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Effects of Starch-entrapped Microsphere Supplementation on Metabolic Phenotype in C57BL/6J Mice

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EFFECTS OF STARCH-ENTRAPPED MICROSPHERE SUPPLEMENTATION ON METABOLIC PHENOTYPE IN C57BL/6J MICE

by
Sean McNabney

A Thesis
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To my beloved family and friends for their kindness, patience, and unwavering support.
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<tbody>
<tr>
<td>ACC</td>
<td>Acetyl CoA carboxylase</td>
</tr>
<tr>
<td>ACL</td>
<td>ATP citrate lyase</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>CACT</td>
<td>Carnitine/acylcarnitine translocase</td>
</tr>
<tr>
<td>CCl₄</td>
<td>Carbon tetrachloride</td>
</tr>
<tr>
<td>CLAMS</td>
<td>Oxymax Comprehensive Laboratory Animal Monitoring System</td>
</tr>
<tr>
<td>CPT-1</td>
<td>Carnitine palmitoyltransferase I</td>
</tr>
<tr>
<td>CPT-2</td>
<td>Carnitine palmitoyltransferase II</td>
</tr>
<tr>
<td>CREBBP/CBP</td>
<td>CREBP binding protein</td>
</tr>
<tr>
<td>CREBP</td>
<td>cAMP-response element-binding protein</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DSS</td>
<td>Dextran sodium sulfate</td>
</tr>
<tr>
<td>EBM</td>
<td>Expressed breastmilk</td>
</tr>
<tr>
<td>ERK-1/2</td>
<td>Extracellular signal-regulated kinase 1/2</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>FFAs</td>
<td>Free fatty acids</td>
</tr>
<tr>
<td>FFAR2</td>
<td>Free fatty acid receptor 2</td>
</tr>
<tr>
<td>FFAR3</td>
<td>Free fatty acid receptor 3</td>
</tr>
<tr>
<td>FIAF</td>
<td>Fasting-induced adipose factor</td>
</tr>
</tbody>
</table>
GLP-1  Glucagon-like peptide-1
GLUT-2  Glucose transporter 2
GLUT-4  Glucose transporter 4
GPR41   G-protein coupled-receptor 41
GPR43   G-protein coupled-receptor 43
HAM-RS2 High-amylose maize resistant starch type II
HAT     Histone acetyltransferase
HbA1C   Glycated hemoglobin
HDAC    Histone deacetylase
HDACi   Histone deacetylase inhibitor
HF      High fat
HFD     High fat diet
HOMA-IR Homeostatic model of insulin resistance
HSL     Hormone-sensitive lipase
IκBα    Inhibitor of nuclear factor kappa B, alpha
IL-6    Interleukin-6
IL-8    Interleukin-8
IP₃     Inositol-1,4,5-triphosphate
JNK     c-Jun N-terminal kinase
LF      Low fat
LFD     Low fat diet
LPL     Lipoprotein lipase
MiRNA-193b MicroRNA-193b
MPC1  Mitochondrial pyruvate carrier 1
mtDNA  Mitochondrial DNA
NaB    Sodium butyrate
NAFLD  Non-alcoholic fatty liver disease
NAS    Non-alcoholic fatty liver disease activity score
NFκB   Nuclear factor kappa B
NRF-2  Nuclear factor (erythroid derived-2)-like 2
OCTN2  Organic cation/carnitine transporter 2
PGC-1α  Peroxisome proliferator-activated receptor gamma coactivator 1-α
PIP₂   Phosphatidylinositol 4,5-bisphosphate
PKB/Akt Protein kinase B
PKC    Protein kinase C
PLCβ   Phospholipase C, beta isoform
PPAR-α  Peroxisome proliferator-activated receptor alpha
PPAR-γ  Peroxisome proliferator-activated receptor gamma
PTF    Preterm formula
RER    Respiratory exchange ratio
ROS    Reactive oxygen species
SCFA(s) Short chain fatty acid(s)
SCMT(s) Sodium-coupled monocarboxylate transporter(s)
SiRNA(s) Small interfering RNA(s)
SIRT-1 Sirtuin-1
SLC5A8  Solute carrier family 5 member 8
SM Starch-entrapped microspheres
SMAD3 Mothers against decapentaplegic homolog 3
SOD Superoxide dismutase
TCA Tricarboxylic acid
TER Transepithelial electrical resistance
TGF-β Transforming growth factor beta
TNF-α Tumor necrosis factor-α
T2DM Type 2 diabetes mellitus
UCP-1 Uncoupling protein-1
UCP-2 Uncoupling protein-2
Wnt Wingless/MMTV integration site
ZO-1 Zona occludens-1
ABSTRACT

Resistant starches are complexes of amylose and/or amylopectin that are indigestible or only partially digestible in the small intestine. When these starches enter the colon, they undergo fermentation via resident bacterial strains to produce the short-chain fatty acids acetate, propionate, and butyrate. Not only does butyrate act as an energy source for colonocytes and regulate luminal pH, but butyrate supplementation has also been demonstrated to confer anti-inflammatory and chemopreventive benefits in cell culture models. Moreover, in murine models of diet-induced obesity and insulin resistance, dietary supplementation of sodium butyrate attenuated weight gain, reduced adipose tissue accumulation, and maintained insulin sensitivity in mice concomitantly fed a high-fat diet. Considering the increasing prevalence of overweight and obesity, estimated to exceed two-thirds of the United States adult population, butyrate has been proposed as a therapeutic candidate to mitigate obesity in humans. Unfortunately, oral sodium butyrate supplementation is not generally feasible in clinical trials due to its unpleasant odor and teratogenic effects at pharmacologic doses. Consequently, current research efforts have been directed toward enhancing endogenous butyrate production in the colon with resistant starch supplementation. These dietary fibers are much better tolerated in the gastrointestinal tract and, because they are present in a variety of foods such as fortified cereals and legumes, can be readily incorporated into the human diet.
More recently, a method has been developed to entrap starch within an alginate matrix. These starch-entrapped microspheres (SM) offer greater protection from small intestinal amylases and ferment more slowly in the colon compared to other classes of resistant starches, thereby maintaining butyrate production over a longer time course. In the present study, we assessed the effects of SM supplementation on body weight, body composition, energy intake, energy expenditure, and insulin sensitivity in a mouse model of diet-induced obesity.
CHAPTER 1. LITERATURE REVIEW

1.1 Introduction

One of the widely recognized benefits of fiber consumption is the production of short chain fatty acids (SCFAs) by the colonic microbiota [1-3]. Butyrate, a four-carbon SCFA derived from bacteria in the colon, is a well-established histone deacetylase (HDAC) inhibitor and modulator of gene accessibility by the transcriptional machinery [4-6]. Butyrate has been strongly implicated in the growth and differentiation of colonocytes, as well as the maintenance and assembly of tight junctions between adjacent cells of the gastrointestinal tract [7,8]. More recently, murine feeding trials have established butyrate involvement in improving insulin sensitivity and reducing adiposity [9,10]. These systemic effects occur following butyrate production in the colon [9,11,12], but transport of butyrate into the systemic circulation remains controversial [13]. Although adipose tissue and skeletal muscle possess surface receptors for which butyrate serves as a ligand (among other SCFAs) [14,15], microbial-derived butyrate is primarily utilized by the colonocytes for energy and measured concentrations in systemic circulation are relatively low, particularly in humans [13,16-19]. These data suggest that butyrate exerts direct effects on the colonic lumen and colonocyte metabolism, whereas its activities in peripheral tissues may be accomplished through metabolic intermediates or indirect mechanisms [20].

This chapter attempts to integrate the available literature relating butyrate to measurable health outcomes. First, it describes the ability of butyrate to mitigate obesity and insulin

Portions of this chapter have been published in:

resistance in several animal models. Second, it describes food products that either contain butyrate or yield SCFAs during fermentation in the colon, including their incorporation into human clinical trials. The chapter then discusses the observed effects of butyrate in the gastrointestinal tract, including its trophic, chemopreventive, and anti-inflammatory roles. Finally, it considers mechanisms by which diet-derived butyrate may influence the activities of the peripheral tissues, with special emphasis on treatment of obesity, type II diabetes mellitus (T2DM), and the metabolic syndrome. Interventions such as microbiota transfer [21,22], dietary fiber supplementation [23-25], and butyrate enemas have all been suggested as potential treatment options for these pathologies, but the consequences of such strategies are subject to significant inter-individual variation [22,26]. An integrated understanding of butyrate action will require contribution from many scientific disciplines, but the development of a working model can better equip medical practitioners to consider feasible interventions with their patients.

1.2 Butyrate and the Obesity Pandemic

Obesity and its associated pathologies represent one of the greatest emerging healthcare challenges in the developed world. In the United States alone, at least two-thirds of the adult population is classified as overweight (defined as a body mass index [BMI] ≥ 25 kg/m²), and greater than one-third of the adult population is obese (BMI ≥ 30 kg/m²) [27-29]. Even more troubling, the prevalence of overweight and obesity in children and adolescents assessed between 2011-2012 was reported as 31.8% [27]. Obesity imposes significant costs on the United States economy, as the aggregate national cost of overweight- and obesity-related medical treatment has been estimated at $113.9 billion [30]. Obesity also exerts financial strain on businesses, as greater absenteeism and workplace fatigue/dampened productivity (“presenteeism”) are
associated with increasing BMI levels [31-33]. Moreover, obesity is associated with several pathologies including cardiovascular disease (CVD) [29,34-37], certain types of cancer [29,38-41], non-alcoholic fatty liver disease (NAFLD) [42,43], reproductive dysfunction [44,45], and T2DM [46,47]. In fact, the grossly elevated risk for T2DM development among overweight/obese individuals has prompted the American Diabetes Association to recommend testing of overweight adults of any age who present with one or more additional risk factors, including physical inactivity [48]. Once T2DM is established, the individual is also susceptible to peripheral neuropathies, retinopathy, and nephropathy [49]. Interestingly, treatments with butyrate or those that increase butyrate production, such as increased dietary fiber or bacterial colonization in the gut, have been shown to prevent or attenuate obesity and insulin resistance [9,11,50-59].

1.2.1 Butyrate and the Reduction of Adiposity

Over the past several years, butyrate supplementation in the diet has been suggested to mitigate weight gain or to reduce adiposity in multiple rodent experiments [9,50,51]. For example, the elegant work of Gao and colleagues supported a protective effect of sodium butyrate supplementation on body weight, as C57BL/6J mice maintained on HFD and 5% wt/wt sodium butyrate gained significantly less body weight during the dietary intervention than the HFD controls [9]. Moreover, sodium butyrate-supplemented mice exhibited lower body fat percentages and higher muscle content [9]. The investigators also examined the effect of sodium butyrate supplementation on the health parameters of C57BL/6J mice after HFD (58% kcal fat) feeding had already occurred for 16 weeks; following 5 weeks of sodium butyrate supplementation and continued consumption of the HFD, sodium butyrate-supplemented mice
exhibited a 10.2% decrease in body weight and 10% reduction in body fat content relative to the HFD controls [9]. These changes were also accompanied by greater insulin sensitivity as assessed by the homeostatic model of insulin resistance (HOMA-IR) [9]. This protective effect was also observed following VSL#3 probiotic supplementation in C57J/B6 mice; overall body weight was significantly lower after 5 weeks of VSL#3 treatment, and decreased fat mass was also observed [50]. Similarly, supplementation with *C. butyricum* not only reduced body weight gain and mitigated fat pad size over the 12-week HFD intervention, but it also reduced free fatty acid content in the liver, suggesting diminished ectopic lipid deposition relative to the HFD controls [51].

Importantly, the anti-obesogenic effects observed following butyrate supplementation can also be achieved through dietary interventions involving resistant starches and other fermentable fibers [52-54]. For example, Keenan and colleagues assessed the differential effects of low-fiber (5% fiber), fermentable high-amylose resistant cornstarch (39.9% fiber, 33% resistant starch), and non-fermentable methylcellulose (37.5% fiber) diets on weight and body composition in 7-month-old female Sprague-Dawley rats [52]. Rats fed the resistant cornstarch or the non-fermentable methylcellulose diets exhibited significantly lower mesenteric, gonadal, and abdominal fat depositions compared to the low-fiber group; the intervention diets also resulted in significantly lower disemboweled body weight relative to the low-fiber controls [52]. Interestingly, the resistant starch diet resulted in elevated gene expression of peptide YY and preproglucagon in both cecal and large intestine samples, whereas the expression of these genes in the methylcellulose diet was not different from the low-fiber control group. Peptide YY and glucagon-like peptide-1 (GLP-1) protein content was also significantly higher in the sera of resistant-starch-fed rats relative to the low-fiber-fed and methylcellulose-fed rats, suggesting that
resistant starch feeding can promote an anorectic (satiety) effect in rodents [52]. To more thoroughly examine the importance of fiber type on these outcomes, the investigators provided 8-week-old male Sprague-Dawley rats with resistant-starch or non-fermentable cellulose diets that were equivalent with regard to metabolizable energy density (3.3 kcal/g) [52]. Resistant-starch-fed rats exhibited lower disemboweled body weight, abdominal fat content, and lower cecal pH relative to their cellulose-fed counterparts; after 3 weeks of feeding, the resistant starch intervention also resulted in greater gene expression of peptide YY and proglucagon, as well as a significantly greater plasma concentration of peptide YY [52].

A similar study utilized a 2x2 factorial design to assess high-amylose maize resistant starch type II (HAM-RS2, ±) and sodium butyrate (±) dietary interventions in male Sprague-Dawley rats (n=60) over the course of 12 weeks [53]. Significantly lower abdominal fat (as a percentage of disemboweled body weight) was reported for the sodium butyrate, resistant starch, and combination treatments relative to the energy control group; moreover, the combination treatment exerted more prominent effects on weight management than either treatment administered individually [53]. Resistant starch treatment significantly increased serum concentrations of GLP-1 and peptide YY, respectively, but the combination treatment significantly diminished the increase. Sodium butyrate treatment alone did not increase serum GLP-1 significantly, and it only marginally increased serum peptide YY levels [53]. According to the investigators, the mechanisms through which dietary butyrate and resistant starches exert their beneficial effects on weight management may differ depending upon the location of metabolism and absorption of these dietary components [53].

Finally, a study was conducted comparing the effects of two dietary fat levels (7% vs. 11% wt/wt) and starch compositions (amylopectin vs. resistant starch [Hi-Maize 260®]) on body
weight and metabolic parameters in C57BL/6J mice and two polygenic murine models of obesity: NONcNZO10/LtJ and Non/ShiLtJ mice, respectively [54]. Although resistant starch treatment improved fasting glucose and HOMA-IR scores for C57BL/6J maintained on a 7% fat diet, the resistant starch (7% dietary fat) intervention did not improve these parameters in the obesity-prone polygenic strains [54]. Moreover, the resistant starch diet was unable to mitigate adiposity in the polygenic strains as assessed via nuclear magnetic resonance (NMR). The investigators asserted that gut microbial diversity may differ widely among the three mouse strains and might account for the inability of obesity-prone polygenic mice to ferment resistant starches in the colon, but such data were not included in the present analysis [54].

1.2.2 Butyrate and the Restoration of Whole-Body Insulin Sensitivity

In addition to its preventive effects on body weight and adiposity, butyrate supplementation has also been associated with the mitigation of insulin resistance in several animal models [9,11,55,56]. For example, sodium butyrate supplementation (5% wt/wt) into the HFD (58% kcal fat) of C57BL/6J mice resulted in lower fasting glucose and insulin levels, as well as greater insulin sensitivity according to HOMA-IR [9]. Additionally, sodium butyrate-supplemented mice exhibited decreased serum triglyceride and total cholesterol levels compared to controls [9]. These data indicate that butyrate can exert beneficial metabolic effects in spite of the challenges posed by an obesogenic (i.e., high-fat) diet. A similar experiment assessed the effects of 1% butyrate supplementation in the drinking water of high-fat-fed (60% kcal fat) and low-fat-fed (10% kcal fat) CD-1 mice [11]. Butyrate was observed to serve a protective role in the HFD-fed mice, as serum insulin and fasting glucose levels were significantly lower when compared to HFD controls [11]. Moreover, overall body weight and liver triglyceride levels were
significantly lower in butyrate-supplemented HFD mice, suggesting that butyrate may prevent or otherwise reduce ectopic deposition of lipids [11]. Khan and Jena examined the effect of differential sodium butyrate injections (200 or 400 mg/kg intraperitoneal doses, twice per day) on streptozotocin-induced diabetic Sprague-Dawley rats maintained on a HFD (58% kcal fat) [55]. Following 10 consecutive weeks of treatment, rats administered the higher dose of sodium butyrate exhibited lower glycated hemoglobin (HbA1C) content, total cholesterol, and plasma glucose levels relative to diabetic controls [55]. Additionally, the ratio of acetylated H3 histone content to total H3 histones was significantly elevated in both sodium butyrate treatment groups relative to the diabetic control, indicating that some of butyrate’s beneficial effects are mediated through HDAC inhibition and, by extension, greater expression of particular genes [55].

Dietary fibers that facilitate butyrate production by the gut microbiome have also been associated with greater insulin sensitivity [57,58]. For example, Zhou and colleagues maintained adult male C57BL/6J mice on a control or resistant starch-rich diet for 10 days; following this period, the mice received intraperitoneal injections of vehicle (citrate buffer) or streptozotocin for 5 consecutive days while continuing their respective diets [57]. At the end of this period, an oral glucose tolerance test was performed. Area under the curve analysis indicated that streptozotocin-injected diabetic mice maintained on the resistant starch diet handled the glucose challenge significantly better than their diabetic control diet counterparts [57]. Likewise, Goldsmith et al. assessed insulin sensitivity and metabolic parameters in male Zucker diabetic fatty (ZDF) rats fed amylopectin-rich corn starch (0% resistant starch), high-amylose maize resistant starch (25% resistant starch), whole grain flour with minimal amylose content (6.9% resistant starch), or whole grain flour with 70% amylose content (25% resistant starch) [58]. All four diets were formulated to be isocaloric (3.2 kcal/g) and were well-tolerated by the rats. After
8 weeks of feeding, serum samples were collected for HOMA-IR analysis. Interestingly, only the high-amylose maize resistant starch treatment resulted in significantly lower HOMA-IR values relative to the controls; insulin sensitivity in the rats maintained on the whole grain, resistant starch-rich diet was not significantly different from the control groups [58]. Nevertheless, both resistant starch-rich diets (amylose vs. whole grain) resulted in higher serum concentration of GLP-1 and altered microbial distribution, with significant increases observed for members of the Bacteroidetes family and decreases with regard to Firmicutes members [58].

In addition to directly supplementing butyrate into the diet, multiple studies have illustrated that supplementing the diet with butyrogenic bacterial strains in the form of a well-tolerated probiotic can exert similar metabolic effects [50,51,59]. For example, C57BL/6 mice were treated with a probiotic containing the butyrogenic bacterium *Clostridium butyricum* in combination with HFD (45% kcal from fat) [51]. In comparison to the mice consuming HFD alone, mice that received *C. butyricum* probiotics in conjunction with the HFD exhibited significantly lower fasting serum insulin levels and lower blood glucose levels at both 30 and 120 minutes following an intraperitoneal glucose tolerance test (GTT) [51]. The investigators also reported lower total serum cholesterol, non-esterified fatty acids, and LDL content for the mice that received *C. butyricum* supplementation [51]. In a similar study, C57J/B6 mice were fed either a LFD (10% kcal fat) or HFD (60% kcal fat) with or without the potent probiotic mixture VSL#3 for 8 weeks [50]. In comparison to the HFD-only condition, mice administered VSL#3 in conjunction with HFD exhibited significantly lower serum insulin, fasting blood glucose, and fed blood glucose levels [50]. Additionally, VSL#3 supplementation in both the LFD and HFD groups significantly increased butyrate production as assessed by fecal sampling, and the VSL#3-HFD combination treatment altered the microbiota composition, with greater DNA
markers for Bacteroidetes and Bifidobacterial strains [50]. In a distinct experimental model, Li and colleagues assessed the effects of live or dead probiotic mixtures on streptozotocin-induced diabetic C57BL/6J mice [59]. Both live and dead probiotic-treated mice exhibited greater insulin sensitivity according to HOMA-IR relative to the diabetic control, but only the live probiotic treatment group exhibited significantly lower insulin levels overall [59]. Moreover, HbA1C content and leptin levels were significantly lower in probiotic-supplemented mice relative to the diabetic controls [59]. Interestingly, only the live probiotic mixture significantly increased butyrate production relative to the nondiabetic and diabetic control groups; although the dead probiotic mixture significantly increased acetate production, it did not have an effect on butyrate or propionate levels [59].

1.2.3 Mitochondrial Adaptations and Beta Oxidation Metabolites/Intermediates

Beta (β) oxidation occurs within the mitochondrial matrix of many cell types, and it represents a significant source of cellular energy, particularly during fasting conditions [60]. β-oxidation refers to the sequential shortening of fatty acyl-CoA chains by two carbon units during each round; this oxidation process generates several acetyl CoA molecules that can be further metabolized in the tricarboxylic acid (TCA) cycle and electron transport chain (ETC) to synthesize ATP [61]. In their activated coenzyme forms, however, long fatty acyl chains are unable to traverse the inner mitochondrial membrane, which is highly impermeable to most substances due to its unique phospholipid composition [62-64]. To facilitate entry into the matrix, fatty acyl chains undergo modification by carnitine palmitoyltransferase-1 (CPT-1), present on the outer mitochondrial membrane, to generate acylcarnitine species [65,66]. These acylcarnitine molecules subsequently traverse the inner membrane through the
carnitine/acylcarnitine translocase (CACT), an antiporter which facilitates entry of acylcarnitines and concomitantly extrudes free carnitine from the matrix [67]. Once the acylcarnitine molecules are present within the mitochondrial matrix, they are converted by CPT-2 into their active fatty acyl-CoA forms, and free carnitine is released [65]. The fatty acyl-CoA molecules may then undergo β-oxidation in the mitochondrial matrix.

Perturbations of β-oxidation have been proposed as a contributor to T2DM and the metabolic syndrome, and metabolomic approaches have identified acylcarnitine profiles which differ between insulin-sensitive/lean and insulin-resistant/obese individuals [67-71]. Taken collectively, these studies have revealed elevated medium- and long-chain acylcarnitines in individuals presenting with obesity, T2DM, or prediabetes, indicating reduced fatty acid oxidation capacity or incomplete β-oxidation following nutrient surplus [69,70,72]. Additionally, Zhang and colleagues observed elevated long-chain acylcarnitines in diabetic and prediabetic individuals [71], potentially indicating that substrate delivery is not responsible for the pathology. More recently, it has been proposed that dysfunction of β-oxidation is not the primary contributor to insulin resistance but rather a supply of macronutrient substrates that greatly exceeds the functional capacity of mitochondrial oxidative enzymes [73]; when the organism experiences chronic overfeeding, the mitochondria gradually exhibit a marked inability to transition from glucose to fatty acid oxidation under physiological conditions [74]. This substrate oversupply ultimately results in the accumulation of acylcarnitine intermediates, production of reactive oxygen species, and ectopic storage of lipids in the liver and skeletal muscle [69,74,75]. Importantly, certain acylcarnitine species may participate in nucleo-mitochondrial crosstalk by downregulating the expression of nuclear-encoded genes related to mitochondrial biogenesis and function [61,66], thereby decreasing oxidative capacity. Acylcarnitine profile changes have
seldom been investigated in conjunction with butyrate treatment, but a recent experiment by Henagan et al. assessed the effect of sodium butyrate (5% wt/wt) supplementation on acylcarnitine metabolomics in male C57BL/6J mice fed HFD (60% kcal fat) for 10 weeks [10]. Whereas the HFD-fed mice exhibited elevated medium- and long-chain acylcarnitine species, reflecting insulin resistance or metabolic inflexibility, butyrate supplementation partially mitigated the accumulation of these acylcarnitine species and, in some respects, resulted in a phenotype reminiscent of the LFD-fed mice [10].

1.3 Dietary Sources of Butyrate

1.3.1 Milk

Bovine milkfat is a particularly rich source of butyrate, with butyrate contributing approximately 4% wt/wt [76,77]. The fatty acid composition of milkfat varies most prominently by season, although stage of lactation and quality of feed are also mediating factors. When incorporated into a triacylglycerol molecule, butyrate is most frequently esterified at the sn-3 position and susceptible to cleavage by pancreatic lipase in the small intestine [78-80]. Butyrate is generally not esterified at the sn-2 position, as very few dibutyryl diacylglycerols have been verified via gas chromatography [79]. This observation suggests a nonrandom esterification of fatty acids within milk triacylglycerols that may be orchestrated by stereospecific enzymes in the bovine mammary gland [78,80,81]. The positioning of butyrate within the triacylglycerol facilitates more rapid cleavage and subsequent uptake by enterocytes because pancreatic lipase liberates free fatty acids (FFAs) from the sn-1 and sn-3 positions [82]. Since the enterocytes preferentially rely upon glutamine and glucose for their energy requirements [83,84], butyrate
released in the small intestine is not as rapidly metabolized and thus exerts beneficial effects on enterocyte proliferation and physiology in porcine, bovine, and murine models [85-89].

Human breastmilk has also been examined as a potential source of butyrate for neonates and a modulator of the colonic microbiota [90-92]. Recent pyrosequencing experiments have identified butyrogenic bacteria in human breastmilk that may facilitate colonization of the neonatal colon [91,93]. Moreover, while human milk triacylglycerols do not contain esterified butyrate, interactions between the colonic bacteria and undigested milk metabolites may result in SCFA production [94,95]. Pourcyrous and colleagues assessed fatty acid distribution in stool samples of preterm infants fed either expressed breastmilk (EBM) or preterm infant formula (PTF) [94]. Although overall butyrate production did not differ significantly between the two conditions, the mean total SCFA concentration (μM/g stool) was significantly higher in the EBM group [94]. Interestingly, mixed model analysis revealed a diet x age interaction for butyrate [94]. Butyrate production was inversely related to postnatal age in the EBM condition, with the highest levels predicted around day 20 and a gradual decline thereafter; in the PTF group, however, butyrate concentrations were lowest when full feed was initiated, and the predicted concentrations increased with postnatal age [94]. Although the authors mentioned that human milk oligosaccharides may have contributed to the observed differences in the EBM group, neither oligosaccharide structure nor potential functions was analyzed from the collected samples. A similar study compared SCFA profiles in premature (gestational age ≥ 33 weeks) and extremely premature (< 33 weeks) infants fed with fortified human milk or lactose-free formula (Nutramigen) [95]. During days 17-21, fecal butyrate concentrations were significantly higher in the formula-fed premature neonates relative to their human milk-fed counterparts [95]. Among the human milk-fed infants, those born extremely prematurely had higher butyrate percentages
than those born at 33 weeks or later (22% vs. 12%), but the effect was nonsignificant [95]. Taken together, these data suggest that the infant microbiome is particularly susceptible to dietary factors that may either encourage or restrict the production of butyrate, among other SCFAs [91,93-95].

1.3.2 Dietary Fiber

Although dietary fiber does not contain butyrate, its fermentation by microbes in the cecum and distal colon generates SCFAs, including butyrate, which can be utilized by the host organism [96,97]. This outcome has been most consistently observed for resistant starches, complexes of amylose and/or amylopectin that have the potential to escape digestion in the small intestine [98]. Resistant starches naturally occur in foods such as cooked and cooled potatoes, raw bananas, legumes, and partly milled seeds [98]. They can also be incorporated into breakfast cereals, tortillas, breads, and corn (maize) through manufacturing techniques as well as fortification [98,99]. Not only do resistant starches facilitate SCFA production by the gastrointestinal microbiota [100], but they also provide systemic benefits, such as improved insulin sensitivity, when incorporated into controlled feeding trials [101,102]. For example, Gower and colleagues assessed insulin sensitivity in healthy, sedentary women following a 4-week dietary intervention with snacks containing either resistant starch (derived from high-amylose maize) in 15 or 30 gram quantities per day, or rapidly digestible starch (waxy corn) [102]. The higher dose of resistant starch significantly improved insulin sensitivity in the insulin-resistant group, but it did not affect those parameters in women classified as insulin sensitive during baseline assessments [102]. It is important to note, however, that total dietary fiber intake was consistently lower in the insulin-resistant group during all phases of the study relative to the insulin-sensitive group, and these preexisting nutritional differences may at least partially
account for the experimental outcomes. A similar study examined SCFA production in healthy adults consuming test meals containing 20-25 grams/day of either soluble corn fiber or resistant starch (high-amylose maize) for one week [103]. Although the soluble corn fiber treatment enhanced total SCFA production, the resistant starch treatment resulted in a greater proportion of colonic-derived butyrate [103]. These data indicate that the structural and chemical properties of dietary fibers are related to their metabolism by the gastrointestinal microbiota, and particular feeding paradigms have the potential to alter microbial diversity and SCFA production in the colon [103-105].

The digestibility of food starches depends not only on chain length and extent of branching, but also upon method of cooking, chemical pretreatments (e.g., esterification), and cooling [99]. For example, cooking at high temperatures can disrupt hydrogen bonds between amylopectin branches, thereby gelatinizing the biopolymer [98]; if allowed to cool, however, these molecules can recrystallize (retrogradation) and become more resistant to digestion by host enzymes [98,99,106]. Manufacturing processes have also been developed to decrease starch digestion in the small intestine, such as entrapping starch within calcium alginate microspheres [107]. Since these starch-entrapped microspheres ferment more slowly in the colon, even compared to other classes of resistant starches, they are less likely to induce bloating and excessive flatulence which often accompany fiber-rich diets [107,108]. Moreover, in vitro fecal fermentation assays indicated that starch-entrapped microspheres generated more butyrate during late-stage fermentation (24-48 h following inoculation) than inulin, psyllium, and corn bran arabinoxylans, respectively; while total butyrate production was higher after treatment with short-chain fructooligosaccharides, long-chain β-glucan, and the resistant fraction of cooked and cooled potato starch, these substrates were metabolized more rapidly and produced the majority
of butyrate within 24 h of inoculation [108]. These data indicate that slowly fermenting fibers, particularly starch-entrapped microspheres, result in more reliable butyrate production in the colon over a longer time course.

The interactions between dietary fiber and the colonic microbiome are likely bidirectional. The efficiency of resistant starch fermentation is dependent on the bacterial communities present in the colon [109], and community profiles are significantly altered by both chronic feeding and single-meal interventions [97,102,103]. Unfortunately, some murine feeding studies have incorporated dietary fiber in proportions that grossly exceed recommended values for optimal human health. For example, Bindels and colleagues reported that resistant starches may comprise between 30-55% of total energy in rodent intervention diets [104]. While such interventions ensure that differences between the control and experimental groups are detected, their generalizability to human populations, particularly those assessed under free-living conditions, may be limited. The 2005 Adequate Intake values for total fiber were established as 38 and 25 grams/day in healthy young men and women, respectively [110], but NHANES data from 1999-2010 revealed an average consumption of only 16.2 grams/day [111]. Unfortunately, the “Western diet” is typically rich in refined carbohydrates that contain significantly less dietary fiber [112], and so the general US population seldom consumes fiber at an optimal level.

1.4 Butyrate Activity in the Gastrointestinal Tract

Recent investigation has identified butyrate as a ligand for G-protein coupled receptors on the enteroendocrine, neutrophil, and colonocyte cell surfaces [15,113]. Due to their high affinities for SCFAs, the “orphaned” G-protein-coupled receptors GPR43 and GPR41 are now described as free fatty acid receptors 2 (FFAR2) and 3 (FFAR3), respectively [17,113]. FFAR2
has the potential to transduce signals through both the $G_{i/o}$ and the $G_{q/11}$ pathways, whereas FFAR3 only utilizes the $G_{i/o}$ pathway [17,114]. Both heterotrimeric G protein families can activate the beta isoform of phospholipase C (PLCβ), which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP$_2$) into diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP$_3$) [115]. DAG and IP$_3$, in turn, can release stored Ca$^{2+}$ from the endoplasmic reticulum, thereby enabling downstream signaling events. In the $G_q$ family, DAG and IP$_3$ may also activate protein kinase C (PKC) to ultimately stimulate the downstream activities of the extracellular signal-regulated kinase 1/ 2 (ERK-1/2) and c-Jun N-terminal kinase (JNK) pathways [115]. As such, the physiologic consequences of butyrate are multivariate, with outcomes dependent upon tissue type, dosage, and time effects. Here, we discuss the more commonly observed effects of butyrate supplementation on the GI tract, with regard to both in vitro cell culture and controlled feeding trials.
As indigestible and partially digestible dietary fibers enter the large intestine, they are fermented by saccharolytic bacterial strains that reside in the colon. Short-chain fatty acids including butyrate, propionate, and acetate are the major by-products of the fermentation process. Butyrate improves gut epithelial integrity by localizing tight junction proteins between adjacent colonocytes. Butyrate also exerts effects intracellularly, as it is absorbed by the colonocytes via a sodium-coupled cotransporter (SLC5A8). Butyrate is the one of the primary energy sources for colonocytes, undergoing β-oxidation within their mitochondria to generate ATP. It can also act as a histone deacetylase inhibitor (HDACi) in the nucleus, increasing gene expression by maintaining chromatin in its relaxed (uncoiled) state.

Figure Legend:
- Dietary fiber
- Butyrogenic colonic bacteria
- Butyrate
- Tight junction proteins
- Adenosine triphosphate
- Sodium-coupled butyrate transporter
- Mitochondrion
- Acetyl group
- Nucleosome
- DNA

(figure continues)
1.4.1 Microbial Production of Butyrate

Although numerous bacterial strains have been analyzed for their butyrate-producing capacities, *Faecalibacterium prausnitzii* (a member of *Clostridium* cluster IV) and *Eubacterium rectale/Roseburia* (*Clostridium* cluster XIVa) have currently received the most attention as they constitute 5-10% of total bacteria in fecal samples collected from healthy adults [116]. In addition to the colonization of the colon by butyrogenic bacteria, it has been proposed that cross-feeding interactions between Bifidobacterial strains and *F. prausnitzii* may ultimately enhance butyrate production [117]. When *F. prausnitzii* was co-cultured with *Bifidobacterium breve* Yakult or *Bifidobacterium adolescentis* and oligofructose (a particular inulin-type fructan) provided as the energy source, butyrate was produced in appreciable quantities (between ~12-30 mM after 48 hours of fermentation) [117]. In contrast to this outcome, the effect of co-culturing *F. prausnitzii* with *Bifidobacterium angulatum* or *Bifidobacterium longum* was dependent upon the energy substrate provided; oligofructose encouraged greater butyrate production than other inulin molecules in both co-culture models, which the investigators attributed to energy competition among the bacterial strains as well as less bioavailable acetate as a co-substrate of butyrate synthesis [117]. SCFA production by the colonic microbiota has also been recognized as an important source of energy for the GI tract cells in the host organism. For example, isolated colonocytes from germ-free C57BL/6 mice exhibited NADH/NAD⁺ ratios that were 16-fold lower than their conventionally raised counterparts, as well as 56% lower ATP levels [5]. When these germ-free colonocytes were colonized with microbes from conventionally raised mice or the butyrogenic bacterium *Butyrivibrio fibrisolvens*, their energy status improved [5]. These data indicate that the microbiome influences energy production in the host organism and may explain weight differences observed between germ-free mice and conventionally raised strains.
In order to distinguish the effects of butyrate from the other SCFAs, Lu and colleagues designed a 16-week feeding trial in C57BL/6J mice with five types of high-fat diet (HFD) [118]. The first HFD (60% kcal, derived equally from lard and soy oils) served as a positive control. The intervention HFDs consisted of sodium acetate, sodium propionate, or sodium butyrate supplementation at 5% wt/wt, and the final HFD included a mixture of these three SCFAs in a 3:1:1 ratio, respectively. Although SCFA supplementation did not influence carnitine palmitoyltransferase I/II (Cpt1 or Cpt2) mRNA content in skeletal muscle, increased acetyl CoA carboxylase (Acc) mRNA was reported in both adipose and liver tissues [118]. Moreover, SCFA supplementation increased expression of hormone-sensitive lipase (Hsl), fasting-induced adipose factor (Fiaf), and adiponectin, whereas SCFA supplementation decreased expression of adipose lipoprotein lipase (Lpl) relative to the HFD condition [118]. Interestingly, sodium butyrate supplementation increased Gpr41 mRNA content in the adipose tissue to a greater extent than any of the other SCFAs and to a level higher than the HFD or low-fat diet (LFD, 10% kcal) controls [118]. These data suggest that SCFA-based dietary interventions can profoundly alter gene expression in metabolically active tissues and that under certain circumstances, butyrate may exert effects that differ from those of acetate and propionate.

1.4.2 Chemopreventive Effects of Butyrate

Butyrate is known to promote growth of the colonic epithelium, yet it exerts a predominately inhibitory effect on colorectal cancers [19] (Figure 2). Emerging evidence suggests that the paradoxical effects of butyrate may be explained by the Warburg effect observed in various cancers [119-121]. Whereas noncancerous colonocytes utilize aerobic respiration to meet energy requirements, cancerous colonocytes rely upon anaerobic glycolysis
even when \( \text{O}_2 \) is plentiful \([119,120]\). Uptake of pyruvate by colonocyte mitochondria also decreases due to a deletion of the mitochondrial pyruvate carrier 1 (\( \text{Mpc1} \)) gene, an outcome that is observed among several cancers \([122]\). Nevertheless, the substrate-level phosphorylation that occurs during anaerobic respiration generates citrate in the mitochondrial matrix. Citrate is then exported to the cytoplasm, converted to acetyl CoA via ATP citrate lyase (ACL), and subsequently used for biosynthesis of lipids involved in cell proliferation \([121]\). Under conditions of anaerobic glycolysis, fatty acid oxidation is limited. Thus, the SCFA butyrate is not used extensively as an energy source by the colonocytes and begins to accumulate in the cytoplasm; this accumulation allows butyrate to act as a histone deacetylase inhibitor (HDACi) and ultimately sensitizes the cancerous colonocytes to apoptotic mechanisms, leading to cellular death \([123,124]\). In noncancerous colonocytes, however, increased \( \beta \)-oxidation of fatty acids (including butyrate) provides a high level of acetyl CoA that can ultimately serve as acetyl group donors for histone acetyltransferase (HAT) proteins such as p300 \([125]\). In addition to cytosolic ACL, Wellen and colleagues demonstrated that ACL is also expressed within the nucleus \([126]\), thereby facilitating the conversion of TCA cycle-derived citrate into acetyl CoA. Nuclear acetyl CoA molecules may serve as acetyl group donors that can hyperacetylate histones \([126]\), thereby increasing chromatin availability to the transcriptional machinery.

The ability of butyrate to accumulate in the cytoplasm of cancerous colonocytes appears to be related to the coordinated downregulation of fatty acid uptake into their mitochondria. Although it was historically established that SCFAs did not require the carnitine palmitoyltransferase proteins (CPT-1, CPT-2) for entry into the mitochondria of hepatic and cardiac tissues \([127]\), it is important to recognize that butyrate only enters these organs in \( \mu \text{M} \) concentrations. In contrast, SCFAs are present in the colonic lumen at a range of 50-100 mM.
[128], and the CPT system appears to have a more prominent role in butyrate uptake at higher concentrations. Mechanistic analysis of the Warburg effect in HCT116 colorectal cancer cells identified decreased intracellular carnitine levels relative to noncancerous fetal human colonocytes [129]; additionally, Western blotting revealed decreased expression of organic cation/carnitine transporter 2 (OCTN2), a sodium-coupled cotransporter for carnitine, in the HCT116 cell line [129]. A similar experiment illustrated that undifferentiated Caco-2 cancer cells exhibited negligible expression of OCTN2, whereas their mature Caco-2 counterparts (which more closely resemble small intestinal enterocytes) expressed OCTN2 at the brush border membrane [130]. Moreover, hypermethylation of the solute carrier family 5 member 8 (Slc5a8) gene, which codes for a sodium-dependent butyrate transporter, has been observed in both cancerous colonocytes as well as aberrant crypt foci [131]. These data suggest that impairment of butyrate uptake and metabolism characterizes early neoplasia in the colon and could potentially contribute to cancer progression [125,129-131]. To this end, multiple lines of research have suggested that a combination treatment of butyrate and carnitine/acetylcarnitine can exert greater effects on cancerous cells than butyrate treatment alone, possibly by enhancing butyrate localization to the mitochondria [132,133]. Oral L-carnitine supplementation also mitigated cancer cachexia symptoms in BALB/c mice injected with adenocarcinoma cells [134,135].

With the HDACi and acetylation effects of butyrate widely recognized, attention has now turned to the identification of signaling pathways through which butyrate may exert anti-proliferative and pro-apoptotic effects in cancerous tissues. For example, the transforming growth factor β (TGF-β) signaling pathway has been implicated in cell sensitization to pro-apoptotic mechanisms in noncancerous colonocytes, but persistent downregulation of its downstream modulators such as mothers against decapentaplegic homolog 3 (SMAD3) has been
implicated in cancer progression [136,137]. In comparison to young adult murine colonocytes, growth of Smad3−/− cells was not markedly inhibited by incubation with TGF-β and 3H-thymidine incorporation only decreased modestly (25% decrease vs. 61% for control) [138]. MicroRNA-193b (MiRNA-193b) has also emerged as a potent inhibitor of SMAD3, and downregulation of MiRNA-193b by small interfering RNAs (siRNAs) was observed to significantly increase SMAD3 protein expression and caspase-3 activity in SW620 cells [139]. Sodium butyrate treatment in RIE-1 cells exhibited a time-dependent effect on Smad3 mRNA content, with the longest incubation period (48 hours) producing the greatest expression relative to control; moreover, sodium butyrate exhibited a dose-dependent effect on SMAD3 protein expression over a 24-hour time course, with intermediate doses (2.5 and 5.0 mM concentrations) having the most potent effects [140]. Interestingly, a combination treatment of TGF-β and sodium butyrate more effectively inhibited anchorage-independent growth of RIE cells overexpressing protein kinase B (PKB/Akt) than TGF-β treatment alone [140]. Pretreatment with sodium butyrate (5 mM) followed by TGF-β treatment (40 pM) was observed to increase DNA fragmentation and the percentage of apoptotic RIE-1 cells to a greater extent than sodium butyrate alone; TGF-β treatment without butyrate did not significantly induce apoptosis or increase DNA fragmentation in comparison to the control [141]. The combination treatment also shifted the distribution of RIE-1 cells within the cell cycle, with a higher percentage of cells arrested in the G0/G1 phase and a lower percentage arrested in the S phase; interestingly, butyrate treatment alone (5 mM) arrested a greater percentage of RIE-1 cells in the G2/M phase of the cell cycle, whereas TGF-β primarily arrested cells in the G0/G1 phase [141]. These data indicate that butyrate exerts unique effects on cell proliferation, but it may also potentiate the effects of TGF-β signaling pathways [141]. In a similar experiment utilizing RKO, HCT-116, and HT-29 cell cultures, butyrate
treatment (5 mM) significantly increased the percentage of cells arrested at the G2/M phase and decreased the percentage of cells arrested in the S phase [6].

Butyrate action has also been implicated in the wingless/MMTV integration site (Wnt) pathway, likely mediated by an increased association of the cAMP-response element-binding protein (CREB) binding protein (CREBBP, or CBP) and the histone acetyltransferase p300, thereby encouraging the transcription of Wnt-related proteins involved in apoptosis of colorectal cancer cells [142,143]. Although moderate Wnt activity has been associated with cancer cell proliferation, the hyperactivation of this pathway by butyrate treatment has been demonstrated to induce apoptosis in multiple cell lines [144,145]. Unfortunately, some cancers can gradually become resistant to the effects of butyrate as well as pharmacologic HDACi [142,146]. This butyrate resistance appears to be marked by a transition from the “canonical” (β-catenin-dependent) Wnt pathway to a modified pathway that does not rely upon β-catenin for its downstream effects [146,147].

Downregulation of the butyrate cotransporter SLC5A8 has also been implicated in the establishment and proliferation of colon cancers [148]. When Li and colleagues assessed Slc5a8 exon status in 64 primary colon cancer tissue samples, methylation-specific PCR revealed exon methylation in 59% of the samples [131]. Moreover, comparison of the 38 methylated tumor samples to noncancerous analogues illustrated that 35 of the 38 healthy tissues (92%) did not display methylation of this region [131]. Interestingly, in addition to facilitating butyrate uptake and subsequent HDACi activity, SLC5A8 appears to independently increase the likelihood of apoptosis in cancerous colonocytes by sequestering and diminishing the activity of survivin [148], a protein that is required for spindle fiber formation during cell division [149].
Cancerous colonocytes do not generally rely upon fatty acid oxidation for their metabolic requirements, allowing butyrate to accumulate in the cytoplasm. Accumulated butyrate can translocate to the nucleus and act as a histone deacetylase inhibitor (HDACi), thereby increasing the transcription of proteins involved in cell cycle regulation or apoptosis. Butyrate also increases expression of SLC5A8. SLC5A8 can independently bind to and sequester the protein survivin, preventing the formation of spindle fibers required for cell division. When supplemented with carnitine, butyrate can more readily enter the mitochondrial matrix and undergo β-oxidation, potentially mitigating metabolic perturbations such as the Warburg effect. Black arrows indicate movement of a metabolite or signify a particular pathway. Green, upward arrows represent an increase in the outcome or product. Red, downward arrows represent a decrease in the outcome or product.
1.4.3 Anti-Inflammatory Effects of Butyrate

Butyrate is not only responsible for the energy requirements of the colonic epithelium [5,150], but it also preserves such tissues by mitigating chronic inflammatory responses. Both fiber-rich diets and SCFA supplementation have been associated with regulation of pro- and anti-inflammatory cytokines [113,151]. One of the most extensively studied cytokines in this regard is interleukin-8 (IL-8), which is frequently elevated in inflammatory bowel disease [152]. While IL-8 is crucial for transient recruitment of neutrophils and other cells of the innate immune system [153,154], persistent elevation of IL-8 has been reported in diabetic and sedentary individuals, and it is associated with poor cardiometabolic outcomes [155,156]. IL-8 induction may also be related to macronutrient consumption. Cultured human vascular smooth muscle cells had significantly higher expression of IL-8 following treatment with palmitate, a saturated fatty acid commonly incorporated into HFDs; the effects of palmitate on IL-8 mRNA and protein content were dose dependent [157].

The effects of butyrate on IL-8 are dependent upon dose and time effects, as well as the cell type under investigation. For example, Gibson and Rosella isolated colonic crypt cells from patients diagnosed with colorectal cancer, Crohn’s disease, or ulcerative colitis and assessed IL-8 secretion in response to butyrate treatment (1 mmol/L) over a 24 hour time course; in all disease groups, butyrate administration significantly lowered IL-8 concentrations in comparison to control [152]. Importantly, butyrate-mediated reduction in IL-8 concentration was also reported for uninflamed colonic mucosa, suggesting that the clinical utility of butyrate may extend beyond pathophysiologic conditions [152]. A similar study examined the effect of butyrate treatment on IL-8 expression following stimulation by Pam3CSK4, a pathogen-associated molecular pattern, in cultured Caco-2 and SW480 cells [158]. Concurrent treatment with butyrate significantly
lowered IL-8 expression for shorter incubation periods (< 9 hours), but when butyrate treatment exceeded 9 hours, expression of IL-8 was significantly higher than the control. Butyrate treatment also increased endogenous expression of A20 [158], a negative feedback regulator of NFκB via ubiquitin-editing mechanisms [159,160]. In contrast to these beneficial effects, butyrate incubation prior to Pam3CSK4 stimulation resulted in greater expression of IL-8 in both cell types [158], indicating that butyrate’s anti-inflammatory effects are restricted to the biochemical milieu of the tissue. Cultured HT-29 adenocarcinoma cells treated with a combination of tumor necrosis factor-α (TNF-α) and sodium butyrate exhibited reduced interleukin-8 (IL-8) secretion in comparison to cells treated with TNF-α alone [161]. The same investigators studied colonic health in Wistar rats that received butyrate enemas in tandem with a diet containing dextran sodium sulfate (DSS), an inducer of colitis in murine models; butyrate treatment resulted in statistically smaller ulcers and decreased myeloperoxidase activity relative to controls [161]. The G-protein coupled receptor GPR43 may also be involved in the mitigation of inflammatory responses. When Gpr43−/− mice are housed with wild-type counterparts, their colitis symptoms improved significantly compared to separately housed mice; the authors attributed these effects to coprophagy of wild-type feces and, by extension, changes in microbiota composition that can alter inflammatory outcomes [151].

Another mechanism by which butyrate has been proposed to reduce systemic inflammation is the maintenance of the intestinal epithelial barrier [7,162]. Recent evidence has suggested that changes in tight junction localization, intestinal permeability, and gut microbial diversity may precede the development of obesity and T2DM [163,164]. As the mucosal layer becomes less capable of repelling unfavorable bacterial strains, these bacteria or their metabolites are able to traverse the intestinal barrier and invade the surrounding tissue, thereby
stimulating an innate immune system response [163]. If poor dietary lifestyle choices, among other factors, prevent full reconstitution of the intestinal barrier, the individual may experience persistent low-grade inflammation, which has been associated with obesity and insulin resistance as well as dysfunction of the peripheral tissues [165]. In a Caco-2 cell model of the intestinal epithelium, Peng and colleagues assessed the effects of butyrate supplementation on tight junction protein expression, localization, and transepithelial electrical resistance (TER) [7]. Although butyrate incubation (2 mmol/L for 72 hours) did not significantly increase protein expression of claudin-1, claudin-4, zona occludens-1 (ZO-1), and occludin, the incubation increased TER and localized the tight junction proteins to the cell periphery during a calcium switch assay [7]. Moreover, butyrate treatment increased the ratio of phosphorylated AMP-activated protein kinase (AMPK) to total AMPK content in a time-dependent manner. The role of AMPK as mediator of these processes was supported with the addition of compound C (10 μM), a known inhibitor of AMPK; when compound C was added to the cell system, butyrate could not induce tight junction assembly even in the presence of Ca^{2+} [7]. The reparative effect of butyrate incubation was also reduced in an analogous experiment by introducing small interfering RNAs (siRNAs) to decrease expression of AMPK [8]. A similar experiment utilized SCFA mixtures with different proportions of butyrate (5, 20, and 50%) to examine the effect of treatment on barrier function with concomitant addition of pro-inflammatory lipopolysaccharide molecules and TNF-α [162]. When the proportion of butyrate was higher in the SCFA mixture (20% or 50%), TER increased significantly despite TNF-α and lipopolysaccharide treatment; moreover, butyrate incubation at the highest concentration increased TER following previous TNF-α and lipopolysaccharide treatment, suggesting that butyrate incubation can exert both protective and reparative effects on the intestinal barrier [162].
1.4.4 Mechanisms of Butyrate Uptake

Due to the overall hydrophobicity and low molecular weights of the SCFAs in their protonated forms, acetate, propionate, and butyrate can be readily absorbed via nonionic diffusion across the apical membrane of colonocytes [166,167]. Yet, the observation that < 10% of SCFAs appear in the feces suggested additional mechanisms for their uptake [96]. Further experimentation led to the identification of sodium-coupled monocarboxylate transporters (SCMTs) that utilize the colonic Na\(^+\) concentration gradient to efficiently sequester SCFAs within colonocytes [18,96,168]. Within this class of transporters, solute carrier family 5 member 8 (SLC5A8) has emerged as the primary transporter of butyrate across the apical membrane of the colonocytes and may also represent an avenue for crosstalk between the microbiome and the host organism (by exchange of metabolized products including SCFAs) [166,168]. In addition to the activity of SLC5A8, proton-coupled monocarboxylate transportation and SCFA-bicarbonate antiporters have also been proposed as viable mechanisms for SCFA uptake as well as regulators of lumen pH [18,169-171]. The functional overlap with regard to SCFA absorption is not surprising, as butyrate metabolism accounts for at least 70% of colonocyte energy requirements [16,166,172].
1.5 Butyrate and the Peripheral Tissues

1.5.1 Liver

Although butyrate is used extensively by the colonocytes [166], the degree to which it may be metabolized by the peripheral tissues is not well-established in humans. In one of the earliest quantification experiments of human SCFA localization, Cummings and colleagues measured SCFAs in portal, hepatic, and peripheral blood during autopsies of sudden death victims (n=6) in the United Kingdom [173]. The average concentrations of butyrate in these blood “compartments” were 29 μmol/L, 12 μmol/L, and 4 μmol/L, respectively [173]. The molar ratio of butyrate was particularly low in the peripheral veins, at only 4%; acetate constituted the most abundant category (91%), and propionate levels were also very low (5%) in the systemic circulation [173]. A more recent study assessed SCFA flux in human patients undergoing major upper abdominal surgery (n=22) in the Netherlands [13]. Unsurprisingly, butyrate was very well absorbed in the gut and could be subsequently released into the portal blood (5.7 μmol/kg body weight/hour). Once this butyrate reached the liver, however, it was almost entirely extracted in an equal proportion to the amount released from the enterocytes and colonocytes [13]. Moreover, the amount of butyrate released from the splanchnic compartment was not significantly different from zero, indicating that the liver metabolized nearly all butyrate that escaped utilization by the colonocytes [13]. This finding does not necessarily preclude butyrate activity in more distal tissues, however, as acetate can be readily converted into butyrate within the colon, which may increase the amount absorbed and subsequently released into circulation [16]. Moreover, the ketone body β-hydroxybutyrate can be formed by the liver and has been observed to bind to FFAR3 and to exert HDACi effects similarly to butyrate [171].
Supplementation of butyrate or butyrogeic bacterial strains has also been associated with the reduction of ectopic lipids in hepatic tissue as well as increased activity of antioxidant enzymes [174-177]. For example, Jin et al. assessed the effect of oral sodium butyrate supplementation (0.6 g/kg body weight/day) in female C57BL/6J mice fed either a liquid control (12% energy from fat) or Westernized diet (25% energy from fat, 50% w/w sucrose supplementation) for 6 weeks [174]. Despite similar body weight to their HFD-fed counterparts, the mice receiving sodium butyrate in conjunction with the Westernized diet exhibited a significant reduction in intrahepatic lipid deposition, decreased liver damage as assessed by the non-alcoholic fatty liver disease activity score (NAS), and dampened inflammatory activity [174]. In a similar study, Mattace Raso and colleagues examined the effect of sodium butyrate gavage (20 mg/kg body weight/day) on male Sprague-Dawley rats consuming HFD (58% kcal fat) for 6 weeks [175]. Butyrate-supplemented rats exhibited lower interleukin-6 (IL-6) mRNA content, reduced nuclear factor κB (NF-κB) protein content, and increased protein content of the inhibitor of nuclear factor kappa B, alpha (IκBα) relative to HFD controls [175]. Proteins related to fatty acid catabolism and greater insulin sensitivity, including peroxisome proliferator-activated receptor α (PPAR-α) and γ (PPAR-γ), were reduced in the HFD-fed rats, but butyrate supplementation significantly increased their expression. Additionally, butyrate-supplemented rats exhibited significantly greater peroxisome proliferator-activated receptor γ coactivator 1-α (PGC-1α) mRNA content relative to their HFD counterparts [175]. In a murine model of autoimmune hepatitis induced by Freund’s complete adjuvant, male C57BL/6 mice receiving 300 mg/kg sodium butyrate as a daily gavage for 3 weeks exhibited reduced mRNA and protein content of NFκB, IL-6, and TNF-α relative to the hepatitis controls [176]. The beneficial effects of butyrate supplementation also extended to the small intestine in this study, as expression of
tight junction proteins including claudin-1, occludin, and ZO-1 was significantly increased relative to the hepatitis-induced controls [176]. Finally, Liu and colleagues administered \( C. \) butyricum (5 x 10^8 CFU) intragastrically in male ICR mice for 5 days prior to acute liver injury induced via carbon tetrachloride (CCL₄) intraperitoneal injection [177]. The pretreatment with \( C. \) butyricum exerted a prophylactic effect against CCL₄ injection: ICR mice that received the bacterial strain had significantly greater nuclear factor (erythroid derived-2)-like 2 (NRF-2) protein content, elevated superoxide dismutase (SOD) and catalase enzyme activity, and reduced TNF-α expression [177].

1.5.2 Skeletal Muscle

Skeletal muscle represents one of the largest insulin-responsive tissues and utilizes a significant proportion of blood glucose during aerobic/endurance exercise [178]. Under fed conditions in healthy individuals, insulin action results in the liberation of glucose transporter 4 (GLUT4) from intracellular storage vesicles and subsequent localization to the plasma membrane (sarcolemma) of skeletal muscle fibers; GLUT4 translocation has also been observed following muscle contraction during exercise, although the underlying mechanisms may differ [179]. In cases of obesity or T2DM, however, uptake of glucose decreases and fatty acid oxidation may also become compromised, leading to increased lipid deposition in the skeletal muscle [180,181]. These changes often occur in conjunction with reduced expression of genes involved in mitochondrial biogenesis and function, thereby decreasing the capacity for oxidative metabolism [61,182]. When incomplete β-oxidation occurs in the mitochondria, acylcarnitine species are produced and may exert repressive effects on nuclear-encoded mitochondrial genes and systemic insulin sensitivity [61,67,183]. Chronic overfeeding has also been associated with
impaired substrate transitioning and generation of reactive oxygen species (ROS) by the mitochondria, which may ultimately interfere with insulin signaling pathways [74,184,185]. In contrast to these deleterious effects, increased expression of PPAR-α has been associated with greater fatty acid oxidation capacity in skeletal muscle [186,187]. Moreover, adenoviral-mediated PGC-1α overexpression in cultured human skeletal muscle myotubes was observed to increase fatty acid oxidation, elevate mitochondrial DNA (mtDNA) content by approximately 72%, and modestly reduce lipid deposition [188].

The beneficial effects of butyrate supplementation on skeletal muscle have been demonstrated in both prevention and treatment-focused interventions (Figure 3). For example, Hong and colleagues examined the effect of oral sodium butyrate administration in C57BL/6 mice that had already been administered HFD (45% kcal fat) for 8 weeks [12]. Half of the HFD-fed mice then received daily gavages of 80 mg sodium butyrate for 10 consecutive days, where the other mice received vehicle. In comparison to the HFD/vehicle treatment, mice that received sodium butyrate in addition to HFD consumption exhibited decreased serum insulin, leptin, and fasting glucose concentrations [12]. Total body weight, liver weight, and epididymal fat pad weight also decreased significantly in butyrate-supplemented mice relative to the HFD controls. It is important to note, however, that butyrate treatment resulted in an intermediate phenotype for weight which was not identical to the standard chow-fed mice [12]. Extending the period of supplementation could potentially increase the observed benefits and result in a phenotype more physiologically similar to the chow-fed mice. Although butyrate supplementation did not alter protein expression of GPR41 or GPR43 in gastrocnemius muscle, chromatin immunoprecipitation (ChIP) analysis revealed elevated acetylation at the gene promoter regions of adiponectin receptors 1 and 2 (Adipor1/Adipor2) and uncoupling proteins 2 and 3.
(Ucp2/Ucp3) in butyrate-supplemented mice [12]. Finally, phosphorylated AMPK content was significantly elevated in butyrate-supplemented mice relative to HFD controls [12]. With regard to preventative benefits of butyrate administration, Gao et al. observed greater weight maintenance and prevention of weight gain in C57BL/6J mice that received sodium butyrate (5% wt/wt) with concurrent HFD (58% kcal fat) feeding as compared to HFD-feeding alone; the butyrate intervention also reduced adiposity, maintained muscle mass, and resulted in greater conversion of type II (glycolytic) muscle fibers to type I (oxidative) fibers [9]. Western blotting also revealed elevated PGC-1α, type I myosin heavy chain, and phosphorylated AMPK protein content in butyrate-treated mice [9]. Henagan and colleagues observed similar effects with concurrent administration of HFD (60% kcal fat) and 5% wt/wt sodium butyrate for 10 weeks, but butyrate supplementation also decreased long- and medium-chain acylcarnitine species, indicating an increase in complete β-oxidation [10].
Figure 3. Effects of Butyrate on Skeletal Muscle Physiology.

Poor lifestyle choices, including physical inactivity and consumption of a high-fat diet (HFD), negatively impact skeletal muscle function (left) [189]. Butyrate supplementation has been observed to mitigate several of the deleterious effects of chronic HFD-feeding in skeletal muscle (right). Green, upward arrows represent an increase in the outcome or product. Red, downward arrows represent a decrease in the outcome or product.

1.6 Future Directions and Summary

While the therapeutic potential of butyrate or resistant starch treatment appears promising, several questions warrant further investigation. A major challenge is to characterize the diverse mechanistic effects of butyrate and to incorporate them into an integrative model. One of the benefits of butyrate supplementation is greater capacity for lipid oxidation; these
effects appear to be related to changes in gene and protein expression of PGC-1α, AMPK, and PPARγ [9,10,77]. Aside from its own role as an energy sensor and inhibitor of fatty acid synthesis, AMPK can activate sirtuin-1 (SIRT-1), which is responsible for the deacetylation and activation of PGC-1α [61]. PGC-1α, in turn, can upregulate other nuclear-encoded mitochondrial genes, thereby exerting downstream effects on mitochondrial biogenesis and function in diverse tissue types [10]. Butyrate treatment has also been observed to increase expression of uncoupling protein-1 (UCP-1) in brown adipose tissue, increasing thermogenesis and perhaps modulating energy expenditure, at least in murine models [9]. In addition to its HDACi activity, butyrate increases transcription of target genes by repositioning nucleosomes away from the transcriptional start site [10]; taken together, these dual mechanisms ensure that chromatin is oriented such that it is accessible by the transcriptional machinery. Finally, butyrate supplementation has been demonstrated to improve insulin sensitivity by maintaining GLUT-2 and GLUT-4 protein content despite HFD feeding [175]. It is likely that members of the insulin signaling pathway are also involved in this outcome, and efforts are ongoing to identify candidate proteins.

There is also some evidence that butyrate may exert its anti-obesogenic effects through a different mechanism than resistant starches. In Sprague-Dawley rats, dietary sodium butyrate was absorbed at the small intestine and thus did not alter cecal concentrations of butyrate, whereas an isocaloric diet containing resistant starch increased butyrate levels as well as empty cecal weight [53]. Moreover, the investigators noted that sodium butyrate treatment did not elevate serum GLP-1 or peptide YY levels relative to the control diet, suggesting that butyrate may influence weight loss or weight maintenance in a manner distinct from changes in satiety hormone profile [53].
Finally, determining the optimal form of butyrate and method of delivery for human use will require continued investigation. Administering butyrate directly to human patients is generally not feasible due to its unpleasant odor; manufacturing efforts have therefore emphasized the production of synthetic triglyceride molecules which contain butyrate and are better tolerated in the GI tract [53,190]. Depending upon the desired treatment outcome (e.g., apoptosis of cancerous colonocytes vs. restoration of insulin sensitivity) it may be more advantageous to use dietary fiber rather than butyrate directly or butyrate-containing triglycerides. Resistant starches ensure that butyrate is produced in the cecum and distal colon, and colonic-derived butyrate appears to act extensively in that particular milieu. If the experimental aim is to deliver butyrate to the peripheral tissues, however, butyrate derivatives may be more appropriate as they are less likely to be utilized by the small intestinal enterocytes for nutritional requirements [85,87,88]. Most clinical studies thus far have utilized resistant starches and other forms of dietary fiber, and so the effects of supplementing butyrate more directly in humans (in the form of triglycerides) are largely unstudied.

Butyrate is essential for proper growth and function of the gastrointestinal epithelium, and it is primarily derived from the fermentation of dietary fibers and resistant starches by the colonic microbiota. Beyond its HDACi action, butyrate serves as a fuel source for enterocytes and colonocytes, facilitates apoptosis of colonic cancer cells, and reduces gut inflammation. In the peripheral tissues, butyrate decreases ectopic lipid storage, reduces inflammation, increases complete β-oxidation of fatty acids, and promotes the expression of genes related to mitochondrial biogenesis and function in the skeletal muscle. Finally, butyrate may mitigate the deleterious effects of obesity and T2DM, which are increasingly prevalent in the United States.
and beyond. Dietary strategies that can facilitate butyrate production, among other SCFAs, might therefore be suggested as one element of a personalized health plan.
CHAPTER 2. EFFECTS OF STARCH-ENTRAPPED MICROSPHERE SUPPLEMENTATION ON METABOLIC PHENOTYPE IN C57BL/6J MICE

2.1 Abstract

The histone deacetylase inhibitor sodium butyrate (NaB) has been previously shown to mitigate weight gain, reduce adipose tissue accumulation, and improve insulin sensitivity in C57BL/6J mice concomitantly fed a high-fat diet (HFD). The translational potential of NaB to humans is restricted, however, due to teratogenic and other adverse effects. The purpose of this study was to compare the effectiveness of NaB on these parameters to that of starch-entrapped microspheres (SM), a type of slowly-fermenting, well-tolerated resistant starch implicated in butyrate production by the gut microbiota. Forty male C57BL/6J mice were divided into four dietary intervention groups (n = 10 mice per treatment): 1) low-fat diet (LFD, 10% kcal from fat), 2) HFD (60% kcal from fat), 3) HFD supplemented with 5% wt/wt NaB (HFD + NaB), and 4) HFD supplemented with 10% wt/wt SM (HFD + SM). Body weight and food consumption were assessed weekly. Body composition was determined weekly using EchoMRI, with scans performed in duplicate. After 7 weeks of feeding, glucose tolerance tests (n = 20) were performed. At 9 weeks, mice were individually housed in metabolic chambers to assess energy expenditure (n = 20). Similarly to previous findings, HFD + NaB and LFD groups did not differ significantly with regard to body weight, lean body mass, fat mass, or glucose tolerance. HFD + SM treatment conferred protection against fat mass accumulation relative to the HFD group, but not to the extent observed in HFD + NaB. Glucose tolerance in HFD + SM did not differ significantly from the LFD group. Both HFD + NaB and HFD + SM groups exhibited significantly greater energy expenditure relative to HFD.
2.2 Introduction

The short chain fatty acid (SCFA) butyrate is produced by colonic bacteria through the fermentation of dietary fibers that are either indigestible or only partially digestible by the host organism [1,150]. Butyrate serves as one of the primary energy sources for the large intestinal colonocytes [5], but it also exerts pleiotropic effects on liver, skeletal muscle, and adipose tissues through its activity as a histone deacetylase inhibitor (HDACi) and ligand for the G-protein-coupled receptors GPR41, GPR43, and GPR109A [4,20,189]. Among other investigators [9,11,12], we have previously demonstrated that sodium butyrate (NaB) supplementation exerts beneficial effects on body composition and metabolic phenotype in C57BL/6J mice concomitantly fed a high-fat diet (HFD) [10]. The NaB treatment also improved mitochondrial function in skeletal muscle by elevating the expression of nuclear-encoded mitochondrial genes such as peroxisome proliferator-activated receptor γ coactivator-1α (Pgc-1α) and by increasing the completeness of β-oxidation [9,10].

Considering the continual rise in overweight and obesity prevalence, estimated to exceed two-thirds of the United States adult population [27], the ability of NaB to mitigate weight gain and to preserve insulin sensitivity despite an obesogenic diet makes it an attractive candidate for medical practitioners. Unfortunately, NaB treatment has been shown in mouse models to be teratogenic at higher doses [191,192], and it is not well-tolerated as a dietary intervention in humans due to its unpleasant odor. To remedy these issues, more recent experiments have used dietary fiber supplementation to increase endogenous butyrate production via the gut microbiota. Resistant starches, complexes of amylose and amylopectin that escape digestion in the small intestine and undergo fermentation in the colon, have illustrated some promise in human clinical
studies [101,102,193]. Moreover, because resistant starches are present in commonly consumed foods such as legumes, partly milled seeds, and fortified breakfast cereals [99], they can be readily incorporated into dietary interventions and generally do not cause gastrointestinal distress in healthy individuals.

The entrapment of starch granules in an alginate matrix represents a novel technique to increase delivery of fermentable carbohydrates to the saccharolytic bacteria of the distal colon [107,108]. As such, we hypothesized that these starch-entrapped microspheres (SM) would function as a resistant starch and elevate endogenous SCFA production, including butyrate, without the teratogenic effects associated with NaB treatment. The aims of the present study were threefold: 1) To assess the feasibility of SM as a dietary supplement and source of resistant starch in an in vivo system; 2) to investigate the utility of SM supplementation in preventing obesity and insulin resistance in C57BL/6J mice, a strain prone to diet-induced obesity [194]; and 3) to compare changes in body weight, body composition, and insulin sensitivity in HFD-fed mice administered SM to those parameters in HFD-fed mice receiving NaB, an intervention previously demonstrated to confer anti-obesity and anti-diabetic effects.

2.3 Materials and Methods

2.3.1 Animals and Diets

All animal care and experimental procedures were evaluated and approved by the Purdue University Animal Care and Use Committee (PACUC) prior to the study period and were in accordance with the National Institutes of Health guidelines for care and use of animals. 40 male C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Upon arrival (4 weeks of age) the mice were placed into quarantine for 1 week and fed a standard chow diet
(Teklad Global Rodent Diet 2018; Envigo; Indianapolis, IN). After the quarantine period, the mice were randomized into four dietary conditions with 10 mice per treatment group: (1) low-fat diet (LFD, 10% kcal from fat; Research Diets #D12450J; New Brunswick, NJ), (2) HFD (60% kcal from fat; Research Diets #D12492), (3) HFD supplemented with 5% wt/wt NaB (HFD + NaB), (4) HFD supplemented with 10% wt/wt SM (HFD + SM). NaB was purchased from Sigma-Aldrich (#303410; St. Louis, MO). SM were prepared by the Hamaker laboratory as described previously [107]. NaB and SM were incorporated into the respective diets via a commercially available food processor (Hamilton Beach Brands, Inc.; Glen Allen, VA).

Mice were singly housed in solid-bottom shoebox cages with corncob bedding on a 12-hour light/dark cycle under constant temperature (22-23°C). The mice had ad libitum access to both food and water, which were changed weekly. Food consumption was measured weekly over a 48-hour period. Weekly body composition was assessed using EchoMRI (EchoMRI™; Houston, TX). Scans were performed in duplicate and did not require anesthesia. Body weight was measured weekly.

2.3.2 Glucose Tolerance Tests

After 7 weeks of feeding with the intervention diets, glucose tolerance tests (GTTs) were performed. Glucose solution was prepared with D-(+)-glucose (Sigma-Aldrich; #G7021) and autoclaved-sterilized PBS. 20 mice were fasted overnight (n = 5 mice per treatment group) and received intraperitoneal injections of 2.5 g glucose/kg body weight. Blood glucose levels were assessed from tail vein samples using a one touch blood glucose monitor.
(Nova Biomedical Corporation; Waltham, MA). Samples were collected prior to the glucose injections and at 30, 60, and 120 minutes following the injections.

2.3.3 Metabolic Phenotyping

After 9 weeks of feeding, 20 mice (n = 5 per treatment group) were house individually in metabolic chambers (Oxymax Comprehensive Laboratory Animal Monitoring System (CLAMS); Columbus Instruments; Columbus, OH) with a 48-hour acclimation period. The temperature in the metabolic chamber was maintained at 23°C. Energy expenditure and substrate utilization were assessed for each mouse over a 4-day period. Respiratory exchange ratio (RER) was calculated as the ratio of oxygen consumed ($V_{O2}$) to carbon dioxide produced ($V_{CO2}$).

2.3.4 Data Analysis

All statistical analyses were performed using GraphPad Prism version 5 (GraphPad Software, Inc.; La Jolla, CA) with the significance level ($\alpha$) set at 0.05. To determine if any outliers were present, extreme studentized deviate (Grubbs’) tests were performed on all data sets prior to analysis. Phenotypic and energy expenditure data were analyzed using repeated measures ANOVA followed by Tukey post-hoc tests. One-way ANVOA with Tukey post-hoc test was performed for GTT data. Data are presented as means ± SEM unless otherwise indicated.
2.4 Results

2.4.1 Body Composition

LFD-fed mice increased their body weight by approximately 5.61 g over the course of the intervention period, from 18.28 ± 0.293 g to 23.89 ± 0.581 g (Figure 4A). The HFD mice gained significantly more weight than their LFD counterparts (P < 0.001), from 18.47 ± 0.292 g to 29.61 ± 0.809 g, representing an average increase of 11.14 g. On average, HFD + NaB-supplemented mice gained 4.43 g during the intervention period, increasing their body weight from 19.38 ± 0.362 g to 23.81 ± 0.593 g. Body weight did not differ significantly between the HFD + NaB mice and their LFD counterparts, but HFD + NaB mice had significantly lower body weight relative to the HFD control group (P < 0.001). HFD + SM-supplemented mice increased their body weight from 19.17 ± 0.293 g to 30.11 ± 0.730 g, representing an increase of approximately 10.94 g during the intervention. HFD + SM-fed mice gained significantly more body weight relative to the LFD control group (P < 0.001), but weight gain did not differ significantly from the HFD controls. HFD + SM-supplemented mice gained significantly more weight than the HFD + NaB intervention group (P < 0.001).

Fat mass increased in the LFD-fed mice from 1.09 ± 0.054 g at week 0 to 2.54 ± 0.246 g at the study conclusion, representing an average increase of 1.45 g during the experiment (Figure 4B). Mice receiving the HFD exhibited significantly greater fat mass accumulation relative to their LFD counterparts (P < 0.001); fat mass in the HFD control group increased by approximately 6.447 g during the intervention period, from 0.903 ± 0.053 g to 7.35 ± 0.412 g. Fat mass increased in NaB-supplemented mice from 1.15 ± 0.085 g to 2.93 ± 0.227 g on average, representing an increase of approximately 1.78 g during the intervention period. Fat mass in the HFD + NaB-supplemented mice did not differ significantly from the LFD controls, but was
significantly lower than the HFD control group (P < 0.001). HFD + SM-supplemented mice increased their fat mass by 5.09 g during the intervention period, from 0.90 ± 0.061 g to 5.99 ± 0.438 g. Although HFD + SM mice had significantly greater fat mass relative to LFD (P < 0.05), they also had less fat accumulation relative to the HFD controls (P < 0.05). Significant differences in fat mass among the intervention groups were detectable after four weeks of feeding.

Fat mass was also evaluated as a percentage of total body weight (Figure 4C). The LFD-fed animals increased their fat percentage from 6.065 ± 0.306% at baseline to 10.568 ± 0.883% by the end of the intervention period. The HFD control group increased their fat percentage from 5.021 ± 0.402% to 23.888 ± 0.848% at the end of the experiment. Fat mass constituted a significantly greater percentage of total body weight in the HFD group as compared to LFD mice (P < 0.001). Mice in the HFD + NaB treatment group increased their fat percentage from 5.828 ± 0.436% at baseline to 12.257 ± 0.848% in the final week of the intervention. Compared to the HFD controls, fat mass in the HFD + NaB group constituted a significantly smaller percentage of body weight (P < 0.001). Fat mass percentage did not differ significantly between the HFD + NaB treatment group and the LFD-fed mice. HFD + SM mice increased their fat percentage from 4.777 ± 0.296% to 20.674 ± 1.254% over the course of the intervention, significantly greater than the LFD (P < 0.05) and HFD + NaB (P < 0.05) groups, respectively. HFD + SM had a significantly smaller fat percentage relative to the HFD controls (P < 0.05).

Over the course of the intervention, lean body mass increased in the LFD mice from 15.278 ± 0.231 g to 18.878 ± 0.481 g, representing an average increase of 3.6 g (Figure 4D). HFD-fed animals increased their lean mass by approximately 5.024 g during the intervention, from 15.707 ± 0.465 g at baseline to 20.731 ± 0.446 g at the end of the experiment. The HFD-fed
mice had significantly greater lean body mass relative to their LFD counterparts (P < 0.001). Lean mass in the HFD + NaB-supplemented mice increased from 16.795 ± 0.408 g to 18.490 ± 0.505 g. The HFD + NaB mice had significantly less lean body mass in comparison to the HFD controls (P < 0.001), but lean mass did not differ significantly from the LFD-fed mice. Mice in the HFD + SM group increased lean mass from 15.789 ± 0.276 g to 20.354 ± 0.140 g over the course of the intervention, representing an increase of 4.565 g on average. HFD + SM mice had significantly greater lean mass in comparison to both LFD (P < 0.001) and HFD + NaB-supplemented mice (P < 0.001), respectively, but lean mass did not differ significantly from the HFD controls.

Lean mass was also assessed as a percentage of total body weight (Figure 4E). Lean percentage in the LFD group decreased from 84.629 ± 1.140% to 79.200 ± 0.695% during the intervention period. The HFD controls decreased their lean percentage from 86.553 ± 1.292% to 67.726 ± 0.873% during the experiment. Lean mass constituted a significantly smaller percentage of total body weight in HFD-fed mice relative to their LFD counterparts (P < 0.001). Mice in the HFD + NaB group decreased their lean mass percentage from 85.180 ± 0.548% at baseline to 77.644 ± 0.773% by the study conclusion. Lean mass represented a significantly greater percentage of total body weight in the HFD + NaB group relative to the HFD controls (P < 0.01), but lean percentage did not differ significantly between the HFD + NaB-supplemented mice and the LFD controls. Mice in the HFD + SM group decreased their lean mass percentage from 83.942 ± 0.647% at baseline to 70.616 ± 1.219% at the end of the intervention period. Lean percent in HFD + SM was significantly lower than that of the LFD controls (P < 0.05), but not different from the HFD controls or the HFD + NaB-supplemented mice.
2.4.2 Energy Intake and Expenditure

Energy intake and expenditure were assessed in order to determine the underlying mechanisms by which NaB and SM supplementation influenced body weight and body composition in HFD-fed mice. On average, LFD-fed mice consumed 2.446 ± 0.09215 g/day (Figure 5A). Mice in the HFD control group consumed approximately 2.266 ± 0.03475 g/day, and intake was not significantly different from the LFD group. HFD + NaB-supplemented mice consumed 2.097 ± 0.08627 g/day, significantly less food in comparison to the LFD-fed mice (P < 0.01) but not different from the HFD controls. Mice in the HFD + SM group consumed 2.61 ± 0.05677 g/day, significantly greater intake compared to both the HFD (P < 0.01) and HFD + NaB-supplemented mice (P < 0.001), respectively, but not significantly different from the LFD control group. Food consumption was also assessed with regard to caloric intake (Figure 5C). Mice in the LFD group consumed approximately 0.4405 ± 0.02103 kcal/g BW/d. The HFD-fed mice consumed 0.5025 ± 0.01255 kcal/g BW/d, significantly greater caloric intake relative to their LFD counterparts (P < 0.05). HFD + NaB-supplemented mice consumed 0.5233 ± 0.0129 kcal/g BW/d on average, significantly more than the LFD controls (P < 0.01) but not significantly different from the HFD control group. HFD + SM-supplemented mice consumed 0.58 ± 0.01644 kcal/g BW/d, significantly greater caloric intake in comparison to both the LFD (P < 0.0001) and HFD groups (P < 0.01). Caloric intake was not significantly different between the HFD + SM-supplemented mice and their HFD + NaB counterparts.

In addition to weekly measures of energy intake, cumulative food consumption was assessed. Over the course of the intervention, LFD-fed mice consumed an average of 22 ± 0.510 g (Figure 5B). The HFD control mice consumed 20.098 ± 0.572 g during the experiment, significantly less than their LFD counterparts (P < 0.01). HFD + NaB-supplemented mice
consumed 18.704 ± 0.670 g, significantly less than the LFD group (P < 0.0001), but not different from the HFD controls. Mice in the HFD + SM group consumed 23.368 ± 0.465 g during the intervention, significantly more than the LFD (P < 0.05), HFD (P < 0.0001), and HFD + NaB-supplemented mice (P < 0.0001), respectively. With regard to cumulative caloric intake, LFD mice consumed an average of 3.851 ± 0.054 kcal/g BW/d (Figure 5D). Over the course of the intervention, HFD-fed mice consumed 4.145 ± 0.073 kcal/g BW/d, significantly more than their LFD counterparts (P < 0.05). Mice in the HFD + NaB treatment group consumed approximately 4.390 ± 0.119 kcal/g BW/d during the experiment, significantly more than the LFD control group (P < 0.01), but not different from the HFD controls. HFD + SM-supplemented mice consumed 4.764 ± 0.114 kcal/g BW/d, a value significantly greater than those observed in the LFD (P < 0.0001), HFD (P < 0.0001), and HFD + NaB groups (P < 0.0001), respectively. Taken together, these data indicate that SM supplementation did not reduce energy intake and that the ability of SM to mitigate adipose tissue accumulation was not attributable to decreased food consumption.

In addition to measures of energy intake, indirect calorimetry was used to determine whether SM supplementation increased overall activity levels or altered substrate utilization in HFD-fed mice. As expected, activity levels were higher for all groups during night periods relative to day periods. On average, LFD-fed mice expended 74.9 ± 2.023 kJ/kg BW/hr (Figure 5E). The HFD controls expended approximately 65.07 ± 1.26 kJ/kg BW/hr, significantly lower expenditure relative to their LFD counterparts (P < 0.001). Mice in the HFD + NaB group expended 73.04 ± 1.677 kJ/kg BW/hr on average, significantly greater expenditure than the HFD control group (P < 0.01) but not different from the LFD-fed mice. HFD + SM-supplemented mice expended 71.48 ± 1.285 kJ/kg BW/hr on average, significantly greater energy expenditure
relative to the HFD control group (P < 0.05). Energy expenditure in the HFD + SM group did not differ significantly from that of the LFD-fed animals or the HFD + NaB intervention group.

Substrate utilization was also assessed for the four groups during the four-day period (Figure 5F). The LFD-fed mice had an RER value of 1.008 ± 0.028, indicative of predominantly carbohydrate utilization. RER in the HFD control group was significantly lower than the LFD control group (P < 0.001), reported as 0.849 ± 0.011 and suggesting greater utilization of lipids for energy requirements rather than carbohydrate. HFD + NaB-supplemented mice had an average RER value of 0.835 ± 0.01, significantly lower than both the LFD (P < 0.001) and HFD (P < 0.01) control groups, respectively. RER in the HFD + NaB-supplemented mice was also significantly lower than that observed in their HFD + SM-supplemented counterparts (P < 0.001). HFD + SM-supplemented mice exhibited an average RER value of 0.852 ± 0.01, significantly lower than the LFD controls (P < 0.001) but not significantly different from the HFD control group. Both NaB and SM supplementation were able to increase overall energy expenditure and utilization of fat as a fuel source. These metabolic alterations might therefore explain how SM treatment alters energy balance such that adipose tissue expansion is prevented despite concomitant HFD feeding.

2.4.3 Glucose Tolerance

T2DM is one of the most severe consequences of obesity and can be readily induced in murine models through HFD feeding [9,10]. To determine whether SM supplementation preserved glucose tolerance in the peripheral tissues, intraperitoneal GTTs were performed after 7 weeks of the intervention diets (Figure 6A). Blood glucose in the LFD control group was reported as 109.2 ± 12.524 mg/dL at baseline, increased to 402.2 ± 47.856 mg/dL 30 minutes
after the glucose injections, decreased to 223.5 ± 14.928 mg/dL one hour after the injections, and ultimately fell to 149.4 ± 9.574 mg/dL after two hours. Baseline blood glucose in the HFD control group was reported as 109 ± 7.232 mg/dL; following the injections, blood glucose increased to 504 ± 27.644 mg/dL at the 30 minute mark, fell to 401.5 ± 22.033 mg/dL after one hour, and further decreased to 202 ± 8.672 mg/dL after two hours. HFD + NaB supplemented mice had average blood glucose levels of 131.6 ± 5.036 mg/dL at baseline. Blood glucose increased to 423.6 ± 47.977 mg/dL 30 minutes following the glucose injections, decreased to 232 ± 13.646 mg/dL after one hour, and further decreased to 167.8 ± 7.165 mg/dL by the two hour mark. For the HFD + SM supplemented mice, blood glucose was reported as 171.76 ± 7.677 mg/dL at baseline, increased to 401.2 ± 27.920 mg/dL by the 30 minute mark, decreased to 315 ± 16.38 mg/dL after one hour, and further fell to 196 ± 10.085 mg/dL by the two hour mark.

Area under the curve (AUC) analysis indicated that plasma glucose levels were significantly elevated in the HFD-fed animals during the GTT relative to the LFD control group (P < 0.001) (Figure 6B). Mice in the HFD + NaB group had significantly better glucose tolerance relative to both the HFD controls (P < 0.001) and the HFD + SM treatment group (P < 0.05), but the glycemic response did not differ significantly from that of LFD-fed mice. HFD + SM-supplemented mice had significantly better glucose tolerance compared to HFD (P < 0.05), and their response during the GTT did not differ significantly from the LFD controls. These data indicate that SM are able to effectively prevent or otherwise mitigate the progressive loss of glucose tolerance that is commonly associated with diet-induced obesity.
Figure 4. Phenotypic Data.

(A) Body weight was measured weekly. (B) Fat mass was assessed weekly using EchoMRI. (C) Percent fat mass relative to total body weight. (D) Lean mass was assessed weekly using EchoMRI. (E) Percent lean mass relative to total body weight. For all graphs, different superscript letters denote significant differences among treatment groups.
Figure 5. Energy Intake and Expenditure.

(A) Food consumption was measured weekly over a 48 hour period. (B) Cumulative food consumption over the course of the intervention. (C) Calorie intake relative to body weight. (D) Cumulative calorie intake relative to body weight. (E) After 9 weeks of feeding, mice were individually housed in metabolic chambers to assess energy expenditure (N = 20). Grey bars depict night periods and open bars depict day periods. (F) Respiratory exchange ratio (RER) was determined during the same period as energy expenditure. For all graphs, different superscript letters denote significant differences among treatment groups.

(figure continues)
Wee k s on Diet
F o o d C o n s u m p tio n (k c a l/g B W /d )

C

D

Food Consumption (kcal/g BW/d)

Weeks on Diet

(figure continues)
Figure 6. Glucose Tolerance Measures.

After 7 weeks of feeding, glucose tolerance tests (N = 20) were performed. (A) Glucose tolerance test. (B) Area under the curve (AUC) analysis for glucose tolerance test. For all graphs, different superscript letters denote significant differences among treatment groups.
2.5 Discussion

Our primary findings indicate that dietary SM supplementation is a safe alternative to NaB that has the potential to maintain insulin sensitivity and diminish adiposity in the C57BL/6J mouse model of diet-induced obesity. SM-supplemented mice also showed significantly higher energy expenditure relative to the HFD-fed controls, as well as lower RER levels relative to mice maintained on LFD. Previous work has illustrated increased utilization of fat in mice receiving butyrate supplementation \cite{9,10}, and our data suggest that SM may function through a similar mechanism to improve body composition. These data are consistent with other experiments utilizing murine models that are particularly susceptible to the development of obesity and insulin resistance or T2DM \cite{195-197}.

In an experimental model of diet-induced obesity, male Wistar rats received HFD in conjunction with resistant starch (derived from high amylose maize), the polysaccharide chitosan, or a starch-chitosan complex \cite{195}. While HFD-fed rats rapidly gained weight in comparison to their LFD-fed counterparts, resistant starch supplementation mitigated body weight gain over the six week intervention period \cite{195}. Although differences in blood glucose levels were not statistically significant among the treatment groups, HFD feeding illustrated a trend toward hyperglycemia that was attenuated by resistant starch supplementation \cite{195}; as noted by the investigators, it is likely that extending the treatment period would accentuate these differences in insulin sensitivity \cite{195}, similar to what we observed in the present study with SM and NaB supplementation.

A similar study assessed the effect of cellulose, inulin, or guar gum supplementation in C57BL/6JRj mice concomitantly receiving HFD over the course of 30 weeks \cite{196}. In comparison to the cellulose and guar gum intervention groups, inulin-supplemented mice gained
significantly less body weight [196]. Although lean mass was similar across all treatments, inulin supplementation greatly reduced total body fat to levels comparable to LFD controls [196]. Energy intake did not differ significantly among the intervention groups, but the respiratory quotient (RQ) values of the intervention groups were lower relative to the LFD-fed mice, suggesting a transition toward greater fat oxidation [196]. Interestingly, we also saw lower RER, representing greater fat oxidation, with NaB supplementation but not with SM. During an oral glucose tolerance test, inulin-supplemented mice exhibited significantly lower blood insulin levels relative to the cellulose treatment at the inflection point of the curve [196]; the inulin treatment group also exhibited significantly lower blood glucose levels relative to the guar gum intervention 60 minutes after glucose load administration [196], suggesting increased insulin sensitivity similar to our NaB and SM groups.

Recent experiments have not only considered resistant starch as a treatment option for obesity and T2DM once they become clinically relevant, but also as a functional supplement that may delay the onset or progression of these disease states [3,14,197]. In this vein, Hedemann and colleagues examined the effect of resistant starch supplementation on preventing or delaying T2DM manifestation in male Zucker diabetic fatty rats [197]. After 9 weeks of feeding, resistant starch-supplemented rats exhibited significantly lower HbA1C and fasting plasma insulin levels relative to all other treatment groups [197]. Moreover, fasting plasma glucose levels in the resistant starch group were lower than those reported for the Glucidex- and EMS-fed groups [197]. Excretion of glucose in the urine, assessed at both the second and ninth week of feeding, was also significantly lower in the resistant starch intervention compared to all other treatments [197]. Importantly, these beneficial metabolic effects occurred despite elevated food consumption in the resistant starch group and greater total body weight at the study conclusion.
These findings are similar to our own observations, as SM supplementation conferred protection against the development of diet-induced adiposity and insulin resistance, but these effects did not occur as the result of caloric restriction among the HFD + SM mice.

It has traditionally been assumed that the beneficial effects of resistant starch supplementation are necessarily mediated through the gut microbiome. To determine whether resistant starch can confer metabolic benefits independently of microbial fermentation, Bindels et al. assessed the effect of HFD feeding (~49% kcal from fat) on both germ-free and conventionalized C3H/HeN mice supplemented with either type 2 (RS-2) or type 4 (RS-4) resistant starch [198]. After 8 weeks of feeding, both fasting plasma glucose and insulin levels were significantly lower in RS-4 treated mice compared to the HFD control mice, irrespective of microbiome status. RS-2 also decreased these parameters relative to HFD, but the trend was not statistically significant. Interestingly, resistant starch supplementation was not associated with significant changes in body weight, white adipose tissue weights, or lean mass (tibialis muscle) weight in this experiment. These data indicate that resistant starches have the potential to exert beneficial effects on peripheral insulin sensitivity independently of their fermentation in the colon.
CHAPTER 3. SUMMARY AND FUTURE DIRECTIONS

Although our findings support the hypothesis that SM can induce anti-obesity and insulin-sensitizing effects in C57BL/6J mice, the translational potential for human health warrants further investigation. Prior clinical trials incorporating dietary fiber, particularly resistant starches, have illustrated some benefit with regard to insulin sensitivity. Some preliminary work with SM has assessed insulin sensitivity in humans [199], and the results of a phase 1 clinical trial have recently been published examining whether SM supplementation can be safely incorporated into the human diet at low doses with minimal GI complications [200].

Previous studies revealed elevated expression of PGC-1α, the transcriptional coactivator involved in regulating mitochondrial adaptations in the skeletal muscle, following NaB treatment. It is possible that SM treatment similarly improved mitochondrial number and the capacity for β-oxidation of fatty acids, and future work can assess these possibilities.

One limitation in the current study pertains to dietary intake. We observed a high level of food consumption for HFD + SM relative to the other treatment groups, but the underlying mechanism for this effect is not clear. A pair-feeding model could ensure that the beneficial effects of SM supplementation occur independently of changes in dietary intake. Our findings could also be attributable to differential SCFA production. For example, it is likely that bacterial fermentation of SM produces an array of SCFAs, each of which may exert its own effects on the peripheral tissues. The receptors GPR41 and GPR43 are responsive not only to butyrate, but also to propionate and acetate [15], and so different SCFA profiles among the treatment groups could potentially contribute to the observed differences in body composition or phenotype.

The increasing prevalence of both obesity and T2DM necessitates the development of new treatment strategies that can ameliorate the consequences of these conditions. Although
further testing is required, SM might ultimately serve as one treatment option in a personalized health plan due to their tolerability in the GI tract, low risk of toxicity compared to dietary NaB or pharmacologic HDACi, and relative ease of storage and use after manufacturing.
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VITA

Sean McNabney was born in Hammond, Indiana. He obtained his Bachelor of Science degree in Biology and Psychology, with minors in Chemistry and Humanities from Valparaiso University in May 2016. In August of the same year, Sean began graduate work in the Interdepartmental Nutrition Program at Purdue University under the supervision of Dr. Tara Henagan. He received several research opportunities as well as experience teaching undergraduate students in an intermediate-level nutrition course. Sean’s research project at Purdue explored the efficacy of resistant starch supplementation to mitigate diet-induced obesity and insulin resistance in the C57BL/6J mouse model. In addition to his research and teaching responsibilities, Sean also served as Vice President of the Nutrition Science Graduate Student Organization (NSGSO) and the Nutrition Science student representative for the Interdisciplinary Graduate Program Student Advisory Board (IGPSA Board).
PUBLICATIONS
