated fat intake in the PWS group (p=0.23, Spearman correlation=0.65) and the fungal genus Alternaria was slightly positively correlated with Saturated fat intake in the PWS group (p=0.16, Spearman correlation=0.73). No other genus was found to be significant after FDR adjustments.

Energy-adjusted intake: Diet data adjusted using the "Nutrient residual (energy-adjusted) model" to control for variation caused by total energy intake. Residual calculation utilized the mean dietary intake values as described in the methodology of Willett et. al (1997). Nutrients of interest selectively presented here from the 3-day dietary records analyzed using Processor SQL (version 11.4, ESHA Research, Salem, OR, 2006)

Abbreviations: Prader-Willi Syndrome (PWS), Control group (CON), False discovery rate (FDR)

Figure 25. CCA model using all collected metadata



	Inertia	Proportion	Rank
Total	12.1267	1.0000	
Constrained	7.1193	0.5871	20
Unconstrained	5.0074	0.4129	16

Figure 25: CCA model using all collected metadata.

The amount of the constrained inertia expressed by each constrained axis was noted on the axis labels. The first CCA dimension (CCA Axis 1) explained 7.4% of variation in the data. The second CCA dimension (CCA Axis 2) explained 7.1% of variation in the data. 58% of variance in the fungal abundance matrix can be explained by the model containing all collected metadata. Vector arrows displayed for only significantly contributing factors as assessed by ANOVA ($P<0.05$).

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Appendix

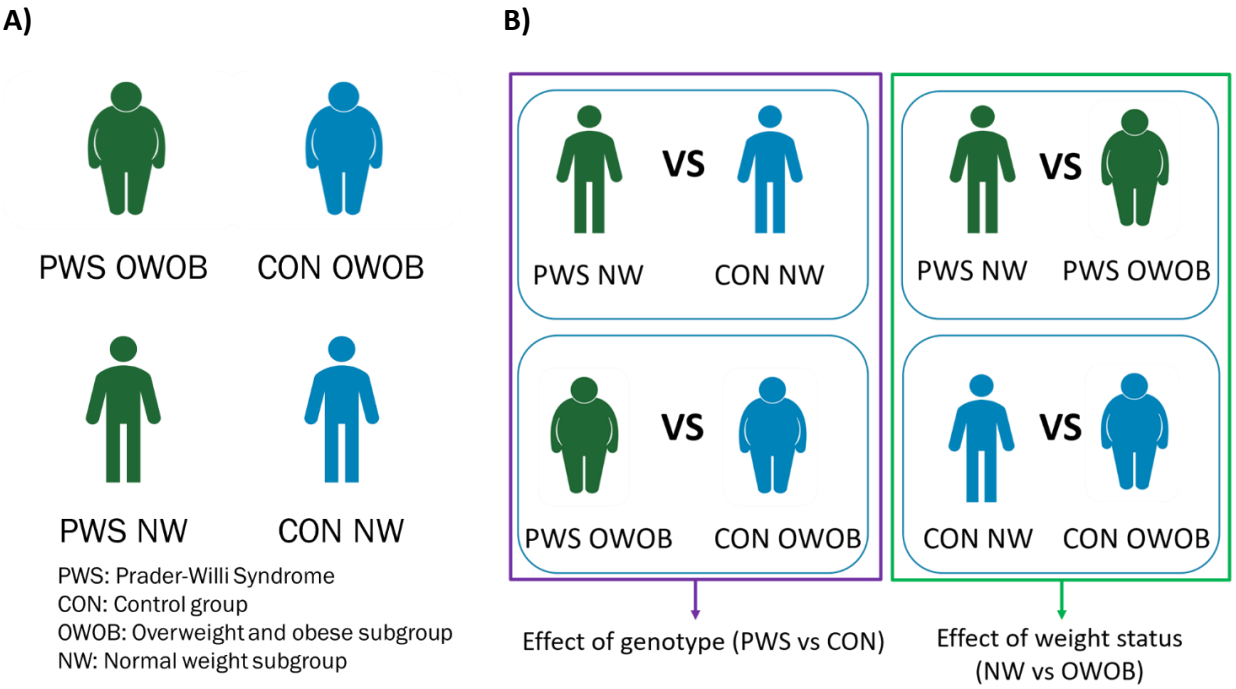
Appendix A – Participant Characteristics
Supplementary Table 1

	Sex	Age	BMI%	Weight group	Genotype	Subgroup	Hyperphagia score				Nutrition intake									
							Behaviour	Drive	Severity	Total	Energy (kcal)	Fib (g)	Prot (g)	Carb (g)	Fat (g)	Sat Fat (g)	Mono Fat (g)	Poly Fat (g)	Trans Fat (g)	Chol (mg)
S005B-0001	F	146	78.5	NW	PWS	NW PWS	7	7	2	16	1814	16	58	271	184	189	180	186	193	154
S005B-0002	M	74	98.9	OWOB	PWS	OWOB PWS	5	7	4	16	1244	18	65	189	214	193	186	188	194	172
S005B-0003	M	36	52.6	NW	PWS	NW PWS	5	5	2	12	1118	16	64	211	201	195	188	188	195	122
S005B-0004	F	79	94.8	OWOB	PWS	OWOB PWS	5	8	4	17	1202	25	82	206	198	192	186	188	194	206
S005B-0005	F	181	65.5	NW	PWS	NW PWS	11	6	11	28	1652	22	60	258	188	191	175	184	194	107
S005B-0006	F	67	86.2	OWOB	PWS	OWOB PWS	8	10	3	21	1361	14	81	198	203	204	183	185	194	346
S005B-0007	F	213	86.2	OWOB	PWS	OWOB PWS	9	8	2	19	1480	19	64	211	204	196	179	182	194	196
S005B-0008	F	159	72.2	NW	PWS	NW PWS	11	11	4	26	1810	18	76	189	211	202	182	183	194	428
S005B-0009	F	65	83.3	NW	PWS	NW PWS	6	14	6	26	847	24	97	175	205	198	187	189	194	293
S005B-0010	M	182	70.6	NW	PWS	NW PWS	7	7	2	16	1058	17	68	195	205	193	187	187	195	216
S005B-0011	F	42	41.5	NW	PWS	NW PWS	14	6	4	24	1046	19	76	159	222	203	202	187	195	383
S005B-0012	M	183	87.3	OWOB	PWS	OWOB PWS	5	7	2	14	1500	14	51	152	240	239	183	186	194	178
S005B-0013	M	66	95.9	OWOB	PWS	OWOB PWS	12	14	4	30	1503	20	96	113	236	213	192	189	197	351
S005B-0014	M	91	98.8	OWOB	PWS	OWOB PWS	5	10	4	19	1299	21	71	195	209	206	181	183	194	364
S005B-0015	F	200	83.9	NW	PWS	NW PWS	8	14	6	28	847	12	65	148	226	204	199	190	195	290
S005B-0016	M	38	71.1	NW	PWS	NW PWS	5	8	4	17	1205	27	71	147	232	208	196	188	194	341
S005B-0017	F	155	28.5	NW	PWS	NW PWS	19	13	7	39	1429	12	64	217	200	197	181	184	194	162
S005B-0018	F	70	40.8	NW	PWS	NW PWS	9	13	4	26	1922	32	70	101	137	175	246	298	229	17
S005B-0019	M	39	30.8	NW	PWS	NW PWS	5	5	2	12	1395	26	75	109	160	183	214	265	224	44

S005B-0020	M	54	70.5	NW	PWS	NW PWS	5	6	3	14	1395	19	93	113	157	189	223	261	218	57
S005B-0021	M	98	95.4	OWOB	PWS	OWOB PWS	5	9	5	19	1644	19	73	129	158	183	208	273	224	12
S005B-0022	F	123	34.2	NW	PWS	NW PWS	10	10	3	23	1327	18	72	192	208	195	190	187	196	306
S005B-0023	F	40	79.3	NW	PWS	NW PWS	5	5	2	12	1176	14	66	149	170	192	219	248	220	70
S005B-0024	F	62	94.1	OWOB	PWS	OWOB PWS	11	13	4	28	1390	23	79	196	210	200	193	186	194	119
S005B-0025	M	69	96.5	OWOB	PWS	OWOB PWS	9	10	3	22	1127	28	65	230	177	193	257	212	200	57
S005B-0026	M	192	81.8	NW	CON	NW CON	5	7	2	14	1048	12	66	225	171	195	304	206	201	62
S005B-0027	M	111	96.7	OWOB	CON	OWOB CON	5	7	2	14	1147	12	76	181	209	208	183	184	195	199
S005B-0028	M	180	51.2	NW	CON	NW CON	5	6	2	13	2043	12	99	172	205	198	174	180	195	475
S005B-0029	M	78	0.9	NW	CON	NW CON	5	7	2	14	1366	15	64	213	202	202	185	185	194	399
S005B-0030	M	127	65.9	NW	CON	NW CON	7	11	2	20	2446	31	73	228	131	195	322	256	225	-34
S005B-0031	M	106	97	OWOB	CON	OWOB CON	12	11	2	25	1914	14	55	243	197	192	187	187	193	267
S005B-0032	M	24	44.6	NW	CON	NW CON	6	5	2	13	826	12	60	189	211	202	190	188	195	221
S005B-0033	M	103	99.6	OWOB	CON	OWOB CON	5	7	2	14	1961	10	67	224	202	197	189	183	193	137
S005B-0034	M	91	82.3	NW	CON	NW CON	6	5	2	13	1889	22	56	243	197	205	181	181	194	115
S005B-0035	F	120	74	NW	CON	NW CON	6	5	2	13	2014	21	61	230	201	205	178	182	195	163
S005B-0036	F	126	86	OWOB	CON	OWOB CON	8	7	3	18	1864	14	49	194	219	200	194	184	193	128
S005B-0037	M	194	49.4	NW	CON	NW CON	9	6	3	18	2672	21	46	241	206	197	178	177	194	66
S005B-0038	F	100	83	NW	CON	NW CON	8	8	2	18	2064	20	31	237	212	194	194	189	195	109
S005B-0039	M	53	74.1	NW	CON	NW CON	7	12	4	23	1480	18	96	177	205	204	187	186	194	344
S005B-0040	M	76	57.1	NW	CON	NW CON	6	8	2	16	2074	19	57	240	198	194	175	178	193	147
S005B-0041	M	105	26.7	NW	CON	NW CON	6	10	2	18	1564	21	51	244	169	214	238	230	216	25
S005B-0042	F	61	76.6	NW	CON	NW CON	11	7	2	20	1101	28	73	378	226	203	195	193	194	181

S005B-0043	F	89	56.4	NW	CON	NW CON	7	5	2	14	1540	20	64	219	202	196	184	187	194	171
S005B-0044	M	117	78.6	NW	CON	NW CON	6	4	2	12	3382	15	109	57	263	218	210	194	191	335
S005B-0045	F	108	91.5	OWOB	CON	OWOB CON	10	10	4	24	2247	16	65	185	223	195	204	195	195	226
S005B-0046	M	152	98.2	OWOB	CON	OWOB CON	6	6	2	14	1912	17	69	222	208	192	194	191	195	524
S005B-0047	M	130	93.9	OWOB	CON	OWOB CON	7	8	3	18	2673	14	92	228	190	194	176	177	193	458
S005B-0048	F	59	35.4	NW	CON	NW CON	7	6	2	15	2051	23	46	289	182	187	176	183	194	49
S005B-0049	F	48	98.1	OWOB	CON	OWOB CON	5	6	2	13	1642	18	73	229	196	200	184	186	194	151
S005B-0050	F	73	19.5	NW	CON	NW CON	8	7	2	17	1722	16	60	193	157	217	238	254	215	26

Appendix B - Graphical depiction of subgroups and pairwise analysis



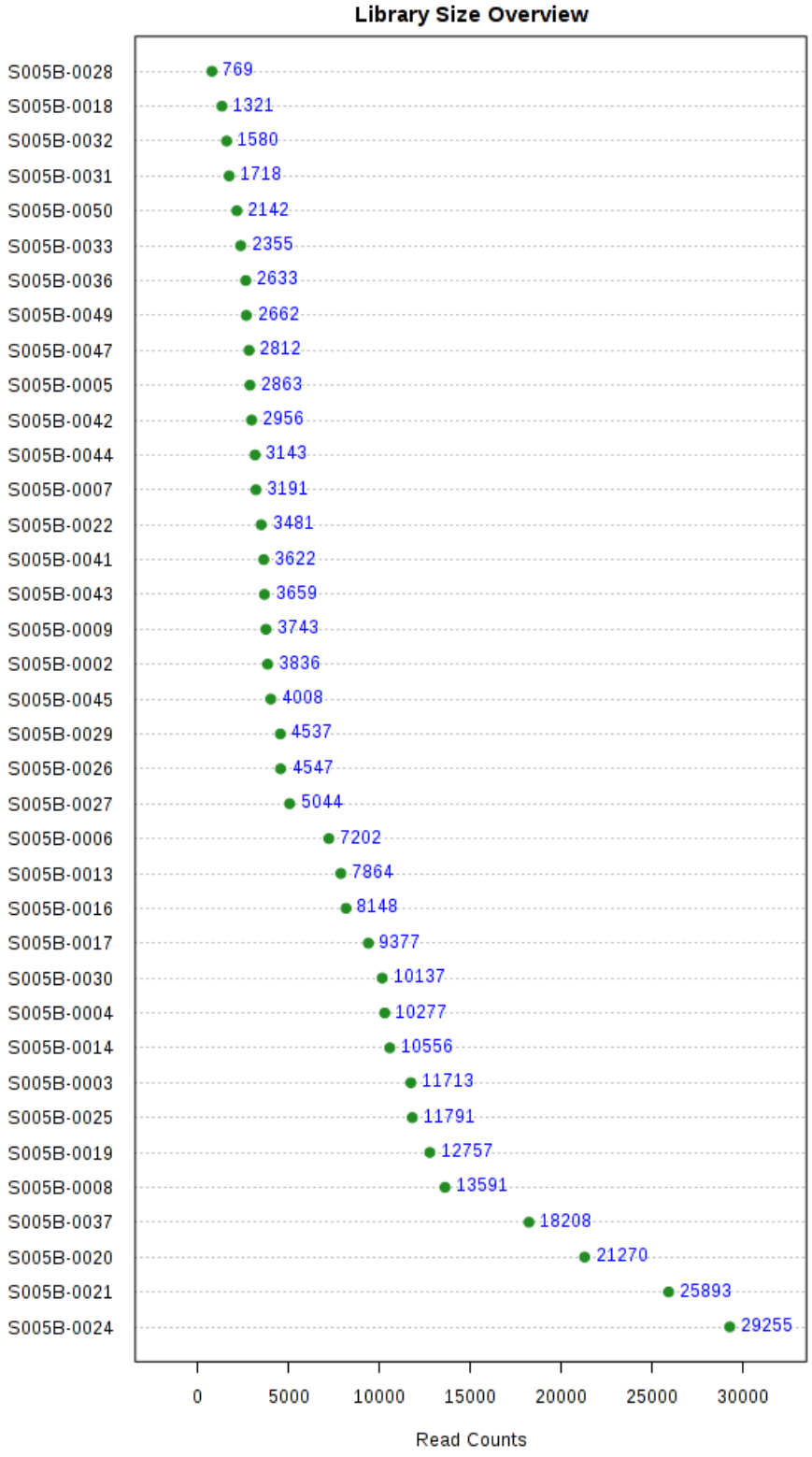
Supplemental figure 1. Graphical depiction of subgroups and pairwise analysis.

A) 4 subgroup (NW PWS, OWOB PWS, NW CON, OWOB CON were considered for pairwise analysis. B) pairwise subgroup analyses (NW CON vs NW PWS; NW OWOB CON vs OWOB PWS; NW PWS vs OWOB PWS; NW CON vs OWOB CON). Pairwise analyses were used to try and gain insight between the effects of weight status (NW vs OWOB) and the effects of group type (PWS vs CON).

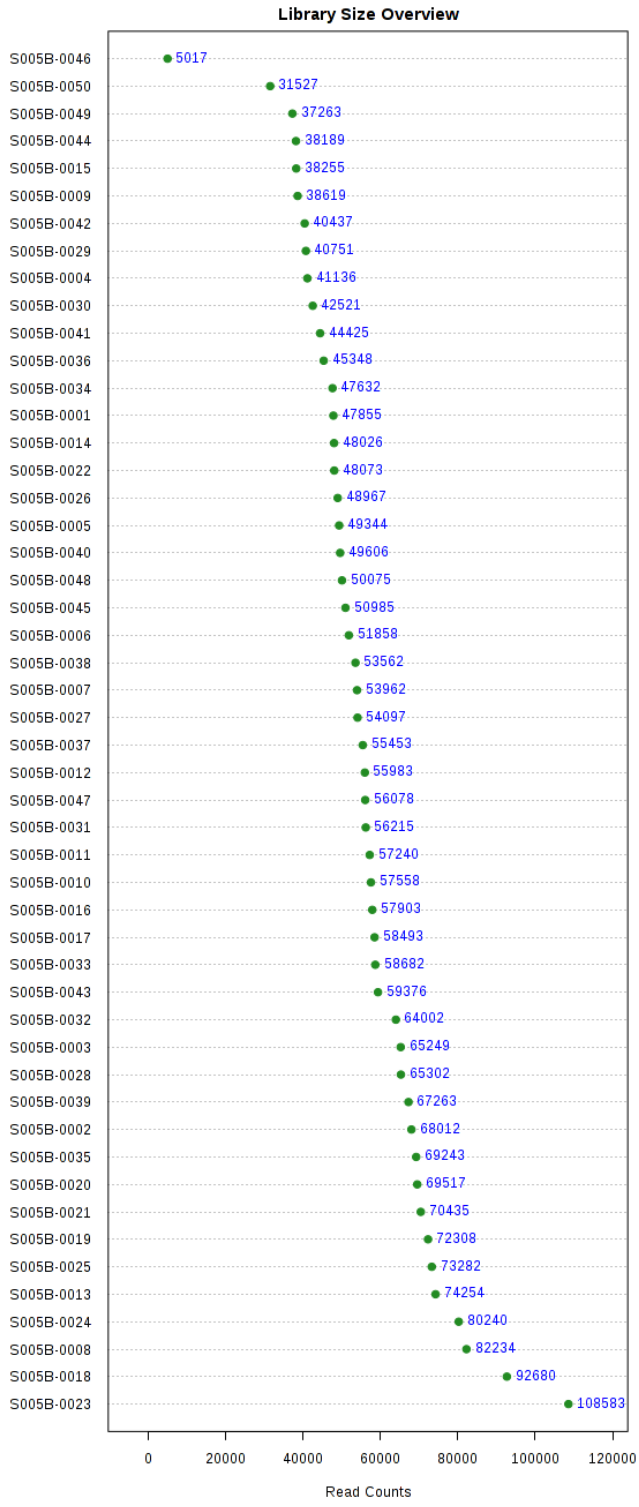
Abbreviations: Overweight/Obese (OWOB), Normal weight (NW), Prader-Willi Syndrome (PWS), Control group (CON)

Appendix C - Sequence read depth summary for A) fungal and B) bacterial sequences

A) Fungal read depth

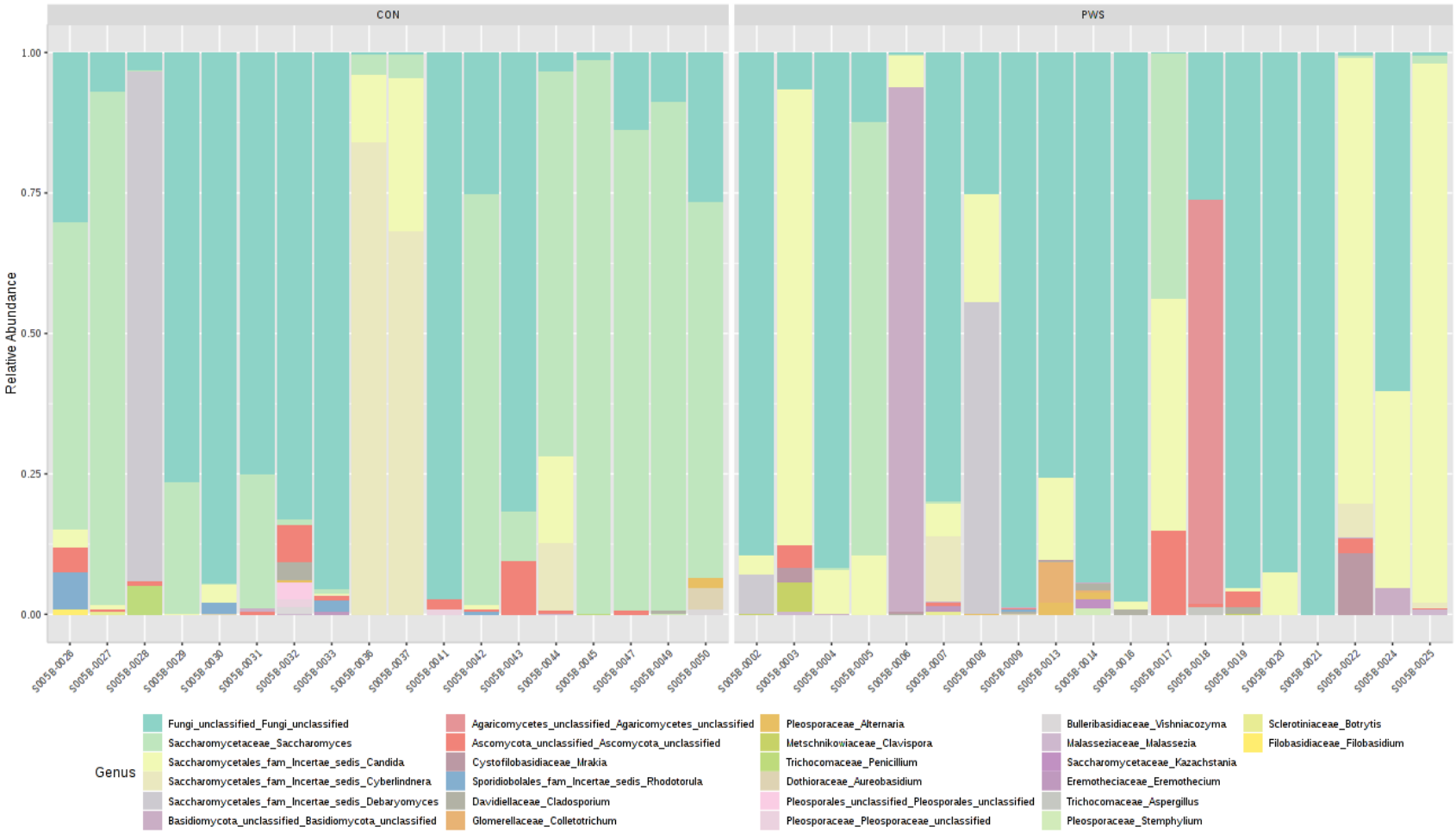


B) Bacterial read depth



Supplemental figure 2. Sequence read depth summary for A) fungal and B) bacterial sequences

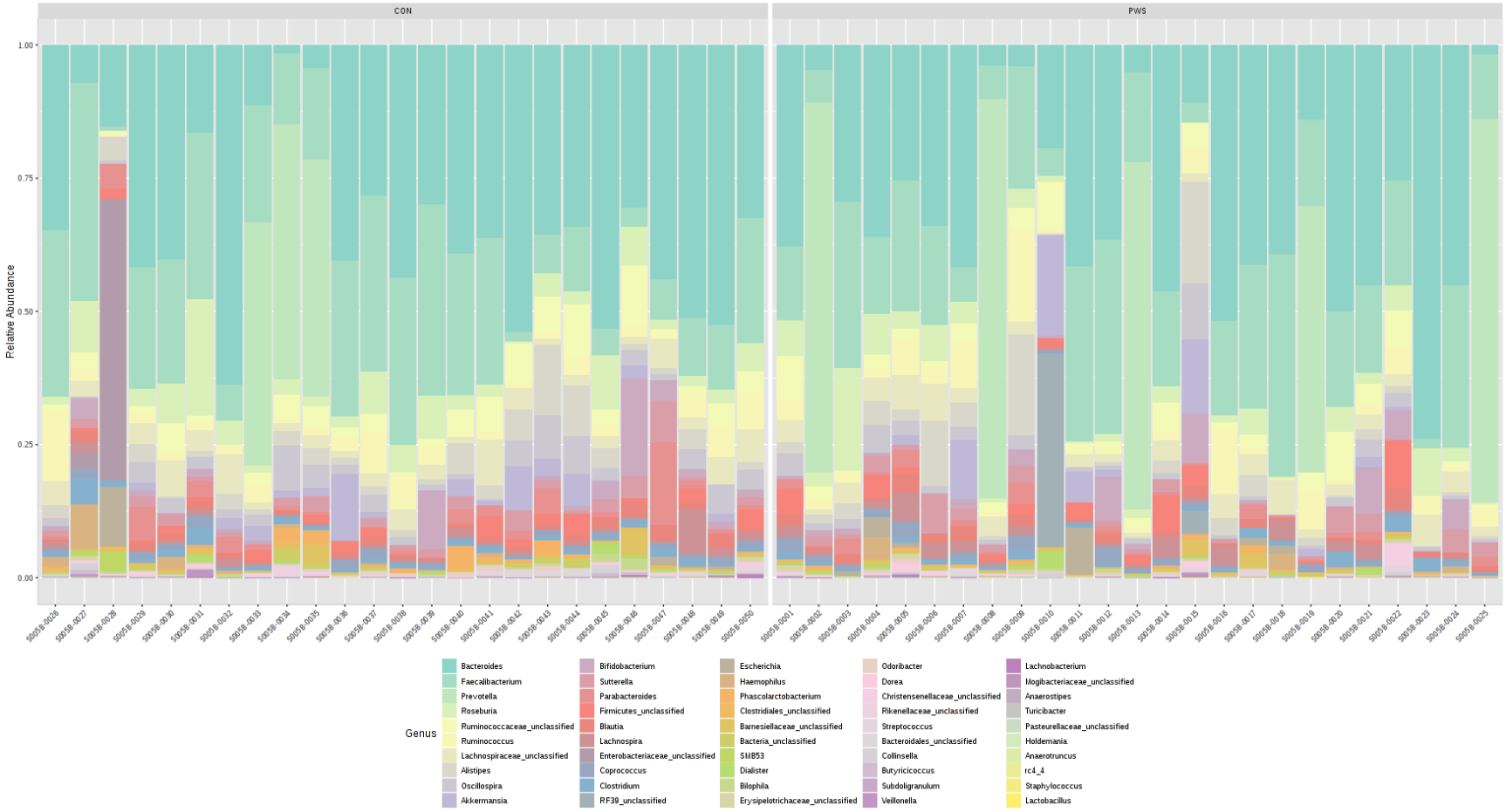
Appendix D - Fungal genus level taxonomic composition



Supplemental figure 3. Fungal genus level taxonomic composition using Stacked bar/area plot

Abbreviations: Prader-Willi Syndrome (PWS), Control group (CON)

Appendix E - Bacterial genus level taxonomic composition



Supplemental figure 4. Bacterial genus level taxonomic composition using Stacked bar/area plot

Abbreviations: Prader-Willi Syndrome (PWS), Control group (CON)

Appendix F - Hyperphagia Questionnaire (Dykens et al., 2007)

Items on the Hyperphagia Questionnaire were rated on a five-point scale (1- not a problem to 5- severe and/or frequent problem).

Date: _____ Study ID#:

EATING BEHAVIORS QUESTIONNAIRE

(1). How upset does your child generally become when denied a desired food?

____ Not particularly upset at all

____ A little upset

____ Somewhat upset

____ Very upset

____ Extremely upset

(2). Once your child has food on their mind, how easy is it for you or others to re-direct your child away from food to other things?

____ Extremely easy, takes minimal effort to do so

____ Very easy, takes just a little effort to do so

____ Somewhat hard, takes some effort to do so

____ Very hard, takes a lot of work to do so

____ Extremely hard, takes sustained and hard work to do so

(3). How persistent is your child in asking or looking for food after being told “no” or “no more”?

____ Lets go of food ideas quickly and easily

____ Lets go of food ideas pretty quickly and easily

____ Somewhat persistent with food ideas

____ Very persistent with food ideas

____ Extremely persistent with food ideas

(4). How often does your child currently get up at night to food seek?

____ 4-7 nights a week

____ 1-3 nights a week

____ 1-2 nights a month

____ A few nights a year

____ Never

(5). How often does your child currently forage through the trash for food?

____ 4-7 times a week

____ 1-3 times a week

____ 1-2 times a month

____ A few times a year

____ Never

(6). How often does your child currently try to bargain or manipulate to get more food at meals?

____ Several times a day

____ Several times a week

____ A few times a week

____ A few times a month

____ A few times a year

(7). How often does your child currently try to steal food (that you are aware of)?

____ Several times a day

____ Several times a week

____ A few times a week

____ A few times a month

____ A few times a year

(8). How "clever" or "fast" is your child at obtaining food?

____ Extremely clever or fast

____ Very clever or fast

____ Somewhat clever or fast

____ A little clever or fast

____ Not particularly clever or fast

(9). How variable is your child's preoccupations or interests in food?

____ Goes up and down all the time

____ Goes up and down quite a lot

____ Goes up and down occasionally

____ Usually stays about the same

____ Hardly ever varies

(10). Outside of normal meal times, how much time does your child spend talking about food or engaged in food-related behaviours?

____ Less than 15 minutes a day

____ 15 to 30 minutes a day

____ 30 minutes to 1 hour a day

____ 1 to 3 hours a day

____ more than 3 hours a day

(11). When others try to stop your child from talking about food or engaging in food-related behaviours, it generally leads to:

____ No distress or upset

____ Mild distress or upset

____ Moderate distress or upset

____ Severe distress or upset

____ Extreme distress or upset

(12). To what extent do food-related thoughts, talk, or behaviour interfere with your child's normal daily routines, self-care, school, or work?

____ No interference

____ Mild, occasional food-related interference in completing school, work or hygiene tasks

____ Moderate, frequent food-related interference in completing school, work of hygiene tasks

____ Severe, almost daily food-related interference in completing school, work of hygiene tasks

____ Extreme, often unable to participate in hygiene tasks or get to school or work due to food-related interference

Appendix G – Summary of dietary fiber selection and dosage rational

Fiber selection rationale

Fiber choice and dosage was determined through extensive review of the literature for 1) benefits of fiber on host and microbiome 2) mechanisms of action of fiber 3) tolerability of fiber 4) ease of incorporation 5) similarity to the previous beneficial PWS intervention. Responses to prebiotic interventions are highly individualized [1, 2]. To maximize the number of responders, a combination of soluble and insoluble fibers will be used, providing a complex and diverse source of nutrients to the gut microbiota and, consequently, to the host [3]. Oligofructose, digestion-resistant maltodextrin, acacia gum and RS2 have all been examined for their ability to promote satiety, elevate circulating anorexigenic hormones, as well as confer other health benefits. These fibers are also associated with alterations in the gut microbiota and increased production of SCFAs which provide system wide benefits to the host. Insoluble fibers found in whole food products such as oat bran and legumes are also well known to aid in appetite regulation and the food matrix provides additional phytochemicals and confers improved tolerability.

Soluble Fibers:

- **Oligosaccharides: Oligofructose & Oligoisomaltose:** Oligofructans have been well documented to provide appetite reducing and anti-obesogenic functions in both adult and pediatric populations. Oligofructose promoted favourable microbial shifts in taxa that promoted SCFA production such as increased abundance of *Bifidobacterium* & *Lactobacillus* and decreased taxa associated with inflammation and disruptions of intestinal barrier function such as *Bacteroides vulgatus* and *Clostridium clostridioforme*. This prebiotic supplementation improved appetite regulation, metabolic & inflammatory outcomes and weight loss in children with overweight and obesity independent of other lifestyle changes [4-6]. Various studies have found improvements of appetite regulation, satiety hormone levels and reduced energy intake [7]. In the Previous PWS intervention trial, these oligosaccharides were consumed daily, providing additional rationale for its selection in the current study [8].
- **Digestion-Resistant Maltodextrin:** Used in the previous PWS intervention. May improve intestinal functions such as GI tolerability, colonic transit time, stool volume and stool consistency [9-11], all of which would aid the digestion of children with PWS. In addition, 10-15 g of maltodextrin has been shown to have satiating effects, and increased levels of PYY and GLP-1 [12]. Promotes increased *Lactobacillus* and *Bifidobacterium* [13] both bacteria implicated in improvement of obesity phenotype through decreasing inflammation and improved glycemic control.

- **Acacia gum [Gum Arabic; Acacia Senegal]:** Studies have found consumption of acacia gum decreased caloric intake and increased subjective ratings of satiety [14]. Other studies have also found weight loss, decreased fat percentage, improved glycemic control, and improved lipid profile benefits to this fiber [15, 16]. The microbiome modulating effects are well established and consumption leads to more beneficial microbial profiles [17].

Insoluble Fibers:

- **Resistant starch (Hi-maize RS2):** Consumption of RS2 is associated with reduced abdominal fat, improved insulin sensitivity, increased serum glucagon-like peptide 1 (GLP-1), increased fasting PYY, lower leptin concentrations, and improved glucose homeostasis [18, 19]. RS2 also reliably modulates the gut microbiome and is shown to promote butyrate production [20, 21]. RS2 is also well tolerated and aids digestion.
- **Whole food sources of insoluble fiber:** Several systematic reviews of the literature consistently find improvements in satiety using non-soluble fibers such as B-glucans [22]. These fibers are better tolerated as whole foods products and whole foods provide a mixture of various non-soluble fibers as well as additional beneficial nutrients, lipids and phytochemicals. The PWS intervention used a partial whole food mixture and the current study aims to adapt this to fit a more Canadian diet; specifically using whole grains, oat bran, legumes, beans, and lentils found in Alberta.

Table: Tolerance data of fibers with limited GI side-effects chosen for the fiber intervention trial

Reference	Population	Type of fiber	Dose	Tolerence outcomes Summary
Calame et al., (2011)	Adults	Acacia Gum	5, 10, or 40 g/d	No reported GI discomfort
Babiker et al., (2018)	Adults	Acacia Gum	30 g/d	**Minor side effects during the first week** of the intervention (viscous sensation (32%), diarrhea (11%), nausea (8%), and abdominal bloating (6%)) **Symptoms subsided within the second week of intake**, with only the discomfort of viscous sensation continuing
Babiker et al., (2012)	Adults	Acacia Gum	30 g/d	**Side effects were mild, experienced only in the first week and then resolved** They included unfavorable viscous sensation in the mouth (100%), early morning nausea

				(81.7%), mild diarrhea (90%) and bloating abdomen (15%)
Cherbut et al., (2003)	Adults	Acacia Gum	10-70 g/d	No difference in tolerance ≤30 g/d. Above this dose, the main complaint was excessive flatulence. However, the mean degree of severity remained mild (<1), even at doses >50 g/d. Other intestinal events were rarely reported.
Weber et al., (2014)	Children	10.5% Oligofructose, 12.5% Inulin, 24% Acacia Gum, 9% Resistant Starch, 33% Soy Polysaccharide, 12% Cellulose	3.8 and 7.6 g/d (based on participant weight; 3.8g/d when <18kg, 7.6 g/d when >18kg)	Adverse events were not observed for either group, and the products were well tolerated
Pedersen et al., (2013)	Adults	Oligofructose	15, 25, 35, 45, and 55 g/d	All doses were well tolerated with no apparent dose–response effect on GI symptom or general wellbeing scores.
Holscher et al., (2014)	Adults	Oligofructose	5 and 7.5 g/d	≤7.5 g/day led to minimal GI upset, with no change in diarrhea, and improved laxation
Liber et al., (2014)	Children	Oligofructose	8 g/d for children 7–11 ys 15 g/d for children 12–18 ys	Adverse effects measured including abdominal pain, flatulence, diarrhoea/loose stool, borborygmi, nausea, and heartburn, did not differ statistically from placebo

Nicolucci et al., (2017)	Children	Oligofructose - enriched inulin	8 g/d	No gastrointestinal side effects were experienced by 70% of the prebiotic group and 61% of the placebo. A mild ↑ in flatulence and bloating was experienced by 25% and 28% of subjects in prebiotic and placebo, respectively. A moderate ↑ in flatulence and bloating was reported by 5% and 11% of subjects in prebiotic and placebo, respectively. No reports of severe effects in either group.
Pasman et al., (2006)	Adults	Resistant Maltodextrin	30 or 45 g/d	Both doses were very well tolerated, and GI complaints did not differ greatly from the placebo treatment. Some habituation and adaptation of the GI symptoms occurred
Ye et al., (2015)	Adults	Resistant Maltodextrin	5 or 10 g/d	None reported
van den Heuvel (2004)	Adults	Resistant Maltodextrin	10, 30, and 60 g/day OR 10, 15, 45, and 80g/day	Well tolerated up to a dose of 45 g daily. Higher daily dosages (60 and 80 g) may result in flatulence but did not result in diarrhea.
Fastinger et al., (2008)	Adults	Resistant Maltodextrin	15 g/d	Very minor effects in gastrointestinal tolerance
Vuksan et al., (2009)	Children	PolyGlycopleX (PGX)	5g	No differences in gastrointestinal tolerance between groups
Carabin et al., (2009)	Adults	PolyGlycopleX (PGX)	2.5-10g/day	Well tolerated with only mild to moderate adverse gastrointestinal effects that did not differ from those seen in the control groups.

Our targeted supplemental fiber mixture (35 g total) will be composed of 6g of fiber from oligofructose + 10g from resistant maltodextrin + 12g from acacia gum + 4g from whole foods + 3g from RS2; and will be split into three meals each day.

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Appendix H – Pediatric dietary fiber tolerance critical review

Preface:

The following review was written as part of the AFNS 675 course requirement in the Fall 2017 semester at the University of Alberta. Knowledge derived from this critical review informed the rational of fiber selection for an upcoming clinical trial (NCT04150991)(Haqq, 2020).

Tolerance of Dietary Fiber Supplementation in Pediatric Populations

Increasing evidence suggests that dietary fiber can be an important factor in the promotion of health and the prevention of disease. Dietary fiber has been shown to have many downstream mediating effects on gastrointestinal (GI) health such as its role in the gut microbiome and modulation of metabolic activity. Unfortunately, there is less known about dietary fiber interventions in children and adolescents, with very few studies having been done on these populations. Dietary fiber recommendations for the pediatric population have been extrapolated from adult energy intake data rather than meticulous evidence-based criteria. In addition, there are several different fiber recommendations that currently exist for pediatric populations including the 'dietary reference intakes' that set the 'adequate intake' levels, the 'American Health Foundation' recommendations and finally the suggestions from the American Academy of Pediatrics[1]. These recommendations vary greatly making it very hard for translation to pediatric populations. Additionally, there are several subcategories that fall under the umbrella of "Dietary Fiber", each with unique properties that may affect both function and tolerability [1, 2]. The age-based fiber recommendations for children based off adult data do not specify the type or properties of the fibers included which may be necessary to support specific health

benefits at the suggested amount of intake. Observational studies have also shown that regardless of the intake guidelines used, a large majority of the pediatric population fail to meet the fiber recommendations. Dietary fibers (especially fermentable fibers) are often associated with undesirable GI symptoms [2]. In adult studies, support has been shown for using tolerance-based recommendations for specific dietary fibers. Tolerance, as described in this review (no significant increase in GI distress/symptoms) is often overlooked in the few fiber intervention studies of the pediatric population. To determine the type and dosage of fiber for intervention studies, several factors must be considered, such as feasibility of administration and tolerance of the fiber. This critical review will review the studies that have fed fiber to pediatric populations and assess how these fibers have been tolerated with the *overall purpose of making recommendations for the design of future trials.*

Fiber type and dosage: An overview- When reviewing the literature, it is apparent that comparing the tolerance of different fiber types and different doses of these fibers is a complicated and challenging issue. As described by Gabritske et al., (2009) [2] several host factors and features of the fiber itself can influence tolerance. Because of differences in manufacturers and overall composition, one may see variance in the chemical and physical properties of a given fiber. There is also the potential of additional ingredients in a mixture that could influence the purity and properties of the fiber content. For example, the glucomannan supplements used in the Horvath, et al. (2013) [3] and Chmielewska, et al., (2011) [4] studies were in a soluble powder format derived from the same source and had the same dosage. In contrast, the glucomannan used in the Martino et al., (2015) [5] study was in capsules made by a different manufacturer and thus precautions must be made when comparing these studies. Maltodextrin or cellulose were

selected as the placebo/control fiber in the majority of studies; these are considered to be suitable control fibers as they are non-fermentable and pass through the digestive tract relatively unchanged. For these reasons, they have been repeatedly found to not lead to GI distress [1, 2].

The objectives of the study ultimately decide the choice of fiber type; the benefits that have been shown or suggested by using the type of fiber, the feasibility of implication and the tolerance are all important factors to consider. Dosages used in the literature were provided as a g/day amount that was supplemented into the diet of participants. The rationale provided for the dosages used in the reviewed pediatric literature was often simply using the same doses as a previously published study of an adult population that had similar primary outcomes, or by using the same dose as another pediatric study that used the aforementioned rationale. Additionally, other than adjusting the doses for different age ranges within the study, no other considerations were mentioned to potentially standardize dosages based on a kg body weight or kcal basis. Determining appropriate dosage is a challenge with the current state of the literature and this is further complicated because different fiber types cannot be easily compared. In this review of tolerance in the pediatric population, oligofructuse supplemented into a participant's diet has been dosed as high as 15g/day (with apparently high tolerance for the measures of tolerance collected), whereas glucomannan is dosed no higher than 5g/day for the same age range (with similar tolerance outcomes). For example, if you compare the Liber[6] and Horvath[3] studies, the measures of abdominal pain (something both studies measured) was 30% and 32% for oligofructose and glucomannan groups respectively; this was using a daily dose of 8-15g of oligofructose compared to 2.5g of glucomannan. This is an indirect comparison as there are several other confounding factors that could impact this result [2], however this example is

meant to exemplify the limitation of assuming at a given dose that all fibers are equals in terms of their tolerability.

Overall, the ranges of doses for a given fiber type in the pediatric studies described were relatively narrow (0.75-15g/day oligofructose; 2-5g glucomannan, see table 1 or 2). This is in contrast to adult studies that have started to attempt to push the upper limits of dosages to the maximum of what can be tolerated comfortably [2, 7]. Taken as a whole, with the limited number of studies, it would not be appropriate to draw any conclusions regarding the tolerable dosages for any of the fiber types discussed in this review. The rationale for dosage in future studies needs to be more robust. Dosages used in the literature will be discussed further in the sections to come.

Measures and definitions of tolerance- A noteworthy obstacle in reviewing the literature is the varied ways of defining and measuring tolerability of the dietary fiber interventions that are presented in each study. In several papers, tolerance was compared between an experimental fiber group (prebiotic group) and a placebo control fiber group. For a robust measure of tolerability, several factors may be considered; the presence of a given symptom, the frequency in which a symptom occurs, the severity of the symptom, and when during the intervention does this symptom occur/for how long of a period does this symptom persist (temporality). Identifying these factors allows for a better understanding of the tolerability, which can be used when planning future trials. Hume et al., (2017) [8] (fed 8g of oligofructose-enriched inulin each day) was one of only three studies to look at tolerance in terms of the severity of symptoms (rankings of either mild, moderate, or severe). In this double-blind placebo-controlled study, only two

measures of tolerance were used, (flatulence and bloating) and these items were not separated when analyzed. Additionally, these measures did not account for the frequency of the GI symptoms and had no temporal component to determine when throughout the intervention these symptoms occurred. The double-blind, randomized, placebo-controlled crossover trial by Francois et al., (2014)[9] also assessed the severity of GI distress (fed 5g of wheat bran extract each day, which contained 3.95g of the active fiber component, arabinoxylan-oligosaccharides) through a daily self report (that was later aggregated into weekly averages for analysis). Three criteria: flatulence, urge to vomit, and abdominal pain/cramps were all assessed on a 5-step scale ranging from no (0), minimal (1), mild (2), moderate (3) and severe (4) distress. Unlike the Hume et al., (2017) study, these items were analyzed and presented separately in the results. Bowel habits were also assessed in this study by monitoring stool frequency, stool consistency, and using the composite Bristol stool scale [10]. Additional adverse effects were identified and categorized according to the 'National Cancer Institute Common Terminology Criteria for Adverse Events (version 3.0)'. These measures (GI distress, bowel habits and adverse effects) were measured during the run-in/baseline period and during the last week of the three-week treatment periods (fiber treatment period and placebo treatment period). Previous reviews of the adult literature suggest the first two weeks of a supplemental fiber intervention is when the most GI symptoms seem to be observed [11]. By only collecting measures during the final week of a three-week intervention, the study authors are observing the tolerance once this predicted period of adjustment has passed and are really studying if the treatment would be tolerated or adapted to in the long-term rather than if it is tolerable earlier in the intervention (when compliance is critical). Thus, the scope of the study is limited in this regard. The use of objective

and standardized measures, such as the Bristol stool scale, and the criteria for adverse events are a strong point of this study and future studies could benefit from similar standardization of measures. In a double-blind, randomized, crossover design trial by Vuksan et al., (2009) [12], subjects were told to consume three different beverages, which varied in fiber type. The beverages contained 5 g of either PGX (combination of xanthan, glucomannan, and sodium alginate dietary fibers), glucomannan (alone) or cellulose. Severity of bloating, belching, diarrhea, flatulence, and nausea were rated on a physical comfort visual analog scale (‘‘low’’ at one end (0) and ‘‘high’’ at the other (100)). An average score was calculated for each gastrointestinal symptom. These measures were taken before drinking the fiber supplemented beverage and in 15-minute intervals for 90 minutes (6 assessments) after consuming the beverage. After the 90 minutes, participants ate an ad libitum lunch and were asked to complete the physical comfort measure again. This procedure was repeated in three study visits (one for each fiber type). With further validation for the pediatric population, the use of a visual analog scale could be a useful alternative to written questionnaires, especially in younger children who would need parents to complete questionnaires for them. A major drawback of this study is that the very short time frame only provides us with an acute look at tolerance of the PGX and glucomannan fibers. More frequent/prolonged use of these fibers at the current dose (5g/day) may have differing GI side effects. Using the information recorded in a daily diary, Chmielewska et al.[4] reported the stool consistency (using the Bristol stool scale), stool frequency, abdominal pain, number of episodes of fecal soiling, number of episodes of painful defecation, and number of episodes of flatulence per week. This paper was one of the only studies to have both a frequency and temporal component to their data reporting, however the investigators did not record the severity of the

GI discomfort experienced (for those measures where this would be applicable). Both the Chmielewska [4] and Horvath [3] studies fed 2.52g of glucomannan daily to the participants. The Horvath [3] study looked at abdominal cramps, abdominal bloating/gassiness, number of episodes of nausea, vomiting, and changes in stool consistency (loose stools & constipated stools). These items are similar to those of the Chmielewska study however the way they are presented/categorized differs and thus the way subjects and researchers interpret these symptoms may differ (example of lack of standardization). The Martino et al., (2015) study with glucomannan (ranging from 2-4.5g/day based on age) did not specify any tolerance measures but instead asked participants to "report any adverse effects", of which none were reported. Liber et al., (2014)[6] (fed 8-15g of oligofructose or maltodextrin control fiber each day, with the dose depending on the age of the participants) considered and measured the following items as adverse effects to be monitored as tolerance measures: abdominal pain, flatulence, diarrhoea/loose stool, borborygmi, feeling of fullness, loss of appetite, nausea, and heartburn. This study also encouraged the participants to report any additional symptoms experienced, though no such additional symptoms were reported by the study authors. The Liber (8-15g/day oligofructose) [6] and the Horvath (2.25g/day glucomannan) [3] studies reported on a larger variety of tolerance measures, however there was no indication to the severity, frequency or temporality of these symptoms). The Moore (2003) study (0.75-3g/day oligofructose) provided a standardized (within the study) daily record log to each parent or caregiver with several GI symptoms to assess and rate ('less than usual', 'about usual' or 'more than usual' for each day of the study). This record was reviewed by the study coordinator during phone interviews each week. These specific results were not provided in the publication. Interestingly, while Liber et al.,

(2014) [6] indicates loss of appetite and feelings of fullness as adverse effects, these are beneficial outcomes for the Hume [8] and Vuksan [12] studies. In summary, there is a large amount of variation in measures and tools used to assess tolerance (see table 3), and several differences in how the same symptoms are categorized and presented. This exemplifies the lack of homogeneity in reports of tolerance in the literature. The lack of standardized and/or validated questionnaires/measures in the majority of studies makes comparison between studies especially challenging.

Tolerance outcomes- All withdrawals from the studies discussed were reported to be unrelated to intolerance of the treatment. No statistical difference between the experimental and control groups in terms of tolerance measures were found in the majority of studies, and all the fermentable fiber interventions (2-5g/day of glucomannan, 0.75-15g/day oligofructose, 3.95g/day arabinoxylan-oligosaccharides; dosages ranging by age groups) were reported to be well tolerated [3, 5, 6, 8, 9, 12-15]. For example, the Hume et al. (2017)[8] study reported no gastrointestinal side effects (flatulence and bloating) were experienced by 70% of the prebiotic group (8g/day of oligofructose-enriched inulin) and 61% of the placebo group (3.3g/day of maltodextrin). There were no reports of any severe ratings of GI distress (only mild and moderate ratings) and there were no statistically significant differences between the study groups. A prominent obstacle in the analysis of the current literature is that several studies do not provide a full breakdown of the analysis of individual tolerance outcome measures. The Moore [15] and Martino [5] studies simple state that there were no significant differences in the tolerance measures between study groups. Conversely, each measure of tolerability was analyzed individually and compared between the prebiotic and placebo groups in the Liber (8-15g/day

Oligofructose), Horvath (2.25g/day glucomannan), and Vuksan (5g/day PGX or 5g/day glucomannan) studies[3, 6, 12]. Very few reported incidents occurred in each of these studies and no significant differences were found between experimental and control groups. The only study that did report differences between the prebiotic and placebo groups was Chmielewska et al. (2011)[4] who found that abdominal pain episodes were more frequent in the glucomannan group (2.52g/day) at week 1 ($P = 0.04$) and week 4 ($P < 0.0001$) but were similar between groups at weeks 2 and 3. Post-hoc analysis determined that the risk of abdominal pain was greater (statistically significant) in the first 2 weeks of treatment for the Glucomannan group. Previous trials with fermentable fibers have shown a greater increased risk for GI distress in the first few weeks of intervention and thus, the result from the post-hoc analysis indicating greater risk in the first two weeks for the glucomannan group are not without precedent[11]. Nonetheless, no difference was found in the frequency of any other GI events measured in this study (episodes of fecal soiling, episodes of painful defecation, and episodes of flatulence). Additionally, despite reaching statistical significance, the incidence rate of the abdominal pain was very low and may not necessarily be biologically/functionally relevant. The other studies using glucomannan (with a dosage range from 2-5g/day in similarly aged populations) did not find significant differences in tolerance outcomes between groups. Several factors may influence the tolerability of dietary fiber. The following sections provides considerations and critical analysis of these additional factors.

Method of administration- The way the fiber is administered is another important factor of the tolerability of a fiber intervention. This can include the time of day, how the fiber is spread throughout the day, the time before/after a meal, if it is given as part of a meal (during), whether

adequate liquid is ingested alongside the fiber, and whether there is a period of time where the dose is reduced from the full dose to ease the participant into the intervention. Hume et al. (2017) [8] standardized the time of administration as well as the amount of liquid consumed along with the fiber across all the participants. They also gave a half dose for the first two weeks of the intervention to help minimize GI distress. Several studies split the dose to be taken twice daily (breakfast and dinner) [3-6, 9]. This has been shown to improve the tolerability of prebiotic interventions [2].

Because of the limited number of studies, it is hard to determine whether the method of administration played a significant role in the reported tolerability of the fiber. Despite differences in administration, 8g/day of oligofructose was tolerated well in overweight 7-11-year-old children. Despite having the exact same administration procedure and dosage (2.52g/day) as the Horvath study, an increase in abdominal pain was noted in the Chmielewska study. These two contrary examples emphasize that methods of administration are only one of many that may impact tolerance.

Compliance- Compliance and tolerance are two variables that are highly interconnected. Without knowing the compliance of the participants to the intervention, it is not possible to accurately make conclusions on the reported overall tolerance to the supplement. Reciprocally, if a treatment is difficult to tolerate, one could expect a decrease in participant compliance to the intervention. Hume et al., (2017) [8] and Liber et al., (2014) [6] assessed compliance by asking participants to return the packages of the fiber. While Hume et al., (2017) [8] asked for both empty and unused/partially used packets to be returned, Liber et al., (2014) [6] only asked for

the empty packets to be returned. In both these studies, compliance was high (87%-90%), indicating a strong adherence to study protocol and suggesting that either the treatments were tolerated, or the GI effects did not impact compliance. Hume et al., (2017)[8] took the tolerance and compliance measures one step further by assessing how participants felt about the ease of incorporation of the fiber into their everyday diet, with 61% reporting it to be very acceptable and 39% reporting it to be moderately acceptable; further supporting the idea that the administration of oligofructose-enriched inulin is tolerable at 8g/day for the population studied (7-12 year old male and female children with BMI-z scores over 85%). This additional survey provides a measure that can be used in the future to support additional intervention trials of this nature. In Horvath et al., (2013) [3] compliance was assessed by direct questioning of the subjects or their caregivers during clinic visits halfway through the study at 2 weeks and again after the full 4 weeks. Similar interview or verbal checks of compliance were done in the Moore, Chimiedwska and Martino studies [4, 5, 15], however the Martino study also had parents confirm that capsules were taken in the daily study diary. The percentage of subjects that complied to the study protocol was not reported in these studies; however, it was said to be statistically the same in both experimental and control groups. This method of measurement is less objective and makes it hard to compare to other studies. There is a widespread continuum of the depth and objectivity of compliance measures used throughout the studies, making it difficult to compare between studies; future trials would benefit from reporting of standardized compliance measures.

Study designs and populations: Other considerations- As with administration, other factors of study design can influence tolerance. The populations used in the reviewed literature vary

greatly (see table 2). Attempting to compare tolerance of a fiber type (e.g. glucomannan) between a healthy population [12] and a population with abdominal pain-related functional gastrointestinal disorders [3] for example, is not a one-to-one comparison and must be done with precaution. Duration of the interventions were long enough to determine if a fiber treatment was tolerated in all studies except for Vuksan et al., (2009)[12] who only had data from a single day (which is not enough to establish long term tolerance)[2, 11, 12]. Sex is another factor that has been identified as influencing tolerability of interventions. Sex differences were accounted for in the majority of studies by trying to have an equal number of males and females in the study groups and checking for sex related differences in outcomes. Again, the Vuksan group had almost 5 times more females to males in their study (25:6, F:M), which may potentially skew tolerability results (may mask potential differences in tolerance) [12]. Doses were not adjusted for weight or sex of participants and only Hume et al. (2017) ensured all participants were in the same pubertal stage (shown to influence tolerance). The sample sizes of the reviewed literature were relatively small, and more data is needed before set conclusions are drawn on tolerability of any doses of fiber presented in this review. Another potential confounding factor is the amount of dietary fiber already in the participant's diet. The only study that looked at baseline fiber intake (through weighed 3-day food records) was Hume et al., (2017) who collected baseline, midpoint, and final fiber intake measures. No statistically significant differences were found between groups. While they did not collect baseline fiber intake, Francois et al., (2014) prohibited the intake of foods containing probiotics and/or prebiotics and participants and their caregivers were asked to read product labels carefully to check for the presence of pro/prebiotics. This was the only study to have this stipulation in their

protocol. This precaution aided to minimize possible confounding factors. In addition, the cross-over designs of the Francois and Vuksan studies minimizes the confounding host factors that may impact tolerability and is therefore an excellent study design for the assessment of tolerance [2, 9, 12].

Conclusions and Future directions- In the reviewed pediatric literature, glucomannan was used and tolerated (as described by study authors) at the following doses: 2-2.25g/day for 3-6-year-old children and 2-5g/day for 7-18-year-old children. Oligofructose was used and tolerated at 3g/day for infants, 8g/day for 7-12-year-old children and at 15g/day for 12-18-year-old children. Wheat bran extract containing arabinoxylan-oligosaccharides was tolerated well at 5g/day (3.95g/day of the oligosaccharide). The lack of congruity between studies makes it difficult to draw conclusions on the tolerance of these fibers at these doses. Even when comparing the same type of fiber, differences in study design and populations make it difficult to aggregate and compare data. The rationale used for dosage is not standardized and often does not consider potential confounding factors (baseline fiber intake, age, sex, etc.). Evaluation of tolerance outcomes is often minimized, and the breakdown of data is not always provided, making it hard to interpret these results fully. Future studies would benefit from standardized and validated daily measures of tolerance to evaluate [2, 7] (for presence, frequency, severity and temporality, with the option to add other symptoms and comments) and a more standardized presentation of tolerance results, all of which could be added to supplementary material if not a primary outcome of the study in question. The standardized daily record log supplied to each participant, parent or caregiver should be reviewed by the study coordinator during phone interviews each week as was done in the Moore study [15]. Ideal measures of

compliance would be similar to Hume et al., (2017) [8] and would include indicating how much of the package participants consumed each day, the ease of incorporation, and the return of all the packets (both fully or partially consumed). Parental verification of the treatment would also be beneficial, especially along-side weekly phone calls to review the tolerance data collected thus far. Repeated trials with the same study design (preferably double-blind randomized placebo-controlled crossover trials) and populations are needed to establish the best methods of administration and to determine tolerable doses of a given fiber. Overall, a better understanding of tolerance can help with future study design and potentially influence future pediatric guidelines.

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Table 2: Specific extraction table view

Study	Population	Age	Prebiotic fiber of interest	Prebiotic dose	Control used	Control dose used	Intervention Duration
Horvath et al., (2013)	Male and female children (7-17) with abdominal pain-related functional gastrointestinal disorders classified according to the Rome Ⅲ diagnostic criteria.	7-17 years old	**Glucomannan**	**2.25g/day**	Maltodextrin	**2.25g/day**	4 weeks
Chmielewska et al., (2011)	Male and female children (3-16) with functional constipation classified according to the Rome Ⅲ diagnostic criteria.	3-16 years old	**Glucomannan**	**2.25g/day**	Maltodextrin	**2.25g/day**	4 weeks
Martino et al., (2015)	Hypercholesterolemic male and female children (4-12)	4-6 years old	**Glucomannan**	<6 years old: 2 (lunch) x 2(dinner) x 0.5g/capsule =**2g /day**	Diet plan without glucomannan	**0g/day**	8 weeks
		7-12 years old	**Glucomannan**	>6 years old: 3(lunch) x 3(dinner) x 0.5g/capsule = **4.5g /day**	Diet plan without glucomannan	**0g/day**	
Vuksan et al., (2009)	Male and female healthy weight adolescents (15-18)	15-18 years old	**Glucomannan**	**5 g**	Cellulose	**5g**	1 study day for each fiber (total of 3 study days)
		15-18 years old	**PGX** (xanthan, glucomannan, and sodium alginate)	**5 g**	Cellulose	**5g**	1 study day for each fiber (total of 3 study days)
Liber et al., (2014)	Male and female children (7-18) with overweight or obesity	7-11 years old	**Oligofructose**	**8g/day**	Maltodextrin	**3.3g/day** *energy equivalant dose (kcal/day) to the prebiotic	12 weeks
		12-18 years old	**Oligofructose**	**15g/day**	Maltodextrin	**6.2g/day** *energy equivalant doses (kcal/day) to the prebiotic	
Hume et al., (2017)	Male and female children (7-12) with overweight or obesity but otherwise healthy and Tanner developmental stage ≤ 3	7–12 years old	**Oligofructose-enriched inulin**	**8g/day**	Maltodextrin	**3.3g/day** *energy equivalant doses (kcal/day) to the prebiotic	16 weeks
Moore et al., (2003)	Male and female healthy term infants (4-12 months)	4-12 Months	**Oligofructose**	minimum of 0.75g/day with a resulting range of **0.75-3.00g/day**	Cereal without oligofructose	0g/day	28 days
Francois et al., (2014)	Healthy male and female children (8-12)	8-12 years old	Wheat bran extract containing **arabinoxylan-oligosaccharides**	5 g/day of Wheat bran extract **3.95g/day** of the active arabinoxylan-oligosaccharide ingrediant	Beverage without prebiotic	0g/day	3 weeks

Table 3: Measures of tolerance considered in each study

Study	Prebiotic fiber of interest	Abdominal bloating	Abdominal pain/cramps	Flatulence	Stool frequency	Changes in stool consistency (constipation)	Changes in stool consistency (loose stools/diarrhea)	Borborygmi	Feeling of fullness	Loss of appetite	Nausea	Heartburn	Vomiting	Belching	Fecal soiling	Painful defication	Crying /colic	Undefined adverse effects
Horvath et al., (2013)	**Glucomannan**																	
Chmielewska et al., (2011)	**Glucomannan**																	
Martino et al., (2015)	**Glucomannan**																	
Vuksan et al., (2009)	**PGX** (xanthan, glucomannan, and sodium alginate) and **Glucomannan**																	
Liber et al., (2014)	**Oligofructose**																	
Moore et al., (2003)	**Oligofructose**																	
Hume et al., (2017)	**Oligofructose-enriched inulin**																	
Francois et al., (2014)	Wheat bran extract containing **arabinoxylan-oligosaccharides**																	

Supplementary material

Inclusion criteria:

1) Added fiber types that had the potential to cause gastrointestinal effects when consumed in excess were identified (based on clinical evidence in the scientific literature.)
2) Studies had to report tolerance in some way.
3) research had to be using a pediatric population (0-18 years old).

The following information was extracted and analyzed to critically review the tolerance outcomes of each study (table1). 1) Overall study Design & Population used, 2) Fibers Used/Assessed, 3) Dosage of fiber, 4) Duration of intervention, 5) Compliance measures, 6) Administration method, 7) Definition of "Tolerance outcomes", 8) Method used to evaluate Tolerance.

Appendix I – Tolerance pilot study journal

The Gut Microbiome in PWS Fiber Intervention Study

Study Journal

Please use this journal to help you keep track of everything you need to do throughout this study.

Thank you for your help!

Date Range of Study: __/__/__ To __/__/__ Fiber dose: ____ g/day

(mm/dd/yy) (mm/dd/yy)

If at any time you have questions, issues, or would like something explained further, please feel free to contact:

Shima Afhami

Telephone Number: [redacted] (text/call) or **Email Address:** [redacted]

How to Add Fiber to your Diet

Do not eat the fiber on its own!

Make sure you eat the fiber with other foods and preferably with your meals.

Spread the fiber out evenly throughout the day.

Try and add it to all your meals in similar amounts.

Drink plenty of fluids!

Make sure to drink fluids not only with the fiber, but also throughout the day.

It is recommended that you drink more than _____ liters per day of fluids.

General Tips for Adding Fiber to your Food

- Just add it to **yogurt** or pudding, mix, and enjoy!
- It can be added to **water**, **juice**, **smoothies** and even **milkshakes**
- **Scrambled eggs** and **omelets** also work
- Try adding it to **hot cereals** like oatmeal
- Add the fiber to **pancakes**, **muffins**, **cakes**, **dinner rolls**, (quick) **breads**, **waffles**, **biscuits**, and **even cookies**!
  - Just replace ~ 1/4 of the flour with the fiber
- Added it to **dips** like hummus or spinach dip
- Try adding to different **sauces**, especially thick sauces like pasta sauce or creamed sauces
- The fiber can easily be added to **mashed potatoes**.
- Add it to your thicker **soups**, **chili**, **curries** or **stew**
- Add it with your condiments on **sandwiches** and **burritos**
- Add it to **ground meats** before cooking them, like hamburgers
- Try adding it to **casseroles** before or after baking. You can even mix it into a smaller portion and bake it separately.
- Add fiber to the **batter** or **bread crumb mix** before coating your meat, poultry, seafood, or vegetables.
- Keep in mind - **be creative** - there are many ways to at the fiber
- For more idea, don't hesitate to **ask us**!

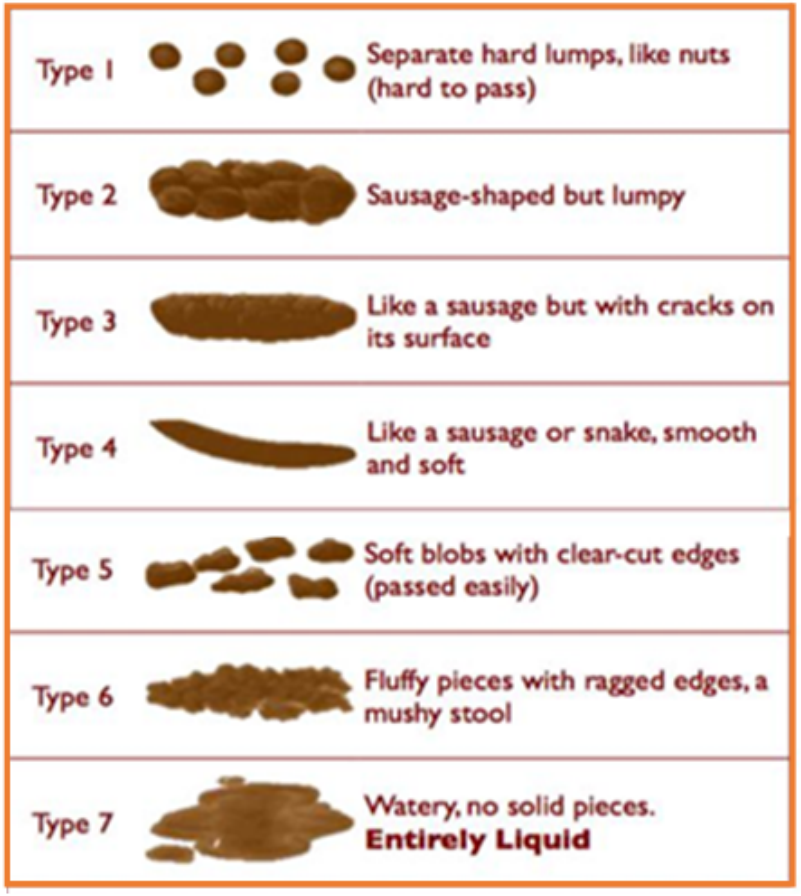
Text/Call: [redacted] or Email: [redacted]

BOWEL HABITS QUESTIONNAIRE

Participant ID:_____

We want to know how participants are tolerating the fiber. This would include any changes in your child's digestive system or related symptoms. Please record and **describe each bowel movement your child has** by using this bowel habits questionnaire. If your child does not have a bowel movement on any of these given days, then just note the date and circle 'no'. If more space is need, please record on another piece of paper.

Bristol Stool Chart



Bowel Movement			Bristol Stool Scale
DATE M/D/Y	Bowel Movement	TIME AM/PM *(hr:min)*	Rate fecal consistency as 1 through 7 per the Bristol Stool Chart
	YES NO	: AM / PM	1 2 3 4 5 6 7
	YES NO	: AM / PM	1 2 3 4 5 6 7
	YES NO	: AM / PM	1 2 3 4 5 6 7
	YES NO	: AM / PM	1 2 3 4 5 6 7
	YES NO	: AM / PM	1 2 3 4 5 6 7
	YES NO	: AM / PM	1 2 3 4 5 6 7
	YES NO	: AM / PM	1 2 3 4 5 6 7
	YES NO	: AM / PM	1 2 3 4 5 6 7
	YES NO	: AM / PM	1 2 3 4 5 6 7

Schedule Overview

Use this calendar to help keep track of everything that needs to be done over the next week. To help even more, **please feel free to write on the calendar** as you see fit.

Day 0	*Day 1*	*Day 2*	*Day 3*	*Day 4*	*Day 5*	*Day 6*	*Day 7*
• Clinic Meeting • Baseline Tolerance Questionnaire • Baseline Bowel Habits Questionnaires	• ½ dose of fiber • Tolerance Questionnaire • Bowel Habits Questionnaires	• ½ dose of fiber • Tolerance Questionnaire • Bowel Habits Questionnaires	• full dose of fiber • Tolerance Questionnaire • Bowel Habits Questionnaires	• full dose of fiber • Tolerance Questionnaire • Bowel Habits Questionnaires	• full dose of fiber • Tolerance Questionnaire • Bowel Habits Questionnaires	• full dose of fiber • Tolerance Questionnaire • Bowel Habits Questionnaires	• full dose of fiber • Tolerance Questionnaire • Bowel Habits Questionnaires • Overall Tolerance Questionnaire • Additional suggestions survey

***Please return the study journal once it is complete via the return envelope provided.**

OVERVIEW-WHAT TO DO?

Participant ID: _____ Date Range: _____

Date	Dosage Taken	Symptoms and Comments
Day 1 ____	○ None of it ○ 25% ○ Half ○ 75% ○ All of it	
Day 2 ____	○ None of it ○ 25% ○ Half ○ 75% ○ All of it	
Day 3 ____	○ None of it ○ 25% ○ Half ○ 75% ○ All of it	
Day 4 ____	○ None of it ○ 25% ○ Half ○ 75% ○ All of it	
Day 5 ____	○ None of it ○ 25% ○ Half ○ 75% ○ All of it	
Day 6 ____	○ None of it ○ 25% ○ Half ○ 75% ○ All of it	
Day 7 ____	○ None of it ○ 25% ○ Half ○ 75% ○ All of it	

For the first 2 days, please the 'Day 1' & 'Day 2' packets to your diet. Consume 1 packet/day for the remainder of the study. In the table below please check the amount you consumed each day, and any symptoms or comments. Please record ***anything*** that changes from adding the fiber, such as side effects or any other observations. If you have **ANY** concerns or issues, please call the study coordinators at [redacted]

Check List

- ☐ Consume the Day 1 & Day 2 packets for the first two days (half dose packets)
- ☐ Eat 1 packet/day for the remainder of the study. At the end of each day, remember to check off the amount that you ate.
- ☐ Write down any symptoms or changes that you noticed.
- ☐ Complete the tolerance questionnaires around the same time of day during the week.
- ☐ Describe each bowel movement your child has during the study period using the bowel habits questionnaire.
- ☐ Complete the Overall Tolerance and additional suggestions surveys.

TOLERANCE QUESTIONNAIRE - Day 0 (No fiber)

Participant ID: _____
Completed Date: __/__/____
(mm/dd/yy)

As part of this study, we want to know how participants are tolerating the dietary fiber. This would include any changes in your digestive system or related symptoms. To complete the questionnaire, please mark **one** response for each question describing your child's tolerance of the dietary fiber. If you have any additional comments, please feel free to write them in the available space. Please complete this questionnaire each time on the **about the same time each day**. Remember to write down the date you completed it. If you have **ANY** concerns or issues with tolerance, please call the study coordinators at [redacted]

1.) Has your child experienced any stomach aches and pains recently, if so how would you rate its severity?

- ☐ 0 - No stomach-aches or pains
- ☐ 1 - Very minor stomach-aches or pains that do not affect daily activities
- ☐ 2 - Mild stomach-aches or pains that affects some daily activities
- ☐ 3 - Moderate stomach-aches or pains that affects many daily activities
- ☐ 4 - Severe stomach-aches or pains that significantly affect daily activities

2.) How would you rate the abdominal distension or bloating experienced by your child recently?

- ☐ 0 - No discomfort
- ☐ 1 - Very minor discomfort with no impact on daily activities
- ☐ 2 - Occasional and brief discomfort that slightly impacts daily activities
- ☐ 3 - Frequent and prolonged discomfort that impacts many daily activities
- ☐ 4 - Severe and continuous discomfort that significantly impacts daily activities

3.) Has your child experienced any flatulence or gas recently, if so how often and to what extent?

- ☐ 0 - No gas
- ☐ 1 - Very minor gas that has no impact on daily activities
- ☐ 2 - Occasional and brief gas that slightly impacts daily activities
- ☐ 3 - Frequent and prolonged gas that impacts many daily activities
- ☐ 4 - Severe and excessive gas that significantly impacts daily activities

4.) How would you rate your child's overall physical well-being?

- ☐ 0 - Very well tolerated with no symptoms
- ☐ 1 - Well tolerated with very minor symptoms
- ☐ 2 - Tolerated with mild symptoms
- ☐ 3 - Poorly tolerated with moderate symptoms
- ☐ 4 - Not tolerated with severe symptoms

Any Additional Comments?

TOLERANCE QUESTIONNAIRE - Day 1 (half dose)

Participant ID: _____
Completed Date: __/__/____
(mm/dd/yy)

As part of this study, we want to know how participants are tolerating the dietary fiber. This would include any changes in your digestive system or related symptoms. To complete the questionnaire, please mark **one** response for each question describing your child's tolerance of the dietary fiber. If you have any additional comments, please feel free to write them in the available space. Please complete this questionnaire each time on the **about the same time each day**. Remember to write down the date you completed it. If you have **ANY** concerns or issues with tolerance, please call the study coordinators at [redacted]

1.) Has your child experienced any stomach aches and pains recently, if so how would you rate its severity?

- ☐ 0 - No stomach-aches or pains
- ☐ 1 - Very minor stomach-aches or pains that do not affect daily activities
- ☐ 2 - Mild stomach-aches or pains that affects some daily activities
- ☐ 3 - Moderate stomach-aches or pains that affects many daily activities
- ☐ 4 - Severe stomach-aches or pains that significantly affect daily activities

2.) How would you rate the abdominal distension or bloating experienced by your child recently?

- ☐ 0 - No discomfort
- ☐ 1 - Very minor discomfort with no impact on daily activities
- ☐ 2 - Occasional and brief discomfort that slightly impacts daily activities
- ☐ 3 - Frequent and prolonged discomfort that impacts many daily activities
- ☐ 4 - Severe and continuous discomfort that significantly impacts daily activities

3.) Has your child experienced any flatulence or gas recently, if so how often and to what extent?

- ☐ 0 - No gas
- ☐ 1 - Very minor gas that has no impact on daily activities
- ☐ 2 - Occasional and brief gas that slightly impacts daily activities
- ☐ 3 - Frequent and prolonged gas that impacts many daily activities
- ☐ 4 - Severe and excessive gas that significantly impacts daily activities

4.) How would you rate your child's overall physical well-being and tolerance of the fiber?

- ☐ 0 - Very well tolerated with no symptoms
- ☐ 1 - Well tolerated with very minor symptoms
- ☐ 2 - Tolerated with mild symptoms
- ☐ 3 - Poorly tolerated with moderate symptoms
- ☐ 4 - Not tolerated with severe symptoms

Any Additional Comments?

TOLERANCE QUESTIONNAIRE - Day 2 (half dose)

Participant ID: _____
Completed Date: __/__/____
(mm/dd/yy)

[redacted]

As part of this study, we want to know how participants are tolerating the dietary fiber. This would include any changes in your digestive system or related symptoms. To complete the questionnaire, please mark **one** response for each question describing your child's tolerance of the dietary fiber. If you have any additional comments, please feel free to write them in the available space. Please complete this questionnaire each time on the **about the same time each day**. Remember to write down the date you completed it. If you have **ANY** concerns or issues with tolerance, please call the study coordinators at [redacted]

1.) Has your child experienced any stomach aches and pains recently, if so how would you rate its severity?

- ☐ 0 - No stomach-aches or pains
- ☐ 1 - Very minor stomach-aches or pains that do not affect daily activities
- ☐ 2 - Mild stomach-aches or pains that affects some daily activities
- ☐ 3 - Moderate stomach-aches or pains that affects many daily activities
- ☐ 4 - Severe stomach-aches or pains that significantly affect daily activities

2.) How would you rate the abdominal distension or bloating experienced by your child recently?

- ☐ 0 - No discomfort
- ☐ 1 - Very minor discomfort with no impact on daily activities
- ☐ 2 - Occasional and brief discomfort that slightly impacts daily activities
- ☐ 3 - Frequent and prolonged discomfort that impacts many daily activities
- ☐ 4 - Severe and continuous discomfort that significantly impacts daily activities

3.) Has your child experienced any flatulence or gas recently, if so how often and to what extent?

- ☐ 0 - No gas
- ☐ 1 - Very minor gas that has no impact on daily activities
- ☐ 2 - Occasional and brief gas that slightly impacts daily activities
- ☐ 3 - Frequent and prolonged gas that impacts many daily activities
- ☐ 4 - Severe and excessive gas that significantly impacts daily activities

4.) How would you rate your child's overall physical well-being and tolerance of the fiber?

- ☐ 0 - Very well tolerated with no symptoms
- ☐ 1 - Well tolerated with very minor symptoms
- ☐ 2 - Tolerated with mild symptoms
- ☐ 3 - Poorly tolerated with moderate symptoms
- ☐ 4 - Not tolerated with severe symptoms

Any Additional Comments?

TOLERANCE QUESTIONNAIRE - Day 3 (full dose)

Participant ID: _____
Completed Date: __/__/____
(mm/dd/yy)

As part of this study, we want to know how participants are tolerating the dietary fiber. This would include any changes in your digestive system or related symptoms. To complete the questionnaire, please mark **one** response for each question describing your child's tolerance of the dietary fiber. If you have any additional comments, please feel free to write them in the available space. Please complete this questionnaire each time on the **about the same time each day**. Remember to write down the date you completed it. If you have **ANY** concerns or issues with tolerance, please call the study coordinators at [redacted]

1.) Has your child experienced any stomach aches and pains recently, if so how would you rate its severity?

- ☐ 0 - No stomach-aches or pains
- ☐ 1 - Very minor stomach-aches or pains that do not affect daily activities
- ☐ 2 - Mild stomach-aches or pains that affects some daily activities
- ☐ 3 - Moderate stomach-aches or pains that affects many daily activities
- ☐ 4 - Severe stomach-aches or pains that significantly affect daily activities

2.) How would you rate the abdominal distension or bloating experienced by your child recently?

- ☐ 0 - No discomfort
- ☐ 1 - Very minor discomfort with no impact on daily activities
- ☐ 2 - Occasional and brief discomfort that slightly impacts daily activities
- ☐ 3 - Frequent and prolonged discomfort that impacts many daily activities
- ☐ 4 - Severe and continuous discomfort that significantly impacts daily activities

3.) Has your child experienced any flatulence or gas recently, if so how often and to what extent?

- ☐ 0 - No gas
- ☐ 1 - Very minor gas that has no impact on daily activities
- ☐ 2 - Occasional and brief gas that slightly impacts daily activities
- ☐ 3 - Frequent and prolonged gas that impacts many daily activities
- ☐ 4 - Severe and excessive gas that significantly impacts daily activities

4.) How would you rate your child's overall physical well-being and tolerance of the fiber?

- ☐ 0 - Very well tolerated with no symptoms
- ☐ 1 - Well tolerated with very minor symptoms
- ☐ 2 - Tolerated with mild symptoms
- ☐ 3 - Poorly tolerated with moderate symptoms
- ☐ 4 - Not tolerated with severe symptoms

Any Additional Comments?

TOLERANCE QUESTIONNAIRE - Day 4 (full dose)

Participant ID: _____
Completed Date: __/__/____
(mm/dd/yy)

As part of this study, we want to know how participants are tolerating the dietary fiber. This would include any changes in your digestive system or related symptoms. To complete the questionnaire, please mark **one** response for each question describing your child's tolerance of the dietary fiber. If you have any additional comments, please feel free to write them in the available space. Please complete this questionnaire each time on the **about the same time each day**. Remember to write down the date you completed it. If you have **ANY** concerns or issues with tolerance, please call the study coordinators at [redacted]

1.) Has your child experienced any stomach aches and pains recently, if so how would you rate its severity?

- ☐ 0 - No stomach-aches or pains
- ☐ 1 - Very minor stomach-aches or pains that do not affect daily activities
- ☐ 2 - Mild stomach-aches or pains that affects some daily activities
- ☐ 3 - Moderate stomach-aches or pains that affects many daily activities
- ☐ 4 - Severe stomach-aches or pains that significantly affect daily activities

2.) How would you rate the abdominal distension or bloating experienced by your child recently?

- ☐ 0 - No discomfort
- ☐ 1 - Very minor discomfort with no impact on daily activities
- ☐ 2 - Occasional and brief discomfort that slightly impacts daily activities
- ☐ 3 - Frequent and prolonged discomfort that impacts many daily activities
- ☐ 4 - Severe and continuous discomfort that significantly impacts daily activities

3.) Has your child experienced any flatulence or gas recently, if so how often and to what extent?

- ☐ 0 - No gas
- ☐ 1 - Very minor gas that has no impact on daily activities
- ☐ 2 - Occasional and brief gas that slightly impacts daily activities
- ☐ 3 - Frequent and prolonged gas that impacts many daily activities
- ☐ 4 - Severe and excessive gas that significantly impacts daily activities

4.) How would you rate your child's overall physical well-being and tolerance of the fiber?

- ☐ 0 - Very well tolerated with no symptoms
- ☐ 1 - Well tolerated with very minor symptoms
- ☐ 2 - Tolerated with mild symptoms
- ☐ 3 - Poorly tolerated with moderate symptoms
- ☐ 4 - Not tolerated with severe symptoms

Any Additional Comments?

TOLERANCE QUESTIONNAIRE - Day 5 (full dose)

Participant ID: _____
Completed Date: __/__/____
(mm/dd/yy)

As part of this study, we want to know how participants are tolerating the dietary fiber. This would include any changes in your digestive system or related symptoms. To complete the questionnaire, please mark **one** response for each question describing your child's tolerance of the dietary fiber. If you have any additional comments, please feel free to write them in the available space. Please complete this questionnaire each time on the **about the same time each day**. Remember to write down the date you completed it. If you have **ANY** concerns or issues with tolerance, please call the study coordinators at [redacted]

1.) Has your child experienced any stomach aches and pains recently, if so how would you rate its severity?

- ☐ 0 - No stomach-aches or pains
- ☐ 1 - Very minor stomach-aches or pains that do not affect daily activities
- ☐ 2 - Mild stomach-aches or pains that affects some daily activities
- ☐ 3 - Moderate stomach-aches or pains that affects many daily activities
- ☐ 4 - Severe stomach-aches or pains that significantly affect daily activities

2.) How would you rate the abdominal distension or bloating experienced by your child recently?

- ☐ 0 - No discomfort
- ☐ 1 - Very minor discomfort with no impact on daily activities
- ☐ 2 - Occasional and brief discomfort that slightly impacts daily activities
- ☐ 3 - Frequent and prolonged discomfort that impacts many daily activities
- ☐ 4 - Severe and continuous discomfort that significantly impacts daily activities

3.) Has your child experienced any flatulence or gas recently, if so how often and to what extent?

- ☐ 0 - No gas
- ☐ 1 - Very minor gas that has no impact on daily activities
- ☐ 2 - Occasional and brief gas that slightly impacts daily activities
- ☐ 3 - Frequent and prolonged gas that impacts many daily activities
- ☐ 4 - Severe and excessive gas that significantly impacts daily activities

4.) How would you rate your child's overall physical well-being and tolerance of the fiber?

- ☐ 0 - Very well tolerated with no symptoms
- ☐ 1 - Well tolerated with very minor symptoms
- ☐ 2 - Tolerated with mild symptoms
- ☐ 3 - Poorly tolerated with moderate symptoms
- ☐ 4 - Not tolerated with severe symptoms

Any Additional Comments?

TOLERANCE QUESTIONNAIRE - Day 6 (full dose)

Participant ID: _____
Completed Date: __/__/____
(mm/dd/yy)

As part of this study, we want to know how participants are tolerating the dietary fiber. This would include any changes in your digestive system or related symptoms. To complete the questionnaire, please mark **one** response for each question describing your child's tolerance of the dietary fiber. If you have any additional comments, please feel free to write them in the available space. Please complete this questionnaire each time on the **about the same time each day**. Remember to write down the date you completed it. If you have **ANY** concerns or issues with tolerance, please call the study coordinators at [redacted]

1.) Has your child experienced any stomach aches and pains recently, if so how would you rate its severity?

- ☐ 0 - No stomach-aches or pains
- ☐ 1 - Very minor stomach-aches or pains that do not affect daily activities
- ☐ 2 - Mild stomach-aches or pains that affects some daily activities
- ☐ 3 - Moderate stomach-aches or pains that affects many daily activities
- ☐ 4 - Severe stomach-aches or pains that significantly affect daily activities

2.) How would you rate the abdominal distension or bloating experienced by your child recently?

- ☐ 0 - No discomfort
- ☐ 1 - Very minor discomfort with no impact on daily activities
- ☐ 2 - Occasional and brief discomfort that slightly impacts daily activities
- ☐ 3 - Frequent and prolonged discomfort that impacts many daily activities
- ☐ 4 - Severe and continuous discomfort that significantly impacts daily activities

3.) Has your child experienced any flatulence or gas recently, if so how often and to what extent?

- ☐ 0 - No gas
- ☐ 1 - Very minor gas that has no impact on daily activities
- ☐ 2 - Occasional and brief gas that slightly impacts daily activities
- ☐ 3 - Frequent and prolonged gas that impacts many daily activities
- ☐ 4 - Severe and excessive gas that significantly impacts daily activities

4.) How would you rate your child's overall physical well-being and tolerance of the fiber?

- ☐ 0 - Very well tolerated with no symptoms
- ☐ 1 - Well tolerated with very minor symptoms
- ☐ 2 - Tolerated with mild symptoms
- ☐ 3 - Poorly tolerated with moderate symptoms
- ☐ 4 - Not tolerated with severe symptoms

Any Additional Comments?

TOLERANCE QUESTIONNAIRE - Day 7 (full dose)

Participant ID: _____
Completed Date: __/__/____
(mm/dd/yy)

As part of this study, we want to know how participants are tolerating the dietary fiber. This would include any changes in your digestive system or related symptoms. To complete the questionnaire, please mark **one** response for each question describing your child's tolerance of the dietary fiber. If you have any additional comments, please feel free to write them in the available space. Please complete this questionnaire each time on the **about the same time each day**. Remember to write down the date you completed it. If you have **ANY** concerns or issues with tolerance, please call the study coordinators at [redacted]

1.) Has your child experienced any stomach aches and pains recently, if so how would you rate its severity?

- ☐ 0 - No stomach-aches or pains
- ☐ 1 - Very minor stomach-aches or pains that do not affect daily activities
- ☐ 2 - Mild stomach-aches or pains that affects some daily activities
- ☐ 3 - Moderate stomach-aches or pains that affects many daily activities
- ☐ 4 - Severe stomach-aches or pains that significantly affect daily activities

2.) How would you rate the abdominal distension or bloating experienced by your child recently?

- ☐ 0 - No discomfort
- ☐ 1 - Very minor discomfort with no impact on daily activities
- ☐ 2 - Occasional and brief discomfort that slightly impacts daily activities
- ☐ 3 - Frequent and prolonged discomfort that impacts many daily activities
- ☐ 4 - Severe and continuous discomfort that significantly impacts daily activities

3.) Has your child experienced any flatulence or gas recently, if so how often and to what extent?

- ☐ 0 - No gas
- ☐ 1 - Very minor gas that has no impact on daily activities
- ☐ 2 - Occasional and brief gas that slightly impacts daily activities
- ☐ 3 - Frequent and prolonged gas that impacts many daily activities
- ☐ 4 - Severe and excessive gas that significantly impacts daily activities

4.) How would you rate your child's overall physical well-being and tolerance of the fiber?

- ☐ 0 - Very well tolerated with no symptoms
- ☐ 1 - Well tolerated with very minor symptoms
- ☐ 2 - Tolerated with mild symptoms
- ☐ 3 - Poorly tolerated with moderate symptoms
- ☐ 4 - Not tolerated with severe symptoms

Any Additional Comments?

TOLERANCE QUESTIONNAIRE - Day 7
(OVERALL IMPRESSION)

Participant ID: _____
Completed Date: __/__/____
(mm/dd/yy)

As part of this study, we want to know how participants are tolerating the dietary fiber. This would include any changes in your digestive system or related symptoms. To complete the questionnaire, please mark **one** response for each question describing your child's tolerance of the dietary fiber ***OVER THE PAST WEEK***. If you have any additional comments, please feel free to write them in the available space. Please complete this questionnaire each time on the **about the same time each day**. Remember to write down the date you completed it. If you have **ANY** concerns or issues with tolerance, please call the study coordinators at [redacted]

1.) Has your child experienced any stomach aches and pains since starting the fiber? If so how would you rate its severity?

- ☐ 0 - No stomach-aches or pains
- ☐ 1 - Very minor stomach-aches or pains that do not affect daily activities
- ☐ 2 - Mild stomach-aches or pains that affects some daily activities
- ☐ 3 - Moderate stomach-aches or pains that affects many daily activities
- ☐ 4 - Severe stomach-aches or pains that significantly affect daily activities

2.) How would you rate the abdominal distension or bloating experienced by your child over the past week?

- ☐ 0 - No discomfort
- ☐ 1 - Very minor discomfort with no impact on daily activities
- ☐ 2 - Occasional and brief discomfort that slightly impacts daily activities
- ☐ 3 - Frequent and prolonged discomfort that impacts many daily activities
- ☐ 4 - Severe and continuous discomfort that significantly impacts daily activities

5.) How would you rate the feasibility and acceptability of incorporating the extra fiber into your child's diet this week?

- ☐ 0 – Very easy to add to daily routine/ very acceptable (neutral)
- ☐ 1 – Relatively easy with very minor difficulty/inconvenience (mild)
- ☐ 2 – Somewhat difficult to add to daily routine (moderate)
- ☐ 3 – Very difficult with major inconvenience (severe)

3.) Has your child experienced any flatulence or gas this week? If so how often and to what extent?

- ☐ 0 - No gas
- ☐ 1 - Very minor gas that has no impact on daily activities
- ☐ 2 - Occasional and brief gas that slightly impacts daily activities
- ☐ 3 - Frequent and prolonged gas that impacts many daily activities
- ☐ 4 - Severe and excessive gas that significantly impacts daily activities

4.) How would you rate your child's overall physical well-being and tolerance of the fiber this week?

- ☐ 0 - Very well tolerated with no symptoms
- ☐ 1 - Well tolerated with very minor symptoms
- ☐ 2 - Tolerated with mild symptoms
- ☐ 3 - Poorly tolerated with moderate symptoms
- ☐ 4 - Not tolerated with severe symptoms

Any Additional Comments?

Fiber Incorporation – Final thoughts

If there were any challenges you faced incorporating the fiber, please describe them below.

- **Any suggestions** and/or additional comments would be greatly appreciated as we would like to incorporate your ideas to improve the acceptability of the intervention for the full trial when the time comes.

If you would prefer to discuss your experience over the phone or in person, please indicate this below and our study coordinator will arrange a time to discuss. You may also write your thoughts below and ask to be contacted for an additional follow-up discussion. Your input is very valuable to us and we sincerely appreciate your time.

I would like to be contacted to discuss my experience over the phone ☐ YES ☐ NO