PRESERVING THE INTESTINAL EPITHELIAL INTEGRITY IMPROVES METABOLIC SYNDROME

by

SUNHYE LEE

(Under the Direction of Claire de La Serre)

ABSTRACT

Metabolic disorders including obesity and its comorbidities are major health problems in the United States where western diets, rich in fat, are widely consumed. Recent studies have shown that increased gastrointestinal (GI) tract permeability in response to high fat (HF) allows for translocation of pro-inflammatory bacterial products to the circulation and promotes systemic inflammation. Chronic inflammation plays a key role in the development of metabolic disorders. HF diet-induced GI inflammation impairs intestinal barrier integrity. Although the causal relationship between increased gut permeability and systemic inflammation has attracted recent interest in the metabolic area, there is little data available on strategies to restore gut epithelial barrier integrity. Thus, the objective of this dissertation is to investigate potential candidates to preserve GI barrier integrity in response to a HF challenge. I hypothesized that preserving the GI barrier integrity will prevent systemic inflammation and consequent deleterious effects in rats fed HF diets. For all studies, Wistar rats were fed a HF diet for 7 to 8 wk to induce inflammation and supplemented with either a bioactive food (blueberry, Chapter 2) or a probiotic (Chapter 3). For the probiotic study, I employed a novel approach of microencapsulation to enhance the efficacy of probiotic delivery to the distal gut. Microbiota composition, intestinal integrity, inflammation,

and glucose homeostasis were assessed. In the first study (Chapter 2), blueberry supplementation decreased inflammation and insulin resistance in HF-fed rats in conjunction with compositional changes in gut microbiota and improved gut integrity. These changes may have prevented bacterial pro-inflammatory product translocation, resulting in reduced systemic inflammation and improved hepatic insulin sensitivity in HF-fed rats. In another study (Chapter 3), although the efficacy of microencapsulation was not verified, probiotic (*Lactobacillus paracasei subsp. paracasei*)-induced improvements in metabolic profiles in HF-fed rats were confirmed to be associated with compositional changes in gut microbiota and improved gut barrier integrity. These effects were primarily associated with alleviation of local and systemic inflammation. Collectively, the studies of this dissertation suggest that preserving the intestinal barrier improves inflammatory conditions in HF-fed rodents and identify potential dietary interventions for the prevention of obesity-associated metabolic abnormalities.

INDEX WORDS: intestinal epithelial barrier, gut microbiota, inflammation, glucose homeostasis, blueberry, probiotic, microencapsulation

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW[1, 2]

Introduction

Obesity prevalence has increased dramatically in the United States (1). Obesity is associated with metabolic syndrome, which has an estimated prevalence of 25% in the United States (2). There is accumulating evidence that increased gastrointestinal tract (GI) permeability can promote systemic inflammation, leading to metabolic disorders (3). The consumption of a high-fat (HF) diets has been shown to induce deleterious changes in microbiota composition, promoting GI inflammation and resulting in disrupted tight junction (TJ) protein network and increased GI permeability (4). Although the association among gut microbiota dysbiosis, gut permeability and inflammatory state has been the recent source of interest in the metabolic area (5-7), there is limited data available on potential strategies to restore gut epithelial barrier integrity. Thus, through a novel approach using animal models treated with dietary interventions

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[1]Adapted from Lee SH, Claire B. Gut microbiome-brain communications regulate host physiology and behavior. J Nutrition 2015:2. Reprinted here with permission of publisher.

[2]Vaughn AC, Cooper EM, DiLorenzo PM, O'Loughlin LJ, Konkel ME, Peters JH, Hajnal A, Sen T, Lee SH, de La Serre CB, et al. Energy-dense diet triggers changes in gut microbiota, reorganization of gut-brain vagal communication and increases body fat accumulation. Acta Neurobiol Exp (Wars) 2017;77:18-30. Reprinted here with permission of publisher.

(blueberry or a probiotic), my dissertation research studies will contribute to filling the void of research on identifying potential pathways to reduce gut permeability in obesity and prevent systemic inflammation.

Microbiota and gastrointestinal health

The human gut contains over 10^{14} microorganisms (8, 9) which are involved in varied functions such as digestion of plant polysaccharides, regulation of host fat storage, and epithelial and innate immune development (10, 11). The intestinal epithelium forms a barrier against potent pathogens but allows regulated active and passive transport (12). The mucosal barrier function is regulated by the apical junctional complex composed of TJs and adherens junctions (13) with TJs being the most critical component as their permeability defines the overall barrier integrity (12). The development of GI inflammation disrupts the epithelial barrier. Incubation of T84 epithelial monolayers with pro-inflammatory cytokines such as interferon γ (IFN-γ) and tumor necrosis factor alpha (TNFα), results in an increased permeability associated with internalization of TJ proteins, occludin in particular (13). Bacterial pathogens can promote gut inflammation; for example, lipopolysaccharide (LPS), a component of the outer-membrane of Gram-negative bacteria, binds to the Toll-like receptor 4 (TLR4) and induces an inflammatory response and synthesis of pro-inflammatory cytokines like interleukin (IL)-1, -6 and TNFα (14-16). Therefore, the microbiota composition can affect the intestinal barrier integrity. Microbiota composition is particularly involved in obesity. Indeed, comparisons between microbiota of lean and obese patients (17) and obese and lean rodents (18, 19) have shown that obesity is associated with changes in microbiota composition, specifically with an increase in the ratio of *Firmicutes* to *Bacteroidetes* (17). Interestingly, genetic (ob/ob and db/db) and diet-induced obese mice also display a lower intestinal resistance than their littermates associated with modification in TJ

protein distribution and/or expression (20-22). Epithelial barrier disruption seems to be tightly linked to the microbiota composition as treatment with prebiotics or antibiotics restores epithelial functions (23, 24). In my recent study (Appendix), I investigated whether normalization of the microbiota composition in HF-fed animals using a broad-spectrum antibiotic (minocycline) would protect from HF diet-induced deleterious metabolic consequences. As a result, minocycline decreased energy intake and body fat accumulation induced by HF feeding (**Figure 1.1**). HF feeding rapidly and significantly altered gut microbiota composition and this was improved by minocycline (**Figure 1.2, 3**). Also, minocycline reduced HF diet-induced metabolic endotoxemia (increases in plasma levels of LPS) (**Figure 1.3**). Taken together, these data confirmed that HF feeding induces gut microbiota dysbiosis, promoting inflammatory responses, and that normalization of the gut microbiota composition via minocycline can blunt the deleterious effects of HF feeding on metabolic functions.

GI permeability and inflammation

The epithelial layer of the GI tract serves as one of the primary interfaces with the outside world (25). The intestinal barrier formed by the epithelial cells and the junctional complex such as TJs exclude the majority of microbes and their metabolites from access to the subepithelial cells (26). However, disruption of the intestinal TJ barrier results in a "leaky" gut and leads to an increase in intestinal permeability (27). A leaky GI tract allows paracellular permeation of luminal antigens that promote inflammation, resulting in metabolic disorders including insulin resistance (28).

Of a variety of factors that can affect mucosal permeability and initiate inflammatory cascades, HF diet consumption has been shown to increase intestinal permeability by reducing the expression of genes coding for TJ proteins and by disturbing the TJ protein network,

allowing translocation of pro-inflammatory factors such as LPS (29). The increased plasma LPS can bind to its receptor, TLR4, expressed on epithelial cells and immune cells to trigger the release of pro-inflammatory cytokines (29, 30). In rodent models, chronic administration of low-dose LPS can trigger weight gain and insulin resistance and the increase in circulating LPS or endotoxemia has notably been shown to promote energy intake via alteration in cholecystokinin signaling (5, 31).

Under normal physiological conditions, LPS does not cross the GI wall and mechanisms are in place to protect the host against LPS-induced inflammation; intestinal alkaline phosphatase (IAP), a duodenal enzyme, is notably able to detoxify LPS (32). In HF diet-induced obesity, the GI tract becomes leaky and IAP activity decreases, resulting in an increase in toxic LPS bioavailability on the basolateral side (31).

HF feeding directly alters intestinal microbiota composition to promote gut inflammation and metabolic endotoxemia (31). Commensal microbiota adhesion to the mucosa is a part of the natural mechanisms that protect the organism from pathogenic microorganisms (33). Under normal physiological conditions, microbiota prevents the attachment of pathogenic microorganisms to mucosal surfaces and their invasion into epithelial cells and the circulation (34). However, diet-driven dysregulated microbiota (dysbiosis) has been linked to chronic low-grade intestinal inflammation and the leakage of gut microbiota-derived LPS to the circulation (35).

The role of a ‘leaky gut’ in the pathogenesis of metabolic disorders is increasingly recognized. Consequently, reducing increased gut permeability is an interesting target for improvement of the clinical status of these diseases.

Blueberry

Blueberries and blueberry components

Blueberries (*Vacciniaceae*) are classified as highbush, lowbush, and rabbiteye (36). Blueberries have one of the highest concentrations of anthocyanins among commonly consumed US fruits (37). Cultivated blueberries have approximately 390 mg of anthocyanins per 100 g fresh weight (37). Blueberries contain other phenolic compounds including phenolic acids, the flavonols kaempferol, myricetin, and quercetin, the flavone luteolin, catechin and epicatechin, and proanthocyanidins. A high percentage of polyphenolics reach the lower intestinal tract due to low bioavailability (38, 39). These compounds are known to have antioxidant and anti-inflammatory properties (40-43) that may reduce oxidative stress and inflammation in the intestinal tract. Anthocyanin and total phenolic concentrations of blueberry are strongly correlated with antioxidant capacity (44). In addition, blueberries have antimicrobial properties against pathogenic bacteria (45). Thus, blueberry polyphenolics may modulate bacterial populations in the gut, protecting the GI barrier. However, the influence of blueberry supplementation on microbiota composition has yet to be determined.

Anthocyanin bioavailability

Anthocyanins can be absorbed in the intestinal tract of rats and humans (46). Glycosylated anthocyanins have been found in the stomach, plasma and urine of rats and humans, suggesting that some can be absorbed intact, and metabolites of anthocyanins, including phenolic acids, have been documented in plasma, bile, urine, jejunum, liver, kidney and brain (47-51). Absorption and metabolism depend on the structures of the anthocyanin aglycones and the attached sugars (37, 48, 52, 53). However, low concentrations of ingested anthocyanins in plasma and urine suggest low bioavailability (46, 49, 52). Thus, high anthocyanin concentrations

are present in the intestinal lumen (38, 53) where they may impact gut health and interact with the commensal microbiota (39, 46, 50, 54).

Blueberry, anthocyanins and microbiota

Blueberries have antimicrobial properties against pathogenic bacteria (45, 55). Lacombe et al. (56) demonstrate *in vitro* selective antimicrobial effects of blueberry fractions on foodborne pathogens and on the probiotic *lactobacillus rhamnosus.* Five different blueberry fractions, including the anthocyanin fraction, inhibited growth of these bacteria, but the probiotic was the least susceptible to inhibition. In contrast, Hidalgo et al. (57) demonstrate that purified anthocyanins increased the growth of beneficial gut bacteria in culture, including *Bifidobacterium spp.* and *Lactobacillus-Enterococcus spp*. Additionally, Molan et al. (58) report that water soluble blueberry extracts increased populations of beneficial gut bacteria *Lactobacillus rhamnosus* and *Bifidobacterium breve in vitro* and in the ceca of healthy rats. In a human model, consumption of wild blueberry juice for 6 wk led to increases in the beneficial *Bifidobacterium spp.* in human feces (59). The effect of blueberry on GI microbiota and bacterial translocation has been studied in models of GI inflammation. Blueberry supplementation (10%) for 21 wk in *mdr*1a$^{-/-}$ mice, a model for inflammatory bowel disease (IBD), altered cecal microbiota, decreasing bacterial populations associated with IBD such as the pathogenic *clostridium perfringens*, and increased intestinal crypt height (43). Supplementation with freeze-dried blueberry powder reduced gut lipid peroxidation, inflammation and bacterial translocation from the intestine to the liver and lymph nodes in rodents with dextran sulfate sodium-induced colitis (60) and reduced liver inflammation and bacterial translocation to the liver from the gut in a model of hepatotoxicity (61). These studies suggest that blueberry may have a beneficial impact on gut microbiota, reduce gut inflammation, and prevent bacterial translocation.

Additionally, blueberry phenolics may decrease bacterial adhesion to GI epithelial cells as berry phenolics can disrupt the outer membrane of pathogenic bacteria (55). Phenolics notably destabilize LPS found on the membrane of Gram-negative bacteria (45, 56).

Blueberry, anthocyanins and metabolic diseases

With the emergence of databases including anthocyanin content of foods, some epidemiological studies have linked higher intakes of anthocyanins with reduced risk for chronic disorders including obesity and type II diabetes (62). Both human double-blind, randomized controlled trials and rodent studies provide supportive evidence that anthocyanin-rich blueberries improve insulin sensitivity associated with obesity (42, 63). Blueberry consumption notably decreases oxidative stress and inflammation (64, 65). Rodent studies suggest that isolated anthocyanin consumption can reduce oxidative stress (66, 67). Whole blueberry supplementation results in a decrease in DNA oxidation (41) and a reduction of biomarkers of lipid peroxidation and LDL-oxidation (68) in human subjects. A study by Dulebohn et al. demonstrates a significant reduction in liver DNA oxidation in rats after 3-wk supplementation of 1% blueberry flavonoids (40) while others report that 8-wk whole blueberry supplementation (8%) reduces DNA oxidation in lymphocytes (50).

With research linking gut microbiota to obesity-associated inflammation and the knowledge that blueberry constituents may decrease populations of pathogenic bacteria or translocation of bacteria, I hypothesized that blueberry supplementation in HF-fed rats would lead to beneficial changes in gut bacterial populations associated with improvement in inflammatory and metabolic status.

Probiotics

Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host (69). For organisms to be considered as probiotics, a bacterial strain should have certain characteristics (70). First, the strain must reach its site of action, usually the gut, and thus survive to physiological stress met during its ingestion; acidic stomach, gut pH, and presence of biliary salts. Then, it must have proved its beneficial effect for the host. Its ingestion must not present any risk for the host. Furthermore, it must maintain its characteristics and remain stable during the manufacturing process and its conservation in the matrix in which it was incorporated.

Mechanisms of action

Probiotics acts via modulation of the intestinal microbial composition by suppressing pathogenic bacteria such as clostridia and/or increasing or protecting beneficial populations such as *bifidobacteria* (71). Probiotics can inhibit growth of enteric pathogens by direct antimicrobial actions via production of inhibitory substances, immune cell stimulation, competitive exclusion for epithelial binding receptors, and improvement of epithelial barrier integrity (72, 73).

Probiotics can have direct antimicrobial properties by producing inhibitory substances such as organic acids and bacteriocins, peptides with antibacterial properties (74, 75). *Lactobacillus* and *Bifidobacterium spp*. exhibit anti-infective effects against enterohemorrhagic *E. coli* O157:H7 in human intestinal cells. Both probiotics produce lactic acid and subsequently decrease luminal pH (76, 77). Corr, et al. (78) demonstrated that *L. Salivarius* UCC118 protected mice from the invasive foodborne pathogen *L. monocytogenes* via stimulation of bacteriocins.

Probiotic bacteria can also act as immunomodulatory agents and alleviate the inflammatory response to infection in the host GI tract (78, 79). They can substantially reduce

pro-inflammatory cytokines such as TNFα and IFN-γ while stimulating anti-inflammatory cytokines such as IL-10 and transforming growth factor beta (78, 80, 81). *B. breve* and *S. thermophilus* have been shown to produce metabolites which inhibit TNFα secretion from peripheral blood mononuclear cells (78, 80-83). Some probiotic strains induce secretory immunoglobulin A (IgA) production, leading to activation of regulatory T and dendritic cells (84). For example, the oral administration of *L. casei* to BALB/c mice induces activation of the gut mucosal immune system by increasing the level of IgA+ cells (85).

Some probiotic bacteria can compete with pathogens for binding sites to the mucous layer or epithelial cells (86, 87). Surface-layer proteins are located outside the bacterial cell wall and are involved in tissue adherence. A study using human epithelial (HEp-2 and T84) cells shows that surface-layer protein extracts from *L. helveticus* block the adherence of *E. coli* O157:H7 to epithelial cells (88).

In addition, probiotics are capable of improving epithelial barrier integrity by upregulating host mucin expression (89). Mucins are primary components of the mucosal layer in the GI tract and form a defensive physicochemical barrier to protect the intestinal epithelium from pathogenic invasion (90). In an *in vitro* study using the Caco-2 cell line, *L. casei* GG is shown to induce mucin 2 (MUC2) expression, inhibiting bacterial translocation to the intestinal epithelium (89). Some probiotics can also stimulate the release of defensins from the host, which act as antimicrobial peptides (91). Several probiotic strains have been found to strengthen intestinal barrier function in Caco-2 cells by upregulating the expression of human β-defensin 2 (DEFβ2). They include *B. longum, B. infantis, B. breve, L. acidophilus, L. casei, L. delbrueckii ssp. bulgaricus, L. plantarum,* and *S. salivarius ssp. thermophilus* (92).

Classification, commercial forms, dosage

There are many different microorganisms currently used as probiotics (93). The most frequently used bacterial genera are lactic bacteria, mainly the *Lactobacillus* and *Bifidobacterium* genera. Other genera are also used such as the *Enterococcus, Streptococcus, Leuconostoc* genera. To better understand how bacteria are named and classified, the following discussion may be helpful. Genus is the first name of a bacterium (e.g., *Lactobacillus*). It is somewhat general and refers to a grouping of organisms based on similarity of qualities, such as physical characteristics, metabolic needs, and metabolic end products. Species is a bacterium's second name (e.g., *paracasei*). It is a much narrower classification based on shared common characteristics that distinguish them from other species. Strain is an even more specific classification that divides members of the same species into subgroups based on several properties that these bacteria have in common that are distinct from other members of the species (e.g., strain LA5) (94).

There are two main forms in which probiotic organisms can be ingested; fermented foods and supplements. Fermented foods can be of both dairy and vegetable origin, with the most commonly known of each being yogurt and sauerkraut, respectively. Probiotic supplements consist of freeze-dried (lyophilized) bacteria in powder, capsule, or tablet forms. Regardless of the form in which the microorganisms are consumed, for clinical efficacy, products containing probiotic organisms must provide live organisms in sufficient numbers to exert therapeutic effects. Both types of fermented foods and supplements are able to do this (95).

The dosage of probiotic foods and supplements is based solely upon the number of live organisms present in the product. Successful results have been attained in clinical trials using between $10^7$and 10^{11} viable bacteria per day (96, 97). Interestingly, it appears that 100 times

fewer viable bacteria need to be given in a dairy medium than in a freeze-dried supplement to achieve similar numbers of live bacteria in the distal gut [1, 58]. Dairy products appear to work as an ideal transport medium for the bacteria, enhancing their survival through the upper GI tract (98).

Probiotics and metabolic diseases

Experimental and clinical studies have found that probiotics can have therapeutic effects on metabolic diseases, suggesting that certain probiotic strains can modulate various aspects of metabolic disorders. For example, oral administration of *L. rhamnosus* (LGG) to HF-fed mice for 13 wk leads to increased expression of fatty acid oxidation genes in the liver and decreased expression of gluconeogenic genes (99). LGG treatment also increases glucose transporter-4 gene expression in skeletal muscle and adiponectin production in adipose tissue (99). In addition, administration of dual probiotic strains (*L. curvatus* HY7601 and *L. plantarum* KY1032) to diet-induced obese mice leads to not only reduced body weight gain and fat accumulation, but also decreased levels of plasma insulin, leptin and total cholesterol (100). Pro-inflammatory genes (including TNFα, IL-6, IL1β and monocyte chemotactic protein (MCP1)) were downregulated in adipose tissue and fatty acid oxidation-related genes were upregulated in the liver (100). The reduction of cytokine expressions in probiotic-treated mice may have been due to decreased levels of pro-inflammatory LPS in the circulation (101) in that probiotic treatment improves intestinal barrier integrity, thereby decreasing LPS translocation to the circulation. Similarly, *Lactobacillus paracasei* subsp. *paracasei* NTU 101 restores the HF, high-fructose diet-induced TJ impairment in the colon, thereby reducing serum LPS and inflammatory cytokine levels (102).

In a clinical setting, *B. infantis* 35624 has been orally administrated for 6 to 8 wk to patients with GI (ulcerative colitis) or non-GI (chronic fatigue syndrome, psoriasis) disorders (103). *B. infantis* 35624 treatment reduced levels of plasma C-reactive protein and pro-inflammatory cytokines (TNFα and IL-6) in three inflammatory disorders when compared to placebo. Furthermore, *B. infantis* 35624 significantly decreased LPS-stimulated secretion of TNFα and IL-6 in *B. infantis* 35624-treated healthy subjects compared to the placebo group. *B. infantis* 35624 appears to have both local (GI) and systemic effects (103). Similarly, administration of *L. paracasei* CNCM I-1572 induces a significant reduction in genus *Ruminococcus*, significant increases in serum acetate and butyrate, and a significant reduction in the pro-inflammatory cytokine IL-15 in patients with irritable bowel syndrome (IBS), suggesting *L. paracasei* CNCM I-1572 can modulate structural and functional composition of the gut microbiota and reduce inflammatory activation in IBS (104).

Probiotics and microencapsulation

In order to exert their functions, probiotic bacteria should be viable in high concentrations when they reach the distal part of the intestine where they can interact with commensal microbiota (105). However, the delivery of orally ingested probiotics may be restricted (loss of viability) by the harsh environmental conditions of the GI tract (e.g. acidic conditions of the stomach, digestive enzymes, and bile salts of the small intestine), limiting the health benefits of the probiotics (106-108). Thus, it is desirable to develop methods that increase the viability of the probiotic cells until the lower GI tract is reached. In this regard, the recent emergence of microencapsulation technique appears to be a potential solution to enhance the survivability of probiotics (109). The use of microcapsules in polymer membranes has notably been shown to successfully protect viable probiotic bacteria following oral delivery in humans and rodents

(110). Formulations for microencapsulation include alginate beads, alginate-chitosan, gellan-alginate, pectin-casein, whey protein, and so forth (110) .

Significance

Metabolic disorders including obesity and its comorbidities are major health problems in the United States where western pattern diets, rich in fat, are widely consumed (1, 111). Although there is evidence that diet-induced increase in gut permeability serves an important role in the pathogenesis of metabolic disorders, there is limited data available on potential strategies to restore gut epithelial barrier integrity. My dissertation studies are significant because identification of interventions to improve intestinal barrier integrity could contribute to reducing the prevalence of metabolic disorders. HF-induced gut dysbiosis is linked to the intestinal epithelial barrier impairments (23, 24) and modulation of gut microbiota can exert protective effects against metabolic disturbances (112). There is evidence that components of blueberry reduce inflammation in obesity models (42). Blueberries are notably rich in polyphenols, particularly anthocyanins (37). Because the gut microbiota impacts systemic inflammation in obesity, I aimed to examine whether high anthocyanin-containing blueberry may improve gut health and systemic inflammation via microbiota manipulation in an obesity model (113). I also examined the beneficial health effects of a probiotic as experimental and clinical studies have found that microbiota manipulation via probiotics has therapeutic effects on metabolic diseases (100, 103). However, their *in vivo* health benefits may be limited depending on their survival and sustenance during gastrointestinal transit, which could be enhanced by a microencapsulation technique. Thus, I employed a novel technique of encapsulating a probiotic with pectin to increase the survivability of the probiotic to the distal gut where most microbiota resides. The

knowledge obtained from these studies will support the development and use of therapeutic strategies aiming to preserve GI barrier integrity in obesity.

Hypotheses and Specific Aims

I hypothesized that preserving the GI barrier integrity will prevent systemic inflammation and metabolic disorders in a rat model of obesity. This hypothesis was tested by feeding male Wistar rats a HF diet for 7 to 8 wk to induce an inflammatory state (114) and supplementing them with either blueberry (bioactive food) or a probiotic.

The specific aims and corresponding hypotheses are:

Specific Aim 1 (Chapter 2). To determine the role of blueberry (anthocyanin-rich food) in promoting gut health. I hypothesized that blueberry consumption would improve microbial composition (increased ratio of beneficial to pathogenic bacteria) and reduce intestinal permeability and inflammation.

Specific Aim 2 (Chapter 3). To investigate a novel approach for the oral delivery of microencapsulated probiotic bacteria *Lactobacillus paracasei subsp. paracasei* L. casei W8® (L. casei W8) and its potentials in the prevention of HF diet-induced metabolic disorders. I hypothesized that probiotic supplementation would improve diet-induced inflammation and epithelial barrier integrity via positive alterations in intestinal microbiota composition. I also anticipated the microencapsulation would significantly enhance delivery and effects of the probiotic.

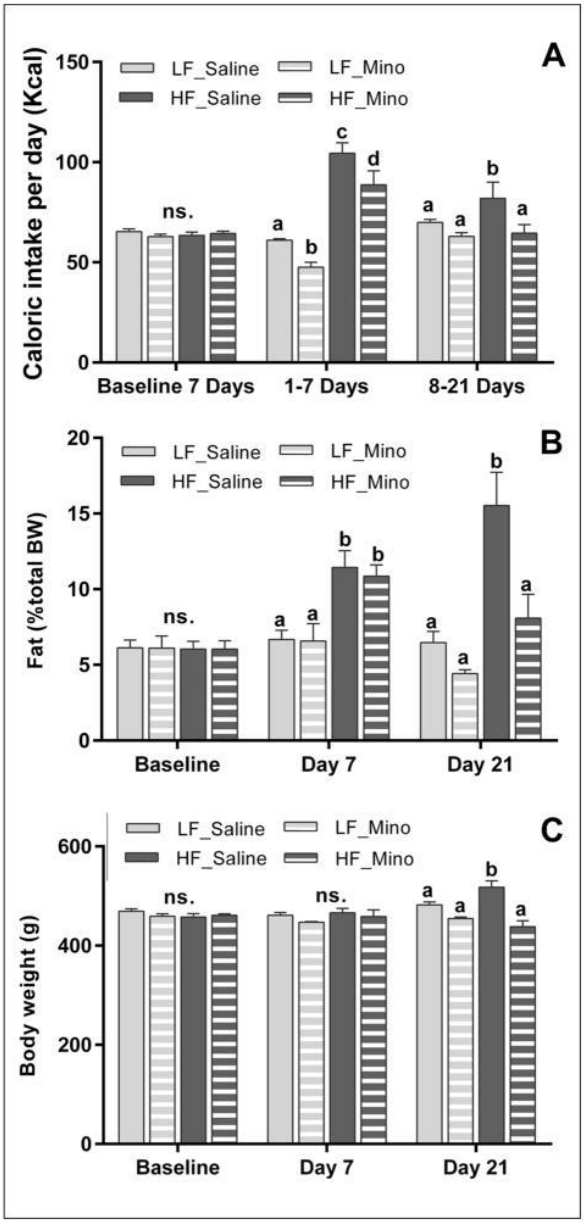

Figure 1.1. Minocycline treatment decreased energy intake and body fat accumulation induced by HFD. Seven days exposure to HFD (High fat diet) significantly increased energy intake (A, $P < 0.001$; n=8/group) and body fat accumulation (B, $P < 0.05$; n=8/group). After 21 days, when compared to saline-treated animals, HFD minocycline-treated rats exhibited a significant decrease in energy intake (A, $P < 0.05$; n=4/group), body fat accumulation (B, $P < 0.05$; n=4/group) and in body weight (C, $P < 0.05$; n=4/group). Minocycline reduced the caloric intake in LFD rats only in the first week (A). However, between the 8th and 21st day of the experiment, this effect was abolished (A). Minocycline did not affect body fat accumulation and body weight of LFD rats (B, C). Bars represent the average value ± SEM; a, b, c – different letters denote significant differences.

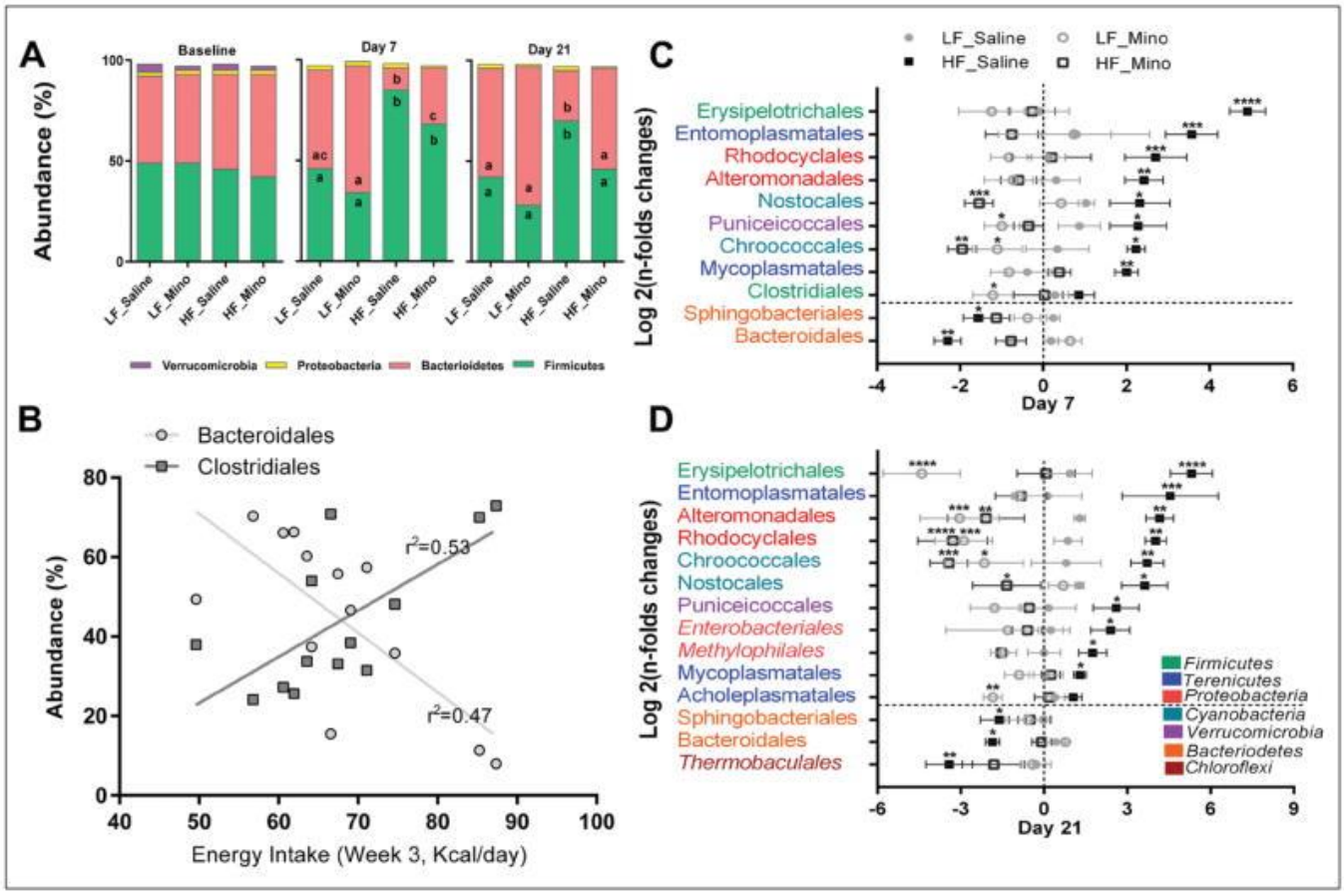

Figure 1.2. Bacterial composition altered by HFD was improved by minocycline treatment. (A) Bacterial phyla abundance was quantified in fecal samples before dietary switch and treatment (baseline) and after 7 and 21 days on the different diets and treatments regimen. *Firmicutes* and *Bacteriodetes* were the most abundant bacterial phyla in all groups and at all time points. In all groups, there was a significant reduction in *Verrucomicrobia* abundance at day 7 ($P < 0.001$) and day 21 ($P < 0.0001$). Seven days of HFD were sufficient to induce a significant increase in *Firmicutes* ($P < 0.0001$) and decrease in *Bacteriodetes* abundances ($P < 0.0001$). Similar changes were observed after 21 days of HFD (A, *Firmicutes*, $p<0.01$; *Bacteriodetes*, $P < 0.001$). Minocycline treatment improved HFD rats' bacterial phyla profile with a significant increase in *Bacteriodetes* abundance after 7 days (A, $P < 0.05$) and a significant reduction in *Firmicutes* (A, $P < 0.01$) and increase in *Bacteriodetes* abundances (A, $P < 0.01$) after 21 days. After 21 days of treatment, there were no significant differences in bacterial phyla abundance between the HFD /minocycline-treated rats and the LFD animals. Minocycline treatment did not significantly affect bacterial phyla abundance in LFD rats (115). a, b, c – different letters denote significant differences. (B) Negative and positive correlations were observed between energy intake and bacterial orders depleted (*Bacteroidales*) and enriched (*Clostridiales*) by HF feeding. Energy intake during the third week of experiment (14 to 21days) was correlated with order abundance measured at day 21 across diets and treatments. *Bacteroidales* abundance was negatively correlated with energy intake ($r^2=0.53$, $P < 0.01$) while *Clostridiales* abundance was positively correlated with intake ($r^2=0.47$, $P < 0.01$). (C and D) Bacterial orders that were significantly enriched or depleted by HFD and/or minocycline

treatment (Log 2-fold changes from baseline). * – denotes significant difference from the LF_Saline group: * – $P < 0.05$, ** – $P < 0.01$, *** – $P < 0.001$, **** – $P < 0.0001$. (B) Consumption of HFD for 7 days led to significant increase in abundance of several bacterial orders belonging to the *Firmicutes* (*Erysipelotrichales*), *Terenicutes* (*Entomoplasmatales, Mycoplasmatales*), *Proteobacteria* (*Rhodocyclales, Altermondales*), *Cyanobacteria* (*Nostocales, Chroococcales*) and *Verrucomicrobia* (*Puniceicoccales*). HFD also led to significant depletion in *Bacteriodetes* orders *Bacteroidales* and *Sphingobacteriales*. C. Similar results were observed after 21 days with additional orders enriched (*Enterobacteriales* and *Methylophilales, Proteobacteria*) and depleted (*Thermobaculales, Chloroflexi*). (B) Minocycline treatment normalized HFD-induced dysbiosis. Seven days of minocycline exposure were sufficient to significantly reduce the HFD-induced proliferation of bacterial orders mentioned above, leading to other normalization of abundance or significant depletion (*Nostocales, Chroococcales*). Minocycline also prevented HFD-induced depletion in *Bacteroidales* and *Sphingobacteriales*. In LFD animals, minocycline alone significantly reduced the abundance of obesity associated *Puniceicoccales, Chroococcales* and *Clostridiales*. (C) Similar results were observed after 21 days of minocycline treatment; minocycline led to normalization or depletion of HFD-associated bacterial orders and restored HFD-depleted orders. Minocycline alone led to a significant reduction in *Erysipelotrichales* (*Firmicutes*) and several *Proteobacteria* and *Cyanobacteria* orders, such as *Rhodocyclales* and *Chroococcales*.

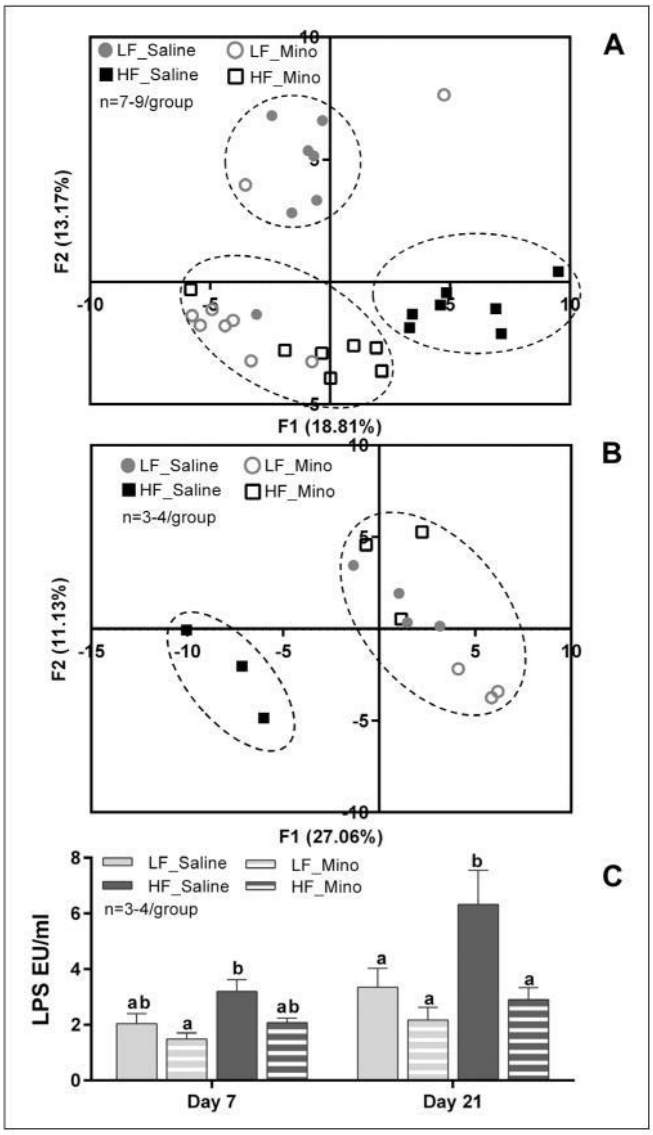

Figure 1.3. Minocycline normalized HFD rats' microbiota profile and reduced HFD-induced metabolic endotoxemia. (A and B) PCA at the order level after 7 or 21 days of diet/minocycline treatments A. PCA showed that 7 days of HFD were sufficient to induce dysbiosis with a distinct microbiota profile while minocycline treatment led in a third profile with more variability, based on the diet consumed (LFD *vs.* HFD). B. HFD-induced dysbiosis was confirmed after 21 days of diet. Minocycline treatment led to normalization of the microbiota profile in HFD rats, with HF_Mino animals clustering with LF_Saline animals. There was no significant effect of minocycline treatment on the LF animals PCA scores. C. Circulating LPS levels in plasma after 7 or 21 days of diet/minocycline treatments. Seven days of HFD a non-significant increase in circulating LPS which became significant after 21 days of HFD ($P < 0.01$). Minocycline treatment reduced circulating LPS in HFD rats and this effect was significant after 21 days ($P < 0.001$). In LFD rats, minocycline treatment did not result in significant changes of LPS plasma levels at any time point. Bars represent the average value ± SEM; a, b – different letters denote significant differences.

CHAPTER 2

BLUEBERRY SUPPLEMENTATION INFLUENCES THE GUT MICROBIOTA, INFLAMMATION, AND INSULIN RESISTANCE IN HIGH-FAT-DIET–FED RATS[3]

[3]Lee S, Keirsey KI, Kirkland R, Grunewald ZI, Fischer JG, de La Serre CB. Blueberry Supplementation Influences the Gut Microbiota, Inflammation, and Insulin Resistance in High-Fat-Diet-Fed Rats. The Journal of Nutrition 2018;148:209-19.Reprinted here with permission of publisher.

Abstract

Background: Gut microbiota dysbiosis has been linked to obesity-associated chronic inflammation. Microbiota manipulation may therefore impact obesity-related comorbidities. Blueberries are rich in anthocyanins, which have anti-inflammatory properties, and may alter gut microbiota.

Objective: We hypothesized that blueberry supplementation would alter gut microbiota, reduce systemic inflammation, and improve insulin resistance in high-fat (HF) diet-fed rats.

Methods: Twenty-four male Wistar rats (260-270 g, n=8/group) were fed low-fat (LF, 10% fat), HF (45% fat) or HF with 10% by weight blueberry powder (HF_BB) diets for 8 wk. LF rats were fed ad libitum while HF and HF_BB rats were pair-fed with diets matched for fiber and sugar contents. Glucose tolerance, microbiota composition (16S rRNA sequencing), intestinal integrity (villus height, gene expression of mucin 2 (*Muc2)* and beta-defensin 2 (*Defb2*)), and inflammation (gene expression of pro-inflammatory cytokines) were assessed.

Results: Blueberry altered microbiota composition with an increase in *Gammaproteobacteria* abundance ($P < 0.001$) compared to LF and HF fed rats. HF feeding led to a ~ 15% decrease in ileal villus height compared to LF rats ($P < 0.05$), which was restored by blueberry supplementation. Ileal gene expression of *Muc2* was ~150% higher in HF_BB rats compared to HF rats ($P < 0.05$), with the LF group expression not being different from either HF or HF_BB. Tumor necrosis factor alpha and interleukin-1 beta gene expression in visceral fat was increased by HF feeding when compared to the LF group (by 300% and 500%, respectively, $P < 0.05$) and normalized by blueberry supplementation. Finally, blueberry improved markers of insulin sensitivity. Hepatic insulin receptor substrate-1 (IRS1) phosphorylation at serine 307/IRS1 ratio

was ~ 35% higher in HF-fed rats when compared to LF ($P < 0.05$) and normalized with blueberry.

Conclusion: In HF-fed male rats, blueberry supplementation led to compositional changes in the gut microbiota associated with improvements in systemic inflammation and insulin signaling.

Key words: blueberry, gut microbiota, epithelial barrier, inflammation, insulin signaling

Introduction

Obesity has been characterized as a low-grade systemic inflammatory state (116). An increase in visceral adiposity is associated with macrophage infiltration, secretion of pro-inflammatory cytokines and decreased insulin sensitivity (116-118). Cytokines, such as tumor necrosis factor alpha (TNFα) and interleukin-1 beta (IL-1β), have been found to impair insulin signaling by promoting insulin receptor substrate-1 (IRS1) phosphorylation at serine 307 (p-IRS1 (Ser307), inhibiting insulin action (118, 119).

There is evidence that obesity-associated inflammation originates, at least partially, from the gastrointestinal (GI) tract (120). Previous studies have demonstrated that gut epithelial barrier integrity is impaired in the distal gut in response to high-fat (HF) feeding (121, 122) with altered expression of gut-protecting mucins and defensins (123). The distal gut is home to over 10^{14} bacteria, and impairment in gut permeability in combination with diet-driven microbiota dysbiosis can lead to translocation of bacterial pro-inflammatory factors such as lipopolysaccharide (LPS) into the circulation (5, 7). LPS activates transcription factor nuclear factor kappa B (NFκB) to promote synthesis of pro-inflammatory cytokines (124). Chronic infusion of LPS in rodents led to weight gain, adipose tissue inflammation and insulin resistance (5, 125). Additionally, manipulation of the microbiota composition using prebiotics or antibiotics restored gut epithelial function and improved metabolic functions, especially insulin sensitivity (126, 127).

There is growing interest in the role of berries in disease prevention. Blueberries are high in anthocyanins, in addition to other polyphenolic compounds (37), and have antioxidant and anti-inflammatory properties (40-42) that may impact disease development. Dietary supplementation with whole blueberry and blueberry polyphenolics reduced biomarkers of

oxidative stress (40, 41) as well as inflammatory gene expression (42). Prior et al. (128-130) showed that purified blueberry anthocyanins reduced body weight and improved glucose tolerance in HF-fed male C57BL/6J mice while whole blueberry powder did not. In contrast, others found that whole blueberry supplementation improved obesity-related insulin sensitivity, even without changes in body weight, in HF-fed mice (42) and Zucker Fatty rats (131). Blueberry supplementation also improved insulin sensitivity in obese, insulin-resistant men and women (63).

Blueberries are a source of fermentable fibers (132). Additionally, high concentrations of anthocyanins have been found in the distal intestine where they can interact with, and be metabolized by, the gut microbiota (133). Therefore, blueberry may improve obesity-related inflammation via alteration of the gut microbiota composition. Berry extracts have exhibited antimicrobial and anti-adhesion properties against pathogenic bacteria (45, 134). In rodents, dietary supplementation with whole blueberry altered microbiota composition (43, 134) and consumption of a wild blueberry powder beverage in men resulted in increases of *Bifidobacterium spp.* (59).

Blueberry-driven changes in gut microbiota could lead to changes in intestinal short chain fatty acids (SCFAs). The most abundant intestinal SCFAs are acetate, propionate, and butyrate (135). Acetate and propionate have been shown to activate G-protein coupled receptors (GPRs), such as GPR43, and promote production of GI peptides including glucagon-like peptide-1 (GLP1), a known incretin (136).

Previous studies investigating whole blueberry supplementation have not examined changes in gut microbiota in conjunction with effects on inflammation and insulin resistance. We hypothesized that consumption of blueberry in HF-fed rats would alter gut microbiota

composition and reduce intestinal permeability, inflammation, and insulin resistance. To test this hypothesis, we fed rats a HF diet supplemented with 10% blueberry powder and investigated changes in gut microbiota composition, inflammation and glucose homeostasis while controlling for food and dietary fiber intake.

Methods

Animals and diets. Twenty-four male Wistar rats (200-220 g) were procured from Envigo (Indianapolis, IN) and single-housed in a temperature-controlled room with a 12-h light-dark cycle. Animals were separated into three groups (n=8) and fed low fat (LF) (10% kcal as fat), HF (45% kcal as fat), or HF with 10 g freeze-dried blueberry powder/100 g (HF_BB) diets for 8 wk (**Supplemental Tables 2.1-5**). The blueberry powder was provided by the US Highbush Blueberry Council (Folsom, CA) and was a Tifblue/Rubel 50/50 blend with 38.39 mg/g phenolics and 21.34 mg/g anthocyanins. HF and HF_BB diets were matched for sugars, soluble and insoluble fibers and were isocaloric (Research Diets, New Brunswick, NJ). All diets were formulated to meet micronutrient requirements. Selection of the 10% blueberry concentration was based on previously published studies (40, 51). Body weight and food intake were monitored daily. The LF group was fed ad libitum while food intake was managed to assure similar intakes between HF and HF_BB-fed rats by pair-feeding the HF-fed group to the HF_BB-fed group. After 8 wk on their respective diets, animals were fasted for 6 h and euthanized via CO_2 inhalation. Sacrifice order was evenly distributed between treatment groups over 2 days and all tissues were removed within 2.5 hours after the beginning of the light cycle. Prior to sacrifice, a 24-hour urine sample was collected and frozen at -80°C to be analyzed for F2-isoprostanes. Blood was collected by cardiac puncture, rested on ice for 15 min, and spun at 1,000 X *g* for 10 min at 4 °C for serum collection. Liver, ileum, cecum, colon, and visceral fat

pads (mesenteric, retroperitoneal, and epididymal) were collected and weighed and an adiposity index was determined. Serum and all tissues were snap frozen and stored at -80°C. All animal care procedures were approved by the Institutional Animal Care and Use Committee of the University of Georgia.

Oral glucose tolerance test (OGTT). After 7 wk, animals were fasted for 5 h before oral gavage with a glucose solution (2 g/kg body weight using 20% glucose, Sigma-Aldrich, St. Louis, MO). Glycemia was measured using a glucometer (Freestyle, Alameda, CA) before (0 min) and after (15, 30, 60, 90, and 120 min) glucose administration. Blood samples (~100 μL) were collected at each time point and centrifuged as described above to obtain serum for insulinemia analysis

Microbiota DNA sequencing. DNA was extracted from cecal contents using the ZR Fecal DNA MiniPrep per the manufacturer's protocol (Zymo Research, Irvine, CA). Briefly, cecal contents were lysed by bead beating and DNA was isolated using fast-spin columns. DNA was filtered to remove humic acids and polyphenols and the eluted DNA was sent to the University of California Davis Genomic Sequencing Center for sequencing. High throughput sequencing was performed with Illumina MiSeq paired end 250 base-pair runs. Amplification was performed on the V4 region of the 16S rRNA genes via polymerase chain reaction (PCR) using the primers: F515 (5′-GTGCCAGCMGCCGCGGTAA-3′) and R806 (5′-GGACTACHVGGGTWTCTAAT-3′). Sequences were subsequently aligned to reference genomes. Bacterial abundance was normalized by log transformation and multivariate statistical analysis and clustering (principal component analysis) were performed using METAGENassist platform (137). Differences in taxa abundance were assessed using a one-way ANOVA (Fisher post-hoc test, METAGENassist).

Linear discriminant analysis effect size analysis was performed on log-transformed abundance using the Galaxy online module to identify discriminant taxa among groups (138).

SCFA analysis. SCFAs were quantified in serum by the Mayo Clinic Metabolomics Core via gas chromatography–mass spectrometry using previously published methods (139).

Blood analyses. LPS-binding protein (LBP; Biometec, Greifswald, Germany) and insulin (Alpco, Salem, NH) in serum were measured via enzyme-linked immunosorbent assay (ELISA) per manufacturers' instructions.

Lipid peroxidation markers. Analysis of urinary F2-isoprostanes was performed in the Vanderbilt University Eicosanoid Core Laboratory with gas chromatography/negative ion chemical ionization mass spectrometry and data was expressed per urinary creatinine. Liver malondialdehyde (MDA) was measured via ELISA per manufacturers' instructions (Oxford Biomedical Research, Oxford, MI).

Intestinal morphology. GI tissues were cryosectioned (5μm; Leica CM1900, Leica Biosystems, Wetzlar, Germany). Sections were stained with Alcian blue and nuclear fast red (Sigma-Aldrich). Villus height (μm) and the number of goblet cells (per crypt) were measured manually in well-oriented sections (5 measurements per ileal section) using a light microscope (BX40, Olympus) equipped with digital camera (DP25, Olympus) and analysis software (DP2-BSW, Olympus).

Liver Histology. Livers were cryosectioned (4 μm; Leica, CM1900). Sections were stained at the UGA College of Veterinary Medicine's Pathology Laboratory using Oil Red O (ORO) with hematoxylin as a counter nuclear stain. Sections were viewed under a light microscope (Nikon Eclipse E400, Nikon, Tokyo, Japan) at 200X magnification. The ORO positive pixels were determined using Scion Image (Scion Corporation, Frederick, MD)

Real-time quantitative PCR (RT-PCR). Gene expression of inflammatory markers was determined in liver, fat and ileum tissues via RT-PCR. Gene expression of gut epithelial function was determined in ileum tissues. mRNA was extracted from liver, ileum and mesenteric fat tissues using the RNeasy Mini Kit or Lipid Tissue Mini Kit (Qiagen, Valencia, CA) per the manufacturer's instruction and assessed for quantity and purity using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). cDNAs were synthesized using the RevertAid™ First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Franklin, MA). qPCR was done on a StepOnePlus real-time PCR system (Thermo Fisher Scientific) using SYBR Green PCR master mix (Thermo Fisher Scientific) with primers purchased from Integrated DNA Technologies (**Supplemental Table 2.6**). Data were analyzed according to the $2^{-\Delta\Delta Ct}$ method (140).

Western blot. The phosphorylation of IRS1 in liver and NFκB p65 in mesenteric fat was determined by western blot. Liver proteins were extracted using lysis buffer (Invitrogen, Carlsbad, CA) and protease and phosphatase inhibitors (Roche Diagnostics, Indianapolis, IN). Nuclear fraction proteins from mesenteric fat were extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fischer Scientific). 20μg (liver) or 30μg (fat) of proteins were loaded in precast Bolt 10% Bis-Tris Mini Gel (Life Technologies, Gaithersburg, MD) for separation before being transferred to a PVDF membrane and probed with primary antibodies (1:1000; Cell Signaling Technology, Beverly, MA); GAPDH, IRS1, phospo-IRS1 Ser307, phospho-NFκB p65, and total NFκB p65. IgG and anti-biotin Rabbit HRP secondary antibodies (Cell Signaling Technology) were then probed onto the membrane. Licor Western Sure chemiluminescent substrate was used as a detection agent. Blots were quantified using a C-DiGit Blot Scanner and Image Studio Software (LiCor, Lincoln, NE).

Statistical analysis. Data are presented as mean ± SEM. Statistical analysis was performed using Prism software (Prism 6.0; GraphPad Software, La Jolla, CA). Two-way repeated measures ANOVA was used to analyze body weight, energy intake, and OGTT. One-way ANOVA was performed to analyze data from adiposity, qPCR, western blot, and biochemical analyses. Differences among groups were analyzed using the Fisher's least significant difference test. Correlations between SCFAs levels and microbiota abundance were determined using the nonparametric Spearman correlation. Differences were considered significant if $P < 0.05$.

Results

Body weight (BW) and glucose tolerance. There was no difference in final BW among LF, HF and HF_BB rats (**Figure 2.1A**). As previously reported (125), HF feeding induced a significant increase in energy intake for the first wk compared to LF and in wk 2 HF_BB rats continued to have significantly higher intakes than LF rats. This may be driven by diet palatability. However, there was no difference in intake among groups throughout the rest of the study and no difference in total energy intake between the HF- and LF-fed animals (**Supplemental Figure 2.1**). Despite no differences in BW, the overall adiposity index was significantly higher in HF and HF_BB rats compared to the LF group ($P < 0.05$; **Figure 2.1B**). While there were no significant differences among groups for mesenteric and epididymal fat depots, the retroperitoneal fat pad weight was significantly higher in HF-BB rats compared to LF rats ($P < 0.05$).

There was no difference in fasting (0 min) glycemia and insulinemia among groups (**Figure 2.1C, D**). Following an OGTT, glycemia increased sharply in three groups and peaked at 15 min post challenge. Peak glycemia at 15 min for HF_BB rats was lower than LF and HF

rats, although comparison with the HF group failed to reach significance ($P = 0.07$). There were no significant differences in AUC among groups. Insulin levels peaked 15 min post oral glucose challenge in the LF and HF_BB groups but peaked at 30 min in HF-fed rats and at that time was significantly higher than the LF and HF_BB groups ($P < 0.05$). There was a 30% reduction in peak insulin in the HF_BB rats compared to the HF animals.

Microbiota composition and metabolites. HF consumption alone did not have a major effect on the microbiota composition. There were no differences in abundance and the ratio of the main phyla *Firmicutes* and *Bacteriodetes* between the HF- and LF-fed animals (**Figure 2.2A**). Blueberry supplementation had a much greater effect on the microbiota composition. At the phylum level, blueberry supplementation led to significant decreases in both *Firmicutes* ($P < 0.001$) and *Bacteriodetes* ($P < 0.001$) abundance and significant increases in *Proteobacteria* ($P < 0.001$) and *Fusobacteria* ($P < 0.05$) abundance compared to HF- and LF-fed rats. *Proteobacteria* represented 37.99% of the identified bacteria in the HF_BB group vs. 2.17% in the LF and 1.56% in the HF rats.

Abundance analysis at all taxonomic levels showed that microbiota composition in LF- and HF-fed rats was very similar, apart from a significant decrease in *Rumminococcus* (Genus, $P < 0.01$) in HF-fed animals when compared to the LF group ($P < 0.01$) (**Figure 2.2E**). The blueberry effect on *Proteobacteria* was driven by an increase in *Gammaproteobacteria* (Class, $P < 0.001$), especially the *Pasteurellales* order ($P < 0.01$), including the genus *Actinobacillus* ($P < 0.001$) and *Aggregatibacter* ($P < 0.001$). The blueberry supplementation-induced increase in *Fusobacteria* abundance was driven by an elevation in *Fusobacteriaceae (*Family, $P < 0.05$). Despite an overall decrease in *Firmicutes*, blueberry supplementation led to increased abundance of *Bacilli* (Class), especially *Lactobacillales* (Order, $P < 0.001$). Similarly, despite an overall

decrease in *Bacteriodetes*, HF_BB displayed a significant increase in the *Porphyromonadaceae* (Family, $P < 0.01$) abundance when compared to both LF and HF rats. Principal component analysis on all taxonomic levels showed that LF- and HF-fed rats had overall a very similar microbiota profile while blueberry supplementation resulted in a radically different profile (**Figure 2.2F**).

Blueberry supplementation was associated with significant changes in serum SCFAs. Acetate was significantly elevated in the HF_BB group compared to the LF and HF groups ($P < 0.05$; **Figure 2.3A**). HF_BB animals had significantly higher levels of propionate than the LF group ($P < 0.01$) but not different from the HF group. Finally, butyrate levels were significantly lower in HF_BB rats when compared to the LF group ($P < 0.05$) but not different from the HF group. There were no significant differences in serum acetate, propionate or butyrate between the LF and HF animals. Correlation analysis showed significant positive relationships between serum acetate and *Proteobacteria* ($_r = 0.42$; $P < 0.05$), *Gammaproteobacteria* ($_r = 0.5$; $P < 0.05$), *Pasteurellales* ($_r = 0.46$; $P < 0.05$), *Actinobacillus* ($_r = 0.59$; $P < 0.01$), and *Aggregatibacter* ($_r = 0.46$; $P < 0.05$) abundance. Serum acetate was negatively correlated with *Bacteriodetes* ($_r = -0.61$; $P < 0.01$) and positively correlated with *Bacilli* ($_r = 0.46$; $P < 0.05$) and *Lactobacillales* ($_r = 0.55$; $P < 0.01$) abundance. Butyrate levels tended to be negatively correlated to *Fusobacterium* ($_r = -0.38$; $P = 0.08$) abundance.

Blueberry supplementation led to a significant three-fold increase in SCFA-target receptor, *Gpr43* gene expression compared to the LF control ($P < 0.01$; **Figure 3B**). *Gpr43* expression in HF_BB rats was also higher than in the HF group, but this difference did not reach significance ($P = 0.1$). HF-fed rats had a significant decrease in *Glp1* gene expression compared to LF and HF_BB rats (LF vs. HF and HF vs HF_BB; $P < 0.05$),

GI barrier integrity and inflammation. HF feeding significantly reduced villus length compared to the LF control group ($P < 0.0001$; **Figure 2.4A**). Blueberry supplementation restored GI integrity; ileal villus length in the HF_BB rats was similar to the LF-fed rats and significantly higher than the HF rats ($P < 0.0001$).

Goblet cell number per crypt was significantly higher in the HF_BB rats compared to the HF animals ($P < 0.05$; **Figure 2.4B**). Goblet cell number in the LF control group did not differ from either the HF or HF_BB rats. Similarly, gene expression of the mucus protein, mucin 2 (*Muc2*), in the ileum was significantly higher (2.5 fold) in the HF_BB compared to the HF-fed animals ($P < 0.05$). *Muc2* expression in the LF group did not differ from either HF or HF_BB animals (**Figure 2.4C**). HF_BB rats exhibited a significant increase in the anti-bacterial peptide beta-defensin 2 (*Defb2**)*** gene expression in the ileum compared to the LF-fed animals ($P < 0.05$). Level of expression in the HF rats was not different from the LF or HF_BB animals.

In the ileum, HF feeding was associated with a significant increase in *Tnfa* gene expression (HF vs. LF; $P < 0.001$) which was normalized by blueberry supplementation (**Figure 2.4D**). There were no differences in other inflammatory genes assessed, including *Il-1b* and *Il-6*.

LBP and Mesenteric fat inflammation. LBP was used as a proxy to assess circulating LPS levels. HF_BB rats had a significant reduction in circulating LBP when compared to the HF-fed rats ($P < 0.05$; **Figure 2.5A**). LBP serum levels in LF-fed rats were not different from either HF or HF_BB. In line with these results, we observed a lower ratio of nuclear phospho- to total NFκB p65 in the mesenteric fat tissue of the HF_BB group than the other groups (**Figure 2.5B**). The difference was not significant ($P = 0.07$) among groups but animals in HF_BB group all clustered within the ratio of less than 0.1 (0.01 - 0.08), while LF and HF groups showed inconsistent patterns of distribution (0.04 - 0.26).

HF feeding significantly upregulated gene expression of *Il-1b* (HF vs. LF; $P < 0.001$) and *Tnfa* (HF vs. LF; $P < 0.05$) in mesenteric fat tissue which were significantly downregulated to LF control levels by blueberry supplementation (**Figure 2.5C**). Also, cluster of differentiation 11d expression (a marker of macrophage infiltration) was significantly lower in blueberry-fed rats compared to the LF and HF groups ($P < 0.05$). Others have reported that blueberry feeding can alter expression of peroxisome proliferator-activated receptor (PPAR) subtypes and impact lipid metabolism (22). Blueberry supplementation increased *Ppara* gene expression in mesenteric fat compared to HF rats (HF_BB vs HF; $P < 0.01$, **Figure 5D**), although *Ppara* gene expression for HF and HF_BB rats was not significantly different than LF rats. *Ppard* expression was significantly decreased in HF rats compared to LF rats ($P < 0.05$) and this was normalized by blueberry treatment (LF vs. HF_BB, $P = 0.08$; HF vs. HF_BB, $P < 0.0001$). We did not find any differences in *Pparg* gene expression (data not shown).

Hepatic Measurements. Compared to LF-fed rats, HF feeding led to a significant increase in liver fat droplets (LF vs. HF, $P < 0.01$, LF vs. HF_BB, $P < 0.05$; **Figure 2.6A, B**) with no significant difference between HF and HF_BB groups. However, hepatic p-IRS1 (Ser307) /IRS1 ratio was significantly increased in HF rats compared with LF rats and reduced to control levels by blueberry treatment ($P < 0.05$; **Figure 2.6C, D**). Hepatic MDA level, a marker of oxidative stress, was significantly reduced in HF_BB rats in comparison with the HF group ($P < 0.05$; **Figure 2.6E**), although neither the HF nor HF_BB groups had higher MDA than the LF group. F2-isoprostanes, a marker of systemic oxidative stress, were elevated in the urine of HF_BB rats relative to LF and HF-fed rats ($P < 0.05$; **Figure 2.6F**).

Discussion

In this study, we investigated the potential effects of blueberry on HF diet-associated metabolic changes. Our hypothesis was that blueberry supplementation would trigger compositional changes in the gut microbiota associated with improved gut epithelial function, decreased systemic inflammation and improved insulin signaling.

Blueberry supplementation resulted in a unique microbiota profile characterized by a high dominance of *Gammaproteobacteria.* These changes were associated with increases in villus height, goblet cell proliferation, and gene expression of *Muc2* and *Defb2* in the ileum, suggesting improvement of gut barrier integrity. Moreover, blueberry treatment suppressed local and systemic inflammation indices and ameliorated hepatic oxidative stress. Finally, blueberry supplementation improved insulin sensitivity, which may be due to a decrease in hepatic p-IRS1 (Ser307) level, a marker of impaired insulin signaling (141), and the upregulation of ileal *Glp1* gene expression.

There were no differences in BW and energy intake between HF- and LF-fed rats while others have found that 8 wk of HF feeding is sufficient to induce hyperphagia in rodents (5, 120). Blueberry supplementation may have reduced energy intake and the pair-feeding paradigm prevented HF diet-induced hyperphagia. Furthermore, all diets were matched for fiber content and a recent study in mice (142) suggested that HF diet-induced hyperphagia was driven by a lack of fibers in the diet. Thus, it is also possible that our diet composition was responsible for the lack of BW difference between LF- and HF-fed groups. We still observed an increase in adiposity in both HF and HF_BB rats compared to the LF group. Adiposity in rats has previously been found to be proportional to dietary fat regardless of body weight (143). Although visceral

fat was not reduced, gene expression of *Ppara* and *Ppard* were significantly increased in HF_BB rats compared with HF rats, suggesting higher fatty acid oxidation in the HF_BB group (144).

HF feeding alone had very little effect on the microbiota composition, HF and LF fed rats' gut microbiota profile was very similar. Noticeable exceptions included a HF diet-associated decrease in *Ruminococcus*, which is a genus of the *Ruminococcaceae* family. Reduced abundance of *Ruminococcaceae* has previously-y bene reported with HF feeding (35). We did not observe changes previously characterized in obese animals such as an increase in *Firmicutes* and a decrease in *Bacteriodetes* abundance (18). This result may have been due to the similar fiber contents of the LF and HF diets since fibers modulate gut microbiota composition (145).

Independently of dietary fiber content, the HF_BB diet induced a shift in the gut microbiota composition characterized by a significant decrease in *Firmicutes* and increases in *Fusobacteria* and *Proteobacteria* abundance. Despite an overall decrease in *Firmicutes,* blueberry supplementation led to increased abundance of *Bacilli* (Class), especially *Lactobacillales*, interestingly blueberry extract have been shown to favor the growth of *Lactobacillus spp.* (57), suggesting this effect may be anthocyanin-driven. The increase in *Proteobacteria* was driven by a dominance of *Gammaproteobacteria* class, especially the *Pasteurellales* order, including the genus *Actinobacillus* and *Aggregatibacter*. *Proteobacteria* has been characterized as the least stable among the major phyla (146). Studies have shown that the relative abundance of *Proteobacteria* in the human gut transiently increases up to 45% without clinical signs (147), highlighting *Proteobacteria*'s sensitivity to environmental factors. Despite being traditionally thought to be pro-inflammatory (148), increases in *Proteobacteria,* especially *Gammaproteobacteria,* have been reported in association with metabolic improvements, notably following roux-en-Y gastric bypass n humans and animals (149, 150). In

these studies, similarly to our results, the abundance of *Aggregatibacter* was significantly increased.

Blueberry-driven changes in microbiota may have improved gut health resulting in reduced translocation of bacterial products such as LPS across the epithelial barrier (151). Epithelial barrier integrity is compromised with inflammation (120) and HF feeding (124). HF diet notably led to increased circulating LPS which induces the transcription of pro-inflammatory cytokines via NFκB activation (124). In this study, we found significantly lower levels of serum LBP (a marker of circulating LPS) (152) in the HF_BB group compared to the HF group and reduced NFκB activation in adipose tissue. Accordingly, *Tnfa* and *IL-1b* gene expression were downregulated in mesenteric fat of the HF_BB rats compared to the HF animals (125). These results confirmed that blueberry had an anti-inflammatory effect. DeFuria et al. (42) similarly reported that blueberry reduced HF diet-induced increases in *Tnfα* gene expression in visceral fat of HF-fed mice.

Polyphenols previously have been shown to strengthen the intestinal epithelial barrier by upregulating the gene expression of MUC2 (153), the primary glycoprotein of the GI mucus layer (154), and stimulating production of antimicrobial peptides, such as DEFβ2 (155). We found that blueberry supplementation had a positive effect on goblet cell count in HF-fed rats and increased *Muc2* and *Defb2* gene expression in the ileum. DEFβ2 is upregulated by inflammation or bacterial stimuli (156). Thus, the observed increase in *Proteobacteria* in the HF_BB group may have acted as a triggering factor. The elevated *Defb2* gene expression in HF rats may have resulted from GI inflammation.

Blueberry may have improved epithelial barrier function through an increase in bacterial fermentation products, namely SCFAs (157). SCFAs have been shown to stimulate the

proliferation and differentiation of enterocytes, ultimately contributing to increases in villus height and goblet cell proliferation (158, 159). Serum concentrations of acetate, propionate, and butyrate are a good proxy for bacterial fermentation (160). We found that blueberry supplementation led to increases in circulating acetate and propionate, while reducing butyrate. The amount and diversity of gut microbiota play a role in SCFA production (160). For example, the cecal concentration of butyrate has been previously correlated with the abundance of several *Firmicutes* taxa (161), which were low in the HF_BB group. In this study there was a positive correlation between serum acetate and *Proteobacteria* taxa including *Gammaproteobacteria (Class), Pasteurellales* (Order), *Actinobacillus* (Genus), and *Aggregatibacter* (Genus), which were primary contributors to the unique microbial composition of HF_BB rats.

Glucose homeostasis was only modestly impaired in HF-fed rats, which could be related to the HF diet's fiber content (37). However, as previously reported (42, 131), blueberry supplementation improved insulin sensitivity in HF-fed rodents. HF_BB rats exhibited lower insulinemia than HF rats, showing that HF_BB rats required less insulin to clear glucose. Enhanced insulin sensitivity may have been due to a reduction in hepatic p-IRS1 (Ser307) level, a marker of cytokine-driven insulin resistance (141). HF feeding increased IRS1 Ser307 phosphorylation in the liver, which was normalized by blueberry treatment. Furthermore, HF diets can promote the production of reactive oxygen species (162) and oxidative stress can alter IRS phosphorylation (163, 164). Similarly to previous research with anthocyanins (165), hepatic MDA, a marker of oxidative stress, was reduced by blueberry supplementation and decreased oxidative stress may have contributed to the normalized IRS1 Ser307 phosphorylation in the HF_BB group.

Another possible mechanism for blueberry-induced changes in insulin sensitivity is through changes in ileal GLP1 expression. GLP1 improves both insulin secretion and sensitivity (166) and has previously been found to be downregulated by HF feeding (167). SCFAs, especially acetate and propionate, have been shown to stimulate GLP1 release via a GPR43-dependent pathway (136). In this study, ileal *Gpr43* gene expression was higher in HF_BB rats than in HF rats and while *Glp1* gene expression was significantly decreased by HF feeding, it was restored by blueberry supplementation.

We also quantified urinary F2-isoprostanes as a biomarker of systemic oxidative stress (168). F2-isoprostanes have previously been shown to decrease with consumption of high anthocyanin foods (168), but were unaltered with blueberry supplementation in a previous study of our laboratory (19). In contrast to the liver MDA results, urinary F2-isoprostanes were slightly increased in the HF_BB group compared to both HF and LF animals, suggesting an increase in systemic oxidative stress. This may be related to specific changes in gut microbiota, although further research would be necessary to confirm this.

There are limitations to this study that deserve consideration. First, we used a rodent model to test our hypothesis and the results cannot be directly extrapolated to humans due to differences in gut microbiota and physiology. Also, we demonstrated the protective effect of blueberry on gut barrier integrity by measuring the level of serum LBP as a proxy to the LPS level in the circulation. A direct assessment of intestinal tight junction permeability would better confirm the role of blueberry in preserving the intestinal epithelial barrier. Lastly, although we demonstrated that metabolic improvements with blueberry supplementation were found in association with compositional changes of the gut microbiota, the use of germ-free models would

be needed to conclusively show that gut microbiota are responsible for changes in inflammation and insulin sensitivity.

In conclusion, we demonstrated for the first time that blueberry-induced reductions of inflammation and insulin resistance in HF-fed rats were found in conjunction with compositional changes in gut microbiota and improved gut integrity. These changes may have prevented LPS translocation, resulting in reduced systemic inflammation and improved hepatic insulin sensitivity in HF-fed rats. Thus, our study provides further support that blueberry may reduce obesity-related inflammation and insulin resistance.

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Supplemental Table 2.S1. Macronutrient composition of LF, HF, and HF_BB diets as a percent of energy[1]

Macronutrient	LF	HF	HF_BB[2]
		% kcal	
Protein	18	18	18.2
Carbohydrate	71.3	35.7	35.3
Dextrose	2.5	2.5	2.5
Sucrose	18.4	18.4	18.4
Fructose	2.6	2.6	2.6
Fat	10.7	46.3	46.5
Total	100	100	100
		Kcal/g diet	
	3.8	4.6	4.6

[1] Diets prepared by Research Diets, Inc. HF, high fat; HF_BB, high fat with 10% blueberry; LF, low fat.

[2]10% freeze-dried blueberry; 93.5 % carbohydrate with 59.3% sugars and 21.5% dietary fiber.

Supplemental Table 2.S2. Dietary fiber in LF, HF, and HF_BB diets[1]

Fiber type	LF	HF	HF_BB
		g/100 g diet	
Insoluble fiber	4.5	5.5	5.5
Soluble fiber	0.3	0.3	0.3

[1] HF, high fat; HF_BB, high fat with 10% blueberry; LF, low fat.

Supplemental Table 2.S3. Diet Composition of LF, HF, and HF_BB diets[1]

Diet Ingredient	LF - D14040205	HF - D14040204	HF_BB - D14040203
		g	
Casein	200	200	200
L-Cystine	3	3	3
Corn Starch[2]	401.29	21.98	6.67
Maltodextrin 10	75	100	100
Sucrose	172.8	172.8	172.8
Dextrose	25.06	25.06	0
Fructose	25.83	25.83	0
Cellulose	47.08	47.08	31.55
Inulin	2.92	2.92	-
Lard	20	177.5	177.5
Soybean Oil	25	25	25
Mineral Mix S10026	10	10	10
Dicalcium Phosphate	13	13	13
Calcium Carbonate	5.5	5.5	5.5
Potassium Citrate, 1 H20	16.5	16.5	16.5
Vitamin Mix V10001[3]	10	10	10
Choline Bitartrate	2	2	2
Freeze Dried Blueberry	-	-	85.815
Red Dye #40, FD&C	0.05	-	-
Blue Dye #1, FD&C	-	-	0.05
Yellow Dye #5, FD&C	-	0.05	-
Total	1055.03	858.22	859.39

[1]Diets prepared by Research Diets, Inc. HF, high fat; HF_BB, high fat with 10% blueberry; LF, low fat.
[2]Corn starch was 88% carbohydrate.
[3]American Institute of Nutrition. Report of the American Institute of Nutrition Ad Hoc Committee on Standards for Nutritional Studies. J Nutr. 1977;107:1340-8.

Supplemental Table 2.S4. Mineral Mix S10026 Composition (Research Diets, Inc.)

Ingredient	g/kg mineral mix
Sodium Chloride 39.3% Na 60.7% Cl	259
Magnesium Oxide, Heavy, DC USP 60.3% Mg	41.9
Magnesium Sulfate, Heptahydrate 9.87% Mg 13.0% S	257.6
Ammonium Molybdate Tetrahydrate	0.3
Chromium Potassium Sulfate 10.4% Cr	1.925
Copper Carbonate 57.5% Cu	1.05
Ferric Citrate 17.4% Fe	21
Manganese Carbonate Hydrate 47.8% Mn	12.25
Potassium Iodate 59.3% I	0.035
Sodium Fluoride 45.2% Fl	0.2
Zinc Carbonate 52.1% Zn	5.6
Sucrose	399.105

Supplemental Table 2.S5. Nutrient Composition of Freeze Dried Blueberry Powder[1]

Nutrient	Per 100 g powder
Protein, g	2.47
Carbohydrate, g	93.5
Fat, g	1.16
Total sugars, g	59.3
Fructose, g	30.1
Glucose, g	29.2
Sucrose, g	<0.1
Maltose, g	<0.1
Lactose, g	<0.1
Insoluble fiber, g	18.1
Soluble fiber, g	3.4
Vitamin C, mg	10.3
Calcium, mg	34.1
Iron, mg	1.73
Potassium, mg	469
Sodium, mg	7.21

[1] Freeze dried blueberry powder #30711 from U.S. Highbush Blueberry Council.

Supplemental Table 2.S6. Primer sequences used for qPCR[1]

Gene*	Forward primers (5' to 3')	Reverse primers (5' to 3')
βactin	ACGGTCAGGTCATCACTATC	GATGCCACAGGATTCCATAC
Cd11d	ACAGATCGGCTCCTACTT	CGGGTCTGCTCATAGTAATG
Cd68	AAACAGTCCAGGCTTCTC	ATGGCTGGGAACCATTAG
Defb2	CGATGTCTAAGAGAGAAAGGG	GGAAACAGGTACCCACAAA
Gapdh	GAGCATCTCCCTCACAATTC	GGGTGCAGCGAACTTTAT
Glp1	CCAAGCAAGGAGAGAGAAAC	GATGACCAAGGCAGAGAAAG
Gpr43	CTGTGGTGACGCTTCTTA	TTGAGGGAACTGAACACC
Il-1b	CATTGTGGCTGTGGAGAA	GCAGTGCAGCTGTCTAAT
Il-6	TGTTGTGGGTGGTATCCT	CCTTCTTGGGACTGATGTTG
Muc2	CTGAGGAAGGCCAAGTTTAC	CAGGTCCCAGAGAGGAAATA
Ppara	CACCCGAGAGTTCCTAAAG	TGTCACTGTCATCCAGTTC
Ppard	GGCCCTGTTCCTAGAATTG	GTATGTGGGAGCAGGAAAG
Pparg	CCTGAAGCTCCAAGAATACC	CATGTGGCCTGTTGTAGAG
Tnfa	TCTACTCCCAGGTTCTCTTC	GCTGACTTTCTCCTGGTATG

[1]*Cd11d*, cluster of differentiation 11d; *Cd68*, cluster of differentiation 68; *Defb2***,** beta-defensin 2; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase; *Glp1*, glucagon-like peptide-1; *Gpr43*, G protein-coupled receptor 43; *Il-1b*, interleukin-1 beta; *Il-6*, interleukin-6; *Muc2*, mucin 2; *Ppara, -d, -g*, peroxisome proliferator-activated receptor alpha, -delta, -gamma; *Tnfa*, tumor necrosis factor alpha.

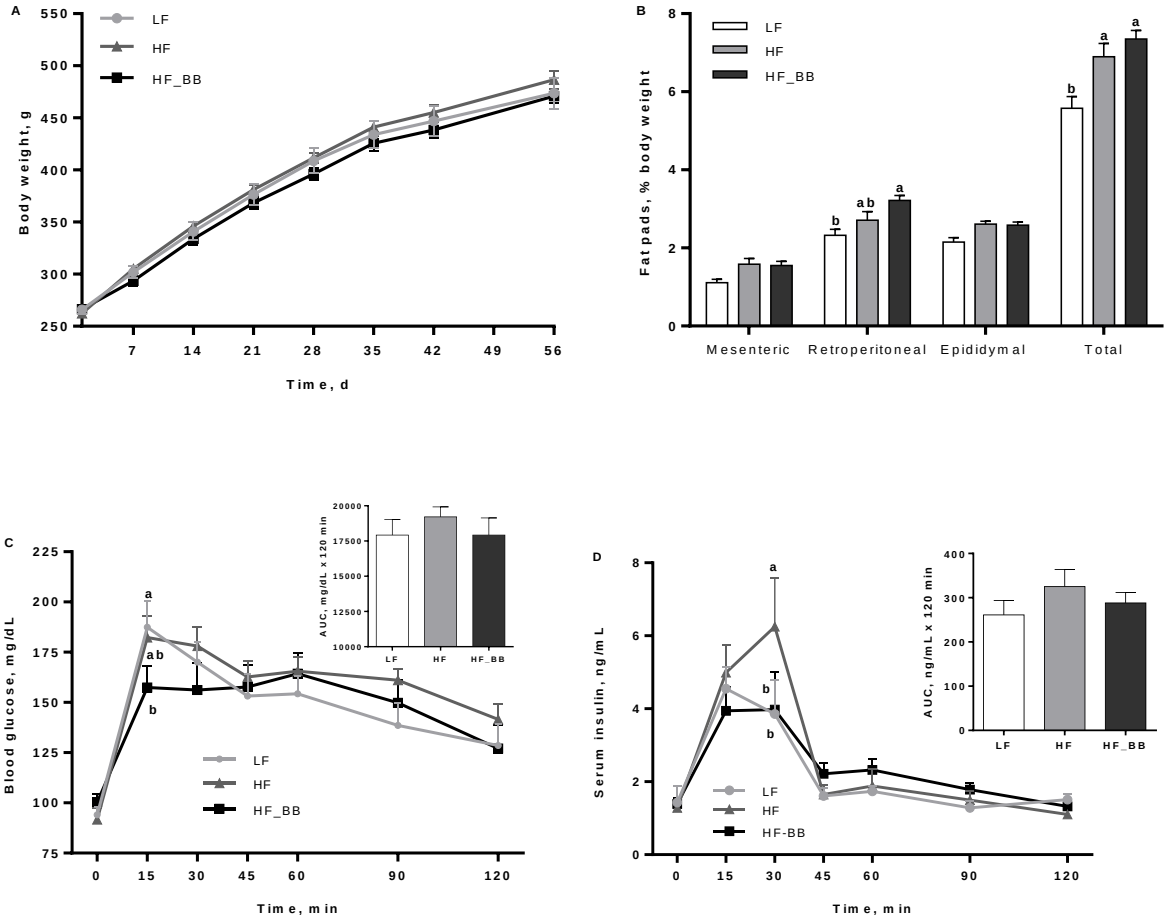
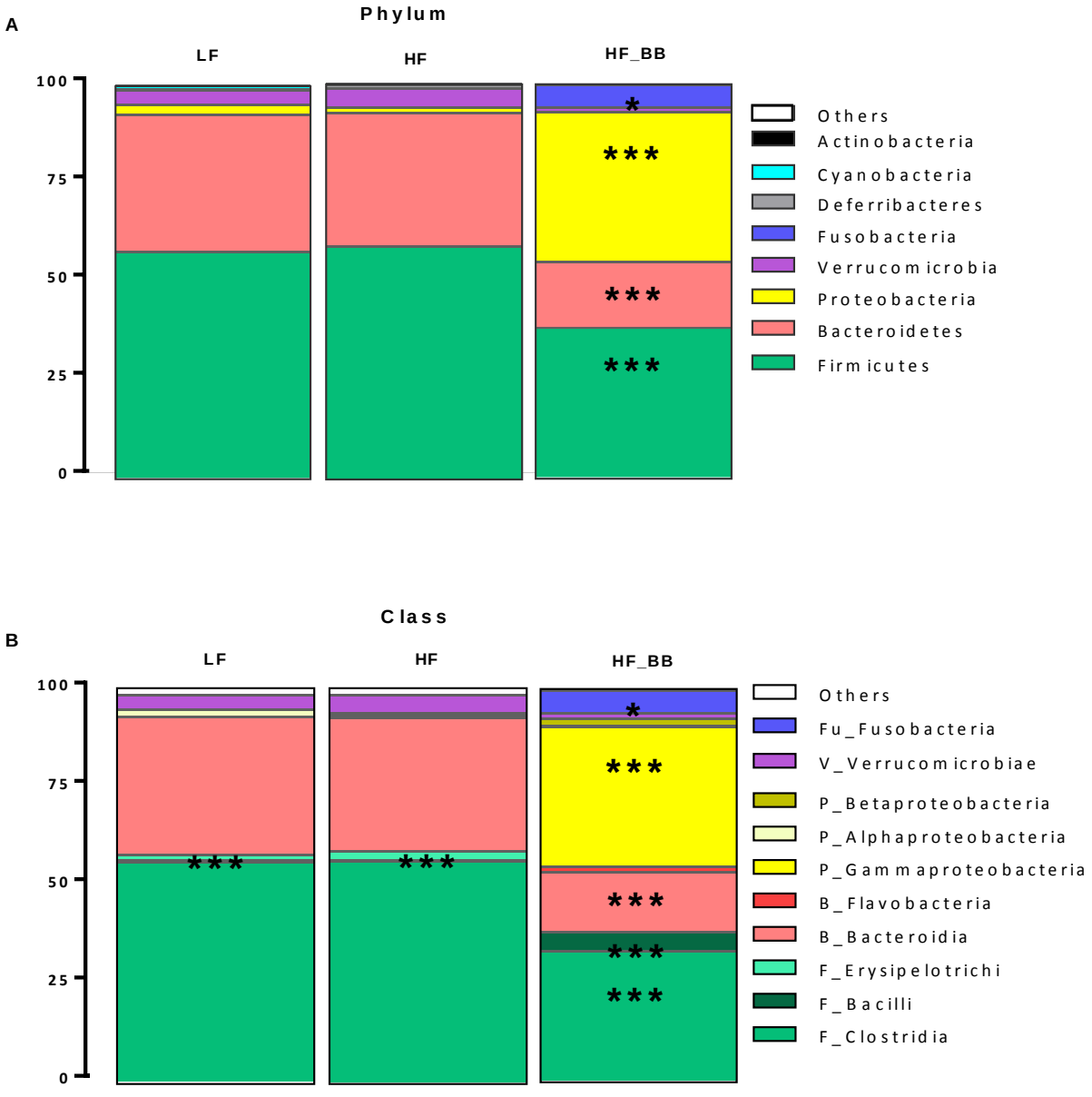
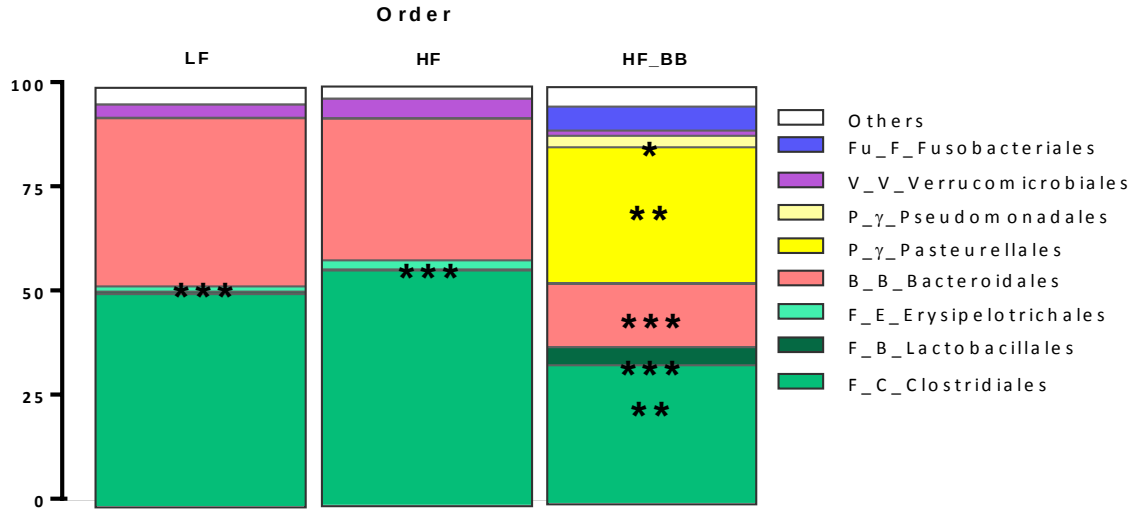


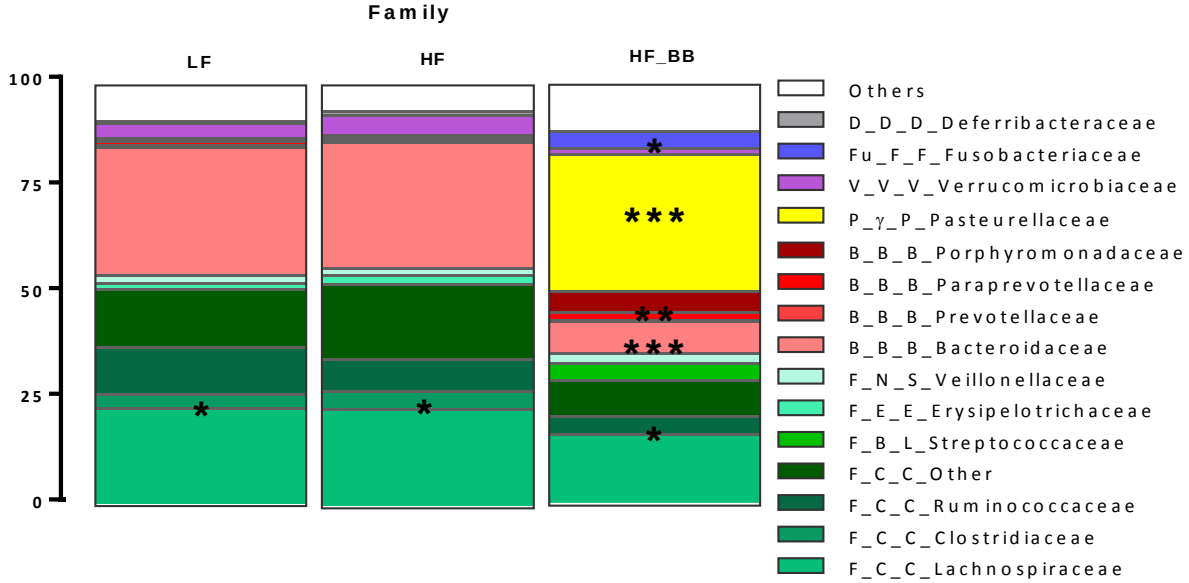
Figure 2.1. Body weight (A), adiposity index (B), and blood glucose (C) and serum insulin (D) during an oral-glucose-tolerance test (2mg/kg) in rats fed an LF, HF, or HF_BB diet for 8 wk. Values are means ± SEMs, n = 8/group. Labeled means at a time without a common letter differ, $P < 0.05$. HF, high fat; HF_BB, high fat with 10% blueberry; LF, low fat.

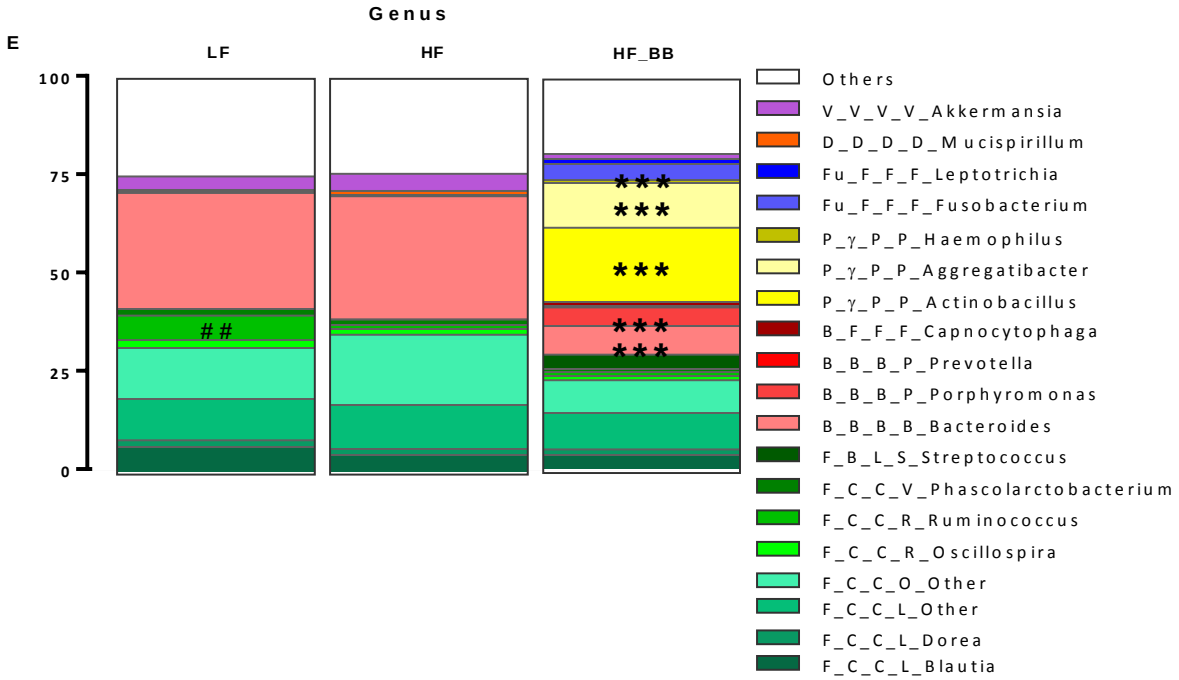

A
Phylum
LF
HF
HF_BB
100
75
50
25
0
*
***
***
***
Others
Actinobacteria
Cyanobacteria
Deferribacteres
Fusobacteria
Verrucomicrobia
Proteobacteria
Bacteroidetes
Firmicutes
B
Class
LF
HF
HF_BB
100
75
50
25
0
***
***
*
***
***
***
***
Others
Fu_Fusobacteria
V_Verrucomicrobiae
P_Betaproteobacteria
P_Alphaproteobacteria
P_Gammaproteobacteria
B_Flavobacteria
B_Bacteroidia
F_Erysipelotrichi
F_Bacilli
F_Clostridia

C


Order
LF
HF
HF_BB
100
75
50
25
0
Others
Fu_F_Fusobacteriales
V_V_Verrucomicrobiales
P_γ_Pseudomonadales
P_γ_Pasteurellales
B_B_Bacteroidales
F_E_Erysipelotrichales
F_B_Lactobacillales
F_C_Clostridiales

D


Family
LF
HF
HF_BB
100
75
50
25
0
Others
D_D_D_Deferribacteraceae
Fu_F_F_Fusobacteriaceae
V_V_V_Verrucomicrobiaceae
P_γ_P_Pasteurellaceae
B_B_B_Porphyromonadaceae
B_B_B_Paraprevotellaceae
B_B_B_Prevotellaceae
B_B_B_Bacteroidaceae
F_N_S_Veillonellaceae
F_E_E_Erysipelotrichaceae
F_B_L_Streptococcaceae
F_C_C_Other
F_C_C_Ruminococcaceae
F_C_C_Clostridiaceae
F_C_C_Lachnospiraceae


E
Genus
LF
HF
HF_BB
100
75
50
25
0
# #
***
***
***
***
***
Others
V_V_V_V_Akkermansia
D_D_D_D_Mucispirillum
Fu_F_F_F_Leptotrichia
Fu_F_F_F_Fusobacterium
P_γ_P_P_Haemophilus
P_γ_P_P_Aggregatibacter
P_γ_P_P_Actinobacillus
B_F_F_F_Capnocytophaga
B_B_B_P_Prevotella
B_B_B_P_Porphyromonas
B_B_B_B_Bacteroides
F_B_L_S_Streptococcus
F_C_C_V_Phascolarctobacterium
F_C_C_R_Ruminococcus
F_C_C_R_Oscillospira
F_C_C_O_Other
F_C_C_L_Other
F_C_C_L_Dorea
F_C_C_L_Blautia

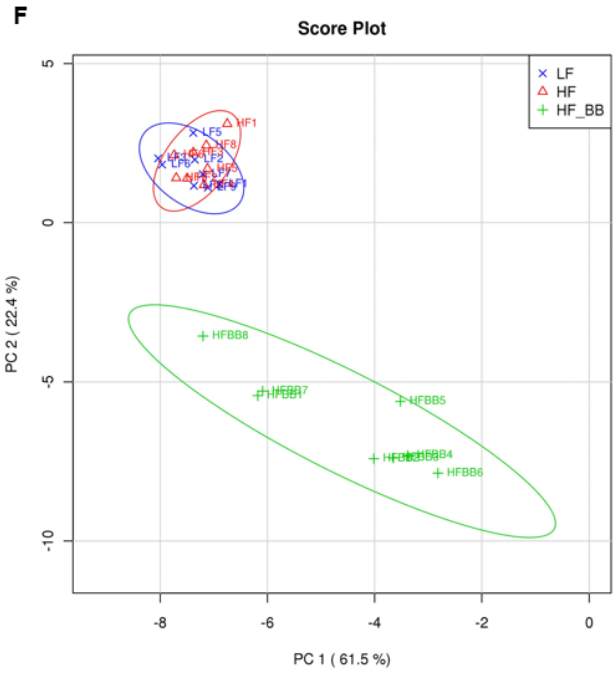

F
Score Plot
LF
HF
HF_BB
PC 2 (22.4 %)
PC 1 (61.5 %)
5
0
-5
-10
-8
-6
-4
-2
0
HFBB8
HFBB5
HFBB6

Figure 2.2. Microbial composition of rats fed an LF, HF, or HF_BB diet for 8 wk. Bacterial relative abundance at the phylum (A), class (B), order (C), family (D), and genus (E) levels and principal component analysis on all taxonomic levels (F). n = 8. * denotes differences between the HF_BB group and the LF and HF groups, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. $^{\#}$ denotes differences between the LF- and HF-fed groups, $^{\#\#}$ $P < 0.01$. Bacteria in any shades of green belong to the *Firmicutes* phylum, shades of red for the *Bacteriodete*s, yellow for the *Proteobacteria*, purple for the *Verrucomicrobia* and blue for the *Fusobacteria*. Phylogenic tree (phylum, class, order, family in order) is indicated by the letters preceding the taxa. **Phylum**: F, *Firmicutes*; B, *Bacteriodetes* Fu, *Fusobacteria*; D, *Deferribacteres*; V, *Verrucomicrobia*; P, *Proteobacteria*. **Class**: C, *Clostridia*; B, *Bacilli*; N, *Negativictes*; E, *Erysipelotrichia*; B, *Bacteriodetes*; F, *Flavobacteria*; γ, *Gammaproteobacteria*;. **Order**: C, *Clostridiales*; L, *Lactobacillales*; S, *Selenomonadales*; E, *Erysipelotrichiales*; B, *Bacteriales*; F, *Flavobacteriales*; F, *Fusobacteriales*; D, *Deferribacteriales*; V, *Verrucomicrobiales*; P, *Pasteurellales*;. **Family**: C, *Clostridiaceae*;R, *Ruminococcaceae*; S, *Streptococcaceae*; V, *Veillonellaceae*; P,*Prevetollaceae*; F, *Flavobacteriaceae*; V, *Verrucomicrobiaceae*; P, *Pasteurellaceae*. HF, high fat; HF_BB, high fat with 10% blueberry; LF, low fat; PC, principal component.

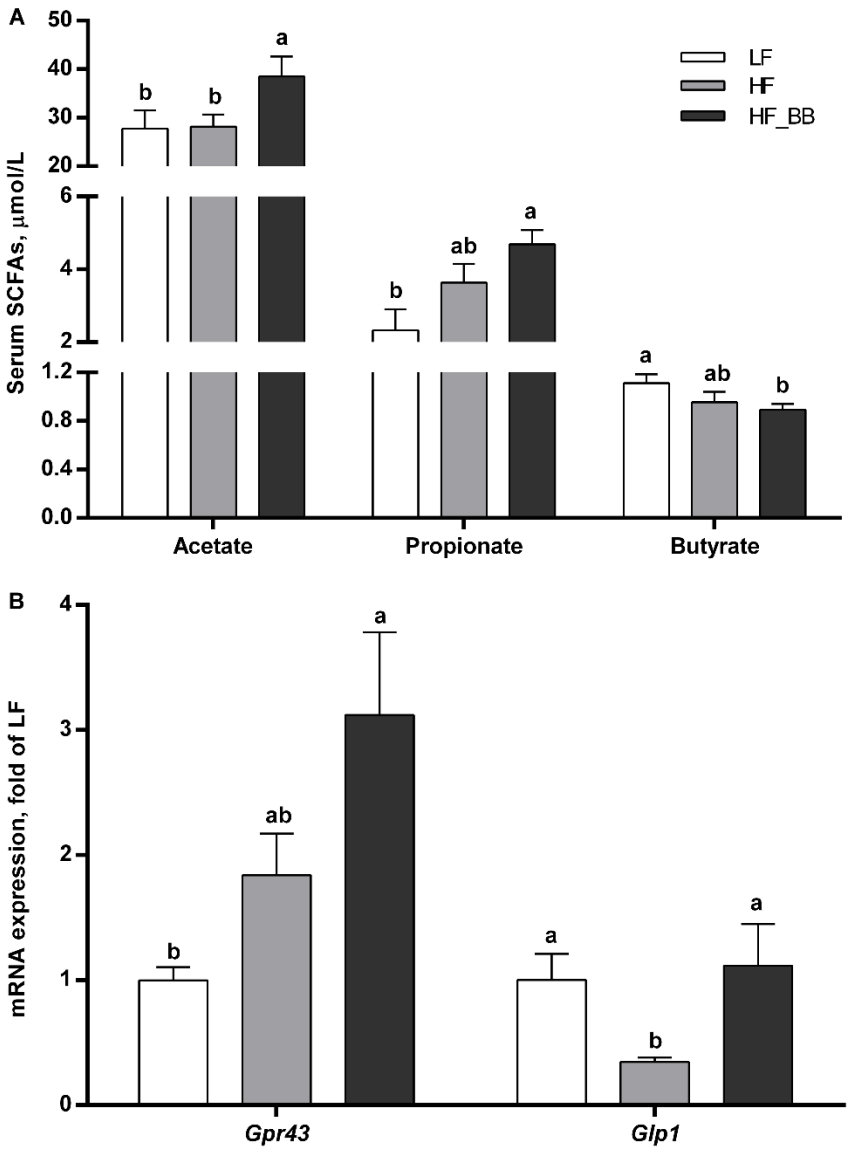


Figure 2.3. Serum SCFAs (A) and gene expression of *Gpr43* and *Glp1* in the ileum (B) of rats fed an LF, HF, or HF_BB diet for 8 wk. Values are means ± SEMs, n = 8. Labeled means without a common letter differ, $P < 0.05$. *Glp1*, glucagon-like peptide 1; *Gpr43*, G protein-coupled receptor 43; HF, high fat; HF_BB, high fat with 10% blueberry; LF, low fat.

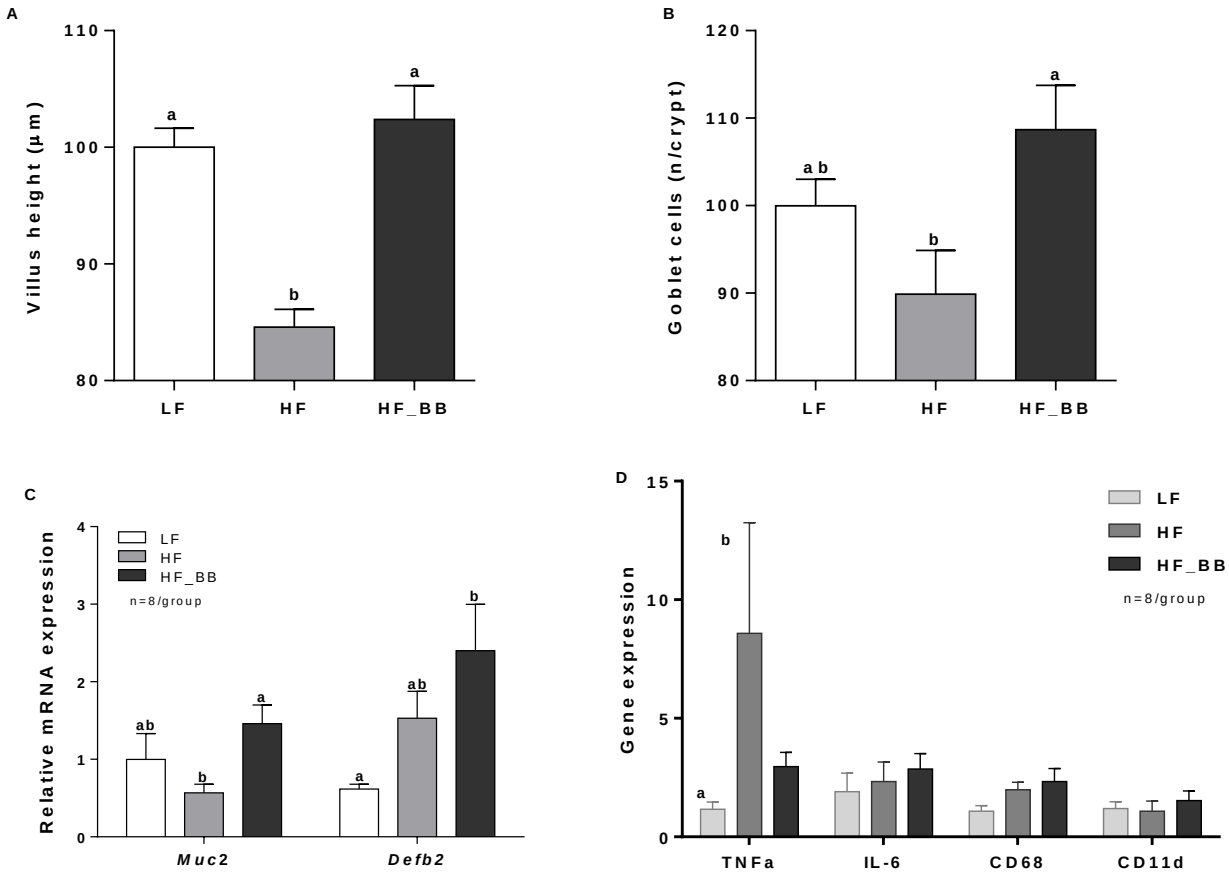


Figure 2.4. Villus length (A), goblet cells/crypt (B), *Defb2* and *Muc2* gene expression (C) and gene expression of inflammatory markers (D) in the ileum of rats fed an LF, HF, or HF_BB diet for 8 wk. Values are means ± SEMs, n = 8. Labeled means without a common letter differ, $P < 0.05$. *Cd11d*, cluster of differentiation 11d; *Cd68*, cluster of differentiation 68; *Defb2*, *β*-defensin 2; HF, high fat; HF_BB, high fat with 10% blueberry; *Il-1b*, interleukin-1 beta; *Il-6*, interleukin-6; LF, low fat; *Muc2*, mucin 2; *Tnfa*, tumor necrosis factor α.

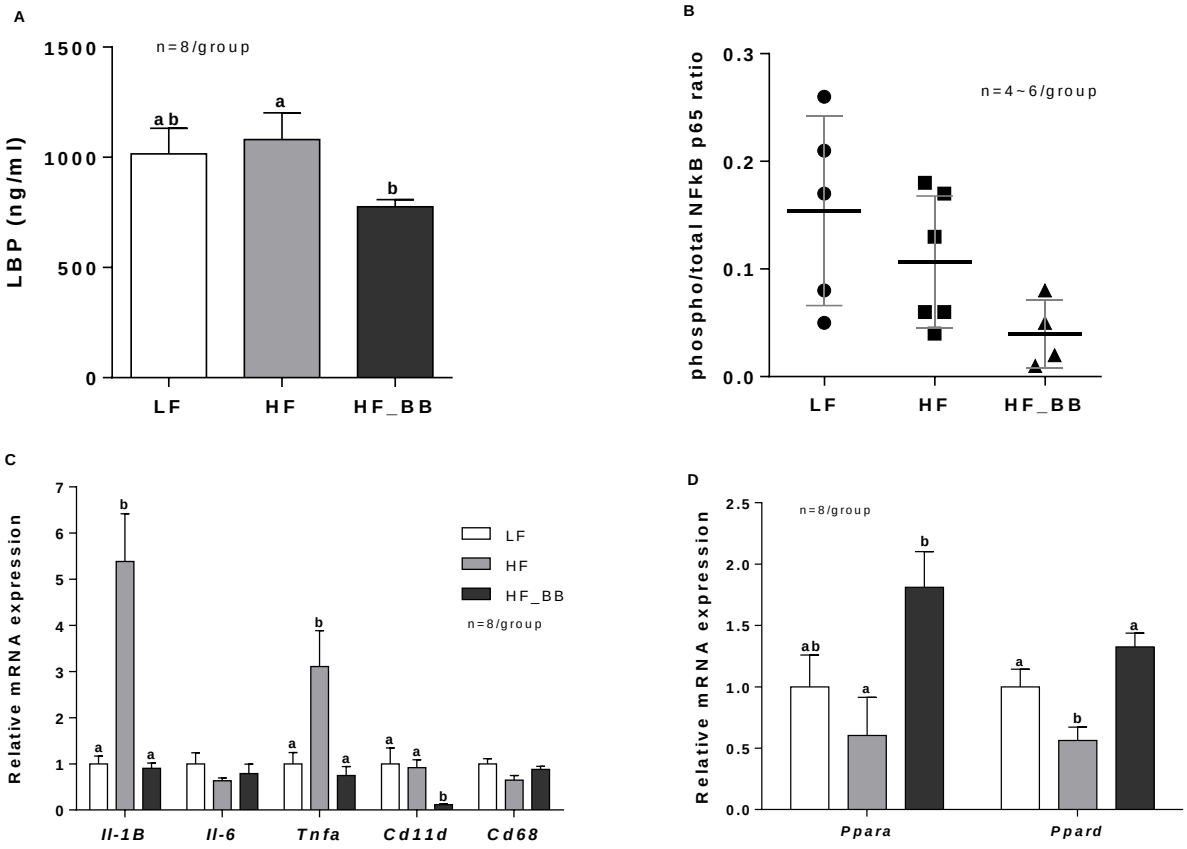


Figure 2.5. Circulating LPS (A), NFκB p65 phosphorylation (B), gene expression of inflammatory markers (C) and gene expression of *Ppara and Ppard* (D) in adipose tissue of rats fed an LF, HF, or HF_BB diet for 8 wk. Values are means ± SEMs; n = 8, except for (B), n = 4~6. Labeled means without a common letter differ, $P < 0.05$. *Cd11d*, cluster of differentiation 11d; *Cd68*, cluster of differentiation 68; HF, high fat; HF_BB, high fat with 10% blueberry; *Il-1b*, interleukin-1β; *Il-6*, interleukin 6; LF, low fat; LBP, LPS-binding protein; *Ppara*, peroxisome proliferator-activated receptor α; *Ppard*, peroxisome proliferator-activated receptor δ; *Tnfa*, tumor necrosis factor α.

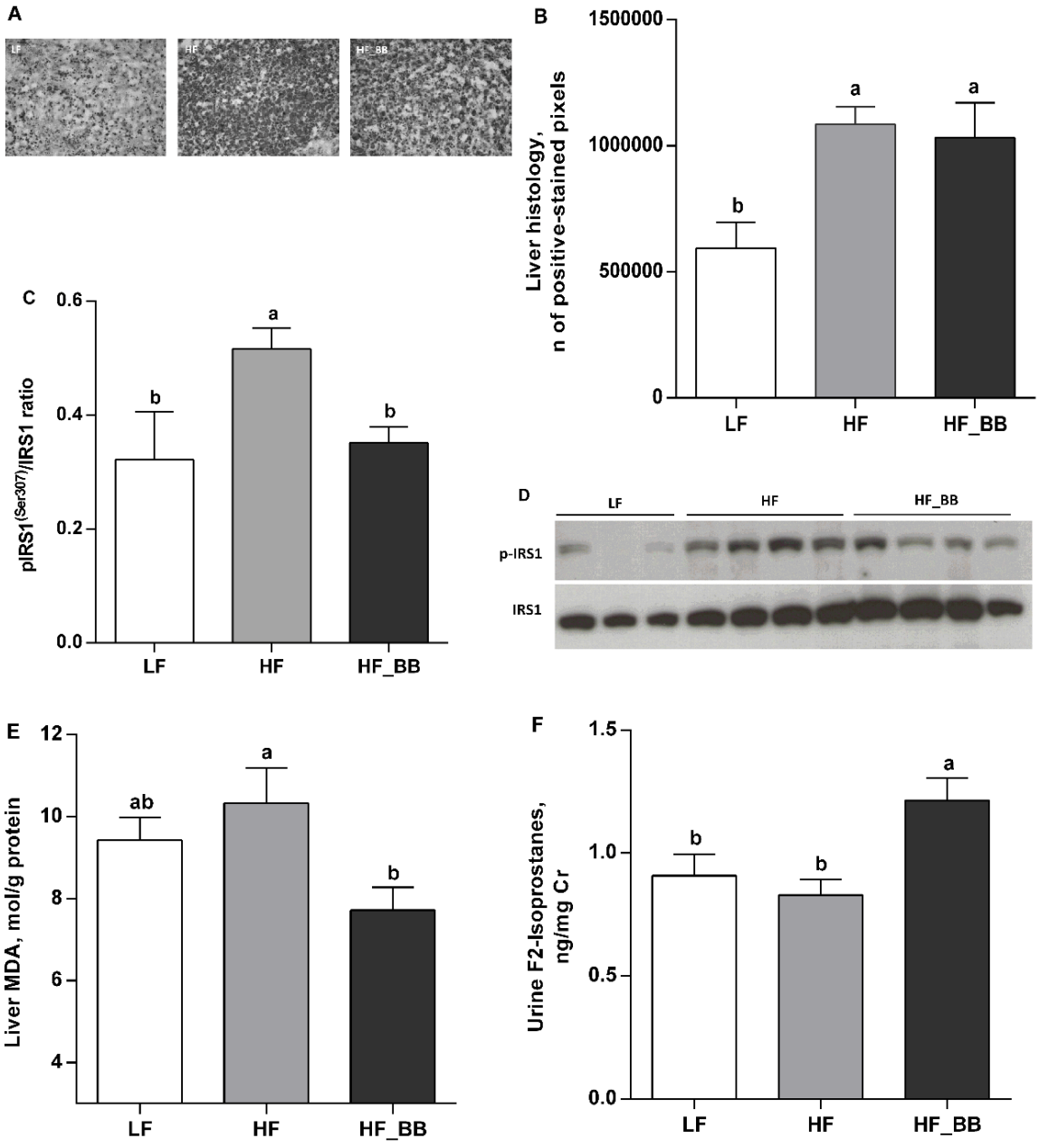
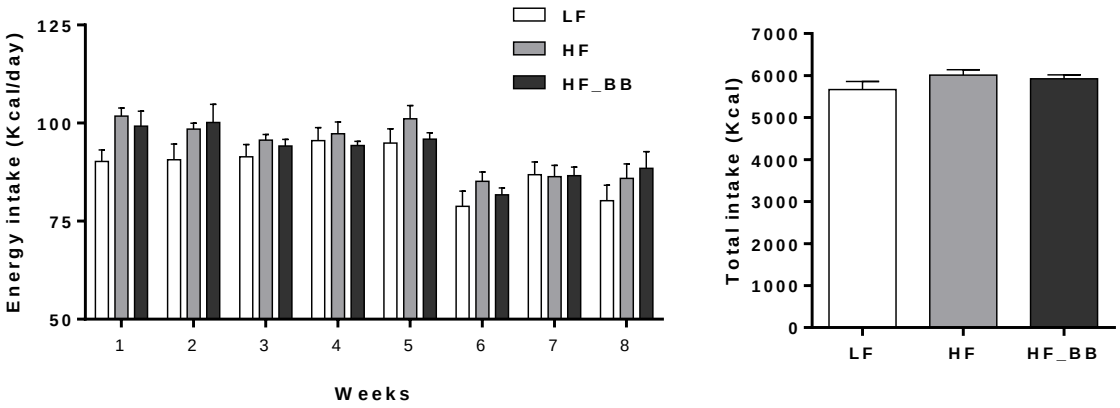
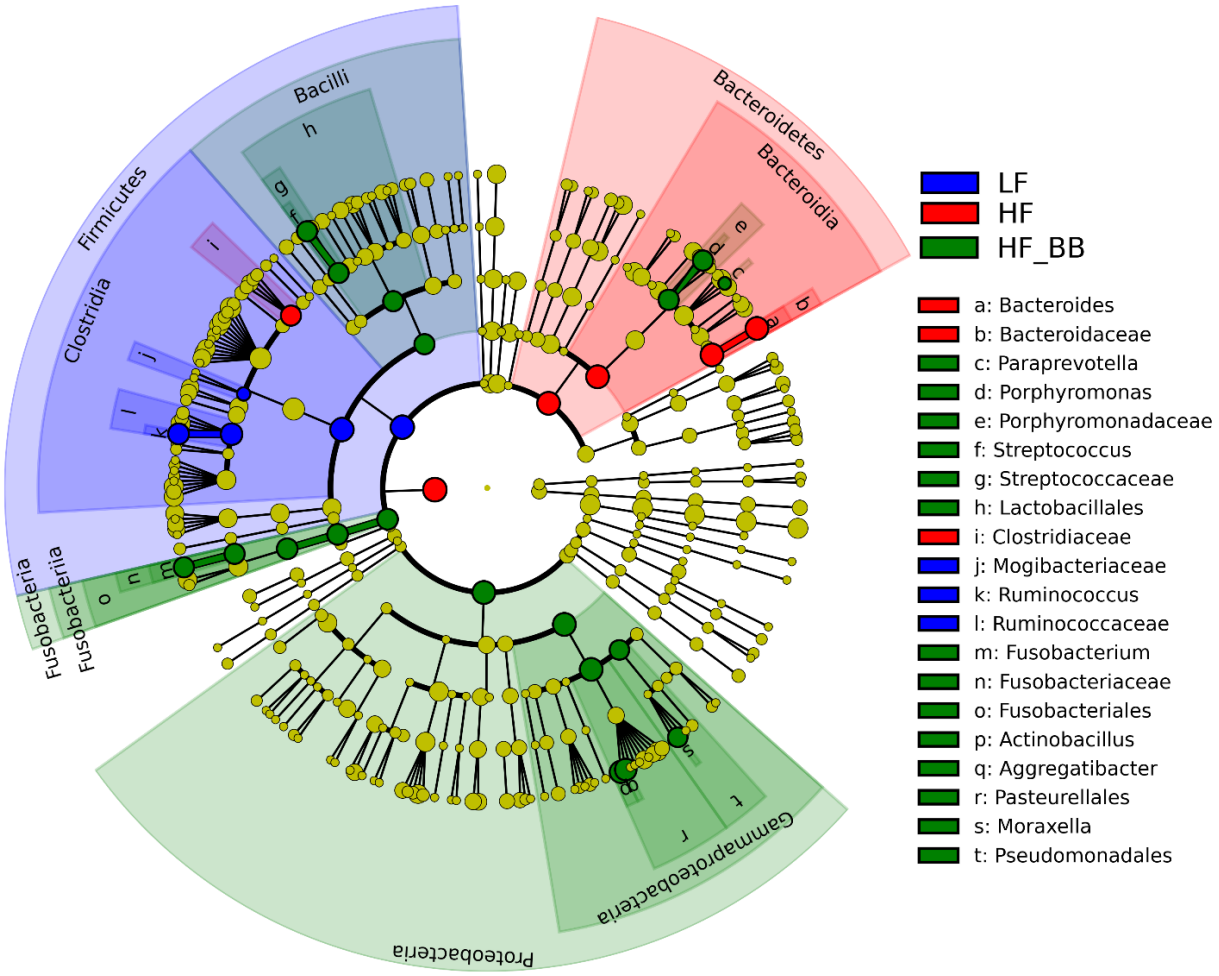


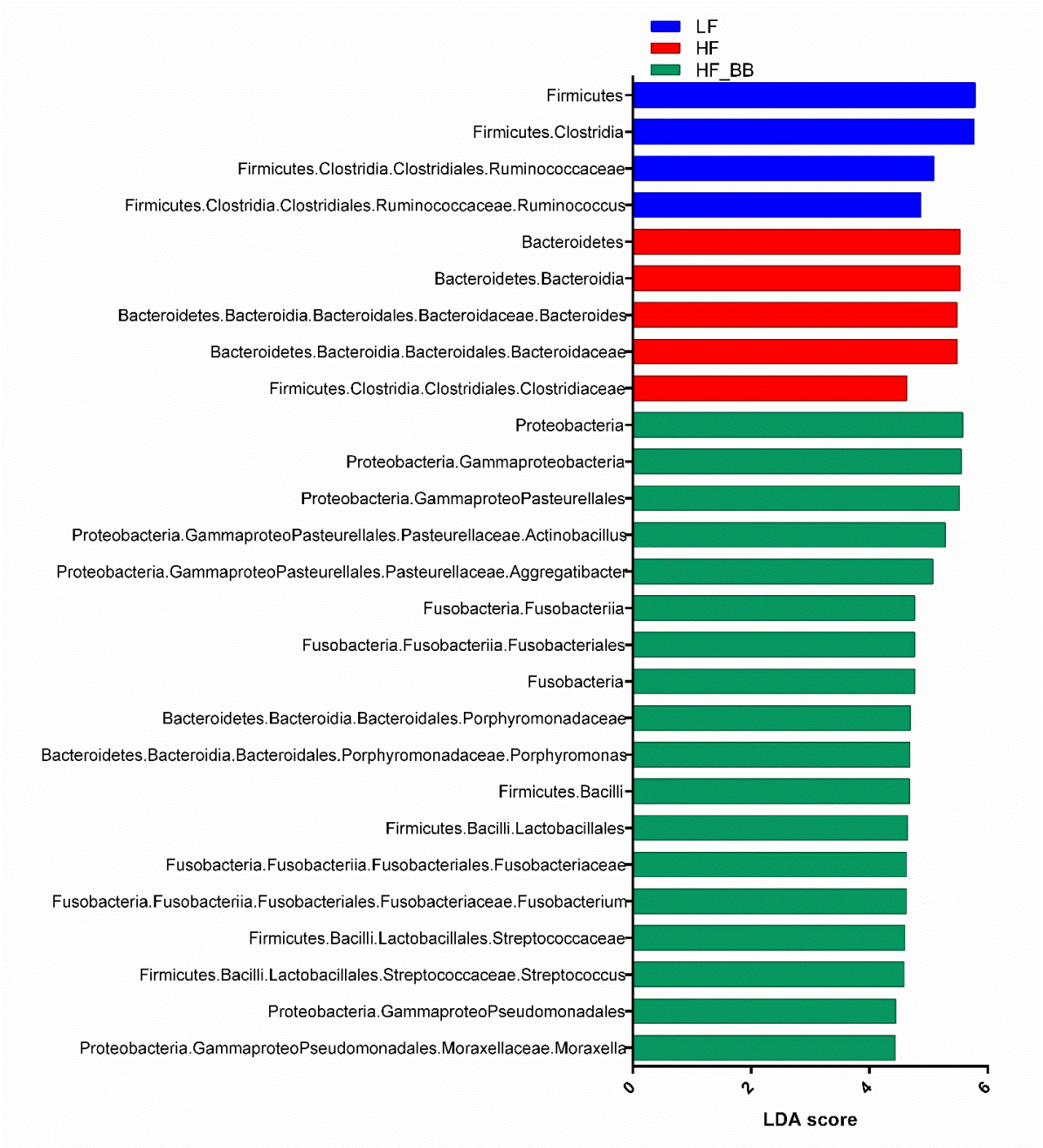
Figure 2.6. Histology (A, B), IRS1 phosphorylation (C, D), and MDA (E) in the liver and urinary F2 isoprostanes (F) of rats fed an LF, HF, or HF_BB diet for 8 wk. Values are means ± SEMs, n = 8; except for (A, B), n = 6~8. Labeled means without a common letter differ, $P < 0.05$. HF, high fat; HF_BB, high fat with 10% blueberry; IRS1, insulin receptor substrate 1; LF, low fat; MDA, malondialdehyde; p-IRS1$^{(Ser307)}$, insulin receptor substrate 1 phosphorylation at serine 307.



Supplemental Figure 2.S1. Daily (A) and total (B) energy intake by rats fed an LF, HF, or HF_BB diet for 8 wk. Values are means ± SEMs, n = 8. Labeled means at a time without a common letter differ, $P < 0.05$. HF, high fat; HF_BB, high fat with 10% blueberry; LF, low fat.



Supplemental Figure 2.S2. Cladogram generated from LEfSe analysis, showing the most differentially abundant taxa enriched in microbiota from rats fed an LF, HF, or HF_BB diet for 8 wk (blue indicating LF, red HF, green HF_BB, and yellow indicating non-significance). n = 8. The six rings of the cladogram stand for domain (innermost), phylum, class, order, family and genus. LEfSe analysis was performed on log-transformed abundances using the Galaxy online module to identify discriminant taxa among groups. The threshold of the logarithmic linear discriminant analysis score was 4.0. HF, high fat; HF_BB, high fat with 10% blueberry; LEfSe, linear discriminant analysis effect size; LF, low fat.



Supplemental Figure 2.S3. Histogram of the LDA scores from LEfSe analysis, showing the most differentially abundant taxa enriched in microbiota from rats fed an LF, HF, or HF_BB diet for 8 wk. n = 8. LEfSe analysis was performed on log-transformed abundances using the Galaxy online module to identify discriminant taxa among groups. The threshold of the logarithmic linear discriminant analysis score was 4.0. HF, high fat; HF_BB, high fat with 10% blueberry; LEfSe, linear discriminant analysis effect size; LF, low fat.

CHAPTER 3

BENEFICIAL EFFECTS OF NON-ENCAPSULATED OR ENCAPSULATED PROBIOTIC SUPPLEMENTATION (LACTOBACILLUS PARACASEI SUBSP. PARACASEI L. CASEI W8®; L. CASEI W8) ON INTESTINAL BARRIER FUNCTIONS, INFLAMMATORY PROFILES, AND GLUCOSE TOLERANCE IN ASSOCIATION WITH MODULATION OF THE GUT MICROBIOTA IN HIGH FAT FED RATS[4]

[4]Sunhye Lee, Rebecca Kirkland, Louise Wicker, Claire B. de La Serre. To be submitted to *Nutrients*.

Abstract

Background: Obesity and its comorbidities are associated with chronic inflammation. Gut microbiota dysbiosis has been linked to diet-induced inflammation, thus modulation of the gut microbiota composition may have therapeutic benefits for metabolic disorders such as insulin resistance. Probiotics exhibit beneficial effects on the host through manipulation of the intestinal microbial composition, suggesting that certain probiotic strains could alleviate aspects of metabolic disorders. However, their *in vivo* health benefits may be limited depending on their survival and sustenance during the gastrointestinal transit, which could be enhanced by a microencapsulation technique.

Objective: The aim of this present study was to examine the potential beneficial effects of non-encapsulated or encapsulated probiotic supplementation (*Lactobacillus paracasei subsp. paracasei* L. casei W8®; L. casei W8) on the gut microbiota composition and metabolic profiles in high-fat (HF) diet-fed rats.

Methods: Thirty-two male Wistar rats (n=8/group) were fed ad libitum with low-fat (LF, 10% fat), HF (45% fat), HF with non-encapsulated L. casei W8 (Pro; $4x10^7$ cfu/g diet), or HF with encapsulated L. casei W8 (Pca; $4x10^7$ cfu /g diet) diet for 7 wk. Microbiota composition (16S rRNA sequencing), intestinal integrity (gut permeability and mucin 2 (MUC2) gene expression), inflammation (gene expression of macrophage infiltration markers and pro-inflammatory cytokines and serum lipopolysaccharide (LPS)), and glucose tolerance were assessed.

Results: HF feeding altered the gut microbiota composition with increases in ratio of *Firmicutes* to *Bacteroidetes* and *Deltaproteobacteria* abundance and decreases in *Cyanobacteria* abundance, which were normalized by probiotic supplementation. Probiotic supplementation led to a significant upregulation of MUC2 gene expression in the ileum, suggesting improvement of gut

barrier integrity. Moreover, probiotic treatment suppressed local (gene expression of cluster of differentiation 68 (CD68), monocyte chemotactic protein 1 (MCP1) and interleukin-1 beta in the ileum) and systemic (gene expression of CD68 and MCP1 in visceral adipose fat and serum LPS-binding protein level) inflammation indices. However, probiotic supplementation had little effect on glucose tolerance, which was improved with a microencapsulation treatment.

Conclusion: Probiotic supplementation led to compositional changes in the gut microbiota associated with improvements in gut barrier functions and local and systemic inflammation in HF-fed male rats. These findings suggest the potential of using an oral probiotic supplementation to ameliorate obesity-associated inflammation.

Key words: probiotics, microencapsulation, gut microbiota, intestinal epithelial barrier, inflammation

Introduction

Metabolic disorders including obesity and its comorbidities are major health problems in the United States where western diets rich in saturated fat and sugars, are widely consumed (111). Obesity has been characterized as a low grade systemic inflammatory state (116). Visceral adiposity, especially the mesenteric depot, is strongly related to metabolic dysfunctions including insulin resistance and systemic inflammation (116, 169). Abnormal expansion of the visceral adipose tissue is associated with macrophage infiltration and secretion of pro-inflammatory cytokines such as interleukin 1β (IL-1β) promoting insulin resistance (117, 118).

There is evidence that obesity-associated inflammation originates, at least partially, from the gastrointestinal (GI) tract (120) and previous studies have shown that propensity to high-fat (HF) diet-induced obesity is associated with an increase in GI permeability (120). Chronic HF feeding has been shown to impair the gut epithelial barrier integrity particularly in the distal gut (ileum, colon), notably by altering the expression of gut-protecting mucins (122). The distal gut harbors more than 10^{14} microbial organisms (170) and increases in gut permeability in combination with diet-driven alteration of the gut microbiota (dysbiosis) allow for translocation of pro-inflammatory bacterial products such as lipopolysaccharide (LPS) to the circulation, promoting systemic inflammation (5, 7). Interestingly, chronic infusion of LPS in rodents is shown to induce weight gain, adipose tissue inflammation and hepatic insulin resistance (5, 125), highlighting a potential causal role for microbiota-driven inflammation in metabolic disorders. Additionally, modulation of the gut microbiota composition restores gut epithelial function and improves metabolic profiles, especially insulin sensitivity (126).

Probiotics are defined by the FAO/WHO as live microorganisms which when administered in adequate amounts confer a health benefit on the host (171). Probiotics exhibit

beneficial effects on the host through modulation of the intestinal microbial composition by suppressing pathogenic bacteria such as *Clostridium perfringens* and protecting beneficial populations such as *bifidobacteria* (172). Probiotics act by inhibiting growth of enteric pathogens via production of inhibitory antimicrobials, competitively adhering to the mucosa and epithelium, strengthening the epithelial barrier integrity via mucin and defensins, and modulating the immune system of the host (173). Furthermore, probiotic-driven changes in gut microbiota can lead to changes in the production of intestinal short chain fatty acids (SCFAs) (174), promoting production of GI peptides including glucagon-like peptide-1 (GLP1) (136). GLP1 is secreted by enteroendocrine L cells in the distal gut in response to feeding and acts as an incretin (175). The therapeutic effects of probiotics on metabolic diseases have been implicated in many experimental and clinical studies (176), showing that certain probiotic strains can modulate various aspects of metabolic disorders. A variety of beneficial strains, especially *Lactobacillus*, *Streptococcus* and *Bifidobacterium*, have been demonstrated to alleviate hyperlipidemia, obesity and insulin resistance in HF diet-fed animals in association with the maintenance of gut integrity and manipulation of the gut microbiota profile (177).

In order to exert their functions, probiotic bacteria should be viable in high concentrations when they reach the distal part of the intestine where they can interact with commensal microbiota. The delivery of orally ingested probiotics may be restricted by the harsh environmental conditions of the GI tract (e.g. acidic conditions of the stomach, digestive enzymes, and bile salts of the small intestine); thus, it is desirable to develop methods that increase the viability of the probiotic cells until the lower GI tract is reached. In this regard, the recent emergence of microencapsulation techniques appears to be a potential solution to enhance the survivability of probiotics (109). The use of microcapsules in polymer membranes has

notably been shown to successfully protect viable probiotic bacteria following oral delivery in humans and rodents (110).

The present study investigated a novel approach for the oral delivery of microencapsulated probiotic bacteria *Lactobacillus paracasei subsp. paracasei* L. casei W8® (L. casei W8) and its potential in the prevention of HF diet-induced metabolic disorders. We hypothesized that probiotic supplementation would improve diet-induced inflammation and epithelial barrier integrity via positive alterations in intestinal microbiota composition. We anticipated the microencapsulation will significantly enhance delivery and effects of the probiotic. To test this hypothesis, Wistar rats were fed a HF diet supplemented with non-encapsulated or encapsulated probiotic for 7 wk and gut microbiota composition, inflammation and glucose tolerance were determined.

Methods

In vitro release. For simulated gastric fluid (SGF), 1 g NaCl was dissolved into 500 ml type II water with the pH adjusted to 2.0 with 10 N HCl. And then, 1.6 g pepsin was added and dissolved completely. For simulated bile fluid (SBF), 1% SBF was prepared by adding 1g porcine bile extract into 100 mL phosphate-buffered saline (PBS). For simulated intestinal fluid (SIF), 2 g pancreatin was dissolved in 400mL PBS. *In vitro* release tests were conducted placing free cells or microcapsules separately or consecutively in SGF for 2 h, SBF for 20 min, and SIF for 2 h. After each process, the number of *Lactobacillus* was calculated by plate counting method.

Animals and treatment regimen. Thirty-two male Wistar rats (200-220 g) were procured from Envigo (Indianapolis, IN) and single-housed in a temperature-controlled room with a 12-h light-dark cycle. Animals were separated into four groups (n=8 per group) and fed low fat (LF;

Research Diets no. 12450H 10% calories from fat, 70% from carbohydrate, and 20% from protein providing 3.85 kcal/g of energy), high fat (HF; 45% calories from fat, 35% from carbohydrate, and 20% from protein providing 4.73 kcal/g of energy), HF supplemented with non-encapsulated L. casei W8 (Pro; $4x10^7$ cfu/g diet), or HF with supplemented with encapsulated L. casei W8 (Pca; $4x10^7$ cfu/g diet) for 7 wk (**Table 3.1**). Body weight and food intake were monitored daily. After 7 wk on their respective diets, animals were fasted for 6 h and euthanized via CO_2 inhalation. Sacrifice order was evenly distributed between treatment groups over 4 days and all tissues were removed within 3 hours after the beginning of the light cycle. Blood was collected by cardiac puncture, rested on ice for 15 min, and spun (3,000 rpm for 10 min at 4 °C) for serum collection. Liver, ileum, cecum, colon, and visceral fat pads (mesenteric, retroperitoneal, and epididymal) were collected and weighed and an adiposity index was determined. Serum and all tissues were snap frozen and stored at -80°C. All animal care procedures were approved by the Institutional Animal Care and Use Committee of the University of Georgia (approval number: A2013 09-002-Y2-A5).

*Oral glucose tolerance test (OGTT)***.** After 6 wk on their respective diets, animals were fasted for 5 h before oral gavage with a glucose solution (2 g/kg body weight using 20% glucose, Sigma-Aldrich, St. Louis, MO). Glycemia was measured using a glucometer (Freestyle, Alameda, CA) before (0 min) and after (15, 30, 60, 90, and 120 min) glucose administration. Blood samples (~100 μL) were collected at each time point for insulin analysis.

Microbiota DNA sequencing. DNA was extracted from cecal contents using the ZR Fecal DNA MiniPrep according to the manufacturer's protocol (Zymo Research, Irvine, CA) and the eluted DNA was sent to SeqMatic LLC (Fremont, CA) for PCR, sequence library preparation, and sequencing. Briefly, polymerase chain reaction (PCR) was used to target the V4 region of

the 16S rRNA genes using the 515F/806R primer set based on the protocol used by the Earth Microbiome project (178). Sequence reads were obtained from a paired end 2x150 Illumina MiSeq run. Sequences were subsequently aligned to reference genomes. Abundance was normalized by log transformation and multivariate statistical analysis were performed using METAGENassist platform (137). Difference in abundance among groups was assessed using one-way ANOVA with Fisher's least-significant difference post hoc test for parametric data and Kruskal–Wallis test with Dunn's post hoc test for non-parametric data.

SCFA analysis. SCFAs were quantified in serum by the Mayo Clinic Metabolomics Core via gas chromatography–mass spectrometry using previously published methods (139).

Biochemical analyses. LPS-binding protein (LBP; Biometec, Greifswald, Germany) and insulin (Alpco, Salem, NH) in serum were measured via enzyme-linked immunosorbent assay (ELISA) according to manufacturers' instructions.

Real-time PCR (qPCR). Gene expression of inflammatory markers was determined in ileum and mesenteric fat tissues via qPCR. Gene expression of gut epithelial function parameters was determined in ileum tissues. mRNA was extracted from ileum and mesenteric fat tissues using the RNeasy Mini Kit or Lipid Tissue Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instruction and assessed for quantity and purity using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). cDNAs were synthesized using the RevertAid™ First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Franklin, MA). Real-time PCR was performed with the StepOnePlus real-time PCR system and software (Thermo Fisher Scientific) using SYBR Green PCR master mix (Thermo Fisher Scientific) with primers purchased from Integrated DNA Technologies (**Table 3.2**). Data were analyzed according to the $2^{-\Delta\Delta Ct}$ method (140).

Statistical analysis. Data are presented as mean ± SEM. Statistical analysis was performed using Prism software (Prism 6.0; GraphPad Software, La Jolla, CA). Two-way repeated measures ANOVA was used to analyze body weight, energy intake, and OGTT. One-way ANOVA was performed to analyze data from adiposity, qPCR, SCFAs, and biochemical analyses. Differences in abundance among groups were assessed using one-way ANOVA with Fisher's least-significant difference post hoc test for parametric data and Kruskal–Wallis test with Dunn's post hoc test for non-parametric data. Correlations between SCFAs levels and microbiota abundance were determined using the nonparametric Spearman correlation. Differences were considered significant if $P < 0.05$.

Results

Survivability of non-encapsulated and microencapsulated L. casei W8 in the simulated gastric tract. The efficacy of microcapsules employed in this study was determined *in vitro* using a gastric simulator (**Table 3.3**). There was no significant difference in initial viability between non-encapsulated and encapsulated probiotics. However, it was clarified that the survivability of microencapsulated cells was significantly higher than that of the free cells after exposure to SGF ($P < 0.05$) and SBF ($P < 0.05$). Both survived the simulated intestinal environment.

Effects of probiotic administration on body weight, energy intake, and adiposity. There was no difference in initial body weight among all treatment groups (**Figure 3.1A**). After 7 wk on respective diets, HF feeding alone did not result in a significant increase in body weight compared to LF feeding. Probiotic-supplemented animals (Pro) had a significantly higher body weight compared to LF rats ($P < 0.05$), but not to HF and Pca groups.

There were no significant differences in energy intake among LF, HF and Pca rats throughout the experiment. However, during the 1st wk, HF-fed groups had a significantly

higher energy intake than the LF group (LF vs. HF, $P < 0.05$; vs. Pro, $P < 0.001$; vs. Pca, $P < 0.05$; **Figure 3.1B**); after 2 wk, the Pro group had higher energy intake than the other groups with (week 3: Pro vs. LF, $P < 0.01$, vs. HF, $P < 0.05$, vs. Pca, $P < 0.01$; week 4: Pro vs. LF, $P < 0.05$, vs. HF, $P < 0.05$, vs. P = Pca, ns; week 7: Pro vs. LF, $P < 0.01$, vs. HF, $P < 0.05$, vs. Pca, ns) or without (week 2, 5, and 6) significance.

Similarly to the increase in body weight, the Pro group had a significantly higher total adiposity index than LF and HF rats (Pro vs. LF, $P < 0.0001$; vs. HF, $P < 0.05$; **Figure 3.1C**) but not than Pca rats, which was mainly driven by the differences in retroperitoneal (Pro vs. LF, $P < 0.01$; vs. HF, $P = 0.08$) and epididymal (Pro vs. LF, $P < 0.001$; vs. HF, $P < 0.01$) fat pads.

Effects of probiotic administration on glucose homeostasis. There was no difference in fasting (0 min) glycemia and insulinemia among groups (**Figure 3.2A, C**). Following an OGTT, glycemia constantly increased for 60 min (LF, HF, and Pca) or 90 min (Pro) post challenge. HF feeding resulted in a non-significant alteration in glucose tolerance. However, the glycemia area under the curve (AUC) over 120 min was significantly higher in the Pro group compared to the LF and Pca groups (Pro vs. LF or Pca, $P < 0.001$) but not to the HF group (**Figure 3.2B**). The response by Pca-fed rats to the OGTT challenge was similar to the LF rats.

During the OGTT, serum insulin levels peaked at 15 min post oral glucose challenge in all groups with a significant elevation in the Pro group compared to the LF group ($P < 0.05$), but not to HF and Pca rats (**Figure 3.2C**). HF feeding significantly increased serum insulin AUC (LF vs. HF, $P < 0.05$), which was reduced by probiotic treatment albeit not significantly (**Figure 3.2D**).

Effects of probiotic administration on the gut microbiota and metabolites. Gut microbiota was analyzed at the levels of phylum, class, order, family, genus, and species (**Figure 3.3**). HF

consumption modestly altered the microbiota composition. At the phylum level (**Figure 3.3A, B, C**), the HF groups had a significantly higher ratio of the main phyla *Firmicutes* to *Bacteroidetes* than the LF group ($P < 0.05$), which was normalized by probiotic supplementation in the Pro group (Pro vs. HF, $P < 0.05$), and partially improved by the Pca treatment. *Cyanobacteria* abundance was significantly reduced by HF feeding (LF vs. HF, $P < 0.05$) and this change was partially restored to the LF level in Pro and Pca groups. There was a significant increase in *Lentisphaerae* abundance in HF-fed groups compared to the LF group (LF vs. HF, $P < 0.01$; vs. Pro, $P < 0.01$; vs. Pca, $P < 0.05$) driven by the family *Victivallaceae* (LF vs. HF, $P < 0.01$, vs. Pro, $P < 0.01$; vs. Pca, $P < 0.05$).

At the class level (**Figure 3.3D, E**), we observed a significant increase in *Deltaproteobacteria* abundance in HF and Pca groups compared to the LF group (LF vs. HF, $P < 0.05$; vs. Pca, $P < 0.01$), which was driven by the order *Desulfovibrionales* (LF vs. HF, $P < 0.05$; vs. Pca, $P < 0.01$), family *Desulfovibrionaceae* (LF vs. HF, $P < 0.05$; vs. Pca, $P < 0.01$), and genus *Bilophila* (LF vs. HF, $P = 0.055$; vs. Pca, $P < 0.01$).

At the order level (**Figure 3.3F, G**), a significant increase was induced by HF feeding in *Coriobacteriales* abundance ($P < 0.001$) along with its family *Coriobacteriaceae* ($P < 0.001$) and genus *Adlercreutzia* ($P < 0.05$).

At the family level (**Figure 3.3H, I**), we observed a lower abundance of *Lactobacillaceae* in HF-fed groups compared to the LF group and this difference reached significance in the probiotic-treated groups (Pro vs. LF, $P < 0.01$; Pca vs LF, $P < 0.05$). This trend was also found in its genus *Lactobacillus* (Pro vs. LF, $P < 0.01$; Pca vs LF, $P < 0.05$).

At the species level (**Figure 3.3L, M, N, O**). There was no significant difference in *Bifidobacterium* abundance among groups but the abundance was significantly higher in the Pca

group compared to the LF group ($P < 0.05$). *L. paracasei* and *L. zeae* were only present in probiotic-treated groups with a significant difference between Pro and Pca groups ($P < 0.05$ and $P < 0.01$, respectively). The presence of *Prevotella* was detected only in LF and Pca groups without a significant difference between them.

Probiotic supplementation was associated with changes in serum concentration of SCFAs (**Figure 3.4A**). HF feeding led to a non-significant decrease in butyrate level in serum, which was increased by probiotic administration with significance in Pca rats (Pca vs. HF, $P < 0.05$) and without significance in Pro rats. In contrast, propionate levels were increased by HF feeding (non-significantly, $P = 0.07$), which was significantly reduced in both the Pro and Pca groups (Pro vs. HF, $P < 0.01$; Pca vs. HF, $P < 0.05$). There was no difference in acetate levels among groups. Correlation analysis showed significant positive relationships between serum butyrate and abundance of *Cyanobacteria* ($_r = 0.47$; $P < 0.01$), *Bifidobacterium* ($_r = 0.38$; $P < 0.05$), and *Prevotella* ($_r = 0.41$; $P < 0.05$).

No significant differences were found among groups for GLP gene expression but the Pca group had the highest GLP1 mRNA expression (**Figure 3.4B**).

Effects of probiotic administration on GI barrier integrity and inflammation. Gene expression of a mucus protein, mucin 2 (MUC2), in the ileum was elevated in the HF-fed groups, but only the Pro group showed a significant difference compared to the LF group ($P < 0.05$; **Figure 3.5A**).

In the ileum, HF feeding was associated with significant upregulation in gene expression of macrophage infiltration markers, CD68 (HF vs. LF, $P < 0.01$) and MCP1 (HF vs. LF, $P < 0.001$), which was normalized by probiotic supplementation (CD68, HF vs. Pro, $P < 0.01$; HF vs. Pca, $P < 0.05$; MCP1, HF vs. Pro, $P < 0.01$; HF vs. Pca, ns; **Figure 3.5B**). A similar trend was

observed in gene expression of inflammatory markers, IL-1B, IL-6 and TNFα, but without significance. However, the increased gene expression of IL-1β by HF feeding was significantly down-regulated in the Pro group ($P < 0.01$).

Serum LBP level was increased by HF feeding and decreased by probiotic treatment compared to the LF group, which were not significant (**Figure 3.5C**).

In mesenteric fat, HF feeding was associated with significant increases in CD68 and MCP1 gene expression (CD68, HF vs. LF, $P < 0.05$; MCP1, $P < 0.001$) with the latter being suppressed by probiotic supplementation (HF vs. Pro, $P < 0.05$; vs. Pca, $P = 0.07$; **Figure 3.5D**).

Discussion

In this current study**,** we investigated the potential effects of L. casei W8 on HF diet-induced metabolic changes with the application of microencapsulation of probiotic bacteria for better survivability. Our hypothesis was that 7 wk of probiotic supplementation as a part of HF diet would improve inflammatory state and glucose tolerance in association with compositional changes in the gut microbiota and improvement in gut barrier function and that there would be differences in probiotic effects between two probiotic-treated groups depending on microencapsulation efficiency.

Microencapsulation of probiotic bacteria was efficacious in enhancing the survivability of the probiotics in the simulated GI tract, notably against gastric and bile acidic environmental conditions. HF-driven increases in ratio of *Firmicutes* to *Bacteroidetes* and *Deltaproteobacteria* abundance and decreases in *Cyanobacteria* abundance were normalized to some extent by non-encapsulated probiotic treatment, but not by the encapsulated probiotic. These changes were associated with a significant upregulation of MUC2 gene expression in the ileum, suggesting improvement of gut barrier integrity. Moreover, non-encapsulated probiotic treatment suppressed

local (CD68, MCP1 and IL-1β in the ileum) and systemic (MCP1 in visceral adipose fat) inflammation indices. Improvements in glucose tolerance and insulin sensitivity were observed only in the encapsulated probiotic-treated group.

Consumption of a HF diet (45% fat) more than 5 wk has been shown in rats to significantly increase body weight, energy intake, and fat mass (114). In this study, it was found that HF feeding for 7 wk brought no significant differences in overall body weight and energy intake between LF and HF fed groups. However, there were significant increases in body weight (the last 3 wk) and energy intake (throughout the experimental period) in the Pro group, but not the Pca group, compared to the LF group. A recent study in rats suggested that diet-induced hyperphagia was driven by high sugar contents in the diet regardless of fat contents (179). Thus, it is possible that our diet composition high in sugars (LF group, 17% kcal; HF groups, 17% kcal) was responsible for the lack of BW difference between the LF and HF groups. We still observed an increase in adiposity in HF-fed rats compared to the LF group. Adiposity in rats has previously been found to be proportional to dietary fat regardless of body weight (143). Unexpectedly, while HF feeding failed to significantly increase visceral adiposity, a significant increase in fat mass was found in probiotic-treated groups compared to the LF group. Thus, these data show that the probiotic treatment, regardless of microencapsulation, was not successful at reducing body weight and adiposity. It is important to note that the effect of *Lactobacillus* species on phenotypic parameters is still controversial due to its species and strain specificity. Previous studies have shown that some *Lactobacillus* species were associated with obesogenic phenotypes (*L. acidophilus, L. ingluviei or L. fermentum*) whereas other specific strains (*L. gasseri* and *L. plantarum*) presented anti-obesity effects (180) . Therefore, no definite conclusion could be made regarding the effect of probiotics on general phenotypes.

HF consumption resulted in modest changes of the gut microbiota composition, which was modulated by probiotic supplementation. The increased ratio of *Firmicutes* to *Bacteroidetes* characterizes obesity-driven disruptions in microbiota, dysbiosis (17). Similarly, we found increased *Firmicutes* and decreased abundance of *Bacteroidetes* with HF feeding, which was normalized by the probiotic to the LF level. *Cyanobacteria* abundance was significantly reduced by HF feeding and this change was returned to the LF level in Pro (partially) and Pca groups. Studies have shown that the relative abundance of *Cyanobacteria* decreases with HF diets and increases in *Cyanobacteria* are associated with metabolic improvements in mice (181, 182)
It is not surprising that *L. paracasei,* the probiotic species treated in this study, was only present in probiotic-treated groups, but it was significantly lower in the Pca group ($P < 0.05$). Evidence is mounting that some *Lactobacillus* species, notably *Lactobacillus plantarum, Lactobacillus rhamnosus,* and *Lactobacillus paracasei,* appear to be transient passengers rather than to be GI commensals, promoting the growth of indigenous beneficial gut microbes such as *Bifidobacteria* and leading to ecological rearrangement of the gut microbiota (183, 184). Similarly, the presence of *L. zeae* was detected only in probiotic-treated groups, whose abundance is significantly increased by *L. casei* BL23 treatment, a probiotic strain well known for its anti-inflammatory properties (185). Furthermore, as one of the most commonly used probiotics, *Bifidobacterium* abundance was highest in the Pca group, which was significantly higher than the LF group. *Bifidobacterium* has been found to be associated with improved glucose tolerance in previous studies, which corresponds to glucose tolerance outcome of this study. As mentioned earlier, *Cyanobacteria* abundance, which is associated with metabolic improvements (181, 182), was significantly reduced by HF feeding and this change returned to the LF level in the Pca group.

Thus, these results suggest the stimulatory effect of *Lactobacillus* on other beneficial bacteria at the expense of its own growth.

We proposed that changes in gut microbiota would be associated with improved gut epithelial integrity and decreases in local and systemic inflammation. HF consumption has been shown to promote intestinal inflammation, leading to altered intestinal barrier function and increases in gut permeability and translocation of LPS into the circulation (31). The increase in LPS in the circulation can promote macrophage infiltration into white adipose tissue, resulting in systemic inflammation (8). In this study, it was found that probiotic supplementation reversed HF diet-induced increases in macrophage accumulation (CD68 and MCP1) and inflammation (IL-1B) in the ileum, suggesting a protective effect of the probiotic against local inflammation. Previous studies have demonstrated that probiotics can improve epithelial barrier integrity by upregulating the expression of mucin from the host (89). Mucin, as the primary glycoprotein of the GI mucus layer in the GI tract, protects the intestinal epithelium from pathogenic invasion by forming a defensive physicochemical barrier (90). In this study**,** we found that probiotic supplementation increased MUC2 gene expression in the ileum. Consistently, MCP1 gene expression was suppressed in mesenteric fat of Pro and Pca rats, indicating an anti-inflammatory effect of the probiotic against systemic inflammation.

Inflammatory cytokines could impair glucose tolerance and insulin sensitivity (186). In contrast with the improved inflammatory profiles of the Pro and Pca groups, probiotic supplementation did not significantly alleviate modestly impaired glucose tolerance by HF feeding. There were little differences in glycemia and insulinemia among HF-fed groups. Interestingly, while no difference was found in insulinemia between probiotic-treated groups, better glycemic control was observed in the Pca group.

Based on metabolic outcomes shown in this study, it is important to point out that while microencapsulation efficacy was confirmed *in vitro* with the use of a gastric simulator, different metabolic outcomes were observed between Pro and Pca groups for gut barrier functions and inflammatory profiles and for glucose tolerance. That is, while both probiotic-treated groups showed improvements in local and systemic inflammation and gut barrier functions against the HF challenge, the effects were only significant in rats receiving non-encapsulated probiotics. In addition, during the OGTT, the HF-induced glucose intolerance was further deteriorated in the Pro group while the impairment was alleviated in the Pca group similarly to LF rats. These differences cast doubt on the efficacy of encapsulation employed in this study. One possible mechanism is that the pectin-based coating may have prevented the complete release of encapsulated probiotics into the distal gut, resulting in excretion through feces without exerting its full effects on the host (187). This possibility raises another question on the metabolic improvements shown in the Pca group without the benefit from the probiotic. As discussed previously, the probiotic was microencapsulated with pectin for better survivability to the distal gut. Pectin is a natural polysaccharide found in most fruits and vegetables. Previous studies demonstrate that pectin supplementation maintains the intestinal barrier integrity and controls glucose metabolism (188, 189). As a main source of soluble dietary fibers, it may have improved epithelial barrier function and glucose tolerance through an increase in bacterial fermentation products, namely SCFAs. Notably, butyrate functions as a key fuel for the intestinal epithelial cells and promotes mucin production, preserving the gut barrier function (190). Studies have shown an increase in butyrate concentration after feeding animals with pectin-containing diets (191, 192). A previous study demonstrated that pectin supplementation maintained the intestinal barrier integrity, which was associated with increases in butyrate concentrations (192). In this

present study, we found that the HF-induced decrease in serum butyrate was restored in Pca rats. The amount and diversity of gut microbiota play a role in SCFA production (160). In this study there were positive correlations between serum butyrate and abundance of *Cyanobacteria*, *Bifidobacterium*, and *Prevotella*, those which characterized the mocrobial profile of the Pca group. For example, we found that *Prevotella* was only present in LF and Pca groups. *Prevotella* abundance increases with carbohydrate-rich diets, especially fibers (193). In this study, the LF group, containing 70% carbohydrates in their diets, had higher abundance of *Prevotella* than the Pca group, which contained 35% CHO in their diets, but it was not significant. Considering that fiber contents were controlled for this study, the increase in *Prevotella* abundance in the Pca group may have been due to the presence of pectin in Pca's diets. In addition, *Prevotella* is known to be associated with improved glucose control (194), which corresponds to glycemia outcome of this study. Thus, improvements in gut functions, inflammatory profiles and glucose tolerance of the Pca group could be attributed to the presence of pectin in their diets. Another possible mechanism for different metabolic outcomes between probiotic-treated groups is that L. casei W8 may have stimulated actions of other beneficial bacteria at the expense of its own growth as previously discussed.

While our study provides evidence for beneficial metabolic effects of probiotic supplementation, some limitations still have to be considered. First, the efficiency of encapsulation was not directly evaluated in this study. We confirmed by qPCR the presence of *Lactobacillus paracasei* in cecum of probiotic-treated groups and no abundance of *L. paracasei* was detected in LF and HF groups. However, qPCR-based detection of microbial DNA does not definitively confirm microbial viability. Also, we did not measure the abundance of *Lactobacillus paracasei* in feces. Thus, our study does not allow a firm conclusion about the

efficacy of encapsulation. Another limitation is that for metagenomics analysis, only cecal contents were collected from animals during sacrifice. However, different patterns of the gut microbiome have been pointed out depending on the source of origin (195, 196). That is, the ecological conditions in the cecum may be not the same as those encountered in the distal gut or feces. For the former, there might be differences in abundance of certain bacterial populations depending on the location in the gut. Some of differences are possibly attributed to the fact that the cecum accommodates the digest from the small intestine, contributing to lower pH and higher concentration of volatile fatty acids (197, 198). For the latter, in a clinical study, Marteau et al. compared the microbial composition from the cecum and feces in healthy subjects and found that the cecum had less numbers of strict anaerobes, *Bacteroides,* and *Clostridium coccoides*/*leptum* groups than the feces. On the other hand, the number of facultative anaerobes was higher in the cecum (25% in proportion) than the feces (1%) (196). In this regard, better confirmation would be made for the effect of probiotic supplementation on microbial profiles by taking into account the spatial differences between cecal and fecal microbiota.

In conclusion, although the probiotic treatment, regardless of microencapsulation, were not successful in reducing body weight and adiposity, we demonstrated that probiotic-induced changes in the gut microbiota composition were associated with improvements in gut barrier functions and local and systemic inflammation in HF-fed rats. These effects were primarily ββassociated with alleviation of local and systemic inflammation characterized by lower levels of CD68, MCP1, and/or IL-1β in the ileum and mesenteric fat. Thus, our findings support further research into the application of probiotics as a dietary intervention for the prevention of obesity-related metabolic disorders. However, it should be noted that the efficacy of encapsulation was

not verified in this study. Thus, extra caution is warranted in interpreting the impact of encapsulation on metabolic effects of the probiotic.

Table 3.1. Diet Composition of LF, HF, Pro and Pca diets[1]

Diet Ingredient	LF - D12450H	HF - D12451	Pro - D12451	Pca - D12451
		g		
Casein, 30 Mesh	200	200	200	200
L-Cystine	3	3	3	3
Corn Starch	452.2	72.8	72.8	72.8
Maltodextrin 10	75	100	100	100
Sucrose	172.8	172.8	172.8	172.8
Cellulose, BW200	50	50	50	50
Soybean Oil	25	25	25	25
Lard	20	177.5	177.5	177.5
Mineral Mix S10026	10	10	10	10
Dicalcium Phosphate	13	13	13	13
Calcium Carbonate	5.5	5.5	5.5	5.5
Potassium Citrate, 1 H20	16.5	16.5	16.5	16.5
Vitamin Mix V10001	10	10	10	10
Choline Bitartrate	2	2	2	2
FD&C Yellow Dye #5	0.04	0	0	0
FD&C Red Dye #40	0.01	0.05	0.05	0.05
Intact L. casei W8			0.0076	
*Microencapsulated L. casei W8				8.58
Total	1055.03	858.15	858.1576	866.7315

[1]Diets L. casei W8 was microencapsulated with pectin beads, consisting of low methyl pectin, water, $CaCl_2$, and L. casei w8.

Table 3.2. Primer sequences used for qPCR[1]

Gene*	Forward primers (5' to 3')	Reverse primers (5' to 3')
β-actin	ACGGTCAGGTCATCACTATC	GATGCCACAGGATTCCATAC
CD68	AAACAGTCCAGGCTTCTC	ATGGCTGGGAACCATTAG
GAPDH	GAGCATCTCCCTCACAATTC	GGGTGCAGCGAACTTTAT
GLP1	CCAAGCAAGGAGAGAGAAAC	GATGACCAAGGCAGAGAAAG
IL-1β	CATTGTGGCTGTGGAGAA	GCAGTGCAGCTGTCTAAT
IL-6	TGTTGTGGGTGGTATCCT	CCTTCTTGGGACTGATGTTG
MCP1	GGCTGGAGAACTACAAGAG	TCTGGACCCATTCCTTATTG
MUC2	CTGAGGAAGGCCAAGTTTAC	CAGGTCCCAGAGAGGAAATA
TNFα	TCTACTCCCAGGTTCTCTTC	GCTGACTTTCTCCTGGTATG

[1]Cd68, cluster of differentiation 68; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GLP1, glucagon-like peptide-1; GPR41, -43, G protein-coupled receptor 41, -43; IL-1β, interleukin-1 beta; IL-6, interleukin-6; MCP1, monocyte chemotactic protein 1; MUC2, mucin 2; TNFα, tumor necrosis factor alpha.

Table 3.3. *In vitro* release test (log cfu)

Sample	Original	SGF 2h	SBF 20min	SIF 3h	SGF-SBF-SIF
Non-encapsulated L. casei W8	11.24 ± 0.81	4.23 ± 0.25*	0.89 ± 0.44*	11.65 ± 0.22	1.23 ± 0.32*
Encapsulated L. casei W8	10.16 ± 0.54	9.26 ± 0.07	9.78 ± 0.32	12.00 ± 0.24	9.03 ± 0.82

Values are means ± SDs. * denotes significant differences between non-encapsulated and encapsulated L. casei W8 at $P < 0.05$ in the same column. SGF, simulated gastric fluid; SBF, simulated bile fluid; SIF, simulated intestine fluid; SGF-SBF-SIF, consecutive treatments with SGF for 2 h, SBF for 20 min and SIF for 2h 40 min.

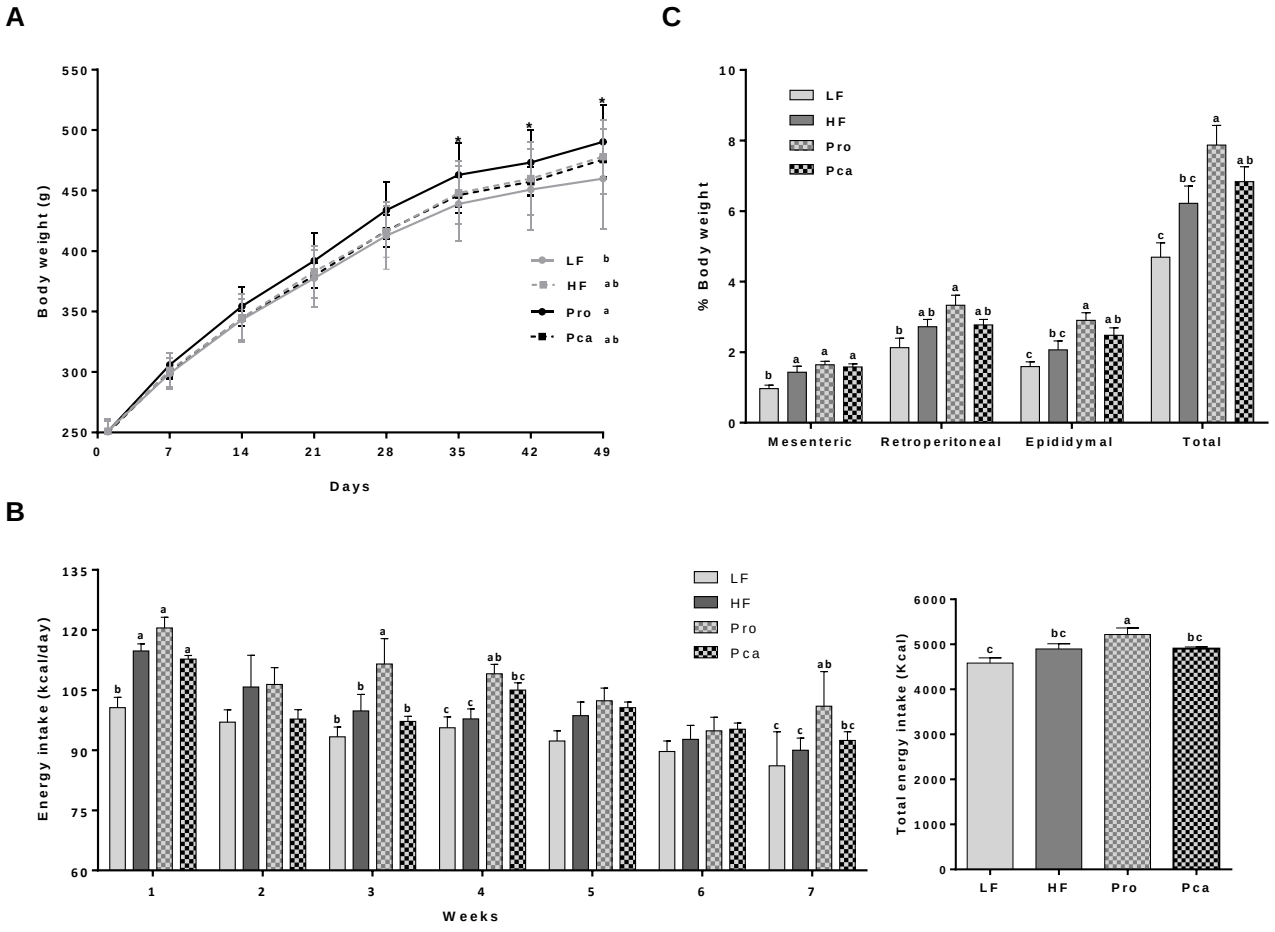


Figure 3.1. Body weight (A), energy intake (B) and adiposity index: fat pad weight/BW (%) (C) of rats fed an LF, HF, Pro, or Pca diet for 7 wk. Values are means ± SEMs; n = 8/group. Means with different letters are statistically significant, $P < 0.05$. HF, high fat; LF, low fat; HF, high fat; Pca, HF with supplemented with encapsulated *L. casei* W8 (Pca; $4x10^7$ cfu /g diet); Pro, HF supplemented with non-encapsulated *L. casei* W8 (Pro; $4x10^7$ cfu/g diet).

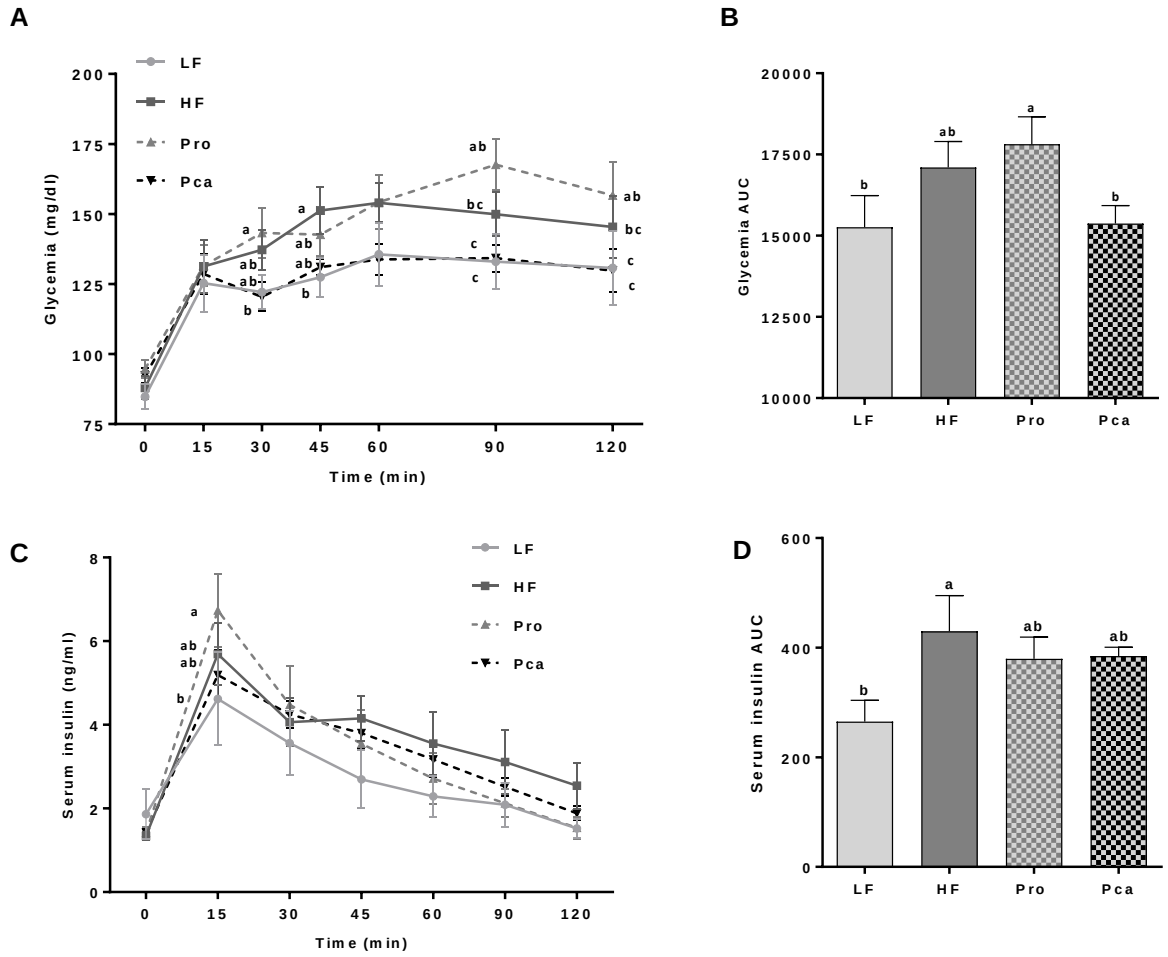
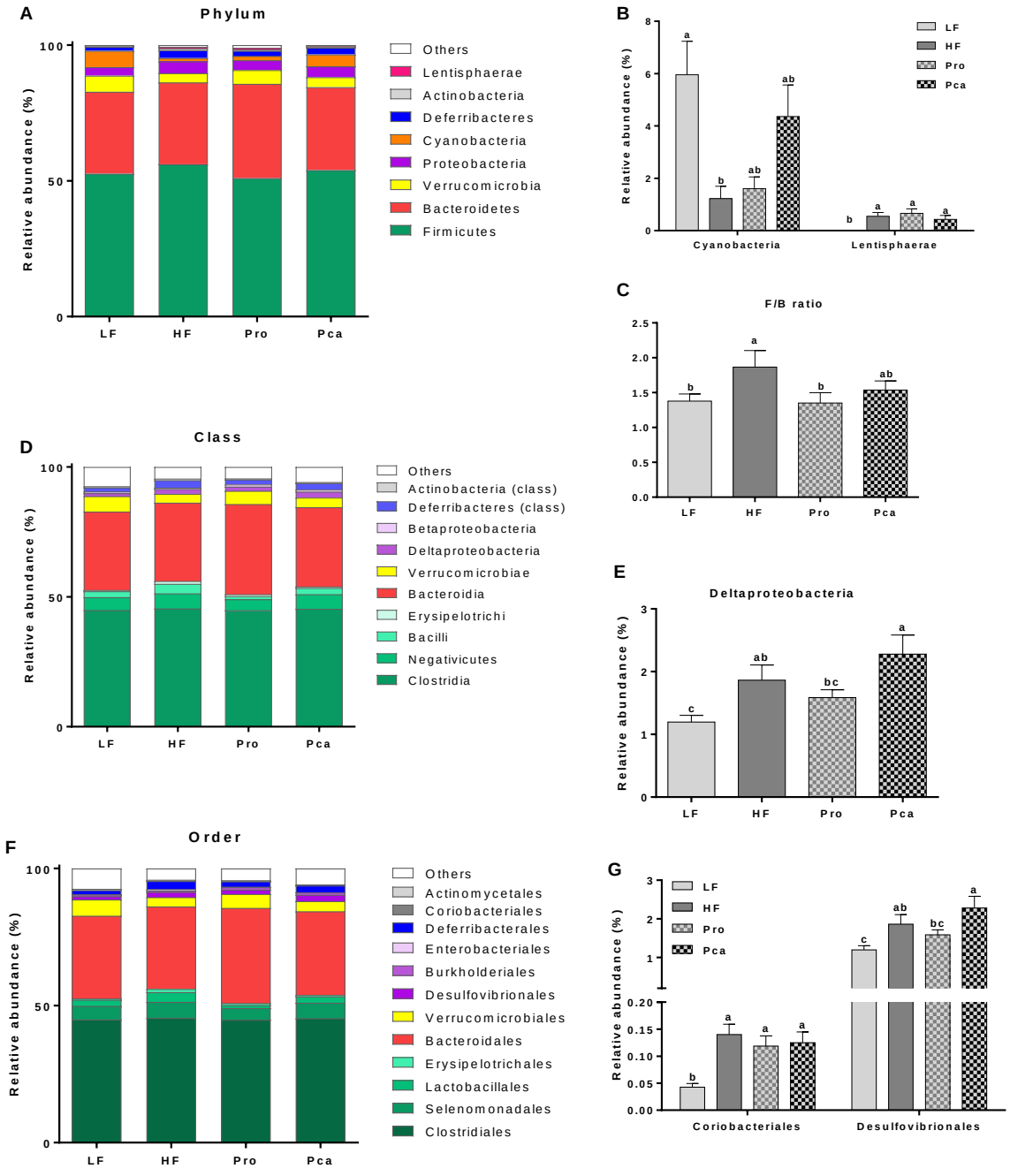


Figure 3.2. Glycemia (A, B) and insulinemia (C, D) were measured during an OGTT (2 mg/kg) in rats fed an LF, HF, Pro, or Pca diet for 7 wk. Values are means ± SEMs; n = 8/group. Means with different letters are statistically significant, $P < 0.05$. HF, high fat; LF, low fat; Pca, HF with supplemented with encapsulated *L. casei* W8 (Pca; $4x10^7$ cfu /g diet); Pro, HF supplemented with non-encapsulated *L. casei* W8 (Pro; $4x10^7$ cfu/g diet).


A
Phylum
Relative abundance (%)
100
50
0
LF
HF
Pro
Pca
Others
Lentisphaerae
Actinobacteria
Deferribacteres
Cyanobacteria
Proteobacteria
Verrucomicrobia
Bacteroidetes
Firmicutes
B
Relative abundance (%)
8
6
4
2
0
a
b
ab
ab
b
a
a
a
LF
HF
Pro
Pca
Cyanobacteria
Lentisphaerae
C
F/B ratio
Relative abundance (%)
2.5
2.0
1.5
1.0
0.5
0.0
b
a
b
ab
LF
HF
Pro
Pca
D
Class
Relative abundance (%)
100
50
0
LF
HF
Pro
Pca
Others
Actinobacteria (class)
Deferribacteres (class)
Betaproteobacteria
Deltaproteobacteria
Verrucomicrobiae
Bacteroidia
Erysipelotrichi
Bacilli
Negativicutes
Clostridia
E
Deltaproteobacteria
Relative abundance (%)
3
2
1
0
c
ab
bc
a
LF
HF
Pro
Pca
F
Order
Relative abundance (%)
100
50
0
LF
HF
Pro
Pca
Others
Actinomycetales
Coriobacteriales
Deferribacterales
Enterobacteriales
Burkholderiales
Desulfovibrionales
Verrucomicrobiales
Bacteroidales
Erysipelotrichales
Lactobacillales
Selenomonadales
Clostridiales
G
Relative abundance (%)
3
2
1
0.20
0.15
0.10
0.05
0.00
b
a
a
a
c
ab
bc
a
LF
HF
Pro
Pca
Coriobacteriales
Desulfovibrionales

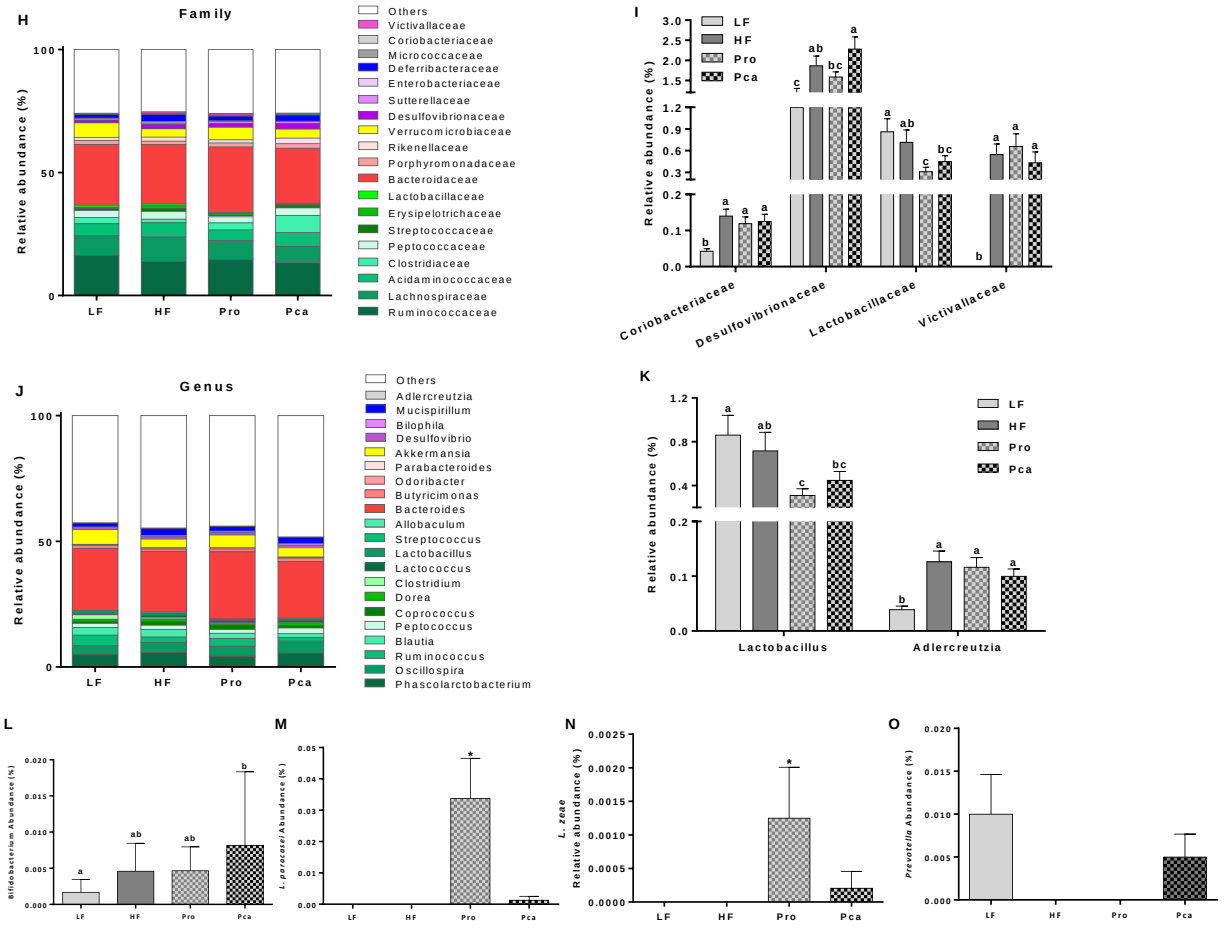


Figure 3.3. Microbial composition at the phylum (A, B, C), class (D, E), order (F, G), family (H, I), genus (J, K), and species (L, M, N, O) of rats fed an LF, HF, Pro, or Pca diet for 7 wk. Values are means ± SEMs; n = 8/group. Means with different letters are statistically significant, $P < 0.05$. HF, high fat; LF, low fat; Pca, HF with supplemented with encapsulated *L. casei* W8 (Pca; $4x10^7$ cfu /g diet); Pro, HF supplemented with non-encapsulated *L. casei* W8 (Pro; $4x10^7$ cfu/g diet).

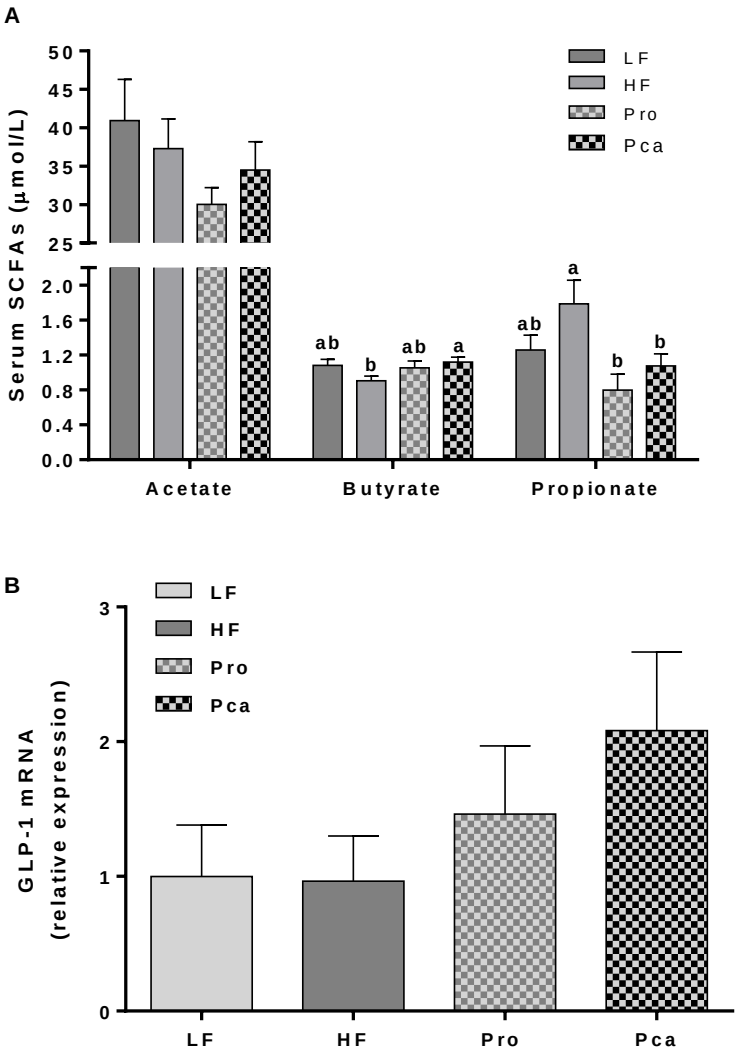


Figure 3.4. Serum SCFAs (A) and gene expressions of GLP1 in the ileum (B) of rats fed an LF, HF, Pro, or Pca diet for 7 wk. Values are means ± SEMs; n = 8/group. Means with different letters are statistically significant, $P < 0.05$. GLP1, glucagon-like peptide-1; HF, high fat; LF, low fat; Pca, HF with supplemented with encapsulated *L. casei* W8 (Pca; $4x10^7$ cfu /g diet); Pro, HF supplemented with non-encapsulated *L. casei* W8 (Pro; $4x10^7$ cfu/g diet); SCFA, short chain fatty acid.

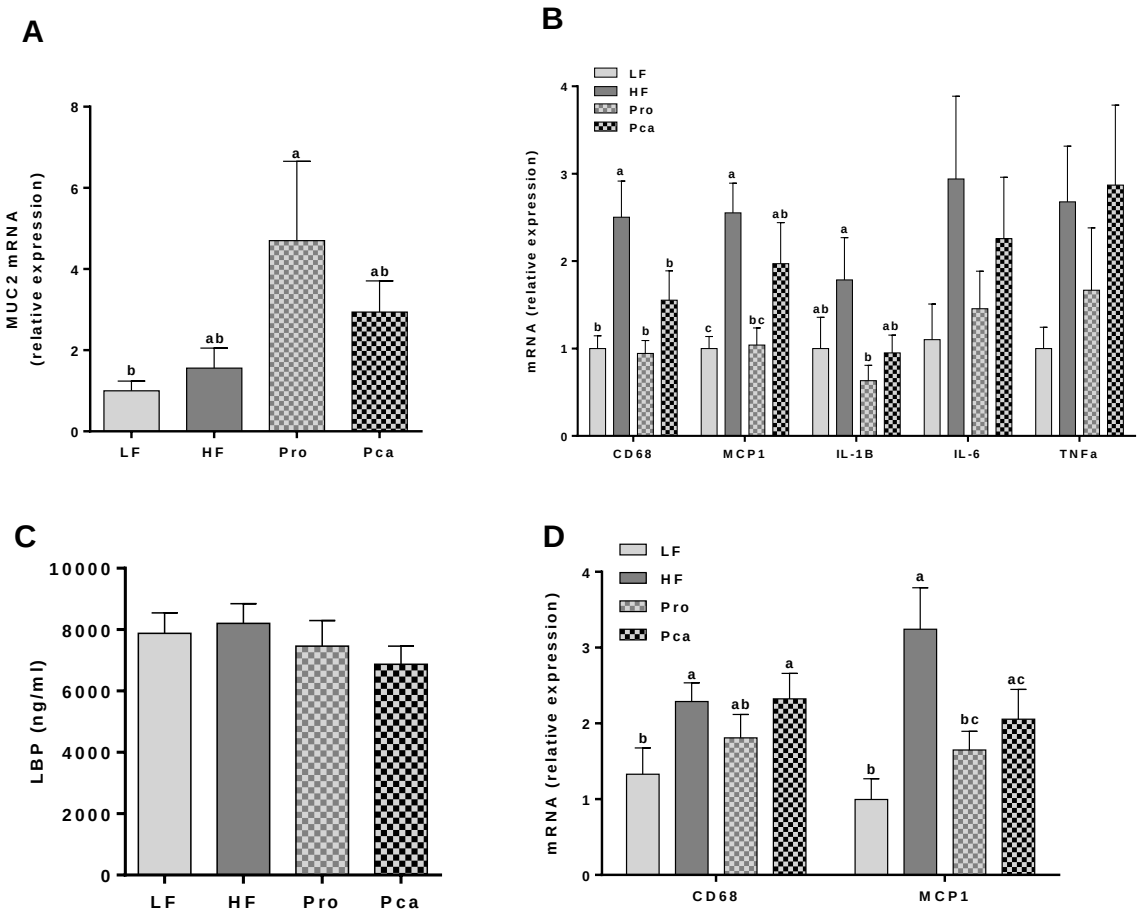


Figure 3.5. Gene expressions of MUC2 (A) and inflammatory markers (B) in the ileum, circulating LPS (C), and gene expression of macrophage infiltration markers (D) in mesenteric fat of rats fed an LF, HF, Pro, or Pca diet for 7 wk. Values are means ± SEMs; n = 8/group. Means with different letters are statistically significant, $P < 0.05$. CD68, cluster of differentiation 68; HF, high fat; LF, low fat; IL-1β, interleukin-1 beta; IL-6, interleukin-6; LPS, lipopolysaccharides; MCP1, monocyte chemoattrantant protein 1; MUC2, mucin 2; Pca, HF with supplemented with encapsulated *L. casei* W8 (Pca; $4x10^7$ cfu /g diet); Pro, HF supplemented with non-encapsulated *L. casei* W8 (Pro; $4x10^7$ cfu/g diet); TNF-α, tumor necrosis factor alpha.

CHAPTER 4

CONCLUSIONS

The objective of this dissertation was to investigate potential strategies to preserve GI barrier integrity in response to HF challenge. Considering the protective role of GI barrier against the inflammatory cascades, I hypothesized that preserving the GI barrier integrity would prevent systemic inflammation and the following deleterious effects in rats fed a HF diet.

The study presented in Chapter 2 was conducted with the primary objective of determining the role of blueberry (anthocyanin-rich food) in promoting gut health. Blueberry treatment altered the microbiota profile with decreases in *Firmicutes* and *Bacteroidetes* abundance compared to LF and HF rats. HF feeding led to a reduction in villus length and goblet cell proliferation in the ileum, which was normalized by blueberry treatment. HF_BB rats showed a significant increase in ileal MUC2 and antimicrobial DEFβ2 gene expression, suggesting an improvement in epithelial health. In line with these results, we found a significant decrease in circulating pro-inflammatory endotoxin in HF_BB rats and downregulation of inflammatory gene expression, such as TNFα and CD11d, in the liver and adipose tissues. Finally, insulin sensitivity was improved by blueberry treatment shown by lower insulinemia during a glucose challenge and a reduction in hepatic Ser307 phosphorylation of insulin receptor substrate 1. Taken together, these data demonstrate a potential anti-inflammatory effect of blueberry associated with improved insulin signaling, possibly via modulation of gut microbiota and epithelial barrier.

Chapter 3 of this dissertation specifically aimed at determining the therapeutic effects of a defined probiotic treatment on high-fat induced intestinal pathophysiology. Although the probiotic treatment, regardless of microencapsulation, was not successful in reducing body weight and adiposity, we demonstrated that probiotic-induced changes in the gut microbiota composition were associated with improvements in gut barrier functions and local and systemic inflammation in HF-fed rats. These effects were primarily associated with alleviation of local and systemic inflammation characterized by lower levels of CD68, MCP1, and/or IL-1B in the ileum and mesenteric fat. Thus, these findings support further research on the application of probiotics as a dietary intervention for the prevention of obesity-related metabolic disorders. However, it should be noted that the efficacy of encapsulation was not verified in this study based on the inconsistent outcomes found in gut barrier functions, inflammatory profiles, and glucose tolerance between encapsulated or non-encapsulated probiotic-treated groups. Thus, extra caution is warranted in interpreting the impact of encapsulation on metabolic effects of the probiotic.

Collectively the data presented in Chapters 2 to 3 provide insights into potential dietary interventions for the prevention of obesity-related metabolic disorders. While our studies provide evidence for the beneficial metabolic effects of blueberry or probiotic supplementation, there are some limitations to these studies that deserve consideration. First, I used rodent models to test my hypotheses and the results cannot be directly extrapolated to humans due to differences in gut microbiota and physiology. Another limitation is that, for metagenomics analysis, only cecal contents were collected from animals during sacrifice. However, there have been several studies that point out different patterns of the gut microbiome depending on the source of origin (195, 196). That is, the ecological conditions in the cecum may be not the same as those encountered

in the distal gut or feces. In this regard, future investigations could confirm the effect of blueberry or probiotic supplementation on host metabolism and microbial profiles by taking into account species specificity and spatial differences between cecal and fecal microbiota in translational or clinical settings. Moreover, the host-gut microbiota interaction would be better elucidated with the use of germ-free animals and ones colonized with microbial strains of interest to determine the effect of specific bacteria on host physiology and related therapeutic strategies.

REFERENCES

1. Ogden CL, Carroll MD, Kit BK, Flegal KM. Prevalence of childhood and adult obesity in the United States, 2011-2012. Jama 2014;311:806-14.

2. McCullough AJ. Epidemiology of the metabolic syndrome in the USA. J Dig Dis 2011;12:333-40.

3. Ma TY, Iwamoto GK, Hoa NT, Akotia V, Pedram A, Boivin MA, Said HM. TNF-alpha-induced increase in intestinal epithelial tight junction permeability requires NF-kappa B activation. Am J Physiol Gastrointest Liver Physiol 2004;286:G367-76.

4. Bischoff SC, Barbara G, Buurman W, Ockhuizen T, Schulzke JD, Serino M, Tilg H, Watson A, Wells JM. Intestinal permeability--a new target for disease prevention and therapy. BMC Gastroenterol 2014;14:189.

5. Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, Neyrinck AM, Fava F, Tuohy KM, Chabo C, et al. Metabolic endotoxemia initiates obesity and insulin resistance. Diabetes 2007;56:1761-72.

6. Delzenne NM, Neyrinck AM, Backhed F, Cani PD. Targeting gut microbiota in obesity: effects of prebiotics and probiotics. Nat Rev Endocrinol 2011;7:639-46.

7. Moreira AP, Texeira TF, Ferreira AB, Peluzio Mdo C, Alfenas Rde C. Influence of a high-fat diet on gut microbiota, intestinal permeability and metabolic endotoxaemia. Br J Nutr 2012;108:801-9.

8. Cani PD, Bibiloni R, Knauf C, Waget A, Neyrinck AM, Delzenne NM, Burcelin R. Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. Diabetes 2008;57:1470-81.

9. Erridge C, Attina T, Spickett CM, Webb DJ. A high-fat meal induces low-grade endotoxemia: evidence of a novel mechanism of postprandial inflammation. Am J Clin Nutr 2007;86:1286-92.

10. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, Gill SR, Nelson KE, Relman DA. Diversity of the human intestinal microbial flora. Science 2005;308:1635-8.

11. Xu J, Gordon JI. Inaugural Article: Honor thy symbionts. Proc Natl Acad Sci U S A 2003;100:10452-9.

12. Turner JR. Molecular basis of epithelial barrier regulation: from basic mechanisms to clinical application. Am J Pathol 2006;169:1901-9.

13. Bruewer M, Luegering A, Kucharzik T, Parkos CA, Madara JL, Hopkins AM, Nusrat A. Proinflammatory cytokines disrupt epithelial barrier function by apoptosis-independent mechanisms. J Immunol 2003;171:6164-72.

14. Backhed F, Hornef M. Toll-like receptor 4-mediated signaling by epithelial surfaces: necessity or threat? Microbes Infect 2003;5:951-9.

15. Akira S, Kiyosi T. Toll-like receptor signalling. Nature Reviews Immunology 2004;4:499-511

16. Shi H, Kokoeva MV, Inouye K, Tzameli I, Yin H, Flier JS. TLR4 links innate immunity and fatty acid-induced insulin resistance. J Clin Invest 2006;116:3015-25.

17. Ley RE, Turnbaugh PJ, Klein S, Gordon JI. Microbial ecology: human gut microbes associated with obesity. Nature 2006;444:1022-3.

18. Ley RE, Backhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI. Obesity alters gut microbial ecology. Proc Natl Acad Sci U S A 2005;102:11070-5.

19. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 2006;444:1027-31.

20. Brun P, Castagliuolo I, Di Leo V, Buda A, Pinzani M, Palu G, Martines D. Increased intestinal permeability in obese mice: new evidence in the pathogenesis of nonalcoholic steatohepatitis. Am J Physiol Gastrointest Liver Physiol 2007;292:G518-25.

21. Cani PD, Delzenne NM, Amar J, Burcelin R. Role of gut microflora in the development of obesity and insulin resistance following high-fat diet feeding. Pathol Biol (Paris) 2008;56:305-9.

22. Cani PD, Possemiers S, Van de Wiele T, Guiot Y, Everard A, Rottier O, Geurts L, Naslain D, Neyrinck A, Lambert DM, et al. Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability. Gut 2009;58:1091-103.

23. Cani PD, Bibiloni R, Knauf C, Waget A, Neyrinck AM, Delzenne NM, Burcelin R. Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. Diabetes 2008.

24. Cani PD, Possemiers S, Van de Wiele T, Guiot Y, Everard A, Rottier O, Geurts L, Naslain D, Neyrinck AM, Lambert DM, et al. Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability. Gut 2009;58 1044-55.

25. Ukena SN, Singh A, Dringenberg U, Engelhardt R, Seidler U, Hansen W, Bleich A, Bruder D, Franzke A, Rogler G, et al. Probiotic Escherichia coli Nissle 1917 inhibits leaky gut by enhancing mucosal integrity. PLoS One 2007;2:e1308.

26. Ulluwishewa D, Anderson RC, McNabb WC, Moughan PJ, Wells JM, Roy NC. Regulation of tight junction permeability by intestinal bacteria and dietary components. J Nutr 2011;141:769-76.

27. Suzuki T. Regulation of intestinal epithelial permeability by tight junctions. Cell Mol Life Sci 2013;70:631-59.

28. Guo S, Al-Sadi R, Said HM, Ma TY. Lipopolysaccharide causes an increase in intestinal tight junction permeability in vitro and in vivo by inducing enterocyte membrane expression and localization of TLR-4 and CD14. Am J Pathol 2013;182:375-87.

29. Lakhan SE, Kirchgessner A. Gut microbiota and sirtuins in obesity-related inflammation and bowel dysfunction. J Transl Med 2011;9:202.

30. Qin H, Roberts KL, Niyongere SA, Cong Y, Elson CO, Benveniste EN. Molecular mechanism of lipopolysaccharide-induced SOCS-3 gene expression in macrophages and microglia. J Immunol 2007;179:5966-76.

31. de La Serre CB, Ellis CL, Lee J, Hartman AL, Rutledge JC, Raybould HE. Propensity to high-fat diet-induced obesity in rats is associated with changes in the gut microbiota and gut inflammation. Am J Physiol Gastrointest Liver Physiol 2010;299:G440-8.

32. Bates JM, Akerlund J, Mittge E, Guillemin K. Intestinal alkaline phosphatase detoxifies lipopolysaccharide and prevents inflammation in zebrafish in response to the gut microbiota. Cell Host Microbe 2007;2:371-82.

33. Kamada N, Chen GY, Inohara N, Nunez G. Control of pathogens and pathobionts by the gut microbiota. Nat Immunol 2013;14:685-90.

34. Tlaskalova-Hogenova H, Stepankova R, Kozakova H, Hudcovic T, Vannucci L, Tuckova L, Rossmann P, Hrncir T, Kverka M, Zakostelska Z, et al. The role of gut microbiota (commensal bacteria) and the mucosal barrier in the pathogenesis of inflammatory and autoimmune diseases and cancer: contribution of germ-free and gnotobiotic animal models of human diseases. Cell Mol Immunol 2011;8:110-20.

35. Daniel H, Gholami AM, Berry D, Desmarchelier C, Hahne H, Loh G, Mondot S, Lepage P, Rothballer M, Walker A, et al. High-fat diet alters gut microbiota physiology in mice. Isme j 2014;8:295-308.

36. Neto CC. Cranberry and blueberry: evidence for protective effects against cancer and vascular diseases. Mol Nutr Food Res 2007;51:652-64.

37. Wu X, Beecher GR, Holden JM, Haytowitz DB, Gebhardt SE, Prior RL. Concentrations of anthocyanins in common foods in the United States and estimation of normal consumption. J Agric Food Chem 2006;54:4069-75.

38. Kahle K, Kraus M, Scheppach W, Ackermann M, Ridder F, Richling E. Studies on apple and blueberry fruit constituents: do the polyphenols reach the colon after ingestion? Mol Nutr Food Res 2006;50:418-23.

39. Williamson G, Clifford MN. Colonic metabolites of berry polyphenols: the missing link to biological activity? Br J Nutr 2010;104 Suppl 3:S48-66.

40. Dulebohn RV, Yi W, Srivastava A, Akoh CC, Krewer G, Fischer JG. Effects of blueberry (Vaccinium ashei) on DNA damage, lipid peroxidation, and phase II enzyme activities in rats. J Agric Food Chem 2008;56:11700-6.

41. Riso P, Klimis-Zacas D, Del Bo C, Martini D, Campolo J, Vendrame S, Moller P, Loft S, De Maria R, Porrini M. Effect of a wild blueberry (Vaccinium angustifolium) drink intervention on markers of oxidative stress, inflammation and endothelial function in humans with cardiovascular risk factors. Eur J Nutr 2013;52:949-61.

42. DeFuria J, Bennett G, Strissel KJ, Perfield JW, 2nd, Milbury PE, Greenberg AS, Obin MS. Dietary blueberry attenuates whole-body insulin resistance in high fat-fed mice by reducing adipocyte death and its inflammatory sequelae. J Nutr 2009;139:1510-6.

43. Paturi G, Mandimika T, Butts CA, Zhu S, Roy NC, McNabb WC, Ansell J. Influence of dietary blueberry and broccoli on cecal microbiota activity and colon morphology in mdr1a(-/-) mice, a model of inflammatory bowel diseases. Nutrition 2012;28:324-30.

44. Sellappan S, Akoh CC, Krewer G. Phenolic compounds and antioxidant capacity of Georgia-grown blueberries and blackberries. J Agric Food Chem 2002;50:2432-8.

45. Puupponen-Pimia R, Nohynek L, Alakomi HL, Oksman-Caldentey KM. The action of berry phenolics against human intestinal pathogens. Biofactors 2005;23:243-51.

46. McGhie TK, Walton MC. The bioavailability and absorption of anthocyanins: towards a better understanding. Mol Nutr Food Res 2007;51:702-13.

47. Cao G, Muccitelli HU, Sanchez-Moreno C, Prior RL. Anthocyanins are absorbed in glycated forms in elderly women: a pharmacokinetic study. Am J Clin Nutr 2001;73:920-6.

48. Talavera S, Felgines C, Texier O, Besson C, Manach C, Lamaison JL, Remesy C. Anthocyanins are efficiently absorbed from the small intestine in rats. J Nutr 2004;134:2275-9.

49. Talavera S, Felgines C, Texier O, Besson C, Gil-Izquierdo A, Lamaison JL, Remesy C. Anthocyanin metabolism in rats and their distribution to digestive area, kidney, and brain. J Agric Food Chem 2005;53:3902-8.

50. Del Bo C, Martini D, Vendrame S, Riso P, Ciappellano S, Klimis-Zacas D, Porrini M. Improvement of lymphocyte resistance against H(2)O(2)-induced DNA damage in

Sprague-Dawley rats after eight weeks of a wild blueberry (Vaccinium angustifolium)-enriched diet. Mutat Res 2010;703:158-62.

51. Xie C, Kang J, Chen JR, Nagarajan S, Badger TM, Wu X. Phenolic acids are in vivo atheroprotective compounds appearing in the serum of rats after blueberry consumption. J Agric Food Chem 2011;59:10381-7.

52. Felgines C, Texier O, Besson C, Fraisse D, Lamaison JL, Remesy C. Blackberry anthocyanins are slightly bioavailable in rats. J Nutr 2002;132:1249-53.

53. He J, Magnuson BA, Giusti MM. Analysis of anthocyanins in rat intestinal contents--impact of anthocyanin chemical structure on fecal excretion. J Agric Food Chem 2005;53:2859-66.

54. Russell WR, Labat A, Scobbie L, Duncan SH. Availability of blueberry phenolics for microbial metabolism in the colon and the potential inflammatory implications. Mol Nutr Food Res 2007;51:726-31.

55. Schmidt BM, Howell AB, McEniry B, Knight CT, Seigler D, Erdman JW, Jr., Lila MA. Effective separation of potent antiproliferation and antiadhesion components from wild blueberry (Vaccinium angustifolium Ait.) fruits. J Agric Food Chem 2004;52:6433-42.

56. Lacombe A, Wu VC, White J, Tadepalli S, Andre EE. The antimicrobial properties of the lowbush blueberry (Vaccinium angustifolium) fractional components against foodborne pathogens and the conservation of probiotic Lactobacillus rhamnosus. Food Microbiol 2012;30:124-31.

57. Hidalgo M, Oruna-Concha MJ, Kolida S, Walton GE, Kallithraka S, Spencer JP, de Pascual-Teresa S. Metabolism of anthocyanins by human gut microflora and their influence on gut bacterial growth. J Agric Food Chem 2012;60:3882-90.

58. Molan A, Lila M, Mawson J, De S. In vitro and in vivo evaluation of the prebiotic activity of water-soluble blueberry extracts.

59. Vendrame S, Guglielmetti S, Riso P, Arioli S, Klimis-Zacas D, Porrini M. Six-week consumption of a wild blueberry powder drink increases bifidobacteria in the human gut. J Agric Food Chem 2011;59:12815-20.

60. Osman N, Adawi D, Ahrne S, Jeppsson B, Molin G. Probiotics and blueberry attenuate the severity of dextran sulfate sodium (DSS)-induced colitis. Dig Dis Sci 2008;53:2464-73.

61. Osman N, Adawi D, Ahrne S, Jeppsson B, Molin G. Endotoxin- and D-galactosamine-induced liver injury improved by the administration of Lactobacillus, Bifidobacterium and blueberry. Dig Liver Dis 2007;39:849-56.

62. Guo H, Ling W. The update of anthocyanins on obesity and type 2 diabetes: experimental evidence and clinical perspectives. Rev Endocr Metab Disord 2015;16:1-13.

63. Stull AJ, Cash KC, Johnson WD, Champagne CM, Cefalu WT. Bioactives in blueberries improve insulin sensitivity in obese, insulin-resistant men and women. J Nutr 2010;140:1764-8.

64. Mink PJ, Scrafford CG, Barraj LM, Harnack L, Hong CP, Nettleton JA, Jacobs DR, Jr. Flavonoid intake and cardiovascular disease mortality: a prospective study in postmenopausal women. Am J Clin Nutr 2007;85:895-909.

65. Wedick NM, Pan A, Cassidy A, Rimm EB, Sampson L, Rosner B, Willett W, Hu FB, Sun Q, van Dam RM. Dietary flavonoid intakes and risk of type 2 diabetes in US men and women. Am J Clin Nutr 2012;95:925-33.

66. Ramirez-Tortosa C, Andersen OM, Cabrita L, Gardner PT, Morrice PC, Wood SG, Duthie SJ, Collins AR, Duthie GG. Anthocyanin-rich extract decreases indices of lipid peroxidation and DNA damage in vitamin E-depleted rats. Free Radic Biol Med 2001;31:1033-7.

67. Prior RL. Fruits and vegetables in the prevention of cellular oxidative damage. Am J Clin Nutr 2003;78:570s-8s.

68. Basu A, Rhone M, Lyons TJ. Berries: emerging impact on cardiovascular health. Nutr Rev 2010;68:168-77.

69. FAO/WHO. Report on Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria. 2001.

70. Dunne C, O'Mahony L, Murphy L, Thornton G, Morrissey D, O'Halloran S, Feeney M, Flynn S, Fitzgerald G, Daly C. In vitro selection criteria for probiotic bacteria of human origin: correlation with in vivo findingsÔÇô. The American journal of clinical nutrition 2001;73:386s-92s.

71. Lahtinen SJ, Boyle RJ, Kivivuori S, Oppedisano F, Smith KR, Robins-Browne R, Salminen SJ, Tang ML. Prenatal probiotic administration can influence Bifidobacterium microbiota development in infants at high risk of allergy. J Allergy Clin Immunol 2009;123:499-501.

72. Corr SC, Hill C, Gahan CG. Understanding the mechanisms by which probiotics inhibit gastrointestinal pathogens. Adv Food Nutr Res 2009;56:1-15.

73. Ohland CL, Macnaughton WK. Probiotic bacteria and intestinal epithelial barrier function. Am J Physiol Gastrointest Liver Physiol 2010;298:G807-19.

74. Cotter PD, Hill C, Ross RP. Bacteriocins: developing innate immunity for food. Nat Rev Microbiol 2005;3:777-88.

75. Asahara T, Shimizu K, Nomoto K, Hamabata T, Ozawa A, Takeda Y. Probiotic bifidobacteria protect mice from lethal infection with Shiga toxin-producing Escherichia coli O157:H7. Infect Immun 2004;72:2240-7.

76. Gopal R, Birdsell D, Monroy FP. Regulation of toll-like receptors in intestinal epithelial cells by stress and Toxoplasma gondii infection. Parasite Immunol 2008;30:563-76.

77. Corr SC, Li Y, Riedel CU, O'Toole PW, Hill C, Gahan CG. Bacteriocin production as a mechanism for the antiinfective activity of Lactobacillus salivarius UCC118. Proc Natl Acad Sci U S A 2007;104:7617-21.

78. Corr SC, Gahan CG, Hill C. Impact of selected Lactobacillus and Bifidobacterium species on Listeria monocytogenes infection and the mucosal immune response. FEMS Immunol Med Microbiol 2007;50:380-8.

79. O'Hara AM, O'Regan P, Fanning A, O'Mahony C, Macsharry J, Lyons A, Bienenstock J, O'Mahony L, Shanahan F. Functional modulation of human intestinal epithelial cell responses by Bifidobacterium infantis and Lactobacillus salivarius. Immunology 2006;118:202-15.

80. Di Giacinto C, Marinaro M, Sanchez M, Strober W, Boirivant M. Probiotics ameliorate recurrent Th1-mediated murine colitis by inducing IL-10 and IL-10-dependent TGF-beta-bearing regulatory cells. J Immunol 2005;174:3237-46.

81. Silva AM, Barbosa FH, Duarte R, Vieira LQ, Arantes RM, Nicoli JR. Effect of Bifidobacterium longum ingestion on experimental salmonellosis in mice. J Appl Microbiol 2004;97:29-37.

82. Menard S, Cerf-Bensussan N, Heyman M. Multiple facets of intestinal permeability and epithelial handling of dietary antigens. Mucosal Immunol 2010;3:247-59.

83. Menard S, Candalh C, Bambou JC, Terpend K, Cerf-Bensussan N, Heyman M. Lactic acid bacteria secrete metabolites retaining anti-inflammatory properties after intestinal transport. Gut 2004;53:821-8.

84. Fukushima Y, Kawata Y, Hara H, Terada A, Mitsuoka T. Effect of a probiotic formula on intestinal immunoglobulin A production in healthy children. Int J Food Microbiol 1998;42:39-44.

85. Galdeano CM, Perdigon G. The probiotic bacterium Lactobacillus casei induces activation of the gut mucosal immune system through innate immunity. Clin Vaccine Immunol 2006;13:219-26.

86. Sherman PM, Johnson-Henry KC, Yeung HP, Ngo PS, Goulet J, Tompkins TA. Probiotics reduce enterohemorrhagic Escherichia coli O157:H7- and enteropathogenic E. coli O127:H6-induced changes in polarized T84 epithelial cell monolayers by reducing bacterial adhesion and cytoskeletal rearrangements. Infect Immun 2005;73:5183-8.

87. Tsai CC, Hsih HY, Chiu HH, Lai YY, Liu JH, Yu B, Tsen HY. Antagonistic activity against Salmonella infection in vitro and in vivo for two Lactobacillus strains from swine and poultry. Int J Food Microbiol 2005;102:185-94.

88. Johnson-Henry KC, Hagen KE, Gordonpour M, Tompkins TA, Sherman PM. Surface-layer protein extracts from Lactobacillus helveticus inhibit enterohaemorrhagic Escherichia coli O157:H7 adhesion to epithelial cells. Cell Microbiol 2007;9:356-67.

89. Mattar AF, Teitelbaum DH, Drongowski RA, Yongyi F, Harmon CM, Coran AG. Probiotics up-regulate MUC-2 mucin gene expression in a Caco-2 cell-culture model. Pediatr Surg Int 2002;18:586-90.

90. Mack DR, Ahrne S, Hyde L, Wei S, Hollingsworth MA. Extracellular MUC3 mucin secretion follows adherence of Lactobacillus strains to intestinal epithelial cells in vitro. Gut 2003;52:827-33.

91. Furrie E, Macfarlane S, Kennedy A, Cummings JH, Walsh SV, O'Neil D A, Macfarlane GT. Synbiotic therapy (Bifidobacterium longum/Synergy 1) initiates resolution of inflammation in patients with active ulcerative colitis: a randomised controlled pilot trial. Gut 2005;54:242-9.

92. Schlee M, Harder J, Koten B, Stange EF, Wehkamp J, Fellermann K. Probiotic lactobacilli and VSL#3 induce enterocyte beta-defensin 2. Clin Exp Immunol 2008;151:528-35.

93. Furrie E. Probiotics and allergy. Proceedings of the Nutrition Society 2005;64:465-9.

94. McKane L, Kandel J. Microbiology: essentials and applications: McGraw-Hill Science, Engineering & Mathematics, 1996.

95. Govender M, Choonara YE, Kumar P, du Toit LC, van Vuuren S, Pillay V. A review of the advancements in probiotic delivery: Conventional vs. non-conventional formulations for intestinal flora supplementation. Aaps PharmSciTech 2014;15:29-43.

96. Shornikova A-V, Casas IA, Mykk ├änen H, Salo E, Vesikari T. Bacteriotherapy with Lactobacillus reuteri in rotavirus gastroenteritis. The Pediatric infectious disease journal 1997;16:1103-7.

97. Hatakka K, Savilahti E, P ├Ânk ├ñ A, Meurman JH, Poussa T, N ├ñse L, Saxelin M, Korpela R. Effect of long term consumption of probiotic milk on infections in children attending day care centres: double blind, randomised trial. Bmj 2001;322:1327.

98. Kailasapathy K, Chin J. Survival and therapeutic potential of probiotic organisms with reference to Lactobacillus acidophilus and Bifidobacterium spp. Immunology & Cell Biology 2000;78:80-8.

99. Kim SW, Park KY, Kim B, Kim E, Hyun CK. Lactobacillus rhamnosus GG improves insulin sensitivity and reduces adiposity in high-fat diet-fed mice through enhancement of adiponectin production. Biochem Biophys Res Commun 2013;431:258-63.

100. Park DY, Ahn YT, Park SH, Huh CS, Yoo SR, Yu R, Sung MK, McGregor RA, Choi MS. Supplementation of Lactobacillus curvatus HY7601 and Lactobacillus plantarum KY1032 in diet-induced obese mice is associated with gut microbial changes and reduction in obesity. PLoS One 2013;8:e59470.

101. Musso G, Gambino R, Cassader M. Interactions between gut microbiota and host metabolism predisposing to obesity and diabetes. Annu Rev Med 2011;62:361-80.

102. Hung S-C, Tseng W-T, Pan T-M. Lactobacillus paracasei subsp. paracasei NTU 101 ameliorates impaired glucose tolerance induced by a high-fat, high-fructose diet in Sprague-Dawley rats. Journal of Functional Foods 2016;24:472-81.

103. Groeger D, O'Mahony L, Murphy EF, Bourke JF, Dinan TG, Kiely B, Shanahan F, Quigley EM. Bifidobacterium infantis 35624 modulates host inflammatory processes beyond the gut. Gut Microbes 2013;4:325-39.

104. Cremon C, Guglielmetti S, Gargari G, Taverniti V, Castellazzi AM, Valsecchi C, Tagliacarne C, Fiore W, Bellini M, Bertani L. Effect of Lactobacillus paracasei CNCM I-1572 on symptoms, gut microbiota, short chain fatty acids, and immune activation in patients with irritable bowel syndrome: A pilot randomized clinical trial. United European Gastroenterology Journal 2017:2050640617736478.

105. Vaughan EE, de Vries MC, Zoetendal EG, Ben-Amor K, Akkermans AD, de Vos WM. The intestinal LABs. Edtion ed. Lactic Acid Bacteria: Genetics, Metabolism and Applications: Springer, 2002:341-52.

106. Nazzaro F, Fratianni F, Coppola R, Sada A, Orlando P. Fermentative ability of alginate-prebiotic encapsulated Lactobacillus acidophilus and survival under simulated gastrointestinal conditions. Journal of Functional Foods 2009;1:319-23.

107. Li XY, Chen XG, Sun ZW, Park HJ, Cha D-S. Preparation of alginate/chitosan/carboxymethyl chitosan complex microcapsules and application in Lactobacillus casei ATCC 393. Carbohydrate Polymers 2011;83:1479-85.

108. Sheehan VM, Sleator RD, Hill C, Fitzgerald GF. Improving gastric transit, gastrointestinal persistence and therapeutic efficacy of the probiotic strain Bifidobacterium breve UCC2003. Microbiology 2007;153:3563-71.

109. Anal AK, Singh H. Recent advances in microencapsulation of probiotics for industrial applications and targeted delivery. Trends in Food Science & Technology 2007;18:240-51.

110. Prakash S, Tomaro-Duchesneau C, Saha S, Cantor A. The gut microbiota and human health with an emphasis on the use of microencapsulated bacterial cells. BioMed Research International 2011;2011.

111. Cordain L, Eaton SB, Sebastian A, Mann N, Lindeberg S, Watkins BA, O'Keefe JH, Brand-Miller J. Origins and evolution of the Western diet: health implications for the 21st century. Am J Clin Nutr 2005;81:341-54.

112. Catalioto RM, Maggi CA, Giuliani S. Intestinal epithelial barrier dysfunction in disease and possible therapeutical interventions. Curr Med Chem 2011;18:398-426.

113. Selma MV, Espin JC, Tomas-Barberan FA. Interaction between phenolics and gut microbiota: role in human health. J Agric Food Chem 2009;57:6485-501.

114. Morin CL, Eckel RH, Marcel T, Pagliassotti MJ. High fat diets elevate adipose tissue-derived tumor necrosis factor-alpha activity. Endocrinology 1997;138:4665-71.

115. Carvalho BM, Guadagnini D, Tsukumo DML, Schenka AA, Latuf-Filho P, Vassallo J, Dias JC, Kubota LT, Carvalheira JBC, Saad MJA. Modulation of gut microbiota by antibiotics improves insulin signalling in high-fat fed mice. Diabetologia 2012;55:2823-34.

116. Sam S, Haffner S, Davidson MH, D'Agostino RB, Sr., Feinstein S, Kondos G, Perez A, Mazzone T. Relation of abdominal fat depots to systemic markers of inflammation in type 2 diabetes. Diabetes Care 2009;32:932-7.

117. Cancello R, Tordjman J, Poitou C, Guilhem G, Bouillot JL, Hugol D, Coussieu C, Basdevant A, Bar Hen A, Bedossa P, et al. Increased infiltration of macrophages in omental adipose tissue is associated with marked hepatic lesions in morbid human obesity. Diabetes 2006;55:1554-61.

118. Jager J, Gremeaux T, Cormont M, Le Marchand-Brustel Y, Tanti JF. Interleukin-1beta-induced insulin resistance in adipocytes through down-regulation of insulin receptor substrate-1 expression. Endocrinology 2007;148:241-51.

119. Aguirre V, Werner ED, Giraud J, Lee YH, Shoelson SE, White MF. Phosphorylation of Ser307 in insulin receptor substrate-1 blocks interactions with the insulin receptor and inhibits insulin action. J Biol Chem 2002;277:1531-7.

120. de La Serre CB, Ellis CL, Lee J, Hartman AL, Rutledge JC, Raybould HE. Propensity to high-fat diet-induced obesity in rats is associated with changes in the gut microbiota and gut inflammation. Am J Physiol Gastrointest Liver Physiol 2010;299:G440-8.

121. Ding S, Chi MM, Scull BP, Rigby R, Schwerbrock NM, Magness S, Jobin C, Lund PK. High-fat diet: bacteria interactions promote intestinal inflammation which precedes and correlates with obesity and insulin resistance in mouse. PLoS One 2010;5:e12191.

122. Hamilton MK, Boudry G, Lemay DG, Raybould HE. Changes in intestinal barrier function and gut microbiota in high-fat diet-fed rats are dynamic and region dependent. Am J Physiol Gastrointest Liver Physiol 2015;308:G840-51.

123. Tomas J, Mulet C, Saffarian A, Cavin JB, Ducroc R, Regnault B, Kun Tan C, Duszka K, Burcelin R, Wahli W, et al. High-fat diet modifies the PPAR-gamma pathway leading to disruption of microbial and physiological ecosystem in murine small intestine. Proc Natl Acad Sci U S A 2016;113:E5934-e43.

124. Lawrence T. The nuclear factor NF-kappaB pathway in inflammation. Cold Spring Harb Perspect Biol 2009;1:a001651.

125. de La Serre CB, de Lartigue G, Raybould HE. Chronic exposure to low dose bacterial lipopolysaccharide inhibits leptin signaling in vagal afferent neurons. Physiol Behav 2015;139:188-94.

126. Cani PD, Knauf C, Iglesias MA, Drucker DJ, Delzenne NM, Burcelin R. Improvement of glucose tolerance and hepatic insulin sensitivity by oligofructose requires a functional glucagon-like peptide 1 receptor. Diabetes 2006;55:1484-90.

127. Carvalho BM, Guadagnini D, Tsukumo DM, Schenka AA, Latuf-Filho P, Vassallo J, Dias JC, Kubota LT, Carvalheira JB, Saad MJ. Modulation of gut microbiota by antibiotics improves insulin signalling in high-fat fed mice. Diabetologia 2012;55:2823-34.

128. Prior RL, Wu X, Gu L, Hager TJ, Hager A, Howard LR. Whole berries versus berry anthocyanins: interactions with dietary fat levels in the C57BL/6J mouse model of obesity. J Agric Food Chem 2008;56:647-53.

129. Prior RL, Wu X, Gu L, Hager T, Hager A, Wilkes S, Howard L. Purified berry anthocyanins but not whole berries normalize lipid parameters in mice fed an obesogenic high fat diet. Mol Nutr Food Res 2009;53:1406-18.

130. Prior RL, S EW, T RR, Khanal RC, Wu X, Howard LR. Purified blueberry anthocyanins and blueberry juice alter development of obesity in mice fed an obesogenic high-fat diet. J Agric Food Chem 2010;58:3970-6.

131. Seymour EM, Tanone, II, Urcuyo-Llanes DE, Lewis SK, Kirakosyan A, Kondoleon MG, Kaufman PB, Bolling SF. Blueberry intake alters skeletal muscle and adipose tissue peroxisome proliferator-activated receptor activity and reduces insulin resistance in obese rats. J Med Food 2011;14:1511-8.

132. Lin Z, Fischer J, Wicker L. Intermolecular binding of blueberry pectin-rich fractions and anthocyanin. Food Chem 2016;194:986-93.

133. McGhie TK, Stevenson DE. Bioavailability and bioabsorption of anthocyanins. Edtion ed. Anthocyanins in Health and Disease: CRC Press, 2013:91-114.

134. Lacombe A, Li RW, Klimis-Zacas D, Kristo AS, Tadepalli S, Krauss E, Young R, Wu VC. Lowbush wild blueberries have the potential to modify gut microbiota and xenobiotic metabolism in the rat colon. PLoS One 2013;8:e67497.

135. Cummings JH, Pomare EW, Branch WJ, Naylor CP, Macfarlane GT. Short chain fatty acids in human large intestine, portal, hepatic and venous blood. Gut 1987;28:1221-7.

136. Tolhurst G, Heffron H, Lam YS, Parker HE, Habib AM, Diakogiannaki E, Cameron J, Grosse J, Reimann F, Gribble FM. Short-chain fatty acids stimulate glucagon-like peptide-1 secretion via the G-protein-coupled receptor FFAR2. Diabetes 2012;61:364-71.

137. Arndt D, Xia J, Liu Y, Zhou Y, Guo AC, Cruz JA, Sinelnikov I, Budwill K, Nesbo CL, Wishart DS. METAGENassist: a comprehensive web server for comparative metagenomics. Nucleic Acids Res 2012;40:W88-95.

138. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, Huttenhower C. Metagenomic biomarker discovery and explanation. Genome Biol 2011;12:R60.

139. Moreau NM, Goupry SM, Antignac JP, Monteau FJ, Le Bizec BJ, Champ MM, Martin LJ, Dumon HJ. Simultaneous measurement of plasma concentrations and 13C-enrichment of short-chain fatty acids, lactic acid and ketone bodies by gas chromatography coupled to mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 2003;784:395-403.

140. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25:402-8.

141. Rui L, Aguirre V, Kim JK, Shulman GI, Lee A, Corbould A, Dunaif A, White MF. Insulin/IGF-1 and TNF-alpha stimulate phosphorylation of IRS-1 at inhibitory Ser307 via distinct pathways. J Clin Invest 2001;107:181-9.

142. Chassaing B, Miles-Brown J, Pellizzon M, Ulman E, Ricci M, Zhang L, Patterson AD, Vijay-Kumar M, Gewirtz AT. Lack of soluble fiber drives diet-induced adiposity in mice. Am J Physiol Gastrointest Liver Physiol 2015;309:G528-41.

143. Boozer CN, Schoenbach G, Atkinson RL. Dietary fat and adiposity: a dose-response relationship in adult male rats fed isocalorically. Am J Physiol 1995;268:E546-50.

144. Varga T, Czimmerer Z, Nagy L. PPARs are a unique set of fatty acid regulated transcription factors controlling both lipid metabolism and inflammation. Biochim Biophys Acta 2011;1812:1007-22.

145. Fak F, Jakobsdottir G, Kulcinskaja E, Marungruang N, Matziouridou C, Nilsson U, Stalbrand H, Nyman M. The physico-chemical properties of dietary fibre determine metabolic responses, short-chain Fatty Acid profiles and gut microbiota composition in rats fed low- and high-fat diets. PLoS One 2015;10:e0127252.

146. Faith JJ, Guruge JL, Charbonneau M, Subramanian S, Seedorf H, Goodman AL, Clemente JC, Knight R, Heath AC, Leibel RL, et al. The long-term stability of the human gut microbiota. Science 2013;341:1237439.

147. Caporaso JG, Lauber CL, Costello EK, Berg-Lyons D, Gonzalez A, Stombaugh J, Knights D, Gajer P, Ravel J, Fierer N, et al. Moving pictures of the human microbiome. Genome Biol 2011;12:R50.

148. Morgan XC, Tickle TL, Sokol H, Gevers D, Devaney KL, Ward DV, Reyes JA, Shah SA, LeLeiko N, Snapper SB, et al. Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment. Genome Biol 2012;13:R79.

149. Shao Y, Ding R, Xu B, Hua R, Shen Q, He K, Yao Q. Alterations of Gut Microbiota After Roux-en-Y Gastric Bypass and Sleeve Gastrectomy in Sprague-Dawley Rats. Obes Surg 2016.

150. Graessler J, Qin Y, Zhong H, Zhang J, Licinio J, Wong ML, Xu A, Chavakis T, Bornstein AB, Ehrhart-Bornstein M, et al. Metagenomic sequencing of the human gut microbiome before and after bariatric surgery in obese patients with type 2 diabetes: correlation with inflammatory and metabolic parameters. Pharmacogenomics J 2013;13:514-22.

151. Osman N, Adawi D, Ahrné S, Jeppsson B, Molin G. Probiotics and Blueberry Attenuate the Severity of Dextran Sulfate Sodium (DSS)-Induced Colitis. Digestive Diseases and Sciences 2008;53:2464-73.

152. Moreno-Navarrete JM, Ortega F, Serino M, Luche E, Waget A, Pardo G, Salvador J, Ricart W, Fruhbeck G, Burcelin R, et al. Circulating lipopolysaccharide-binding protein (LBP) as a marker of obesity-related insulin resistance. Int J Obes (Lond) 2012;36:1442-9.

153. Paturi G, Butts CA, Bentley-Hewitt KL, Ansell J. Influence of green and gold kiwifruit on indices of large bowel function in healthy rats. J Food Sci 2014;79:H1611-20.

154. Johansson ME, Ambort D, Pelaseyed T, Schutte A, Gustafsson JK, Ermund A, Subramani DB, Holmen-Larsson JM, Thomsson KA, Bergstrom JH, et al. Composition and functional role of the mucus layers in the intestine. Cell Mol Life Sci 2011;68:3635-41.

155. Ling K-h, 凌加豪. Polyphenol-mediated protective effects against bacterial translocation across intestinal epithelial cells and their mechanisms. HKU Theses Online (HKUTO) 2015.

156. Wehkamp J, Harder J, Wehkamp K, Wehkamp-von Meissner B, Schlee M, Enders C, Sonnenborn U, Nuding S, Bengmark S, Fellermann K, et al. NF-kappaB- and AP-1-mediated induction of human beta defensin-2 in intestinal epithelial cells by Escherichia coli Nissle 1917: a novel effect of a probiotic bacterium. Infect Immun 2004;72:5750-8.

157. Henningsson AM, Bjorck IM, Nyman EM. Combinations of indigestible carbohydrates affect short-chain fatty acid formation in the hindgut of rats. J Nutr 2002;132:3098-104.

158. Frankel WL, Zhang W, Singh A, Klurfeld DM, Don S, Sakata T, Modlin I, Rombeau JL. Mediation of the trophic effects of short-chain fatty acids on the rat jejunum and colon. Gastroenterology 1994;106:375-80.

159. Park JH, Kotani T, Konno T, Setiawan J, Kitamura Y, Imada S, Usui Y, Hatano N, Shinohara M, Saito Y, et al. Promotion of Intestinal Epithelial Cell Turnover by Commensal Bacteria: Role of Short-Chain Fatty Acids. PLoS One 2016;11:e0156334.

160. Wong JM, de Souza R, Kendall CW, Emam A, Jenkins DJ. Colonic health: fermentation and short chain fatty acids. J Clin Gastroenterol 2006;40:235-43.

161. Zhong Y, Nyman M, Fak F. Modulation of gut microbiota in rats fed high-fat diets by processing whole-grain barley to barley malt. Mol Nutr Food Res 2015;59:2066-76.

162. Matsuzawa-Nagata N, Takamura T, Ando H, Nakamura S, Kurita S, Misu H, Ota T, Yokoyama M, Honda M, Miyamoto K, et al. Increased oxidative stress precedes the onset of high-fat diet-induced insulin resistance and obesity. Metabolism 2008;57:1071-7.

163. Rains JL, Jain SK. Oxidative stress, insulin signaling, and diabetes. Free Radic Biol Med 2011;50:567-75.

164. Vinayagamoorthi R, Bobby Z, Sridhar MG. Antioxidants preserve redox balance and inhibit c-Jun-N-terminal kinase pathway while improving insulin signaling in fat-fed rats: evidence for the role of oxidative stress on IRS-1 serine phosphorylation and insulin resistance. J Endocrinol 2008;197:287-96.

165. Wu T, Yin J, Zhang G, Long H, Zheng X. Mulberry and cherry anthocyanin consumption prevents oxidative stress and inflammation in diet-induced obese mice. Mol Nutr Food Res 2016;60:687-94.

166. Vella A, Rizza RA. Extrapancreatic effects of GIP and GLP-1. Horm Metab Res 2004;36:830-6.

167. Duca FA, Swartz TD, Sakar Y, Covasa M. Decreased intestinal nutrient response in diet-induced obese rats: role of gut peptides and nutrient receptors. Int J Obes (Lond) 2013;37:375-81.

168. Milne GL, Dai Q, Roberts LJ, 2nd. The isoprostanes--25 years later. Biochim Biophys Acta 2015;1851:433-45.

169. Miyazaki Y, Glass L, Triplitt C, Wajcberg E, Mandarino LJ, DeFronzo RA. Abdominal fat distribution and peripheral and hepatic insulin resistance in type 2 diabetes mellitus. Am J Physiol Endocrinol Metab 2002;283:E1135-43.

170. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, Nielsen T, Pons N, Levenez F, Yamada T, et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature 2010;464:59-65.

171. FAO W. Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. 2001. Report of a joint FAO/WHO expert consultation on evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria 2016.

172. Markowiak P, ┼Üli┼╝ewska K. Effects of probiotics, prebiotics, and synbiotics on human health. Nutrients 2017;9:1021.

173. Lee SH, Claire B. Gut microbiome-brain communications regulate host physiology and behavior. J Nutrition 2015:2.

174. Yadav H, Lee JH, Lloyd J, Walter P, Rane SG. Beneficial metabolic effects of a probiotic via butyrate-induced GLP-1 hormone secretion. J Biol Chem 2013;288:25088-97.

175. Greiner TU, Backhed F. Microbial regulation of GLP-1 and L-cell biology. Mol Metab 2016;5:753-8.

176. Erejuwa OO, Sulaiman SA, Ab Wahab MS. Modulation of gut microbiota in the management of metabolic disorders: the prospects and challenges. Int J Mol Sci 2014;15:4158-88.

177. Marchesi JR, Adams DH, Fava F, Hermes GD, Hirschfield GM, Hold G, Quraishi MN, Kinross J, Smidt H, Tuohy KM. The gut microbiota and host health: a new clinical frontier. Gut 2015:gutjnl-2015-309990.

178. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, Owens SM, Betley J, Fraser L, Bauer M. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. The ISME journal 2012;6:1621.

179. Sen T, Cawthon CR, Ihde BT, Hajnal A, DiLorenzo PM, de La Serre CB, Czaja K. Diet-driven microbiota dysbiosis is associated with vagal remodeling and obesity. Physiol Behav 2017;173:305-17.

180. Million M, Angelakis E, Paul M, Armougom F, Leibovici L, Raoult D. Comparative meta-analysis of the effect of Lactobacillus species on weight gain in humans and animals. Microb Pathog 2012;53:100-8.

181. Everard A, Lazarevic V, Gaia N, Johansson M, Stahlman M, Backhed F, Delzenne NM, Schrenzel J, Francois P, Cani PD. Microbiome of prebiotic-treated mice reveals novel targets involved in host response during obesity. Isme j 2014;8:2116-30.

182. Henning SM, Yang J, Shao P, Lee RP, Huang J, Ly A, Hsu M, Lu QY, Thames G, Heber D, et al. Health benefit of vegetable/fruit juice-based diet: Role of microbiome. Sci Rep 2017;7:2167.

183. Reuter G. The Lactobacillus and Bifidobacterium microflora of the human intestine: composition and succession. Curr Issues Intest Microbiol 2001;2:43-53.

184. Stolaki M, De Vos WM, Kleerebezem M, Zoetendal EG. Lactic acid bacteria in the gut. Lactic acid bacteria: microbiological and functional aspects 2012:385-401.

185. Jacouton E, Chain F, Sokol H, Langella P, Bermudez-Humaran LG. Probiotic Strain Lactobacillus casei BL23 Prevents Colitis-Associated Colorectal Cancer. Front Immunol 2017;8:1553.

186. Dandona P, Aljada A, Bandyopadhyay A. Inflammation: the link between insulin resistance, obesity and diabetes. Trends Immunol 2004;25:4-7.

187. Chen J, Wang Q, Liu CM, Gong J. Issues deserve attention in encapsulating probiotics: Critical review of existing literature. Crit Rev Food Sci Nutr 2017;57:1228-38.

188. Sanchez D, Muguerza B, Moulay L, Hernandez R, Miguel M, Aleixandre A. Highly methoxylated pectin improves insulin resistance and other cardiometabolic risk factors in Zucker fatty rats. J Agric Food Chem 2008;56:3574-81.

189. Palou M, Sanchez J, Garcia-Carrizo F, Palou A, Pico C. Pectin supplementation in rats mitigates age-related impairment in insulin and leptin sensitivity independently of reducing food intake. Mol Nutr Food Res 2015;59:2022-33.

190. Canani RB, Costanzo MD, Leone L, Pedata M, Meli R, Calignano A. Potential beneficial effects of butyrate in intestinal and extraintestinal diseases. World J Gastroenterol 2011;17:1519-28.

191. Andoh A, Bamba T, Sasaki M. Physiological and anti-inflammatory roles of dietary fiber and butyrate in intestinal functions. JPEN J Parenter Enteral Nutr 1999;23:S70-3.

192. Licht TR, Hansen M, Bergstrom A, Poulsen M, Krath BN, Markowski J, Dragsted LO, Wilcks A. Effects of apples and specific apple components on the cecal environment of conventional rats: role of apple pectin. BMC Microbiol 2010;10:13.

193. Ivarsson E, Roos S, Liu HY, Lindberg JE. Fermentable non-starch polysaccharides increases the abundance of Bacteroides-Prevotella-Porphyromonas in ileal microbial community of growing pigs. Animal 2014;8:1777-87.

194. Kovatcheva-Datchary P, Nilsson A, Akrami R, Lee YS, De Vadder F, Arora T, Hallen A, Martens E, Bjorck I, Backhed F. Dietary Fiber-Induced Improvement in Glucose Metabolism Is Associated with Increased Abundance of Prevotella. Cell Metab 2015;22:971-82.

195. Macfarlane GT, Macfarlane S. Bacteria, colonic fermentation, and gastrointestinal health. J AOAC Int 2012;95:50-60.

196. Marteau P, Pochart P, Dore J, Bera-Maillet C, Bernalier A, Corthier G. Comparative study of bacterial groups within the human cecal and fecal microbiota. Appl Environ Microbiol 2001;67:4939-42.

197. Sharon G, Garg N, Debelius J, Knight R, Dorrestein PC, Mazmanian SK. Specialized metabolites from the microbiome in health and disease. Cell Metab 2014;20:719-30.

198. Topping DL, Clifton PM. Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. Physiol Rev 2001;81:1031-64.

APPENDIX

ENERGY-DENSE DIET TRIGGERS CHANGES IN GUT MICROBIOTA, REORGANIZATION OF GUT-BRAIN VAGAL COMMUNICATION AND INCREASES BODY FAT ACCUMULATION

Vaughn AC, Cooper EM, DiLorenzo PM, O'Loughlin LJ, Konkel ME, Peters JH, Hajnal A, Sen T, Lee SH, de La Serre CB, et al. Energy-dense diet triggers changes in gut microbiota, reorganization of gut-brain vagal communication and increases body fat accumulation. Acta Neurobiol Exp (Wars) 2017;77:18-30. Reprinted here with permission of publisher.

Abstract

Obesity is associated with consumption of energy-dense diets and development of systemic inflammation. Gut microbiota play a role in energy harvest and inflammation and can influence the change from lean to obese phenotypes. The nucleus of the solitary tract (NTS) is a brain target for gastrointestinal signals modulating satiety and alterations in gut-brain vagal pathway may promote overeating and obesity. Therefore, we tested the hypothesis that high-fat diet-induced changes in gut microbiota alter vagal gut-brain communication associated with increased body fat accumulation. Sprague-Dawley rats consumed a low energy-dense rodent diet (LFD; 3.1 kcal/g) or high energy-dense diet (HFD, 5.24 kcal/g). Minocycline was used to manipulate gut microbiota composition. 16S Sequencing was used to determine microbiota composition. Immunofluorescence against IB4 and Iba1 was used to determine NTS reorganization and microglia activation. Nodose ganglia from LFD rats were isolated and co-cultured with different bacteria strains to determine neurotoxicity. HFD altered gut microbiota with increases in *Firmicutes/Bacteriodetes* ratio and in pro inflammatory *Proteobacteria* proliferation. HFD triggered reorganization of vagal afferents and microglia activation in the NTS, associated with weight gain. Minocycline-treated HFD rats exhibited microbiota profile comparable to LFD animals. Minocycline suppressed HFD-induced reorganization of vagal afferents and microglia activation in the NTS, and reduced body fat accumulation. Proteobacteria isolated from cecum of HFD rats were toxic to vagal afferent neurons in culture. Our findings show that diet-induced shift in gut microbiome may disrupt vagal gut-brain communication resulting in microglia activation and increased body fat accumulation.

Key words: microglia, obesity, vagus, bacteria, plasticity

Introduction

Obesity rate in the United States climbed to nearly 38 percent of adults in 2013–2014, up from 32 percent about a decade earlier. Among contributing factors is an increase in the intake of energy-dense diets leading to body weight gain (Little and Feinle-Bisset 2011, Prentice and Jebb 2003). Obesity is considered to be a state of chronic inflammation (Cox et al. 2015) originating, at least partially, from the gastrointestinal (GI) tract (Cani et al. 2008). There is evidence that the GI microbiota contribute to the development of obesity (Montiel-Castro et al. 2013). Conventionalization of germ-free animals with microbiota from lean or obese donors results in recapitulation of the donor phenotype (Ridaura et al. 2013). The mechanisms and pathways by which GI microbiota may affect regulation of feeding are not well understood. Interestingly, diet composition rather than adiposity affects the microbiota composition (de La Serre et al. 2010, Hildebrandt et al. 2009).

Changes in satiation have been linked to diet composition. Protein induces supercaloric compensation; carbohydrate leads to approximate caloric compensation; and fat generates subcaloric compensation and hence promotes excess energy intake (Rolls 1995, 2009). At first, consumed fat changes meal patterns coherent with stimulation of a short-term satiety. This effect is reduced with long-term exposure to fat in the diet, independently of calorie intake or body weight. This alteration in short-term satiety leads to an increase in meal size and contributes to development of high fat diet-induced obesity (Paulino et al. 2008). Moreover, vagally-mediated effects (decreased response to cholecystokinin) reported in high fat diet-induced obesity are due to the diet composition because they appear before the increased body fat accumulation leading to obesity (Troy et al. 2016).

Meal size is primarily regulated by gut-brain neural signaling. GI signals inform the brain about the quantity and quality of food being consumed to regulate satiety and food intake (Ritter 2004). Information from GI-borne signals is relayed to the brain via vagal afferents (Dockray 2003). The nucleus of the solitary tract (NTS), located in the caudal brainstem is the site at which the vagal afferents make their first central synapses; while GI-projecting motor neurons are located at the dorsal motor nucleus of the vagus (DMV) (Berthoud et al. 1991, Peters et al. 2013). Neurobiological insights into vagal gut-brain crosstalk have revealed that gut-brain signaling is critical for maintaining adequate adiposity and preventing obesity (Berthoud et al. 2011, Ritter 2004).

Vagal afferents can relay microbiota signals to the brain to alter host behavior. Bacteria-driven hippocampal activation and modulation of anxiety are notably abolished by vagotomy (Wang et al. 2002). We have recently shown that a pro-inflammatory bacteria product, lipopolysaccharide (LPS), can activate vagal afferent neurons and impair satiety signaling (de La Serre et al. 2015). Therefore, diet-shifted microbiota may alter vagal satiety signaling to the hindbrain feeding centers to increase energy intake and adiposity (de Lartigue et al. 2011).

The goal of our study was to determine whether HFD-induced (High-fat-diet-induced) changes in gut microbiota composition can drive remodeling of the vagal pathway, to stimulate body fat accumulation.

Methods

Male Sprague-Dawley rats (~460 g BW, Simonsen Laboratories, CA) were individually housed in a temperature-controlled vivarium with ad libitum access to food and water (12-hour light/dark schedule). Individual housing was necessary for food intake measurements and fecal pellet collection. Animal procedures were approved by the Washington State University

Institutional Animal Care and Use Committee and conformed to National Institutes of Health guidelines for the use of vertebrate animals (publication 86-23). Appropriate measures were taken to minimize pain and discomfort of the animals.

Following a seven-days baseline (low energy-dense rodent diet; LFD; Teklad F6; 3.1 kcal/g; 19% fat), animals were assigned to their respective diet groups. LFD rats (n=16) were maintained on LFD for additional 7 (n=8) or 21 days (n=8). High fat diet (HFD) fed animals (n=16) were switched to a high energy-dense diet (Research Diets, D12492; 5.24 kcal/g; 60% fat) for 7 days (n=8) or 21 days (n=8). Beginning at dietary switch and continuing until sacrifice, LFD and HFD rats received daily injections of a broad-spectrum antibiotic, minocycline, (n=8/group; 20 mg/kg i.p.; Sigma-Aldrich) or sterile 0.9% NaCl (n=8/group; 0.5 ml). Body weight and food intake were monitored daily. Fecal pellets were collected before introducing the HFD and 7 and 21 days after dietary switch. Body composition was determined by Dual-Energy X-ray Absorptiometry (DEXA) at baseline and 7 and 21 day after dietary switch. After 21 days, rats were euthanized by CO2 and blood samples were collected. Animals were then transcardially perfused with 0.1 M PBS (pH 7.4) followed by 4% paraformaldehyde and hindbrains were collected. Additionally, the nodose ganglia (NG) from a separate group of LFD rats (n=20) were isolated and co-cultured with different bacterial strains.

Microbiome analysis. Bacterial DNA was extracted from fecal samples using a kit following the recommendation of the manufacturer (Zymo research, CA). Fecal contents were lysed by bead beating and DNA was isolated using fast-spin columns. DNA was filtered to remove humic acids and polyphenols and the eluted DNA was sent to SeqMatic facility (Fremont, CA) for sequencing. A library was generated by targeting the 16S V4 region. Sequencing was performed via Illumina MiSeq and sequences aligned to reference genomes. Illumina BaseSpace software

was used for data analysis. Diversity was determined using Shannon-Weiner index. Abundance at the Phylum, Class, Order, Family, Genus and Species levels were calculated and changes in composition from baseline were expressed as Log2 (n-folds change). Principal component analysis (PCA) at the order levels was run using XLSTAT (Addinsoft, NY).

To identify and isolate the cecal bacteria strains from HF rats, the samples were normalized by weight, and serially-diluted lysates were plated on three separate culture media (MHB, Mueller-Hinton Blood agar plate; MRS, deMan, Rogosa and Sharpe agar plate; LB, Luria-Bertani agar plate). Identification of microbial taxa was based on 16S sequencing of colony types that were enriched in the animals given a high fat diet. DNA was extracted from individual colony isolates and PCR of the 16S ribosomal RNA subunit was performed using the 27F primer: 5′-AGAGTTTGATCMTGGCTCAGAACG-3′ and 1435R primer: 5′-CGATTACTAGCGATTCCRRCTTCA-3′, where M=A or C and R=A or G. The primers used for sequencing included the 27F primer, 1435R primer, 533F primer: 5′-GTGCCAGCMGCCGCGGTAA-3′, and 519R primer: 5′-GTATTACCGCGGCTGCTGG-3′. Sequences were trimmed and analyzed by BLAST for identification. The Genus and species classification of an individual isolate was based on 100% sequence identify of >1000 nucleotides.

Quantification of serum LPS. Serum LPS was measured as described previously (de La Serre et al. 2015). LPS was quantified using a Pyrochrome Lysate Mix, a quantitative chromogenic reagent, (Associate of Cape Cod, MA) diluted in Glucashield buffer which inhibits cross-reactivity with (1→3)-β-D-Glucans. Briefly, serum samples were diluted 1:10 in Pyrogen free water (Lonza, Switzerland) and heated for 10 min at 70°C. Samples and reactive solution were incubated at 37° C for 30 min and absorbance was read at 405 nm.

Immunofluorescence. Hindbrains were cryosectioned at 20 μm thickness and stained for selected antigens. After blocking in 10% normal horse serum in Tris-phosphate buffered saline (TPBS, pH 7.4) sections were incubated overnight in a primary antibody against ionized calcium binding adaptor molecule 1 (Iba1, 1:1000; 019-19741, Dako, GA) followed by an Alexa-488 secondary antibody (1:400; A21206, Invitrogen, CA) to visualize microglia activation as previously described (Gallaher et al. 2012). For visualization of vagal afferents, the hindbrain sections were incubated with isolectin B4 biotin-conjugated antibody (IB4, 1:400, cat# B-1205, Vector Laboratories, CA) for 12 h at room temperature (Shehab 2009), followed by ExtrAvidin-CY3 (1:600, E-4142, Sigma-Aldrich) for 2 h. Negative controls were performed by omission of primary antibodies. Sections were mounted in ProLong (Molecular Probes, OR) and examined under Nikon 80-I fluorescent microscope as previously described (Gallaher et al. 2012, Peters et al. 2013).

NG primary cultures. NG were isolated from LFD fed animals 3 hours after light onset under deep anesthesia (Ketamine, 25 mg/100 g; plus Xylazine, 2.5 mg/100 g). Once isolated, NG were digested in Ca2+/Mg2+ free Hank's Balanced Salt Solution containing 1 mg/mL of Dispase II and Collagenase Type 1A (120 min at 37°C in 95% air/5% CO2). Dispersed cells were plated onto polylysine-coated coverslips and maintained in DMEM+10% FBS (37°C in 95% air/5% CO2). Cultures were infected with one of the following facultative anaerobic bacteria strains identified in HFD rats' cecal contents: Streptococcus mitis, Proteus mirablis, Lactobacillus animalis or Enterococcus faecalis (n=4 rats/strain) at a concentration of ~5×10(7). Culture medium without bacteria was added to control plates (n=4 rats). Thirty min later all cultures were fixed in paraformaldehyde and stained with beta III-tubulin antibody (1:500, ab 78078, Abcam, UK) to

identify neuronal profiles. For neurons quantification, beta-III tubulin-stained neuronal perikarya were counted in 3 randomly assigned frames per culture (frame size 3×3 mm).

Statistics. GraphPad prism (GraphPad, CA) was used for statistical analysis. A two-way ANOVA with a Tukey post hoc test was used to analyze the data. Differences were considered significant if $p<0.05$.

Results

Minocycline decreased energy intake and body fat accumulation induced by HFD. During the baseline we observed no statistically significant differences in energy intake, body fat and body weight between rats (Figs 1A–1C). Seven-day exposure to HFD was sufficient to significantly increase energy intake and body fat accumulation, but not the body weight. After 21 days on HFD rats significantly increased the energy intake, body fat accumulation and body weight (Figs 1A–1C). Minocycline reduced energy intake after one week of treatment and there was no effect of minocycline on body fat or body weight gain at day 7 (Figs 1A–1C). However, after 21 days on HFD minocycline-treated rats exhibited a significant decrease in energy intake, in body fat accumulation and in body weight gain (Figs 1A–1C). Body fat and body weight in rats fed a HFD and treated with minocycline were significantly lower than HF_Saline rats (8.1±1.6% *vs.* 15.5±2.2%; Fig. 1B and 438.275 g±11.8 g *vs.* 517.7g±13.2; Fig. 1C) and normalized to the level of control LF_Saline animals (6.5±0.7%; Fig. 1B and 469.087 g±5.0 g; Fig. 1C). Minocycline reduced the caloric intake in LFD rats only in the first week (Fig. 1A). However, between the 8th and 21st day of the experiment, this effect was abolished (Fig. 1A). Minocycline did not affect body fat accumulation and body weight of LFD rats (Figs 1B, 1C).

Bacterial composition altered by HFD was improved by minocycline. *Firmicutes* and *Bacteroidetes* were the most abundant phyla and represented over 85% of the bacteria identified

(Fig. 2A). In all groups, there was a significant reduction in *Verrucomicrobia* abundance at day 7 and day 21. Bacterial composition was changed after 7 days on HFD. HFD consumption led to a significant increase in *Firmicutes* abundance (LF_Saline 46.8±3.2% *vs.* HF_Saline 85.5±3.4%) and a reduction in *Bacteroidetes* (LF_Saline 49.3±3.5% *vs.* HF_Saline 11.3±3.2%) in the fecal pellets. The same changes were present after 21 days of HFD consumption (LF_Saline *Firmicutes* 42.2±4.8% vs. HF_Saline 70.7±12.4%; LF_Saline *Bacteroidetes* 54.4±4.9% vs. HF_Saline 25.4±12.7%) in the fecal pellets.

In LFD animals, minocycline (Mino) did not affect the microbiota composition, at the phylum level; however, it significantly improved the microbiome profile of the HFD rats. After 7 days of minocycline, there was a reduction in *Firmicutes* abundance in the HF_Mino rats compared to the HF_Saline rats but it did not reach significance. There was a significant increase in *Bacteriodetes* abundance in the HF_Mino groups compared to the HF_Saline rats (HF_Saline 11.3±3.2% *vs.* HF_Mino 28.4±7.8%). After 21 days of minocycline, *Firmicutes* and *Bacteriodetes* abundance levels were normalized in the HFD rats. *Firmicutes* abundance in the HF_Mino rats (Day 21: 46.9±6.3%) was not different from the LF_Saline group and significantly lower than in the HF_Saline animals. *Bacteroidetes* abundance in the HF_Mino rats (Day 21:50.9±6.4%) was not different from the LF_Saline group and was significantly higher than in HF_Saline animals. *Clostridiales* (the main Firmicutes order) and *Bacteroidales* (the main *bacteriodetes* order) abundances were positively and negatively correlated with energy intake across diets and treatments (Fig. 2B).

Principal component analysis (PCA) was run to identify the main contributors to variance (at the order level). Log2 fold changes from baseline were calculated to specifically identify bacterial orders that were enriched or depleted by HFD and/or minocycline (Figs 2C, 2D).

Interestingly, for the majority of bacterial orders identified by PCA, the relative abundance was significantly positively or negatively correlated with energy intake and/or adiposity (Fig. 2B).

Seven days on HFD led to a significant increase in the abundance of several orders belonging to the Firmicutes (*Erysipelotrichales*), *Terenicutes* (*Entomoplasmatales*, *Mycoplasmatales*), *Proteobacteria* (*Rhodocyclales*, *Altermondales*), *Cyanobacteria* (*Nostocales*, *Chroococcales*) and *Verrucomicrobia* (*Puniceicoccales*). The orders classified as *Terenicutes* all belong to the *Mollicutes* class which has previously been classified as *Firmicutes*. HFD consumption led to a significant depletion in *Bacteriodetes*, especially *Sphingobacteriales* and *Bacteroidales* (Fig. 2C). Similar results were observed after 3 weeks on HFD, additional orders were found to significantly contribute to variance, such as *Enterobacteriales* and *Methylophilales* (*Proteobacteria*), which were enriched on HFD and *Thermobaculales* (*Chloroflexi*) was depleted by HFD (Fig. 2D).

Minocycline had the opposite modulatory effects; 7 days of minocycline exposure were sufficient to significantly reduce the HFD-induced proliferation of bacterial orders mentioned above, leading to normalization of abundance or significant depletion (*Nostocales*, *Chroococcales*). Minocycline prevented HFD-induced depletion of *Bacteroidales* and *Sphingobacteriales*. In LFD animals, minocycline alone significantly reduced the abundance of obesity-associated *Puniceicoccales*, *Chroococcales* and *Clostridiales.* Similar results were observed after 21 days on minocycline. Minocycline led to normalization or depletion of HFD-associated bacterial orders and restored HFD-depleted orders. Minocycline alone led to significant reduction in *Erysipelotrichales* and several *Proteobacteria* and *Cyanobacteria* orders, (Figs 2C, 2D).

PCA showed that after 7 days, LFD and HFD rats had different microbiota profile; minocycline treatment resulted in a third profile with more variabilities, based on the diet consumed (LFD *vs.* HFD; Fig. 3A). After 7 days, the HF_Mino microbiota profile showed more similarities with the HF_Saline rats than the LF_Saline rats (Fig. 3A). However, after 21 days, HF_Mino microbiota profile overlap with LF_Saline groups. While minocycline did affect abundance of some specific species it did not result in a different microbiota profile in LFD rats (Fig. 3B).

HFD-related increases in plasma levels of LPS were suppressed by minocycline. HFD led to an increase in circulating LPS after 7 days when compared to LFD animals, and LPS levels were significantly elevated in HFD rats after 21 days on the diet (6.3 EU±1.2 *vs.* 3.3 EU±0.7; Fig. 3C). Minocycline reduced circulating LPS in HFD rats. This effect was not significant after 7 days of treatment, however, after 21 days, minocycline significantly decreased LPS levels in HFD rats (6.3 EU±1.2 *vs.* 2.9 EU±0.4; Fig. 3C). In LFD rats, minocycline did not result in changes of LPS levels at any time point (2.0 EU±0.4 *vs.* 1.5 EU±0.2 and 3.3 EU±0.7 *vs.* 2.2 EU±0.5 for 7 and 21 days; Fig. 3C).

HFD-induced activation of microglia in the NTS and DMV was suppressed by minocycline. Immunostaining against Iba-1 revealed that HFD increased microglia activation in the NTS and the DMV. After 7 days on HFD microglia activation was observed only in the DMV (Figs 4A–4E). The area fraction populated by fluorescent staining against Iba1 in the DMV was increased in HFD when compared to LFD rats (0.0292±0.0092 *vs.* 0.0096±0.0011). After 21 days on HFD microglia activation was observed in both the DMV and the NTS (Figs 4A, 4F–4I). The area fraction populated by the fluorescent staining against Iba1 in the DMV and

the NTS was increased in HFD rats when compared to LFD (0.0423±0.0083 *vs.*0.0180±0.005 and 0.0512±0.0143 *vs.* 0.0206±0.0057 respectively).

Minocycline attenuated the HFD-induced increases in the microglia activation in both the DMV and the NTS. The area fraction populated by the fluorescent staining against Iba1 in the DMV was significantly smaller in HF_Mino rats when compared to HF_Saline rats (0.0179±0.0052 vs. 0.0292±0.0092 and 0.0083±0.0024 vs. 0.0423±0.0083 at 7 and 21 days respectively; Fig. 4A). The area fraction populated by Iba1 staining in the NTS was significantly smaller in HF_Mino rats when compared to HF_Saline rats only at day 21 (0.0156±0.0039 vs. 0.0512±0.0143; Fig. 4A). Minocycline did not significantly change the microglia activation in the DMV and the NTS in rats fed LF diet at any time point (Fig. 4A).

HFD-induced vagal remodeling in the NTS and DMV was suppressed by minocycline. Binary analysis of the area fraction of IB4-labeled vagal afferents in the intermediate NTS and DMV revealed significant differences in the density of labeled vagal terminals between rats fed different diets (Fig. 5). After 7 days the HFD decreased total IB4 labeling in the NTS and the DMV when compared to LFD (0.0566±0.0089 *vs.* 0.2611±0.0232 for NTS and 0.0013±0.0003±0.0005 vs. 0.0031 for DMV; Figs 5A, 5B–5E). This effect was dampened by minocycline only in the NTS (Figs 5A–5E) but failed to reach significance. At day 21, we observed a significant increase in density of IB4-labeled vagal afferents projecting to the NTS and DMV in HFD rats when compared to LFD rats (1.0187±0.2895 *vs.*0.2622±0.0411 for NTS and 0.0064±0.0021 *vs.* 0.0022±0.0008 for DMV; Figs 5A, 5F–5I). This effect was significantly decreased in both the NTS and DMV by minocycline (Figs 5A, 5F–5I). There were no significant differences between LF_Saline and LF_Mino rats at any time point.

Gram-negative bacteria isolated from HFD rats significantly reduced the number of NG neurons in culture. Co-culture of NG primary sensory neurons isolated from LFD rats with *Streptococcus mitis, Lactobacillus animalis or Enterococcus faecalis (Firmicutes, Bacilli, Lactobacillales)* isolated from the cecum of HFD rats did not change the number of surviving neurons when compared to NG cultures without the bacteria (238.5±20.5; 214.8±26.8; 243.5±56.6 *vs.* 205.3±61.5; Fig. 6). Adding *Proteus mirablis (Proteobacteria, Gammaproteobacteria, Enterobacteriales)* to NG cultures induced a dramatic loss of neurons when compared to NG cultures without the bacteria (29.3±4.2 *vs.* 205.3±61.5; Fig. 6)

Discussion

This study provides evidence for dynamic diet-influenced and bacteria-driven neural plasticity in the NTS and DMV, the first relay stations for nutritionally relevant information from the GI tract (Rogers and McCann 1993). We show that a HFD rapidly and significantly changed gut microbiota composition. HFD led to an increase in circulating pro-inflammatory LPS and was associated with neuronal damage to the NTS and DMV. This damage was reflected by induced activation of microglia and vagal remodeling in the NTS and DMV. Normalization of the microbiota composition in HFD rats using minocycline was protective against HFD-induced neuronal damage and increase in adiposity, showing that gut microbiota dysbiosis is necessary for HFD-driven deleterious effects on gut-brain signaling. Therefore, bacteria-driven brain plasticity in response to HFD may be responsible for increased energy intake, body fat accumulation, and body weight promoting obesity.

Results show that HFD induced an inflammatory response reflected by microglia activation in NTS and DMV. The pro-inflammatory action of the HFD has been also reported in the NG and hypothalamus (Waise et al. 2015, Yi et al. 2012). Taken together, these results

suggest that a HFD weakens neuroprotective signaling and promotes inflammation in the brain feeding centers, which may lead to increased food intake and obesity.

We found that a HFD triggered reorganization of vagal afferents in the NTS and DMV. HFD induced transient withdrawal of vagal afferents from the hindbrain feeding centers following by an increase in the density of vagal afferents in NTS and DMV. The increase in the density may reflect a sprouting of vagal inputs into the hindbrain feeding centers. Interestingly, our previous studies revealed that damage to peripheral axons of the vagus (subdiaphragmatic vagotomy) also induced transient decrease in the density of vagal afferents projecting to the NTS (Ballsmider et al. 2015, Peters et al. 2013) followed by vagal inputs sprouting. Vagal damage also resulted in microglia activation in the NTS and DMV (Gallaher et al. 2012). We previously found that vagal afferent remodeling led to alterations in frequency and amplitude of glutamate release in the NTS (Peters et al. 2013). Satiety peptide CCK triggers glutamate release in the NTS while hunger signal ghrelin inhibits glutamate inputs to the NTS. Therefore, HFD driven vagal remodeling may lead to alteration in satiety signaling and overeating.

Dietary fat-induced neuronal remodeling has previously been reported in the hypothalamus (Benani et al. 2012) and obesity has been found to have neurodegenerative effects in this nucleus (McNay et al. 2012). Microglia activation is involved in neuronal circuit remodeling (Kettenmann et al. 2013) and has been found to control HFD-induced hypothalamic inflammation and loss of neuronal function (Valdearcos et al. 2014). Interestingly, HFD-induced hypothalamic inflammation is significantly reduced by vagotomy (Lee et al. 2015, Waise et al. 2015) suggesting that vagal afferents may transfer bacterial-derived inflammatory signals to the brain feeding centers to trigger microglia activation and neural remodeling.

Supporting this hypothesis, we found that microbiota normalization blunted the inflammatory effect of HFD on hindbrain, showing the dysbiosis is necessary for HFD-induced vagal remodeling. While our study did not establish whether gut microbiota dysbiosis is sufficient to promote vagal remodeling, colonization of germ free animals with dysbiotic microbiota has previously been found to induce hypothalamic inflammation (Duca et al. 2014). Taken together, these data point towards a causal role for the microbiota in HFD-driven hindbrain inflammation and neural remodeling. This pathway is supported by previous studies showing that obesity-associated inflammation originates, at least partially, from bacterial endotoxin in the GI tract (de La Serre et al. 2010, Lassenius et al. 2011). LPS is a constituent of the gram-negative bacterial cell wall and circulating LPS levels have been found to be chronically increased in diet-induced obesity (Cani et al. 2007, Moreira et al. 2014). Interestingly, constant infusion of low doses of LPS results in weight gain (Cani et al. 2007). An increase in circulating LPS could be caused by overpopulation of gram-negative bacteria in the gut (Nguyen et al. 2004) and/or an increase in GI permeability (de La Serre et al. 2010).

We found that HFD led to rapid changes in microbiota composition with a bloom in several orders, belonging mostly to the *Firmicutes* and *Proteobacteria* phyla; notably *Erysipelotrichales* and *Enterobacteriales*. Both orders have previously been linked to obesity (de La Serre et al. 2010) and systemic inflammation (Dinh et al. 2015). We also found positive correlations between the abundance of bacterial orders enriched by HFD and energy intake and adiposity. Conversely, orders that were depleted on HFD were negatively correlated with intake, body fat mass and body weight gain. *Erysipelotrichales* are gram-positive anaerobic bacteria while *Enterobacteriales* are gram-negative, potential LPS producer, and facultative anaerobic bacteria. HFD rats exhibited a significant increase in circulating LPS that was

normalized by minocycline. LPS, is a potent inducer of inflammation and activator of microglia (Herrera et al. 2000). Dietary-driven microglia activation in the retina is notably dependent on LPS receptor activation (Lee et al. 2015). Interestingly, vagal sensitivity to GI originating signals is altered in LPS treated animals, identifying LPS as a potential trigger in HFD-induced neuronal inflammation and hindbrain remodeling (de La Serre et al. 2015). Neurotoxic effects of bacteria and/or bacterial components were confirmed by culture experiments in our study. *Streptococcus mitis*, *Lactobacillus animalis* and *Enterococcus faecalis*belong to the *Lactobacillales* order (*Firmicutes, Bacilli*), are gram-positive bacteria, and there was no difference in *Lactobacillales* abundance with diet or minocycline treatment. Co-culture of NG neurons with these bacteria did not affect neuron growth. Conversely, co-culture with *Proteus mirablis*, a gram-negative *Enterobacteriales* that was enriched in the HFD rats, led to neuronal death. *Proteus mirablis* is an LPS producing bacteria and its abundance has previously been correlated to metabolic changes induced by HFD (Lecomte et al. 2015). Culture experiments were limited to facultative anaerobic bacteria. We were unable to assess the potential neurotoxicity of anaerobic *Erysipelotrichales* because it is not possible to culture NG neurons without oxygen. Another limitation of the culture experiments was an isolation of the bacteria from cecum, rather than fecal samples. The rationale for this was the fact that facultative anaerobes represent 25% of total bacteria in the cecum versus only 1% in the fecal samples (Marteau et al., 2001).

While evidence from this study points towards bacteria-driven damage to the hindbrain, we cannot rule out an additional effect of minocycline alone. The anti-inflammatory action of minocycline has notably been reported in the brainstem after damage to the vagus nerve (Gallaher et al. 2012) and minocycline may directly reduce HFD-driven inflammation and body

fat accumulation. It has been previously reported that minocycline can suppress body weight gain (Sun et al. 2015). It should be noted the above study minocycline was administered directly into the spinal cord at a dose of 10, 50 or 100 μg/kg. Direct anti-inflammatory effects of minocycline have been reported for doses ranging from 20 to 100 mg/kg (Amin et al. 2015, Gallaher et al. 2012, Kumar and Addepalli 2011). Minocycline may alleviate inflammation via a decrease in bacteria-driven cytokine production. Minocycline has been shown to decrease expression of the LPS receptor, Toll-like receptor 4 (TLR4), in dorsal horn of spinal cord (Nazemi et al. 2015). In our study, minocycline alone, at 20 mg/kg, was not sufficient to induce significant changes in hindbrain inflammation, intake or adiposity in the LFD rats. The absence of significant differences between LF_Saline and LF_Mino animals supports a microbiota-driven pathway. It should be noted that while non-significant, hindbrain inflammation and adiposity were lowered in the LF_Mino rats compared to the LF_Saline animals. However, microbiota analysis revealed that minocycline led to a reduction in obesity-associated bacterial orders, such as *Erysipelotrichales* and *Enterobacteriales* in LFD rats. Therefore, the potential effect of minocycline in LFD rats may be related to modulation in microbiota composition rather than a direct anti-inflammatory effect.

Antibiotics-driven modulation of gut microbiota has traditionally been conducted via oral delivery of broad spectrum antibiotics at doses ranging from 40 to 500 mg daily (Membrez et al. 2008, Mikkelsen et al. 2015, Vrieze et al. 2014). These treatments led to acute and dramatic decreases in microbiota diversity and abundance, with *Firmicute*s and *Bacteriodete*s not being the most abundant phyla (Carvalho et al. 2012). While effective in promoting weight loss, such treatment did not result in normalization of the HFD-associated microbiota, hence in this study we chose to use a lower dose and a different rout of administration to avoid “wiping out” the gut

microbiota. Minocycline has been reported to have potential weight-loss effect (Sun et al. 2015) which may be related to its targeting specific obesity-associated bacterial strains. Minocycline and HFD had opposite modulatory effects on several orders abundance, including *Erysipelotrichales* and *Enterobacteriales* and HFD/minocycline rats exhibited normalized microbiota composition, with microbiome profile comparable to the LFD rats.

Our data support a diet-associated and bacteria-driven gut to brain pathway promoting hindbrain inflammation; however, descending brain to gut communication may also modulate the gut microbiota. Vagal efferent neurons located in the DMV send motor information from the brain to the GI tract (de Lartigue et al. 2014). In HFD-rats, we have found that minocycline increased microglia activation in the DMV and vagal remodeling. Vagal efferent fibers modulate gut motility (Chang et al. 2003), and motility has been demonstrated to modify microbiota composition (Kashyap et al. 2013); therefore, we cannot rule out a possible indirect effect of minocycline on microbiota composition in HFD rats via changes in vagal efferent output.

Conclusions

Taken together, the results show that HFD induces changes in the diversity of the intestinal microbiota, and alters the gut-brain communication resulting in inflammation of the hindbrain feeding centers associated with overeating, overweight and increased in body fat accumulation. Normalization of microbiota composition via minocycline blunted the effects of HFD on hindbrain inflammation, energy intake and adiposity, which let us to conclude that a HFD triggered shift in gut microbiome may disrupt vagal gut-brain communication resulting in microglia activation and increased body fat accumulation leading to obesity.

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References

1. Amin B, Hajhashemi V, Hosseinzadeh H. Minocycline potentiates the anti-hyperalgesic effect of ceftriaxone in CCI-induced neuropathic pain in rats. Res Pharm Sci. 2015;10:34–42.
2. Ballsmider LA, Vaughn AC, David M, Hajnal A, Di Lorenzo PM, Czaja K. Sleeve gastrectomy and Roux-en-Y gastric bypass alter the gut-brain communication. Neural Plast. 2015;2015:601985.
3. Benani A, Hryhorczuk C, Gouaze A, Fioramonti X, Brenachot X, Guissard C, Krezymon A, Duparc T, Colom A, Nedelec E, Rigault C, Lemoine A, Gascuel J, Gerardy-Schahn R, Valet P, Knauf C, Lorsignol A, Penicaud L. Food intake adaptation to dietary fat involves PSA-dependent rewiring of the arcuate melanocortin system in mice. J Neurosci. 2012;32:11970–11979.
4. Berthoud HR, Carlson NR, Powley TL. Topography of efferent vagal innervation of the rat gastrointestinal tract. Am J Physiol. 1991;260:R200–R207.
5. Berthoud HR, Shin AC, Zheng H. Obesity surgery and gut-brain communication. Physiol Behav. 2011;105:106–119.
6. Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, Neyrinck AM, Fava F, Tuohy KM, Chabo C, Waget A, Delmee E, Cousin B, Sulpice T, Chamontin B, Ferrieres J, Tanti JF, Gibson GR, Casteilla L, Delzenne NM, Alessi MC, Burcelin R. Metabolic endotoxemia initiates obesity and insulin resistance. Diabetes. 2007;56:1761–1772.
7. Cani PD, Delzenne NM, Amar J, Burcelin R. Role of gut microflora in the development of obesity and insulin resistance following high-fat diet feeding. Pathol Biol (Paris) 2008;56:305–309.
8. Carvalho BM, Guadagnini D, Tsukumo DM, Schenka AA, Latuf-Filho P, Vassallo J, Dias JC, Kubota LT, Carvalheira JB, Saad MJ. Modulation of gut microbiota by antibiotics improves insulin signalling in high-fat fed mice. Diabetologia. 2012;55:2823–2834.
9. Chang HY, Mashimo H, Goyal RK. Musings on the wanderer: what's new in our understanding of vago-vagal reflex? IV. Current concepts of vagal efferent projections to the gut. Am J Physiol Gastrointest Liver Physiol. 2003;284:G357–G366.
10. Cox AJ, West NP, Cripps AW. Obesity, inflammation, and the gut microbiota. Lancet Diabetes Endocrinol. 2015;3:207–215.
11. de Lartigue G, de La Serre CB, Raybould HE. Vagal afferent neurons in high fat diet-induced obesity; intestinal microflora, gut inflammation and cholecystokinin. Physiol Behav. 2011;105:100–105.
12. de Lartigue G, Ronveaux CC, Raybould HE. Vagal plasticity the key to obesity. Mol Metab. 2014;3:855–856.
13. de La Serre CB, de Lartigue G, Raybould HE. Chronic exposure to low dose bacterial lipopolysaccharide inhibits leptin signaling in vagal afferent neurons. Physiol Behav. 2015;139:188–194.
14. de La Serre CB, Ellis CL, Lee J, Hartman AL, Rutledge JC, Raybould HE. Propensity to high-fat diet-induced obesity in rats is associated with changes in the gut microbiota and gut inflammation. Am J Physiol Gastrointest Liver Physiol. 2010;299:G440–G448.

15. Dinh DM, Volpe GE, Duffalo C, Bhalchandra S, Tai AK, Kane AV, Wanke CA, Ward HD. Intestinal microbiota, microbial translocation, and systemic inflammation in chronic HIV infection. J Infect Dis. 2015;211:19–27.
16. Dockray GJ. Luminal sensing in the gut: an overview. J Physiol Pharmacol. 2003;54(Suppl 4):9–17.
17. Duca FA, Sakar Y, Lepage P, Devime F, Langelier B, Dore J, Covasa M. Replication of obesity and associated signaling pathways through transfer of microbiota from obese-prone rats. Diabetes. 2014;63:1624–1636.
18. Gallaher ZR, Ryu V, Herzog T, Ritter RC, Czaja K. Changes in microglial activation within the hindbrain, nodose ganglia, and the spinal cord following subdiaphragmatic vagotomy. Neurosci Lett. 2012;513:31–36.
19. Herrera AJ, Castano A, Venero JL, Cano J, Machado A. The single intranigral injection of LPS as a new model for studying the selective effects of inflammatory reactions on dopaminergic system. Neurobiol Dis. 2000;7:429–447.
20. Hildebrandt MA, Hoffmann C, Sherrill-Mix SA, Keilbaugh SA, Hamady M, Chen YY, Knight R, Ahima RS, Bushman F, Wu GD. High-fat diet determines the composition of the murine gut microbiome independently of obesity. Gastroenterology. 2009;137:1716–1724.
21. Kashyap PC, Marcobal A, Ursell LK, Larauche M, Duboc H, Earle KA, Sonnenburg ED, Ferreyra JA, Higginbottom SK, Million M, Tache Y, Pasricha PJ, Knight R, Farrugia G, Sonnenburg JL. Complex interactions among diet, gastrointestinal transit, and gut microbiota in humanized mice. Gastroenterology. 2013;144:967–977.
22. Kettenmann H, Kirchhoff F, Verkhratsky A. Microglia: new roles for the synaptic stripper. Neuron. 2013;77:10–18.
23. Kumar BL, Addepalli V. Minocycline with aspirin: an approach to attenuate diabetic nephropathy in rats. Ren Fail. 2011;33:72–78.
24. Lassenius MI, Pietilainen KH, Kaartinen K, Pussinen PJ, Syrjanen J, Forsblom C, Porsti I, Rissanen A, Kaprio J, Mustonen J, Groop PH, Lehto M. Bacterial endotoxin activity in human serum is associated with dyslipidemia, insulin resistance, obesity, and chronic inflammation. Diabetes Care. 2011;34:1809–1815.
25. Lecomte V, Kaakoush NO, Maloney CA, Raipuria M, Huinao KD, Mitchell HM, Morris MJ. Changes in gut microbiota in rats fed a high fat diet correlate with obesity-associated metabolic parameters. PLoS One. 2015;10:e0126931.
26. Lee JJ, Wang PW, Yang IH, Huang HM, Chang CS, Wu CL, Chuang JH. High-fat diet induces toll-like receptor 4-dependent macrophage/microglial cell activation and retinal impairment. Invest Ophthalmol Vis Sci. 2015;56:3041–3050.
27. Little TJ, Feinle-Bisset C. Effects of dietary fat on appetite and energy intake in health and obesity – oral and gastrointestinal sensory contributions. Physiol Behav. 2011;104:613–620.
28. Marteau P, Pochart P, Doré J, Béra-Maillet C, Bernalier A, Corthier G. Comparative study of bacterial groups within the human cecal and fecal microbiota. Appl Environ Microbiol. 2001;67(10):4939–4942.
29. McNay DE, Briancon N, Kokoeva MV, Maratos-Flier E, Flier JS. Remodeling of the arcuate nucleus energy-balance circuit is inhibited in obese mice. J Clin Invest. 2012;122:142–152.

30. Membrez M, Blancher F, Jaquet M, Bibiloni R, Cani PD, Burcelin RG, Corthesy I, Mace K, Chou CJ. Gut microbiota modulation with norfloxacin and ampicillin enhances glucose tolerance in mice. FASEB J. 2008;22:2416–2426.
31. Mikkelsen KH, Frost M, Bahl MI, Licht TR, Jensen US, Rosenberg J, Pedersen O, Hansen T, Rehfeld JF, Holst JJ, Vilsboll T, Knop FK. Effect of antibiotics on gut microbiota, gut hormones and glucose metabolism. PLoS One. 2015;10:e0142352.
32. Montiel-Castro AJ, Gonzalez-Cervantes RM, Bravo-Ruiseco G, Pacheco-Lopez G. The microbiota-gut-brain axis: neurobehavioral correlates, health and sociality. Front Integr Neurosci. 2013;7:70.
33. Moreira AP, Teixeira TF, Alves RD, Peluzio MC, Costa NM, Bressan J, Mattes R, Alfenas RC. Effect of a high-fat meal containing conventional or high-oleic peanuts on post-prandial lipopolysaccharide concentrations in overweight/obese men. J Hum Nutr Diet. 2014;29(1):95–104.
34. Nazemi S, Manaheji H, Noorbakhsh SM, Zaringhalam J, Sadeghi M, Mohammad-Zadeh M, Haghparast A. Inhibition of microglial activity alters spinal wide dynamic range neuron discharge and reduces microglial Toll-like receptor 4 expression in neuropathic rats. Clin Exp Pharmacol Physiol. 2015;42:772–779.
35. Nguyen MD, D'Aigle T, Gowing G, Julien JP, Rivest S. Exacerbation of motor neuron disease by chronic stimulation of innate immunity in a mouse model of amyotrophic lateral sclerosis. J Neurosci. 2004;24:1340–1349.
36. Paulino G, Darcel N, Tome D, Raybould H. Adaptation of lipid-induced satiation is not dependent on caloric density in rats. Physiol Behav. 2008;93:930–936.
37. Peters JH, Gallaher ZR, Ryu V, Czaja K. Withdrawal and restoration of central vagal afferents within the dorsal vagal complex following subdiaphragmatic vagotomy. J Comp Neurol. 2013;521:3584–3599.
38. Prentice AM, Jebb SA. Fast foods, energy density and obesity: a possible mechanistic link. Obes Rev. 2003;4:187–194.
39. Ridaura VK, Faith JJ, Rey FE, Cheng J, Duncan AE, Kau AL, Griffin NW, Lombard V, Henrissat B, Bain JR, Muehlbauer MJ, Ilkayeva O, Semenkovich CF, Funai K, Hayashi DK, Lyle BJ, Martini MC, Ursell LK, Clemente JC, Van TW, Walters WA, Knight R, Newgard CB, Heath AC, Gordon JI. Gut microbiota from twins discordant for obesity modulate metabolism in mice. Science. 2013;341:1241214.
40. Ritter RC. Gastrointestinal mechanisms of satiation for food. Physiol Behav. 2004;81:249–273.
41. Rogers RC, McCann MJ. Intramedullary connections of the gastric region in the solitary nucleus: a biocytin histochemical tracing study in the rat. J Auton Nerv Syst. 1993;42:119–130.
42. Rolls BJ. Carbohydrates, fats, and satiety. Am J Clin Nutr. 1995;61:960S–967S.
43. Rolls BJ. The relationship between dietary energy density and energy intake. Physiol Behav. 2009;97:609–615.
44. Shehab SA. Acute and chronic sectioning of fifth lumbar spinal nerve has equivalent effects on the primary afferents of sciatic nerve in rat spinal cord. J Comp Neurol. 2009;517:481–492.
45. Sun JS, Yang YJ, Zhang YZ, Huang W, Li ZS, Zhang Y. Minocycline attenuates pain by inhibiting spinal microglia activation in diabetic rats. Mol Med Rep. 2015;12:2677–2682.

46. Troy AE, Simmonds SS, Stocker SD, Browning KN. High fat diet attenuates glucose-dependent facilitation of 5-HT3-mediated responses in rat gastric vagal afferents. J Physiol. 2016;594:99–114.
47. Valdearcos M, Robblee MM, Benjamin DI, Nomura DK, Xu AW, Koliwad SK. Microglia dictate the impact of saturated fat consumption on hypothalamic inflammation and neuronal function. Cell Rep. 2014;9:2124–2138.
48. Vrieze A, Out C, Fuentes S, Jonker L, Reuling I, Kootte RS, van NE, Holleman F, Knaapen M, Romijn JA, Soeters MR, Blaak EE, Dallinga-Thie GM, Reijnders D, Ackermans MT, Serlie MJ, Knop FK, Holst JJ, van der Ley C, Kema IP, Zoetendal EG, de Vos WM, Hoekstra JB, Stroes ES, Groen AK, Nieuwdorp M. Impact of oral vancomycin on gut microbiota, bile acid metabolism, and insulin sensitivity. J Hepatol. 2014;60:824–831.
49. Waise TM, Toshinai K, Naznin F, NamKoong C, Md Moin AS, Sakoda H, Nakazato M. One-day high-fat diet induces inflammation in the nodose ganglion and hypothalamus of mice. Biochem Biophys Res Commun. 2015;464:1157–1162.
50. Wang X, Wang BR, Zhang XJ, Xu Z, Ding YQ, Ju G. Evidences for vagus nerve in maintenance of immune balance and transmission of immune information from gut to brain in STM-infected rats. World J Gastroenterol. 2002;8:540–545.
51. Yi CX, Tschop MH, Woods SC, Hofmann SM. High-fat-diet exposure induces IgG accumulation in hypothalamic microglia. Dis Model Mech. 2012;5:686–690.

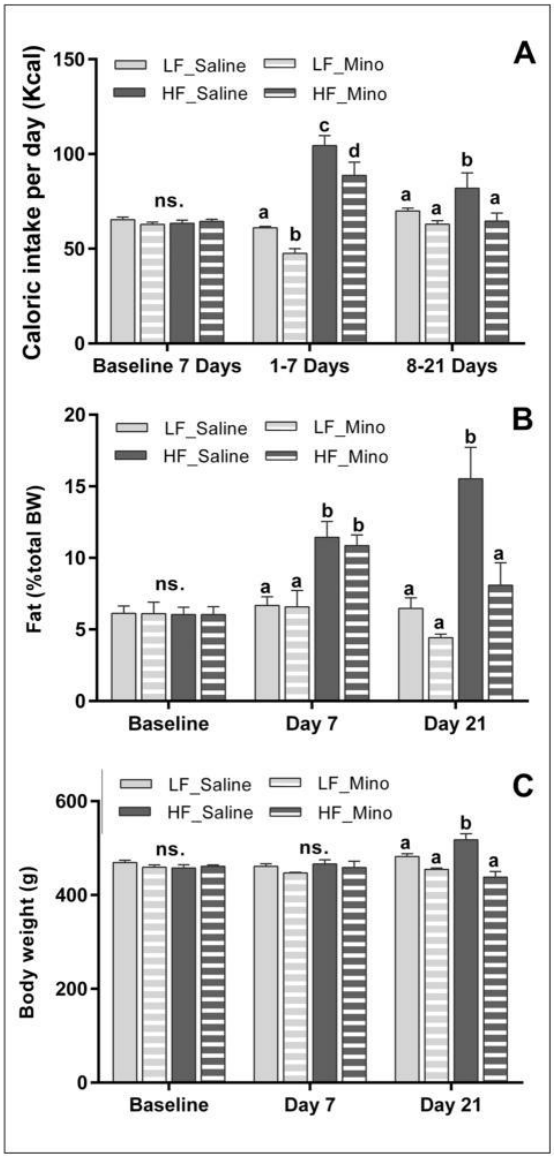

Fig. 1
Minocycline treatment decreased energy intake and body fat accumulation induced by HFD. Seven days exposure to HFD (High fat diet) significantly increased energy intake (A, $p<0.001$; n=8/group) and body fat accumulation (B, $p<0.05$; n=8/group). After 21 days, when compared to saline-treated animals, HFD minocycline-treated rats exhibited a significant decrease in energy intake (A, $p<0.05$; n=4/group), body fat accumulation (B, $p<0.05$; n=4/group) and in body weight (C, $p<0.05$; n=4/group). Minocycline reduced the caloric intake in LFD rats only in the first week (A). However, between the 8th and 21st day of the experiment, this effect was abolished (A). Minocycline did not affect body fat accumulation and body weight of LFD rats (B, C). Bars represent the average value ±SEM; a, b, c – different letters denote significant differences.

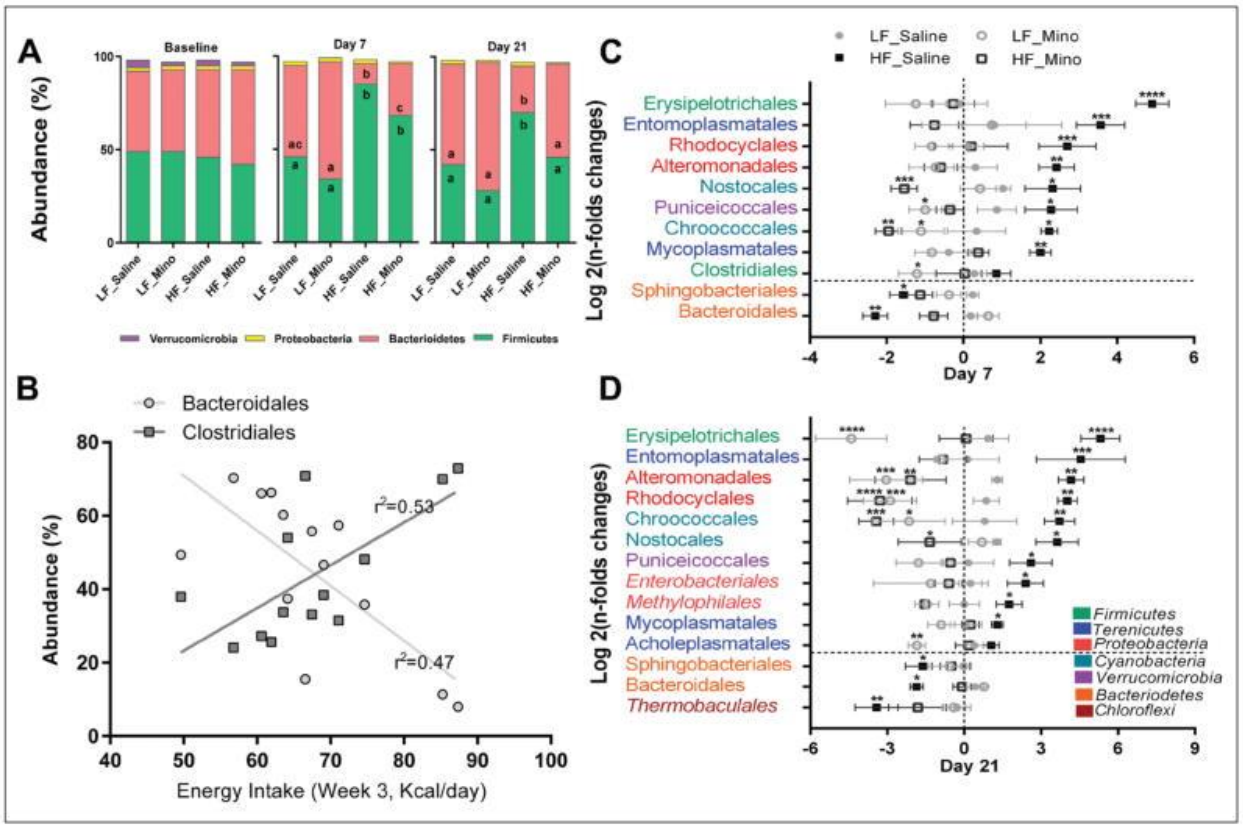

Fig. 2
Bacterial composition altered by HFD was improved by minocycline treatment. (A) Bacterial phyla abundance was quantified in fecal samples before dietary switch and treatment (baseline) and after 7 and 21 days on the different diets and treatments regimen. *Firmicutes* and *Bacteriodetes* were the most abundant bacterial phyla in all groups and at all time points. In all groups, there was a significant reduction in *Verrucomicrobia* abundance at day 7 ($P < 0.001$) and day 21 ($P < 0.0001$). Seven days of HFD were sufficient to induce a significant increase in *Firmicutes* ($P < 0.0001$) and decrease in *Bacteriodetes* abundances ($P < 0.0001$). Similar changes were observed after 21 days of HFD (A, *Firmicutes*, $p<0.01$; *Bacteriodetes*, $P < 0.001$). Minocycline treatment improved HFD rats' bacterial phyla profile with a significant increase in *Bacteriodetes* abundance after 7 days (A, $P < 0.05$) and a significant reduction in *Firmicutes* (A, $P < 0.01$) and increase in *Bacteriodetes* abundances (A, $P < 0.01$) after 21 days. After 21 days of treatment, there were no significant differences in bacterial phyla abundance between the HFD /minocycline-treated rats and the LFD animals. Minocycline treatment did not significantly affect bacterial phyla abundance in LFD rats (115). a, b, c – different letters denote significant differences. (B) Negative and positive correlations were observed between energy intake and bacterial orders depleted (*Bacteroidales*) and enriched (*Clostridiales*) by HF feeding. Energy intake during the third week of experiment (14 to 21days) was correlated with order abundance measured at day 21 across diets and treatments. *Bacteroidales* abundance was negatively correlated with energy intake ($r^2=0.53$, $P < 0.01$) while *Clostridiales* abundance was positively correlated with intake ($r^2=0.47$, $P < 0.01$). (C and D) Bacterial orders that were significantly enriched or depleted by HFD and/or minocycline treatment (Log 2 fold changes from baseline). * – denotes significant difference from the

LF_Saline group: * – $P < 0.05$, ** – $P < 0.01$, *** – $P < 0.001$, **** – $P < 0.0001$. (B) Consumption of HFD for 7 days led to significant increase in abundance of several bacterial orders belonging to the *Firmicutes*(*Erysipelotrichales*), *Terenicutes* (*Entomoplasmatales*, *Mycoplasmatales*), *Proteobacteria* (*Rhodocyclales*, *Altermondales*), *Cyanobacteria*(*Nostocales*, *Chroococcales*) and *Verrucomicrobia* (*Puniceicoccales*). HFD also led to significant depletion in *Bacteriodetes* orders *Bacteroidales* and *Sphingobacteriales*. C. Similar results were observed after 21 days with additional orders enriched (*Enterobacteriales* and *Methylophilales*, *Proteobacteria*) and depleted (*Thermobaculales, Chloroflexi*). (B) Minocycline treatment normalized HFD-induced dysbiosis. Seven days of minocycline exposure were sufficient to significantly reduce the HFD-induced proliferation of bacterial orders mentioned above, leading to other normalization of abundance or significant depletion (*Nostocales, Chroococcales*). Minocycline also prevented HFD-induced depletion in *Bacteroidales* and *Sphingobacteriales*. In LFD animals, minocycline alone significantly reduced the abundance of obesity associated *Puniceicoccales*, *Chroococcales* and *Clostridiales*. (C) Similar results were observed after 21 days of minocycline treatment; minocycline led to normalization or depletion of HFD-associated bacterial orders and restored HFD-depleted orders. Minocycline alone led to a significant reduction in *Erysipelotrichales* (*Firmicutes*) and several *Proteobacteria* and *Cyanobacteria* orders, such as *Rhodocyclales* and *Chroococcales*.

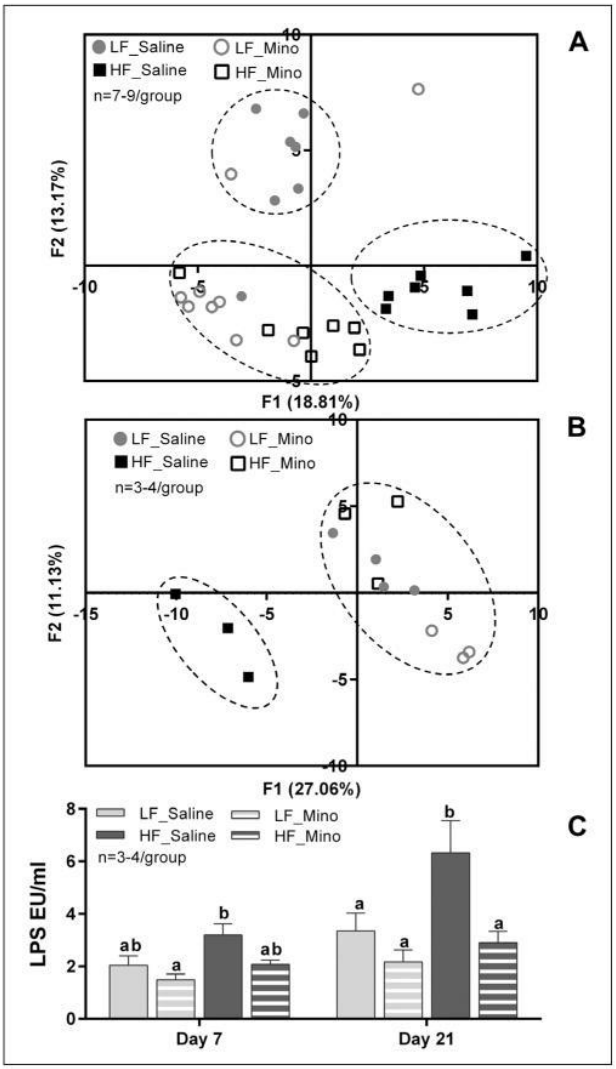

Fig. 3

Minocycline normalized HFD rats'microbiota profile and reduced HFD-induced metabolic endotoxemia. (A and B) PCA at the order level after 7 or 21 days of diet/minocycline treatments A. PCA showed that 7 days of HFD were sufficient to induce dysbiosis with a distinct microbiota profile while minocycline treatment led in a third profile with more variability, based on the diet consumed (LFD *vs.* HFD). B. HFD-induced dysbiosis was confirmed after 21 days of diet. Minocycline treatment led to normalization of the microbiota profile in HFD rats, with HF_Mino animals clustering with LF_Saline animals. There was no significant effect of minocycline treatment on the LF animals PCA scores. C. Circulating LPS levels in plasma after 7 or 21 days of diet/minocycline treatments. Seven days of HFD a non-significant increase in circulating LPS which became significant after 21 days of HFD ($P < 0.01$). Minocycline treatment reduced circulating LPS in HFD rats and this effect was significant after 21 days ($P < 0.001$). In LFD rats, minocycline treatment did not result in significant changes of LPS plasma levels at any time point. Bars represent the average value ± SEM; a, b – different letters denote significant differences.

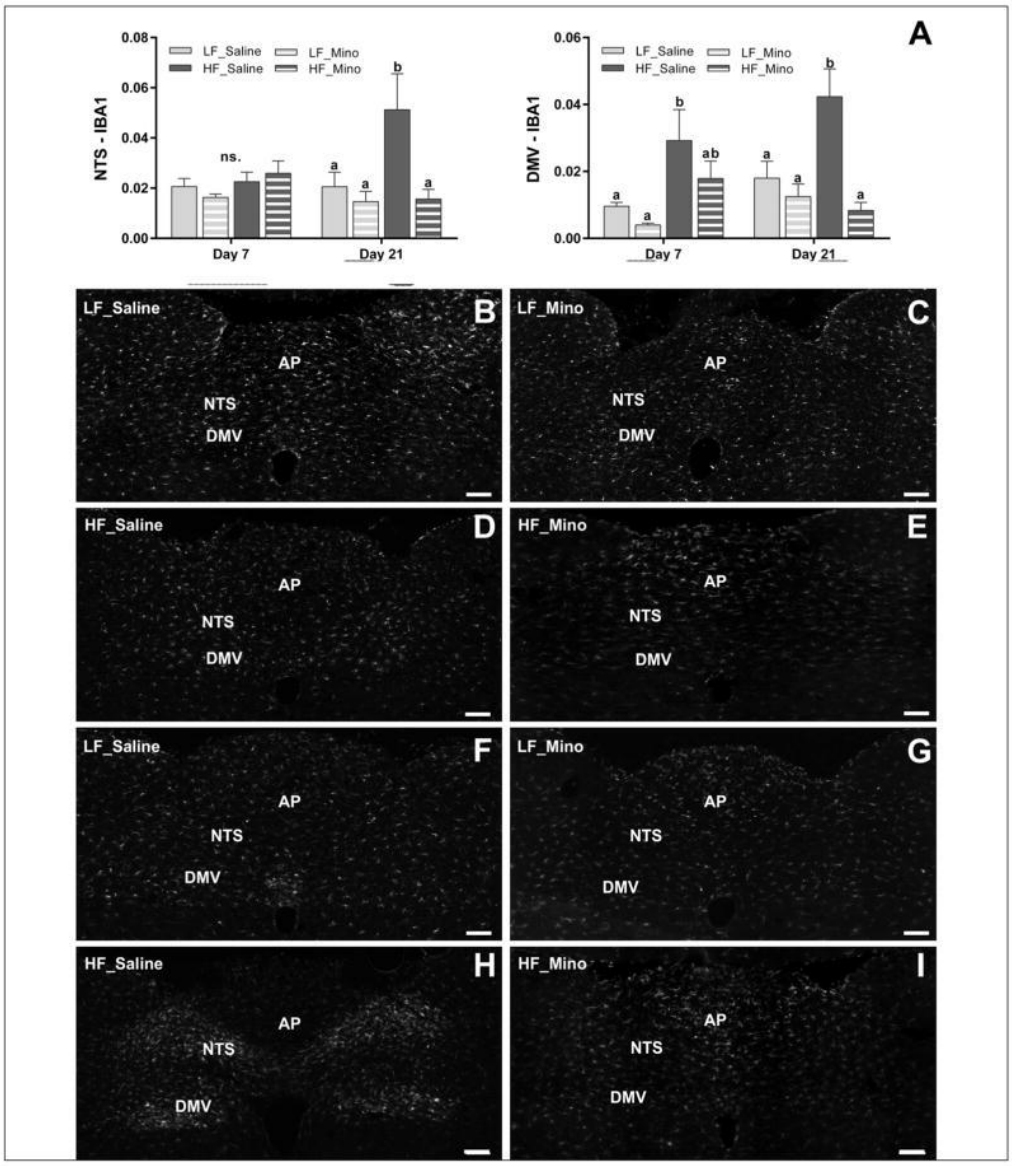

Fig. 4

High-fat diet-induced activation of microglia in the NTS and DMV was suppressed by minocycline treatment. (B–I) Representative images of Iba1 immunoreactivity in the hindbrain between bregma −13.10 and −14.10 mm. Immunostaining against Iba-1 revealed that HFD increased microglia activation in the NTS and the DMV. After 7 days on HFD microglia activation was observed only in the DMV (A, B–E) ($p<0.05$). After 21 days on HFD microglia activation was observed in both the DMV ($p<0.01$) and the NTS ($p<0.01$) (A, F–I). Minocycline treatment attenuated the HFD-induced increases in the microglia activation in both the DMV ($p<0.001$) and the NTS ($p<0.01$) at day 21 (A, F–I). Minocycline treatment did not significantly change the microglia activation in the DMV and the NTS in rats fed LFD at any studied time point. Bars represent the average value ±SEM; a, b – different letters denote significant differences. NTS: nucleus of the solitary tract; DMV: dorsal motor nucleus of the vagus; AP: area postrema; scale bar =200 μm.

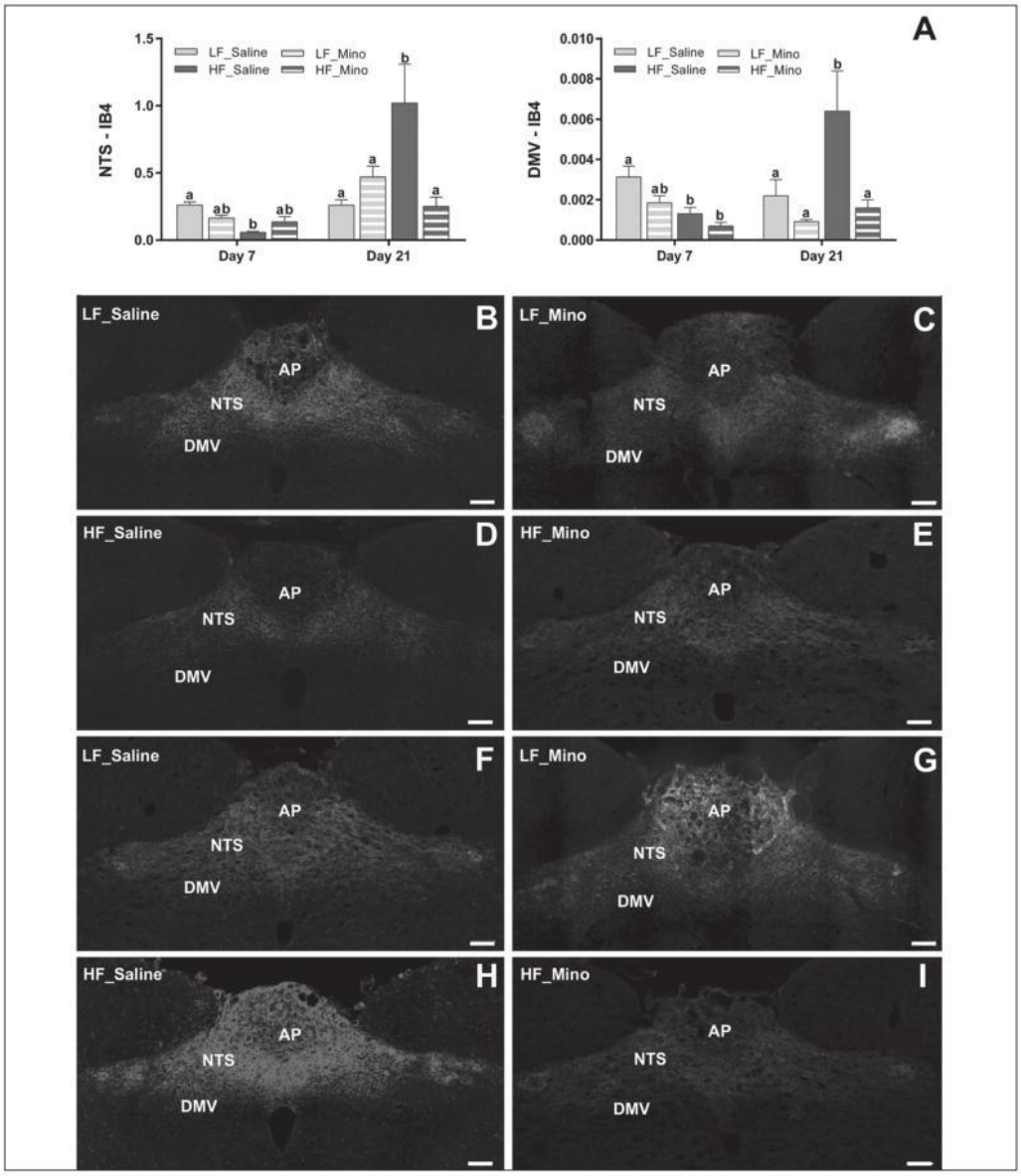

Fig. 5

HFD-induced vagal remodeling in the NTS and DMV was suppressed by minocycline. Binary analysis of the area fraction of IB4-labeled vagal afferents in the intermediate NTS and DMV revealed significant differences in the density of labeled afferent terminals between rats fed different diets. After 7 days HFD decreased the total IB4 labeling in the NTS ($p<0.001$) and the DMV ($p<0.05$) with compare to LFD (A, B–E). This effect was dampened by minocycline treatment only in the NTS but failed to reach significance. At day 21, we observed significant increase in density of IB4-labeled vagal afferents projecting to the NTS ($p<0.001$) and DMV ($p<0.01$) in HFD rats with compare to LFD rats (A, F–I). This effect was significantly decreased in both the NTS ($p<0.001$) and DMV ($p<0.01$) by minocycline treatment (A, F–I). There were no significant differences between LF_Saline and LF_Mino rats at any studied time point. Bars represent the average value ±SEM; a, b – different letters denote significant differences. NTS: nucleus of the solitary tract; DMV: dorsal motor nucleus of the vagus; AP: area postrema; scale bar =200 μm.

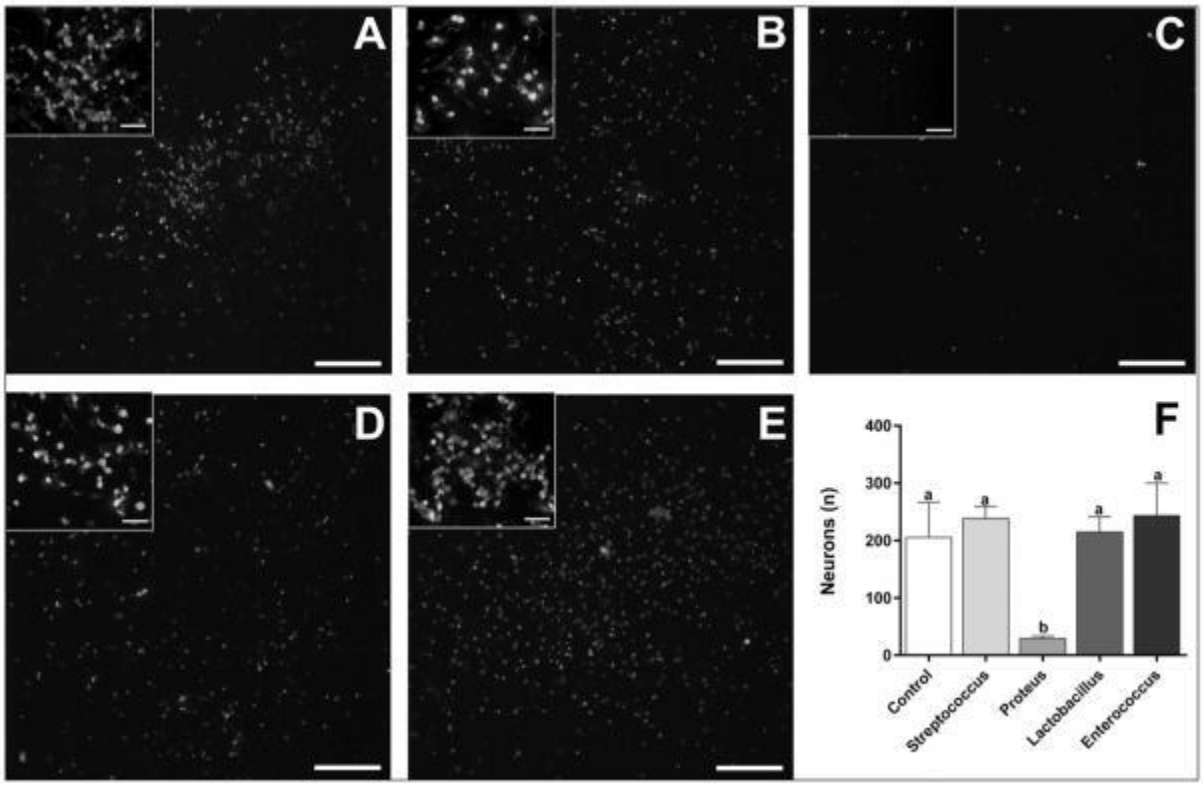

Fig. 6
Gram-negative bacteria isolated from HFD rats significantly reduced the number of NG neurons in culture. Co-culture of NG primary sensory neurons isolated from LFD rats with *Streptococcus* mitis, *Lactobacillus animalis* or *Enterococcus faecalis* (B, D and E respectively; *Firmicutes, Bacilli, Lactobacillales*) isolated from HFD rats did not change the number of surviving neurons with compare to NG cultures without the bacteria (A, F). Adding *Proteus mirablis* (C; *Proteobacteria, Gammaproteobacteria, Enterobacteriales*), isolated from HFD rats, to NG cultures from LFD rats induced a dramatic loss of primary sensory neurons with compare to NG cultures without the bacteria (A, F, $p<0.05$). Inserts show high magnification images from the same section demonstrating neuronal morphology. Scale bar =200 μm or 20 μm in inserts. Bars represent the average value ±SEM; a, b – different letters denote significant differences.