Fecal Microbial Composition in Relation to Diet and Body Mass Index

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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Emerging evidence from animal and human studies has indicated a potential link between a certain pattern of gut microbiota and adiposity. In the current study, the relationship between gut microbial composition and BMI was further investigated in human with the incorporation of macronutrient consumption and quantification of methanogenic Archaea. BMI was found to be negatively associated with the amount of fecal Bacteroides/Prevotella, E. coli and total bacteria. The intake of carbohydrate and dietary fiber were negatively associated with the amount of C. coccoides, and polyunsaturated fat intake was inversely correlated with the amount of fecal C. leptum, Bacteroides/Prevotella, and total bacteria. Fecal Archaea measured by breath CH₄ was positively associated with the amount of fecal E.coli but negatively associated with C. coccoides and log Firmicutes/Bacteroidetes ratio. In conclusion, our findings suggest that fecal bacterial composition is associated with BMI, diet and fecal Archaea.
Acknowledgments

Firstly, I would like to express my sincere gratitude to my supervisor, Dr. Elena Comelli, for her persistent help and invaluable guidance along every stage of this Masters project. I am grateful for her knowledgeable, insight, and inspiration, and it is my greatest fortune to have her as my mentor both in science and in life, and will be indebted to her forever for her support.

Special thanks to my co-supervisor, Dr. Thomas Wolever, for his unfailing support and tremendous insights towards this project, and I benefited considerably from his feedback.

Also, I want to extend my gratefulness to my advisory committee member, Dr. Ahmed El-Sohemy for his expertise and encouragement during my graduate studies.

Thanks to all members (past or present) of the Comelli lab who supported me in this project: Angela Wang, Christopher Villa, Natasha Singh, Raha Jahani, and Andrea Glenn.

I also want to thank Judyln Fernandes, Sari Rosenbloom, and Kervan Rivera-Rufner for their collaboration and help.

Finally, I would like to take this opportunity to thank my family and friends. This thesis would not have been possible without their constant love and support.
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List of Abbreviation

AAC- Acetyl-CoA carboxylase

AMPK- AMP-activated protein kinase

Arch-- Subjects without detectable amount of Archaea

Arch+- Subjects with detectable amount of Archaea

BMI- Body mass index

CFUs- Colony forming units

CH$_4$+- Subjects with detectable amount of breath CH$_4$

CH$_4$--Subjects without detectable amount of breath CH$_4$

ChREBP- Carbohydrate responsive element binding protein

DM- *Drosophila melanogaster*

F/B- Firmicutes/Bacteroidetes

FAS- Fatty acid synthase

Fiaf- Fasting-induced adipose factor

FISH- Fluorescence in situ hybridization

GF- Germ-free

GI- Gastrointestinal

GRP- G-protein coupled receptor

HMG-CoA- 3-hydroxy-3-methylglutaryl-CoA

LAGB- Laparoscopic adjustable gastric banding
LPL- Lipoprotein lipase
LPS- Lipopolysaccharides
PUFAs- Polyunsaturated fatty acids
qPCR- Quantitative PCR
RYGB- Roux-en-Y Gastric Bypass
SCFAs- Short chain fatty acids
SRB- Sulfate-reducing bacteria
SREBP-1c- Sterol regulatory element binding protein-1c
TLR- Toll-like receptor
Yrs- Years
Chapter 1
Introduction
1 Introduction

The human gastrointestinal (GI) tract is populated by more than $10^{14}$ microorganisms. The number of bacterial cells per gram of luminal content increases along the length of GI tract, and reaches the highest level in the proximal region of the colon where substrate availability is the greatest. Residing in one of the most metabolically active regions of the human body, the gut microbiota influence a wide array of host biological events including intestinal development, epithelial function, immune response, and nutrient processing. In particular, during colonic fermentation, the gut microbiota extract energy from the otherwise indigestible part of human diet such as dietary fibers and resistant starches, and at the same time, generate short chain fatty acids (SCFAs) and gases. SCFAs not only provide a source of energy to host, but are also involved in a variety of physiological responses such as assisting cholesterol synthesis in the adipose tissue, promoting gluconeogenesis in the liver, and modulating gut hormones to limit weight gain. These suggest an important role of gut microbiota in obesity.

Obesity is an emergent epidemic, and recognized as one of the biggest global public health challenges not only because of its rapidly growing rate, but also because of its increasing prevalence. In Canada, according to the World Health Organization, more than 60% of adults over the age of 20 years are either overweight or obese. This poses a significant public health concern in Canada due to its associated co-morbidities. For example, more than 8000 deaths in Canada were linked to obesity in 2004. In 2006, the direct cost associated with obesity was approximately 6 billion annually [1]. The fundamental cause of excessive weight gain in obese people is the positive imbalance between energy intake and expenditure.

Evidence raised from in vitro, and human studies has suggested a link between the gut microbial composition and obesity. Through the use of gnotobiotic animals, it has been found that the
presence and absence of gut microbiota is associated with remarkable difference in terms of body fat content, and that Archaea may be an important player in fat deposition processes [2][3]. On the other hand, clinical studies have shown that obesity is associated with an abnormal fecal microbial composition, and an altered Firmicutes to Bacteroidetes (F/B) ratio [4][5][6].

Overall, these studies suggest an association between the gut microbiota and body weight regulation. However, it is worth pointing out that the results from subsequent human studies are not always in accordance with each other. Therefore, the working hypothesis of this study was that fecal microbial composition correlates with body mass index (BMI), diet and Archaea. To address this hypothesis, this study investigated the dominant bacterial species in relation to dietary intake, and Archaea in a study population with different BMIs.
Chapter 2
Background
2 Background

2.1 Gut microbiota

2.1.1 Gut microbiota diversity

The human GI tract is populated by an immense number of microorganisms collectively known as the gut microbiota. This microbial community is composed of at least $10^{14}$ residents from 500-1000 species encompassing all three domains of life (Archaea, Bacteria and Eukarya), whose collective genome, commonly referred to as the microbiome, contains 100 times more genes than the human genome [7]. Along the cephalo-caudal axis of human GI tract, there is a substantial increase of the gut microbiota concentration from the stomach to the colon, and a shift of microbial composition from aerobes to anaerobes. Depending on whether a microorganism is able to establish itself within a given niche in the GI tract, the gut microbiota can be divided into two distinct groups: resident bacteria and transient, non-resident bacteria. The colonization of the microorganisms in the GI tract is controlled by a number of factors such as gastric, pancreatic and biliary secretions, pH, oxygen, transit time, peristalsis, redox potential, mucin secretion, bacterial adhesion, diet, and nutrient availability [8][9]. Evidences from classic culture-dependent studies have indicated that the bacterial count is higher in the distal portion of the intestine, which is predominantly populated by anaerobic bacteria, than in the proximal portion of the intestine, where most aerobic and facultative aerobic bacteria are found [10][11]. Indeed, in the distal portion of the GI tract and in particular in the colon, which are characterized by slow turnover and high redox potential and a relatively high concentration of SCFAs, microorganisms reach the highest density with up to $10^{11}$ to $10^{12}$ colony forming units (CFUs) of bacteria per mL of luminal content [12][9].
2.1.1.1 Dominant and sub-dominant groups of intestinal bacteria

Dominant and sub-dominant groups of bacteria are defined according to their relative presence in the entire fecal bacteria. A dominant group of bacteria represents ≥1% of total fecal bacteria, whereas a sub-dominant group represents less than 1% [13]. Metagenomic studies have showed that Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria are the 4 main phyla found in human colon [14]. Harmsen et al. and Lay et al. have shown 80-90% of fecal bacteria in healthy adults can be detected with fluorescence in situ hybridization (FISH), with 54-75% being Gram-positive bacteria [15][16]. *Clostridium leptum* (*C. leptum*) group and *Clostridium coccoides-Eubacterium rectale* (*C. coccoides*) group are the Gram-positive bacterial groups found in higher numbers in human feces, and they represent 21.1-25.2% and 22.7-28.0% of total bacteria, while *Bacteroides-Prevotella* group is the most abundant Gram-negative bacterial group accounting for 8.5-28.0% of total bacteria [17]. In line with this, Eckburg et al. found that in human colon, Firmicutes and Bacteroidetes make up more than 90% of all bacterial phylotypes [14].

Actinobacteria such as Bifidobacteria are another group of dominant bacteria commonly found in feces, and Proteobacteria such as Enterobacteria are widely considered a sub-dominant group [13][18]. Interestingly, although one’s gut microbiota varies at the species taxonomic level, it is not the case at the phylum level [14]. In a recently published paper, three distinct human gut microbiome enterotypes have been identified from the analysis of fecal samples obtained from 39 volunteers which can be used to classify human beings [19]. Enterotype 1 is characterized by high level of *Bacteroides* and co-occurring *Parabacteroides*. Enterotype 2 has an enriched number of *Prevotella* and co-occurring *Desulfovibrio*. Whereas, enterotype 3 is the most common type characterized by a high level of *Ruminococcus* and co-occurring *Akkermansia*. This finding suggested the stratified nature of gut intestinal microbiota variation other than continuous. Interestingly, the 3 enterotypes are mostly species driven, and independent of BMI,
age, or gender, while long-term dietary pattern is strongly associated with alternative enterotype states [20]. Despite the relative abundance at phyla level, most people share a gut microbial profile comprised of 50 to 100 core species, and a core microbiome consisting of more than 6000 functional gene groups [21].

2.1.1.2 Acquisition of the gut microbiota in early life

The GI tract of a fetus *in utero* is considered sterile. The first exposure of a naturally delivered infant to microbes takes place in the vaginal canal and continues through adulthood. The acquisition of the gut microbiota is a rapid process influenced by a series of elements such as, mode of delivery, diet, gestational age, drug treatment, health status, and surrounding environment [22][23]. A study conducted by Pettker *et al.* showed that a detectable amount of microbes are present not only in the amniotic fluid and placenta from the mothers, but also in umbilical cord blood of healthy neonates, suggesting that maternal microbiota may contribute to the first set of residents in GI tract of the new born [24]. The mode of delivery is an important factor which shapes composition of the infant microbiota [23][25][26]. For instance, in contrast to vaginally born infants, newborns delivered by caesarean section have a different enteric microbiota characterized by delayed colonization and lower number of strict anaerobes such as *Bacteroides* [27][23]. In addition, the feeding regime is a potent factor in the determination of gut microbiota in infants. Formula-fed newborns have a broader spectrum of microorganisms than those who are breast-fed [28]. Furthermore, preterm infants have been reported to have an altered developmental scheme of enteric microbes characterized by increased pathogenic bacterial species accompanied by delayed beneficial bacterial colonization [29]. In general, during the first 3 weeks of life, the GI tract of infants is colonized by a significant number of facultative bacterial species such as *Escherichia coli* (*E. coli*) and streptococci which in turn create a reduced environment favoring the establishment of strictly anaerobic bacterial strains.
including *Bacteroides*, *Bifidobacterium*, and *Clostridium* later on [30][31]. Weaning represents an important stage during such transition when a carbohydrate-rich diet is introduced to replace high-fat milk-based diet supporting the establishment of carbohydrate-fermenting bacteria [32]. The intestinal microbiota undergoes a continuous regulated shaping until two years of age when it resembles that of an adult which is considered to be relatively stable over time [33].

### 2.1.1.3 Archaea

Intestinal Archaea are methanogens, utilizing various substrates such as CO2, H2, acetate, and methylamines for methanogenesis [34][35]. *Methanobrevibacter smithii* (*M. smithii*) is the predominant archaeon in the human gut [14][36][37][38]. It was first identified from the human intestinal contents through microbiological, physiological, and immunological methods [39]. It is the most dominant methanogenic species in human GI tract, and can comprise up to 10% of all anaerobes residing in the colon of healthy individuals [40][41]. Interestingly, despite dietary change and the wide use of antibiotics, the percentage of methane excretors (36.4%) in the population, and the average concentration of methane (16.6 ppm), is close to what were measured 35 years ago, 33.6% and 15.2 ppm respectively [42]. This is partly due to the fact that methanogens belonging to the genus *Methanobrevibacter* have not been found in the human food chain, and the distribution frequency of methane producers is related to geographical and cultural origins. [43]. Typically, the acquisition of the methanogens starts in children after 27 months of age with the incidence of methanogenic Archaea host gradually increasing to 40% at 3 years and 60% at 5 years [44]. The distribution of methanogens also shows spatial difference within the human intestine. For methanogens carriers, the distal region of the colon is heavily populated by methanogens reaching up to 12% of total anaerobes in contrast to only 0.003% in the proximal colon [45]. Methanogens are known to interact with other bacteria in the colon. For example, methanogens and sulfate-reducing bacteria (SRB) coexist in the colon, and compete for H2. Up
regulating the level of SRB by increasing dietary sulfate is able to limit the number of methanogens, and lower breath methane [46]. In addition, since methanogens are able to generate methane by utilizing the H2, they essentially remove the product of polysaccharide fermentation, and promote the growth of H2 producing microbes such as Bacteroides thetaiotaomicron (B. thetaiotaomicron). Indeed, Samuel et al found that the co-colonization of germ-free (GF) mice with only M. smithii and B. thetaiotaomicron significantly enhanced the serum acetate level and liver triglycerides compared to GF mice colonized with either B. thetaiotaomicron or M. smithii alone [3]. However, no difference was observed when M. smithii was co-colonized with Desulfovibrio piger which is a SRB. Moreover, although the isolation of certain Archaea species is currently not feasible, evidence based on molecular analysis supports the existence of other groups of Archaea such as Methanosarcina, Thermoplasma, Crenarchaeota and halophilic Archaea in the human GI tract [47]. Therefore, the level of Archaea is of great importance in the examination of gut microbiota with which they interact.

2.1.2 Metabolic activity of gut microbiota

The human large intestine is about 150 cm in length with a corresponding surface area of 1300 cm², and one can have somewhere between 58g and 980g of contents within this region [48]. In a typical person consuming a western diet, bacteria represent 40-55% of fecal solid matter. The gut microbiota is considered by many as a true organ in the human body performing functions we have not evolved ourselves.

2.1.2.1 Diet and gut microbiota

Diet is considered the most important factor shaping the gut microbiota composition. Most nutrients available for colonic fermentation are the leftover from the digestion occurring in the upper GI tract. Unlike fat which undergoes more complete digestion, 20-60g of available
carbohydrate, and 5-20g of protein are estimated to pass onto the colon on a daily basis [49][50][51]. Among all sources of fermentation, resistant starches are considered the major substrates for colonic fermentation followed by dietary fiber, unabsorbed sugars, and modified cellulose [52]. Furthermore, non-dietary components such as mucus, digestion enzymes, and salvaged host cell and bacterial cells are additional substrates for colonic fermentation [53]. For the purpose of this thesis, effects of diet are discussed more in detail.

Diet can affect the gut microbial composition by modulating the substrate availability for colonic fermentation. Although a link exists between diet and the gut microbiota composition, a few intervention studies are available showing association between diets and gut microbial composition (Table 1). It has been found that despite the type of calorie-restricted diet, the number of Bacteriodetes is positively correlated with the amount of weight loss [4]. In particular, the combination of low-calorie diet and exercise has been reported to reduce the counts of C. coccoides, C. histolyticum, B. longum and B. adolescentis, but to increase the concentration of B. fragilis, Lactobacilli and Bacteroides [54][55]. In addition, it has been shown that glycated pea protein promotes the growth of gut commensal bacteria such as lactobacilli and bifidobacteria [56]. Higher intakes of resistant starch and dietary fiber have been shown to promote the growth of Firmicutes such as Ruminococcus, E. rectale-C. coccoides, F. prausnitzii and Roseburia [57][58]. Moreover, high dietary fiber intake has also been associated with increased presence of beneficial bacteria groups such as Bifidobacteria and Lactobacilli [58]. For instance, inulin, a prebiotic, is a common food additive known for its bifidogenic property in human [59][60]. Furthermore, when taking enterotype into consideration, carbohydrate-based diet has been linked to enterotypes 2 characterized by high level of Prevotella, while western diet with high animal protein and saturated fat consumption is associated with enterotype 1 dominated by Bacteroides [20]. Such finding paralleled a recent paper where it was found that European microbiome was
dominated by taxa typical of *Bacteroides* enterotype, whereas African microbiome was
dominated by the *Prevotella* enterotype [61].

On the other hand, micronutrients can also affect the gut microbiota. Dietary selenium has been
shown to impact on the intestinal microorganism by increasing their overall diversity and
exerting differential effects on the bacterial colonization of specific taxonomic groups [62]. In a
long-term randomized control study in African children, iron fortification has been shown to
result in lower level of fecal lactobacilli and higher level of fecal enterobacteria [63]. Vitamins
have also been suggested to influence gut microbial composition. For example, a clinical study
composed of African American and Caucasian American subjects has suggested that different
intake of vitamins such as vitamin A, C, and D was associated with distinct fecal microbial
compositions between the two groups [6]. In animal studies, rats fed with a vitamin A-deficient
diet had an altered microbiota characterized by an elevated total number of bacteria in the GI
tract, a proportional decrease in *Lactobacillus* spp. and the simultaneous increased appearance of
*E. coli* strain [64]. Mice with vitamin D deficiency were found to have a 50-fold increase of
bacteria in the colonic tissue compared to mice on vitamin D sufficient diet [65].

In summary, intervention studies over the last few years have indicated potential link between
the intake of specific macronutrients and the composition/function of the gut microbiota.
However, there is lack of cross-sectional studies showing the association between multiple
 macronutrients and gut microbiota. Such research is needed to understand the interplay between
different macronutrients and the microbiota.
Table 1. Studies investigating the role of diet in relation to gut microbiota

<table>
<thead>
<tr>
<th>Diet</th>
<th>Subject character</th>
<th>Bacteria increased</th>
<th>Bacteria decreased</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low caloric &amp; fat restricted</td>
<td>Obese</td>
<td>Bacteroidetes</td>
<td>Firmicutes</td>
<td>[4]</td>
</tr>
<tr>
<td>Low caloric &amp; carbohydrate restricted</td>
<td>Obese</td>
<td>Bacteroidetes</td>
<td>Firmicutes</td>
<td>[4]</td>
</tr>
<tr>
<td>Low carbohydrate &amp; high protein</td>
<td>Overweight</td>
<td><em>Oscillibacter valerigens</em></td>
<td><em>Roseburia, E. rectal</em></td>
<td>[57]</td>
</tr>
<tr>
<td></td>
<td>Obese</td>
<td></td>
<td><em>Bifidobacterium</em></td>
<td>[66]</td>
</tr>
<tr>
<td></td>
<td>Obese</td>
<td></td>
<td><em>Roseburia, E. rectal</em></td>
<td>[67][57]</td>
</tr>
<tr>
<td>High resistant starch</td>
<td>Overweight</td>
<td><em>Ruminococcus bromii, Oscillibacter valerigens, Roseburia and E. rectale</em></td>
<td></td>
<td>[57]</td>
</tr>
<tr>
<td>Calorie restriction &amp; exercise</td>
<td>Overweight</td>
<td><em>B. fragilis, Lactobacillus</em></td>
<td><em>C. coccoides, B. longum, B. adolescentis</em></td>
<td>[54]</td>
</tr>
<tr>
<td></td>
<td>Adolescents</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Obese</td>
<td><em>Bacteroides</em></td>
<td><em>C. histolyticum, E. rectal-C. coccoides</em></td>
<td>[55]</td>
</tr>
<tr>
<td></td>
<td>Adolescents</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- Subjects are adults unless otherwise indicated
2.1.2.2 Products of colonic fermentation

Due to their relatively high abundance of substrates in the proximal colon, carbohydrates, as the more energetically efficient choice to generate ATP, are preferentially fermented [68]. Consequently, the proximal region of the colon is predominantly populated by saccharolytic species. As the available carbohydrates drop along the length of the colon, the colonic microbiota gradually changes into a more proteolytic, methanogenic and sulphate-reducing phenotype [49][69]. Proteolysis is common in the distal part of colon where low substrate availability and neutral pH are persistent [69]. In contrast to the benign end products of carbohydrate fermentation, some of metabolites formed through proteolytic fermentation are potential toxins [70]. The endpoint of protein fermentation is the generation of SCFAs and branched-chain fatty acids together with the production of potentially toxic compounds such as ammonia, amines, phenols, thiols, and indoles [71][72]. While some of the metabolites are utilized by the bacteria as a nitrogen source, others such as phenols and indoles are metabolized by hepatic enzymes and excreted in urine [70].

It is estimated that colonic fermentation gives rise to up to 10% of energy absorbed by human body [73]. SCFAs are a group of organic fatty acids comprised by no more than 6 carbon atoms, and they are considered the principal anions and the major products of colonic fermentation [74][75][49]. The production of SCFAs is influenced by a number of factors including but not limited to nutrient availability, regional microbiota composition, transit time, and pH [76][77][78]. Depending on the types of substrates available for fermentation, bacteria in proximal colon consume soluble carbohydrates which give rise to linear SCFAs together with H₂ and CO₂ [79][80]. Instead of linear SCFAs, bacteria in the distal colon generate branched
SCFAs, H₂, CO₂, CH₄, phenols, and amines by proteolytic fermentation [80]. Population survey data has shown that acetate, butyrate, and propionate are the 3 major SCFAs (Figure 1) found in human feces with acetate being the most abundant (60% of total SCFA) followed by propionate (20%) and butyrate (20%) [81]. They are efficiently absorbed through primarily simple diffusion or ion exchange in the colon, and only 5-10% is excreted in the feces [82][83].

![Chemical structure of SCFAs](image)

**Acetic acid** | **Propionic acid** | **Butyric acid**

Figure 1. Chemical structure of 3 major short chain fatty acids found in human feces.

Acetate is the major SCFA present in the colon. It is less metabolised, and rapidly absorbed and transported to liver [76]. Acetate is important for cholesterol synthesis. In the systemic circulation, acetic acid is converted by the cytosolic acetyl-CoA synthetase of adipose tissue and mammary gland to acetyl-CoA which is utilized for fatty acid synthesis.

Propionate can be found in a few naturally occurring foods, and it is also available as a food preservative due to its anti-fungal and anti-bacteria effect [84][85]. However, the main source of propionate comes from microbial fermentation in the colon [86]. Compared to acetate, propionate is more readily absorbed and utilized in the human liver, and therefore, colonic propionate reaches systemic circulation to a lesser extent [87][88]. It is a substrate for hepatic
gluconeogenesis, and exhibits hypocholesterolemic effect by inhibiting cholesterol synthesis in hepatic tissue [89]. Indeed, studies using animal models have suggested that propionate inhibits both 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase which catalyzes the reaction of acetoacetyl-CoA to HMG-CoA, and HMG-CoA reductase which catalyzes a rate limiting step of the conversion from HMG-CoA to mevalonic acid in the mevalonate pathway for the production of cholesterol and other isoprenoids [90].

Butyrate is preferred over propionate, acetate, glucose, and glutamine as the main energy source of colonic epithelial cells [76][81][91]. It is often considered the most important SCFA in colonocyte metabolism with up to 90% of butyrate metabolized by colonocytes [76]. Evidence from animal and cell line studies has pointed out the anti-proliferative effect of butyrate, and its protective role against colorectal adenoma and cancer [92]. However, the underlying mechanism of butyrate anti-carcinogenetic action is not clear. Although acetate and propionate have also been shown to provoke apoptosis, they are less effective than butyrate [93][94].

What is common to all three major SCFAs is that they all exert trophic effect in the small and large intestine, and have been shown to stimulate epithelial cell proliferation and differentiation in vivo [95].

2.1.2.3 Gut microbiota impacts on energy homeostasis

2.1.2.3.1 Obesity epidemic

Obesity is defined as a BMI of 30 or higher [96], and it can be further categorized into Class I (30 ≤ BMI < 35), Class II (35 ≤ BMI < 40), and Class III (BMI ≥ 40) [97]. Obesity is a very common metabolic condition in developed countries, and it has reached epidemic proportions
According to the Canadian Community Health Survey conducted in 2004, 23.4% of Canadian adults between the age of 20 and 64 are considered obese, and in addition, 35.4% are overweight ($25 \leq \text{BMI} < 30$) [99].

Obesity is known for its detrimental effect on the quality of life [100]. A recent report estimated that 10% premature mortality among Canadian adults from age 20 to 64 years is directly linked to obesity [101]. Obesity is also accompanied by a low-grade chronic inflammation state characterized by a systematic increase level of CRP and circulating cytokines including TNF-$\alpha$ and IL-6 [102][103]. Indeed, being obese or overweight is a well-established risk factor for a number of associated morbidities including Type 2 diabetes, cardiovascular diseases, cancer, nonalcoholic fatty liver disease, and other disorders [104]. In Canada, the direct cost associated with obesity was approximately 6 billion in 2006, which represented 4.1% of total health spending in 2006 [1].

2.1.2.3.2 Treatment of obesity

The fundamental cause of obesity is the long-term imbalance among the energy intake, expenditure, and storage, which in turn contributes to weight gain in the form of growing fat deposit [105]. The basic treatment of obesity targets the persistent positive energy imbalance by putting obese people on calorie-restricted diets together with increasing physical activity [106]. Unfortunately, long-term adherence towards the lifestyle intervention is a common problem in such approach because compliance is not only difficult to maintain, but also hard to assess [107]. Moreover, effect from such remedy is usually transient, and most patients tend to regain part, if not all, of their lost weight once the treatment is discontinued [108]. On the other hand, pharmaceutical treatment of obesity has been disappointing because of its limited utility in long-
term weight loss, compared to diet and exercise [109][110]. Orlistat (Xenical, Roche), which blocks fat absorption in the body, is the only FDA approved long-term anti-obesity medication currently available on the market, while other medications have been withdrawn due to safety concerns. Moreover, Orlistat is considered only modestly efficacious in terms of weight loss, and known for its side effect such as steatorrhea [111]. In the case of morbid obesity (BMI>40), bariatric surgery is performed, when the appropriate conservative approaches do not result in substantial weight loss. Complications may occur after such invasive approaches including but not limited to gastric dumping syndrome, incisional hernia, infections and pneumonia [112][113]. For example, among frequently performed surgical managements of obesity, laparoscopic adjustable gastric banding (LAGB) is considered medium efficacy with a lower rate of overall and major complications [114]. However, patients undergo LAGB may experience symptoms such as pouch enlargement, band slip, and port breakage.

2.1.2.3.3 Environmental factors involved in obesity

A twin study from a total of 15,017 monozygotic and dizygotic twin pairs has shown that genetic variation is significantly positively correlated with the prevalence of obesity [115]. Although one’s predisposition towards excessive weight gain is genetically determined, the role of environmental components certainly adds another layer of complexity to the current obesity epidemic [116]. Factors such as the accessibility of energy-dense foods, time spent on physical activities, and the ease of technology advance in modern life, all are considered the building blocks of the obesity labyrinth. For instance, the availability of green space is linked to physical activities; therefore, it also links to obesity. However, the results from evaluating the association between obesity and green space have been inconsistent, and conclusion has not yet been reached
Other studies suggested that suboptimal micronutrient status such as calcium or dairy intake may also contribute to positive energy metabolism [118]. Nevertheless, the results from several clinical trials of dietary components in relation to weight management are far from conclusive [119]. Enlightened by the recent finding on gut microbiota and obesity, an essential role of intestinal microbial community in energy and metabolic homeostasis has been proposed [120-124].

### 2.1.2.3.4 Animal studies

Gut microbiota is an important environmental factor that contributes to the development of metabolic syndrome and obesity [2]. Backhed et al. were the first to show that 8 to 10 week old conventional mice have 40% higher body fat content and 47% higher gonadal fat content than their GF counterparts despite lower food consumption. In other words, GF mice are protected against diet-induced obesity. Besides, the obese phenotype of the conventional microbiota was also shown to be transmissible, as it induced 60% increase in body fat mass and development of insulin resistance upon transplantation in GF recipient animals. The same was true, at an even greater extent, when the transplanted gut microbiota was harvested from genetically obese (ob/ob) mice [125].

On the other hand, obesity is also associated with an altered gut microbiota composition. A study using 5,088 bacterial 16S rRNA gene sequences showed that compared to lean mice, obese animals had a 50% reduction in the amount of Bacteroidetes and a proportional increase in the Firmicutes [126]. In addition, a recent study has shown that diet-induced obesity in mice resulted in an increased proportion of a single uncultured clade within the Mollicutes class of Firmicutes, which was diminished by subsequent dietary intervention to restrict the weight gain [127].
Studies from both Backhed *et al.* and Ley *et al.* imply the potential effect of manipulating gut microbial community to regulate energy homeostasis in obese animals. Then the question arises, what about the intrinsic difference of gut microbiota in humans in comparison to mice, and what is the relevance of the animal studies towards human conditions? Although the majority of species of mouse gut microbiota is specific to mice, mice and humans do share similar gut microbiota structure at the division level, with predominantly Firmicutes and Bacteroidetes [126].

### 2.1.2.3.5 Clinical studies

#### 2.1.2.3.5.1 Role of Bacteria in obesity

In a pioneer study in 2006, the relation between fat storage and microbiota composition in 12 obese people who were put on a weight-loss program for a year was investigated [4]. Obese individuals were found to carry significantly more Firmicutes and less Bacteroidetes than the lean control participants. Furthermore, after weight-loss, the ratio of Bacteroidetes and Firmicutes increased, and the abundance of Bacteroidetes in obese participants increased from approximately 3% to approximately 15% [4]. This withstanding, this study could not explain why obese people have a higher proportion of Firmicutes. While this study has the merit of being the first to show a connection between microbiota and obesity, the findings presented were not consistently confirmed in subsequent research from other groups (Table 2a). For instance, a clinical study compared the fecal microbial composition over a 4 week period between a group of obese and non-obese subjects undergoing a weight maintenance diet and group of obese subjects undergoing a weight-loss diet has shown a significant dietary-dependent reduction of butyrate producing Firmicutes in the feces for those subjects put on a weight-loss diet [128].
However, neither a difference in the percentage of Bacteroidetes between the obese and non-obese subjects, nor a change of the proportion of Bacteroidetes before or after weight-loss diet was detected. In other words, the author was unable to identify a relation between BMI and altered proportion of Bacteroidetes and Firmicutes in their obese cohort.

Results from cross-sectional studies are even more mixed (Table 2b). According to Brignardello et al., compared to lean subjects, the levels of *C. coccoides* and *Bacteroides* were decreased in obese subjects. However, in contrast to its finding, Collado et al. reported an increased level of *Bacteroides* in obese subject, whereas, Schwiertz et al. found an higher number of *C. leptum*, *C. coccoides* and *Bacteroides* in both lean and obese subjects than overweight subjects. Other groups reported no difference between lean and obese people in terms of these three bacterial species [129][130]. *Faecalibacterium prausnitzii* (*F. prausnitzii*) is a member of the Firmicutes, and a lower level of *F. prausnitzii* has been associated with an increased low-grade inflammation state in obesity and diabetes [131]. However, the role of *F. prausnitzii* is not clear based on the contradicting results from two studies. Balamurugan et al. claimed that obesity is associated with increased *F. prausnitzii* level, whereas Brignardello et al. had the opposite conclusion [130][132].

In summary, previous studies have provided interesting observations indicating possible correlation between prevalence of bacterial groups and host weight, yet discrepancies were frequently found among these studies. Such discrepancies could have been due to different methodologies used between the different researchers or, more likely, as a result of the inherent differences among the participants in each study (eg., different geographical region, population, gender, diet, definition of obesity, etc.). As such, further studies with large sample size based on
a geographically defined heterogenous population/diet habits will be of interest to bridge between these population-dependent results.

2.1.2.3.5.2 Role of Archaea in obesity

In the context of Archaea, in the obese subject, Brignardello et al. reported a decreased level of \textit{M. smithii} in obesity, while Zhang et al. declared a higher level of \textit{M. smithii} in both the lean and obese people in contrast to post-gastric bypass (PGB) patients [132][133]. Others reported that there is no difference between the obese patient and normal people in terms of their Archaea level [129].

One limitation commonly found in studies of the role of Archaea in obesity lies in the usually small sample. For example, the study by Zhang et al. recruited only 9 subjects. We have reported detectable breath methane in only 39% of 100 participants in a recent study, suggesting that findings by Zhang et al. could have been due to chance alone. As such, further studies with a more prudent sample size are needed in order for a consensus on this issue.
Table 2a. Fecal microbiota and to BMI: Intervention studies

<table>
<thead>
<tr>
<th>Sample characteristic</th>
<th>Intervention</th>
<th>Methodology</th>
<th>Finding</th>
<th>After</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 obese 2 normal weight</td>
<td>Low-calorie diets (52 wks) ♦ Fat-restricted (30%) ♦ Carb-restricted (25%)</td>
<td>16S rRNA gene sequencing</td>
<td>Obese people have fewer Bacteroidetes &amp; more Firmicutes</td>
<td>↑ F/B ♦ Bacteroidetes correlated with percentage of weight loss</td>
<td>[4]</td>
</tr>
<tr>
<td>30 obese</td>
<td>Before &amp; after RYGB</td>
<td>qPCR</td>
<td>↓ Bacteroides/Prevotella in obese ‡ F. prausnitzii in diabetes</td>
<td>↑ Bacteroides/Prevotella &amp; E. coli ↓ Bifidobacterium, Lactobacillus /Leuconostoc/Predicoccus ‡ F. prausnitzii ▼ C. coccoides, B. longum, B. adolescentis ‡ Bacteroides fragilis, Lactobacillus</td>
<td>[131]</td>
</tr>
<tr>
<td>36 overweight adolescents</td>
<td>Calorie-restricted diet (10-40% reduction) Increased physical activity (15 to 23 kcal/kg of body weight per week) (10 weeks)</td>
<td>qPCR</td>
<td>↓ Bacteroides/Prevotella &amp; E. coli ↓ Bifidobacterium, Lactobacillus /Leuconostoc/Predicoccus ‡ F. prausnitzii ▼ C. coccoides, B. longum, B. adolescentis ‡ Bacteroides fragilis, Lactobacillus</td>
<td>[54]</td>
<td></td>
</tr>
<tr>
<td>33 obese 14 non-obese</td>
<td>23 out of 33 obese subjects undergo weight loss regimes (&lt; 2031.5 Kcal per day) (8 weeks)</td>
<td>FISH</td>
<td>No relation between BMI and the proportion of Bacteroides For obese subjects in weight loss diets ♦ No change in the percentage of Bacteroidetes Diet-dependent reductions in a group of butyrate producing Firmicutes</td>
<td>[128]</td>
<td></td>
</tr>
</tbody>
</table>

- Subjects are adults unless otherwise indicated
- ↑, increase; ↓, decrease
- RYGB: Roux-en-Y Gastric Bypass
Table 2b. Fecal microbiota and BMI: Cross-sectional studies

<table>
<thead>
<tr>
<th>Sample characteristic</th>
<th>Methodology</th>
<th>Finding</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 obese, 9 anorexia, 20 normal weight</td>
<td>qPCR</td>
<td>Similar Firmicutes data in all groups, ↑<em>Lactobacillus</em> in some obese subjects, ↑<em>M. smithii</em> in anorexia patients</td>
<td>[129]</td>
</tr>
<tr>
<td>33 obese, 35 overweight, 30 normal weight</td>
<td>qPCR</td>
<td>↑F/B ratio in overweight &amp; obese subjects</td>
<td>[5]</td>
</tr>
<tr>
<td>3 obese, 3 post-gastric bypass, 3 normal weight</td>
<td>16S rRNA gene sequencing, qPCR</td>
<td>↓Firmicutes, proportional ↑ Gammaproteobacteria in PGB, ↑H₂-producing Prevotellaceae in the obese, ↑Archaea in obese than in normal weight or PGB</td>
<td>[133]</td>
</tr>
<tr>
<td>13 obese, 11 normal weight</td>
<td>G+C profiling</td>
<td>↓<em>Bacteroides, C. coccoides, F. prausnitzii, Bifidobacterium</em> in obese subject, ↑low G+C Clostridium cluster, some species of <em>Lactobacillus</em> in obesity</td>
<td>[132]</td>
</tr>
<tr>
<td>Children, 15 obese, 13 normal weight</td>
<td>qPCR</td>
<td>Higher level of <em>F. prausnitzii</em> in obese than in non-obese children</td>
<td>[130]</td>
</tr>
<tr>
<td>Children, 25 overweight &amp; obese, 24 normal weight</td>
<td>FISH, qPCR</td>
<td>Higher bifidobacterial number in normal than in overweight during infancy, Greater number of <em>Staphylococcus aureus</em> in children becoming overweight than in children remaining normal weight</td>
<td>[134]</td>
</tr>
<tr>
<td>52 African American, 46 Caucasian American</td>
<td>Comet assay</td>
<td>BMI was not associated with proportions of <em>Bacteroides</em> or Firmicutes</td>
<td>[6]</td>
</tr>
</tbody>
</table>
### Table 2b. Continued

<table>
<thead>
<tr>
<th>Sample characteristic</th>
<th>Methodology</th>
<th>Finding</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnant women</td>
<td>qPCR</td>
<td>↓<em>Bifidobacterium</em> &amp; <em>Bacteroides</em> number in overweight compared to controls</td>
<td></td>
</tr>
<tr>
<td>16 overweight</td>
<td></td>
<td>↑<em>Staphylococcus</em>, <em>Enterobacteriaceae</em> &amp; <em>E. coli</em> in overweight compared to controls</td>
<td>[135]</td>
</tr>
<tr>
<td>34 normal weight</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18 overweight women</td>
<td>FISH</td>
<td>↑<em>Bacteroides</em> &amp; <em>Staphylococcus</em> in overweight</td>
<td></td>
</tr>
<tr>
<td>36 normal weight</td>
<td>qPCR</td>
<td>Higher concentration of <em>Bacteroides</em>, <em>Clostridium</em> &amp; <em>Staphylococcus</em> correlated with mother weight before pregnancy</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>↑<em>Bacteroides</em> was associated with excessive weight gain over pregnancy</td>
<td>[136]</td>
</tr>
</tbody>
</table>

- Subjects are adults or otherwise indicated
- ↑, increase; ↓, decrease
- qPCR: Quantitative PCR
- FISH: Fluorescence in situ hybridization
2.1.2.3.6 Gut microbiota and host energy balance

Several mechanisms have been proposed to explain the links between composition of the gut microbial community and energy homeostasis. The first pathway was suggested by Jeffrey I. Gordon’s group. In a study focusing on developmental regulation of intestinal angiogenesis in mice, they found adult GF mice had arrested capillary network formation in the small intestinal villi, and such process could be revived and completed within 10 days once the GF mice were colonized with a complete microbiota derived from conventional mice, thereby improving nutrient absorption in the small intestine [137].

The second pathway involves energy extraction from fermentable food components, which in turn gives rise to SCFAs, and eventually, leads to de novo hepatic lipogenesis. This can be achieved through the expression of several mediators such as acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) [138]. Both ACC and FAS are the downstream targets for carbohydrate responsive element binding protein (ChREBP) and sterol regulatory element binding protein-1c (SREBP-1c) [139]. According to a study performed by Backhed and colleagues, conventionalized GF mice have an increased hepatic ChREBP mRNA and SREBP-1c mRNA expression [2]. The liver has several ways of dealing with enhanced delivery of calories, and one of them is to relocate them in the form of fat deposit in the peripheral tissues. However, up-regulated ChREBP and SREBP-1c are not the only players. Lipoprotein lipase (LPL) is a key enzyme responsible for the hydrolysis of circulating triglycerides to free fatty acids [140]. Enhanced LPL activity results in increased cellular uptake of fatty acids and triglyceride accumulation in adipose tissue. Fasting-induced adipose factor (Fiaf) is a circulating inhibitor of LPL. It has been show that conventionalization of adult GF mice leads to suppression
of Fiaf expression in ileum, and therefore fat is deposited as a consequence of liberated LPL activity [2].

A third mechanism has been proposed to explain the resistance to diet-induced obesity in GF mice, which was shown to be associated with elevated level of skeletal muscle and liver phosphorylated AMP-activated protein kinase (AMPK) and its downstream targets involving fat oxidation [141][98]. Therefore, gut microbiota colonization causes the down regulation of AMPK related fat oxidation in liver and skeletal muscle.

The fourth mechanism involves SCFAs as the substrates for a group of G-protein coupled receptor (GPR) namely GPR41 and GRP 43 [142]. A study by Samuel et al. has shown that compared to wild type littermates, GPR41 knock out mice are associated with a specific pattern of gut microbiota expression, and increased resistance to diet-induced obesity [143]. Another study looking into GRP43 knock out mice draws a similar conclusion [144].

Low-grade inflammation is a common characteristic of obese people. A recent study explored the gut microbiota composition in relation to bariatric surgery-induced weight loss in obese patients [131], and found that increased numbers of the Firmicute F. prausnitzii are directly linked to the reduction in low-grade inflammation state in obesity and diabetes, independently of dietary intake patterns. In other words, reduced numbers of F. prausnitzii correlate with the establishment of a low-grade inflammatory state in obese and diabetic patients. Others suggest that an antibiotic-induced reduction of endotoxins such as lipopolysaccharides (LPS) leads to reduced glucose intolerance, weight gain, fat disposition, oxidative stress, and inflammation [145][142]. In line with this, a recent study showed that in mice gut Bacteroides-derived LPS correlates with metabolic endotoxemia in mice on a high-fat diet for 4 weeks [146]. Interestingly, the same authors demonstrate a role of toll-like receptor 4 (TLR4) as a mediator in this process,
highlighting a role for bacterial-sensing molecules expressed on intestinal epithelial cells.

Consistently with this, Vijay-Kumar and colleagues recently found that conventionalization of wild-type GF mice conventionalized with gut microbiota harvested from TLR5-deficient mice conferred many features resembling metabolic syndrome to the hosts [147]

2.1.2.4 Gut microbiota influences on other host functions

Gut microbiota has been also found to impact on various aspects of host function other than nutrient processing and energy homeostasis. For example, a healthy gut microbiota has been associated with increased longevity in humans, worms and insects by modulating the host immune system, influencing systemic metabolic effects, and limiting the colonization and growth of pathogenic species [148]. Surprisingly, the mating preference of Drosophila melanogaster (DM) is also affected by gut microbiota [149]. This study employed DM feeding on molasses based and starch based medium has shown that molasses-fed flies were more likely to mate with other molasses-fed flies, and similar mating pattern was also observed among starch-fed flies. However, antibiotic treatment was able to abolish the mating preference, indicating that the commensal bacteria is responsible for the establishing such preference. What is more, gut microbiota is also responsible for mammalian brain development and behaviour [150]. Compared to GF mice, specific pathogen free mice with a normal gut microbiota have reduced motor activity and increased anxiety, suggesting that the colonization of the gut microbiota alters the signalling pathway such as second messenger and long-term synaptic potential in regions of brain dedicating to motor control and anxiety.

2.2 Summary and rationale

The human gut microbiota has been recognized as an important metabolic organ whose composition and function are driven by the force of continuous competition and selection. A
number of factors such as dietary habits, age, and race have been shown to contribute to the variation in the makeup of intestinal microbiota between individuals. Accordingly, the exact functions provided by such a “super organ” are still poorly understood partially due to its great inter-individual difference at the species level. The gut microbiota possesses the enzymes that are essential for the catabolism of the indigestible component of the human diet (i.e. dietary fiber).

Indeed, evidence from recent animal and human studies suggests a role for the gut microbiota as an environmental factor impacting host energy homeostasis. However, no consensus has been reached in the literature on the characterization of microbial dysbiosis in the feces of overweight and obese population as compared to their lean counterparts. Although the fundamental cause of obesity is a positive energy balance, it results from a complicated interaction between environmental and genetic factors with the former playing a more decisive role. Overall, current literature suggests a link between adiposity, diet and a certain pattern of gut microbiota.

However, a ratio of Firmicutes/Bacteroidetes alone is often not sufficient to describe the tie between obesity and gut microbial composition. For example, Archaea which is the sole methane producer in the human gut has been shown to make up to 10% of total gut microbiota, yet despite being recognized as potential contributor to determination of host weight, evidence on the direction of the influence of Archaea is often contradictory, and further studies with larger samples size are warranted. Furthermore, relationship between prevalence of certain bacterial groups and host adiposity requires further investigation to bridge the discrepancies frequently observed in published results. Finally, as discussed above, the effects of the interplay between multiple macronutrients in shaping the composition and function of gut microbiota remains unclear; whereas published studies usually were mostly intervention studies which primarily focused on either the types of diet or specific macronutrient. Therefore, a cross sectional study featuring multiple macronutrients and gut microbial composition is of great interest. As such,
this study was initiated with the hypothesis that the observed link between obesity and gut microbial composition is likely a result of interactions among bacterial composition, diet and Archaea.
Chapter 3
Hypothesis and objectives
3   Hypothesis and objectives

3.1 Hypothesis

Fecal microbial composition correlates with BMI, diet and Archaea.

3.2 Objectives

1) To quantify dominant and sub-dominant microbial groups in the feces of lean, overweight and obese subjects

2) To determine the association among macronutrient intake, fecal microbial composition, and BMI

3) To investigate whether fecal Archaea correlate with microbial composition in the feces
Chapter 4
Materials and methods
4 Materials and methods

4.1.1 Subjects and study design

Ninety-nine males and non-pregnant females over the age of 17 years were recruited by advertisement in the form of posters on the University of Toronto campus. Subjects were excluded from the study if any of the following conditions were present: diabetes, BMI<18.0 or >39.9, use of any oral hypoglycaemic agent, insulin sensitizer or insulin, use of antibiotics within the last 3 months, GI diseases, motility disorder or malabsorption, liver or kidney disease or major medical or surgical event within 6 months, high fiber intake (>30 g/day) or other abnormal dietary pattern, and unwilling or unable to give informed consent and/or comply with study protocol. This study was approved by Research Ethics Board, University of Toronto, and all subjects gave written informed consent to participate in the study. Prior to the study, eligible individuals were screened for their medical history and therapeutic agents use, and were asked to visit the laboratory twice at any convenient time of their choice. At the first visit, subjects were asked to provide demographic parameters such as height, weight, waist circumference, blood pressure, two breath samples and other routine biochemistry parameters. In addition, they were also given instructions on how to compile a 3-day dietary record and collect stool samples while maintaining usual diet and activities. At the second visit, subjects returned with 3-day dietary record, stool samples and provided two more breath samples. Freshly passed stools from participants were gathered in a plastic container and placed on dry ice until delivery which occurred within 24 hours from defecation. Once the stool sample was received in the lab, it was homogenized using a stomacher, and stored in aliquots at -20 °C until further analysis.

Easy Sampler™ with tube holder (Quintron Instrument Company, Milwaukee, WI) was used for collecting breath samples. Breath methane and hydrogen were measured by gas chromatography
(Quintron Microlyzer, Model SC, Milwaukee, WI) while calibrating simultaneously with room air, as previously described [151]. In the current study, the detection limit is 2 ppm for breath CH₄ and H₂, and subjects with no detectable breath CH₄ and H₂ were assigned a value of 0.

The participants were categorised as lean, overweight or obese based on BMI < 25 kg/m², 25 kg/m² ≤ BMI < 30 kg/m², and BMI ≥ 30 kg/m², respectively.

4.1.2 Enumeration of fecal microbes

4.1.2.1 Fecal DNA extraction

Total DNA was extracted from 80 mg of stool with E.Z.N.A.® Stool DNA Isolation Kit (OMEGA Bio-tek, Norcross, GA) according to the manufacturer’s protocol modified to include an additional lysozyme digestion step at 37°C for 30 minutes before the proteinase digestion step and eluted in 100 µl of UltraPure™ DNase/RNase-Free Distilled Water (Life Technologies Inc., Burlington, ON). DNA concentration (µg/µl) and purity (OD₂₆₀/₂₈₀, 2₆₀/₂₃₀) were assessed by Thermoscientific’s Nanodrop 1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) and DNA samples were stored at -20 °C until processing.

4.1.2.2 Quantitative analysis of fecal microbial composition

Fecal bacteria and Archaea were enumerated in triplicates by quantitative real-time PCR using 50 ng of DNA for the specific TaqMan® custom assay or SYBR green assay, in conjunction with the TaqMan® Gene Expression Master Mix (Applied Biosystems, Foster City, CA) or Power SYBR Green Master Mix (Applied Biosystems, Foster City, CA), respectively, and a 7900 HT fast real-time PCR system equipped with a 384 wells block (Applied Biosystems, Foster City, CA). Primer and probe sets and their corresponding efficiency and detection limits for the bacteria genera and/or groups of interest are listed in Table 3. Numbers of microbial cells in each sample were calculated by interpolation using pre-constructed standard curves, and were
expressed as log number of cell counts per gram of wet feces and log number of \textit{16S rRNA} gene copies per gram of wet feces for bacteria and Archaea, respectively. In addition, F/B ratio was calculated by dividing the sum of absolute amounts of \textit{C. coccoides} and \textit{C. leptum} by the absolute amount of \textit{Bacteroides/Prevotella}. The specificity of primer and probe sets were tested using DNA from pure bacterial cultures (Table 4). In addition, a non-template control was used in all qPCR runs. In all cases, only the DNA from target species successfully generated amplification product.
Table 3. List of specific 16S rRNA gene targeted primers and probes used in this study

<table>
<thead>
<tr>
<th>Target</th>
<th>Primers and Probes (5’ - 3’)</th>
<th>Detection limit (cells/ng of DNA)</th>
<th>Efficiency</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lower</td>
<td>Upper</td>
<td></td>
</tr>
<tr>
<td>Total Bacteria</td>
<td>Forward: CGGTGAATAACGTCCCAGG&lt;br&gt;Reverse: TACGGCTACTTGGTTACGACTT&lt;br&gt;Probe: CTTGTACACACCAGCCGTC</td>
<td>8.07E+02</td>
<td>2.52E+06</td>
<td>100.75%</td>
</tr>
<tr>
<td>Bacteroides/Prevotella</td>
<td>Forward: CCTTCGTGGATAGGGGTT&lt;br&gt;Reverse: CACGCTACTTGGCTGTTTCAG&lt;br&gt;Probe: AAGGTCCCCACACATTG</td>
<td>1.25E+01</td>
<td>1.25E+07</td>
<td>93.71%</td>
</tr>
<tr>
<td>C. coccoides</td>
<td>Forward: GACGGCCGCGTGAAGGA&lt;br&gt;Reverse: AGCCCCAGCCTTTCACATC&lt;br&gt;Probe: CGGTACCTGACTAAGAAG</td>
<td>3.09E+00</td>
<td>3.09E+06</td>
<td>99.93%</td>
</tr>
<tr>
<td>C. leptum</td>
<td>Forward: CCTTCCGTGCCGAGTTA&lt;br&gt;Reverse: GAATTTAACACATACATCAGCTGGTT&lt;br&gt;Probe: CACAATAGTAATCCACC</td>
<td>1.97E+02</td>
<td>1.97E+07</td>
<td>100.78%</td>
</tr>
<tr>
<td>Bifidobacteria</td>
<td>Forward: CGGGTGAGTAATGCCTGGGACC&lt;br&gt;Reverse: TGATAGGACGCGACA&lt;br&gt;Probe: CTCCTGGAAAACGGGTG</td>
<td>5.12E-01</td>
<td>5.12E+06</td>
<td>94.83%</td>
</tr>
<tr>
<td>E. coli</td>
<td>Forward: CATGCGCGCTGTATGAAGAA&lt;br&gt;Reverse: CGGGTAACGTCAATGAGCAAA</td>
<td>1.82E+00</td>
<td>1.82E+06</td>
<td>100.70%</td>
</tr>
<tr>
<td>Archaea</td>
<td>Forward: ATTAGATACCCGGGTAGTCC&lt;br&gt;Reverse: GCCATGCACCTCCTCT&lt;br&gt;Probe: AGGAATTGGCGGAGGACAC</td>
<td>2.51E+02</td>
<td>2.51E+10</td>
<td>100.52%</td>
</tr>
</tbody>
</table>

-Efficiency was calculated using the equation: Efficiency = -1+10^(-1/slope)
Table 4. Specificity of primer and probe sets for qPCR

<table>
<thead>
<tr>
<th>Strain</th>
<th>Primer sets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total Bacteria</td>
</tr>
<tr>
<td>Total bacteria</td>
<td>+</td>
</tr>
<tr>
<td>C. coccoides</td>
<td>+</td>
</tr>
<tr>
<td>C. leptum</td>
<td>+</td>
</tr>
<tr>
<td>Bacteroides/Prevotella</td>
<td>+</td>
</tr>
<tr>
<td>Bifidobacteria</td>
<td>+</td>
</tr>
<tr>
<td>E. coli</td>
<td>+</td>
</tr>
<tr>
<td>Archaea</td>
<td>-</td>
</tr>
</tbody>
</table>

+-, positive; -, negative
The standard curves for each TaqMan® custom assay and SYBR green custom assay were obtained through 9 serial 10-fold dilutions of pure bacterial DNA extracted from a given pure bacterial culture. The detection limit for each primer used in the study was calculated based on the upper and lower limit of the linearity range of the corresponding standard curve (Table 3). In the case of Archaea, the standard curve was obtained from serial dilutions prepared from plasmid DNA, as previously described [152]. Bacteria and culture conditions used in this study are listed in Table 5. Pure *M. smithii* (ATCC 35061) DNA was obtained from DSMZ, Braunschweig, Germany.

### Table 5. Bacteria and grow condition used in this study

<table>
<thead>
<tr>
<th>Species</th>
<th>Medium</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacteroides thetaotaomicron</em></td>
<td>PRAS cooked meat glucose medium (Remel, Lenexa, KS)</td>
<td>48-72 hrs 37 °C Anaerobiosis</td>
</tr>
<tr>
<td>ATCC 29148</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Clostridium coccoides</em></td>
<td>PRAS cooked meat glucose medium (Remel, Lenexa, KS)</td>
<td>48-72 hrs 37 °C Anaerobiosis</td>
</tr>
<tr>
<td>ATCC 29236</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Clostridium leptum</em></td>
<td>Reinforced Clostridal Medium, 10 g/L maltose (Oxoid, Nepean, ON)</td>
<td>4-7 days 37 °C Anaerobiosis</td>
</tr>
<tr>
<td>ATCC 29065</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bifidobacterium longum</em></td>
<td>MRS Broth, HCL-cysteine 0.05% (Oxoid, Nepean, ON)</td>
<td>24-48 hrs 37 °C Anaerobiosis</td>
</tr>
<tr>
<td>NCC2705</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**4.1.3 Diet analysis**

Dietary records were analyzed using The Food Processor SQL, Version 10.6.3 (ESHA Research; Salem, OR).
4.1.4 Data Analysis

All data collected was first tested for normality using Shapiro-Wilk test. The choices of parametric or non-parametric statistical tests were based on the normality test results using SPSS (SPSS, Inc., Somers, NY). The differences in terms of microbiology data, demographic and anthropometric parameters, and macronutrient intake among the groups that are classified according to BMI, the presence of Archaea, or breath CH₄, were tested using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). One-way ANOVA, Kruskal-Wallis test, t-test, or Mann-Whitney test was applied based on the distribution and nature of data sets. Chi-square test was applied to compare the distributions of gender and ethnicity among the groups using SPSS. GraphPad Prism was also used for examining the association among the counts of specific microbial group or count-derived ratio, demographic and anthropometric data, and macronutrient intake using Pearson’s correlation test. Multiple linear regression analysis (SPSS) was used to assess whether the observed associations persist after excluding the confounder effect of BMI, age and gender. All the graphs presented in this thesis were made using GraphPad Prism. For all statistical tests, p<0.05 was considered significant.
Chapter 5
Results

The recruitment of study subjects and the collection of their demographic data, anthropometric data, dietary record, and collection of fecal samples were done by Judlyn Fernandes, and Sari Rosenbloom.

Part of data from objective 3 is included in a manuscript under submission.
5 Results

5.1 Group comparison

5.1.1 Subject characteristics

In total, 99 subjects were recruited in the study, 46 males and 53 females (Table 6). The age range for the participants was from 18 to 67 years old, and the range for BMI was from 18.14 to 37.79 kg/m². The subjects were from different ethnic backgrounds with more than half of them being Caucasians. In addition, approximately, 89% (88/99) and 26% (26/99) subjects had detectable amount of breath H₂ and CH₄, respectively, and 19 subjects had detectable amount of both breath H₂ and CH₄. The ranges of breath H₂ and CH₄ in detectable subjects were found to vary from 0.3 to 53.8 ppm and 0.5 to 94.3 ppm, respectively.

Table 6. Characteristics of the studied subjects

<table>
<thead>
<tr>
<th>Total subjects</th>
<th>N=99</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Yrs)</td>
<td>18-67</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>18.14-37.79</td>
</tr>
<tr>
<td>Gender (Male:Female)</td>
<td>46:53</td>
</tr>
<tr>
<td>Breath CH₄ (ppm)</td>
<td>0.5-94.3 (n=26)</td>
</tr>
<tr>
<td>Breath H₂ (ppm)</td>
<td>0.3-53.8 (n=88)</td>
</tr>
<tr>
<td>Ethnicity (Caucasian:Asian:Black:Spanish)</td>
<td>51:40:5:3</td>
</tr>
</tbody>
</table>

-Data are shown as ranges except for gender and Ethnicity which are shown as counts.
5.1.1.1 Subject characteristics based on BMI

To determine the relationship between subject BMI and other characteristics, the 99 subjects were separated into 3 groups with 55 people in the lean group, 26 people in overweight group and 18 people in the obese group. The 3 groups were matching for gender and ethnicity. Kruskal-Wallis test was used to test whether the age, breath CH\textsubscript{4} and H\textsubscript{2} were different among the groups. As shown in Figure 2, the age of obese group was significantly higher than that of lean group. However, there were no significant differences in breath H\textsubscript{2} or CH\textsubscript{4} among the groups or groups containing only subjects with detectable amount of breath H\textsubscript{2} or CH\textsubscript{4}.
Figure 2. Demographic and anthropometric comparison among lean, overweight, and obese subjects. * and *** denote significance at p<0.05 and p<0.001 levels, respectively.
5.1.1.2 Subject characteristics based on the presence of Archaea

To assess whether specific subject characteristics are affected by the presence of Archaea, the 99 subjects were divided into 2 groups based on the presence (Arch+) or the absence of Archaea (Arch-). The Arch+ and Arch- groups included 37 and 62 subjects, respectively, and were also matching for gender and ethnicity. Mann-Whitney test revealed that, compared to Arch- groups, Arch+ subjects had a significantly higher level of breath CH₄ when including subjects with non-detectable breath CH₄ (Figure 3). No difference was found in age, BMI, and breath H₂ between the groups.
Figure 3. Demographic and anthropometric comparison between Arch+ and Arch- subjects. *** denotes significance at p<0.001 level.
5.1.1.3 Subject characteristics based on the presence of breath CH$_4$

To assess the relationship between subject breath CH$_4$ and other characteristics, subjects were categorized as methane producers (CH$_4^+$) or non-producers (CH$_4^-$) based on whether he or she had detectable amount of breath CH$_4$ (i.e higher than 2 ppm). Of 99 subjects, 26 were CH$_4^+$, and 73 were identified as CH$_4^-$. CH$_4^-$ subjects had a significantly higher breath H$_2$ concentration than CH$_4^+$ subjects including or excluding subjects without detectable H$_2$ (Figure 4). CH$_4^+$ and CH$_4^-$ subjects were matching for their gender and ethnicity, and no difference was found in terms of age and BMI between those two groups.

![Figure 4](image-url)

* and *** denote significance at p<0.05 and p<0.001 levels, respectively.

Figure 4. Demographic and anthropometric comparison between CH$_4^+$ and CH$_4^-$ subjects.
5.1.2 Energy and macronutrient intake

Totally, 97 dietary records were collected from the participants (subject No. 62 had his dietary record misplaced; subject No. 73 is considered an outlier for his dietary data). The intake of total energy, percentage of total energy intake from carbohydrate, protein and fat, and other macronutrients such as carbohydrates, protein, fat, sugar, dietary fiber, soluble fiber, saturate fat, and polyunsaturated fat were included for dietary analysis.

5.1.2.1 Energy and macronutrient intake based on BMI

To evaluate the relationship between BMI and energy/macronutrient intake, total energy intake, percentage of total energy intake from carbohydrate, protein and fat, and macronutrient intake were compared among the 3 BMI groups by Kruskal-Wallis test or one-way ANOVA according to normality test. The total energy intake among lean, overweight and obese groups was not significantly different from each other (Table 7). Moreover, carbohydrate, fat, sugar, dietary fiber, soluble fiber, saturate fat, polyunsaturated fat, and cholesterol intakes were not different among the groups. Nevertheless, obese groups had a higher intake of protein compared to lean or overweight groups. However, the percentages of total energy intake from protein as well as from carbohydrate and fat were similar in the 3 BMI groups (Table 7).

5.1.2.2 Energy and macronutrient intake based on the presence of Archaea

To assess the relationship between Archaea concentration and energy/macronutrient intake, total energy intake, percentage of total energy intake from carbohydrate, protein and fat, and macronutrient intake were compared between Arch+ and Arch- groups by Mann-Whitney test or t-test based on the normality of the distribution. Total energy intake, percentage of total energy
intake from carbohydrate, protein and fat as well as macronutrient intakes were not significantly
different from each other (Table 8).

5.1.2.3 Energy and macronutrient intake based on the presence of
breath CH₄

Next, to determine if there is an association between breath CH₄ levels and energy/macronutrient
intake, energy and dietary intake were compared between CH₄+ and CH₄- groups. Based on the
normality test, Mann-Whitney test or t-test was used. CH₄+ subjects had significant higher level
of dietary fiber intake than subjects in CH₄- (Table 9). Other dietary parameters were not
significantly different between the groups.
Table 7. Energy and macronutrient intake: Lean, overweight, and obese subjects

<table>
<thead>
<tr>
<th></th>
<th>Lean (n=53)</th>
<th>Overweight (n=26)</th>
<th>Obese (n=18)</th>
<th>p-value(^1)</th>
<th>p-value(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total energy intake (Kcals)</td>
<td>2066±93</td>
<td>1966±106</td>
<td>2265±168</td>
<td></td>
<td>0.2325</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>260.99±14.25</td>
<td>238.09±15.36</td>
<td>262.22±21.91</td>
<td></td>
<td>0.6284</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>84.50±4.76(^a)</td>
<td>77.23±4.51(^a)</td>
<td>105.01±8.61(^b)</td>
<td>0.0188*</td>
<td></td>
</tr>
<tr>
<td>Fat (g)</td>
<td>76.00±3.86</td>
<td>78.39±5.53</td>
<td>88.51±9.15</td>
<td></td>
<td>0.2928</td>
</tr>
<tr>
<td>Sugar (g)</td>
<td>88.83±6.05</td>
<td>86.13±8.98</td>
<td>90.80±13.50</td>
<td>0.9777</td>
<td></td>
</tr>
<tr>
<td>Dietary fiber (g)</td>
<td>21.52±1.77</td>
<td>20.18±1.36</td>
<td>20.68±1.70</td>
<td>0.6913</td>
<td></td>
</tr>
<tr>
<td>Soluble fiber (g)</td>
<td>0.57±0.09</td>
<td>0.87±0.16</td>
<td>0.74±0.20</td>
<td>0.1692</td>
<td></td>
</tr>
<tr>
<td>Saturated fat (g)</td>
<td>24.14±1.49</td>
<td>25.48±2.36</td>
<td>29.66±3.50</td>
<td>0.3994</td>
<td></td>
</tr>
<tr>
<td>Polyunsaturated fat (g)</td>
<td>10.67±0.81</td>
<td>11.96±1.56</td>
<td>12.72±1.66</td>
<td>0.3411</td>
<td></td>
</tr>
<tr>
<td>% of total energy intake - carbohydrate</td>
<td>50.34±0.01</td>
<td>48.48±0.02</td>
<td>46.23±0.02</td>
<td>0.2252</td>
<td></td>
</tr>
<tr>
<td>% of total energy intake - protein</td>
<td>16.46±0.01</td>
<td>16.10±0.01</td>
<td>19.41±0.01</td>
<td>0.1884</td>
<td></td>
</tr>
<tr>
<td>% of total energy intake - fat</td>
<td>33.19±0.01</td>
<td>35.42±0.02</td>
<td>34.37±0.02</td>
<td>0.5134</td>
<td></td>
</tr>
</tbody>
</table>

-Values are Mean ± SEM
-\(^*\) donates significance at p<0.05 level
-\(^1\)Kruskal-Wallis test
-\(^2\)One-way ANOVA
<table>
<thead>
<tr>
<th></th>
<th>Arch+ (n=36)</th>
<th>Arch- (n=61)</th>
<th>p-value(^1)</th>
<th>p-value(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total energy intake (Kcals)</td>
<td>1988±94</td>
<td>2127±91</td>
<td>0.4502</td>
<td></td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>234.32±12.33</td>
<td>267.22±13.56</td>
<td>0.1325</td>
<td></td>
</tr>
<tr>
<td>Protein (g)</td>
<td>80.75±5.25</td>
<td>89.59±4.47</td>
<td>0.2411</td>
<td></td>
</tr>
<tr>
<td>Fat (g)</td>
<td>80.91±5.31</td>
<td>77.78±3.88</td>
<td></td>
<td>0.5708</td>
</tr>
<tr>
<td>Sugar (g)</td>
<td>83.40±8.11</td>
<td>91.42±5.96</td>
<td>0.4418</td>
<td></td>
</tr>
<tr>
<td>Dietary fiber (g)</td>
<td>19.60±1.08</td>
<td>21.83±1.61</td>
<td>0.9643</td>
<td></td>
</tr>
<tr>
<td>Soluble fiber (g)</td>
<td>0.61±0.15</td>
<td>0.72±0.09</td>
<td>0.1505</td>
<td></td>
</tr>
<tr>
<td>Saturated fat (g)</td>
<td>27.75±2.32</td>
<td>2421±1.41</td>
<td>0.1730</td>
<td></td>
</tr>
<tr>
<td>Polyunsaturated fat (g)</td>
<td>10.90±1.10</td>
<td>11.67±0.88</td>
<td>0.6012</td>
<td></td>
</tr>
<tr>
<td>% of total energy intake - carbohydrate</td>
<td>47.51±1.46</td>
<td>50.01±1.05</td>
<td></td>
<td>0.1952</td>
</tr>
<tr>
<td>% of total energy intake - protein</td>
<td>16.50±0.76</td>
<td>17.15±0.61</td>
<td></td>
<td>0.4135</td>
</tr>
<tr>
<td>% of total energy intake - fat</td>
<td>35.99±1.33</td>
<td>32.84±0.87</td>
<td></td>
<td>0.0529</td>
</tr>
</tbody>
</table>

- Values are Mean ± SEM
- \(^1\) t-test
- \(^2\) Mann-Whitney test
Table 9. Energy and macronutrient intake: CH₄+ and CH₄- subjects

<table>
<thead>
<tr>
<th></th>
<th>CH₄+ (n=25)</th>
<th>CH₄- (n=72)</th>
<th>p-value¹</th>
<th>p-value²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total energy intake (Kcals)</td>
<td>2012±128</td>
<td>2098±78</td>
<td>0.5748</td>
<td></td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>247.17±19.48</td>
<td>257.86±11.29</td>
<td>0.6343</td>
<td></td>
</tr>
<tr>
<td>Protein (g)</td>
<td>82.04±7.00</td>
<td>87.81±3.92</td>
<td>0.1480</td>
<td></td>
</tr>
<tr>
<td>Fat (g)</td>
<td>77.25±6.45</td>
<td>79.50±3.56</td>
<td>0.7530</td>
<td></td>
</tr>
<tr>
<td>Sugar (g)</td>
<td>82.51±10.78</td>
<td>90.51±5.26</td>
<td>0.2625</td>
<td></td>
</tr>
<tr>
<td>Dietary fiber (g)</td>
<td>23.87±1.88</td>
<td>20.03±1.30</td>
<td>0.0202*</td>
<td></td>
</tr>
<tr>
<td>Soluble fiber (g)</td>
<td>0.68±0.16</td>
<td>0.68±0.09</td>
<td>0.7099</td>
<td></td>
</tr>
<tr>
<td>Saturated fat (g)</td>
<td>25.02±2.55</td>
<td>25.67±1.41</td>
<td>0.8163</td>
<td></td>
</tr>
<tr>
<td>Polyunsaturated fat (g)</td>
<td>11.86±1.62</td>
<td>11.22±0.74</td>
<td>0.8930</td>
<td></td>
</tr>
<tr>
<td>% of total energy intake - carbohydrate</td>
<td>48.90±2.06</td>
<td>49.16±0.91</td>
<td>0.8965</td>
<td></td>
</tr>
<tr>
<td>% of total energy intake - protein</td>
<td>16.72±0.94</td>
<td>16.97±0.55</td>
<td>0.8037</td>
<td></td>
</tr>
<tr>
<td>% of total energy intake- fat</td>
<td>34.39±1.77</td>
<td>33.87±0.80</td>
<td>0.7623</td>
<td></td>
</tr>
</tbody>
</table>

-Values are Mean ± SEM
-* donates significance at p<0.05 level
¹t- test
²Mann-Whitney test
5.1.3 Fecal microbial composition

All 99 subjects had quantifiable *C. coccoides* (7.50-10.18 log cell counts/g of wet feces), *C. leptum* (7.61-10.49 log cell counts/g of wet feces) and *Bacteroides/Prevotella* (5.61-9.84 log cell counts/g of wet feces) corresponding to 0.29% to 72.72%, 0.28% to 55.05% and 0.002% to 29.26% of total bacteria, respectively. 95 and 86 subjects had quantifiable number of *Bifidobacteria* (5.01-10.64 log cell counts/g of wet feces) corresponding to less than 0.00% to 82.19% of total bacteria and *E. coli* (3.81-10.42 log cell counts/g of wet feces) corresponding to less than 0.00% to 2.04% of total bacteria, respectively. Archaea were detected in 37 subjects (6.29-10.24 log copies/g of wet feces).

5.1.3.1 Fecal microbial composition based on BMI

According to normality test, Kruskal-Wallis test or one-way ANOVA were chosen over the other to analyze microbiology data. There was no difference in terms of amount of specific microbial groups quantified in the study, nor the log F/B ratio among the 3 BMI groups (Figure 5).
Figure 5. Fecal microbial composition comparison among lean, overweight, and obese subjects. ¹ indicates the use of one-way ANOVA instead of Kruskal-Wallis test.
5.1.3.2 Fecal microbial composition based on the presence of Archaea

Either Mann-Whitney or t-test was performed for data analysis. When Arch+ and Arch- groups were compared, there were significantly higher levels of *C. leptum* and total bacteria in the Arch+ group (Figure 6). On the other hand, the amount of other microbes and log F/B ratio were similar between the groups.
Figure 6. Fecal microbial composition comparison between Arch+ and Arch- subjects. 

indicates the use of t-test instead of Mann-Whitney test. * denotes significance at p<0.05 level.
5.1.3.3 Fecal microbial composition based on the presence of breath CH$_4$

When only the presence of breath CH$_4$ was considered, CH$_4^+$ subjects had significant higher level of Archaea than CH$_4^-$ participants (Figure 7). The bacterial composition was not significantly different between the groups.
Figure 7. Fecal microbial composition comparison between CH₄+ and CH₄- subjects. ¹
indicates the use of t-test instead of Mann-Whitney test. * denotes significance at p<0.05 level.
5.2 Correlation analysis

Next, to assess the association among fecal microbial composition, demographic data, anthropometric data, and dietary intake, Pearson’s r was used for correlation analyses. In addition, multiple regression analysis was applied to assess the strength of relationship after adjusting for confounding factors such as BMI, age, and gender.

5.2.1 Fecal microbial composition vs. demographic and anthropometric parameters

BMI was inversely correlated with the counts of *Bacteroides/Prevotella* and Total Bacteria (Figure 8). Also, there was a significant negative association between BMI and *E. coli* counts for the 86 subjects with detectable amount of *E. coli*. When correlating breath CH$_4$ with microbiology data (n=26), breath CH$_4$ was found to be negatively correlated with *C. coccoides* and log F/B ratio, and positively correlated with the amount *E.coli* and fecal Archaea (Figure 9). In particular, breath CH$_4$ was found to be positive correlated with the amount of fecal Archaea after adjusting for BMI, age and gender (Table 10).
Figure 8. Association between fecal microbial composition and BMI.
Figure 9. Association between fecal microbial composition and breath CH4.
5.2.2 Fecal microbial composition vs. dietary intake

There were significant negative correlations between carbohydrate, dietary fiber intake and *C. coccoides* group independently of BMI, age and gender (Figure 10) (Table 11). For *C. leptum* group, there was a significant negative correlation with polyunsaturated fatty acids (PUFAs) intake, which was not significant after adjusting for BMI, age and gender (Table 11). The counts of *Bacteroides/Prevotella* phylum and total bacteria were negatively associated with PUFAs intake, but were no longer significant after adjusting for BMI, age and gender (Table 11). In the case of Bifidobacteria and *E. coli*, they were not significant association with any of the macronutrients.
Figure 10. Association between macronutrient intakes and fecal microbial composition.
5.2.3  Anthropometric parameters vs. dietary intake

There were significantly positive correlations between total energy intake, protein intake, fat intake, saturate fat intake and BMI (Figure 11). In addition, a positive association was observed between breath CH$_4$ and dietary fiber consumption with or without adjusting for BMI, age and gender (Table 12).
Figure 11. Association between macronutrient intakes and BMI or breath CH₄.
5.2.4 Other correlations

There was a positive correlation between breath CH$_4$ and the age of participants (Figure 12).

Figure 12. Association between age and breath CH$_4$.
Table 10. Multiple linear regression: microbiology data and breath CH$_4$ and H$_2$

<table>
<thead>
<tr>
<th>Dependent variables</th>
<th>C. coccoides</th>
<th>C. leptum</th>
<th>Bacteroides /Prevotella</th>
<th>Bifidobacteria</th>
<th>E. coli</th>
<th>Archaea</th>
<th>All bac</th>
<th>log F/B</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH$_4$</td>
<td>-.139, .153</td>
<td>-.054, .573</td>
<td>.023, .814</td>
<td>.053, .574</td>
<td>.173, .067</td>
<td>.305, .001*</td>
<td>.065, .502</td>
<td>-.081, .393</td>
</tr>
<tr>
<td>H$_2$</td>
<td>.158, .136</td>
<td>.057, .585</td>
<td>-.069, .510</td>
<td>.110, .283</td>
<td>.001, .992</td>
<td>-.137, .183</td>
<td>.050, .636</td>
<td>.127, .221</td>
</tr>
</tbody>
</table>

- Values are expressed as standardized coefficients Beta, and significance
- Controlling for Age, BMI and sex
- * denotes significance at p<0.05 level
### Table 11. Multiple linear regression: dietary intake and microbiology data

<table>
<thead>
<tr>
<th>Dependent variables</th>
<th>Total E</th>
<th>Carb</th>
<th>Protein</th>
<th>Fat</th>
<th>DT_fiber</th>
<th>Sat_fat</th>
<th>Poly_fat</th>
<th>% Carb</th>
<th>% Protein</th>
<th>% Fat</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. coccoides</td>
<td>-.166,.103</td>
<td>-.214,.030*</td>
<td>-.121,.255</td>
<td>-.027,.789</td>
<td>-.242,.014*</td>
<td>.019,.856</td>
<td>-.068,.500</td>
<td>-.150,.135</td>
<td>.028,.773</td>
<td>.149,.130</td>
</tr>
<tr>
<td>C. leptum</td>
<td>-.043,.679</td>
<td>-.057,.576</td>
<td>-.061,.575</td>
<td>.008,.940</td>
<td>-.016,.876</td>
<td>.065,.534</td>
<td>-.155,.126</td>
<td>.000,.998</td>
<td>-.068,.504</td>
<td>.042,.675</td>
</tr>
<tr>
<td>Bacteroides/Prevotella</td>
<td>-.028,.789</td>
<td>-.001,.989</td>
<td>-.100,.355</td>
<td>-.020,.852</td>
<td>-.023,.821</td>
<td>.032,.763</td>
<td>-.197,.052</td>
<td>.107,.295</td>
<td>-.076,.458</td>
<td>-.073,.471</td>
</tr>
<tr>
<td>Bifidobacteria</td>
<td>-.076,.481</td>
<td>-.019,.855</td>
<td>-.077,.494</td>
<td>-.120,.265</td>
<td>.029,.784</td>
<td>-.091,.370</td>
<td>-.120,.253</td>
<td>.070,.504</td>
<td>.001,.992</td>
<td>-.079,.445</td>
</tr>
<tr>
<td>E. coli</td>
<td>.014,.895</td>
<td>-.026,.803</td>
<td>.153,.157</td>
<td>.001,.990</td>
<td>-.082,.422</td>
<td>-.039,.711</td>
<td>.056,.586</td>
<td>-.059,.565</td>
<td>.156,.126</td>
<td>-.027,.792</td>
</tr>
<tr>
<td>Archaea</td>
<td>-.075,.705</td>
<td>-.092,.636</td>
<td>-.117,.554</td>
<td>-.003,.987</td>
<td>.210,.266</td>
<td>.100,.596</td>
<td>-.100,.592</td>
<td>-.019,.917</td>
<td>-.030,.867</td>
<td>.038,.834</td>
</tr>
<tr>
<td>All bac</td>
<td>-.096,.354</td>
<td>-.092,.363</td>
<td>-.079,.466</td>
<td>-.059,.571</td>
<td>-.101,.315</td>
<td>.007,.949</td>
<td>-.173,.087</td>
<td>.020,.847</td>
<td>-.001,.996</td>
<td>-.023,.823</td>
</tr>
<tr>
<td>log F/B</td>
<td>-.018,.861</td>
<td>-.067,.516</td>
<td>.079,.470</td>
<td>.018,.862</td>
<td>-.041,.689</td>
<td>.001,.992</td>
<td>.142,.168</td>
<td>-.164,.112</td>
<td>.090,.383</td>
<td>.127,.211</td>
</tr>
</tbody>
</table>

*Values are expressed as standardized coefficients Beta, and significance*

*Controlling for Age, BMI and sex*

* denotes significance at p<0.05 level

- Total E: total energy; Carb: carbohydrate; DT_fiber: dietary fiber; Sat_fat: saturated fat; poly_fat: polyunsaturated fat
- %Carb: % of total energy from carbohydrate; % Protein: % of total energy from protein; % Fat: % of total energy from fat
Table 12. Multiple linear regression: dietary intake and breath CH₄ and H₂

<table>
<thead>
<tr>
<th>Dependent variables</th>
<th>Total E</th>
<th>Carb</th>
<th>Protein</th>
<th>Fat</th>
<th>DT_fiber</th>
<th>Sat_fat</th>
<th>Poly_fat</th>
<th>% Carb</th>
<th>% Protein</th>
<th>% Fat</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH₄</td>
<td>-0.06, 0.952</td>
<td>0.021, 0.821</td>
<td>0.030, 0.765</td>
<td>-0.058, 0.546</td>
<td>0.221, 0.016*</td>
<td>-0.038, 0.697</td>
<td>-0.070, 0.445</td>
<td>0.063, 0.504</td>
<td>0.046, 0.625</td>
<td>-0.098, 0.290</td>
</tr>
<tr>
<td>H₂</td>
<td>0.061, 0.564</td>
<td>0.056, 0.583</td>
<td>0.046, 0.673</td>
<td>0.041, 0.700</td>
<td>0.037, 0.715</td>
<td>0.056, 0.605</td>
<td>0.003, 0.978</td>
<td>0.054, 0.605</td>
<td>-0.008, 0.938</td>
<td>-0.055, 0.586</td>
</tr>
</tbody>
</table>

- Values are expressed as standardized coefficients Beta, and significance
- Controlling for Age, BMI and sex
- * denotes significance at p<0.05 level
- Total E: total energy; Carb: carbohydrate; DT_fiber: dietary fiber; Sat_fat: saturated fat; poly_fat: polyunsaturated fat
- %Carb: % of total energy from carbohydrate; % Protein: % of total energy from protein; % Fat: % of total energy from fat
Chapter 6
Discussion
6 Discussion

This cross-sectional study investigated the relative importance of demographic and anthropometric measurements, energy and macronutrient intake and fecal microbial composition in adults. In particular, this analysis focused on the impact of BMI, the intakes of energy and macronutrient, and the amount of Archaea on fecal microbial profiles.

BMI is considered to be one of the most important factors modulating the gut microbial composition [153]. In the current study, 99 subjects were categorized into lean, overweight and obese groups based on BMI, and then, demographic and anthropometric measurements, microbiology data, energy and macronutrient intake were compared among the 3 groups. Data indicated that the obese people were significantly older than the lean subjects enrolled in the study. Similar finding has been reported in a number of publications [154][155][156]. This suggests that weight changes are closely linked to age-related events such as sedentary life style, muscle loss and decreased metabolic activities [157][158].

A number of early studies looked into the connection between BMI, skin fold measurements, waist to hip ratio and/or waist circumference and energy intake, and suggested the potential role of macronutrients (carbohydrate, protein and fat) in the development of obesity in adulthood ([159][160][161]. In this study, in addition to carbohydrate, protein and fat, subjects’ intakes of sugar, dietary fiber, soluble fiber, saturate fat, polyunsaturated fat, cholesterol were also evaluated as part of macronutrient analysis. While the total energy intakes among the 3 BMI groups were similar to each other, which is consistent with published literature [162][163], there was a positive correlation between total energy intake and BMI in the study, suggesting that people with higher calorie intake tend to also have higher BMI. Moreover, no evidence of significant associations between the percentage energy intake from carbohydrate, protein or fat
and BMI was found, which is in accordance with previous reports [164]. In the current study, the 3 BMI groups had similar intake of macronutrients except for protein intake that was significantly higher in the obese group compared to lean or overweight subjects. This is expected, given that moderately elevated protein intake increases thermogenesis, satiety, and retains fat-free mass, which may be part of the weight management some of these obese participants are subject to [165]. On the other hand, it has been reported that total dietary fat intake including saturated fatty acids, and PUFAs is positively associated with BMI [166][167]. In line with this, data from current study demonstrated that there was a significant positive correlation between total energy, protein, fat, or saturated fat intake and BMI suggesting that total calorie intake and the energy density of the food are of great importance in one’s weight status. Furthermore, it has been shown that obesity is related to low-grade inflammation possibly caused by elevated level of LPS. In animal studies it was demonstrated that compared to control, the plasma concentration of endotoxin in 4 week high-fat-fed mice was 2 to 3 times higher compared to control, which was accompanied by reduced number of C. coccoides group and the anti-inflammatory Bifidobacteria, suggesting an increased proportion of LPS-containing bacterial population in the gut [146]. Also, it has been shown that consuming Western diet characterized by high fat content for 1 month period led to a 71% increase in plasma levels of endotoxin activity measured using a specially designed monoclonal antibody targeting both soluble and bound LPS [168]. Such rise in endotoxin activity could result from either an altered intestinal epithelial barrier function or a change in gut microbial composition; both can be caused by the consumption of Western diet. Consistently, it was found in the current study that there was a positive correlation between BMI and fat intake, potentially as a result of elevated LPS in response to high fat intake, which in turn leads to higher BMI. In accordance with mice studies, similar findings have been observed in humans as reported in intervention study [169] and
population-based cross-sectional survey [170]. Increased levels of inflammatory makers such as TNF-α and IL-6 could impair insulin sensitivity by suppressing insulin signal transduction that in turn leads to higher blood sugar level and increased fat production [171][172].

While current study suggested that being lean, overweight or obese based on BMI cut-offs does not necessarily transform into differences in the levels of fecal *C. coccoides, C. leptum, Bacteroides/Prevotella*, Bifidobacteria, total bacteria, log F/B ratio, and Archaea, this study indicated that the amount of *Bacteroides/Prevotella* and total bacteria present in the feces are inversely correlated with BMI. Numbers of Firmicutes and Bacteroidetes as the two most predominant groups of bacteria found in human gut have been shown to be linked to weight changes in both human and animal models. It has been suggested that obese humans and animals have higher percentage of their gut bacteria coming from Firmicutes counterbalanced by relative less bacteria from the phylum of Bacteroidetes. Indeed, the proportion of Firmicutes tended to decrease overtime when the patient went through weight loss program [4]. On the other hand, in adults, the proportion of Bacteroidetes was found to be significantly higher in lean than obese subjects by using qPCR and *16S rRNA* gene sequencing [129][173]. A decrease of *Bacteroides* caused by the death of such predominant Gram-negative bacteria has been proposed to be pro-inflammatory by releasing endotoxin in the gut. LPS is an endotoxin and a primary constituent of the outer membrane of Gram-negative bacteria, which is mobilized into the gut upon the death of these bacterial cells [174]. Once LPS goes into intestinal capillaries, it binds to lipopolysaccharide-binding protein in serum and exerts pro-inflammatory effect through TLR4 dependent cascade. Nevertheless, circulating levels of LPS were not measured in the current study, and should be evaluated in future studies. Though based on the current study, it can be speculated that high fat intake damages the intestinal environment making it unfavorable for bacteria.
Since the interaction between Firmicutes and Bacteroidetes has been suggested to play an important role in weight changes, the log F/B ratio provides a way to evaluate Firmicutes and Bacteroidetes simultaneously, and it is a maker of dysbiosis. In this study, the log F/B ratio was not significantly different among the 3 BMI groups, which is in line with the finding of other groups [6][128], but in disagreement with others [4][5]. The contradiction could rise from the difference in methodology (qPCR vs 16S rRNA gene based sequencing) and the nature of study population. Also, such ratio has been found to be diet-related. Ley et al. has shown that F/B ratio was positively associated with the changes in body weight (%) regardless of the type of low calorie diets [4]. In addition, a comparative study of gut microbiota of 1 to 6 year old from two different regions has indicated that, rural African kids consuming diet characterized by high fiber content had a significantly increased proportion of Bacteroidetes and a decreased proportion of Firmicutes in contrast to that of European kids following a western diet [61]. What’s more, the F/B ratio has also been shown to be age-related with 0.4 in the infants, 10.9 in the adults and 0.6 in the elders [175].

Although the amount of fecal E. coli was also not significantly different among the three BMI groups, this study has shown that BMI is negatively associated with the level of E. coli. In contrast, Santacruz et al. reported an increased E. coli levels in overweight pregnant women (BMI>25 kg/m²) [135]. The discrepancy can be partially explained by the distinct study designs and subjects of interest (pregnant vs. general population) analyzed in each study. Given its gram-negative property, a reduced level of E. coli caused by its death may relate to low-grade inflammatory status in overweight and obese subjects via a similar mechanism as Gram-negative Bacteroidetes.
Given that age and protein intake are higher among the obese subjects in current study, a partial correlation analysis that controlled for age and protein intake was applied to see whether the association of BMI with other variables is still valid. After adjusting for age and protein intake, BMI was found to be negatively associated with the *C. coccoides*, *E. coli* counts and breath CH₄ concentration. *C. coccoides* group together with *C. leptum* group are the two most important butyrate-producing bacteria clusters present in human fecal samples [176][177]. Butyrate is a SCFA derived from colonic fermentation of dietary fiber by the gut bacteria. It has been suggested to be beneficial in both intestinal and extra-intestinal conditions. In particular, butyrate is considered as an anti-inflammatory agent, and has the ability to alleviate oxidative stress. Since obesity is associated with a low-grade inflammatory state, decrease level of *C. coccoides* could lead to less butyrate being produced which may in turn contribute to the inflammatory state. The negative association observed over BMI and breath CH₄ is opposite to what has been reported by other group [178]. However, there were major limitations for the cited study. In contrast to current study, the observed positive association by Basseri *et al.* was based on a small sample size with only obese subjects whose BMI range from 30.3 to 57.2 kg/m², and 12 out of the total 58 participants were methane-positive based on the criteria implemented in the study. Moreover, the subjects in that study were all seeking surgical or medical option for weight loss, and may not be a good proxy for general obese population as well as the general public.

Dietary habits, especially calorie and macronutrient intakes, are important environmental factors that modulates the diversity and density of human gut microbiota. While most animal studies support a positive correlation between carbohydrate intake or dietary fiber intake and *C. coccoides* after adjusting for energy intake, finding from current study suggested otherwise.
An increased proportion of *C. coccoides* has been associated with increased BMI, weight, body fat, fat mass percentage, serum triglycerides, and decreased high-density lipoprotein HDL [181]. In this study, higher carbohydrate or dietary fiber intake were associated with lower counts of *C. coccoides*, and the observed relationships were valid even after adjusting for BMI, age and sex. Interestingly, an *in vitro* three-stage colonic model has shown that high level of fiber intake is associated with up-regulated level of *C. coccoides* in the vessel 1 that is designed to resemble the proximal colon [58]. The different observation generated from the current study and study done by Shen *et al.* could result from the fact that the bacterial content of proximal colon content is different from the bacterial content of stools. On the other hand, a comparison study investigating the fecal microbial composition in vegetarians and omnivores has implicated that the type of diet rather than specific macronutrient component may be a more relevant approach to formulate relationship with specific microbial groups [182]. Moreover, it has been shown that long-term dietary pattern correlate with enterotypes clustering in a way that animal protein/fat and carbohydrates were associated with *Bacteroides* enterotype and *Prevotella* enterotype, respectively [183]. This study was not designed to quantify *Bacteroides* and *Prevotella* separately; therefore, no conclusion can be drawn in the context of enterotypes.

Interestingly, data generated from current study demonstrated a number of negative associations between *C. leptum*, total bacteria, *Bacteroides/Prevotella* and PUFAs intake. PUFAs are fatty acids containing more than one double bond and considered to be beneficial in both human and animal health. Most of the genes present in gut microbiome do not normally engage in fatty acid metabolism [184]. However, there are reports indicating that PUFAs affect the gut microbiota. *In vitro* studies have revealed that modulatory effect of PUFAs concentrations in the growth and adhesion of different *Lactobacillus* strains. A concentration of 10-40 μg/ml of PUFAs stunned the growth and adhesion of several lactobacilli, whereas 5μg/ml of PUFAs promoted the growth
and adhesion to mucus of *Lactobacillus casei* Shirota (*L. casei* Shirota) [185]. The observed link suggested that dietary fatty acids can alter the fatty acid composition of intestinal wall, and modify the attachment site to promote or inhibit the colonization of indigenous microbiota. In addition, high monounsaturated fatty acids intake has been associated with decreased total bacteria, but did not affect individual bacterial groups, in a metabolic syndrome prone population [186].

The third objective of this study was to assess if the presence of Archaea is a determinant of fecal microbial composition. Fecal Archaea are methanogens, and utilize the H₂ produced by colonic fermentation to generate CH₄ that is excreted as flatus or diffuses into portal vein and is excreted in breath. *M. smithii* is the dominant species of Archaea found in human feces [187]. Animal studies suggest that methanogenic Archaea could indirectly promote the energy extraction from the food in the colon by improving colonic fermentation through their ability of utilizing H₂ in the gut [3]. Out of 99 subjects, 37 and 62 of them were Arch+ and Arch-, respectively. In contrast to the study of Zhang *et al.* which had a small sample size of only 3 subjects in each group, and found that normal weight subjects had no detectable amount of fecal Archaea, the percentage of Arch+ subjects in this study was not significantly different among the lean, overweight and obese groups [133]. Most bacteria groups were not affected by the presence of Archaea except for the counts of *C. leptum* and total bacteria. The two groups did not differ in any of the demographic and anthropometric measurement, total energy and macronutrient intakes, except for breath CH₄ which was higher in the Arch+. This is in line with Archaea being the only methane producer in the human gut. Moreover, the increased presence of Archaea was positively associated with breath CH₄ level that stands after adjusting for BMI, age and gender.
Compared to 46% of Arch+ subjects did not have detectable breath CH$_4$ in the study, only 23% of CH$_4$+ did not have detectable Archaea supporting that qPCR quantification as a more sensitive tool to detect Archaea than breath CH$_4$ measurement. Moreover, the presence of oral Archaea can also confound with the breath CH$_4$ measurement weakening the connection between fecal Archaea and breath CH$_4$.

In this study, 26 subjects has detectable amount of breath CH$_4$ (CH$_4$+), and the rest are without detectable breath CH$_4$ (CH$_4$-). The CH$_4$+ and CH$_4$- subjects were roughly matched for everything except that CH$_4$+ group had low concentration of breath H$_2$, higher amount of Archaea, and higher dietary fiber intake. CH$_4$+ subjects are expected to have a lower concentration of breath H$_2$ given that H$_2$ is utilized as a reducing agent to reduce CO$_2$ to CH$_4$. Moreover, in accordance with the finding by Fernandes et al., the concentration of breath CH$_4$ in this study was also positively associated with dietary fiber intake [188]. The positive relationship stands even after adjusting for BMI, age and gender. Interestingly, the level of Bacteroides has been shown to be elevated by dietary fiber, thus high dietary fiber intake could indirectly impact the breath CH$_4$ production of Archaea by providing more substrate for methanogenesis [189]. In addition, there was a clear positive correlation between breath CH$_4$ and age. Such association has been confirmed in a number of published studies [190][191]. Human infants are devoid of any detectable breath CH$_4$, and gradually obtaining the breath CH$_4$ level of adult at age of 10 [192]. Also, it has been indicated that the percentage of breath CH$_4$ producers in the population increases throughout adulthood [191]. Such association can be in part explained by the changes in gastrointestinal tract function over time: as we age, breath CH$_4$ excretion is likely enhanced by decreased colonic transit time and increased lactose intolerance [193]. Current study also suggested a linkage among breath CH$_4$ and log F/B ratio such that increased level of breath CH$_4$ was inversely associated with log F/B ratio. In line with this finding, Samuel et al. have shown
that compared to colonization with *M. smithii* or *B. thetaiotamicron* alone, GF mice colonization with both *M. smithii* and *B. thetaiotamicron* enhanced the representation of both species in the cecum and distal region of the colon [3]. In addition, we found that *E. coli* was positively associated with breath CH₄, perhaps because an increased level of *E. coli* provides more substrate (CO₂, H₂) for methanogenesis by Archaea in the gut. Also, *C. coccoides* was found to inversely correlate with breath CH₄ concentration. Unfortunately, no clear explanation can be given at this point. However, what really came to one’s attention is that most associations observed with breath CH₄ were not readily explained by the corresponding Archaea level(s). It has been shown that the concentration of subject’s Archaea in the colon has to exceed 10⁸ cells per gram of dry weight feces to have detectable amount of breath CH₄ [194]. One explanation is that the concentration of Archaea becomes physiologically relevant to human only when it is higher than a certain threshold that may vary from one to another. Further studies are needed to investigate this aspect.

6.1 Conclusion

1) Although the abundance of dominant and sub-dominant microbial groups was not different among lean, overweight and obese subjects, BMI was negatively associated with the *C. coccoides, E. coli* counts and breath CH₄ concentration after controlling for age and protein intake.

2) The total energy and micronutrient intake were similar among the 3 BMI groups but obese subjects had higher intake of protein. Increased presence of *C. coccoides* was inversely associated with carbohydrate and dietary fiber intake, whereas increased PUFAs intake was inversely associated with the abundance of *C. leptum, Bacteroides/Prevotella* and total bacteria.
Although the cause of such observation was not directly addressed in the study, it suggests a potential role of macronutrients in the modulation of gut microbiota.

3) The number of *C. leptum* and total bacteria were higher in Arch+ groups than Arch- subjects. However, the abundance of fecal Archaea was not associated with the gut microbial composition at genus or higher taxonomic level. On the other hand, breath CH₄, not fecal Archaea, was useful in evaluating the relationship between intestinal methanogenesis activity and its impact on gut microbial composition.

### 6.2 Limitation and future research

There are limitations to the current study that can be reconciled in future research.

Firstly, SCFAs are the major product of colonic fermentation. However, neither the absorption of SCFAs, nor SCFAs in the feces were measured. Those data can help towards a more insightful understanding of factors involved in colonic fermentation. It will be interesting to see how changes in the absorption or production of SCFAs are related to dietary intake and gut microbiota, thereby further substantiating the argument that dietary intake can influence physiological function of host through metabolic product generated by gut microbiota.

Secondly, the current study did not measure fecal energy loss, which can be used to derive true energy intake by subtracting fecal energy loss from total energy intake. This may help strengthen the power of the study to detect energy-intake-related changes in gut microbiota.
Lastly, in the current study, the number of bacteria was quantified based on wet weight of feces. Since the water content of feces varies among individuals, this may introduce potential error when calculating bacterial counts from qPCR data.

6.3 Significance

The obesity epidemic has become increasingly prevalent in Canada, and represents a huge burden to the Canadian healthcare system with approximately 3.96 (CDN) billion spending each year [195]. Emerging knowledge suggests more of an active role of gut microbial community in shaping the host’s overall health. Animal studies have provided insight and interest in the subject, but the ultimate measures in humans are urgently needed. To our knowledge, this is the first cross-sectional study conducted in Canadian population comprised of a group of people living in the same area with a very broad ethnicity, genetic background and various diet types. Overall, this study contributes to the knowledge of interplay between macronutrient intakes, gut microbial composition and adiposity, and may assist in the design of dietary intervention to better substantiate the effect of weight-control regime.
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