The Role of Short Chain Fatty Acids in Obesity

By

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Abstract

Short-chain fatty acids (SCFA) are produced by colonic microbiota, mostly from dietary fiber. Mice studies have shown that excess production of SCFA by the ‘obese microbiota’ (increased Firmicutes and decreased Bacteroidetes), may promote obesity via increased colonic energy availability. However, other studies have shown that colonic fermentation of dietary-fiber to SCFA may play a role in preventing obesity and diabetes.

Thus, the primary goals of this work were to investigate whether overweight and obese (OWO) humans produce SCFA more efficiently than lean (LN) humans, and whether SCFA from colonic fermentation of two different fibers affect appetite hormone and glycemic responses. To achieve these goals, 2 studies in LN and OWO human subjects were performed.

The first study confirmed higher fecal SCFA concentrations in OWO than LN humans. This could have been due to more efficient SCFA production by the ‘obese microbiota’, since colonic SCFA absorption, dietary intakes and markers for gut transit time were similar in LN and OWO subjects and, therefore, could not explain the difference in fecal SCFA between the groups. However, the second study did not confirm the higher SCFA concentrations in feces or in blood (either at fasting or postprandially following fiber consumption) in OWO compared to LN humans.

Unexpectedly the increase in SCFA from inulin or resistant-starch’s fermentation did not affect glycemia or appetite hormone responses (excluding a possible effect of inulin on ghrelin). On the other hand, both fibers induced early effects that were not likely related to colonic fermentation:
resistant-starch significantly reduced free-fatty acid rebound and second-meal glucose and insulin responses, while inulin significantly elevated the satiety hormone PYY.

The results do not support the hypothesis that overweight/obesity is associated with increased colonic SCFA production. The acute effects of consuming resistant starch to reduce glycemic responses and of inulin to improve appetite hormones profiles are not likely a result of their fermentation to SCFA. However, a longer adaptation to high fermentable fiber intakes may yield different results. Therefore, longer term and larger scales human studies are warranted.
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<td>BMI</td>
<td>body mass index</td>
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<td>CH₄</td>
<td>methane</td>
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<td>CHO</td>
<td>carbohydrates</td>
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<td>F:B</td>
<td>Firmicutes to Bacteroidetes ratio</td>
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<td>FFA</td>
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<td>GLP-1</td>
<td>glucagon-like peptide-1</td>
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<td>GLU</td>
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<td>g-protein coupled receptors</td>
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<td>iAUC</td>
<td>incremental area under curve</td>
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<td>monocarboxylate transporter</td>
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<td>overweight and obese</td>
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<td>peptide YY</td>
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<td>resistant-starch</td>
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<td>SCFA</td>
<td>short-chain fatty acids</td>
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<td>SEM</td>
<td>standard error of mean</td>
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<td>SMT1</td>
<td>sodium-coupled monocarboxylate transporter 1</td>
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<td>tAUC</td>
<td>total area under the curve</td>
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CHAPTER 1:
INTRODUCTION
1.1 Introduction

The alarming worldwide increase in the prevalence of obesity and type 2 diabetes mellitus (T2DM) has posed an urgent need for strategies to prevent and treat these epidemics. Among a few strategies that have been researched and developed over the last few decades (i.e., bariatric surgeries and pharmacological options), dietary interventions are the most frequent strategy at the population level (Rosa Fortin et al. 2014). Most dietary interventions include the recommendation to increase the daily fiber intake, since epidemiological and clinical data has shown its beneficial effect on reducing weight and hyperglycemia (McKeown et al. 2002, 390-398; Du et al. 2010, 329-336; Yao et al. 2014, 79-88; Delzenne and Cani 2005, 636-640). Although the exact mechanism as to how dietary fiber regulates these beneficial effects is not clear, one potential mechanism is through their fermentation into SCFA by the colonic microbiota.

Since the early 2000s, two major findings have led to an extensive research in the field of SCFA; in 2003, the receptors G-protein coupled receptors 41 (GPR41) and GPR43 have been identified as receptors for SCFA (Nilsson et al. 2003, 1047-1052). Since then, multiple functions for these receptors have been implicated in rodents, showing beneficial effects of colonic fermentation on improving glycemic responses and appetite regulation (Kimura et al. 2013, 1829; Samuel et al. 2008, 16767-16772; Brown et al. 2003, 11312-11319; Lin et al. 2012).

These findings were hampered in 2005 by Ley et al. who, based on mice studies, suggested that obesity is associated with changes in the relative abundance of the two dominant bacterial divisions, the Firmicutes and the Bacteroidetes (Ley et al. 2005, 11070-11075). They showed that the microbiota of obese mice differs from the lean microbiota, with greater ratio of Firmicutes to Bacteroidetes. In a following metagenomic analysis, it was shown that compared to the lean microbiota, the obese microbiota is enriched for genes encoding enzymes involved in breaking down indigestible dietary carbohydrates. Consequently, the obese microbiome is more efficient in fermenting SCFA (energy) from a given diet and may cause weight gain (Turnbaugh et al. 2006a, 1027-1031). A following human study confirmed the relative lower proportion of Bacteroidetes and higher Firmicutes in obese than lean individuals (Ley et al. 2006, 1022-1023).
Human studies comparing SCFA in lean and obese are sparse, and human studies comparing microbiota from lean and obese are inconsistent with the mice studies’ findings. However, considering the potentially conflicting hypotheses, we need to know if, and how, colonic SCFA are involved in the pathogenesis of obesity and T2DM, so that interventions to prevent or treat obesity can be aimed at the appropriate targets. For instance, if a more efficient fermentation of fiber due to the presence of certain colonic bacteria causes obesity, then fiber intake may not be effective in preventing and treating obesity because this would provide even more substrate for fermentation. On the other hand, increased SCFA may decrease appetite and blood glucose. Thus, my main hypotheses were that fecal SCFA concentration will be increased in overweight/obese compared to lean humans. Moreover, colonic fermentation of fermentable fibers (inulin and resistant starch) will elicit higher postprandial SCFA responses and have less effect in reducing serum FFA, glucose and insulin responses in overweight/obese compared to lean humans. These SCFA responses will also have less effect in reducing serum ghrelin and increasing GLP-1 and PYY responses in overweight/obese compared to lean humans.
CHAPTER 2:

LITERATURE REVIEW
2.1 Obesity

Overweight and obesity are defined by the World Health Organization (WHO) as “abnormal or excessive fat accumulation that presents a risk to health” (World Health Organization). A common measurement of obesity is the body mass index (BMI), calculated by dividing the individual’s weight (in kilograms) by the square of his or her height (in metres). Generally, a normal BMI is considered to be between 18.5 and 25 kg/m², a BMI between 25 and 30 is considered overweight, and a BMI above 30 considered obese. Obesity has been described as a global epidemic, with the highest prevalence (67.3%) of overweight and obese people living in the United States (WHO, 2014). Canada is not far behind, with 54% of adults classified as overweight (Canadian Community Health Survey, 2014 Statistics Canada). Obesity dramatically increases the risk of morbidity from chronic diseases including, diabetes, CVD, and certain types of cancer (Kopelman 2007, 13-17). Obesity results from a combination of causes and contributing factors; however, the primary factors are environmental, namely the increase in availability of energy-dense foods, and the decrease in energy expenditure due to the increasing sedentary nature of life (Selassie and Sinha 2011, 1-9). Other factors that may contribute to the obesity epidemic could be assortative mating, mainly among parents of lean or obese offspring that may suggest that genes and mutations predisposing to obesity are more prevalent among obese parents and vice versa (Katzmarzyk, Hebebrand, and Bouchard 2002, 241-246).

Epigenetic effects (i.e., as a result of maternal obesity), sleep debt, drug-induced weight gain (i.e., psychotropic medications, steroid), decline in cigarette smoking, endocrine disruptors chemical that have been implicated as contributing to obesity possibly by interfering with estrogen and androgen signaling (i.e., bisphenol A, pesticides) could all use as potential contributors to the obesity epidemic (McAllister et al. 2009, 868-913).

2.2 Type 2 Diabetes Mellitus

Type 2 diabetes mellitus (T2DM) is a chronic disease causing substantial morbidity and mortality and thus is associated with enormous health care costs (Yang et al. 2013, 1033-1046). It is a metabolic disorder characterized by hyperglycemia that develops after the establishment of insulin resistance. High insulin secretion to overcome the resistant may cause an exhaustion of pancreatic beta cell function and thus a future relative lack of insulin production. Obesity is an
established major risk factor for T2DM (Kodama et al. 2012, 959-969). The expression ‘diabesity’, although originating in the early 70’s to describe how weight gain could lead to a deterioration of glycemic control (Sims, Danforth Jr, and Horton 1973, 457-496), has recently been reused to describe the co-existence of the epidemics of T2DM and obesity. Type 2 DM has also become a global epidemic that is only projected to worsen in the coming decades (Lam and LeRoith 2012, 93-96); the number of people with T2DM at the beginning of this century was estimated to be 171 million individuals, and it is expected to increase to 366 million by 2030 (Wild et al. 2004, 1047-1053). The prevalence of adults considered diabetic in Canada is 9.2%, which is the third highest compared with the global prevalence of T2DM (Shaw, Sicree, and Zimmet 2010, 4-14).

2.3 Dietary fibers

Although ‘dietary fiber’ has no universally accepted definition, most existing definitions recognize it to be a group of carbohydrate polymers and oligomers that escape digestion in the small intestine and pass into the large bowel and promote beneficial physiological effects. Some chief dietary fibers are resistant starches, celluloses, hemicelluloses, and non-digestible oligosaccharides. The dietary fiber could be categorized according to their sources, solubility, fermentability and physiological effects. In the past, dietary fibers were mainly classified as soluble and insoluble, as it was believed that the main beneficial physiological effects of the fibers are attributed to this physical property. However, nowadays the physiologic effects of dietary fibers are also attributed to their viscosity, fermentation, and in some cases to their prebiotic effects (inulin and galacto-oligo-fructose).

The beneficial effects of dietary fibers on health may include increased fecal bulk and laxation, reduced blood pressure, reduced serum LDL cholesterol, decreased intestinal transit time, increased SCFA production, improving colonic microbiota profiles (particularly increasing Bifidobacteria), improve mineral absorption and a protective effect on colon cancer (Fuller et al. 2016, 1-12). In addition, two extensively researched effects of dietary fibers are their ability to lower postprandial glycaemia and insulinaemia, and to reduce weight gain and hunger. In the following section I will discuss the latter two effects.

2.4 Dietary Fiber, Obesity and Diabetes
Much scientific effort is being invested to find effective treatments for obesity and T2DM, including, pharmacotherapy options, surgical treatments and physical activities. However, a modification in lifestyle in the form of appropriate diet was, and still is, the most common available treatment to decrease the likelihood of developing these diseases. One element of nutrition that has been identified as an important player is dietary fiber. Epidemiological studies have shown that high intake of dietary fiber is associated with reduced risk for obesity and T2DM (McKeown et al. 2002, 390-398; Du et al. 2010, 329-336). Findings from prospective studies have shown that high intake of dietary fiber is associated with improved glucose homeostasis, insulin sensitivity, body weight and food intake (Yao et al. 2014, 79-88; Delzenne and Cani 2005, 636-640). There are multiple mechanisms by which dietary fibers may affect obesity and T2DM. For example, dietary fibers reduce the energy density of foods, which may directly lead to reduced caloric intake (Drewnowski 1998, 347-353). Dietary fiber may generally take longer to chew, which may increase sensory satiety and reduce meal size (Zijlstra et al. 2009, 269-275; Zijlstra et al. 2008, 676-683). Furthermore, viscous dietary fibers may lead to a more gradual nutrient absorption and prolonged feelings of satiety (Dikeman and Fahey Jr. 2006, 649-663). One rather important mechanism of dietary fiber, which will be discussed in the following sections, relates to the effects of the fermentation of the fiber into short-chain fatty acids on obesity and T2DM.

2.5 Short Chain Fatty Acids

Short-chain fatty acids (SCFA) are the principal anions in the colonic content that arise from bacterial fermentation of dietary macronutrients reaching the colon (Scott et al. 2013b, 52-60). They are saturated aliphatic organic acids that consist of one to six carbons. The main types of SCFA present in the feces are acetate (C2), propionate (C3), and butyrate (C4), in approximate molar ratios of 60:20:20, respectively. Besides SCFA, bacterial fermentation also yields gases like CO₂, hydrogen (Roy et al. 2006, 351-366). Moreover, 30-80% of the population (depend on the study) would also produce methane (CH₄), a gas produced by archaea, which is another domain of single-celled microorganisms that harbors the human colon (Conway de Macario and Macario 2009, 99-108).
SCFA metabolism involves production, absorption, hepatic uptake and peripheral clearance:

### 2.5.1 SCFA Production

The SCFA production is divided into exogenous production (by colonic bacteria) and endogenous production (by different metabolic pathways).

#### 2.5.1.1 Exogenous Production

Exogenous production of SCFA arise from bacterial fermentation of carbohydrates (CHO) and proteins escaping absorption in the small bowel, either because they are not digestible (like dietary fiber) or because they short-circuit the digestive/absorptive apparatus (CHO overload, altered motility or disease, etc). Other substrates for colonic fermentation are endogenous substrates like host enzymes, sloughed intestinal cells and the host’s mucin (Macfarlane and Macfarlane 2012, 50-60). The major part of SCFA that is produced in the colon depends on the types and amounts of substrates available from the diet and on the composition of the colonic microbiota. The process involved in the fermentation of the substrates into SCFA involves complex enzymatic pathways that are active in a broad number of bacterial species.

#### 2.5.1.2 Endogenous Production

Acetate and butyrate are produced endogenously by fat oxidation, while propionate comes from branched-chain amino acid and methaionine metabolism (Brindle et al. 1988, 10653-10657). Thus, plasma SCFA rise during states of starvation (Scheppach et al. 1991, 177-182), T2DM (Smith, Humphreys, and Hockaday 1986, 285-291) and glucose intolerance (Wolever et al. 1997,

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**Figure 2.1: The chemical structure of the three major short chain fatty acids (adapted from):**

It is generally considered that under normal circumstances colonic fermentation is the primary source of colonic acetate (Scheppach et al. 1991, 177-182). However, plasma acetate falls after breakfast, rebounds after lunch, and then falls again after lunch (Wolever et al. 1997, 805-811), a response pattern similar to that of free-fatty acids which are suppressed by insulin (Robertson et al. 2005, 559-567). Thus, a substantial portion of colonic acetate may be endogenously produced.

### 2.5.2 SCFA Absorption

Approximately 5-10% of the SCFA produced in the colon appear in feces, due to colonic absorption that is responsible for the major decline in concentrations along the large bowel (Ruppin et al. 1980, 1500-1507; McNeil, Cummings, and James 1978, 819-822). Four proposed mechanisms were suggested for the transport of SCFA across the apical membrane of the colonocytes (figure 2.2). One mechanism has been suggested to be through the unnamed SCFA⁻ HCO₃⁻ antiporter, that exchanges an SCFA anion for a bicarbonate ion (named ‘?’ figure 2.2). Second mechanism is through the monocarboxylate transporter (MCT1) which transports SCFA in an H⁺-dependent electroneutral manner (Roy et al. 2006, 351-366). It was shown that increased butyrate concentrations may induce an increase in MCT1 expression. This increase might serve as a mechanism to maximize intracellular availability of butyrate and other SCFA (Cuff, Lambert, and Shirazi-Beechey 2002, 361-371; Borthakur et al. 2008, 1452-1463). The third mechanism is the sodium-dependent monocarboxylate transporter 1 (SMCT1). Anions of SCFA are transported by SMCT1 are coupled to Na⁺ transport, thus SCFA absorption by this simporter stimulates

![Figure 2.2: Schematic overview of the proposed transport mechanisms of SCFAs in colonocytes. Adapted from Den Besten, G. 2013](image-url)
sodium and consequently water absorption. The last mechanism is through passive diffusion of protonated SCFA, however, at the pH of the lumen, which is approximately 5.6 to 6.6 (Cummings et al. 1987, 1221-1227), more than 95% of the SCFA are in the anionic form (Cummings 1981, 763-779), thus this method of absorption is less likely to occur.

The SCFA that are absorbed through the apical membrane, but not metabolized by the colonic cells will be transported across the colonic basolateral membrane. The basolateral SCFA\(^{-}\)-HCO\(_3\)^{-} antiporter and the basolateral MCT4 are quite similar to the apical SCFA\(^{-}\)-HCO\(_3\)^{-} antiporter and the MCT1, respectively. However, they have distinct affinities for their substrates. The role of MCT5 in SCFA transportation is yet to be determined (Den Besten et al. 2013, 2325-2340).

Absorption rates are the same in all the regions of the colon, while colonic retention time and other metabolites present in the feces, such as bicarbonate or sodium may affect the absorption (Queenan et al. 2007). The concentration of fermentation substrates is highest in the proximal colon, and decreases aborally, with an average concentrations of SCFA of 70 to 140 mM in the proximal colon to 20 to 70 mM in the distal colon (depending on the diet) (Topping and Clifton 2001, 1031-1064).

Studying colonic SCFA absorption in humans is limited by the mechanical difficulties in accessing this organ. Most studies have measured SCFA absorption by rectal infusions of SCFA solutions that were retained in the distal colon for about 30 min and then voided for SCFA measurements. Adding propionate to a rectal infusion of acetate in a ratio of 1:3, respectively, significantly raised acetate absorption compared to an infusion of acetate alone (Wolever, Trinidad, and Thompson 1995, 393-398). However, this combination effect on SCFA absorption was not seen in other studies. When a rectal solution of acetate, propionate and butyrate was infused in a physiological molar ratio of 60:20:20, respectively, the mean percentage of absorption of butyrate was greater than that of acetate (Vogt and Wolever 2003, 3145-3148). When acetate, propionate and butyrate were infused together in equimolar concentrations (40 mmol/l) (Saunders 1991, 841-847) butyrate absorption was also greater than that of acetate. Vogt and Wolever have also found a positive correlation between SCFA absorption and SCFA concentration in the rectal solution. However, above 80 mmol/l SCFA solution, the SCFA absorption was higher than the regression line (Vogt and Wolever 2003, 3145-3148) suggesting
another absorption mechanism may be active at these concentrations. The authors suggested that the concentration dependent absorption together with the chain length effect (i.e., greater butyrate absorption than that of acetate) point to a passive diffusion as the predominant mechanism of absorption, or a transporter mechanism that has a greater affinity for butyrate than acetate.

Figure 2.3: An overview of the relationship between transit of food through the human gastrointestinal tract and the digestion of nutrients in the small intestine and fermentation in the cecum and colon. Food components and endogenous secretions not absorbed into the small intestine, reach the colon where they are fermented by the colonic microbiota. Fermentation is high in the proximal large bowel as is the SCFA production. Absorption of SCFA and of water and minerals is high in this viscus. On passage of the fecal stream, fermentation declines through substrate depletion, and SCFA values fall. The distal large colon and rectum are the regions of the large bowel with the most limited supply of SCFA. Bacteria and unfermented components of low fermentability are voided in the feces.

2.5.3 Colonic, Hepatic and Peripheral Uptake of SCFA

Once the SCFA are absorbed, they are metabolized by the different organs. Butyrate is the major fuel for colonocytes, thus 70-90% of the absorbed butyrate is metabolized by the colonocytes (Cook and Sellin 1998, 499-507), and the liver clears most of the butyrate that is transferred to the portal vein. Thus, butyrate concentration in the peripheral blood is extremely low. Propionate is abundant in portal blood but is largely (50%) removed by the liver and used as a precursor for
gluconeogenesis. Acetate is 50-70% taken up by the liver, where it is used as an energy source, and as a substrate for the synthesis of cholesterol, long-chain fatty acids, glutamine and glutamate (Den Besten et al. 2013, 2325-2340). Therefore, the molar ratio of acetate to propionate to butyrate in the colonic content is 57:22:21, while in the peripheral blood it is 91:5:4 (Cummings et al. 1987, 1221-1227). The amount of acetate taken up by the liver is higher in fed than fasted animals, and is directly related to the portal acetate concentration (Rémésy C., Demingé C., Morand C. 1995). It has been shown by Dr. Wolever’s group that hepatic acetate uptake is similar in normal and hyper-insulinemic individuals (Fernandes, Vogt, and Wolever 2014).

The clearance of SCFA from circulating blood is done by organs like the heart, adipose tissue and muscles that metabolize them (Knowles et al. 1974, 401-411). Dr. Wolever’s group has shown that acetate clearance is similar in normal and hyperinsulinemic subjects (Fernandes, Vogt, and Wolever 2012, 1029-1034), but higher in subjects with T2DM than those without (Akanji, Derek, and Hockaday 1990, 112-118; Price, Dehal, and Arkin 2009, 1641-1650).

2.6 Challenges in Understanding SCFA Metabolism in Humans

The metabolism of SCFA includes several ‘phases’: production, absorption, and utilization by the different organs. Studying each of these metabolic ‘phases’ separately is difficult as this demands invasive methods. For example, in order to study hepatic SCFA uptake, blood sampling from the hepatic and portal vein is needed. Consequently, most human studies measure either serum or fecal SCFA, which reflects a net result of SCFA production from the diet, absorption, hepatic uptake and clearance.

Previously, a few studies have used unique methods to better understand the different ‘phases’ of SCFA metabolism. One study sampled SCFA from different parts of the colon, and from the portal, hepatic and peripheral blood of humans after a sudden death (Cummings et al. 1987, 1221-1227). Another study measured interorgan SCFA exchange in humans undergoing major upper abdominal surgery, by sampling blood flow and SCFA from a radial artery, the portal and a hepatic vein (Bloemen et al. 2009, 657-661). Another approach was to study SCFA production in healthy humans by using stable isotopes technique (Pouteau et al. 2003, 87-93; Boets et al. 2015, 8916-8929). Although these methods allow a better understanding of the different phases...
of SCFA metabolism, they have some limitations. For instance, the stable isotope technique is cumbersome, complicated and expensive. For the sudden death subjects and for the surgery patients, no nutritional background was provided. In the surgery method, the subjects were operated upon for malignant disease of the gastrointestinal tract. Both the surgery and the disease might have an effect on the SCFA metabolism. Also, these studies were done on a very small sample sizes.

Notably, as far as I know, differences in SCFA metabolism between lean and obese humans have not been previously explored.

2.7 **Factors affecting colonic SCFA concentration**

There are a variety of factors that may affect colonic SCFA concentrations in healthy humans; however, the main factors include diet, microbial profile, gut transit time and SCFA absorption into the colon. In inflammatory bowel disease like Crohn’s disease and ulcerative colitis, SCFA were found to be in lower concentrations in parallel with a dysbiosis of the gut microbiota compared to healthy humans (Huda-Faujan et al. 2010, 53-8; Marchesi et al. 2007, 546-551). In the sections below I will expand on the main factors that relates to a healthy state.

2.7.1 **Diet**

The colonic microbiota derives its growth substrates mainly from carbohydrates and proteins reaching the colon undigested. Dietary carbohydrates are fermented by saccharolytic bacteria primarily in the proximal colon producing linear SCFA, H₂, and CO₂, while dietary proteins and amino acids are fermented by proteolytic bacteria producing branched SCFA along with potentially toxic metabolites like phenols, and amines (Roberfroid 2007, 2493S-2502S). Quantitatively, resistant starches and non-starch polysaccharides are the main substrates for colonic fermentation, with daily amounts of 8 to 40g and 8 to 18g that enter the colon, respectively (Bingham 1990, 153-171).

Most dietary fibers are fermented to some extent; dietary fibers like pectin, inulin, β-glucans, resistant starch and oligosaccharides tend to be more readily fermented than cereal fibers like cellulose and lignins (Flint et al. 2008, 121-131; Wong et al. 2006, 235-243). As such, different
types and amounts of substrates may produce varying types and amounts of colonic SCFA. Differences in SCFA production from different fiber types have been shown in vitro, using human feces as inocula in a batch-culture system, though SCFA productions were not always consistent between studies (Velázquez et al. 2000, 87-92; Yang et al. 2013, 74-81). Moreover, different types and amounts of fermentable fiber may change SCFA concentrations indirectly, through their prebiotic effect, by selectively stimulating the growth or activity of specific bacteria.

2.7.2 Colonic transit time

Colonic transit time refers to the time required for the digested food to pass through the colon. This time differs between individuals, ranging between 20 to 120 h, with mean values of 60hrs (Cummings et al. 1992, 1783-1789). The rate of movement of colonic content through the colon is an important factor contributing to the rate and extent of colonic SCFA production, as it can alter microbial growth and its metabolic activities and it can also affect SCFA absorption into the colonocytes. Most data show that a rapid transit time results in higher fecal SCFA concentration, and vice versa. However, whether increased SCFA concentrations are a result of an increased SCFA production or decreased SCFA absorption has not been determined from current data (El Oufir et al. 2000, 603-609; El Oufir et al. 1996, 870-877; Stephen, Wiggins, and Cummings 1987, 601-609; Lewis and Heaton 1997, 245-251). Yet, measuring colonic transit time when exploring fecal SCFA appears to be an important confounder.

2.7.3 Colonic SCFA absorption

About 90-95% of SCFA produced in the colon are absorbed from the lumen, enter the portal vein and are transported to the liver and metabolized there, while the remaining SCFA are secreted in the feces. However, if SCFA absorption varies in different individuals, then this is important for us to know for two reasons. First, if, for example, SCFA absorption is increased in some people, then the metabolic effects attributed to SCFA (and will be mentioned below) will be greater in these people. Second, if increased SCFA absorption results in reduced fecal SCFA, then one cannot interpret fecal SCFA as a marker for colonic production. Indeed, acetate absorption from a rectal infusion of SCFA of acetate, propionate and butyrate in a molar ratio of 60:20:20,
respectively, in lean healthy subjects, varied from 10% to 70% absorption. Across the latter range, there was an inverse relationship between acetate absorption and fecal acetate concentration (Vogt and Wolever 2003, 3145-3148). In a more recent study, Dr. Wolever’s group did not find a difference in SCFA absorption from a rectal solution of 90 mmol/l acetate between normal and hyper-insulinemic individuals (Fernandes, Vogt, and Wolever 2014). However, it is not known if SCFA absorption differs in lean compared to obese humans, and whether the increased fecal SCFA seen in obese compared to lean humans, as will be discussed in section 2.9, is a result of increased production, or a decreased absorption. Furthermore, it is not clear if the large variations in SCFA absorption seen in the latter studies are a result of drawbacks of the rectal infusion model (i.e. the inability to recover all of the unabsorbed control material infused rectally in some participants), or a real variation between people.

2.7.4 Microbiota

The colonic microbiota is a dynamic ecosystem containing hundreds of bacterial species that represents approximately 1 kg of our body weight. There are at least three distinct sub-ecosystems in the colon. The cecum, where rapidly fermentable substrates (i.e. sugars, inulin) are present, the ascending and transverse colon, where only more slowly fermented substrates (i.e. non-starch polysaccharides) are available, and the distal colon, where fermentable substrates have largely been exhausted by the bacteria. The preferred substrates and products of fermentation vary in different bacterial species. Moreover, some bacteria metabolize the gases or SCFA produced by others (cross feeding), and thus, the presence of some bacteria may alter the function of others.

The type of bacteria present in the colon may be determined by many factors including microbial species acquired at birth, antibiotic usage, dietary intake, age, genetics, physical activities and by host factors such as the type of intestinal mucin and the immunological properties of the colonic epithelium (Scott et al. 2013a, 52-60; Samuel et al. 2007, 10643-10648; Turnbaugh et al. 2009, 480-484). The most abundant phyla in the colon of healthy humans are Bacteroidetes (gram-negative), Firmicutes (gram-positive), and Actinobacteria (gram-positive) (Scott et al. 2013a, 52-60) that account for more than 90% of all bacteria in the colon. The Bacteroidetes mainly produces acetate and propionate, while the Firmicutes mainly produces butyrate as its primary
metabolic end product. Formation of H₂ and CO₂ varies widely between species in pure culture. But in the mixed communities, these products are partially converted to acetate (i.e. by acetogenic bacteria), methane (by the methanogenic-archaea) or hydrogen sulfide (by sulfate-reducing bacteria) (Russell et al. 2013, 246-254).

2.8 The effects of SCFA on Glycemia and Energy Balance

The role of SCFA as major players in maintenance of colonic health and integrity of the gut has been highlighted over the past several decades. More recently, their physiological roles have been shown to be broader than a local effect on the colonocytes and on digestive function; SCFA may also systemically and locally effect the regulation of lipid and glucose metabolism, inflammation, immune and endocrine homeostasis. Two major SCFA signaling mechanisms have been identified (though other mechanisms may also exist): through activation of G-protein-coupled receptors, and through inhibition of histone deacetylase. Through these mechanisms SCFA may change cell proliferation and function; alter chemotaxis and phagocytosis; induce anti-inflammatory, antitumorigenic, and antimicrobial effects; regulate lipid metabolism; improve glycemia; increase satiety hormones; and alter gut integrity. However, in this work, I will focus on the SCFA effects that are relevant to my scope of work: the effects of SCFA on energy balance and glycemia that will be further expanded in the following sections (Figure 2.5).

2.8.1 The caloric contribution of SCFA

As previously mentioned (section 2.5.3), when SCFA are absorbed, they are used as a source of energy. In humans, available carbohydrates provide 4kcal/gram when metabolized and absorbed from the small intestine. However, unavailable carbohydrates such as dietary fibers provide approximately 2Kcal/g, if they are completely fermented into SCFA in the colon (Livesey G. 1995, 424). It has been estimated that up to 10% of the energy requirements of the average western diet are provided from colonic fermentation of SCFA (McNeil 1984, 338-342). This proportion (10%) may increase in the presence of higher than average intake of dietary fiber.

2.8.2 SCFA Receptors: GPR41 and GPR43
Beside the role of SCFA as substrates for energy, they also have a role as regulatory molecules, as they are sensed by two specific G protein coupled receptors (GPR) 41 and 43 (Nilsson et al. 2003, 1047-1052; Le Poul et al. 2003, 25481-25489) that are involved in energy, glucose and lipid regulation (Canfora, Jocken, and Blaak 2015, 577-591) (Figure 2.5). G-protein coupled receptor 41 and GPR43 (named also free fatty acid receptor 3 (FFAR3) and FFAR2, respectively) are expressed in a variety of tissues including the colon, adipose (Covington et al. 2006, 770-773) immune cells, skeletal muscle (Brown et al. 2003, 11312-11319), and within the peripheral nervous system (De Vadder et al. 2014, 84-96). This wide expression indicates that SCFA might also affect cellular responses in peripheral tissues.

As discussed below, the effects of SCFA through their receptors appear to be involved in regulation of appetite hormones and glucose metabolism.

2.8.3 Regulation of Glycemia by SCFA

A few mechanisms have been suggested for the effects of SCFA on glycemic responses. However, the mechanism that I chose to explore in this work is the lowering effect of SCFA on plasma FFAs that may consequently improve insulin sensitivity (Robertson et al. 2003, 659-665).

It is well established that high blood FFA concentrations induce insulin resistant and thus decrease insulin stimulated glucose uptake into the skeletal muscle, impair insulin mediated inhibition of hepatic glucose production in the liver, and reduce the ability of insulin to inhibit lipolysis in the adipose tissue. In the long term, high FFA concentration may also reduce insulin secretion leading in most cases to T2DM (Capurso and Capurso 2012a, 91-97). However, the mechanisms by which FFA cause insulin resistance are still not fully understood.

Three main hypotheses have been proposed to explain how increased FFA concentrations can affect insulin signaling (Capurso and Capurso 2012b, 91-97). The lipid metabolite hypothesis suggests that increased plasma FFA concentration results in intramyocellular and intrahepatic accumulation of triglycerides as well as several metabolites of the FFA re-esterification pathway. These metabolites may consequently activate protein kinase C isoforms that induce inhibition of insulin signaling and action, and thus induce insulin resistance. The inflammation hypothesis suggests that high FFA concentration may activate the proinflammatory NF-kB pathway. This
pathway may result in increased expression of several proinflammatory cytokines (Boden et al. 2005, 3458-3465) which can interfere with binding of insulin receptor substrate 1/2 to the insulin receptor (Lebrun and Van Obberghen 2008, 29-36). *The oxidative and endoplasmic reticulum stress hypothesis* suggests that in response to converting excessive amounts of FFA and other nutrients into fat, the adipocyte can develop signs of oxidative and endoplasmic reticulum stress. These proinflammatory cytokines may then be able to produce insulin resistance (Capurso and Capurso 2012b, 91-97).

Increased colonic SCFA have been suggested to activate fatty acid oxidation and inhibit de novo synthesis and lipolysis, resulting in a reduction in plasma FFA concentration. The molecular mechanism for how SCFA may lower FFA concentrations has yet to be clarified in humans, though a few mechanisms were suggested from animal and *in vitro* studies, as briefly detailed in the figure below (Figure 2.4).
Figure 2.4: Schematic overview of the proposed mechanisms by which SCFAs increase fatty acid oxidation in liver, muscle and brown adipose tissue. In muscle and liver, SCFAs phosphorylate and activate AMPK (pAMPK) directly by increasing the AMP/ATP ratio and indirectly via the GPR41 (FFAR2) - leptin pathway in white adipose tissue. In white adipose tissue, SCFAs decrease insulin sensitivity via GPR41 (FFAR2) and thereby decrease fat storage. In addition, binding of SCFAs to GPR41 (FFAR2) leads to the release of the Gi/o protein, subsequent inhibition of adenylate cyclase (AC) and an increase of the ATP/cAMP ratio. This, in turn, leads to the inhibition of protein kinase A (PKA) and subsequent inhibition of hormone-sensitive lipase (HSL), leading to a decreased lipolysis and reduced plasma free fatty acids. Adapted from Den Besten et al. 2013.
In humans, healthy lean subjects who consumed a dinner containing low-glycemic index meal had a reduced glucose concentration in the next morning compared to high glycemic index meal (Wolever et al. 1988, 1041-1047). The reduced blood glucose on the following morning was possibly due to raised colonic SCFA concentrations leading to lower FFAs and reduced hepatic glucose output (Thorburn, Muir, and Proietto 1993, 780-785). In an acute study, healthy individuals who consumed an inulin drink in the morning had lower plasma FFA concentrations and increased SCFA concentration after 4 hrs (Tarini and Wolever 2010, 9-16). A reduction in FFA concentration was also seen in another acute study in hyper-insulinemic and noramal-insulinemic individuals who consumed an inulin drink or control. However, this reduction occurred before an increase in serum SCFA was seen (Fernandes, Vogt, and Wolever 2011, 1279-1286). Moreover, no differences in serum glucose and insulin responses were seen following the inulin drinks in the latter acute studies. Thus, more human studies are needed to explore the effect of SCFA on FFA, and glycemic responses. In addition, it is not known if colonic SCFA reduce plasma FFAs to the same extent in lean and obese subjects.

![Figure 2.5: SCFA and inter-organ crosstalk](image)

Figure 2.5: SCFA and inter-organ crosstalk. Fermentation of indigestible foods in the colon results in the production of SCFA. In the colon, SCFA bind to GPR41 and GPR43, which leads to the production of the gut hormones PYY and GLP-1 and affects satiety and glucose homeostasis. Furthermore, propionate...
2.8.4 Regulation of Appetite by SCFA

2.8.4.1 Central Nervous System Regulation of Energy Intake and Appetite

The main brain regions responsible for the regulation of energy homeostasis are the hypothalamus and the brainstem. They receive neural and hormonal signals from the gut regarding the acute nutritional state of the periphery, which enable them to regulate short-term appetite. Gut hormones can stimulate ascending vagal pathways from the gut to the brainstem or act directly on neurons in the brain (Murphy and Bloom 2006, 854-859). We will focus on the hormones: Glucagon-like peptide-1 (GLP-1), Peptide YY (PYY) and ghrelin, three widely researched gut hormones and their role in the regulation of energy homeostasis.

2.8.4.2 GLP-1

GLP-1 (7-36) amide is a 30 amino-acid peptide made by post-translational processing of proglucagon produced in the L-cells of the intestine (Bell et al. 1983, 368-371). Plasma GLP-1 begins to rise 10-20 min after a meal and peaks at ~60 min. The initial rise in GLP-1 is stimulated by the peptide GIP secreted in response to glucose or fat absorption (Song and Wolfe 2007, 46-51), whereas later increases reflect the time it takes nutrients to reach the ileum where L-cells are more abundant. GLP-1 and GIP are incretins; i.e., they account for the excess of insulin secreted after oral vs intravenous glucose (Scrocchi et al. 1996, 1254-1258; Collier et al. 1988, 323-326; Kreymann et al. 1987, 1300-1304). Thus, GLP-1 analogues and inhibitors of DPP4 (which inactivates circulating GLP-1), are used to treat T2DM. GLP-1 also inhibits gastric motility and secretion (Layer and Holst 1993, 385-386; Read, French, and Cunningham 1994, 1-10) thereby promoting satiety and reducing energy intake in animals (Tang-Christensen et al. 1996, R848-R856) and humans (Flint et al. 1998, 515-520). Morbidly obese subjects have low plasma GLP-1
levels (Holst et al. 1983, 529-538), and reduced GLP-1 responses after high carbohydrate, but not high fat meals (Ranganath et al. 1996, 916-919). Thus, reduced GLP-1 secretion could be involved in the pathogenesis of obesity and T2DM. Enhancing endogenous GLP-1 secretion by fiber supplementation might be helpful for the prevention/treatment of obesity and T2DM.

2.8.4.3 Peptide YY

PYY is a 36 amino acid peptide secreted from intestinal L-cells present in high concentrations in the terminal ileum and colon with maximal concentrations in the rectum. PYY (3-36), the active form, reduces food intake in humans and animals. Serum PYY increases within 30min of eating, suggesting neural regulation of secretion. PYY secretion is directly proportional to energy intake, and appears to be stimulated particularly by fat. PYY infusions reduce food intake by equivalent amounts in lean and obese subjects. However, obese subjects have low fasting and postprandial serum PYY levels, so that more energy intake is required to obtain a normal response (Huda, Wilding, and Pinkney 2006, 163-182).

2.8.4.4 Ghrelin

Ghrelin is a 28 amino acid peptide produced in the stomach and small intestine. Unlike other gut hormones, ghrelin is orexigenic (high levels induce food intake). Thus, serum ghrelin is high in the fasting state and falls after eating. Serum ghrelin is suppressed more by carbohydrates than protein and fat and the degree of suppression is directly related to the calorie load. The effect of insulin on ghrelin is unclear, but PYY appears to reduce serum ghrelin in mice (Cani, Dewever, and Delzenne 2004, 521-526). Serum ghrelin is reduced in obesity and increased by diet-induced weight loss (Huda, Wilding, and Pinkney 2006, 163-182). Ghrelin infusion stimulates gastric emptying and gastrin release (Perez-Tilve et al. 2006, 61-71) raises serum glucose and FFA and increases food intake in both lean and obese subjects (Vestergaard et al. 2007, E1829-E1836). Taken together, the available evidence suggests an emerging role for ghrelin in the central (body weight) and peripheral (β cell function) control of glucose homeostasis.
Figure 2.6: Hormonal changes during the fasting versus fed states. Several hormones are released from the GI tract. The “hunger hormone” ghrelin is secreted from cells in the gastric fundus, whereas PYY and GLP-1 are secreted from L cells, primarily from the ileum and colon. During fasting, decreased food intake suppresses the release of PYY and GLP-1 from the gut while stimulating the secretion of ghrelin by the stomach. These changes are detected by the brain, leading to hunger. Dieting results in a gut hormone profile that mimics the fasted state. During and after regular feeding, there is a reduction in the production of ghrelin by the stomach. In contrast, production of PYY and GLP-1 from the gut is increased. These changes, which are detected by the brain, result in decreased appetite and a feeling of satiety. Adapted from Weiss et al. 2015.

2.8.5 SCFA and Appetite Hormones

There are a few gut hormones currently receiving much attention due to their relevance to obesity and T2DM: GLP-1, PYY (satiety hormones), and ghrelin (a hunger hormone). These hormones influence the central circuits in the hypothalamus and brain stem to produce a negative or positive effect on energy balance, and also influence glucose homeostasis through their actions on peripheral target organs (Drucker 2007, 24-32).
In vitro and animal studies have shown that the colonic SCFA receptors, GPR43 and GPR41, are coupled to enteroendocrine L-cells. L cells are endocrine cells located in the highest density in the colon and secret the anorexigenic hormones PYY and GLP-1 (Kimura et al. 2013, 1829; Karaki et al. 2006, 353-360; Karaki et al. 2008, 135-142; Tolhurst et al. 2012, 364-371; Zhou et al. 2008, E1160-E1166; Keenan et al. 2006, 1523-1534). Rats models have shown that a long term consumption of fermentable fibers like inulin-type fructans or resistant starch, resulted in a decrease in energy intake, and increase in caecal and portal pool of PYY and GLP-1 due to caecal tissue proliferation, and an increase in the precursor of GLP-1 (proglucagon mRNA), and a decrease in serum ghrelin (Cani, Dewever, and Delzenne 2004, 521-526; Keenan et al. 2006, 1523-1534; Delzenne et al. 2005, S157-S161; So et al. 2007). Interestingly, 4wk supplementation of an inulin-type fructant also increased the numbers of GPR43 in rat proximal colon in parallel with an increase in the numbers of GLP-1 containing L-cells (Kaji et al. 2011, 27-38).

Not much is known about the relation between SCFA and gut hormones in humans. In hyperinsulinaemic females, rectal or intravenous acetate infusion increased PYY and GLP-1, with no change in ghrelin levels (Freeland and Wolever 2010, 460-466). It was also shown that inulin ingestion acutely increased postprandial serum SCFA with a corresponding reduction in plasma ghrelin (Tarini and Wolever 2010, 9-16). Although, acute elevation of circulating SCFA by administration of rectal or intravenous infusions of acetate resulted in increased PYY and GLP-1 responses, there was no effect on ghrelin (Freeland and Wolever 2010, 460-466).

Postprandial GLP-1 and PYY responses were also raised by acute elevation of circulating propionate (using a novel dietary molecule that delivers propionate into the colon), followed by a reduced food intake at a subsequent ad libitum meal (Chambers et al. 2015, 1744-1754). Cani et al. (Cani et al. 2009b, 1236-1243) showed that after a 2-wk supplementation of 16g/d mixture of Inulin and oligofructose, a standardized breakfast increased breath hydrogen AUC between 0-3hrs and increased GLP-1 and PYY concentrations at 10 min, but not at other times compared to control. Similarly, supplementation of 30g/d oligofructose for 6wks compared to cellulose (non fermentable fiber) increased PYY (0-7hrs), breath hydrogen (0-7hrs), and acetate concentrations (at 6 and 7 hrs) following a standardized meal. However, there were no effects on postprandial GLP-1 during the 7hrs, or on energy intakes in a following ad libitum meal, or on body weight at
the end of the study (Daud et al. 2014, 1430-1438). We showed that a daily intake of 24g wheat fiber for 1 year increased serum SCFA and GLP-1 responses, but that these effects took 9 to 12mo to occur (Freeland, Wilson, and Wolever 2010, 82-90). All in all, though most evidence regarding the effect of dietary fiber on appetite and appetite hormones show positive results, there is inconsistency regarding which specific hormone is being influenced, and paucity of data exploring if SCFA modulate these responses as seen in animal studies. Therefore, the questions addressed are whether SCFA generated during colonic fermentation may mediate effects on food intake regulation in humans and, whether these effects of SCFA are the same in lean and obese subjects.

2.8.6 The effects of the fermentable fibers inulin and resistant starch on glucose responses and appetite hormones

It has been speculated that the ability of a fiber to produce SCFA in the colon may be a key component in its metabolic link with obesity and diabetes risk reduction. Therefore, it would appear logical to explore inulin and resistant starch, which are two highly fermented fibers that have been largely explored regarding their metabolic effects on obesity and T2DM in animal and humans.

2.8.6.1 Inulin

Inulin is a fermentable soluble dietary fiber. It belongs to a family of linear poly-fructans which are composed predominantly of \(\beta (2\rightarrow 1)\) fructosyl/fructosyl linkages. Human digestive enzymes are specific for \(\alpha\)-glycosidic bonds, thus, inulin resists hydrolysis in the small intestine. Inulin is found naturally in small amounts in onion, leek, garlic, banana, wheat, rye and barley, and in larger amount in Jerusalem artichoke, chicory root and garlic (Mensink et al. 2015, 405-419). It has been estimated that the average inulin consumption of North Americans is 2.6 g per day, while Europeans consume an average of 7 g inulin per day (Roberfroid 2007, 2493S-2502S). Chicory root is most commonly used in industry for the extraction of inulin. The degree of polymerization of chicory inulin is 2-60 units with an average of 12 units. Oligofructose is another member of this family, extensively used in studies in this field, with a degree of
polymerization < 10 units. Inulin and oligofructose produces relatively more acetate \textit{in vitro}, however, human studies are equivocal (Cummings, Macfarlane, and Englyst 2001, 415S-420S).

Animal studies have shown that inulin is capable of reducing blood glucose concentrations (Diez et al. 1997, 1238-1242; Rozan et al. 2008, 1192-1199; Diez et al. 1998, 91-96; Beylot 2005, S163-S168), however, a systematic review exploring the effect of inulin of blood glucose in humans has revealed that only 4 out of 13 randomized controlled studies reported a decrease in serum glucose concentrations (Bonsu, Johnson, and Mcleod 2011, 58-66). Recently, a systematic review has suggested that long-term administration of inulin-type fructans may also contribute to weight reduction in humans (Liber and Szajewska 2013, 42-54).

2.8.6.2 Resistant Starch

Resistant starch is any starch or starch digestion product that resists, to varying degrees, amylase digestion in the small intestine, and thus enters to the colon. RS is a good substrate for colonic fermentation that can increase SCFA concentrations in animals (Ferguson et al. 2000, 230-237; Henningsson et al. 2003, 319-327) and humans (Muir et al. 2004, 1020-1028). Some studies show that starch favors butyrate production (Vidrine et al. 2014b, 344-348; Li et al. 2015, 1997-2004), however, not consistently (Nugent 2005a, 27-54), possibly since individuals differ in their microbial profiles, with some being incapable of metabolizing some types of RS (Walker et al. 2011, 220-230).

As will be discussed in chapters 4 and 5, the potential of both resistant starch and inulin to improve glucose metabolism and appetite regulation have shown promising results in animal studies, however, human studies are equivocal. Thus, further human studies are needed in order to explore the effects of these fermentable fibers on glycemia and appetite regulation.

2.9 SCFA and Obesity

In 2005, it was first shown that the types of bacteria in the colon of obese mice differs from those in lean mice, with obese mice showing a lower relative abundance of the phylum Bacteroidetes, and a proportional higher relative abundance of the phylum Firmicutes compared to the lean counterparts (Ley et al. 2005, 11070-11075). In a following study, the germ-free mice that were colonized with ‘obese microbiota’ taken from obese mice, gained more weight than those
colonized with ‘lean microbiota’ although consuming the same amount of food. The amounts of cecal SCFA in the obese transplanted mice were increased compared to the lean transplanted mice (Ley et al. 2005, 11070-11075; Ley et al. 2006, 1022-1023). A comparative metagenomic analysis has suggested that the microbiota taken from the obese transplanted mice has an increased capacity to harvest energy from the same diet than the microbiota of the lean transplanted mice (Turnbaugh et al. 2006a, 1027-1031). These new findings have led to extensive further research, to investigate whether the proposed role of SCFA and microbiota composition in obesity, which was based on proof of principle experiments, can be confirmed in further studies.

In humans, some studies (Armougom et al. 2009; Santacruz et al. 2010, 83-92; Furet et al. 2010, 3049-3057) have confirmed the obese and lean profiles of Firmicutes and Bacteroidetes as seen in mice, however, others did not report any differences in these profiles, or even found an opposite profile (Collado et al. 2008, 894-899; Duncan et al. 2008, 1720-1724; Jumpertz et al. 2011, 58-65; Schwiertz et al. 2010a, 190-195). Studies that have looked at differences in fecal SCFA concentrations between lean and obese are sparse. Most of them confirmed the increased SCFA concentrations in obese compared to lean humans (Schwiertz et al. 2010a, 190-195; Fernandes et al. 2014; Teixeira et al. 2013, 914-919), though one study has found that the obese cohort had a reduced fecal SCFA compared to the lean cohort (Murugesan et al. 2015, 1337-1346) (Table 2.1). Differences in plasma SCFA concentration between lean and obese cohorts have not been tested before. In in vitro studies using fecal inocula derived from lean and obese humans to test for differences in SCFA production between lean and obese were inconsistent. These studies have either found a similar SCFA production in lean versus obese samples (Li et al. 2015, 1997-2004; Yang, Keshavarzian, and Rose 2013, 862-867), or an increased /decreased SCFA production in lean versus obese samples, depend on the fiber type that was fermented (Aguirre et al. 2014). For example, galactooligosaccharides and lactulose, resulted in lower SCFA production with the lean fecal sample compared to the obese, while this was reversed for the pectin fiber.

In conclusion, most current evidence shows that obese humans have increased fecal SCFA concentrations compared to lean humans. However, data is limited and it is not clear if the increased fecal SCFA concentrations in obese humans are due to increased SCFA colonic
production, as seen in mice, or due to other factors. Moreover, evidence from human studies regarding the obese and lean microbial profiles is inconsistent with evidence from mice. Thus, the relationship between SCFA, obesity and microbiota is yet to be elucidated in human studies.
### Table 2.1: The observational clinical studies that measured SCFA concentrations and gut microbiota composition between lean and overweight and obese humans

<table>
<thead>
<tr>
<th>Study participants</th>
<th>Microbial profiling technique</th>
<th>Dietary intake</th>
<th>Microbial composition at phylum level in obesity</th>
<th>Fecal SCFA concentration in obesity</th>
<th>Concluding remarks</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal weight (n = 30), Overweight (n = 35), Obese (n = 35),</td>
<td>Real-time qPCR</td>
<td>not reported</td>
<td>↓ Firmicutes/Bacteroidetes</td>
<td>↑Total SCFA and ↑propionate in obese compared to lean</td>
<td>SCFA metabolism play a role in obesity, though the contribution of bacterial composition remains controversial</td>
<td>(Schwiertz et al. 2010a, 190-195)</td>
</tr>
<tr>
<td>Lean (BMI&lt;19), normal (BMI 18–24), obese (BMI 25–53), and treated obese (BMI 25–36, individuals regressing to normal BMI after bariatric surgeries),</td>
<td>Sequencing 16S rRNA</td>
<td>Not reported</td>
<td>No trend in the distribution of Bacteroidetes and Firmicutes. While treated-obese individuals had ↓ Bacteroides</td>
<td>Compared to normal group, obese had ↑SCFA. While treated-obese individuals had ↓SCFA.</td>
<td>The study representative microbial diversity in the Indian obese individuals; nevertheless, further studies are essential to understand their role in obesity.</td>
<td>(Ppatil et al. 2012, 647-657)</td>
</tr>
<tr>
<td>Lean (n = 52), overweight/obese (n = 42)</td>
<td>Real time qPCR</td>
<td>Dietary intake reported</td>
<td>↑ Firmicutes:Bacteroidetes and ↑ SCFA ↓ Bacteroidetes</td>
<td>↓ SCFA in overweight and obese group</td>
<td>Colonic fermentation patterns may be altered, leading to different fecal SCFA concentrations in obese compared with lean humans</td>
<td>(Fernandes et al. 2013, 1269-75)</td>
</tr>
<tr>
<td>190 unrelated children: n = 81 normal BMI, n = 29 overweight BMI, and n = 80 obese BMI</td>
<td>16S rDNA by Ion Torrent</td>
<td>Dietary intake reported</td>
<td>no significant dysbiosis in the bacterial phylum population</td>
<td>Overweight and obese children had ↓propionate and ↓butyrate than lean</td>
<td>Imbalance in the abundance of at least nine different bacteria (not at phylum level) as well as altered SCFA concentration in is associated to the obese conditions of Mexican children.</td>
<td>(Murugesan et al. 2015, 1337-1346)</td>
</tr>
<tr>
<td>Lean (n = 20) and obese (n = 20) women</td>
<td>NA</td>
<td>Dietary intake reported</td>
<td>NA</td>
<td>Obese had ↑butyrate and ↑acetate than the lean group</td>
<td>The higher fecal SCFA concentration is associated with metabolic risk factors and thus may influence metabolic homeostasis.</td>
<td>(Teixeira et al. 2013, 914-919)</td>
</tr>
</tbody>
</table>
2.10 **Summary Rationale**

Recent evidence suggests that colonic fermentation plays an important role in obesity and diabetes, yet current hypotheses related to this are apparently contradictory: one hypothesis is that increased dietary fiber intake may prevent obesity and diabetes; while the other suggests that the presence of colonic bacteria which more efficiently ferments fiber may cause obesity. This discrepancy may be reconciled if fiber and/or the obese microbiota influence energy balance by mechanisms other than colonic SCFA. Or, if the beneficial effects of SCFA are somehow compromised in obese subjects. Or, if the beneficial effects of SCFA are simply not strong enough to compensate for other factors that may cause obesity.

It is important to study the metabolism of SCFA in humans for several reasons. First, most of the knowledge about the role of SCFA in obesity and in appetite and glucose regulation is based on animal studies; there is a need to see if the knowledge gained in animals applies to humans. Second, the effect of increased SCFA production on energy balance is likely to vary in different individuals because of differences in SCFA absorption, metabolism, and differences in the ability to compensate for the energy absorbed. Third, if colonic SCFA are involved in the pathogenesis of obesity and T2DM, we need to know the mechanism(s) involved so that interventions to prevent or treat obesity can be aimed at the appropriate target(s) and their effectiveness assessed. This is particularly important with the current potentially conflicting hypotheses. If efficient fermentation of fiber due to the presence of certain colonic bacteria causes obesity, then fiber intake may not be effective in preventing and treating obesity because it would provide even more substrate for fermentation. If, however, obese subjects have the ability to compensate for increased energy provided by SCFA because of reduced absorption or increased hormone responses, then increased fiber intake may be beneficial to help normalize glucose and appetite hormones.

2.11 **Overall Hypothesis**

1. Fecal SCFA concentration will be increased in overweight/obese compared to lean humans
2. RS and IN will elicit higher postprandial SCFA responses and have less effect in reducing serum FFA and second-meal glucose and insulin responses in overweight/obese compared to lean humans.

3. RS and IN will elicit higher postprandial SCFA responses and have less effect in reducing serum ghrelin and increasing GLP-1 and PYY responses in overweight/obese compared to lean humans.

2.12 Objectives

1. To compare fecal SCFA concentrations, rectal SCFA absorption, dietary intake and fecal microbial profile in healthy overweight/obese vs lean participants.
2. To compare the effects of inulin and resistant-starch on postprandial SCFA and second-meal glycemic response in healthy overweight/obese vs lean participants.
3. To compare the effects inulin and resistant-starch on postprandial SCFA, GLP-1, PYY, and ghrelin responses in healthy overweight/obese vs lean participants.

2.13 Outline of Thesis

Two experiments were performed to address these objectives. The first objective was addressed by an experiment reported in Chapter 3. A second experiment was designed to address objectives 2 and 3; the methods used and the results of which address objective 2 are described in Chapter 4 and the results which address objective 3 are described in Chapter 5.
CHAPTER 3:
EVIDENCE FOR GREATER PRODUCTION OF COLONIC SHORT CHAIN FATTY ACIDS IN OVERWEIGHT THAN LEAN HUMANS

Adapted from:

3.1 ABSTRACT

**Background:** Short-chain fatty acids (SCFA) are produced by colonic microbiota from dietary carbohydrates and proteins that reach the colon. It has been suggested that SCFA may promote obesity via increased colonic energy availability. Recent studies suggest obese humans have higher fecal SCFA than lean, but it is unclear if this difference is due to increased SCFA production or reduced absorption.

**Objectives:** To compare rectal SCFA absorption, dietary intake and fecal microbial profile in lean (LN) versus overweight and obese (OWO) individuals.

**Design:** Eleven (11) LN and 11 OWO individuals completed a 3-day diet record, provided a fresh fecal sample and had SCFA absorption measured using the rectal dialysis bag method. The procedures were repeated after two weeks.

**Results:** Age-adjusted fecal SCFA concentration was significantly higher in OWO than LN (81.3 ± 7.4 vs. 64.1 ± 10.4 mmol/kg, P = 0.023). SCFA absorption (24.4 ± 0.8 vs 24.7 ± 1.2%, respectively, P =0.787) and dietary intakes were similar between the groups, except for a higher fat intake in OWO. However, fat intake did not correlate with SCFA or bacterial abundance. OWO had higher relative Firmicutes abundance (83.1 ± 4.1 vs 69.5 ± 5.8%, respectively, P = 0.008) and a higher Firmicutes:Bacteriodetes ratio (P = 0.023) than LN. There was a positive correlation between Firmicutes and fecal SCFA within the whole group (r =0.507, P =0.044), with a stronger correlation after adjusting for available carbohydrate (r = 0.615, P =0.005).

**Conclusions:** The higher fecal SCFA in OWO subjects is not due to differences in SCFA absorption or diet. Our results are consistent with the hypothesis that OWO subjects produce more colonic SCFA than LN due to differences in colonic microbiota. However, further studies are needed to prove this.
3.2 INTRODUCTION

Short-chain fatty acids (SCFA): acetate, propionate and butyrate are anions in human feces, present in a molar ratio of ~ 60:20:20, respectively (Cummings 1981, 763-779). SCFA are produced by the colonic microbiota through anaerobic fermentation. The chief substrates for colonic fermentation are undigested carbohydrates, namely dietary fiber and resistant starch and, in smaller amounts, proteins (Topping and Clifton 2001, 1031-1064). Short-chain fatty acids are readily absorbed from the lumen (Topping and Clifton 2001, 1031-1064) and are used as metabolic precursors; acetate is utilized for lipogenesis in the liver and as a fuel source once it enters the peripheral circulation. Propionate is largely taken up by the liver, and is used as a substrate for hepatic gluconeogenesis. Butyrate is the major fuel source for colonocytes (Wong et al. 2006, 235-243).

Recently, the role of SCFA as a contributor to obesity has been highlighted. Studies in mice suggest that the obese microbiota, characterized by a high Firmicutes to Bacteriodetes ratio (F:B) (Ley et al. 2005, 11070-11075), promotes obesity because of excess SCFA production and thus increased colonic energy availability that may contribute to weight gain (Turnbaugh et al. 2006a, 1027-1031). In agreement with this, some obese humans have been found to have higher fecal SCFA concentrations than lean individuals (Schwiertz et al. 2010a, 190-195; Teixeira et al. 2013, 914-919); however the results for F:B ratio have been inconsistent (Duncan et al. 2008, 1720-1724; Schwiertz et al. 2010a, 190-195).

An increase in fecal SCFA concentration could be due to several factors, including reduced colonic SCFA absorption (Vogt and Wolever 2003, 3145-3148), reduced colonic transit time (El Oufir et al. 2000, 603-609), or increased SCFA production due to differences in dietary intake (Haenen et al. 2013b, 274-283; Cummings et al. 1996, 733-747) or colonic microbiota (Duncan et al. 2007, 1073-1078; Bourriaud et al. 2005, 201-212). A previous study from our lab found a large range of SCFA absorption rates in lean participants and, across this range there was an inverse relationship between rectal acetate absorption and fecal acetate concentration (Vogt and Wolever 2003, 3145-3148). Therefore, a higher SCFA concentration in obese individuals may be a result of lower SCFA colonic absorption. However, this possibility has not been investigated. Moreover, there has been little research examining differences in dietary intakes, microbial
profile and gut transit time, factors which may determine the difference in fecal SCFA concentrations between these groups. Therefore, in this pilot observational study, our primary objectives were to compare the rate of SCFA absorption from the rectum in LN and OWO individuals and to examine the relationship between rectal SCFA absorption and fecal SCFA concentrations. Our secondary objectives were to compare dietary intake, microbial profile and gut transit time in LN and OWO subjects, and to see if these variables relate to fecal SCFA.

3.3 SUBJECTS AND METHODS

3.3.1 Subjects

Twenty two (22) male or non-pregnant, non-lactating females over the age of 17 years were recruited via advertisements posted around the University of Toronto campus and from a pool of subjects previously involved in studies by our group. Participants were divided into two groups based on their BMI. There were 11 participants in the LN group (BMI ≤ 25) and 11 participants in the OWO group (BMI > 25). Subjects were excluded for any of the following reasons: regular user of antibiotics (≥1 course per year over the last 5 years), any use of antibiotics, laxatives, pre/probiotics or other drugs known to influence gastrointestinal function within three months of the study, presence of inflammatory bowel disease, malabsorption, gastrointestinal infection, short bowel, or other condition affecting gastrointestinal function or any recent (3 months prior to the study) illness or surgery requiring hospitalization. Ethical approval was obtained from the Research Ethics Board, University of Toronto. Written informed consent was obtained from all participants.

Eligible participants completed questionnaires that provided demographics, medical history, drug use, and physical activity data (Ainsworth et al. 1993, 71-80; Anonymous 1997, S73-S78). Their height and weight were measured and they were given instructions on how to complete a 3-day dietary intake history, fill out a 3-day bowel habit diary and collect a fecal sample in a plastic bag (Ziploc, S.C. Johnson &Son, Inc.) using the Fisher brand commode specimen collection system (Fisher Scientific, Ottawa, ON). After this, subjects participated in two 4-day study periods separated by a 2-week washout period.
3.3.2 **Study period protocol**

For the first 3 days of each study period subjects kept a diet record and filled out the bowel habit diary (figure 3.1). On the 3rd day, after picking up a Styrofoam box full of dry ice and fecal-collection kit, subjects collected a fresh stool sample which was immediately placed onto dry ice. Subjects came to the laboratory on the morning (between 8 to 10am) of the 4th day after eating their normal breakfast. After handing in their diet record, bowel habit questionnaire and fecal sample, 2 breath samples were collected as described elsewhere (Fernandes et al. 2013, 1269-75). Subjects then underwent a procedure to measure rectal SCFA absorption using a rectal dialysis bag method that has been validated previously by others (McNeil, Cummings, and James 1978, 819-822; Edmonds 1971, 356-362). First, subjects put ~5cm of the end of a piece of Tygon flexible plastic tubing (Norton Performance Plastics, Akron, OH; o.d. 5mm) into their rectum, infused 500mL of water, and emptied their colon; they repeated this step if the return was not clean. Subjects then waited 15 min to ensure that there was no further urge to void, and inserted a dialysis bag containing SCFA solution into their rectum. The bag consisted of dialysis tubing (o.d. 16mm; Dialysis Tubing Cellulose Membrane AVG, Sigma-Aldrich Canada, Ltd) which had been knotted at one end and filled with 10 ml of a solution containing 150mmol/L of SCFA in a molar ratio of 90:30:30 mmol/L AC:PR:BU, respectively. After expelling any air bubbles, the opposite end was tied and the filled dialysis bag weighed. The length of the filled portion of the dialysis bags was 5cm, and an 8-10cm length of empty tubing was left attached to facilitate removal of the tubing from the rectum. After careful instructions, each participant inserted a dialysis bag, covered with a small amount of water-soluble lubricant (K-Y Jelly®), into his or her rectum until the distal end was 2cm from the anal sphincter. After 30 min the dialysis bag was gently removed by pulling the protruding long end and immediately processed for analysis. Approximately, 2 weeks after completing the first 4-day study period, subjects underwent a second study period using the same protocol, including the diet record, bowel habit diary, fecal sample collection and rectal SCFA absorption procedure.
Figure 3.1: Schematic diagram of study #1 design
3.3.3 Sample Handling

After retraction from the rectum, the dialysis bag was weighed to detect volume changes and a 3-ml syringe with a 21G 1½ inches long needle was then used to collect 1 ml of the solution from the dialysis bag. The rest of the solution was carefully emptied out of the bag and the empty bag was weighed again. The net solution weight was calculated by subtracting the weight of the bag before and after the procedure. The 1 ml samples were immediately stored at -70°C for later analysis. Frozen fecal samples were kept at -20°C prior to analysis.

3.3.4 pH and SCFA analysis

After fecal samples were thawed, they were homogenized. pH was determined by pH meter and fecal SCFA concentrations were determined by gas chromatography (GC) as previously described (Fernandes et al. 2013, 1269-75). After thawing the supernatants from the rectal dialysis bag, they were diluted 1:500 with double distilled water, centrifuged and subsequently analyzed by GC.

3.3.5 Questionnaires

Intake of nutrients was calculated from the 3-day diet records using ESHA Research's Food Processor® SQL, Version 10.9.0. A self-administered bowel habit questionnaire provided a subjective assessment of bowel function. Participants assessed the following characteristics for each bowel movement during the 3-day diet record: ease of movement (scale: from 0 easy to pass, to 6 difficult to pass); stool consistency (scale: 0-watery, 2-soft, 4-formed, 6-very hard); frequency of flatulence, presence of cramping, presence of bloating (scale: 0-none, 2-mild, 4-moderate, 6-severe).

3.3.6 Analysis of Fecal Microbiota

DNA extraction and Ion Torrent V6 16S rRNA sequencing were performed as described elsewhere (Petrof et al. 2013, 3).

3.4 STATISTICAL ANALYSIS
The percentage of SCFA absorption was calculated as the mmol of SCFA which disappeared from the dialysis bag divided by the mmol of SCFA present at baseline (SCFA_{bl}) multiplied by 100:

\[ \% \text{ SCFA absorption} = \left[ \frac{\text{SCFA}_{\text{end}} - \text{SCFA}_{\text{bl}}}{\text{SCFA}_{\text{bl}}} \right] \times 100 \]

A Mixed-models approach was used for all statistical tests to account for the repeated measurements (two visits for each participant) performed by SAS Version 9.3. Residuals from final models were assessed to ensure that model assumptions were met. Differences in fecal SCFA concentration, SCFA absorption and bacterial composition between the LN and OWO groups were assessed in 3 models: without adjustment, with adjustments for age and with adjustments for both age and carbohydrate intake. The effect of fecal SCFA concentration on SCFA absorption and the effect modification of the group were tested.

To examine our secondary objective, dietary intakes and microbial profile were used as predictors for fecal SCFA concentration using mixed-models analysis. For microbial relative abundance, dietary intakes were used as predictors. A combination of dietary intakes and microbial abundance were used to test for a better model, and presented only if the model was still significant (i.e., Firmicutes and CHO predicted fecal SCFA better than Firmicutes alone). The correlation coefficients between the SCFA, dietary intake and bacterial relative abundance were calculated by a Pearson’s r test (IBM SPSS Statistics version 21) using the average value of the two study periods. The P-value of the correlation was calculated by SAS Proc-Mixed to control for the repeated measurements. In all the correlation tests, inclusion of the effect modification of the group tested our hypothesis, because a significant interaction provides evidence for a different pattern of effect between the LN and the OWO groups. Only significant interactions are presented. Differences with P-values ≤0.05 (2-tailed) were considered to be statistically significant. The results are expressed as means ±SEM.

To examine whether changes in energy, fat, carbohydrate and fiber of habitual intakes were associated with similar changes in fecal SCFA concentrations in LN vs OWO participants, we compared fecal SCFA concentrations after the diet period with the lower intake in each subject with fecal SCFA after the diet period with the higher intake.
3.4.1 Sequencing Analysis

The weighted UniFrac distances were calculated in QIIME (Bittinger et al. 2010, 335+) by using a phylogenetic tree of OTU sequences built with FastTree (Price, Dehal, and Arkin 2009, 1641-1650) and based on an OTU sequence alignment with MUSCLE (Edgar 2004, 113). The QIIME pipeline was also used to calculate Shannon’s diversity index (logarithms with base 2). A modified version of the ALDEx R package (Fernandes et al. 2013, e67019) was used to compare relative abundances of ribotypes at different taxonomic levels between the LN and OWO groups. Microbiome data are best represented as compositional distributions, and are thus not independent of each other (Fernandes et al. 2013, e67019; Friedman and Alm 2012, e1002687). The correct interpretation of data of these types is to state that the relative abundance of A is greater or less than B (Aitchison 1986). Therefore to compare relative abundances we used the ALDEx R package that estimates the technical variation inherent in high-throughput sequencing by Monte-Carlo sampling from a Dirichlet distribution (Jaynes 2003). The Monte-Carlo replicates are transformed using the centred log-ratio transformation (Aitchison 1986) that takes the base2 logarithm of the Monte-Carlo estimates of organism abundances in each sample divided by the per-sample geometric mean organism abundance. This transformation has several desirable properties that do not exist in proportional data; notably subcomposition coherence and linear sample independence. Data transformed in this way permit the use of standard statistical tests to determine significance (Aitchison 1986). We used the median centred log-ratio value output by ALDEx2 to conduct statistical significance for these comparisons and for the F:B ratio using a SAS Proc-Mixed with p<0.05 (Strimmer 2008, 1461-1462).

3.5 RESULTS

3.5.1 Participant characteristics

OWO subjects had significantly higher BMI (P<0.0001) and breath methane (P=0.043) than LN, but there was no significant difference between the two groups in age (P=0.262), gender (χ²=0.669), ethnicity (χ²=0.665), physical activity (P=0.838), fecal consistency (P=0.317), frequency of evacuation (p=0.425), and breath hydrogen (P=0.129) (Table 3.1).
Table 3.1: Characteristics of the study participants

<table>
<thead>
<tr>
<th></th>
<th>LN (n = 11)</th>
<th>OWO (n = 11)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI, kg/m²</td>
<td>22.6 ± 0.6</td>
<td>30.1 ± 0.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Gender, Male: Female</td>
<td>6:5</td>
<td>6:5</td>
<td>0.669</td>
</tr>
<tr>
<td>Age, y</td>
<td>35.8 ± 4.2</td>
<td>42.5 ± 3.9</td>
<td>0.262</td>
</tr>
<tr>
<td>Ethnicity, C:O</td>
<td>7:4</td>
<td>6:5</td>
<td>0.665</td>
</tr>
<tr>
<td>Activity Level, METs</td>
<td>27.0 ± 8.1</td>
<td>29.7 ± 10.4</td>
<td>0.838</td>
</tr>
<tr>
<td>Faecal consistency¹</td>
<td>3.3</td>
<td>3.0</td>
<td>0.292</td>
</tr>
<tr>
<td>Breath methane, ppm</td>
<td>24.1 ± 10.5</td>
<td>1.3 ± 7.5</td>
<td>0.043</td>
</tr>
<tr>
<td>Breath Hydrogen, ppm</td>
<td>3.9 ± 1.7</td>
<td>6.5 ± 1.2</td>
<td>0.129</td>
</tr>
</tbody>
</table>

Abbreviations: BMI, body mass index; C, Caucasian; O, others; METs, Metabolic Equivalents. Values are means ± SEM. ¹calculated from Bowel Habit Diary (see Methods). Independent t-test, test was used to calculate differences between the groups, χ² was used for categorical variables. SAS Proc Mixed was used to control for repeated measurements for faecal consistency, breath methane and breath hydrogen.

3.5.2 Fecal SCFA and Microbiota

The OWO group had significantly higher total fecal acetate, butyrate and total SCFA concentrations compared to the LN group after adjusting for age (Table 3.2). Without age adjustment, the differences were not significant (Table 3.2). No significant differences were seen in iso-butyrate, iso-valerate and valerate concentrations between the groups. Mean SCFA absorption from the dialysis bags was similar between the OWO and the LN groups (Table 3.2). Among all bacterial phyla, only the relative abundance of the Firmicutes phylum was significantly different between the LN and the OWO group (Figure 3.2) with the aggregated relative abundance of Firmicutes being increased 2.8 fold in OWO compared to LN (P=0.008) (Table 3.2). There was a 5 fold difference in the ratio between the relative abundances of Firmicutes and Bacteroidetes (P=0.023, or P=0.0098 when adjusted for age) (Table 3.2).
Table 3.2: Fecal SCFA concentrations, SCFA absorption, fecal bacterial composition and fecal pH in LN and OWO groups

<table>
<thead>
<tr>
<th></th>
<th>LN</th>
<th>OW</th>
<th>P-value</th>
<th>P-value(^1)</th>
<th>P-value(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC, mmol/kg wet wt</td>
<td>35.1 ± 6.1</td>
<td>45.3 ± 4.3</td>
<td>0.109</td>
<td>0.048</td>
<td>0.016</td>
</tr>
<tr>
<td>PR, mmol/kg wet wt</td>
<td>12.7 ± 2.8</td>
<td>15.4 ± 2.0</td>
<td>0.350</td>
<td>0.103</td>
<td>0.083</td>
</tr>
<tr>
<td>BU, mmol/kg wet wt</td>
<td>11.1 ± 2.4</td>
<td>15.4 ± 1.7</td>
<td>0.089</td>
<td>0.017</td>
<td>0.015</td>
</tr>
<tr>
<td>Iso-BU, mmol/kg wet wt</td>
<td>1.5 ± 0.5</td>
<td>1.4 ± 0.3</td>
<td>0.833</td>
<td>0.946</td>
<td>0.970</td>
</tr>
<tr>
<td>Iso-VA, mmol/kg wet wt</td>
<td>2.1 ± 0.7</td>
<td>2.0 ± 0.5</td>
<td>0.889</td>
<td>0.909</td>
<td>0.945</td>
</tr>
<tr>
<td>VA, mmol/kg wet wt</td>
<td>1.6 ± 0.5</td>
<td>1.9 ± 0.4</td>
<td>0.627</td>
<td>0.283</td>
<td>0.295</td>
</tr>
<tr>
<td>tSCFA, mmol/kg wet wt</td>
<td>64.1 ± 10.4</td>
<td>81.3 ± 7.4</td>
<td>0.114</td>
<td>0.023</td>
<td>0.011</td>
</tr>
<tr>
<td>SCFA absorption, %</td>
<td>24.7 ± 1.2</td>
<td>24.4 ± 0.8</td>
<td>0.787</td>
<td>0.639</td>
<td>0.745</td>
</tr>
<tr>
<td>Firmicutes, %</td>
<td>69.5 ± 5.8</td>
<td>83.1 ± 4.1</td>
<td>0.008</td>
<td>0.0007</td>
<td>0.001</td>
</tr>
<tr>
<td>Bacteroidetes, %</td>
<td>19.4 ± 6.1</td>
<td>6.4 ± 4.3</td>
<td>0.335</td>
<td>0.329</td>
<td>0.335</td>
</tr>
<tr>
<td>F:B Ratio(^3)</td>
<td>6.8 ± 1.0</td>
<td>34.3 ± 1.6</td>
<td>0.023</td>
<td>0.010</td>
<td>0.010</td>
</tr>
<tr>
<td>Faecal pH</td>
<td>6.7 ± 0.1</td>
<td>6.4 ± 0.1</td>
<td>0.037</td>
<td>0.011</td>
<td>0.005</td>
</tr>
</tbody>
</table>

Abbreviations: AC, Acetate; BU, Butyrate; F:B, Firmicutes to Bacteroidetes; PR, Propionate; tSCFA, total short chain fatty acids; VA, Valerate. Values are means ± SEM. \(^1\)adjusted for age; \(^2\)adjusted for age and available carbohydrate (g/day). All calculated by SAS Proc-Mixed to account for repeated measurements. \(^3\)The F:B ratio is expressed as a base 2 logarithm derived from the median centred log-ratio transformed values of each sample (n=22)
Table 3.3: Daily dietary macronutrient intakes in lean and overweight participants

<table>
<thead>
<tr>
<th>Macronutrient</th>
<th>LN (n = 11)</th>
<th>OW (n = 11)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy, kcal</td>
<td>1 995 ± 119</td>
<td>2 231 ± 90</td>
<td>0.13</td>
</tr>
<tr>
<td>Protein, g/d</td>
<td>77 ± 6</td>
<td>97 ± 9</td>
<td>0.09</td>
</tr>
<tr>
<td>% energy</td>
<td>15 ± 0.8</td>
<td>17 ± 1.1</td>
<td>0.20</td>
</tr>
<tr>
<td>AvCHO, g/d</td>
<td>244 ± 17</td>
<td>238 ± 14</td>
<td>0.79</td>
</tr>
<tr>
<td>% energy</td>
<td>49 ± 1.8</td>
<td>43 ± 2</td>
<td>0.03</td>
</tr>
<tr>
<td>Fat, g/d</td>
<td>76 ± 6</td>
<td>95 ± 4</td>
<td>0.01</td>
</tr>
<tr>
<td>% energy</td>
<td>35 ± 1.7</td>
<td>39 ± 1.7</td>
<td>0.09</td>
</tr>
<tr>
<td>Alcohol, g/d</td>
<td>3.4 ± 1.8</td>
<td>4.6 ± 3.1</td>
<td>0.73</td>
</tr>
<tr>
<td>Fiber g/d</td>
<td>23.8 ± 3.4</td>
<td>21.7 ± 1.7</td>
<td>0.60</td>
</tr>
<tr>
<td>g/1000 kcal</td>
<td>12 ± 1.6</td>
<td>9.9 ± 0.7</td>
<td>0.25</td>
</tr>
</tbody>
</table>

Values are means ± SEM; Abbreviation: AvCHO, available carbohydrate; BMI, body mass index; Calculated by independent t-test
3.5.3 Dietary intake

The average consumption of all dietary nutrients did not differ significantly between the groups, except that fat intake (g/d, but not % energy) was higher in the OWO group (P=0.014). Available carbohydrate intake was similar between the groups when expressed as g/d, but when expressed as % energy was higher in the LN group (P=0.028) (Table 3.3).

3.5.4 Group interactions

The model for SCFA absorption from the dialysis bag and the fecal SCFA concentrations in the LN compared to the OWO group showed a significant interaction between fecal SCFA concentration and group (P=0.04). This interaction indicated a general trend for OWO individuals to absorb more SCFA from the dialysis bag when their fecal SCFA concentrations are higher, while this trend did not exist in the lean group (Figure 3.3).

Figure 3.3: Faecal SCFA concentration by Group interaction with SCFA absorption

P value for the interaction (P = 0.04) was calculated by SAS Proc Mixed model to account for repeated measurements. Each point represents one visit.
The mean changes within participants in fecal acetate concentration associated with increases in dietary intakes (energy, dietary fiber and fat) differed significantly between LN and OWO subjects. In the OWO group, the diet period with the higher energy intake (2430±123 vs 2030±82 kcal/d) was associated with higher fecal acetate (50.8±5.5 vs 39.8±4.7 mmol/kg) while in the LN group, the diet period with the higher energy intake (2120±117 vs 1870±126 kcal/d) was associated with lower fecal acetate (33.6±3.8 vs 36.5±33.6 mmol/kg; diet×group interaction, p=0.009) (Figure 3.4A). Similarly, in the OWO group, the diet period with the higher dietary fiber intake (23.7±1.7 vs 19.7±1.9 g/d) was associated with higher fecal acetate (48.5±5.3 vs 42.1±5.0 mmol/kg) while in the LN group, the diet period with the higher dietary fiber intake (25.4±3.4 vs 22.1±3.5 g/d) was associated with lower fecal acetate (32.1±4.0 vs 38.1±4.0 mmol/kg; diet×group interaction, P=0.03) (Figure 3.4B). Finally, in the OWO group, the diet period with the higher fat intake (106.2±5.0 vs 84.5±4.3 g/d) was associated with higher fecal acetate (49.7±5.7 vs 41.0±4.8 mmol/kg) while in the LN group, the diet period with the higher fat intake (82.6±5.8 vs 69.7±6.2 g/d) was associated with lower fecal acetate (32.2±4.1 vs 37.9±3.9 mmol/kg; diet×group interaction, P=0.03) (Figure 3.4C).

3.5.5 Correlations between Fecal SCFA, microbiota and dietary intakes

Significant predictors for fecal SCFA concentrations and for Firmicutes abundance are presented in Table 3.4. Significant predictors for fecal SCFA were protein (g/day) (r=0.408, P=0.004) and the Firmicutes relative abundance (r=0.507, P=0.044) and the result was more significant after adjusting for available carbohydrate (r=0.615, P=0.005). Significant predictors for the Firmicutes relative abundance were available carbohydrate (r= -0.185, P=0.008), fiber (r= -0.477, P=0.015) and BMI (r= 0.502, P=0.017).
Table 3.4: Pearson correlation coefficients among faecal SCFA (mmol/kg wet wt), microbial composition, F:B ratio and their predictors

<table>
<thead>
<tr>
<th></th>
<th>Total SCFA</th>
<th>Acetate</th>
<th>Propionate</th>
<th>Butyrate</th>
<th>Firmicutes</th>
<th>F:B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calories, Kcal</td>
<td>r</td>
<td>0.370</td>
<td>0.416</td>
<td>0.333</td>
<td>0.251</td>
<td>0.293</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.053</td>
<td>0.028</td>
<td>0.079</td>
<td>ns</td>
<td>0.089</td>
</tr>
<tr>
<td>Fat, g/d</td>
<td>r</td>
<td>0.139</td>
<td>0.147</td>
<td>0.183</td>
<td>0.139</td>
<td>0.567</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Available CHO, g/d</td>
<td>r</td>
<td>0.306</td>
<td>0.445</td>
<td>0.141</td>
<td>0.131</td>
<td>-0.185</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>ns</td>
<td>0.066</td>
<td>ns</td>
<td>ns</td>
<td>0.008</td>
</tr>
<tr>
<td>Fibres, g/d</td>
<td>r</td>
<td>-0.295</td>
<td>-0.297</td>
<td>-0.243</td>
<td>-0.249</td>
<td>-0.477</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>ns</td>
<td>ns</td>
<td>0.088</td>
<td>ns</td>
<td>0.015</td>
</tr>
<tr>
<td>Protein, g/d</td>
<td>r</td>
<td>0.408</td>
<td>0.300</td>
<td>0.511</td>
<td>0.310</td>
<td>0.370</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.004</td>
<td>0.023</td>
<td>0.002</td>
<td>0.015</td>
<td>ns</td>
</tr>
<tr>
<td>Age</td>
<td>r</td>
<td>-0.383</td>
<td>-0.237</td>
<td>-0.437</td>
<td>-0.370</td>
<td>-0.293</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.079</td>
<td>ns</td>
<td>0.042</td>
<td>0.091</td>
<td>ns</td>
</tr>
<tr>
<td>BMI</td>
<td>r</td>
<td>0.292</td>
<td>0.288</td>
<td>0.182</td>
<td>0.347</td>
<td>0.502</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>0.017</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>r</td>
<td>0.507</td>
<td>0.471</td>
<td>0.454</td>
<td>0.468</td>
<td>-----</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.044</td>
<td>0.057</td>
<td>0.065</td>
<td>0.053</td>
<td>-----</td>
</tr>
<tr>
<td>Firmicutes&lt;sup&gt;1&lt;/sup&gt;</td>
<td>r</td>
<td>0.615</td>
<td>0.646</td>
<td>0.501</td>
<td>0.511</td>
<td>-----</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.005</td>
<td>0.002</td>
<td>0.020</td>
<td>0.032</td>
<td>-----</td>
</tr>
<tr>
<td>F:B Ratio</td>
<td>r</td>
<td>0.244</td>
<td>0.105</td>
<td>0.277</td>
<td>0.331</td>
<td>-----</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>-----</td>
</tr>
</tbody>
</table>

Abbreviations: AC, Acetate; BMI, body mass index; BU, Butyrate; CHO, carbohydrate; F:B, Firmicutes to Bacteriodetes; ns, non significant; PR, Propionate; r, correlations coefficient; tSCFA, total short chain fatty acids. Correlations are Pearson’s Correlations Coefficient; P Values calculated by SAS Proc-Mixed to account for repeated measurements; <sup>1</sup> adjusted for available carbohydrate (g/day). n=22, (P > 0.1 are presented)
Figure 3.4: Group and dietary components interactions with fecal acetate concentration

Group by Energy-intake interaction with faecal acetate concentration (A), Group by dietary-fiber (DF) intake with fecal acetate concentration (B), Group by fat-intake interaction with fecal acetate concentration (C). Low, visit with lower intake; High, visit with higher intake. § adjusted for energy.

3.6 DISCUSSION

Our findings suggest that the OWO have higher fecal SCFA than LN, with no difference in rectal SCFA absorption. Therefore, the higher fecal SCFA concentration in the OWO group is unlikely to be due to lower colonic SCFA absorption. The higher SCFA concentration is also not likely to be due to different dietary intake. Although fat intake was higher in the OWO individuals compared to the LN individuals, no significant correlations were observed between fat intake and fecal SCFA concentration. We found a higher relative abundance of Firmicutes and F:B ratio in the OWO group. These findings, together with the positive correlation between Firmicutes and fecal SCFA, are consistent with the hypothesis that OWO have higher fecal SCFA concentrations due to the obese microbial profile which is more efficient in fermenting substrates than the lean microbial profile.

Consistent with other studies (Schwiertz et al. 2010a, 190-195; Teixeira et al. 2013, 914-919; Ppatil et al. 2012, 647-657), the OWO individuals in the present study had a higher fecal SCFA
concentration compared to their LN counterparts after adjusting for age. We adjusted for age as ageing may indirectly influence SCFA concentrations in other animal and human studies as well (Murphy et al. 2010, 1635-1642; Gomes et al. 2011, S187-190; Wolever, Fernandes, and Rao 1996, 2790-2797). The current results are consistent with this in demonstrating a negative trend between the individual and total SCFA and age (p<0.05 for propionate and valerate, p<0.09 for total SCFA and butyrate).

Fecal SCFA are typically measured to reflect the colonic production of the SCFA; however, they are also a surrogate measurement of the SCFA absorption from the colon. The similar capability of the LN and OWO groups to absorb SCFA in this study suggests that reduced SCFA absorption is not the reason why OWO subjects have a higher fecal SCFA concentration than LN individuals. This conclusion was supported by the finding that there was no association between fecal SCFA and SCFA absorption within the whole study group. In our study, the total and individual SCFA were absorbed in proportion to their concentration inside the dialysis bag (data not shown). The concentration-dependent absorption points to passive diffusion as a predominant mechanism, as has been previously suggested (Stein, Zores, and Schröder 2000, 121-125; Fleming, Choi, and Fitch 1991, 1787-1797). However, since the pH of the lumen is approximately 5.6 to 6.6 (Cummings et al. 1987, 1221-1227) (6.6 in our participants), more than 95% of the SCFA are in the anionic form (Cummings 1981, 763-779), and anionic SCFA cannot be absorbed via simple passive diffusion but rather by a facilitated passive diffusion. The Monocarboxylate Transporters (MCT1) and the sodium-coupled MCT (SMCT) were suggested to facilitate the transport of SCFA from the lumen into the colonocytes and blood (Gill et al. 2005, C846-C852; Iwanaga et al. 2006, 243-254). A possible explanation for the group × fecal SCFA interaction seen for SCFA absorption could be the ability of the colonic SCFA to up-regulate the MCT gene expression and function (Haenen et al. 2013b, 274-283; Borthakur et al. 2012, G1126-G1133; Zhou et al. 2006, 683-689). Within the OWO group, individuals with a higher colonic SCFA concentration had more SCFA molecules to up-regulate more transporters in their rectum, and consequently had higher SCFA absorption than OWO individuals with lower fecal SCFA concentration. While within the LN group, the SCFA concentration may not be enough to up-regulate as many transporters, and therefore this association did not exist. Vogt and Wolever (Vogt and Wolever 2003, 3145-3148) concluded from rectal infusion studies using a
SCFA concentration of 100mmol/L that a mechanism other than simple diffusion might play a role in the absorption of colonic SCFA. This finding is comparable with our suggestion that the higher range of colonic SCFA concentration may result in a more efficient absorption.

A weakness of our study is that we measured SCFA absorption from the rectum which may not represent that in the proximal colon where most SCFA are absorbed. However, the rectum is the continuation of the colon and was previously shown to contain the same SCFA transporters as the rest of the colon (Iwanaga et al. 2006, 243-254). Moreover, we have shown that rectal infusion of SCFA increases serum SCFA concentrations (Freeland and Wolever 2010, 460-466). Thus, although differences in the transport rate between the cecum and the rectum might occur, there is a biological plausibility to extend our findings to the rest of the colon. Another weakness is that the validity of our results depends on the assumption that the disappearance of the SCFA from the dialysis bag represents rectal absorption, which could be criticized. However, studies that validated the technique to measure rectal mucosal transport in rats and humans, although not proven for SCFA, showed that only 5-9% of the labeled sodium that disappeared from the bag was recovered from the human lumen, they also showed that at least 90% of the bag’s solution was absorbed (McNeil, Cummings, and James 1978, 819-822; Edmonds 1971, 356-362).

Dietary macronutrients may affect SCFA concentration either directly, as substrates for fermentation or indirectly, through their influence on colonic microbiota and thereby on the SCFA concentration (De Filippo et al. 2010, 14691-14696). We found protein intake to be positively associated with fecal SCFA. However, since protein intake was similar in the LN and OWO groups, it does not explain the higher SCFA in the OWO group. The only difference in dietary intake between groups was that fat intake (g/d) was higher in the OWO group. Fat is not a substrate for bacterial fermentation although a small amount of dietary fat can reach the colon (Gabert et al. 2011, 2697-2703) and animal studies suggest that high fat diets are associated with alteration in microflora, though not necessarily with altered SCFA concentration (Murphy et al. 2010, 1635-1642; Hildebrandt et al. 2009, 1716-1724.e2; Kim et al. 2012; Mujico et al. 2013, 711-720). However, we found no correlations between fat intake and bacterial phyla or between fat intake and SCFA concentration suggesting that fat did not determine the higher fecal SCFA concentration in the OWO compared to the LN group. The findings of Teixeira et al. were consistent with ours, showing no correlations between fat intake and fecal SCFA in lean and
obese women (Teixeira et al. 2013, 914-919). Fava et al. showed in overweight individuals that high monounsaturated fat diets did not change fecal SCFA, but that a high saturated fatty acid diet increased SCFA concentration (Fava et al. 2013, 216-223). By contrast, Brinkworth et al. found that an energy-restricted high-fat diet decreased the fecal SCFA concentrations in overweight participants compared to an energy-restricted low fat diet (Brinkworth et al. 2009, 1493-1502). However, in the latter study carbohydrate and fiber intakes were extremely low in the high fat diet which may make the results difficult to compare.

Our study design, which included two visits for each individual, allowed us to investigate how changes within individuals’ diets affect their fecal SCFA concentration. Interestingly, an increase in individual’s energy, fiber or fat intakes from visit to visit was correlated with an increase in acetate within OWO individuals, but a decrease within LN individuals. These interactions suggest that LN and OWO individuals respond to changes in habitual diets differently, possibly due to differences in colonic bacteria between the groups. Different bacteria employ different metabolic pathways which may result in altered colonic SCFA (Mahowald et al. 2009, 5859-5864). However, there was no correlation between the bacterial changes and the SCFA changes (data not shown). Jumpertz et al. demonstrated that lean and obese individuals have differences in fecal energy losses when increasing their energy intake (Jumpertz et al. 2011, 58-65). The higher relative abundance of the Firmicutes was observed to be due largely to a higher relative abundance of the aggregated abundance of members of the family Erysipelotrichaceae (data not shown). Notably, members of this family are found in increased abundance in mice fed a high fat diet (Zhang et al. 2010, 232-241). The higher relative Firmicutes abundance and the higher F:B ratio we observed in the OWO group compared to LN, together with the positive correlation between Firmicutes and fecal SCFA (CHO-adjusted), and with the similar dietary intakes between the groups support the early hypothesis that the obese-microbiome is more efficient in harvesting energy (SCFA) from the diet (Ley et al. 2005, 11070-11075; Turnbaugh et al. 2006a, 1027-1031; Ley et al. 2006, 1022-1023; Murphy et al. 2010, 1635-1642; Zhang et al. 2009, 2365-2370). We adjusted for carbohydrate because carbohydrates had strong negative association with Firmicutes. Though dietary fiber also had a strong association with Firmicutes, fiber was not a significant predictor when added to this model.
Gut transit time can influence fecal SCFA concentration and colonic microbiota (El Oufir et al. 2000, 603-609; El Oufir et al. 1996, 870-877; Stephen, Wiggins, and Cummings 1987, 601-609; Lewis and Heaton 1997, 245-251). A reasonably accurate qualitative assessment of gut transit time is fecal consistency (Saad et al. 2010, 403-411), and since fecal consistency was similar between the groups, we assume that differences in gut transit time did not contribute to the differences in fecal SCFA concentrations and colonic bacteria between the groups. However, a more accurate technique of measuring gut transit time (i.e., using radiopaque markers and serial abdominal radiographs) may yield different results.

It is of note that measures of fecal SCFA and microbiota, represent the status in the rectum and not other regions of the colon where most SCFA are produced. Thus, our results need to be interpreted with caution. It is possible that fecal SCFA and microbiota may differ in OWO subjects due to factors we did not measure such as differences in the small intestinal absorption of nutrients, or in the rate of fermentation in the proximal colon resulting in different substrates reaching the distal colon, or other unknown causes.

We conclude that the higher fecal SCFA concentration seen in OWO compared to LN individuals is unlikely a result of differences in SCFA absorption or dietary intakes. The fact that increases in energy, dietary fiber and fat intakes within individuals were associated with an increase in fecal acetate in OWO but a decrease in LN subjects may be explained by higher Firmicutes abundance and higher F:B ratio in OWO compared LN subjects. These differences, together with the positive correlation between Firmicutes abundance and fecal SCFA concentration, are consistent with the hypothesis that OWO subjects produce more colonic SCFA than LN due to differences in colonic microbiota. However, larger studies looking at the effect of diet and colonic flora on fecal SCFA in LN and OWO participants are warranted.

3.7 Acknowledgements
We are thankful to Jean M Macklaim for support in statistical analysis of microbial data. Supported by grant no.OOP-64648 from the Canadian Institutes for Health Research (CIHR), Institute of Nutrition, Metabolism and Diabetes.
CHAPTER 4:

THE ACUTE EFFECTS OF INULIN AND RESISTANT STARCH ON POSTPRANDIAL SERUM SHORT-CHAIN FATTY ACIDS AND SECOND-MEAL GLYCEMIC RESPONSE ARE SIMILAR IN LEAN AND OVERWEIGHT SUBJECTS: A RANDOMIZED, CONTROLLED TRIAL
4.1 ABSTRACT

**Background:** Excess production of colonic short-chain-fatty-acids (SCFA) has been implicated in the promotion of obesity, but colonic fermentation of dietary-fiber to SCFA may play a role in preventing obesity and diabetes.

**Objectives:** This study aimed to compare the effects of inulin and resistant-starch on postprandial SCFA and second-meal glycemic response in healthy overweight or obese (OWO) vs lean (LN) participants.

**Methods:** Using a randomized, single-blind, crossover design, 13 OWO and 12 LN overnight fasted participants consumed 300mL water containing 75g glucose (Control), or 75g glucose plus 24g inulin (IN) or 28.2g resistant-starch (RS) on 3 separate days. A standard lunch was served 4h after the test-drink.

**Results:** Within the entire cohort, IN significantly increased serum SCFA but had no effect on serum FFA or second-meal glucose and insulin responses compared to control. By contrast, RS had no significant effect on SCFA but significantly reduced FFA rebound and second-meal glucose and insulin responses. Compared to LN participants, OWO had similar serum SCFA and glucose concentrations but significantly greater insulin and FFA. However, the effects of IN and RS on SCFA, glucose, insulin and FFA responses were similar in LN and OWO.

**Conclusions:** Inulin increased postprandial SCFA but did not reduce the second-meal glycemic response whereas resistant-starch had no significant effect on serum SCFA but reduced serum FFA and second-meal glucose and insulin responses. These effects were similar in lean and overweight/obese subjects. These results do not support the hypothesis that overweight/obesity is associated with increased colonic SCFA production, but the study had insufficient power. Thus, substantial difference in SCFA responses between OWO and LN could have been missed.
4.2 INTRODUCTION

High intake of dietary fibers is associated with reduced risk for T2DM and has been shown to improve glycemic control in T2DM (Weickert and Pfeiffer 2008, 439-442). The benefit from dietary fibers has been suggested to be due, at least in part, to its fermentation by colonic bacteria into the short-chain fatty acids (SCFA) acetate, propionate and butyrate, which are readily absorbed from the lumen and transported to the liver and peripheral blood.

SCFA may be involved in glucose homeostasis by modulating the secretion of gut hormones such as PYY and GLP-1 (Kimura et al. 2013, 1829). However, in this study, we focus in particular on another mechanism by which SCFA may improve glucose control, namely by reducing serum FFA (Den Besten et al. 2013, 2325-2340; Tarini and Wolever 2010, 9-16; Higgins 2004, 761-768). High FFA levels induce peripheral and hepatic insulin resistance (Brighenti et al. 2006a, 817-822; Nilsson et al. 2006, 1092-1099; Wolever, Bentum-Williams, and Jenkins 1995, 962-970), and contribute to oxidative stress and thus deteriorate pancreatic β-cell function as well as insulin sensitivity (Nilsson et al. 2008, 712-720). Reductions of FFA have been demonstrated in humans after oral SCFA ingestion (Crouse et al. 1968, 509-512), rectal SCFA infusion (Wolever et al. 1989, 1027-1033) and dietary fibers consumption (Tarini and Wolever 2010, 9-16; Brighenti et al. 2006a, 817-822). These effects were often, but not always, associated with reduced glycemic responses.

Different types of dietary fibers are fermented into different types and amounts of SCFA (Slavin et al. 2010, 177-191; Jenkins et al. 1998, 609-616), therefore, different dietary fibers may affect glycemic responses differently. Two fermentable dietary fibers of particular interest in this respect are resistant starch (RS) and inulin (IN). RS is an insoluble type of cereal fiber while IN is a soluble fiber that can be found in many types of plant foods. In vitro fermentations of RS and IN were shown to produce different profiles of SCFA (Yang et al. 2013, 74-81). RS has been shown to increase insulin sensitivity in short and long term consumption studies (Robertson et al. 2005, 559-567; Robertson et al. 2003, 659-665; Johnston et al. 2010, 391-397; Robertson et al. 2012, 3326-3332) however, the mechanism is not clear. On the other hand, although animal studies evaluating the effects of IN on glucose control have shown positive results, the results of
human studies have been equivocal (Tarini and Wolever 2010, 9-16; Bonsu, Johnson, and Mcleod 2011, 58-66; Raninen et al. 2011, 9-21).

The amount of SCFA produced from the fermentation of dietary fibers in the colon depends on the nature of the colonic microflora. The microbiota of obese mice differs from lean mice and produces SCFA more efficiently (Turnbaugh et al. 2006b, 1027-1031); however, evidence from human studies is inconsistent. While some human studies have confirmed the obese and lean profiles of Firmicutes and Bacteroidetes as seen in mice (Armougom et al. 2009; Santacruz et al. 2010, 83-92; Furet et al. 2010, 3049-3057), others did not report any differences in these profiles, or even found an opposite profile (Collado et al. 2008, 894-899; Duncan et al. 2008, 1720-1724; Jumpertz et al. 2011, 58-65; Schwiertz et al. 2010a, 190-195). Furthermore, studies that have looked at differences in fecal SCFA concentrations between lean and obese are few, and tend confirmed the increased SCFA concentrations in obese compared to lean humans (Schwiertz et al. 2010a, 190-195; Fernandes et al. 2014; Teixeira et al. 2013, 914-919), though one study have found the opposite results (Murugesan et al. 2015, 1337-1346). Differences in SCFA production between lean and obese were also explored in vitro, by using fecal inocula derived from lean and obese humans. These studies have either found a similar SCFA production in lean versus obese samples (Li et al. 2015, 1997-2004; Yang, Keshavarzian, and Rose 2013, 862-867), or an increased/decreased SCFA production in lean versus obese samples, depend on the fiber type that was fermented (Aguirre et al. 2014).

Current data from human studies is inconsistent, however, if obese humans ferment SCFA more efficiently than lean humans, then excess SCFA production may contribute extra calories to diet, and thus, may enhance weight gain and increase risk for T2DM. On the other hand, excess SCFA production may lower FFA concentrations and improve glucose control, and therefore decrease risk for T2DM.

Therefore, our objective was to compare the effects of RS and IN on postprandial responses of SCFA, glucose, insulin and FFA in overweight vs. lean participants. We hypothesized that, in overweight relative to lean subjects, RS and IN would elicit higher postprandial SCFA responses and have less effect in reducing serum FFA and second-meal glucose and insulin responses.
4.3 METHODS

4.3.1 Participants

Male and non-pregnant, non-lactating females aged 18–65 years with body mass index (BMI) \( \geq 20 \) and \( \leq 35 \) kg/m\(^2\) were recruited from a pool of participants previously involved in similar studies. Participants were excluded for any of the following reasons: presence of diabetes, cardiovascular, bowel, kidney or liver disease; use of medications which affect blood glucose or insulin sensitivity (such as diuretics); any use of antibiotics, laxatives, pre/probiotics or other drugs known to influence gastrointestinal function in the 3 months before the study; smoking; following any unusual dietary practices (such as weight loss diet, Atkins diet, vegan diet); abnormal plasma blood glucose (\( \geq 7.0 \) mmol/L); or anemia (based on serum hematocrit within the normal range: for male 0.39-0.49L/L and female 0.345-0.45L/L). Eligible participants were then divided prospectively into two groups based on their BMI; 12 participants in the LN group (BMI<25) and 13 participants in the OWO group (BMI\( \geq 25 \)). All tests were conducted at the Glycemic Index Laboratories, Toronto. Ethical approval for the study was obtained from the Research Ethics Board, University of Toronto. Participants gave written informed consent to participate in the study.

4.3.2 Phase 1

Participants completed questionnaires related to demographics, medical history, drug use, bowel habit, physical activity (Ainsworth et al. 1993, 71-80; Anonymous 1997, S73-S78). They were given instructions on how to record their dietary intake and asked to keep a 3-day diet record. Participants were also given a fecal collection kit which consisted of the Fisher brand commode specimen collection system (Fisher Scientific, Ottawa, ON) and plastic bags. On the third day of the diet record or the day after, participants collected a fecal sample. The completed 3-day diet record and the plastic bag containing the fecal sample was immediately placed on dry ice, and brought to the lab within 24 h of being collected. The frozen fecal samples were stored at -20 °C until they were processed (Figure 4.1).
Figure 4.1: The design of study 2 visits

- **WK 3**
- **WK 2**
- **WK 1**
- **DAY 4**
- **DAY 1-3**

**0**

- **Visit 1**
- **Visit 2**
- **Visit 3**
- **Visit 4, 5, 6**

- Start

Activities:
- Screening, Consent form, Blood sample
- Personal information forms, 3-D-diet-record
- Test drink: Glucose / Glucose + Inulin / Glucose + Resistant Starch
Figure 4.2: The design of study 2 - Focus on a Specific Study Day

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>-5</th>
<th>0</th>
<th>30</th>
<th>60</th>
<th>90</th>
<th>120</th>
<th>180</th>
<th>240</th>
<th>270</th>
<th>300</th>
<th>330</th>
<th>360</th>
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<tr>
<td>Sample</td>
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<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
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</tr>
<tr>
<td>Test Drink</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>●</td>
<td>●</td>
<td>●</td>
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<td>●</td>
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</tr>
<tr>
<td>Lunch</td>
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<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
</tr>
</tbody>
</table>

- SCFA, gut hormones (GLP-1, PYY, Ghrelin), Glucose, Insulin, C-Pep, FFA
- Breath sample (Hydrogen, Methane)
- Glucose (GTT) / Glucose + Inulin / Glucose + Resistant Starch (RS)
- Lunch: sandwich, juice and cookies
4.3.3 Phase 2

A week after completing phase 1, participants began phase 2. In phase 2, participants came to the laboratory on 3 separate occasions (3 treatments), separated by a 1-week washout (Figure 4.2). Subjects arrived at 8:00am on the morning of the study after fasting for 12 hours. When participants arrived, they were seated and had their forearms warmed with a heating pad. An indwelling cannula was inserted into a forearm vein; it was kept clear with periodic saline flushes. After a fasting blood sample was drawn, participants consumed a test drink within 5 min, and further blood samples were drawn at 0.5, 1, 1.5, 2, 3, and 4 h after the start of the test drink. Immediately after the 4-h blood sample, a standard lunch was provided. Participants ate the lunch within 15 min, and further blood samples were drawn at 4.5, 5, 5.5, and 6 h. Participants remained seated and awake for the duration of the study. Breath samples were collected at 1, 2, 3, 4, 5 and 6 h in order to measure breath hydrogen and methane.

4.3.4 Test drinks

Each subject consumed all 3 treatments in random order. The test drinks consisted of 75 g glucose (Glucose; Grain Process Enterprises Ltd, Scarborough, ON, Canada) or 75 g glucose+24 g Oliggo-Fiber Instant Inulin (Inulin; 90% dietary fiber, 10% free fructose, glucose and saccharose; Inulin; Cargill Inc., Wayzata, MN, USA) or 75 g glucose + 28 g resistant starch (RS) (total fiber content of ~ 85%; Nutriose® FB06, Roquette, France) dissolved in 300 mL water on the morning of the study. The dose of 24g was based on a previous study from our lab that looked at serum SCFA, glycemia and hormone responses in lean humans (Tarini and Wolever 2010, 9-16).

4.3.5 Standard lunch

The standard lunch consisted of a cheese and tomato sandwich (2 slices Dempster’s Original Whole Wheat Bread, 65g Hot House tomato, 45g Cracker Barrel Medium Cheddar Cheese, 15g Miracle Whip), a drink of apple juice (200mL Allen’s apple juice), a bottle of water (500mL), and two chocolate cookies (Dare Simple Pleasures Chocolate Thins). The lunch provided 588 Cal, 49.8% as CHO, 13.6% as protein and 36.7% as fat.
4.3.6 Analytical methods

The fecal sample was weighed and homogenized in a 400 series masticator (IUL Instruments, S.A., Barcelona, Spain) for 1 min. Aliquots of feces were then transferred to individual vials for determination of pH and SCFA. Fecal pH was measured using a FE20 pH meter with a MT InLab Solids Pro electrode (Mettler-Toledo, Columbus, OH, USA). Fecal SCFA were analyzed by gas chromatography as previously described (Rahat-Rozenbloom et al. 2014, 1525-1531).

DNA extraction and Ion Torrent V6 16S rRNA sequencing were performed as described elsewhere (Petrof et al. 2013, 3).

Blood for glucose, insulin, C-peptide and FFA was drawn into tubes containing spray-coated silica and a polymer gel for serum separation (BD Canada Inc., Oakville, Ont.). Blood for SCFA was drawn into serum tube with no additive and an uncoated interior (BD Canada Inc., Oakville, Ont.). Serum glucose was measured by a glucose oxidase method. Serum insulin was measured by immunoassay using the ALPCO Insulin EIA, and C-peptide by a sandwich type immunoassay using the ALPCO C-peptide ELISA. Serum FFA was measure using Wako NEFA-HR (2) reagents (VWR Canada). All blood samples were allowed to clot at room temperature for 30 min, centrifuged at 3000 rpm for 15 minutes at 4 °C, and the serum aliquoted and stored at −70 °C before analysis.

Serum SCFA was measured by gas chromatography after microfiltration and vacuum distillation. A 1.2–1.5 ml aliquot of serum was filtered through a micropartition system with a 30-kDa MWCO Vivaspin RC (VS02H22) filters (Sartorius Inc., Mississauga, ON, Canada) by centrifugation at 5000 g at 4 °C for 90 min. The protein-free filtrate was stored at −20 °C before vacuum distillation (Tollinger, Vreman, and Weiner 1979, 1787-1790). Distillation was performed by using a 225-μl sample of protein-free serum to which was added a 25-μl internal standard solution consisting of 1.25 mm valeric acid and 1.06 m formic acid. An automatic sampler (HP 7673; Hewlett-Packard, Mississauga, ON, Canada) was used to inject 1 μl aliquots of sample into a gas chromatograph (HP 5890 Series II; Hewlett-Packard) equipped with a direct cool, on-column inlet, an Agilent HP-FFAP column (30 m × 0.53 mm × 1.0 μm film), Agilent 19095F-123 (Agilent Technologies Canada Inc., Mississauga, ON, Canada), and a flame ionization detector.
Participants collected breath samples using the Easy Sampler with tube holder (Quintron Instrument Company, Milwaukee, WI, USA). Methane and hydrogen were measured by gas chromatography (Quintron Microlyzer, Model SC, Milwaukee, WI, USA) in breath samples and simultaneously obtained room air. Breath hydrogen and methane concentrations reported were adjusted by subtracting the hydrogen and methane of room air from that of each breath sample collected.

### 4.3.7 Statistical analysis

Results are given as means ±SEM. For glucose, insulin and c-peptide, incremental areas under the curve (iAUC; subtracting area below the baseline) over 0-2h and 2-4h were calculated using the fasting concentration as the baseline. For FFA, iAUC from the nadir to 4h (iAUCmin4) was calculated using the minimum concentration achieved over the first 4h as the baseline. For glucose, insulin, c-peptide and FFA, total areas under the curve (tAUC) were calculated over the 4-6h periods using the trapezoidal method. For SCFA, tAUC were calculated over the 0-4 and 4-6h periods, and iAUC from the nadir to 6h (iAUCmin6) were calculated using the minimum concentration achieved over the first 4h as the baseline. All AUCs were calculated using a computer spreadsheet (Microsoft Office Excel 2007).

To examine for the main effects of groups and treatments, and interactions between these effects, a statistical analysis was performed with STATA 13.0 (College Station, TX), using the mixed-effects (random-effects) model. Since the interaction term was not significant for any of the outcomes (probably due to small sample size), the interaction term was dropped from the statistical model to save power for the main effects.

To investigate a possible relationship between serum FFA and SCFA and second-meal glucose responses, the difference in response between IN and Control and between RS and Control were calculated, and the differences in FFA and SCFA were correlated with the differences in glucose tAUC from 4-6 hrs.

Independent t-tests were performed to analyse differences in baseline data between the groups using IBM SPSS Statistics version 22 (SPSS Inc., Chicago, IL, USA). Differences were taken to be statistically significant if 2-tailed $p < 0.05$. 
4.4 RESULTS

4.4.1 Baseline characteristics

OWO participants were significantly older than LN but OWO and LN did not differ significantly with respect to sex and ethnicity (Table 4.1). Concentrations of fecal SCFA (Table 4.2) and fasting serum SCFA (Table 4.1) were not significantly different between the groups. However, after age adjustment, fecal propionate was significantly increased in the LN group compared to the OWO group. All other individual fecal and serum SCFA did not differ between the groups after age adjustments (Table 4.2). OWO had significantly higher fasting insulin, total- and low-density lipoprotein-cholesterol and triglycerides than LN, but similar glucose, aspartate transaminase, C-reactive protein and high-density lipoprotein-cholesterol concentrations.

OWO had higher mean intakes of fat (g), protein (g), CHO (g) sugars (g) and total calories than the LN group, but the differences were not significant.

4.4.2 Serum SCFA

Serum acetate, propionate and butyrate concentrations began to increase 2 to 4h after IN compared to GLU and were significantly higher than GLU from 4 to 6h (Figure 4.3); thus, both the incremental AUC and the total AUC of all 3 SCFA after IN were significantly greater than those after GLU (Table 4.4). By contrast, it took 5-6h for mean serum SCFA concentrations after RS to begin to exceed those after GLU; by 6h after RS the concentrations of all 3 SCFA were significantly greater those after GLU by paired t-test when uncorrected for multiple comparisons (Figure 4.3), but the differences were small and there were no significant differences between RS and GLU in incremental or total AUC (Table 4.4). There were no significant differences in serum SCFA responses between LN and OWO subjects (Table 4.6).
### Table 4.1: Characteristics of study participants at screening

<table>
<thead>
<tr>
<th></th>
<th>Whole group (n=25)</th>
<th>Lean (n=12)</th>
<th>Overweight/obese (n=13)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Mass Index (kg/m²)</td>
<td>27.5 ± 1.0</td>
<td>23.2 ± 0.4</td>
<td>31.5 ± 1.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Age (y)</td>
<td>39.8 ± 2.8</td>
<td>33.4 ± 3.7</td>
<td>45.8 ± 3.5</td>
<td>0.025</td>
</tr>
<tr>
<td>Ethnicity A:C:O (n)**</td>
<td>5:16:4</td>
<td>2:9:1</td>
<td>3:7:3</td>
<td>0.494</td>
</tr>
<tr>
<td>Sex (M:F) (n)</td>
<td>12:13</td>
<td>7:5</td>
<td>5:8</td>
<td>0.320</td>
</tr>
</tbody>
</table>

**Blood Samples**

<table>
<thead>
<tr>
<th></th>
<th>Whole group (n=25)</th>
<th>Lean (n=12)</th>
<th>Overweight/obese (n=13)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate (μmol/L)</td>
<td>36 ± 4</td>
<td>42 ± 6</td>
<td>32 ± 5</td>
<td>0.230</td>
</tr>
<tr>
<td>Propionate (μmol/L)</td>
<td>1.1 ± 0.5</td>
<td>1.2 ± 0.2</td>
<td>1.0 ± 0.1</td>
<td>0.347</td>
</tr>
<tr>
<td>Butyrate (μmol/L)</td>
<td>0.40 ± 0.04</td>
<td>0.39 ± 0.04</td>
<td>0.41 ± 0.06</td>
<td>0.756</td>
</tr>
<tr>
<td>Fasting Glucose (mmol/L)</td>
<td>4.87 ± 0.84</td>
<td>4.76 ± 0.12</td>
<td>4.98 ± 0.11</td>
<td>0.202</td>
</tr>
<tr>
<td>Fasting Insulin (pmol/L)</td>
<td>43.1 ± 5.0</td>
<td>25.7 ± 3.0</td>
<td>57.77 ± 6.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>0.40 ± 0.01</td>
<td>0.39 ± 0.01</td>
<td>0.40 ± 0.01</td>
<td>0.710</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>22.4 ± 1.4</td>
<td>21.7 ± 1.44</td>
<td>23.0 ± 2.31</td>
<td>0.657</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>3.3 ± 1.5</td>
<td>0.7 ± 0.2</td>
<td>5.7 ± 2.8</td>
<td>0.098</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>4.70 ± 0.22</td>
<td>4.23 ± 0.19</td>
<td>5.11 ± 0.35</td>
<td>0.046</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>0.94 ± 0.10</td>
<td>0.68 ± 0.08</td>
<td>1.18 ± 0.15</td>
<td>0.009</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.47 ± 0.05</td>
<td>1.53 ± 0.09</td>
<td>1.41 ± 0.06</td>
<td>0.300</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>2.85 ± 0.18</td>
<td>2.46 ± 0.16</td>
<td>3.19 ± 0.28</td>
<td>0.038</td>
</tr>
</tbody>
</table>

Values are means ±SEM except for Ethnicity and sex which are number of subjects.

* Significance of difference between Lean and Overweight/obese groups; ** A=Asian; C=Caucasian; O=Other
### Table 4.2: Fecal SCFA concentrations in lean and Overweight/obese groups

<table>
<thead>
<tr>
<th>Fecal SCFA (mmol/kg)</th>
<th>Whole group (n=25)</th>
<th>Lean (n=12)</th>
<th>Overweight/obese (n=13)</th>
<th>P value*</th>
<th>P value*1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>48.4 ± 3.5</td>
<td>48.7 ± 3.3</td>
<td>48.0 ± 6.2</td>
<td>0.929</td>
<td>0.205</td>
</tr>
<tr>
<td>Propionate</td>
<td>15.7 ± 1.2</td>
<td>17.4 ± 1.5</td>
<td>14.1 ± 1.8</td>
<td>0.181</td>
<td>0.015</td>
</tr>
<tr>
<td>Iso-Butyrate</td>
<td>1.5 ± 0.2</td>
<td>1.7 ± 0.3</td>
<td>1.4 ± 0.2</td>
<td>0.475</td>
<td>0.283</td>
</tr>
<tr>
<td>Butyrate</td>
<td>15.2 ± 1.2</td>
<td>15.2 ± 1.3</td>
<td>15.2 ± 2.0</td>
<td>0.984</td>
<td>0.411</td>
</tr>
<tr>
<td>Iso-Valerate</td>
<td>2.1 ± 0.3</td>
<td>2.2 ± 0.5</td>
<td>1.9 ± 0.3</td>
<td>0.541</td>
<td>0.359</td>
</tr>
<tr>
<td>Valerate</td>
<td>2.1 ± 0.2</td>
<td>2.3 ± 0.3</td>
<td>1.9 ± 0.4</td>
<td>0.393</td>
<td>0.128</td>
</tr>
</tbody>
</table>

Values are means ± SEM. * Significance of difference between Lean vs. Overweight/Obese groups; 1 adjusted for age

### Table 4.3: Daily dietary macronutrient intakes

<table>
<thead>
<tr>
<th></th>
<th>Whole group (n=24)</th>
<th>LN Group (n=11)</th>
<th>OWO Group (n=13)</th>
<th>P value LN vs. OWO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calories (kcal)</td>
<td>Mean 2060.6</td>
<td>Mean 1929.9</td>
<td>Mean 2171.2</td>
<td>0.141</td>
</tr>
<tr>
<td></td>
<td>SEM 81.1</td>
<td>SEM 97.5</td>
<td>SEM 119.7</td>
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</tr>
<tr>
<td>Fat (g)</td>
<td>Mean 81.7</td>
<td>Mean 72.6</td>
<td>Mean 89.5</td>
<td>0.086</td>
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<tr>
<td></td>
<td>SEM 4.9</td>
<td>SEM 6.4</td>
<td>SEM 6.7</td>
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</tr>
<tr>
<td>% Fat</td>
<td>Mean 35.4</td>
<td>Mean 33.7</td>
<td>Mean 36.9</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>SEM 1.5</td>
<td>SEM 2.6</td>
<td>SEM 1.7</td>
<td></td>
</tr>
<tr>
<td>Protein (g)</td>
<td>Mean 83.7</td>
<td>Mean 82.7</td>
<td>Mean 84.6</td>
<td>0.876</td>
</tr>
<tr>
<td></td>
<td>SEM 6</td>
<td>SEM 10.7</td>
<td>SEM 6.8</td>
<td></td>
</tr>
<tr>
<td>% Pro</td>
<td>Mean 16.2</td>
<td>Mean 16.8</td>
<td>Mean 15.7</td>
<td>0.594</td>
</tr>
<tr>
<td></td>
<td>SEM 1</td>
<td>SEM 1.6</td>
<td>SEM 1.2</td>
<td></td>
</tr>
<tr>
<td>CHO (g)</td>
<td>Mean 257</td>
<td>Mean 243.8</td>
<td>Mean 268.1</td>
<td>0.311</td>
</tr>
<tr>
<td></td>
<td>SEM 11.7</td>
<td>SEM 17.1</td>
<td>SEM 16</td>
<td></td>
</tr>
<tr>
<td>Av CHO (g)</td>
<td>Mean 233.9</td>
<td>Mean 219.9</td>
<td>Mean 245.8</td>
<td>0.239</td>
</tr>
<tr>
<td></td>
<td>SEM 10.8</td>
<td>SEM 15.1</td>
<td>SEM 14.9</td>
<td></td>
</tr>
<tr>
<td>% Av CHO</td>
<td>Mean 45.5</td>
<td>Mean 45.7</td>
<td>Mean 45.3</td>
<td>0.872</td>
</tr>
<tr>
<td>Fiber (g)</td>
<td>Mean 23.1</td>
<td>Mean 23.9</td>
<td>Mean 22.3</td>
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</tr>
<tr>
<td></td>
<td>SEM 1.9</td>
<td>SEM 3.1</td>
<td>SEM 2.3</td>
<td></td>
</tr>
<tr>
<td>Sugar (g)</td>
<td>Mean 94.8</td>
<td>Mean 84.6</td>
<td>Mean 103.5</td>
<td>0.182</td>
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<tr>
<td></td>
<td>SEM 7</td>
<td>SEM 9.2</td>
<td>SEM 9.9</td>
<td></td>
</tr>
<tr>
<td>Alcohol (g)</td>
<td>Mean 7.8</td>
<td>Mean 9.5</td>
<td>Mean 6.4</td>
<td>0.564</td>
</tr>
<tr>
<td></td>
<td>SEM 2.6</td>
<td>SEM 4.6</td>
<td>SEM 3.1</td>
<td></td>
</tr>
<tr>
<td>% Alcohol</td>
<td>Mean 2.9</td>
<td>Mean 3.8</td>
<td>Mean 2.1</td>
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<tr>
<td></td>
<td>SEM 5.3</td>
<td>SEM 2.1</td>
<td>SEM 1</td>
<td></td>
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</tbody>
</table>

1Values are means ± SEM; Av CHO, available carbohydrate; LN, lean; OWO, overweight and obese; In the LN group, one diet record was misplaced.
Figure 4.3: Serum short-chain fatty acid responses elicited by the test meals

Values are means±SEM for n=25 subjects. * Significant difference between Inulin and Glucose by related samples Wilcoxon signed rank test (p<0.05). † Significant difference between resistant starch and glucose by related samples Wilcoxon signed rank test (p<0.05).
<table>
<thead>
<tr>
<th>Variable</th>
<th>Time after treatment (h)</th>
<th>Treatment</th>
<th>Glucose</th>
<th>Inulin</th>
<th>Resistant Starch</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate (μmol×h/L)</td>
<td>iAUC min-6h</td>
<td>24.4±2.8</td>
<td>94.5±13.1 a</td>
<td>36.1±3.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>tAUC 0-4h</td>
<td>79.7 ± 7.6</td>
<td>108.4 ± 12.4 a</td>
<td>80.4 ± 8.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>tAUC 4-6h</td>
<td>41.9 ± 3.8</td>
<td>102 ± 11.9 a</td>
<td>52.9 ± 4.2</td>
<td></td>
</tr>
<tr>
<td>Propionate (μmol×h/L)</td>
<td>iAUC min-6h</td>
<td>1.01±0.09</td>
<td>1.71±0.20 a</td>
<td>1.09±0.13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>tAUC 0-4h</td>
<td>3.98 ± 0.21</td>
<td>4.25 ± 0.29</td>
<td>4.05 ± 0.27</td>
<td></td>
</tr>
<tr>
<td></td>
<td>tAUC 4-6h</td>
<td>2.26 ± 0.11</td>
<td>3.04 ± 0.24 a</td>
<td>2.48 ± 0.17</td>
<td></td>
</tr>
<tr>
<td>Butyrate (μmol×h/L)</td>
<td>iAUC min-6h</td>
<td>0.27±0.03</td>
<td>1.11±0.15 a</td>
<td>0.40±0.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>tAUC 0-4h</td>
<td>1.4 ± 0.1</td>
<td>1.67 ± 0.14 a</td>
<td>1.49 ± 0.14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>tAUC 4-6h</td>
<td>0.71 ± 0.05</td>
<td>1.51 ± 0.16 a</td>
<td>0.83 ± 0.06</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean±SEM for n=25; iAUC, incremental area under the curve; tAUC, total area under the curve; min6, measured from nadir to 6hrs. a mean for Inulin significantly different from mean for Glucose.
Table 4.5: Serum glucose, insulin, C-peptide and free-fatty acid responses elicited by the test meals

<table>
<thead>
<tr>
<th>Time after treatment (h)</th>
<th>Glucose (mmol×h/L)</th>
<th>Treatment</th>
<th>Glucose</th>
<th>Inulin</th>
<th>Resistant Starch</th>
</tr>
</thead>
<tbody>
<tr>
<td>iAUC 0-2h</td>
<td></td>
<td></td>
<td>3.73±0.49</td>
<td><strong>4.60±0.55</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.93±0.56</td>
</tr>
<tr>
<td>iAUC 2-4hr</td>
<td></td>
<td></td>
<td>-1.13±0.23</td>
<td>-0.89±0.25</td>
<td>-0.66±0.30</td>
</tr>
<tr>
<td>tAUC 4-6h</td>
<td></td>
<td></td>
<td>11.6±0.3</td>
<td>11.5±0.3</td>
<td><strong>10.9±0.3</strong>&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>iAUC 0-2h</td>
<td>Insulin (pmol×h/L)</td>
<td></td>
<td>111.0±18.8</td>
<td>114.9±21.0</td>
<td>106.8±16.8</td>
</tr>
<tr>
<td>iAUC 2-4hr</td>
<td></td>
<td></td>
<td>23.7±7.2</td>
<td>33.3±11.6</td>
<td>34.0±10.1</td>
</tr>
<tr>
<td>tAUC 4-6h</td>
<td></td>
<td></td>
<td>77.8±10.5</td>
<td>76.7±9.2</td>
<td><strong>66.7±8.9</strong>&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>iAUC 0-2h</td>
<td>C-peptide (pmol×h/L)</td>
<td></td>
<td>2660±238</td>
<td>2770±212</td>
<td>2720±203</td>
</tr>
<tr>
<td>iAUC 2-4h</td>
<td></td>
<td></td>
<td>857±151</td>
<td>1055±179</td>
<td><strong>1290±241</strong>&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>tAUC 4-6h</td>
<td></td>
<td></td>
<td>2632±211</td>
<td>2617±188</td>
<td><strong>2444±197</strong>&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>tAUC 0-4h</td>
<td>FFA (mEq×h/L)</td>
<td></td>
<td>1.05±0.07</td>
<td>1.06±0.08</td>
<td><strong>0.94±0.06</strong>&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>tAUC 4-6h</td>
<td></td>
<td></td>
<td>0.53±0.03</td>
<td>0.52±0.03</td>
<td><strong>0.48±0.03</strong>&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>iAUCmin4</td>
<td></td>
<td></td>
<td>0.21±0.03</td>
<td>0.19±0.03</td>
<td><strong>0.15±0.03</strong>&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are mean ± SEM for n=25; iAUC, incremental area under the curve; min4, measured from nadir to 4 hrs; tAUC, total area under the curve; a, inulin treatment significantly different from Glucose treatment; b, resistant starch treatment significantly different from Glucose treatment.
### 4.4.3 Breath Gases

Breath hydrogen 2h after IN was significantly greater than after GLU and increased exponentially to reach a peak concentration at 5h which was about 60 ppm greater than that after GLU (Figure 4.4). Breath hydrogen after RS increased gradually to became significantly greater than GLU at 4hr, reaching a peak concentration at 5h of approximately 10 ppm. There was no difference in breath hydrogen responses between LN and OWO subjects (not shown). There were 8 methane producers, and their mean breath methane concentration tended to fall throughout the day with no difference among treatments (Figure 4.4).

**Figure 4.4: Breath hydrogen and methane responses elicited by the test meals**

Values are means±SEM in n=25 subjects for hydrogen and n=8 methane producers for methane. * Significant difference between Inulin and Glucose by related samples Wilcoxon signed rank test (p<0.05). † Significant difference between resistant starch and glucose by related samples Wilcoxon signed rank test (p<0.05).
### Table 4.6: The mean serum SCFA, glucose, insulin, C-peptide and free-fatty acid (FFA) responses elicited by the test meals of the 3 visits in lean vs. overweight and obese participants

<table>
<thead>
<tr>
<th>Variable</th>
<th>Time</th>
<th>Lean (n=12)</th>
<th>Overweight and obese (n=13)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>iAUC min6</td>
<td>46.2 ± 6.1</td>
<td>56.7 ± 9</td>
<td>0.319</td>
</tr>
<tr>
<td>(μmol×h/L)</td>
<td>tAUC 0-6h</td>
<td>158.2 ± 14</td>
<td>152.2 ± 19.8</td>
<td>0.799</td>
</tr>
<tr>
<td>Propionate</td>
<td>iAUC min6</td>
<td>1.3 ± 0.2</td>
<td>1.2 ± 0.2</td>
<td>0.386</td>
</tr>
<tr>
<td>(μmol×h/L)</td>
<td>tAUC 0-6h</td>
<td>6.9 ± 0.5</td>
<td>6.5 ± 0.5</td>
<td>0.254</td>
</tr>
<tr>
<td>Butyrate</td>
<td>iAUC min6</td>
<td>0.5 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>0.78</td>
</tr>
<tr>
<td>(μmol×h/L)</td>
<td>tAUC 0-6h</td>
<td>2.3 ± 0.1</td>
<td>2.7 ± 0.3</td>
<td>0.617</td>
</tr>
<tr>
<td>Glucose</td>
<td>iAUC 0-2h</td>
<td>3.3±0.5</td>
<td>4.8±0.8</td>
<td>0.076</td>
</tr>
<tr>
<td>(mmol×h/L)</td>
<td>iAUC 2-4hr</td>
<td>-1.1±0.2</td>
<td>-0.7±0.4</td>
<td>0.663</td>
</tr>
<tr>
<td></td>
<td>tAUC 4-6h</td>
<td>11.7±0.4</td>
<td>11.0±0.3</td>
<td>0.208</td>
</tr>
<tr>
<td>FFA</td>
<td>tAUC 0-4h</td>
<td>0.97±0.11</td>
<td>1.07±0.08</td>
<td>0.425</td>
</tr>
<tr>
<td>(mEq×h/L)</td>
<td>tAUC 4-6h</td>
<td>0.45±0.03</td>
<td>0.57±0.04</td>
<td>0.038</td>
</tr>
<tr>
<td></td>
<td>iAUC min4</td>
<td>0.19±0.05</td>
<td>0.17±0.03</td>
<td>0.699</td>
</tr>
<tr>
<td>Insulin</td>
<td>iAUC 0-2h</td>
<td>65.5±738</td>
<td>152.8±30.6</td>
<td>0.005</td>
</tr>
<tr>
<td>(pmol×h/L)</td>
<td>iAUC 2-4hr</td>
<td>16.1±3.1</td>
<td>43.5±16.5</td>
<td>0.166</td>
</tr>
<tr>
<td></td>
<td>tAUC 4-6h</td>
<td>49.5±5.3</td>
<td>96.2±14.4</td>
<td>0.002</td>
</tr>
<tr>
<td>C-peptide</td>
<td>iAUC 0-2h</td>
<td>2139±138</td>
<td>3250±324</td>
<td>0.001</td>
</tr>
<tr>
<td>(pmol×h/L)</td>
<td>iAUC 2-4hr</td>
<td>698±108.7</td>
<td>1410±304.6</td>
<td>0.136</td>
</tr>
<tr>
<td></td>
<td>tAUC 4-6h</td>
<td>2030±133.5</td>
<td>3056±291</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Values are mean ± SEM; *, P value for the difference between the lean group to the overweight and obese group; FFA, free fatty acids; iAUC, incremental area under the curve; min4/6, measured from nadir to 4/6hrs; tAUC, total area under the curve
4.4.4 Serum Glucose

During the 0-2h period the serum glucose response was significantly greater after IN than GLU but was not different after RS compared to GLU (Figure 4.5, Table 4.5). However, after the standard lunch, tAUC 4-6h was significantly less after RS than GLU but there was no difference in tAUC 4-6h between IN and GLU (Figure 4.5, Table 4.5). There were no significant differences in glycemic response between the LN and OWO groups (Table 4.6).

Figure 4.5: Serum glucose, insulin, c-peptide and FFA responses elicited by the test meals

Values are means±SEM in n=25 subjects. * Significant difference between Inulin and Glucose by related samples Wilcoxon signed rank test (p<0.05). † Significant difference between resistant starch and glucose by related samples Wilcoxon signed rank test (p<0.05).
4.4.5 Serum Insulin and C-peptide

There was no difference among treatments in serum insulin or c-peptide responses between 0-2h; however, the mean iAUC from 2-4h was greater after RS compared to GLU, and the difference was significant for c-peptide. During the 4-6-h period, serum insulin and c-peptide responses after IN were similar to those after GLU, but, after RS, tAUC 4-6h for serum insulin and c-peptide were significantly less than those after GLU (Figure 4.5, Table 4.5). Serum insulin and c-peptide responses between 0-2h and 4-6h were significantly greater in the OWO group compared to the LN group (Table 4.6).

4.4.6 Serum FFA

Serum FFA responses were similar after IN compared to GLU (Figure 4.5, Table 4.5). However, over the 0-4h and 4-6h periods the total AUC for FFA was significantly less after RS than GLU, due to a significantly smaller rebound between the nadir and 4h (Figure 4.5, Table 4.5). FFA responses in LN did not differ significantly from those in OWO (Table 4.6).

4.4.7 Correlations between SCFA, FFA and glucose responses

The changes in serum butyrate iAUC from minimum (nadir) to 6hr elicited by IN were negatively related to the changes in glucose tAUC at 4-6hr (p=0.055) (Table 4.7A). The changes in serum FFA rise (minimum nadir to peak nadir) elicited by RS tent to negatively relate to glucose tAUC 4-6hr (p=0.059)(Table 4.7A). The changes in acetate tAUC at 0-4hr elicited by IN were negatively correlated to the changes in FFA rise (p=0.034) (Table 4.7B).
### Table 4.7: Relations between serum SCFA, glucose and FFA after inulin or after resistant-starch treatments

#### A) Correlations between serum SCFA and FFA and Glucose AUC after lunch

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Variable</th>
<th>Glucose tAUC 46</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inulin</td>
<td>Acetate iAUC min6</td>
<td>r</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p -.240 .248</td>
</tr>
<tr>
<td></td>
<td>Propionate iAUC min6</td>
<td>r</td>
</tr>
<tr>
<td></td>
<td>Butyrate iAUC min6</td>
<td>r</td>
</tr>
<tr>
<td></td>
<td>FFA Peak rise</td>
<td>r</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p .108 .606</td>
</tr>
<tr>
<td></td>
<td>Acetate iAUC min6</td>
<td>r</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p .266 .199</td>
</tr>
<tr>
<td></td>
<td>Propionate iAUC min6</td>
<td>r</td>
</tr>
<tr>
<td></td>
<td>Butyrate iAUC min6</td>
<td>r</td>
</tr>
<tr>
<td></td>
<td>FFA Peak rise</td>
<td>r</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p .383 .059</td>
</tr>
</tbody>
</table>

#### B) Correlations between serum SCFA and pre-lunch FFA peak before lunch

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Variable</th>
<th>FFA Peak rise</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inulin</td>
<td>Acetate tAUC 04</td>
<td>r -.425</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p .034</td>
</tr>
<tr>
<td></td>
<td>Propionate tAUC 04</td>
<td>r</td>
</tr>
<tr>
<td></td>
<td>Butyrate tAUC 04</td>
<td>r</td>
</tr>
<tr>
<td></td>
<td>Acetate tAUC 04</td>
<td>r</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p .052 .807</td>
</tr>
<tr>
<td></td>
<td>Propionate tAUC 04</td>
<td>r</td>
</tr>
<tr>
<td></td>
<td>Butyrate tAUC 04</td>
<td>r</td>
</tr>
</tbody>
</table>

Abbreviations: FFA, free fatty acids; iAUC, incremental areas under the curves; iAUC min6, incremental areas under the curves measured from nadir (minimum) to 6hrs; p, significant; r, correlation; SCFA, short chain fatty acid; tAUC 46, total areas under the curves from 4 to 6 hrs. a pre-lunch FFA peak (Spearman’s Correlation Test) .
4.5 DISCUSSION

It has been suggested that not only is excess production of colonic SCFA a cause of obesity, but also that colonic fermentation of dietary fibers improves insulin sensitivity, and therefore reduces the risk for T2DM (Robertson et al. 2005, 559-567; Robertson et al. 2003, 659-665; Freeland, Wilson, and Wolever 2010, 82-90). Unlike previous reports from ourselves and others (Fernandes et al. 2014; Rahat-Rozenbloom et al. 2014, 1525-1531; Schwiertz et al. 2010b, 190-195), significant differences in fecal SCFA concentrations between LN and OWO participants were not seen in this study and neither were significant differences in postprandial SCFA responses. This does not support the hypothesis that excess SCFA production is a cause of obesity. However, the results do not rule out increased colonic SCFA production in OWO compared to LN participants since, if this was also accompanied by increased hepatic and peripheral SCFA clearance, postprandial serum SCFA responses could be similar in the 2 groups. Our results also suggest that any effect of the colonic fermentation of dietary fibers on glycemic responses is not due to SCFA per se, since IN increased serum SCFA without reducing the second-meal glycemic response, whereas RS reduced the second-meal glycemic response without increasing serum SCFA. In the case of RS, the second-meal effect is more likely due to a reduced FFA rebound (Brighenti et al. 2006b, 817-822), possibly related to prolonged or increased carbohydrate absorption.

Although both IN and RS are fermentable, only IN elicited a significant increase in SCFA. The earlier elevation of acetate after IN compared to RS could reflect faster gastric emptying (which may also explain the increased glucose concentrations we saw after the IN breakfast), a more rapid transit of IN than RS through the small intestine, or more rapid and more proximal fermentation of IN than RS in the large intestine (James et al. 2003, 291-296). The latter is suggested by that fact that serum SCFA after RS did not reach a peak by 6h, while after IN serum SCFA peaked at approximately 5h (Figure 5.1). To our knowledge, no acute human studies have compared the fermentation of IN and RS. However, 12-hr fermentation of fecal inocula from humans (Yang et al. 2013, 74-81) showed that RS and IN produced similar amounts of total SCFA, with increased butyrate production from IN. Therefore, a study period longer that 6hr may be required to detect rises in serum SCFA after RS.
In this study, the reduced insulin and c-peptide responses together with the lower glucose response after RS at 4-6 hrs compared to control are consistent with other studies showing that RS treatment improves insulin sensitivity (Robertson et al. 2003, 659-665; Johnston et al. 2010, 391-397; Bodinham et al. 2014, 75-84; Klosterbuer, Thomas, and Slavin 2012, 11928-11934). The reduced glycemic response we observed is likely not related to SCFA (as there was no significant increase in SCFA after RS), but rather to the reduced FFA rebound (Wolever, Bentum-Williams, and Jenkins 1995, 962-970; Wolever, Bentum-Williams, and Jenkins 1995, 962-970; Jenkins et al. 1989, 929-934). This suggestion is supported by the positive correlation between the FFA rise elicited by RS before lunch and glucose concentrations after lunch (p=0.059). The fact that both c-peptide and insulin AUCs were lower after RS suggests that the reduced insulin response was more likely due to reduced pancreatic insulin secretion than to reduced hepatic insulin extraction. Robertson et al. demonstrated that supplementation of 30 g/day of RS for 4wks in healthy subjects significantly improved insulin sensitivity, reduced subcutaneous abdominal adipose tissue FFA (but not systemic FFA) and increased serum SCFA concentrations (Robertson et al. 2005, 559-567). However, in other studies in subjects without diabetes showing a beneficial effect of RS on insulin sensitivity SCFA and FFA were either not measured (Bodinham et al. 2013, 1429-1433) or showed no significant change (Robertson et al. 2003, 659-665). In people with T2DM, 12 weeks of RS supplementation resulted in lower postprandial glucose concentrations, and a reduction in fasting and postprandial FFA. However, no effect on insulin sensitivity or SCFA concentrations was seen (Bodinham et al. 2014, 75-84). The acute reduction in FFA we observed after RS was not associated with increased serum SCFA, but, could have been explained by the presence of a larger than expected amount of available, but slowly digested starch in the RS ingredient used, since both prolonged carbohydrate absorption (Jenkins et al. 1989, 929-934) and an increase in the amount of carbohydrate absorbed (Wolever, Bentum-Williams, and Jenkins 1995, 962-970) have been shown to reduce postprandial FFA rebound.

The inability of IN to elicit a significant reduction in postprandial FFA rebound, despite a large increase in serum SCFA concentrations, was unexpected and not consistent with the results of several previous studies from our laboratory (Tarini and Wolever 2010, 9-16; Fernandes, Vogt, and Wolever 2011, 1279-1286). The increased glucose response before lunch was also
unexpected and inconsistent with our previous studies which showed that the glycemic response after adding 24 g IN to either high-fructose corn syrup (Tarini and Wolever 2010, 9-16) or to 75g glucose (Fernandes, Vogt, and Wolever 2012, 1029-1034) was virtually identical to that after the control. The inverse relation between butyrate (min-6h) and glycemic responses at 4-6h may show that butyrate has a major role in reducing glucose, though its elevation might not be consistent and high enough to significantly reduce blood glucose. Though animal studies have shown a positive effect of the inulin-type fructans on glucose control (Diez et al. 1997, 1238-1242; Rozan et al. 2008, 1192-1199; Diez et al. 1998, 91-96), a recent systematic review in humans did not find a conclusive result (Bonsu, Johnson, and Mcleod 2011, 58-66). The inconsistency in human studies was suggested to be due to the differences in fructan types, doses, durations of studies, health status of the participants, and differences in analytical methods. Further investigations are needed in order to determine whether inulin has any effect on glycemic control.

Although previous studies tend to show that obese humans have higher fecal SCFA than LN people, the correlation with microbial profiles is less clear (Fernandes et al. 2014; Teixereira et al. 2013, 914-919; Murugesan et al. 2015, 1337-1346; Rahat-Rozenbloom et al. 2014, 1525-1531; Schwiertz et al. 2010b, 190-195). We found no differences in serum and /or fecal SCFA between LN and OWO groups. However, there was large inter-individual variation in serum SCFA responses with a coefficient of variation of 69% for serum acetate iAUC. This is not surprising given the many factors such as diet, human and microbial genes and physical activity can affect fecal and serum SCFA concentrations. The non-significant difference in fecal and serum SCFA between our LN and OWO groups is consistent with the lack of difference in daily dietary intakes and in fecal microbial profile (at least at the phylum level; data not shown). However, a post-hoc calculation shows that the study had 80% power to detect a difference of 74 μmol×h/L in serum acetate response after inulin between LN and OWO; while we found a difference of 27 μmol×h/L. Thus, a substantial difference in SCFA responses between OWO and LN could have been missed. Therefore, in future studies a larger sample size may be needed to detect differences in serum SCFA responses between LN and OWO subjects.

It is concluded that IN and RS differ in their acute postprandial effects: IN is rapidly fermented in the colon eliciting prompt and large increases in breath hydrogen and serum SCFA.
concentrations, whereas RS is more slowly fermented with much smaller increases in breath hydrogen and serum SCFA for 6hr after consumption. The results provide no evidence that acute elevations in SCFA per se influence glucose metabolism. In addition, the results do not support the hypothesis that overweight/obesity is associated with increased colonic SCFA production. However, the study only did not have enough statistical to detect a difference in SCFA response between lean and overweight subjects, thus a substantial difference may have been missed.
CHAPTER 5:
ACUTE INCREASES IN SERUM COLONIC SHORT-CHAIN FATTY ACIDS ELICITED BY INULIN DO NOT INCREASE GLP-1 OR PYY RESPONSES BUT MAY REDUCE GHRELIN IN LEAN AND OVERWEIGHT AND OBESE HUMANS
5.1 Abstract

**Background:** colonic fermentation of dietary-fiber to short-chain-fatty-acids (SCFA) beneficially influences appetite hormone secretion in animals, but SCFA production is excessive in obese animals. This suggests there may be resistance to the effect of SCFA on appetite hormones in obesity.

**Objectives:** to determine the effects of inulin (IN) and resistant-starch (RS) on postprandial SCFA, GLP-1, PYY, and ghrelin responses in healthy overweight/obese (OWO) vs lean (LN) humans.

**Methods:** overnight fasted participants (13 OWO, 12 LN) consumed 300 mL water containing 75g glucose (Control) or 75g glucose plus 24g IN, or 28.2g RS using a randomized cross-over design. A standard lunch was served 4 h after the test drink.

**Results:** Relative to Control, IN and RS, respectively, increased PYY (p=0.003 and p=0.069) and reduced ghrelin (p=0.068 and p=0.088) areas-under-the-curve (AUC) from 0-4 h (AUC$_{0-4}$). Relative to Control, IN, but not RS, significantly increased SCFA AUC from 4-6 h (AUC$_{4-6}$). Neither IN nor RS affected GLP-1 or PYY AUC$_{4-6}$, however, IN, but not RS, reduced serum-ghrelin at 6hr (p<0.05). After IN, the changes in SCFA AUC$_{0-4}$ and AUC$_{4-6}$ were negatively related to the changes in ghrelin AUC$_{0-4}$ (p=0.057) and AUC$_{4-6}$ (p=0.017), respectively. SCFA and hormone responses did not differ significantly between LN and OWO.

**Conclusions:** acute increases in colonic SCFA do not affect GLP-1 or PYY responses in LN or OWO subjects, but may reduce ghrelin. The results do not support the hypothesis that SCFA acutely stimulate PYY and GLP-1 secretion; however, a longer adaptation to increased colonic fermentation may yield different results.
5.2 INTRODUCTION

High intakes of dietary fiber are associated with reduced weight gain (Liu et al. 2003, 920-927; Howarth, Saltzman, and Roberts 2001, 129-139). One suggested mechanism behind this effect attributes to the fermentation of the dietary fibers into short-chain fatty acids (SCFA) by the colonic bacteria. The SCFA, namely acetate, propionate and butyrate, are readily absorbed from the lumen into the colonocytes, transported through the portal vein to the liver and then to peripheral circulation (Kasubuchi et al. 2015, 2839-2849). *In vitro* and animal studies have shown that enteroendocrine L-cells, located in the highest density in the colon, are stimulated by SCFA to secrete anorectic hormones like peptide tyrosine tyrosine (PYY) and glucagon-like peptide (GLP)-1. Hormone secretions were demonstrated directly via SCFA administration, or indirectly via fermentable dietary fibers supplementation to diet (Zhou et al. 2008, E1160-E1166; Keenan et al. 2006, 1523-1534), and are postulated to be mediated by SCFA receptors (free-fatty acid receptors 2 and 3 (FFAR2/3)) that are coupled to the L-cells (Kimura et al. 2013, 1829; Tolhurst et al. 2012, 364-371). Animal studies have also suggested that the hunger hormone ghrelin decreases by colonic fermentation, though the underlying mechanism has not been clarified (Cani, Dewever, and Delzenne 2004, 521-526; Delzenne et al. 2005, S157-S161).

Some human studies have shown that fermentable dietary fibers improve PYY, GLP-1 and ghrelin secretion, however, results are inconsistent regarding which specific hormone is being affected (Tarini and Wolever 2010, 9-16; Klosterbuer, Thomas, and Slavin 2012, 11928-11934; Nilsson et al. 2013; Cani et al. 2009a, 1236-1243; Johansson et al. 2013). Furthermore, most studies have not measured SCFA responses, so whether the effects of the dietary fibers on gut hormones were mediated by SCFA, or by other effects of the fiber, is not clear. Two fermentable fibers of particular interest in this respect are resistant starch (RS) and inulin (IN). Resistant-starch is an insoluble dietary fiber while IN is a soluble dietary fiber, both found naturally in many types of plant foods, or extracted and add to food products for their additional health benefits (Nugent 2005b, 27-54). Inulin and RS yield different SCFA profiles (amounts and rates) during fermentation (Slavin et al. 2010, 177-191; Jenkins et al. 1998, 609-616), therefore, we wanted to compare the effects of different SCFA profiles on gut hormones.

The profiles of colonic SCFA depend also on the nature of the colonic microbiota (Den Besten et al. 2013, 2325-2340; Fernandes et al. 2014). Obese animals have a colonic microbiota that is
more efficient in fermenting SCFA from a given diet (Turnbaugh et al. 2006a, 1027-1031), and is characterized by increased relative abundance of the phylum Firmicutes than the phylum Bacteroidetes, compared to the lean one (Ley et al. 2005, 11070-11075; Ley et al. 2006, 1022-1023); however, this has not been observed consistently in human studies (Fernandes et al. 2014; Rahat-Rozenbloom et al. 2014, 1525-1531; Ridaura et al. 2013; Ismail et al. 2011, 501-507). If this is true, excess SCFA production may contribute extra calories to diet, and thus may enhance weight gain. On the other hand, excess SCFA production can contribute to increased satiety by stimulation gut hormone secretion, although there may be resistance to the effect of SCFA on appetite hormones in obesity.

Therefore, our objective was to compare the SCFA responses after acute RS and IN consumption, and their effects on postprandial responses of PYY, GLP-1 and ghrelin in overweight and obese (OWO) vs. lean (LN) individuals. We hypothesized that: RS and IN would increase postprandial SCFA responses differently, with a different stimulation effect on PYY, GLP-1 and ghrelin; and that OWO subjects would have higher SCFA responses than LN but be less sensitive to the effects of colonic SCFA on postprandial gut hormones.

5.3 METHODS

Subject characteristics and methods were described in detail in section 3.3.

5.3.1 Analytical methods

In addition to the analytical methods described at section 3.3, in this part of the study blood for PYY and ghrelin was drawn into tubes containing spray-coated silica and a polymer gel (BD Canada Inc., Oakville, Ont.), and 1 mL of blood for ghrelin analysis was immediately transferred and mixed into plain tubes containing 10 10µl AEBSF(4-(2-Aminoethyl)-Benzene Sulfonyle Fluoride, Hydrochloride, Amresco). Blood for GLP-1 active and total was drawn into P700 Blood Collection System for Plasma GLP-1 Preservation (BD Canada Inc., Oakville, Ont.) and was immediately centrifuged. All other tubes were left to clot for 30 min before centrifuge and the serum was removed and stored at -70°C prior to analysis. Serum for the analysis of ghrelin was additionally treated with HCl before freezing. Plasma total and active GLP-1 were measured using GLP-1 (Active 7-36) ELISA kit (ALPCO, NH, USA); Serum ghrelin was measured by The
5.3.2 Statistical analysis

Results are given as means ±SEM. For GLP-1 total and active and for PYY, net incremental areas under the curve (iAUC; subtracting area below the baseline) over 0-4 h were calculated using the trapezoid rule with the fasting concentration as the baseline. For ghrelin, iAUC from the nadir to 4h (iAUCmin4) was calculated using the minimum concentration achieved over the first 4h as the baseline. For SCFA, iAUC from the nadir to 6h (iAUCmin6) was calculated using the minimum concentration achieved over the first 4h as the baseline. For all variables, total areas under the curve (tAUC) were calculated over the 4-6h period.

To examine for the main effects of groups and treatments, and interactions between these effects, a statistical analysis was performed with STATA 13.0 (College Station, TX), using the mixed-effects (random-effects) model. Since the interaction term was not significant for any of the outcomes (possibly due to small sample size), the interaction term was dropped from the statistical model to save power for the main effects.

Independent t-tests were performed to analyse differences in baseline data between the groups using IBM SPSS Statistics version 22 (SPSS Inc., Chicago, IL, USA). Differences were taken to be statistically significant if 2-tailed $p < 0.05$.

5.4 RESULTS

5.4.1 Baseline characteristics

The baseline characteristics of the subjects are given in section 4.4.1.

5.4.2 Serum SCFA

The postprandial serum SCFA results are given in section 4.4.2. Briefly, serum acetate, propionate and butyrate concentrations began to increase 3 to 4h after IN compared to GLU and were significantly higher than GLU from 4 to 6h, by related samples Wilcoxon signed rank test (uncorrected for multiple comparisons;
Figure 5.1). Thus, both the incremental AUC and the total AUC of all 3 SCFA after IN were significantly greater than those after GLU (Table 4.4). By contrast, it took 5-6h for mean serum SCFA concentrations after RS to begin to exceed those after GLU; by 6h after RS the concentrations of all 3 SCFA were significantly greater those after GLU by related samples Wilcoxon signed rank test (uncorrected for multiple comparisons;
Figure 5.1), but the differences were small and there were no significant differences between RS and GLU in incremental or total AUC (Table 4.4). There were no significant differences in serum SCFA responses between LN and OWO subjects (Table 4.6).
Figure 5.1: Serum short-chain fatty acid responses elicited by inulin and resistant starch

Values are means±95% confidence intervals of the differences from control for n=25 subjects. * Significant difference between Inulin and Glucose by related samples Wilcoxon signed rank test (p<0.05). † Significant difference between resistant starch and glucose by related samples Wilcoxon signed rank test (p<0.05)
5.4.3 Breath Gases

Breath gas results are given in section 4.4.3.

5.4.4 Serum GLP-1 Total and Active

There were no differences among treatments in serum GLP-1 total and active responses (Figure 5.2, Table 5.1). Relative to the GLU, changes in serum GLP-1 did not correlate with changes in serum SCFA from 0-4hr or from 4-6hr after either IN or RS (Figure 5.4, Figure 5.5). Serum GLP-1 active response between 0-2h was significantly greater in the OWO group compared to the LN group (Table 5.2, Figure 5.3).

5.4.5 Serum PYY

Incremental AUC during the 0-4h period for serum PYY response was greater after IN than GLU (p=0.003) and after RS compared to GLU (p=0.069)(Figure 5.2, Table 5.1). However, after the standard lunch, there were no differences in tAUC 4-6h between IN and GLU and between RS and GLU (Figure 5.2, Table 5.1). Relative to GLU, changes in PYY AUC after IN and RS were not related to changes in SCFA either from 0-4h or from 4-6h (Figure 5.4, Figure 5.5), except for the difference in PYY tAUC from 4-6h between GLU and IN which was negatively related to the difference in SCFA tAUC (p=0.029); i.e. PYY was lower in those subjects with higher SCFA responses. There were no significant differences in PYY response between the LN and OWO groups (Table 5.2, Figure 5.3).

5.4.6 Serum Ghrelin

Incremental serum ghrelin responses between the nadir and 4h, relative to GLU, were non-significantly lower after IN (p=0.068) and RS (p=0.088) (Figure 5.2, Table 5.1). Over the 4-6h period the total AUC for ghrelin did not differ significantly between IN and GLU nor between RS and GLU (Figure 5.2, Table 5.1). However, by Wilcoxon signed rank test, serum ghrelin at 6h was significantly lower after IN than GLU. In addition, the differences in ghrelin AUC between GLU and IN were negatively related to the differences in SCFA both between 0-4h (p=0.057) and 4-6h (Figure 5.4, Figure 5.5). Ghrelin responses in LN did not differ significantly from those in OWO (Table 5.2, Figure 5.3).
Table 5.1: Serum GLP1 (Active and Total), PYY and Ghrelin responses elicited by the test meals.

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Variable</th>
<th>Glucose</th>
<th>Inulin</th>
<th>P value a</th>
<th>Resistant starch</th>
<th>P value b</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLP-1 active (pmol×h/L)</td>
<td>iAUC04</td>
<td>3.07 ± 0.68</td>
<td>2.47 ± 1.17</td>
<td>0.574</td>
<td>3.82 ± 0.72</td>
<td>0.297</td>
</tr>
<tr>
<td></td>
<td>tAUC46</td>
<td>11.3 ± 3.07</td>
<td>10.8 ± 3.05</td>
<td>0.102</td>
<td>10.7 ± 2.95</td>
<td>0.085</td>
</tr>
<tr>
<td>GLP-1 total (pmol×h/L)</td>
<td>iAUC04</td>
<td>6.45 ± 1.3</td>
<td>6.61 ± 1.4</td>
<td>0.893</td>
<td>7.82 ± 1.5</td>
<td>0.286</td>
</tr>
<tr>
<td></td>
<td>tAUC46</td>
<td>16.3 ± 3.86</td>
<td>15.6 ± 3.86</td>
<td>0.099</td>
<td>16.2 ± 3.9</td>
<td>0.793</td>
</tr>
<tr>
<td>PYY (pmol×h/L)</td>
<td>iAUC04</td>
<td>1.06 ± 2.00</td>
<td>5.69 ± 1.96</td>
<td>0.003</td>
<td>3.94 ± 1.79</td>
<td>0.069</td>
</tr>
<tr>
<td></td>
<td>tAUC46</td>
<td>42.4 ± 7.5</td>
<td>42.1 ± 7.5</td>
<td>0.837</td>
<td>43.3 ± 7.6</td>
<td>0.579</td>
</tr>
<tr>
<td>Ghrelin (pmol×h/L)</td>
<td>iAUCmin4</td>
<td>453 ± 84</td>
<td>379 ± 58</td>
<td>0.068</td>
<td>383 ± 64</td>
<td>0.088</td>
</tr>
<tr>
<td></td>
<td>tAUC46</td>
<td>1062 ± 178</td>
<td>984 ± 164</td>
<td>0.099</td>
<td>1009 ± 155</td>
<td>0.294</td>
</tr>
</tbody>
</table>

Values are mean±SEM for n=25. GLU, glucose drink; iAUC, net incremental area under the curve; tAUC, total area under the curve; iAUCmin4, from nadir to 4 hrs. a, P value for the difference between glucose treatment and inulin treatment; b, P value for the difference between glucose treatment and resistant starch treatment.
Figure 5.2: Serum gut hormone responses elicited by the test meals

Values are means±SEM for n=25 subjects. For GLP-1, the upper curves are total GLP-1 and the bottom curves are active GLP-1. * Mean for Inulin significantly different from Glucose by related samples Wilcoxon signed rank test (p<0.05). † Mean for RS significantly different from Glucose by related samples Wilcoxon signed rank test (p<0.05).
Table 5.2: Mean serum GLP-1 active and total, PYY and Ghrelin responses (means for the 3 test meals) in lean vs. overweight and obese participants

<table>
<thead>
<tr>
<th>Hormone (pmol×h/L)</th>
<th>Variable</th>
<th>Lean (n=12)</th>
<th>Overweight and Obese (n=13)</th>
<th>P value a</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLP-1 Active</td>
<td>iAUC04</td>
<td>1.8 ± 1.1</td>
<td>4.3 ± 0.9</td>
<td>0.053</td>
</tr>
<tr>
<td></td>
<td>tAUC46</td>
<td>15.0 ± 4.5</td>
<td>7.5 ± 4.0</td>
<td>0.199</td>
</tr>
<tr>
<td>GLP-1 Total</td>
<td>iAUC04</td>
<td>5.5 ± 1.6</td>
<td>8.2 ± 1.8</td>
<td>0.215</td>
</tr>
<tr>
<td></td>
<td>tAUC46</td>
<td>1 ± 0.7</td>
<td>0.8 ± 0.5</td>
<td>0.836</td>
</tr>
<tr>
<td>PYY</td>
<td>iAUC04</td>
<td>0.11 ± 0.10</td>
<td>0.2 ± 0.1</td>
<td>0.595</td>
</tr>
<tr>
<td></td>
<td>tAUC46</td>
<td>2.1 ± 0.6</td>
<td>1.4 ± 0.2</td>
<td>0.231</td>
</tr>
<tr>
<td>Ghrelin</td>
<td>iAUCmin4</td>
<td>483 ± 122</td>
<td>333 ± 52</td>
<td>0.226</td>
</tr>
<tr>
<td></td>
<td>tAUC46</td>
<td>1228 ± 300</td>
<td>825 ± 142</td>
<td>0.206</td>
</tr>
</tbody>
</table>

Values are mean±SEM; iAUC, net incremental area under the curve; tAUC, total area under the curve; a P value for the difference between the LN group and OWO group.
Figure 5.3: Serum GLP1 (Active and Total), PYY and Ghrelin responses elicited by the test meals in Lean and overweight and obese groups

Values are means±SEM for n=12 lean and n=13 overweight/obese subjects. For GLP-1, the upper curves are total GLP-1 and the bottom curves are active GLP-1.
Values for hormones are differences in incremental AUC from 0-4h between Glucose and Inulin (left) and Glucose and Resistant Starch (right). Values for SCFA are differences in total AUC from 0-4h between Glucose and Inulin (left) and Glucose and Resistant Starch (right). SCFA tAUCs are the sum of the tAUCs for acetate, propionate and butyrate. Closed circles are lean subjects and open circles are overweight/obese subjects. Spearman’s correlation coefficients and approximate p-values are shown in each panel.
Figure 5.5: Relationships between changes in gut hormone responses and changes in SCFA responses between 4 and 6 hours

Values for hormones are differences in total AUC from 4-6h between Glucose and Inulin (left) and Glucose and Resistant Starch (right). Values for SCFA are differences in total AUC from 4-6h between Glucose and Inulin (left) and Glucose and Resistant Starch (right). SCFA tAUCs are the sum of the tAUCs for acetate, propionate and butyrate. Closed circles are lean subjects and open circles are overweight/obese subjects. Spearman’s correlation coefficients and approximate p-values are shown in each panel.
5.5 DISCUSSION

Animal and cell-cultures studies suggest that colonic SCFA increase PYY and GLP-1 secretion and, hence, may promote weight loss (Zhou et al. 2008, E1160-E1166; Keenan et al. 2006, 1523-1534). However, our results do not support this mechanism in humans since GLP-1 responses after IN and RS were not different from the GLU control throughout the 6h study period, despite evidence of large increases in colonic fermentation. In addition, the early increase in PYY after IN (and to a lesser extent after RS) was not associated with increased colonic SCFA, whereas after lunch, when serum SCFA concentrations after IN were highest compared to the GLU, mean PYY response was no different from GLU; indeed, if anything, the change in PYY response after IN was negatively related to the change in colonic SCFA.

We chose two types of fermentable dietary fibers in order to examine how two different fermentation profiles affect appetite hormones responses in LN and OWO subjects. IN was chosen because it is known to be rapidly fermented in humans, eliciting high SCFA responses within a few hours of consumption (Boets et al. 2015, 8916-8929; Tarini and Wolever 2010, 9-16) and, hence, considered suitable to test the hypothesis that OWO subjects produce more colonic SCFA than LN and that colonic SCFA increase GLP-1 and PYY secretion. RS was chosen because animal studies have demonstrated its beneficial effects on gut hormones via SCFA (Zhou et al. 2008, E1160-E1166; Belobrajdic et al. 2012; Zhou et al. 2015, 1000-1003), but its acute effects have not been documented in humans, though few longer term studies have shown inconsistent results as discussed below (Robertson et al. 2005, 559-567; Nilsson, Johansson-Boll, and Björck 2015, 899-907). While IN elicited robust increases in colonic fermentation, as evidenced by increased breath H₂ and serum SCFA, RS elicited only small and marginally significant increases in markers of colonic fermentation over the 6hr course of this study. Thus, a longer study period may be needed to demonstrate an effect of RS on colonic fermentation and gut hormone responses in humans. The reduced response of breath H₂ and serum SCFA after RS, as compared to IN, could be due to a slower intestinal transit time, a slower fermentation, and/or a more distal fermentation site (James et al. 2003, 291-296).

Although we were unable to demonstrate a statistically significant effect of IN on GLP-1 and ghrelin, our results are consistent with those of Tarini and Wolever (Tarini and Wolever 2010, 9-16) who showed that, compared to consuming 56g high-fructose corn-syrup (HFCS) alone, 56g
HFCS plus 24g IN after an overnight fast significantly increased serum GLP-1 at 30min, and decreased serum ghrelin concentrations 1-2h after a subsequent lunch. In our results, mean serum GLP-1 30min after IN was higher than after GLU, but this was not statistically significant and, although IN reduced ghrelin tAUC from 4-6h insignificantly (P = 0.099), serum ghrelin 6hr after IN was significantly less than that after GLU, and the changes in serum ghrelin after IN were negatively related to the changes in SCFA. This, at least, is consistent with the suggestion that colonic fermentation reduces ghrelin secretion (Cani, Dewever, and Delzenne 2004, 521-526), though the mechanism is yet to be explored.

The inability of IN to increase GLP-1 or PYY in the face of large increases in serum SCFA after lunch seems inconsistent with studies showing that acute elevation of circulating SCFA by administration of rectal or intravenous infusions of acetate resulted in increased PYY and GLP-1 responses, but had no effect on ghrelin (Freeland and Wolever 2010, 460-466). However, the changes in serum acetate after rectal and IV infusion respectively were 4-times and 15-times higher than the change elicited by oral IN in the present study. Postprandial GLP-1 and PYY responses were also raised by acute elevation of circulating propionate (using a novel dietary molecule that delivers propionate, chemically bound to 10g inulin, into the colon), followed by a reduced food intake at a subsequent ad libitum meal (Chambers et al. 2015, 1744-1754). In the latter study, the change in serum propionate was 12-times higher than the change elicited in the present study. Surprisingly, the effects on PYY and GLP-1 were not seen after consuming this dietary molecule for 24 wks, despite a reduced subjective postprandial appetite and a reduced weight gain at the end of the study. The author suggested that in the long run, propionate may mediate satiety by other mechanisms (Chambers et al. 2015, 1744-1754).

Studies examining the long term effects of IN supplementation have generally shown positive effects on at least one of the appetite hormones (Verhoef, Meyer, and Westerterp 2011, 1757-1762; Parnell and Reimer 2009, 1751-1759; Pedersen et al. 2013, 44-53), however, few studies assessed if these effects were SCFA-mediated. Cani et al. (Cani et al. 2009b, 1236-1243) showed that after a 2-wk supplementation of 16g/d mixture of Inulin and oligofructose, a standardized breakfast increased breath hydrogen AUC between 0-3hrs and increased GLP-1 and PYY concentrations at 10 min, but not at other times compared to control. This suggests that a chronic increase in colonic fermentation may upregulate GLP-1 and PYY secretion. Similarly, a
supplementation of 30g/d oligofructose for 6wks compared to cellulose (a poorly-fermentable fiber) increased PYY (0-7hr), breath hydrogen (0-7hr), and acetate concentrations (at 6 and 7hr) following a standardized meal, but there were no effects on postprandial GLP-1 during the 7hrs, and no effect on energy intakes in a following *ad libitum* meal, or on body weight at the end of the study (Daud et al. 2014, 1430-1438). However, the chronic effects of non-soluble fiber may take longer than 4-6 weeks to occur; for example, we showed that 24g wheat fiber daily for 1 year increased serum SCFA and GLP-1 responses, but that these effects took 9 to 12mo to occur (Freeland, Wilson, and Wolever 2010, 82-90). All in all, though most evidence regarding the effect of IN on appetite and appetite hormones show positive results, there is inconsistency regarding which specific hormone is being influenced, and paucity of data exploring if SCFA modulate these responses as seen in animal studies.

The early increase in PYY responses after IN seems most likely related to the significantly increased early glycemic response as reported in Chapter 4, however the reason for this is not clear. IN also increased SCFA and tended to reduce ghrelin between 0-4h (p=0.068) with the reduction in ghrelin being negatively associated with the increase in serum SCFA (p=0.057). On the other hand, RS non-significantly increased PYY (p=0.069) and reduced ghrelin (p=0.088) between 0-4h, in the absence of any change in SCFA. We believe the latter effects are due to the presence of a larger than expected amount of available carbohydrate in the RS ingredient used, since the reduction in serum FFA after RS was significantly prolonged compared to that after GLU (Figure 4.5, Table 4.5) and both PYY and ghrelin have been shown to respond proportionally to the caloric load and macronutrient content (Field, Chaudhri, and Bloom 2010, 444-453; Müller et al. 2015, 437-460). Previous acute studies have demonstrated either no effect of RS on postprandial gut hormones (Gentile et al. 2015), or a reduced postprandial GLP-1 response following breakfast meal (Raben et al. 1994, 544-551), but not lunch (Bodinham et al. 2013, 1429-1433). Adding 60g RS to diet for 24h increased SCFA but did not change postprandial GLP-1 responses to a standard test meal (Robertson et al. 2003, 659-665). 3-day consumption of barley kernel bread, rich in RS, increased fasting serum acetate, propionate and GLP-1, but not fasting serum PYY and ghrelin. However, following a standard breakfast, postprandial PYY and breath hydrogen increased in the barley group, but had no effect on GLP-1 and ghrelin responses (Nilsson, Johansson-Boll, and Björck 2015, 899-907). Surprisingly, 4 wk
consumption of 30g/d RS did not change fasting serum SCFA and GLP-1 concentrations, but increased fasting serum ghrelin. Following a meal tolerance test, RS increased postprandial serum acetate and propionate from 0 to 5 hrs, but had no effect on GLP-1 and ghrelin responses (Robertson et al. 2005, 559-567). In summary, though animal research has shown consistent increases in gut hormones following RS fermentation (Zhou et al. 2008, E1160-E1166; Zhou et al. 2015, 1000-1003; Vidrine et al. 2014a, 344-348), human data are inconsistent.

Some of the inconsistencies in human studies could be explained by the large variations in study designs; such as the different fiber types used (i.e., oligofructose vs. inulin) (Delzenne et al. 2005, S157-S161), the different doses, the different time points that are being measured and the different duration of the studies. The latter could be especially important because IN (and potentially RS) is prebiotics that promote gradual changes in gut microbiota composition over time and thus alter SCFA production (Salazar et al. 2015, 501-507). SCFA were shown to increase the number of L-cells in the intestinal epithelium, and as a result increase GLP-1 and PYY secretion (Petersen et al. 2014, 410-420); but both of these are time-related effects, i.e., no alteration in microbiota or L-cells would be seen in acute studies, as opposed to longer term studies. In addition, the within- and between-individual variability in gut hormone concentrations may mask the effect of SCFA on gut hormones as seen in animal studies. Therefore, a larger sample size may be needed to explore this question.

We hypothesized that OWO individuals would have higher SCFA responses than LN but would be less sensitive to the effects of colonic SCFA on gut hormones. However, this was not confirmed in our study, as there were no differences between the groups in serum SCFA and hormone responses during fermentation. To our knowledge, previous studies have not compared circulating SCFA after fermentable fibers consumption between LN and OWO individuals; however, in non-obese individuals the BMI did correlate with serum SCFA after acute consumption of 15g inulin (Boets et al. 2015, 8916-8929). Generally, SCFA production, in terms of rate, type and amount, is a complex interplay between diet, microbial profile and gut transit time (Den Besten et al. 2013, 2325-2340). Thus, the similar relative abundances of Firmicutes (F) and Bacteroidetes (B) and F:B ratio, and similar dietary intakes in our LN and OWO groups, together with the observation that no differences in fecal SCFA concentrations were measured, strengthen our assumption that SCFA production did not differ in our LN and OWO groups.
These results are inconsistent with mice studies, although human studies done so far have not yet reached to a firm conclusion, as reviewed elsewhere (Conterno et al. 2011, 241-260; Rosenbaum, Knight, and Leibel 2015, 493-501). Interestingly, recent in vitro fermentation studies using fecal inocula from lean vs. obese donors with different dietary fiber has not confirmed the mice studies, showing that 4 different types of RS produced similar SCFA concentrations in LN and OWO inocula (Li et al. 2015, 1997-2004), while other types of fibers showed either increased or decreased SCFA production in OWO compared to LN (Aguirre et al. 2014). Therefore, further research is needed to fully elucidate whether the SCFA production and metabolism differ in lean and obese humans.

It is important to note that circulating SCFA may not adequately reflect colonic SCFA production, as they are the net result of endogenous and exogenous production, absorption, hepatic extraction of the colonic SCFA. However, the comparison of the IN and the RS treatments to the GLU treatment, allowed us to control, at least in part, for the endogenous SCFA production.

We conclude that acute increases in colonic fermentation do not affect GLP-1 or PYY responses in LN or OWO subjects, but increases in serum SCFA may reduce ghrelin. The results do not support the hypothesis that colonic SCFA acutely stimulate PYY and GLP-1 secretion in humans, but a longer adaptation to increased colonic fermentation may yield different results. The similarity of SCFA responses in LN and OWO subjects does not support the hypothesis that colonic SCFA production is increased in obesity; however, increased SCFA production may not occur in all overweight/obese subjects and serum SCFA responses may not necessarily reflect colonic SCFA production.
CHAPTER 6:
GENERAL DISCUSSION
6.1 Overall findings

The overall objectives of this work were to compare fecal SCFA concentrations, rectal SCFA absorptions, dietary intakes and fecal microbial profiles in healthy OWO vs LN participants, and to compare the effects of IN and RS on postprandial SCFA, appetite hormones and second-meal glycemic responses in healthy OWO vs LN participants. The following paragraphs will discuss the overall findings and conclusions, in relation to these objectives.

Objective 1 (chapter 3): To compare fecal SCFA concentrations, rectal SCFA absorption, dietary intake and fecal microbial profile in healthy overweight/obese vs lean participants

The first study showed that the OWO participants had an increased fecal SCFA compared to the LN humans (age adjusted), with increased Firmicutes to Bacteroidetes ratio. Using a previously validated dialysis rectal bag technique, we were the first to demonstrate that LN and OWO individuals do not differ in their capacity to absorb SCFA from a given solution, suggesting that the increased fecal SCFA concentrations in OWO than LN individuals is not likely a result of reduced colonic absorption. The findings that our groups did not differ in their dietary intakes, stool consistency (a marker for gut transit time), ethnicity and habitual physical activity (effect gut transit time), further reinforced our conclusion, that the higher fecal SCFA in the OWO individuals is not due to differences in SCFA absorption or diet, but possibly an increased production. These results were consistent with our hypothesis that OWO humans produce more colonic SCFA than LN humans, possibly due to differences in colonic microbiota.

Objective 2 (chapter 4): To compare the effects of inulin and resistant-starch on postprandial SCFA and second-meal glycemic response in healthy overweight/obese vs lean participants

In the second study, overnight fasted participants consumed water containing 75g glucose (Control), or 75g glucose plus IN or RS on 3 separate days. A standard lunch was served 4h after the test-drink. Surprisingly, relative to Control, IN significantly increased serum SCFA, but had no effect on serum FFA or second-meal glucose and insulin responses. Compared to control, RS increased serum SCFA only at ~5.5 hrs; hence, their effect could not be tested in our study. However, due to this late fermentation, we could conclude that the decreases in postprandial FFA, glucose C-peptide and insulin responses after RS compared to IN were not a result of
colonic fermentation, as frequently argued (Bindels, Walter, and Ramer-Tait 2015, 559-565), but due to a different property of RS that effected insulin sensitivity. In order to further analyze the effect of RS fermentation on glycemia, SCFA should be measured for longer duration (i.e., 10 hrs study instead of 6 hrs).

To our knowledge, this is the first study to compare serum SCFA between LN and OWO individuals. Our LN and OWO participants did not differ in serum SCFA concentrations either at fasting or postprandially after IN or RS. This does not necessarily rule out increased SCFA production in the OWO group, since it is possible that the excess SCFA produced were taken up by the colonocytes and/or liver. However, none of the other variables tested in this study indicated that the OWO participants were more efficient at producing SCFA than the LN participants; they had no difference in fecal Firmicutes or Bacteroidetes, and no differences in fecal SCFA (propionate was even decreased in OWO after age adjustment), habitual physical activity, ethnicity and dietary intakes; all this strengthens our conclusion that in this cohort, the OWO participants were not more efficient in fermenting SCFA compared to the LN participants, either from their habitual diet (represented by similar fecal and serum SCFA concentrations at fasting), or from a given amount of fibers (represented by their postprandial SCFA responses after IN or RS). Thus, these findings were not consistent with our hypothesis that RS and IN will elicit higher postprandial SCFA responses and have less effect in reducing serum FFA and second-meal glucose and insulin responses in overweight/obese compared to lean humans. The discrepancy between the first and the second study in fecal SCFA concentrations that were increased in the OWO group in the first study but not in the second study will be discussed below (section 6.2).

**Objective 3 (chapter 5):** To compare the effects of inulin and resistant-starch on postprandial SCFA GLP-1, PYY, and ghrelin response in healthy overweight/obese vs lean participants

In addition to the above, the second study also showed that relative to control, IN significantly increased PYY right after breakfast, and decreased ghrelin at 6hrs, but had no effect on serum GLP-1 responses. Importantly, the effect on PYY was too early to occur due to colonic fermentation, but the effect on ghrelin was possibly a result of the increased SCFA concentration. By contrast, compared to control, RS had no effects on appetite hormones.
throughout the study. The OWO and LN participants did not differ in appetite hormones responses at the time when fermentation peaked. Thus, these findings were not consistent with our hypothesis that RS and IN will elicit higher postprandial SCFA responses and have less effect on appetite hormones responses in overweight/obese compared to lean humans.

6.2 Other Considerations

As detailed in previous chapters, there is a strong evidence from *in vitro* and in vivo animal studies that colonic fermentation is increased in OWO than LN animals, and that administration of SCFA or dietary fibers increase GLP-1 and PYY, and decrease ghrelin and glycemic responses. However, evidence from human studies is controversial. Moreover, evidence regarding whether OWO has increased production of SCFA than LN humans, and whether this is associated with increased Firmicutes and decreased Bacteroidetes abundance is also controversial. Hence, in the following section, I will discuss some of the major factors that could account for the inconsistencies.

**What accounts for the discrepancies in SCFA concentrations, microbial profiles and in the effect of SCFA on glycemia and gut hormone responses between studies in this field?**

It is possible that there is no consistent increase in SCFA production in all types of overweight and obese people, as the etiology for obesity may vary between individuals, for instance, over eating vs. genetic background (Bouret, Levin, and Ozanne 2015, 47-82). Each type of obesity may alter SCFA production and/or microbial profiles differently, if at all.

It is possible that an increase in SCFA production in OWO than LN individuals is not consistent with all types of fibers. This was demonstrated in *in vitro* models (Aguirre et al. 2014), when lactulose fermentation was increases after incubating with OWO compared to LN microbiota, as opposed to pectin that was increased after incubating with LN than OWO microbiota. While fermentation of resistant starches, resulted in similar SCFA concentrations after incubating with either LN or OWO microbiota (Li et al. 2015, 1997-2004).

Inconsistency in SCFA production and therefore their effects on glycemia and gut hormones may also be due to the differences in fiber types between studies (i.e., oligofructose vs. inulin, or RS
type 2 vs. type 3) and in doses tested. Any change in type or dose may change the end point results. Thus, comparing studies with different protocols may not be correct in this field.

The specific time point, at which metabolites (i.e., SCFA, glucose, FFA or gut hormones) have been measured, may increase the inconsistencies between studies; for instance, we measure a peak in GLP-1 at 30 min, however, this important peak could have been missed in studies that measured GLP-1 only after 60 min.

Unmeasured confounders may also obscure true underlying differences in SCFA concentrations and thus cause inconsistencies between studies. For example, Schwiertz et al. observed an increased fecal SCFA in OWO than in LN participants. However, potential influencing factors, such as dietary intakes, ethnicity and overall fitness (Schwiertz et al. 2010a, 190-195) have not been taken into account. These factors, especially the dietary regimens of participants, have major effects on microbial profiles and SCFA concentration, and thus it could be that is possible that the increased SCFA in the OWO group is a matter of different diet, and not increased production. Another example for a confounder that has generally been neglected in most studies so far is gut transit time (as described in section 2.7.2). Gut transit time can be estimated by fecal consistency as a surrogate maker (Saad et al. 2010, 403-411). Recently, fecal consistency has also been demonstrated to have a profound association with colonic microbiota composition (Vandeputte et al. 2016, 57-62), which emphasizes the crucial importance of stool consistency assessment in this field.

Recently, additional confounders have been found to also affect the interplay between obesity, SCFA and microbiota, like genetic and environmental components (Ussar et al. 2015, 516-530; Goodrich et al. 2014, 789-799). The impact and the variety of these factors present a great challenge to conducting studies in this field, particularly in humans.

The presence of Archaea as part of the colonic milieu can also cause for inconsistencies between studies. Archaea is a domain of anaerobic microorganisms that represent 0–12% of the total colonic anaerobic microbiota, while the most abundant Archaea in humans is the methanogenic Methanobrevibacter smithii. It uses H₂ to reduce CO₂ to methane, therefore, it coexist in a syntrophic relationship with hydrogen-producing bacteria (Goodrich et al. 2014, 789-799).
Consequently, Archaea may modify the metabolic pathways of fermentative bacteria, and alter the SCFA profiles (Gaci et al. 2014, 16062-16078). A recent study from our group (that included our first study’s cohort) has shown a negative correlation between breath methane and fecal SCFA concentrations in healthy participants that harbored Archaea in their colon (Fernandes et al. 2013, 1269-75). In agreement, Abell et al. found a negative correlation between mean fecal butyrate and methanogen abundance (Abell, Conlon, and Mcorist 2006, 154-160). Though, in a group of individuals with impaired glucose tolerance, the methane producers (MP) had increases postprandial serum acetate compared to methane non-producers (MNP) (Fernandes, Rao, and Wolever 1999, 361-372). Thus, there is accumulating evidence in humans that Archaea may influence colonic fermentation and microbial profiles by altering SCFA metabolism and fecal SCFA profiles, however, not all previous studies have accounted for methane producing status (Teixeira et al. 2013, 914-919; Murugesan et al. 2015, 1337-1346), thus adding inconsistencies in literature.

In our work, there were no differences in breath methane between the LN and OWO groups. However, interestingly enough, in a post hoc analysis of our second study cohort (in the study there were too few methane producers for meaningful analysis), grouping the participants into MP (n=8) and MNP (n=17), based on their presence or absence of breath methane, showed that, consistent with literature (Fernandes et al. 2013, 1269-75; Abell, Conlon, and Mcorist 2006, 154-160), MP had less fecal butyrate (p=0.013) and propionate (p=0.057) concentrations than MNP, and no differences in BMI between the groups (Table 6.1).
Table 6.1: Mean fecal and serum fasting and postprandial SCFA, glucose, insulin, C-peptide and ghrelin in Methane producers (MP) and Methane non Producers (MNP)

<table>
<thead>
<tr>
<th></th>
<th>MNP (n=17)</th>
<th>MP (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fecal Acetate</strong></td>
<td>50.2 ± 19.1</td>
<td>44.2 ± 13.6</td>
</tr>
<tr>
<td><strong>Fecal Propionate</strong></td>
<td>17.2 ± 1.4</td>
<td>12.4 ± 1.5</td>
</tr>
<tr>
<td><strong>Fecal butyrate</strong></td>
<td>16.8 ± 1.5†</td>
<td>11.8 ± 1.1</td>
</tr>
</tbody>
</table>

**Serum**

<table>
<thead>
<tr>
<th></th>
<th>MNP (n=17)</th>
<th>MP (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose iAUC02</td>
<td>4.6 ± 0.5† †</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>Insulin iAUC02</td>
<td>135.5 ± 25.3 † †</td>
<td>58.7 ± 10.5</td>
</tr>
<tr>
<td>CPEP iAUC02</td>
<td>3049.8 ± 300.6 † †</td>
<td>1832.6 ± 154.7</td>
</tr>
<tr>
<td>Ghrelin tAUC04</td>
<td>1505.1 ± 249.1 † †</td>
<td>3554.1 ± 702.5</td>
</tr>
<tr>
<td>Ghrelin tAUC46</td>
<td>759.5 ± 137.3 † †</td>
<td>1705.3 ± 400.9</td>
</tr>
<tr>
<td>Fasting Ghrelin</td>
<td>405.7 ± 58.0 † †</td>
<td>851.1 ± 149.1</td>
</tr>
<tr>
<td>Fasting acetate</td>
<td>29.4 ± 3.7 †</td>
<td>51.5 ± 8.8</td>
</tr>
<tr>
<td>Fasting propionate</td>
<td>1.1 ± 0.1 †</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>Fasting butyrate</td>
<td>0.37 ± 0.05</td>
<td>0.45 ± 0.06</td>
</tr>
<tr>
<td>Fasting ALT u/L</td>
<td>26.3 ± 3.9 †</td>
<td>16.8 ± 1.3</td>
</tr>
<tr>
<td>Fasting Triglycerides</td>
<td>1.1 ± 0.1 †</td>
<td>0.59 ± 0.06</td>
</tr>
</tbody>
</table>

Values are mean±SEM; iAUC, net incremental area under the curve; tAUC, total area under the curve; †, Significance of difference between MP and MNP individuals, p<0.05; ††, Significance of difference between MP and MNP individuals, p<0.01; Mann Whitney test; ††

Moreover, within the MNP group, significant negative correlations (age-adjusted) were seen between total fiber intake (g/d) and fecal iso-butyrate and iso-valerate, while within the MP group, significant negative correlations (age-adjusted) were seen between total fiber intake and fecal acetate, propionate, butyrate and valerate (Table 6.2), which is consistent with literature (Fernandes et al. 2013, 1269-75). Fascinatingly, glucose, insulin and C-peptide responses at 0-2
hrs were decreased in MP than in MNP, and ghrelin was increased at fasting and during the 6 hrs in MP compared to MNP. Altered glucose responses have been recently associated with methane status in humans with T1DM (Cesario et al. 2014, 201-207) and in pre-diabetic humans (Mathur et al. 2016). But ghrelin was never studied before.

Table 6.2: Age adjusted correlations between fiber intakes and fecal SCFA in methane producers (MP) and non producers (MNP)

<table>
<thead>
<tr>
<th>Fiber Intake</th>
<th>Acetate</th>
<th>Propionate</th>
<th>Butyrate</th>
<th>Iso-Butyrate</th>
<th>Iso-Valerate</th>
<th>Valerate</th>
<th>Total SCFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>MNP (n=17)</td>
<td>r</td>
<td>-0.089</td>
<td>0.219</td>
<td>-0.516</td>
<td>-0.532</td>
<td>-0.116</td>
<td>0.094</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.505</td>
<td>0.753</td>
<td>0.434</td>
<td>0.049</td>
<td>0.041</td>
<td>0.681</td>
</tr>
<tr>
<td></td>
<td>df</td>
<td>13</td>
<td>13</td>
<td>13</td>
<td>13</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>MP (n=8)</td>
<td>r</td>
<td>-0.908</td>
<td>-0.796</td>
<td>-0.92</td>
<td>-0.482</td>
<td>-0.343</td>
<td>-0.871</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.005</td>
<td>0.032</td>
<td>0.003</td>
<td>0.273</td>
<td>0.451</td>
<td>0.011</td>
</tr>
<tr>
<td></td>
<td>df</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

Abbreviations: df, Degrees of Freedom; p, P-value; r, Correlation Coefficient (Spearman’s Rank Test); SCFA, Short Chain Fatty Acids.

These preliminary findings raise intriguing questions about the effect of methane on glycemia, BMI, SCFA, and interactions with diet and microbiota. Therefore, the role of methane and Archaea should be further investigated in future well powered studies.

Durations of studies also appear to have a dominant effect on results. This could be due to the gradual changes in gut microbiota compositions and in SCFA productions that are promoted over time by prebiotics supplementations (Salazar et al. 2015, 501-507). Durations could also change the results due to the gradual increase in the numbers of the colonic L-cells (Petersen et al. 2014, 410-420; Kuhre, Holst, and Kappe 2016, 79-91) SCFA receptors (Kaji et al. 2011, 27-38), and SCFA transporters (Haenen et al. 2013a, 274-283), as seen in *in vitro* and *in vivo* animal studies following SCFA administration or long term fiber consumption. If these adaptations occur also in humans, then the duration of studies could contribute to more inconsistencies between studies that differ only in their durations. For instance: acute consumption of 24g inulin significantly
increased postprandial SCFA responses (Tarini and Wolever 2010, 9-16), while a similar inulin dose following a 6-day supplementation did not increase postprandial SCFA at all (Darzi et al. 2016, 142-149). Likewise, a similar dose of propionate that was delivered into the colon by a novel supplement, significantly increased GLP-1 and PYY after acute consumption, but had no effect after 24 wks supplementation (Chambers et al. 2015, 1744-1754).

Inconsistencies in microbial analyses could be a result of technical differences, such as characterizing the microbial profiles by different platforms (i.e. Illumina, Roche-454, Ion Torrent) and/or by using different materials and methods (i.e., choosing different primers or regions of 16S rRNA gene) that appear to have differential effects across studies (Ley 2010, 5-11; Kim and Yu 2014, 355-365). Furthermore, the statistical approaches that are used for data analysis vary, and do not always translate across experiments (Fernandes et al. 2014).

6.3 Future research and implications

The inconsistencies between human studies highlight how each component in this complex environment can change the outcomes to unexpected directions, and how our current understanding of the interrelationship between SCFA, microbiota, and host physiology in humans is still in its infancy. However, in light of the emerging evidence regarding the health benefits of prebiotics to human obesity and T2DM (Kellow, Coughlan, and Reid 2014, 1147-1161; Druart et al. 2014, 624S-633S; Dewulf et al. 2013, 1112-1121), a clearer picture of the physiology of human SCFA metabolism and its interactions with glucose and weight control is of pivotal importance. As this knowledge will help designing novel treatments and better nutritional strategies to prevent or decrease obesity and T2DM.

More specifically, the metabolic benefits of resistant starch and inulin in improving insulin sensitivity and weight management for humans have been well established (Liber and Szajewska 2013, 42-54; Bindels, Walter, and Ramer-Tait 2015, 559-565), however, whether the mechanisms underpinning their effects on obesity and T2DM are related to colonic SCFA, remain to be further elucidated.

In order to increase our knowledge in this field, integrating a few types of research methods are required: 1) In vitro models – to further elucidate the mechanisms of actions in the molecular level of SCFA on human colonic-cells, gene expression of hormones, receptors, etc. 2) Animal
models – to further investigate metabolic pathways that require relatively invasive procedures, such as examining the effects of different fibers on the colonic SCFA in the different parts of the colon, liver and blood. 3) Large-scale human studies - that may provide a clearer picture of the major predictors affecting SCFA, microbiome and obesity, by integrating comprehensive data on life style, diet, demographics, clinical parameters, and microbiome in conjunction with clinical interventions. Small-scale studies do not provide enough power to explore this complex environment. 4) Fecal microbial transplantations (FMT) - may be an effective approach for exploring the effects of the microbiome on SCFA and obesity. FMT is a relatively new method, involves the transfer of feces from a healthy donor to a recipient, and thus modify the host’s microflora. FMT is becoming the treatment of choice for recurrent Clostridium difficile infection (Jayasinghe et al. 2016, 15). The influence of FMT on obesity and diabetes may provide us with valuable information regarding the interplay between microbiota, SCFA, diabetes and obesity.

6.4 Conclusions

In relation to the objectives of this thesis, the following conclusions can be made:

1) Overweight/obese individuals have increased fecal SCFA concentrations than LN individuals, possibly because of increased production by the obese microbial profile, and not because of differences in rectal SCFA absorption, or dietary intakes. However, increased fecal SCFA is not universal in all OWO individuals, possibly because of other etiologies of obesity.

2) Overweight/obese and LN individuals respond similarly to inulin and resistant starch with respect to postprandial SCFA, second-meal glycemic responses, GLP-1, PYY, and ghrelin responses.

3) Inulin and resistant starch consumptions have no acute effect on second-meal glycemic responses, GLP-1 and PYY responses through SCFA in LN and OWO individuals; however, increase in SCFA after inulin may reduce ghrelin.

Other types of fermentable fiber, a larger sample size and a longer duration of the study may yield different results.
CHAPTER 7:
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