IMPACT OF SASKATOON BERRY POWDER ON METABOLISM AND GUT MICROBIOTA IN DIET-INDUCED INSULIN RESISTANT MICE

by

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Abstract

Type 2 diabetes is one of the most common and prevalent metabolic disorders with an ever-increasing rate globally. Metabolic syndromes are characterized by low-grade inflammation and insulin resistance, and are usually accompanied with alteration in the gut microbiota profile. Evidence in recent decades shows that the gut microbiota plays a significant role in the development of metabolic syndromes, and obese individuals have abnormal gut microbiota in composition, abundance and metabolic functions compared to lean individuals. Saskatoon berry is a type of fruit-bearing shrub native in North America. Previous studies found that Saskatoon berry powder (SBp) is capable of reducing inflammation in leptin receptor-knockout (*db/db*) diabetic mice. Among the anthocyanins in SBp, cyanidin-3-glucoside (C3G) has 3 times higher anti-inflammatory capacity compared to cyanidin-3-galactoside (C3Ga) according to endothelial cell studies. However, research on the effects of Saskatoon berries or contained active components on diabetes and gut microbiota still remains limited. In the present study, the effects of SBp and C3G on diabetes-related physiological indicators and gut microbiota were explored. The result shows that both SBp and C3G significantly decreased elevated levels of glucose, cholesterol, triglyceride, insulin, insulin resistance and inflammatory markers caused by high fat-high sucrose (HFHS) diet. Certain bacteria in the mouse gut, such as *Muribaculaceae*, was significantly increased by 5% SBp (w/w) or equivalent amount of C3G in 5% SBp. Correlation results revealed that some bacterial families, such as *Muribaculaceae* and *Akkermansiaceae*, are negatively associated with hyperglycemia, hypercholesteremia, hyperlipidemia, insulin resistance and low-grade inflammation. Dose-response study found that addition of 1-2.5% (w/w) SBp into HFHS diet already showed an anti-diabetic and anti-inflammatory effects. These findings suggest that Saskatoon berries or related products may have the potential to be applied as a functional food for the prevention or treatment of type 2 diabetes.

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Table of Contents

List of tables ... vii

List of figures ... viii

List of abbreviations ... xi

CHAPTER 1: INTRODUCTION ... 1

Saskatoon berries and distribution ... 1

Growth pattern and characteristics ... 1

Commercial applications ... 2

Phytochemicals of Saskatoon berries ... 3

Anthocyanins of Saskatoon berries ... 3

Health benefits of Saskatoon berries and anthocyanins ... 4

Diabetes ... 5

Pathogenesis of diabetes ... 6

Insulin and insulin resistance ... 6

Inflammation ... 7

Microbial community and gut microbiota ... 8

Gut microbiota and diseases ... 9

Gut microbiota mechanism for diseases ... 9

Gut microbiota and diets ... 9

CHAPTER 2: HYPOTHESIS AND OBJECTIVES ... 11

Study Hypothesis ... 11

Specific Objectives ... 11

CHAPTER 3: METHODS AND RESULTS ... 12

Part 1: Effects of Saskatoon berry powder on high fat-high sucrose diet-induced insulin resistance and gut dysbiosis in mice ... 12

Methods and Materials ... 12

Preparation of Saskatoon berry powder ... 12

Diet preparation ... 12

Animal preparation ... 12

Dietary experimental design 13
Measurement of biochemical indicators 13
Measurements of inflammatory markers 13
Fecal sample collection and DNA extraction...14
16S rRNA sequencing 14
Bioinformatics analysis and statistics...14
Results 15
Effects of SBp on body weights, food intake, plasma glucose, cholesterol and triglycerides...15
Effects of SBp on insulin and HOMA-IR 16
Effects of SBp on inflammatory markers...16
Effects of SBp on gut microbiota 16
Correlations of gut bacteria with physiochemical results 17
Part 2: Effects of cyanidin-3-glucoside on high-fat high-sucrose diet-induced insulin resistance and gut dysbiosis in mice 28
Materials and methods 28
Dietary Intervention...28
Food intake and body weight...28
Blood collection..28
Diabetes-related biochemical parameters 29
Measurement of Inflammatory markers..29
Fecal sample collection 29
DNA extraction and sequencing 29
Bioinformatic analysis and statistics 30
Results 31
Body weight and food intake 31
Effects of C3G and SBp on glucose, cholesterol and triglycerides 31
Effects of C3G and SBp on insulin and HOMA-IR 31
Effects of C3G and SBp on gut microbiota 32

Part 3: Dose-response of Saskatoon berry powder on high-fat high-sucrose diet-induced insulin resistance and gut dysbiosis in mice 47
Material and method 47
Dietary Intervention 47
Food intake and body weight 47
Blood collection 47
Diabetes-related biochemical parameters 48
Measurement of Inflammatory markers 48
Fecal sample collection 48
DNA extraction and sequencing 48
Bioinformatic analysis and statistics 48
Results 48
Effects of 1% and 2.5% SBp on plasma levels of glucose, cholesterol and triglycerides 48
Effects of 1% SBp and 2.5% SBp on insulin and HOMA-IR 49
Effects of 1% and 2.5% SBp on levels of inflammatory markers 49
Effects of 1% and 2.5% SBp on gut microbiota 49
Comparison of effects of 1%, 2.5% and 5% SBp supplemented into HFHS diets 50
CHAPTER 4: DISCUSSION 61
Major novel findings of the studies 61
Hypoglycemic effects of berries and possible mechanism 63
Hypolipidemic effects of berries and possible mechanism 65
Inhibition of inflammation and possible mechanisms 67
Modulation of gut microbiota and implications 69
Conclusion 73
Limitations of the researches in the present study 73
Future directions and knowledge translation 74
References 75

List of tables

Table 3-1-1 Impact of HFHS and HFHS+SBp diets on the abundances of bacteria on phylum level in the mouse gut.. 18
Table 3-1-2 Impact of HFHS and HFHS+SBp diets on the abundances of bacteria on family level in the mouse gut.. 19

List of figures

Figure 3-1-1 Fasting plasma glucose, body weight and food intake in mice fed with HFHS diet or HFHS+SBp diet.. 20
Figure 3-1-2 Effects of HFHS and HFHS+SBp on cholesterol and triglycerides in mice 21
Figure 3-1-3 Effects of HFHS and HFHS+SBp diets on insulin and insulin resistance in mice....22
Figure 3-1-4 Effects of HFHS and HFHS+SBp diets on inflammatory markers in mouse plasma. 23
Figure 3-1-5 Impact of HFHS and HFHS+SBp diets on the diversity of intestinal microbiota in mice....24
Figure 3-1-6 The effects of HFHS and HFHS+SBp diets on the abundance of gut bacteria on phylum level....25
Figure 3-1-7 The effects of HFHS and HFHS+SBp diets on the abundance percentage of gut bacteria on family level....26
Figure 3-1-8 Correlations between the abundances of family-level gut bacteria and body weight, metabolic variables or inflammatory markers. 27
Figure 3-2-1 Effects of HFHS diet supplemented with or without SBp or C3G on the body weight and food intake of mice....36
Figure 3-2-2 Levels of glucose, cholesterol and triglycerides in plasma of mice fed with HFHS diets supplemented with or without SBp or C3G. 37
Figure 3-2-3 Effects of HFHS diet supplemented with or without SBp or C3G on insulin and insulin resistance in mice. 38
Figure 3-2-4 Effects of HFHS diet supplemented with or without SBp or C3G on inflammatory markers....39
Figure 3-2-5 Effect of HFHS diet supplemented with or without SBp or C3G on β-diversity of gut microbiota in mice. PCA was based on Bray–Curtis dissimilarities between all sample sets....40

Figure 3-2-6 Effects of HFHS diet supplemented with or without SBp or C3G on the relative abundance of *Bacteroidetes* and *Firmicutes* 41
Figure 3-2-7 Effect of HFHS diet supplemented with or without SBp or C3G on the relative abundance of gut microbiota on family level 42
Figure 3-2-8 Correlations between the abundances of gut bacteria and body weight, metabolic variables or inflammatory markers 43
Figure 3-2-9 Linear regression analysis of correlation between the relative abundance of three families and three key diabetes-related indicators 44
Figure 3-2-10 Comparison of the effects of SBp and C3G on HFHS-induced gut microbiota profiles 45
Figure 3-2-11 Function prediction of HFHS diet supplemented with or without SBp or C3G through PICRUSt 46
Figure 3-3-1 Levels of glucose, cholesterol and triglycerides in plasma of mice fed with HFHS diets supplemented 1% or 2.5% SBp..52
Figure 3-3-2 Effects of HFHS diets supplemented with 1% or 2.5% SBp on insulin and insulin resistance in mice 53
Figure 3-3-3 Levels of inflammatory markers in the plasma of mice receiving the HFHS diet supplemented with 1% or 2.5% SBp 54
Figure 3-3-4 Effect of the HFHS diet supplemented with 1% or 2.5% SBp on β-diversity in mice. 55
Figure 3-3-5 Effects of HFHS diet supplemented with 1% or 2.5% SBp on the relative abundance of *Bacteroidetes* and *Firmicutes*. 56
Figure 3-3-6 Effect of HFHS diet supplemented with 1% or 2.5% SBp on the relative abundance of gut microbiota on family level. 57
Figure 3-3-7 Linear regression analysis of correlations between the relative abundance of *Muribaculaceae* and metabolic variables or inflammatory markers in plasma of the studied mice 58
Figure 3-3-8 Comparison of relative changes in glucose, insulin and HOMA-IR of HFHS group, HFHS+1% SBp group, HFHS+2.5% SBp group and HFHS+5% SBp

group. 59
Figure 3-3-9 Linear regression analysis of correlation between the relative abundance of *Muribaculaceae* and relative changes of glucose, insulin and HOMA-IR, respectively 60

List of abbreviations

ASV – Amplicon Sequence Variants

C3G – Cyanidin-3-Glucoside

C3Ga – Cyanidin-3-Galactoside

CoA – Coenzyme A

CTL – Control

FMT – Fecal Microbiota Transplantation

HDL– High-Density Lipoproteins

HFHS – High Fat-High Sucrose

HLA – Human Leukocyte Antigen

HOMA-IR – Homeostatic Model Assessment of Insulin Resistance

IL-1β – Interleukin-1β

IL-6 – Interleukin-6

LDL – Low-Density Lipoproteins

LDL-C – Low-Density Lipoproteins Cholesterol

HDL-C – High-Density Lipoproteins Cholesterol

LPS – Lipopolysaccharide

MCP-1 – Monocyte Chemotactic Protein 1

ORAC – Oxygen Radical Absorbance Capacity

PAI-1 – Plasminogen Activator Inhibitor-1

PCR – Polymerase Chain Reaction

PCA – Principal Component Analysis

PICRUSt – Phylogenetic Investigation of Communities by Reconstruction of Unobserved States

QIIME – Quantitative Insights Into Microbial Ecology

SBp – Saskatoon Berry powder

SCFAs – Short Chain Fatty Acids

SOD – Superoxide Dismutase

T1D – Type 1 Diabetes

T2D – Type 2 Diabetes

TNF-α – Tumor Necrosis Factor-α

CHAPTER 1: INTRODUCTION

Saskatoon berries and distribution

Saskatoon berry, also known as Saskatoon, juneberry or serviceberry, is a fruit-bearing shrub native to North American continent, and naturally grow in southern Yukon and Northwest Territories in Canada as well as northwestern states in the United States (Mazza & Davidson, 1993). Indigenous people used to use it to make pies, juice and pemmican (Leighton, 1997). This berry fruit was also adopted by early landed European settlers as an effective remedy for ascorbic acid insufficiency. In spite of a long history of consuming Saskatoon berry, agricultural cultivation did not start until the 1900s when pioneers transplanted it from the wild (Turner & Aderkas, 2012).

The fruit of the Saskatoon berry shrub has similar appearance as that of the blueberry shrub. However, in fact, unlike blueberry and cranberry which belong to *Vacciniaceae*, Saskatoon berry is a pome fruit of *Rosaceae*, showing its closer affinity to apple and pear than traditional 'berries' (Mazza, 2005).

Growth pattern and characteristics

The Saskatoon berry shrub is self-fertile and thus does not need cross-pollination (Olson, 1984). It usually takes up to 8-12 years to grow into a mature plant with a height of 3-4 meters (Laughlin et al., 1996) (Figure 1-1-1). In spite of being able to grow in a wide range of conditions, it prefers lower temperature and alkaline environment rather than warm and acid places (St-Pierre et al., 2005), because it can sustain more drought and require much less irrigation compared to most other berries (Laughlin et al., 1996).

Figure 1-1-1 Photos of Saskatoon berry shrubs and fruit clusters. Pictures were taken in Saskatoon berry orchard in Portage la Prairie, Manitoba

Plants begin to bear fruit at three to five years of age, coming into full production by seven to eight years (Loeppky, 1984). Berries often ripen in late July, 6-8 weeks after flowering. Depending on cultivars, yields and fruit sizes may differ. And the harvest of fruits can range from 0.72 to 4.16 kg per shrub (Bieniek et al., 2019). The largest size of berry fruits can reach up to 13.9 mm in diameter for 'Martin' cultivar, while other cultivars such as 'Success' and 'Bluff' have relatively small sizes (St-Pierre et al., 2005).

Commercial applications

Saskatoon berries have been applied into the food industry in diverse forms. Fruits with

lower quality are processed in jams and purees where intact appearance is not a selling point. Pigment from dark color skin is made into food colorant for edible additives (Reddy, 2006). Due to the easy rottenness, present markets have only approximately 10-12% of Saskatoon berries sold fresh with the majority of them sold frozen or canned (Reddy, 2006). Since fruits often have a longer storage period after dehydration, industry has resorted to different dehydrating techniques, such as freeze drying, vacuum drying and microwave drying, to process Saskatoon berries (Lachowicz, Michalska, et al., 2019). However, loss of bioactive compounds inevitably happens in the process of dehydration. Compared to vacuum dried and microwave dried ones, freeze-dried berries have higher contents of total phenolics, anthocyanins and polyphenols as well as antioxidant activities (Alfaro et al., 2014).

Phytochemicals of Saskatoon berries

Saskatoon berries reportedly contain at least 48 phytochemicals, such as polyphenols, triterpenoids, carotenoids, chlorophylls and tocopherols (Lachowicz et al., 2017). Phytochemical components in leaves include quercetin, epicatechin and chlorogenic acid, while stems contain flavanone, catechins, and hydroxybenzoic acids (Bakowska-Barczak & Kolodziejczyk, 2008). Seeds were found to have polymeric procyanidins, triglycerides (containing mainly linoleic acid and oleic acid), tocopherol and sterol (Bakowska-Barczak et al., 2009). In terms of fruits, soluble solids and flavonols are the predominant components in the flesh, while peels have the greatest concentrations of phytochemicals which include anthocyanins, polymerized compounds, triterpenoids and hydroxycinnamic acid (Lachowicz et al., 2020).

Anthocyanins of Saskatoon berries

Among all the phenolic compounds in Saskatoon berries, cyanidin-based anthocyanins predominate and account for 63% of the total phenols. The composition of anthocyanins can vary from 258.7 to 517.9 mg/100 g fresh weight according to different cultivars (Lavola et al., 2012). Types of anthocyanins identified in Saskatoon berries consist of

cyanidin-3-galactoside (C3Ga), cyanidin-3-glucoside (C3G), cyanidin-3-arabinoside, cyanidin-3-xyloside, cyanidin-3,5-diglucoside. C3Ga and C3G are the two leading anthocyanins in Saskatoon berries (Hu et al., 2005; Meczarska et al., 2017). In terms of the 'Smoky' cultivar, only four cyanidin-based anthocyanins were detected: C3Ga (3708.2 mg/kg dry weight basis), C3G (903.4 mg/kg dry weight basis), cyanidin-3-arabinoside (345.0 mg/kg dry weight basis) and cyanidin-3-xyloside (314.5 mg/kg dry weight basis) (Hosseinian & Beta, 2007; Zhao, Le, et al., 2014).

Within the two most abundant anthocyanins in SBp, cyanidin-3-glucoside (C3G) was found to have 3 times higher anti-inflammatory capacity compared to cyanidin-3-galactoside (C3Ga) in a study using cultured endothelial cells (Zhao et al., 2015). The function variation may lie in the difference of chemical structures that the hydroxyl group at the C4 position of the ring attached to cyanidin is equatorially oriented for glucoside but axial for galactoside (Ahmadi et al., 2017).

Concentrations of anthocyanins are closely related to fruit ripeness which also affects total phenolics and antioxidant capacity (Green & Mazza, 1986). Oxygen radical absorbance capacity (ORAC) assay indicated that berry fruit-derived anthocyanins significantly decreased intracellular peroxyl radical-induced oxidation in proportion to ripeness and storage period (Elisia et al., 2007; Kalt et al., 2003).

Health benefits of Saskatoon berries and anthocyanins

Saskatoon berries showed strong antioxidant capacity in previous research which reported that Saskatoon berry syrup significantly increased the antioxidant capacity of rooibos tea (Grant et al., 2017; Hu et al., 2005); however, the contributive components remained unidentified. A study, with a focus on structure, pointed out that components in glycoside form such as anthocyanins are more likely to reduce oxidative stress (Hu et al., 2005). This hypothesis was further supported by a human study using anthocyanin-containing fruit juice. In this study, consuming 750 mL of anthocyanin-

rich fruit juice for 55 days not only increased superoxide dismutase (SOD) activity but also reduced total DNA breaks, with other beneficial results such as a decrease in the low-density lipoprotein (LDL) (Bakuradze et al., 2019).

In addition to inhibiting oxidation, both Saskatoon berries and anthocyanins were found to suppress the growth of certain microorganisms. The activity of several microbial enzymes in *Enterococcus hirae*, such as α-amylase and α-glucosidase, can be inhibited by Saskatoon berries (Lachowicz, Wiśniewski, et al., 2019). And supplementation of anthocyanin-containing mixed berry extracts (blueberry, elderberry and wild bilberry) was found to inhibit the growth of *Helicobacter pylori* (Bagchi, 2004).

Other health-contributing effects found only in anthocyanins imply similar functions expected in Saskatoon berries. Research showed that intake of anthocyanins was reported to improve vision and alleviate metabolic syndrome-related symptoms (Turrini et al., 2017). Specifically, clinical study on visual function showed that consuming black currant anthocyanin at a daily dose of 50 mg improved dark adaption of the eyes (Nakaishi et al., 2000). And adding 11 g freeze-dried wild blueberry powder into the diet for over 28 days increased vascular dilation and decreased ambulatory systolic blood pressure (Rodriguez-Mateos et al., 2019).

Diabetes

Diabetes mellitus is a group of metabolic diseases mainly characterized by hyperglycemia due to insufficient production of insulin or insulin resistance by the body (Mellitus, 2005). Multimorbidity, defined as multiple health-affecting diseases cooccurring with diabetes in the same individual, may appear in the later stage if blood glucose is poorly-controlled. Diabetic complications may include heart disease, kidney failure and blindness, which can lead to a deteriorating life quality and a increasing mortality (Gijsen et al., 2001). The prevalence of diabetes has become a global public health burden with a growing number of people diagnosed with either diabetes or

prediabetes. Recent research using logistic regression model predicted that the world will have 693 million diabetic patients by 2045 with half of them undiagnosed (Cho et al., 2018).

Pathogenesis of diabetes

Based on pathogenesis, diabetes can be categorized into two types: type 1 diabetes and type 2 diabetes. These two types are disproportionally distributed, since type 2 diabetes accounts for 90% of global diabetes cases (Zheng et al., 2018). Type 1 diabetes is caused by impairment of insulin-producing pancreatic islet β cells due to attack from autoimmune antibodies. Although type 1 diabetes possibly occurs later in life, it more often starts from childhood or young adults with predisposing factors including genetics, infections and medical conditions. For example, individuals with variations of human leukocyte antigen (HLA) region on chromosome 6 have increased risk of autoimmunity, and mutations that cause changes in the insulin structure could lead to decreased affinity between receptors and insulin (Krischer et al., 2017). On the other hand, a different pathogenesis is attributed to the onset of type 2. Insulin resistance turns out to be the most symbolic feature in patients with type 2 diabetes which is characterized by reduced glucose uptake and utilization, regardless of how much insulin pancreatic islet β cells secret (Reaven, 2005; Reusch, 2002). The major risk factors for type 2 diabetes are unhealthy dietary habits and sedentary life styles (Astrup, 2001; Narayan et al., 2002; Sami et al., 2017). A large volume of experimental and clinical studies demonstrated that high fat and high sugar diets are closely associated with symptoms of type 2 diabetes: hyperglycemia, hyperlipidemia, insulin resistance and chronic inflammation (K. A. Kim et al., 2012; Panchal et al., 2011; Shoelson et al., 2006). However, diets with low fat and less carbohydrate reportedly reversed high fat and/or high sugar diets induced metabolic disorders (Arora & McFarlane, 2005; Samaha et al., 2003).

Insulin and insulin resistance

Insulin is a peptide hormone secreted by the pancreas when the islet β cells are

stimulated by endogenous or exogenous substances (DeFronzo, 1982). As the only hormone in the body that lowers plasma glucose level and promotes glucose transformation into advanced carbohydrate structure or other biological macromolecules, insulin evidently plays an important part in glucose metabolism (Mayer et al., 2007).

Skeletal muscles directly utilize glucose for energy expenditure and thus are sensitively affected by insulin concentration (Groop et al., 1989). In hepatocytes, gluconeogenesis was found to be inhibited by insulin (Dentin et al., 2007). And in adipocytes, insulin stimulates free fatty acid uptake, inhibits lipolysis and contributes to the de novo fatty acid synthesis (Morigny et al., 2016).

Once insulin resistance occurs, the uptake of plasma glucose starts to decrease in the skeletal muscle (Petersen & Shulman, 2002). Gluconeogenesis in the liver is enhanced with insulin resistance, leading to an elevated glucose level in the plasma (Bock et al., 2007). Insulin resistance in adipose tissue causes increased free fatty acids in the plasma, which in turn exacerbates insulin resistance due to fatty acids occupying insulin receptors on the cell membrane (Petersen & Shulman, 2002).

Inflammation

Inflammation in general is the response of the body tissue to external stimuli through immune system and often found enhanced in type 2 diabetes (Calle & Fernandez, 2012). Proinflammatory mediators induce chronic inflammation in which proinflammatory cytokines cause a series of intracellular signal transductions and cellular responses (Dinarello, 2000). For example, monocyte chemotactic protein-1 (MCP-1) which attract monocytes to migrate and adhere to the inflammatory spots are increased in the activated monocytes and endothelium (Hartung et al., 2007). Those activated monocytes also generate superoxide radicals, causing the transformation of LDL into oxidized or glycated LDL. Furthermore, the oxidized or glycated LDL increase the

production of inflammatory mediators such as plasminogen activator inhibitor-1 (PAI-1) in vascular endothelial cells, which is considered as a factor that leads to the development of thrombosis (Zhao, Ren, et al., 2014).

Microbial community and gut microbiota

Microbial community is a collective of microorganisms that live closely together in the same environment (Fuhrman, 2009). Compared to individually isolated colonies, microbial community is a more universal and common form for microorganisms to perform functions in the environment (Kaeberlein et al., 2002; Singh, 2010). For example, marine microbial communities maintain the marine carbon, nitrogen and sulfur cycles (Fuhrman, 2009); soil microbial communities decompose organic matter and help form mycorrhiza (Marschner et al., 2003). Commonly studied microbial communities are from gut, mouth, skin, soil, active sludge and fermented food (Gibbons & Rinker, 2015; Kent & Triplett, 2002; Reid et al., 2011; Xia et al., 2018). Structural balance for a microbial community is possible to be maintained when conditions such as nutrients, oxygen contents, temperature and humidity remain unchanged (Shade et al., 2012).

The microbial community living in the intestine is gut microbiota. The human gut microbiota is called human 'second genome' due to 3.3 million genes (150 times more than human genes) found in the genomes of gut microbes (Grice & Segre, 2012; Zhu et al., 2010). Research revealed the metabolism-regulating roles of gut microbiota through a series of findings, pointing out that many chronic metabolic diseases, such as obesity and diabetes, are associated with altered gut microbiota (Fan & Pedersen, 2020). The composition of gut microbiota is not only changeable when exposed to a new environment but also has a resilient ability that allows it to recover (Shade et al., 2012).

Gut microbiota and diseases

Bacteroidetes (B), *Firmicutes* (F) and *Actinobacteria* are three dominant phyla that account for almost 80% of total gut microbiota in adults (Mariat et al., 2009). Difference in their proportions was often found between healthy individuals and those with metabolic syndromes, characterized by a lower B/F ratio in diabetic patients compared to lean individuals (Larsen et al., 2010). However, changes in gut microbiota were considered as a concomitant result rather than a contributive factor for a period of time until new research techniques, such as fecal microbiota transplantation (FMT), were applied to prove the role of gut microbiota (Alang & Kelly, 2015; Bakken et al., 2011; Smits et al., 2013). For example, research indicated that transplantation of gut bacteria from obese mice into lean germ-free mice could cause the occurrence of obesity in germ-free mice (Alang & Kelly, 2015).

Gut microbiota mechanism for diseases

Lipopolysaccharide-induced inflammation is a widely accepted mechanism for the roles of gut microbiota in the development of metabolic syndromes (Manco et al., 2010; Sun et al., 2018). Lipopolysaccharide (LPS) is a structural component of the Gram-negative bacterial cell wall which impairs intestinal permeability and causes LPS to transfer across intestinal barrier into blood circulation (Guerville & Boudry, 2016). Research has found that high levels of plasma LPS were associated with endotoxemia-induced inflammation in individuals with obesity and type 2 diabetes (Cani et al., 2008).

Gut microbiota and diets

Components of diets and medicines alter the gut microbial profile by promoting or inhibiting the growth and metabolism of gut bacteria (Eid et al., 2017; Jia et al., 2008). Ingredients that were unable to be digested by the human body are possible to be utilized by gut bacteria (Chassard et al., 2012). Many berry-derived compounds that change gut microbiota profile may potentially attenuate the symptoms of metabolic syndromes for the host. For example, berberine, a unique compound in barberries with

a low absorbing rate in the human body, was found to improve diabetic symptoms as well as increase *Lachnospiraceae* and *Erysipelotrichidae* (Zhang et al., 2012).

The beneficial functions of Saskatoon berries and relevant mechanisms have not been fully explored. Previous research found that Saskatoon berries alleviated inflammation levels in *db/db* mice but did not decrease fasting plasma glucose in the same study (Zhao, Le, et al., 2014). Given that *db/db* genetic diabetic mice are caused by the deficiency of leptin receptor rather than high fat-high sugar diets for the majority of type 2 diabetes patients, the present study used a diet-induced mouse model. Specifically, high fat-high sucrose (HFHS) diet-induced obese and insulin resistant mice were used in this study to explore the effects of Saskatoon berries on both diabetes-related metabolic features and gut microbiota.

CHAPTER 2: HYPOTHESIS AND OBJECTIVES

Study Hypothesis

1. Saskatoon berries have the ability to lower high fat-high sucrose (HFHS) diet-induced hyperglycemia, hyperlipidemia, insulin resistance and inflammation in mice.
2. Cyanidin-3-glucoside (C3G), an anthocyanin in Saskatoon berries, is one of the key components that contribute to the anti-diabetic and anti-inflammatory effects of Saskatoon berries.
3. Those effects of Saskatoon berries and C3G are associated with the changes in the profile of gut microbiota.

Specific Objectives

1. To explore the effects of Saskatoon berry powder (SBp) on glucose, lipids, insulin, insulin resistance, inflammatory markers and gut microbiota in HFHS diet-induced mice.
2. To identify the effective components in SBp by exploring the above effects of C3G and comparing those with SBp.
3. To explore the dose response of SBp on the above effects in the same animal model.

CHAPTER 3: METHODS AND RESULTS

Part 1: Effects of Saskatoon berry powder on high fat-high sucrose diet-induced insulin resistance and gut dysbiosis in mice

This is the first documented study on the effect of Saskatoon berries on glucose metabolism and gut microbiota in diet-induced obese and insulin resistant animals. The manuscript has been published on 'The Journal of Nutritional Biochemistry' in 2019 (https://doi.org/10.1016/j.jnutbio.2019.03.023). I was involved in data analysis of gut microbiota of this study and listed as the 4th author.

Methods and Materials

Preparation of Saskatoon berry powder

Saskatoon berries ('Smoky' cultivar) were obtained from Prairie Lane Saskatoon (Portage la Prairie, MB). Fresh berries were cleaned and dehydrated using a freeze-dryer, and then milled into powder which was subsequently stored at -20°C.

Diet preparation

Control low-fat diet (D12450K) was purchased from Research Diets (New Brunswick, NJ) containing 4.3% of fat, 19.2% of protein and 67.3% of carbohydrate without sucrose. HFHS (D12492) diet was also purchased from Research Diets (New Brunswick, NJ) containing 35% of fat, 26% of protein and 26% of carbohydrate containing 9% sucrose (w/w). SBp diet was made by adding 5% SBp (w/w) into HFHS diet, mixing and shaping into pellets with diameters of 2-3 cm.

Animal preparation

Twenty-four male C57 BL/6J mice at age of 6 weeks were purchased from Jackson Laboratory (Bar Harbor, ME). Once arrived, they were housed in an air-conditioned room with regular day/night light cycle for stabilization for one week with regular

mouse chow and tap water.

Dietary experimental design

The mice were randomized into 3 groups. Each group had 8 mice with 4 mice in each cage. Control (CTL) group received D12450K diet. HFHS group received D12492 diet, and HFHS+SBp group receiving D12492 diet plus supplemented 5% SBp (w/w). All groups of mice received assigned diets and tap water ad libitum for 15 weeks. Food intake (per cage) and body weight (per mouse) were measured once a week. Overnight fasting was performed to avoid the fluctuation caused by food ingestion. Approximately 200 μL of blood was collected from saphenous vein every 2-3 weeks for measurement of plasma biochemical indicators. The blood used for measuring lipids and inflammatory markers was withdrawn through the cardiac puncture under deep terminal anesthesia.

Measurement of biochemical indicators

The level of plasma glucose of each mouse was measured using Sekisui Diagnostics SL reagent kits (Charlottetown, PEI). Plasma insulin level was measured using enzyme-linked immunosorbent assay (ELISA) kits from EMD Millipore (Billerica, MA, USA, for insulin). Homeostatic model assessment of insulin resistance (HOMA-IR) was calculated based on the levels of fasting plasma insulin and glucose detected in simultaneously collected specimens using the following formula: fasting insulin concentration (μU/mL) × fasting plasma glucose level (mmol/L)/22.5 (Miranda et al., 2018). Plasma cholesterol was measured using Sekisui Diagnostics SL cholesterol reagent kits (Charlottetown, PEI). Plasma triglyceride level was measured using BioAssay Systems reagents (Hayward, CA, USA).

Measurements of inflammatory markers

Inflammatory factors, including MCP-1, PAI-1 and tumor necrosis factor-α (TNF-α)

were measured. Level of PAI-1 was assessed via enzyme-linked immunosorbent assay (ELISA) using kits for mouse PAI-1 from Oxford Biomedical Research (Oxford, MI), MCP-1 using kits for mouse MCP-1 from Thermo Fisher Scientific (Ottawa, ON) and TNF-α using kits for mouse TNF-α from BD Bioscience (San Diego, CA).

Fecal sample collection and DNA extraction

Fecal pellets were collected in the morning from cages of individually hosted mice and the cages were changed at 4 pm of the previous day. DNA extraction was conducted using ZYMO Fecal DNA Extraction Kit (Zymo Research, Irvine, CA). DNA was quantified through a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA). 27F (5'-GAAGAGTTTGATCATGGCTCAG-3') and 342R (5'-CTGCTGCCTCCCGTAG-3') were used as primers for polymerase chain reaction (PCR) amplification followed by 1% agarose gel electrophoresis to verify DNA amplicons.

16S rRNA sequencing

With primers of 338F (5'-ACTCCTACGGGAGGCAGCA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'), the V3-V4 region of 16S rRNA gene was targeted for PCR amplification. PCR reactions contained 1 μL of pre-normalized 20ng/μL DNA, 1 μL of primers (10 μM) and 10 μL of Prime Hot MasterMix (Thermo). Reactions started with an initial denaturing step at 94°C for 3 min followed by 30 cycles at 94°C for 45 sec, 62°C for 60 sec, and 72°C for 90 sec; finalized by an extension step at 72°C for 10 min. PCR products were then purified using a Zymo Research DNA Clean-up Kit (Zymo). An aliquot of 200 ng of each DNA sample was sequenced using established protocols on an Illumina MiSeq system in the Gut Microbiome Laboratory in the Department of Animal Science at the University of Manitoba.

Bioinformatics analysis and statistics

The output raw data were analyzed using downstream computational pipelines of the

open-source software package Quantitative Insights Into Microbial Ecology (QIIME) version 1.9.0. Assembled reads were demultiplexed according to the barcode sequences and exposed to additional quality-filters and those with quality scores (Q-scores) below 20 were discarded. Chimeric reads were filtered using UCHIME and sequences were assigned to Operational Taxonomic Units (OTU) using UCLUST at 97% pairwise identity threshold. Taxonomies were assigned using Ribosomal Database Project classifier and aligned with the Greengenes Core reference database (gg_13_5). α-diversity analysis is to reveal diversity within community while β-diversity is to compare microbial compositions between samples. α-diversity was assessed through Shannon index and β-diversity was assessed through Bray–Curtis metric based on the abundances of species (without considering the relatedness of species) to compare microbial compositions between samples. Normal distribution was assessed through Anderson-Darling test, followed by one-way analysis of variance (ANOVA). Tukey's HSD was used in post hoc test.

Results

Effects of SBp on body weights, food intake, plasma glucose, cholesterol and triglycerides

Increases in fasting plasma glucose were detected in mice in the HFHS group for ≥7 weeks compared to the control group for corresponding periods (P<.01). The HFHS+SBp diet reduced fasting plasma glucose in mice after ≥7 weeks of intervention compared to the HFHS diet (P<.05 or .01) (Figure 3-1-1 A). Mice in both HFHS group and HFHS+SBp group had significant increases in body weight compared to the control group. However, no significant difference in body weight between HFHS group and HFHS+SBp group was observed (Figure 3-1-1 B). And food intake of all groups fluctuated without significant difference (Figure 3-1-1 C). HFHS diet increased the levels of both total cholesterol and triglycerides in the mouse fasting plasma before the end of the dietary intervention compared to the control diet. HFHS+SBp group significantly reduced the levels of plasma cholesterol and triglycerides compared to the

HFHS diet alone ($P<.01$) (Figure 3-1-2).

Effects of SBp on insulin and HOMA-IR

Significant increases were observed in the levels of plasma insulin of mice fed with the HFHS diet for ≥13 weeks compared to control diet-fed mice ($P<.05$ or .01). However, the HFHS+SBp diet reduced the levels of insulin in mice after ≥9 weeks compared to HFHS diet-fed mice ($P<.05$ or .01). Besides, the HFHS diet also increased HOMA-IR compared to the control diet after ≥9 weeks ($P<.05$ or .01), while HFHS+SBp diet significantly reduced HOMA-IR compared to HFHS diet after 11 weeks of dietary intervention ($P<.05$ or .01) (Figure 3-1-3).

Effects of SBp on inflammatory markers

The plasma levels of inflammatory markers, including MCP-1, PAI-1 and TNFα, were higher in HFHS-fed mice than control diet fed mice ($P<.01$). However, the increases in the inflammatory markers in the circulation of mice induced by the HFHS diet were significantly suppressed by the addition of 5% SBp ($P<.01$) (Figure 3-1-4).

Effects of SBp on gut microbiota

As is shown in Figure 3-1-5**,** the HFHS diet increased the Shannon index, a common index for α-diversity of gut microbiota, compared to the control diet. However, the HFHS+SBp group had a higher level of Shannon index compared to that in the HFHS group (Figure 3-1-5 A). The β-diversity result demonstrated that the compositions of gut microbiota in three groups were well separated, suggesting that the three diets caused distinct profiles of gut microbiota (Figure 3-1-5 B).

Bacteroidetes and *Firmicutes* represented two major bacteria on phylum level in mouse feces. Although HFHS diet did not significantly alter the relative abundance of *Firmicutes* compared to the control or HFHS+SBp diet, it reduced that of *Bacteroidetes* compared to the control diet (Figure 3-1-6 and Table 3-1-1**)**. The relative abundance of

Bacteroidetes phylum in the HFHS+SBp diet (16%) was not significantly different from the other two groups (Table 3-1-1). In terms of *Actinobacteria*, the relative abundance in both HFHS and HFHS+SBp groups was greater than that in the control group, with HFHS+SBp group having a higher level than HFHS group (Figure 3-1-6 and Table 3-1-1).

On family level, the HFHS diet decreased the relative abundances of *S24-7*, *Lactobacillaceae*, *Peptostreptococcaceae*, and *Clostridiaceae*, but increased those of *Ruminococcaceae*, *Lachnospiraceae*, *Coriobacteriaceae* and *Mogibacteriaceae* compared to the control diet. The HFHS+SBp diet significantly decreased the relative abundance of *Lactobacillaceae* compared to the control diet and increased that of *Ruminococcaceae*, *Dehalobacteriaceae* and *Coriobacteriaceae* compared to the HFHS group. Although HFHS+SBp increased the relative abundance of *S24-7* compared to HFHS group, no significant difference was detected between the two groups (Figure 3-1-7 and Table 3-1-2).

Correlations of gut bacteria with physiochemical results

Correlations between bacterial families and physiochemical results in the mice were analyzed in terms of body weights, fasting plasma glucose, cholesterol, triglycerides, insulin, HOMA-IR, TNF-α, PAI-1 or MCP-1. The relative abundance of *S24-7* in the stool of mice was negatively correlated with all the studied physiochemical variables, which suggested that the higher level of *S24-7* was associated with lower levels of glucose, lipids, inflammatory markers and body weight in the mice. Positive correlations between bacteria and physiochemical variables also existed. Those bacteria included *Lachnospiraceae, Streptococaceae* and *Peptococaceae* (Figure 3-1-8).

Phylum bacteria	CTL (%)	HFHS (%)	HFHS +SBp(%)	HFHS vs. CTL	HFHS +SBp vs. CTL	HFHS +SBp vs. HFHS
Others	29.08	6.4	1.06	**	**	
Spirochaetes	0	0	0.003			
Proteobacteria	0.29	0.23	0.03			
Firmicutes	47.72	82.17	81.27			
Bacteroidetes	22.68	10.24	16.26	**		
Actinobacteria	0.23	0.96	1.36	**	**	++

Table 3-1-1 Impact of HFHS and HFHS+SBp diets on the abundances of bacteria on phylum level in the mouse gut. Values in the tables were expressed as averages (%, n=6-8). *, **: P<.05 or 0.01 vs. CTL group; +, ++: P<.05 or 0.01 vs. HFHS group.

Family bacteria	Control (%)	HFHS (%)	HFHS+ SBp (%)	HFHS vs. CTL	HFHS +SBp vs. CTL	HFHS +SBp vs. HFHS
Veillonellaceae	0.004	0.001	0.001			
Streptococcaceae	0.23	1.64	1.48	**	**	
Staphylococcaceae	0.001	0.02	0.008			
Spirochaetaceae	0	0	0.003			
S24-7	21.14	10.05	14.64	**	*	
Ruminococcaceae	3.53	11.37	14.7	**	**	+
Rikenellaceae	0.21	0.12	0.24			
RF16	0.001	0.001	0			
Prevotellaceae	0.008	0.01	0.005			
Peptostreptococcaceae	0.004	0	0	*	*	
Peptococcaceae	0	0.04	0.03			
Lactobacillaceae	31.30	24.11	11.46	*		
Lachnospiraceae	3.16	15.69	16.65	**	**	
Erysipelotrichaceae	0.003	0	0			
Enterococcaceae	0.003	0.008	0.004			
Dehalobacteriaceae	0.07	0.10	0.18		*	+
Coriobacteriaceae	0.23	0.96	1.36	**	**	++
Clostridiaceae	4.15	0.63	0.30	**	**	
Bacteroidaceae	1.26	0.005	1.30			
Alcaligenaceae	0.29	0.23	0.03			
Paraprevotellaceae	0.001	0.004	0.002			
Mogibacteriaceae	0.08	0.48	0.56	**	**	
Others	34.33	34.53	37.05			

Table 3-1-2 Impact of HFHS and HFHS+SBp diets on the abundances of bacteria on family level in the mouse gut. Values in the tables were expressed as averages (%, n=6-8). *, **: P<.05 or .01 vs. CTL group; +, ++: P<.05 or .01 vs. HFHS group.

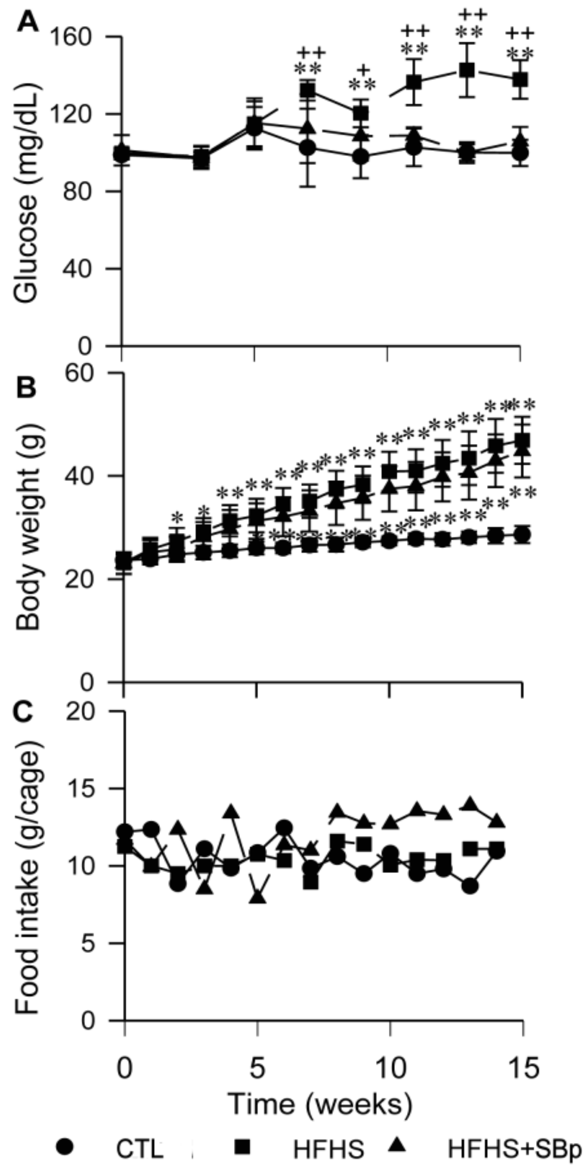

Figure 3-1-1 Fasting plasma glucose, body weight and food intake in mice fed with HFHS diet or HFHS+SBp diet. A: Fasting plasma glucose every 2–3 weeks expressed in mean ± standard deviation (SD) (n=8/group). B: Body weight expressed in mean ± SD. C: Food intake of mice/cage (n=4/cage) was presented in average (g) of two cages/group. *, ** P<.05 or .01 vs. CTL group; +, ++ P<.05 or .01 vs. HFHS+SBp group.

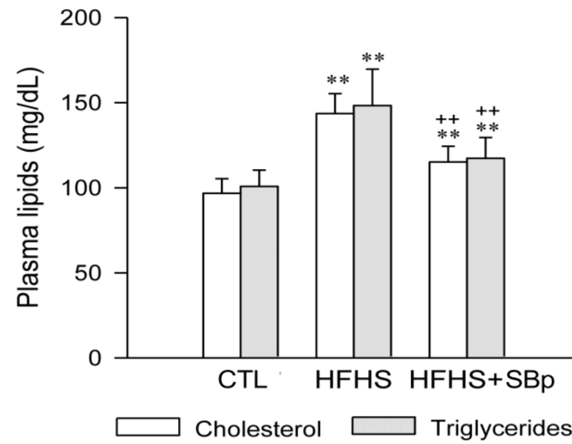

Figure 3-1-2 Effects of HFHS and HFHS+SBp on cholesterol and triglycerides in mice. The levels of fasting plasma cholesterol and triglycerides were analyzed at the 15th week after the initiation of the dietary experiment. Values were expressed in mean ± SD (n=8/group). *, **P<.05 or .01 vs. CTL group; +, ++P<.05 or .01 vs. HFHS group.

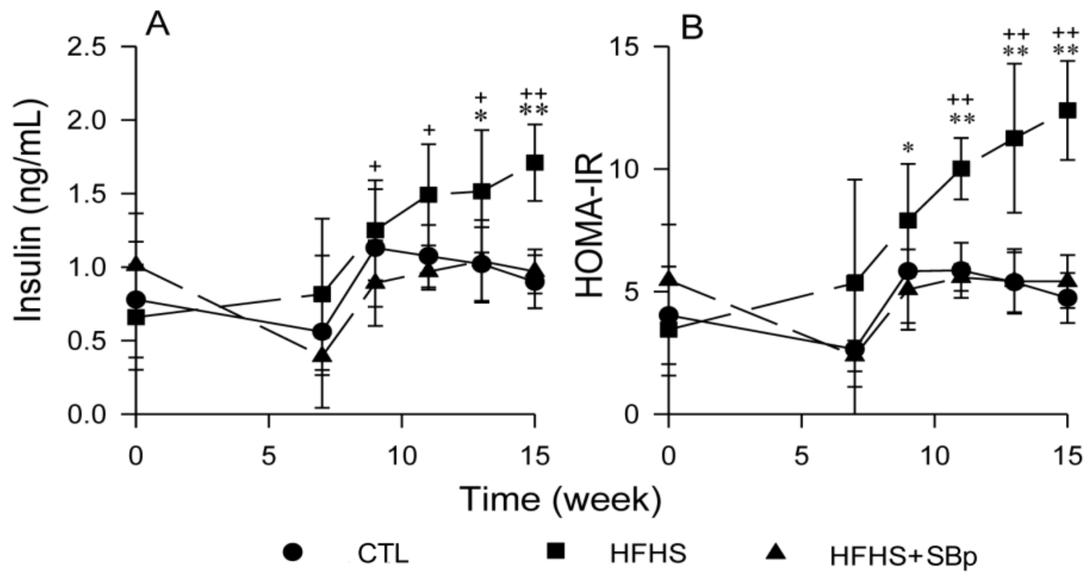

Figure 3-1-3 Effects of HFHS and HFHS+SBp diets on insulin and insulin resistance in mice. A: Insulin. B: HOMA-IR. The levels of fasting plasma insulin (ng/mL) were measured at ≥7 weeks after the start of the dietary intervention every 2 weeks using mouse insulin ELISA kit. Values were expressed in mean ± SD (n=8/group). *, **P<.05 or .01 vs. CTL group; +, ++P<.05 or .01 vs. HFHS+SBp group.

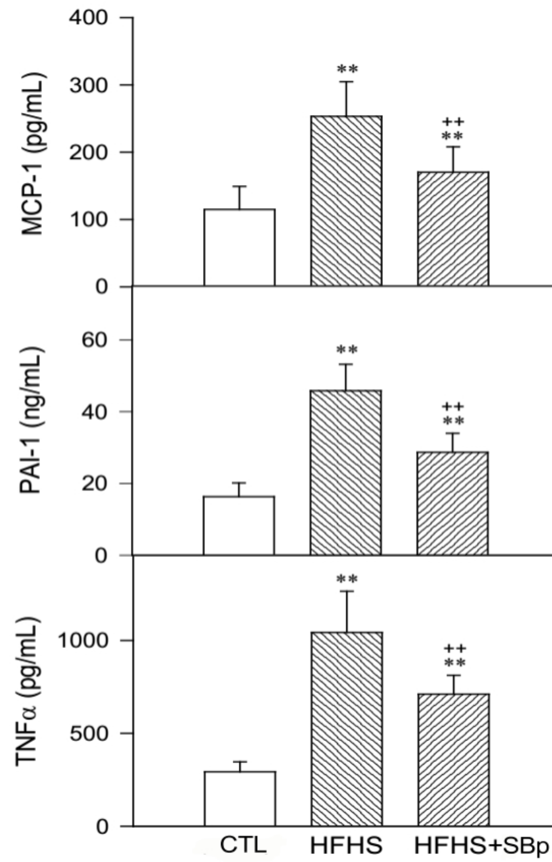

Figure 3-1-4 Effects of HFHS and HFHS+SBp diets on inflammatory markers in mouse plasma. Values were expressed in mean ± SD (n=8/group). *, **P<.05 or .01 vs. CTL group; +, ++P<.05 or 0.01 vs. HFHS group.

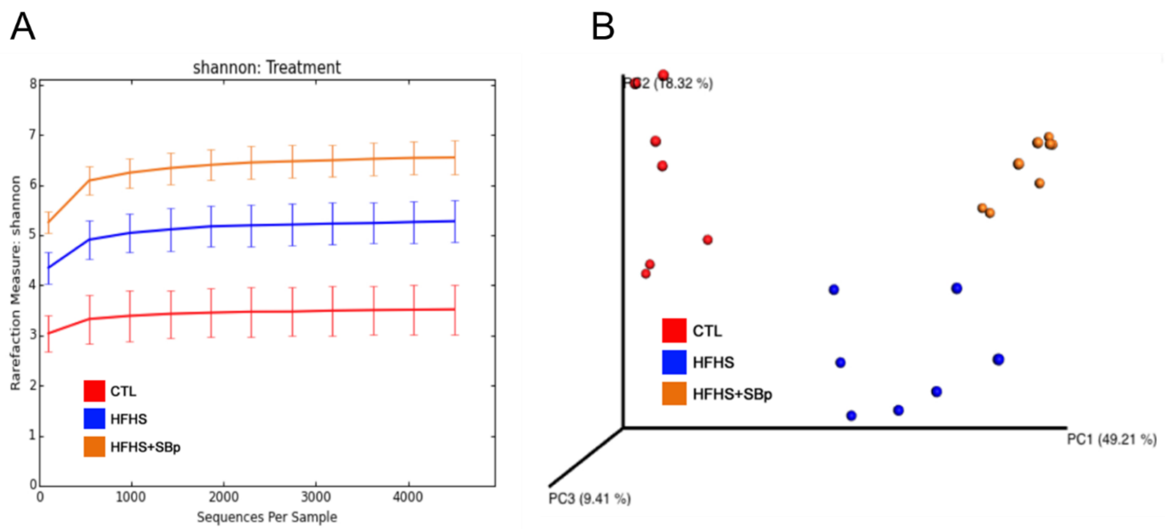

Figure 3-1-5 Impact of HFHS and HFHS+SBp diets on the diversity of intestinal microbiota in mice. A: Shannon diversity Index for α-diversity; B: Principal coordinates analysis (PCA) based on Bray–Curtis dissimilarity for β-diversity.

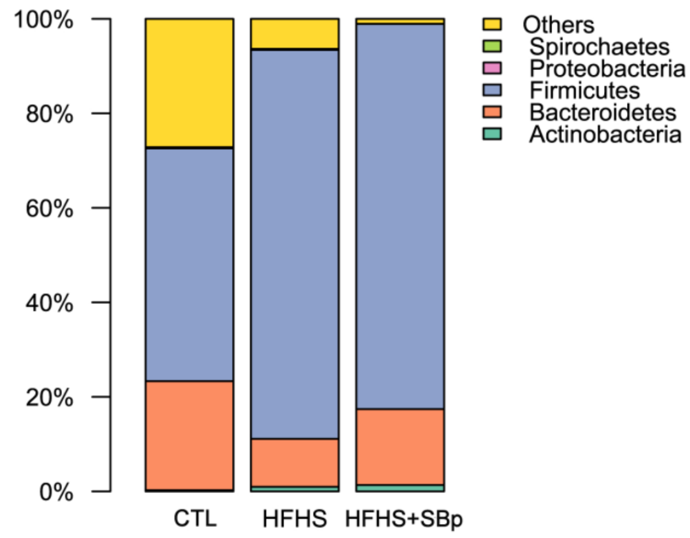

Figure 3-1-6 The effects of HFHS and HFHS+SBp diets on the abundance of gut bacteria on phylum level.

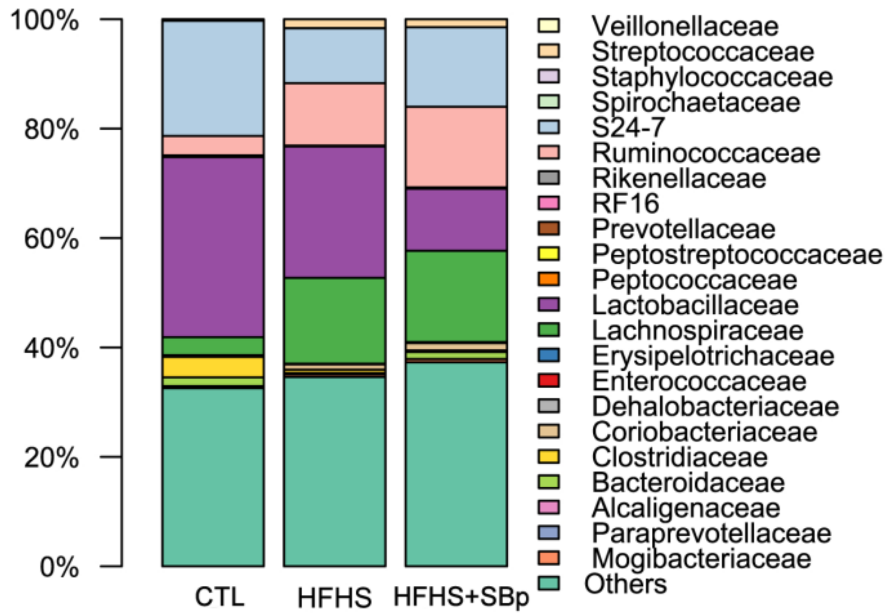

Figure 3-1-7 The effects of HFHS and HFHS+SBp diets on the abundance percentage of gut bacteria on family level.

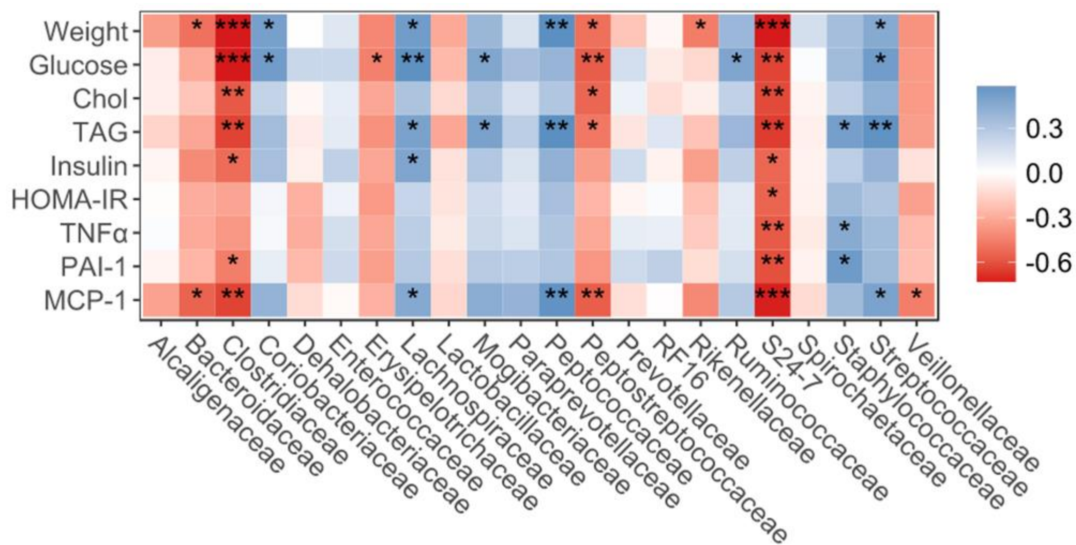

Figure 3-1-8 Correlations between the abundances of family-level gut bacteria and body weight, metabolic variables or inflammatory markers. Blue: positive correlations; red: negative correlations. The darkness of color in the squares represented the strength of correlation as indicated in the legend. The significances of correlation were displayed with *, **, ***P<.05, .01 or .001 (n=21).

Part 2: Effects of cyanidin-3-glucoside on high-fat high-sucrose diet-induced insulin resistance and gut dysbiosis in mice

In the subsequent dietary experiment, we examined the effect of a major anthocyanin in Saskatoon berry powder, cyanidin-3-glucoside (C3G), on glucose, lipids, inflammatory markers and gut microbiota. The manuscript has been published online on 'Microorganisms' in 2020 (https://doi.org/10.3390/microorganisms8081238). I was involved in animal experiments, sample collection, microbiota data analysis and manuscript draft and listed as the first author in the article.

Materials and methods

Dietary Intervention

A total of 32 male C57BL/6J mice (6 weeks of age) were randomized into 4 groups with 8 mice in each group after stabilization. The control, HFHS and HFHS+SBp groups received the same diets described in Part 1. C3G was obtained from Polyphenols (Sandnes, Norway). HFHS+C3G diet was made by adding equivalent-to- 5% SBp amount of C3G into HFHS diet. Specifically, SBp group received HFHS diet supplemented with 8 g/kg/day of SBp (5% SBp) and C3G group received HFHS diet supplemented with 7.2 mg/kg/day of C3G, which equal to the amount of C3G contained in 8 g/kg/day of SBp. Mice had access to food and distilled water *ad libitum* during 11-week dietary intervention.

Food intake and body weight

Food intake of 24 hours in each cage was measured every 2 weeks. Body weight of each mouse was also recorded every two weeks during the experiment.

Blood collection

Blood was collected as described in Part 1 for the measurement of biochemical indicators.

Diabetes-related biochemical parameters

The plasma levels of fasting plasma glucose, insulin, cholesterol and triglyceride were measured using the same kits as described in Part 1. HOMA-IR was still calculated using the measured data from fasting plasma insulin and glucose.

Measurement of Inflammatory markers

The plasma levels of inflammatory markers were measured using the same kits as described in Part 1.

Fecal sample collection

In order to collect feces of individual mouse, each mouse was transferred into a new cage and housed independently overnight, followed by carefully collecting fecal pallets from each cage. All the fecal samples were stored at -80°C and kept frozen until subsequent DNA extraction.

DNA extraction and sequencing

The target region of 16S rDNA was switched to V4-V5 region due to service option. Differences also included kits and methods used for DNA extraction and amplification. DNA extraction and 16S rRNA gene sequencing of fecal samples were submitted to Integrative Microbiome Resource in Dalhousie University for subsequent experiments.

DNA extraction was achieved using PowerFecal DNA Isolation Kit (QIAGEN, Germantown, MD). DNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific). Polymerase chain reaction (PCR) was performed on DNA samples using modified primers containing 515F(5'-GTGYCAGCMGCCGCGGTAA-3') and 926R(5'-CCGYCAATTYMTTTRAGTTT-3') targeting 16S rDNA V4-V5 region. Modified primers were composed of three regions: Illumina adaptors, indices and primers targeting V4-V5 region. A high-throughput Hamilton Nimbus Select robot

using Coastal Genomics Analytical Gels was run to verify the quality of PCR products visually. Failed amplicons with spurious bands were repeatedly amplified after modifying conditions for PCR until qualified bands were produced. The PCR amplicons were normalized by using a high-throughput Charm Biotech Just-a-Plate 96-well Normalization Kit, then pooled to construct a library and quantified before sequencing on an Illumina MiSeq platform.

Bioinformatic analysis and statistics

Freshly generated raw data in the form of fastq file were demultiplexed according to the barcode sequences, followed by trimmed using Cutadapt (version 1.17) for primers to be remove from reads. Trimmed reads were imported into an open-source bioinformatics pipeline of decentralized microbiome analysis package: Quantitative Insights Into Microbial Ecology 2 (QIIME2, version: 2018. 8). After joining of paired-end reads and filtering out of low-quality reads, amplicon sequence variants (ASVs) were obtained through DADA2 workflow. Taxonomies were assigned to ASVs using a Naive-Bayes approach and SILVA database. Diversity metrics (Core-metrics-phylogenetic) within QIIME2 were used for evaluating α-diversity and β-diversity. Average and standard deviation of physiological (food intake and body weight) and biochemical (glucose, cholesterol, triglyceride, insulin, HOMA-IR) values were calculated. Normal distribution was assessed through three methods: Shapiro-Wilk test, Kolmogorov-Smirnov test and Anderson-Darling test, followed by one-way ANOVA. Tukey's HSD was also used this time in post hoc test. Relative abundances of ASVs assigned with taxonomy in feature table were correlated with physical and clinical parameters using Pearson correlation. Metagenomic functions were predicted with the Galaxy web application and Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt).

Results

Body weight and food intake

All mice involved in the study began with similar body weights (approximately 23 g) during stabilization. Body weights in all groups kept increasing until the end of dietary intervention at 11th week. Body weights in the 3 groups of mice receiving HFHS diet were significantly greater than mice receiving CTL diet after ≥2 weeks of dietary intervention ($P<.05$ or .01). No significant difference in body weight were found among mice in the HFHS, SBp or C3G groups (Figure 3-2-1 A**)**. Food intake in mass among four groups fluctuated but was not significantly different during the dietary experiment (Figure 3-2-1 B).

Effects of C3G and SBp on glucose, cholesterol and triglycerides

Fasting plasma glucose levels in HFHS group was significantly higher than that in the control group ($P<.01$). Mice receiving HFHS diet supplemented with SBp or C3G had significantly lower levels of plasma glucose compared to those fed with HFHS diet alone ($P<.05$ or .01). However, their glucose levels were still significantly higher than those in the control group ($P<.01$). No significant difference in plasma glucose was found between SBp and C3G group (Figure 3-2-2). Similar patterns of changes were detected in fasting plasma cholesterol and triglycerides of mice fed with HFHS diet, HFHS+SBp or HFHS+C3G diet compared to mice in CTL group (Figure 3-2-2).

Effects of C3G and SBp on insulin and HOMA-IR

Results of both fasting plasma insulin and HOMA-IR showed that the control group had the lowest levels of plasma insulin concentration and insulin resistance. The levels of plasma insulin and HOMA-IR in HFHS group were significantly higher than the other three groups ($P<.01$). Supplementation of SBp or C3G significantly reduced the increase in the levels of plasma insulin and HOMA-IR in mice induced by HFHS diet ($P<.01$). No significant difference in insulin or HOMA-IR was detected in mice fed

with HFHS+SBp or HFHS+C3G diet (P<.01) (Figure 3-2-3).

The results of plasma concentrations of inflammatory markers showed the HFHS group had the highest levels of MCP-1 and PAI-1 compared to other 3 groups (P<.01). However, supplementation of SBp or C3G significantly reduced the two inflammatory markers in mice compared to mice exclusively fed with HFHS diet. No significant difference was detected in mice fed with HFHS diet containing either SBp or C3G (Figure 3-2-4).

Effects of C3G and SBp on gut microbiota

The results of β-diversity demonstrated that the gut microbiota in the four dietary groups were well separated (Figure 3-2-5). Comparison of the relative abundance showed *Bacteroidetes* in mice fed with HFHS diets was significantly lower than that in mice fed with either SBp or C3G diet (P<.01) (Figure 3-2-6 A). Meanwhile, *Firmicutes* in the HFHS group showed a significantly higher relative abundance compared to the other three groups (P<.01) (Figure 3-2-6 B). With an addition of either SBp or C3G into the HFHS diet tended to increase the abundance of *Bacteroidetes* and decreased the levels of *Firmicutes* (P<.01 or .05). Besides the relative abundance, the control group and HFHS group also significantly differed in ratios of B/F and F/B. Group receiving SBp had a significantly increase in B/F ratio (P<.05) and a lowered F/B ratio compared to the HFHS group (P<.01). Mice receiving C3G had a higher but not significant different B/F ratio and significantly lower F/B ratio (P<.01) compared to the HFHS group (Figure 3-2-6).

Relative abundance analysis of family bacteria demonstrated that several families of bacteria, including *Akkermansiaceae*, *Bacteroidaceae*, *Erysipelotrichaceae*, *Lachnospiraceae*, *Lactobacillaceae*, *Muribaculaceae* and *Ruminococcaceae*, predominated in the gut microbiota structure. Mice fed with HFHS diet had a different microbial profile with an increase in abundances of *Erysipelotrichaceae*,

Lachnospiraceae and *Peptostreptococcaceae* but a decrease in the abundance of *Muribaculaceae* (*S24-7*) compared to mice in the other three groups. In spite of low abundances in microbial profiles for certain bacteria, such *Atopobiaceae*, *Clostridiaceae 1* and *Clostridiales vadinBB60 group*, ANOVA results revealed those significant difference among mice fed with different diets. The correlation result showed that *Defluviitaleaceae*, *Eggerthellaceae*, *Erysipelotrichaceae*, *Lachnospiraceae* and *Peptostreptococcaeae* were mainly positively correlated with the biochemical variables, while *Clostridiaceae* 1, *Clostridiales* vadinBB60 group, *Muribaculaceae* and *Lactobacillaceae* were negatively correlated with those variables. The relative abundance of *Muribaculaceae* was the lowest in mice fed with HFHS diet but the highest in control group. Both SBp and C3G significantly increased the level of *Muribaculaceae*, with HFHS+SBp group having an even higher level of *Muribaculaceae* than that in HFHS+C3G group (Figure 3-2-7).

The correlation heatmap demonstrated the existence of association between the relative abundance of family bacteria and some diabetes-related biochemical parameters. The abundances of *Defluviitaleaceae*, *Eggerthellaceae*, *Erysipelotrichaceae*, *Family XIII*, *Lachnospiraceae*, *Peptpcoccaceae*, *Peptostreptococcaeae*, *Rununococcaceae* and *Streptococcaceae* family bacteria were positively correlated with body weight, glucose/lipid metabolism and inflammatory markers in the mice, while *Akkermansiaceae, Clostridiaceae 1*, *Clostridiales vadinBB60 group*, *Muribaculaceae* and *Lactobacillaceae* were negatively correlated with the physical, metabolic and inflammatory variables (Fig. 3-2-8).

In order to further examine relationships between gut bacteria and glucose metabolism, linear regression plots for selected bacterial families and fasting plasma glucose, insulin and HOMA-IR were displayed (Figure 3-2-9). *Akkermansiaceae* and *Muribaculaceae* were two families that were negatively associated with T2D-related biochemical variables in the correlation heatmap (Figure 3-2-8). Mice receiving the control diet had

the highest abundances of *Akkermansiaceae* and *Muribaculaceae*, while mice receiving HFHS diet had the lowest levels of *Akkermansiaceae* and *Muribaculaceae*. The relative abundances of *Akkermansiaceae* and *Muribaculaceae* in HFHS+SBp or HFHS+C3G group again showed negative correlations with the two groups partially overlapping with HFSH group (Figure 3-2-9 A-F). On the other hand, the relative abundance of *Lachnospiraceae* had positive correlations with T2D-related biochemical variables in the correlation heatmap (Figure 3-3-8). The control group had the lowest abundance of *Lachnospiraceae*, while the HFHS group had the highest abundance of this family. And the level of *Lachnospiraceae* decreased with the supplementation of SBp or C3G in HFHS diet (Figure 3-2-9 G-I).

The comparison between SBp and C3G showed that they have a similar effect on some bacteria, such as *Muribaculaceae*, *Lachnospiraceae* and *Erysipelotrichaceae*. *Muribaculaceae* was the family increased by both SBp and C3G, while both SBp and C3G lowered *Erysipelotrichaceae*. The relative abundance of *Lachnospiraceae* was significantly lowered by both SBp and C3G groups, while *Ruminococcaceae* was found significantly different between HFHS and SBp groups (Figure 3-2-10). The relative abundance of *Ruminococcaceae* in HFHS group was significantly higher than that in HFHS+SBp group. However, no significant difference was detected on *Ruminococcaceae* abundance between HFHS and C3G groups. Overall, C3G had the potential to shape the gut microbiota profile in a similar way as SBp. Both significantly increased *Muribaculaceae* and decreased *Erysipelotrichaceae* and *Lachnospiraceae*.

Function prediction by PICRUSt showed that pathways related to multiple cellular and metabolic processes differed among the four groups. At least two types of distribution patterns were found. In the pathways with the first pattern, mice in the HFHS group showed the highest level of activity among 4 groups. The supplementation of SBp or C3G inhibited the activation of those pathways caused by HFHS diet. Examples of this pattern included membrane transport, cell motility and transcription. In the pathways

with the second pattern, mice in the HFHS group showed the lowest level of activity among the 4 groups, while adding SBp or C3G groups activated those pathways which included amino acid metabolism, glycan biosynthesis and metabolism, lipid metabolism and energy metabolism (Figure 3-2-11).

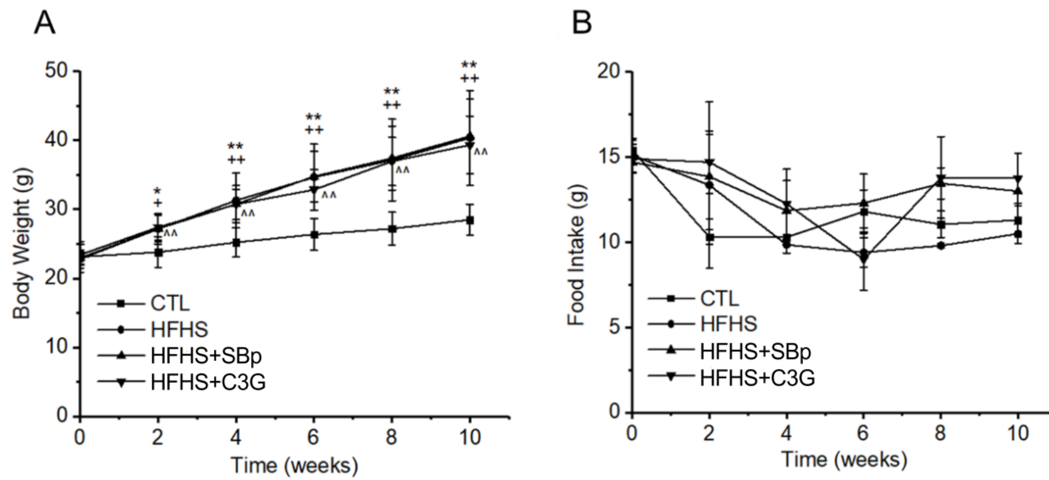

Figure 3-2-1 Effects of HFHS diet supplemented with or without SBp or C3G on the body weight and food intake of mice. A: Body weight. B: Daily food intake. The values were expressed as mean ± SD (n = 8/group). *, **: P<.05 or .01 HFHS group vs. CTL group, +, ++: P<.05 or .01 SBp group vs. CTL group, ˆ, ˆˆ: P<.05 or .01 C3G group vs CTL group.

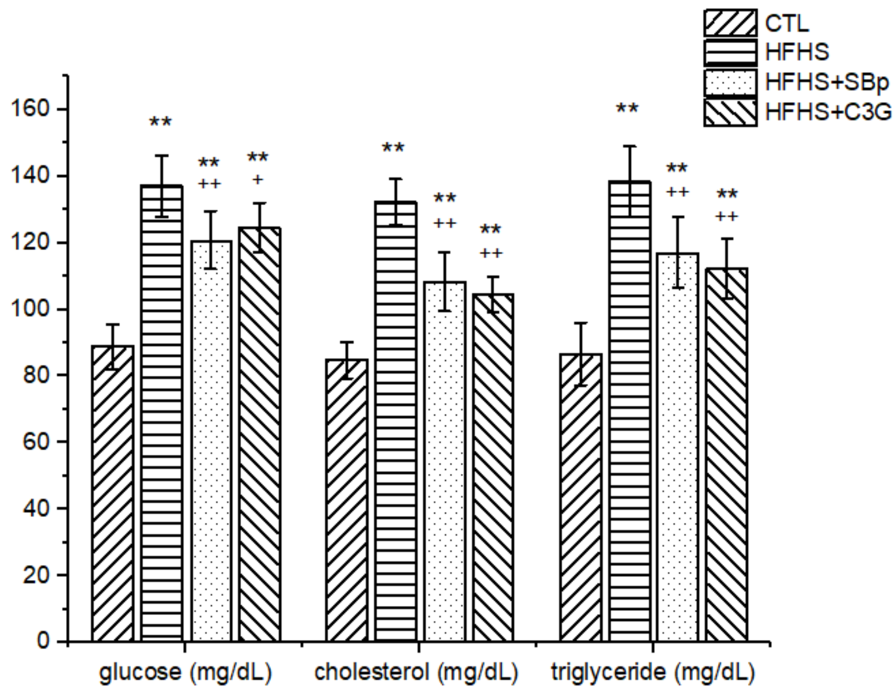

Figure 3-2-2 Levels of glucose, cholesterol and triglycerides in plasma of mice fed with HFHS diets supplemented with or without SBp or C3G. The values were expressed as mean ±SD (n = 8/group). *, **: P<.05 or .01 vs. CTL group, +, ++: P<.05 or .01 vs. HFHS group.

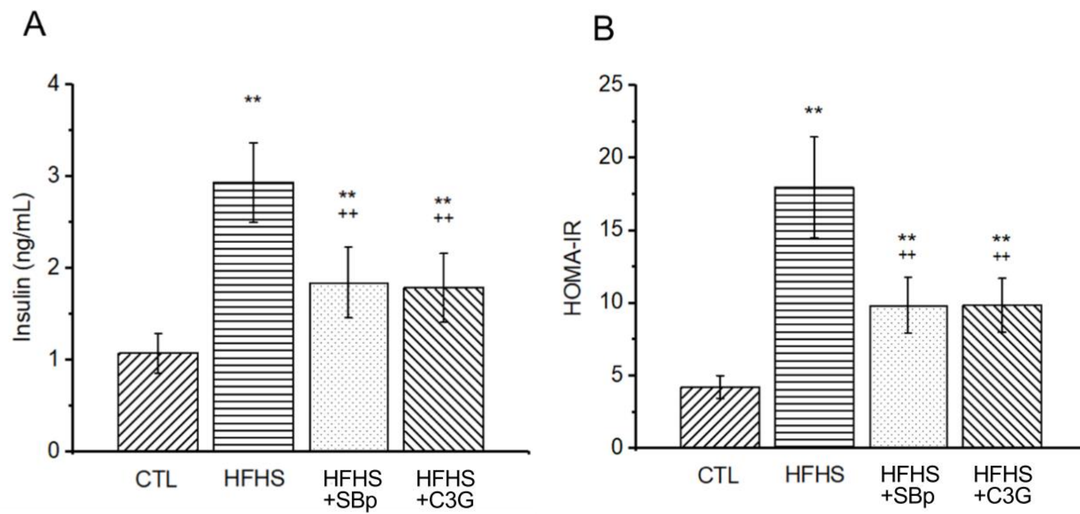

Figure 3-2-3 Effects of HFHS diet supplemented with or without SBp or C3G on insulin and insulin resistance in mice. A: Insulin. B: HOMA-IR. The values were expressed as mean ±SD (n = 8/group). *, **: P<.05 or .01 vs. CTL group, +, ++: P<.05 or .01 vs. HFHS group.

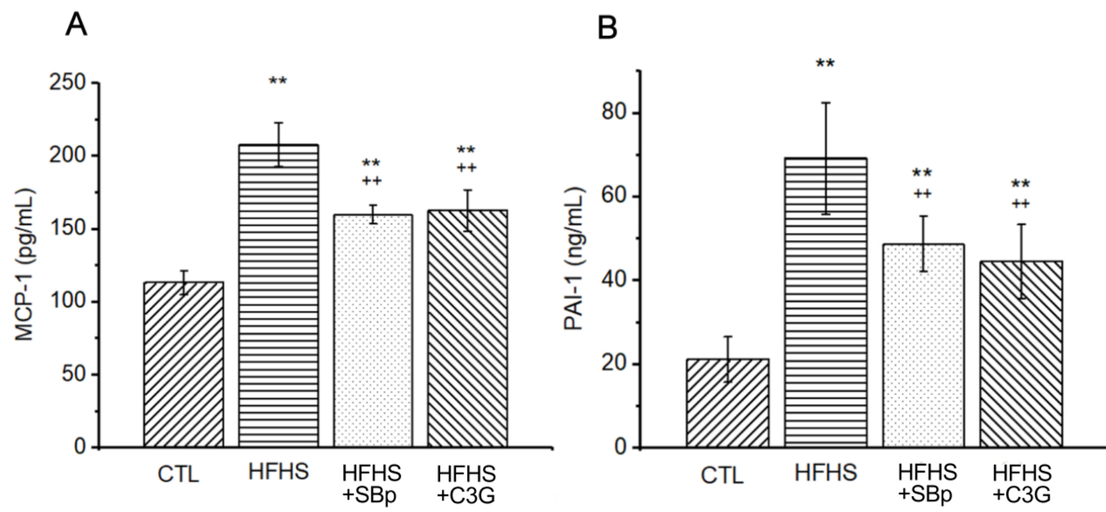

Figure 3-2-4 Effects of HFHS diet supplemented with or without SBp or C3G on inflammatory markers. A: MCP-1. B: PAI-1. The values were expressed as mean ±SD (n = 8/group). *, **: P<.05 or .01 vs. CTL group, +, ++: P<.05 or .01 vs. HFHS group.

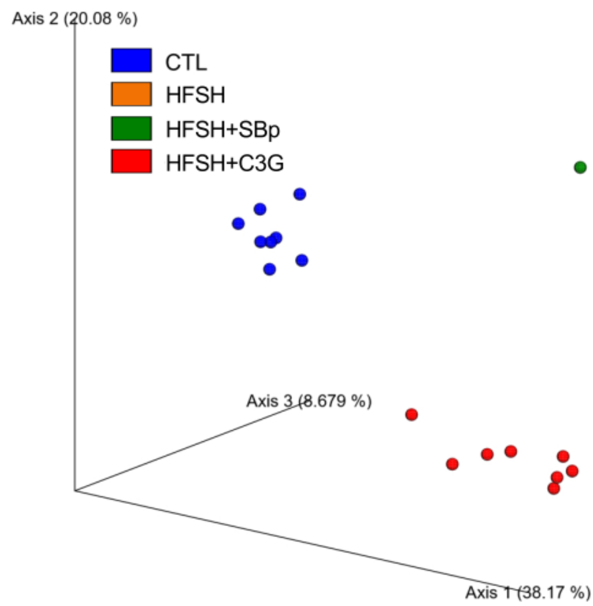

Figure 3-2-5 Effect of HFHS diet supplemented with or without SBp or C3G on β-diversity of gut microbiota in mice. PCA was based on Bray–Curtis dissimilarities between all sample sets.

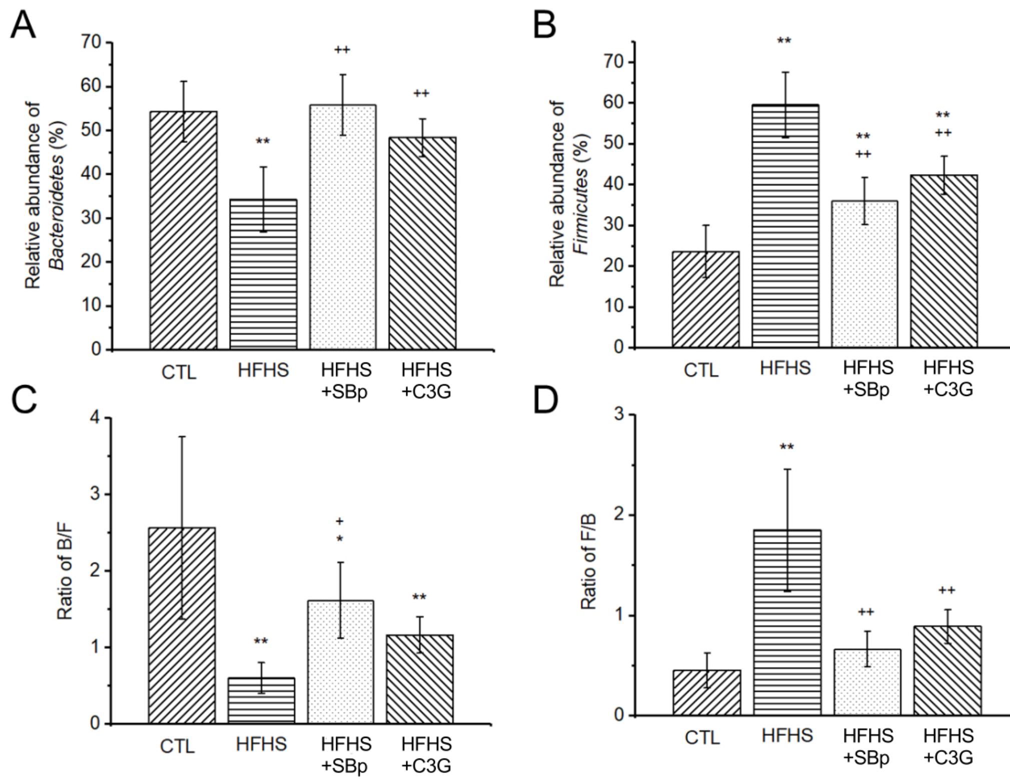

Figure 3-2-6 Effects of HFHS diet supplemented with or without SBp or C3G on the relative abundance of *Bacteroidetes* and *Firmicutes*. A: Relative abundance of *Bacteroidetes*. B: Relative abundance (%) of *Firmicutes* C: Ratio of *Bacteroidetes* over *Firmicutes* (B/F). D: Ratio of *Firmicutes* over *Bacteroidetes* (F/B). The values were expressed as mean ±SD g (n = 8/group). *, **: P<.05 or .01 vs. CTL group, +, ++: P<.05 or .01 vs. HFHS group.

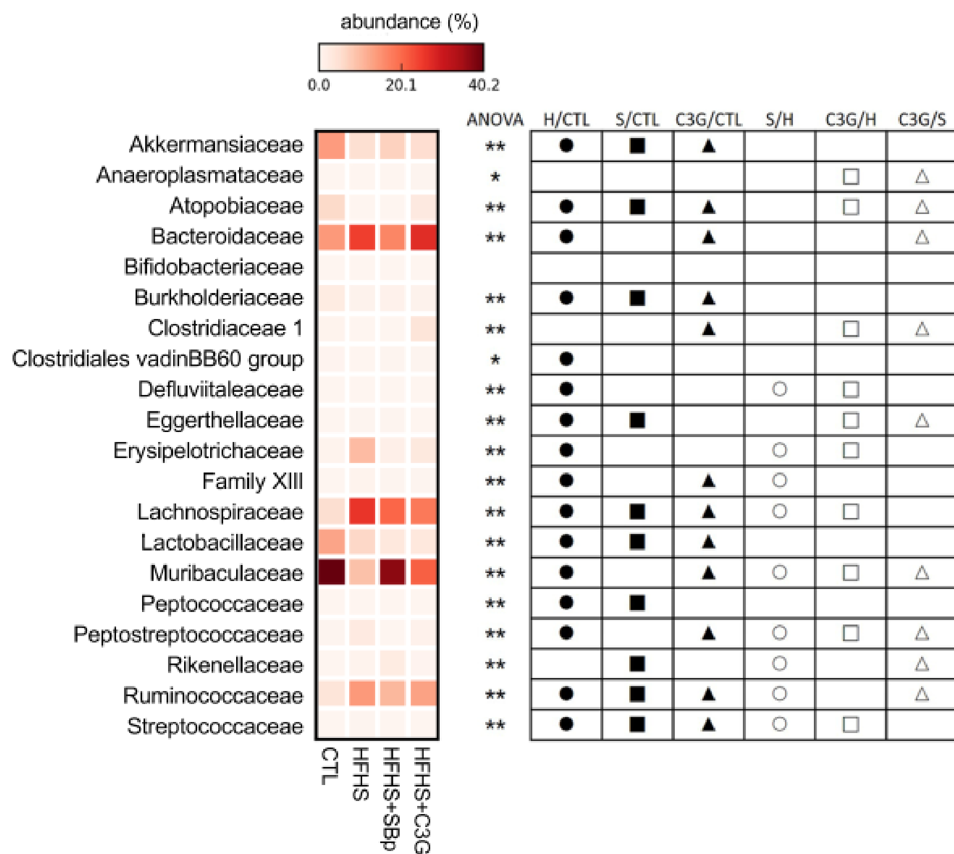

Figure 3-2-7 Effect of HFHS diet supplemented with or without SBp or C3G on the relative abundance of gut microbiota on family level. Results of ANOVA and post-hoc (Tukey) test were presented. *, **: P<.05 or .01 for overall ANOVA result; ●: P<.05 for HFHS group vs. CTL group (H/CTL); ■: P<.05 for SBp group vs. CTL group (S/CTL); ▲: P<.05 for C3G group vs. CTL group (C3G/CTL); ○: P<.05 in SBp group vs. HFHS group (S/H); □: P<.05 for C3G group vs. HFHS group (C3G/H); △: P<.05 for C3G group vs. SBp group (C3G/S).

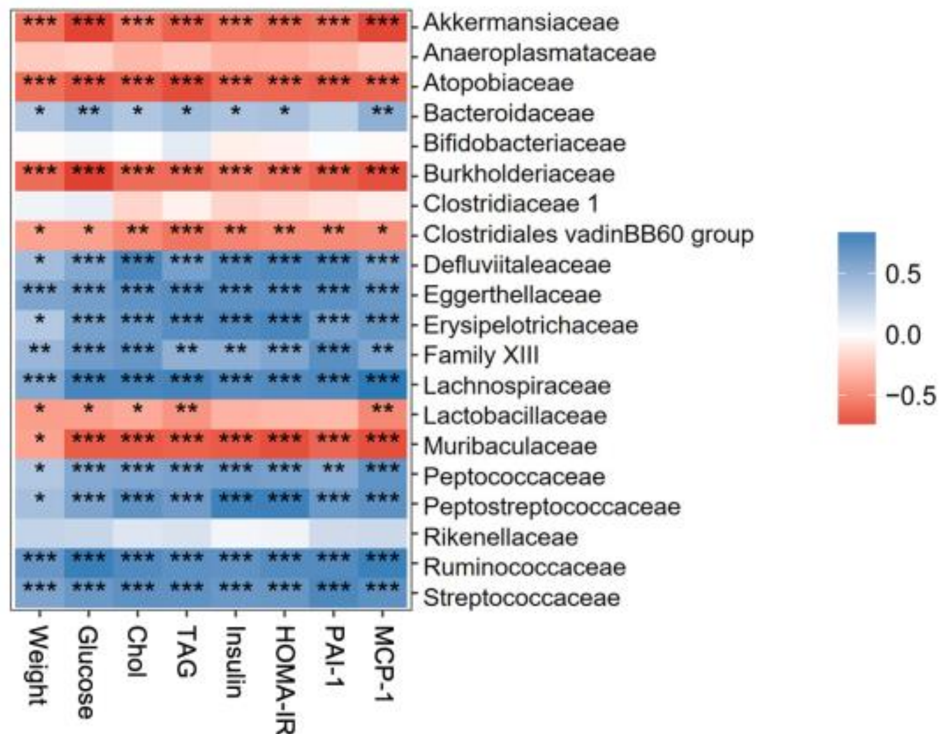

Figure 3-2-8 Correlations between the abundances of gut bacteria and body weight, metabolic variables or inflammatory markers. Blue: positive correlations. Red: negative correlations. The darkness of color in the squares represented the strength of correlation as indicated in the legend. The significances of correlation were displayed with *, **, ***P<.05, .01 or .001 (n=32).

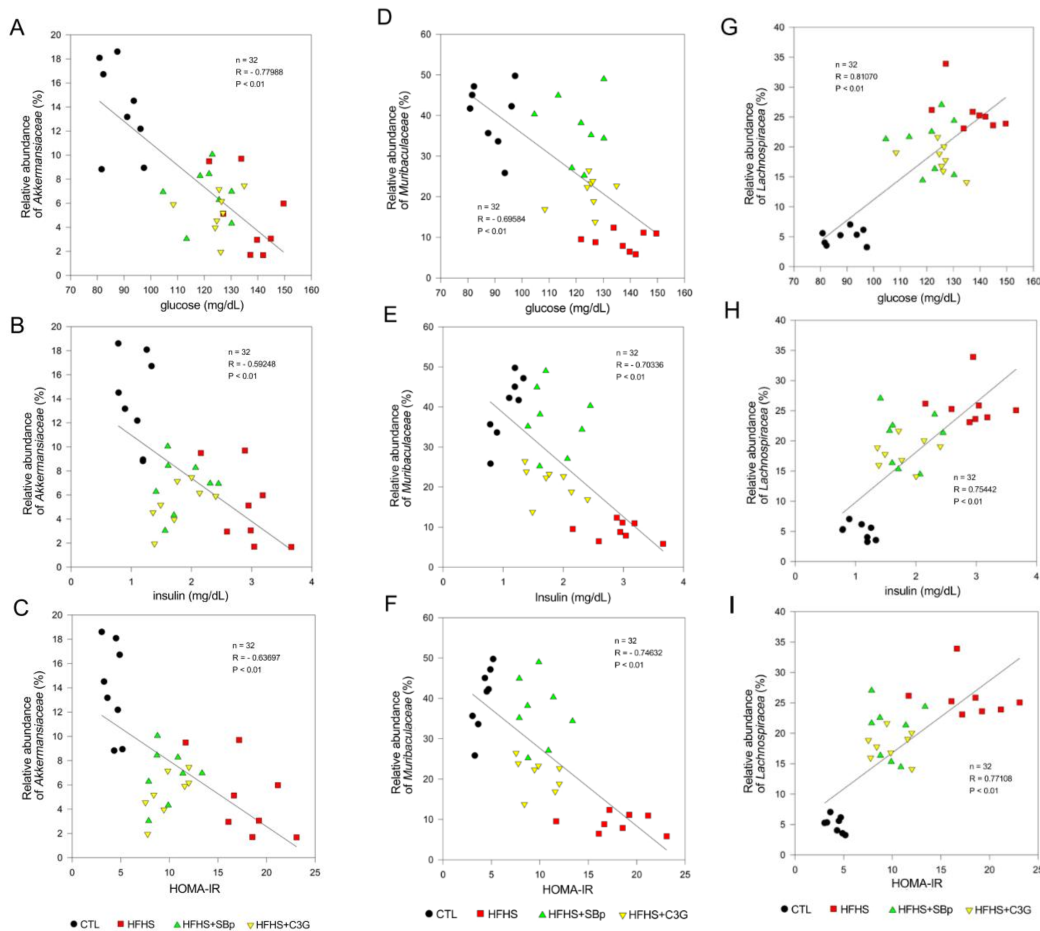

Figure 3-2-9 Linear regression analysis of correlation between the relative abundance of three families and three key diabetes-related indicators. A-C: Correlation between the relative abundance of *Akkermansiaceae* with glucose, insulin and HOMA-IR, respectively. D-F: Correlation between the relative abundance of *Muribaculaceae* with glucose, insulin and HOMA-IR, respectively. G-I: Correlation between the relative abundance of *Lachnospiraceae* with glucose, insulin and HOMA-IR, respectively (n=32).

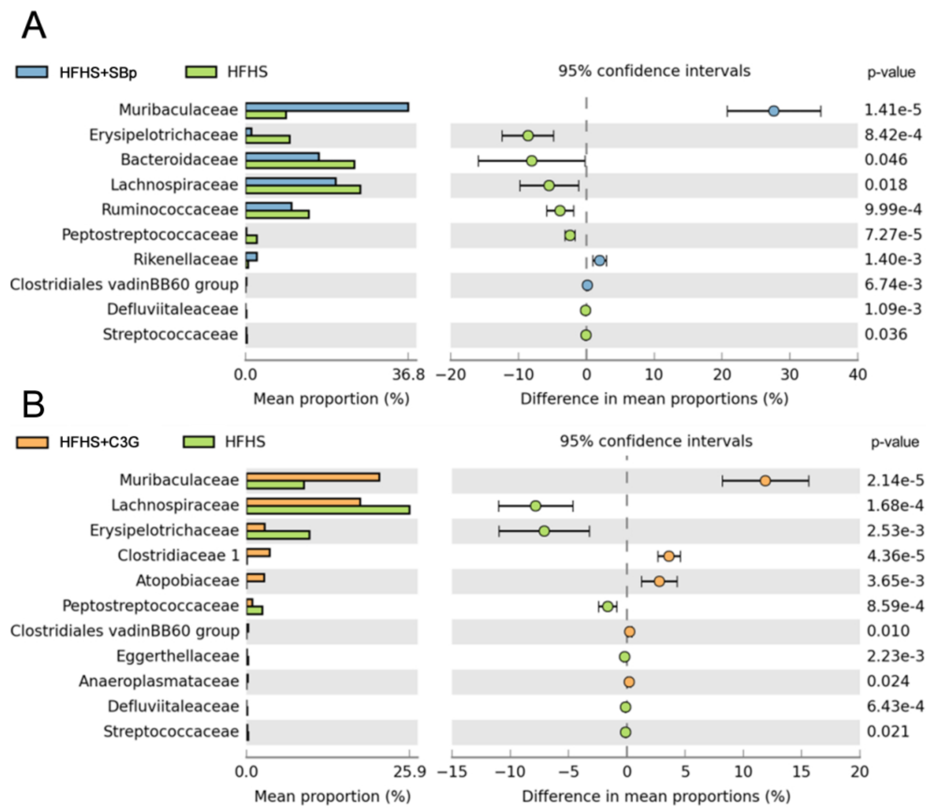

Figure 3-2-10 Comparison of the effects of SBp and C3G on HFHS-induced gut microbiota profiles. A: Extended error bar plot (STAMP tool) showing difference in mean relative abundance between SBp group and HFHS group. B: Extended error bar plot (STAMP tool) showing difference in mean relative abundance between C3G group and HFHS group.

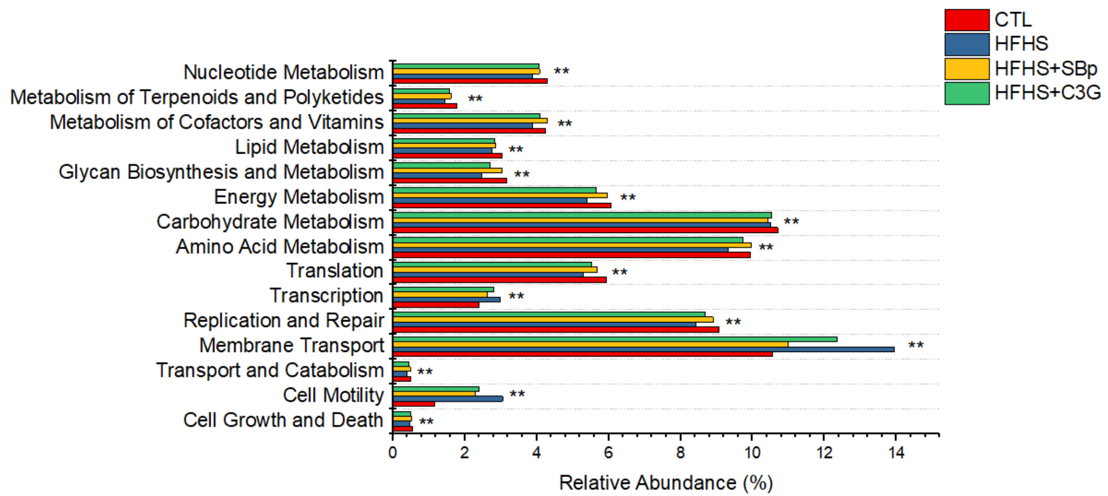

Figure 3-2-11 Function prediction of HFHS diet supplemented with or without SBp or C3G through PICRUSt. Differences in relative abundance (%) in metabolic pathways among four groups were in the form of bar plot. Values were expressed as averages (n=8/group). *, **: P<.05 or .01 showing ANOVA result among four groups.

Part 3: Dose-response of Saskatoon berry powder on high-fat high-sucrose diet-induced insulin resistance and gut dysbiosis in mice

Our previous study found that 5% SBp inhibited HFHS diet-induced hyperglycemia, hyperlipidemia, insulin resistance and altered gut microbiota composition in male C57 BL/6J mice (Huang et al., 2020; Zhao et al., 2019). Since the dose-response of SBp supplemented in HFHS diet on glucose or lipid metabolism has not been documented, we examined the effects of HFHS diet supplemented with 1% and 2.5% of SBp on glucose, lipids, insulin resistance and gut microbiota in mice in the following experiment. In this part, I was involved in animal experiments, sample collection and microbiota data analysis.

Material and method

Dietary Intervention

A total of 32 male C57 BL/6J mice (6 weeks of age) were obtained from Jackson Laboratory (Bar Harbor, ME). After arrival, the mice were housed and stabilized as described above. Mice were randomized into 4 groups (n=8/group) for following diets: control group fed with D12450K low-fat diet; HFHS group with D12492 HFHS diet; 1% SBp group fed with HFHS diet supplemented with 1% SBp (w/w); 2.5% SBp group fed with HFHS diet supplemented with 2.5% SBp (w/w). Mice had access to food and distilled water *ad libitum* during 11-week dietary intervention.

Food intake and body weight

Food intake and body weight were measured as described in Part 2.

Blood collection

Blood was collected as described in Part 1 for the measurement of biochemical indicators.

Diabetes-related biochemical parameters

The plasma levels of fasting glucose, insulin, cholesterol and triglycerides were measured using the commercial assay kits as described in Part 1. HOMA-IR was calculated using the measured data from fasting plasma insulin and glucose as described in part 1.

Measurement of Inflammatory markers

The plasma levels of inflammatory markers were also measured using the same kits as described in Part 1.

Fecal sample collection

Fecal samples were collected as described in Part 2.

DNA extraction and sequencing

DNA was extracted and sequenced as described in Part 2.

Bioinformatic analysis and statistics

Sequence data generated were analyzed using the same pipeline and statistical methods described in Part 2.

Results

Effects of 1% and 2.5% SBp on plasma levels of glucose, cholesterol and triglycerides

Fasting plasma glucose levels were different among the four groups. Mice fed with HFHS diet had the highest level of glucose, while mice in control group had the lowest glucose level among all groups. Mice received 1% SBp had a significantly lower level of glucose compared to those fed with HFHS diet only ($P<.01$). The level of glucose

was further decreased in mice treated with 2.5% SBp; however, no significant difference was found between 1% and 2.5% SBp groups. The changes in the levels of cholesterol and triglycerides in fasting plasma among the 4 groups had a similar pattern as that for glucose. The HFHS group had the highest levels of cholesterol or triglycerides among the 4 groups, and control group had the lowest. Plasma levels of cholesterol and triglycerides in both 1% SBp and 2.5% SBp group were significantly lower than HFHS group with no significant difference detected between 1% SBp group and 2.5% SBp group (Figure 3-3-1).

Effects of 1% SBp and 2.5% SBp on insulin and HOMA-IR

The control group had the lowest levels of plasma insulin concentration and HOMA-IR. And the plasma insulin level in the HFHS group was significantly higher than the other three groups. The supplementation of 1% or 2.5 % SBp in HFHS diet significantly reduced plasma insulin compared to mice fed with HFHS diet alone. The level of HOMA-IR in the HFHS group was the highest among four groups. A significant decrease in HOMA-IR was detected in mice fed with diet containing either 1% or 2.5% SBp compared to mice receiving HFHS diet alone ($P<.01$) Figure 3-3-2).

Effects of 1% and 2.5% SBp on levels of inflammatory markers

The result indicated that the control group mice had the lowest levels of inflammation based on their lowest levels of MCP-1 and PAI-1 compared to other 3 groups. The addition of 1% or 2.5 % SBp to HFHS diet significantly reduced the two inflammatory markers compared to mice exclusively fed with HFHS diet. No significant difference was detected between 1% SBp group and 2.5% SBp (Figure 3-3-3).

Effects of 1% and 2.5% SBp on gut microbiota

The β-diversity showed that groups were separate well except for the HFHS group and 1% SBp group, in which around half dots in each group were overlapped (Figure 3-3-4). On phylum level, microbial composition was dominantly constituted by

Bacteroidetes and *Firmicutes* in the gut of the mice from all four groups. Opposite to the control group, mice fed with HFHS diet had a microbial community with a low level of *Bacteroidetes* and a high level of *Firmicutes*. Addition with either 1% SBp or 2.5% SBp into HFHS diet did not significantly increase the abundance of *Bacteroidetes* or decrease the level of *Firmicutes*. The control group and HFHS group also significantly differed in the ratios. However, Groups with 1% or 2.5% SBp did not show any significant change in the ratios compared to HFHS group (Figure 3-3-5).

Relative abundance analysis showed that several bacteria, including *Akkermansiaceae*, *Bacteroidaceae*, *Lachnospiraceae*, *Lactobacillaceae*, *Muribaculaceae* and *Ruminococcaceae*, predominated in the gut microbiota profiles. Mice fed with HFHS diet had a different microbial profile with higher relative abundances in *Bacteroidaceae*, *Lachnospiraceae*, *Lactobacillaceae* and lower relative abundances in *Muribaculaceae*, compared to control group. Adding 1% SBp or 2.5% SBp to HFHS diet increased the *Lachnospiraceae*, *Muribaculaceae* and *Ruminococcaceae* compared to the HFHS diet alone. However, the relative abundances of those family bacteria in mice receiving SBp were not significantly different from the mice fed with HFHS diet alone (Figure 3-3-6).

The relative abundance of *Muribaculaceae* was a key bacterial family upregulated by SBp or C3G supplementation in HFHS-fed mice (Part 1 and Part 2). In the study on the dose-response of SBp, *Muribaculaceae*, negatively correlated with fasting plasma glucose, cholesterol, triglycerides, insulin, HOMA-IR and MCP-1 when control group was included ($P<.01$). The results were consistent with the previous experiments, and suggested that *Muribaculaceae/S24-7* may be a family of bacteria targeted by SBp (Figure 3-3-7).

Comparison of effects of 1%, 2.5% and 5% SBp supplemented into HFHS diets

Exclusive HFHS group had the highest relative increase in plasma glucose compared to control group (about 40% increase), while the other three groups with SBp added

(HFHS+1% SBp and HFHS+2.5% SBp from part 3, and HFHS+5% SBp from part 1) had significantly lower relative changes in plasma glucose (P<.01), with no significant difference detected among groups (Figure 3-3-8 A). Exclusive HFHS diet had the highest relative change in insulin among groups. Compared to HFHS+1% SBp and HFHS+2.5% SBp, HFHS+5% SBp had a significant lower relative change (P<.01) in plasma insulin level (Figure 3-3-8 B). HFHS+5% SBp group also had a lower relative change in HOMA-IR compared to HFHS and HFHS+1% SBp groups (P<.01), while no significant difference was found between HFHS+2.5% SBp and HFHS+5% SBp (Figure 3-3-8 C).

The linear regression results showed significant negative correlations between relative changes of insulin and the relative abundance of *Muribaculaceae* in mice treated with HFHS diet supplemented with different dosages of SBp (P<.01). The significant negative correlations were also found in the relative changes of HOMA-IR (P<.05). However, the relative change of glucose showed the tendency of negative correlation with the relative abundance of *Muribaculaceae* but the correlation was not significant (P>.05). Significantly negative correlation between *Muribaculaceae* and insulin or HOMA-IR in the mice implied a potential causal role of *Muribaculaceae* in the reduction of insulin resistance in mice receiving SBp-containing HFHS diet (Figure 3-3-9).

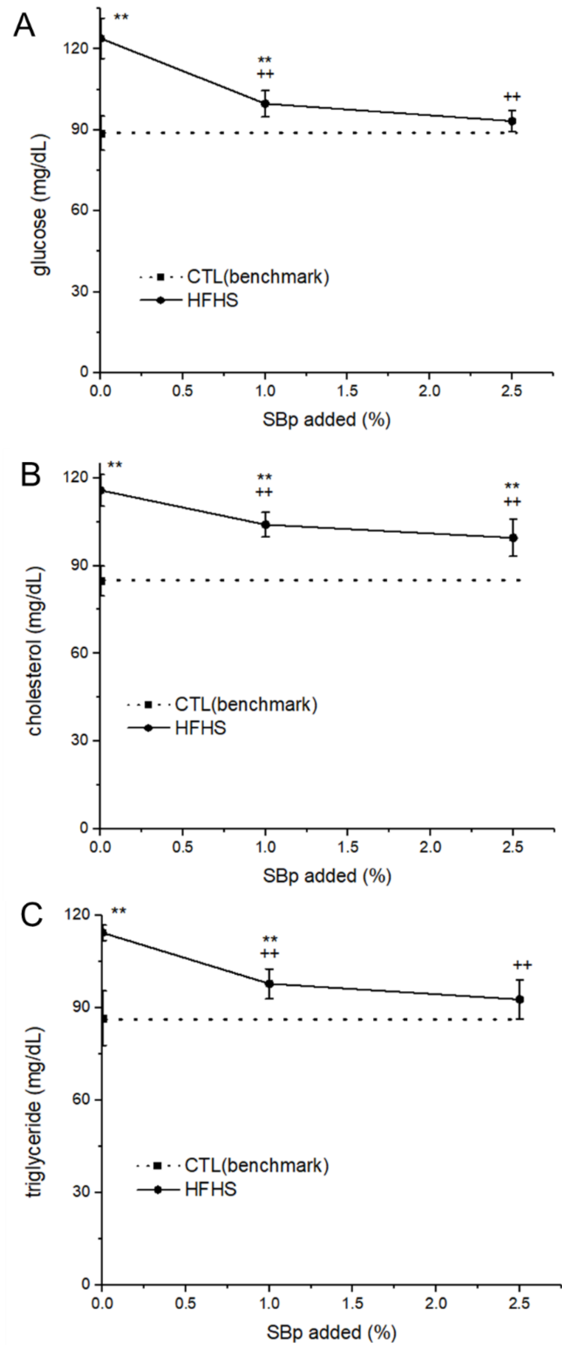

Figure 3-3-1 Levels of glucose, cholesterol and triglycerides in plasma of mice fed with HFHS diets supplemented 1% or 2.5% SBp. Values were expressed as mean ± SD (n = 8/group). *, **: P<.05 or 01 vs. CTL group; +, ++: P<.05 or .01 vs. HFHS group.

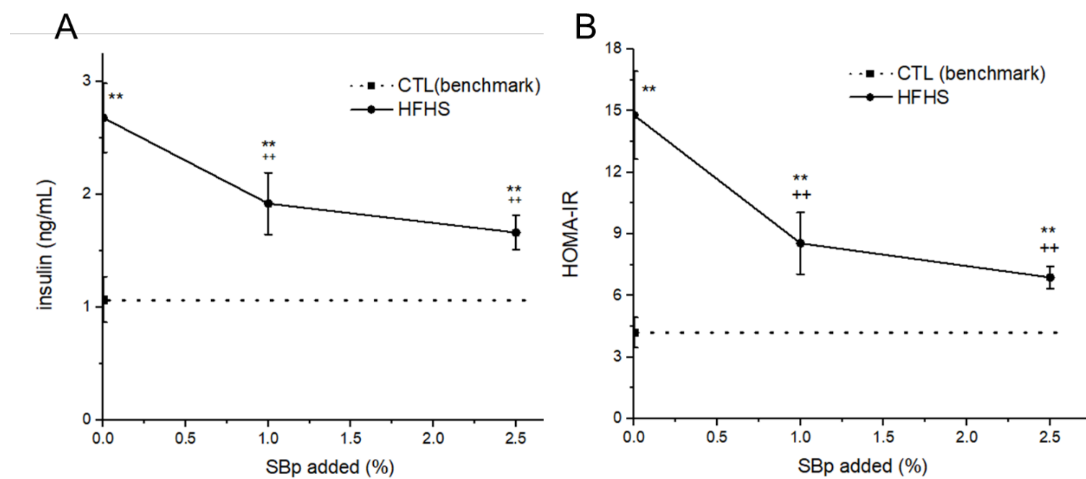

Figure 3-3-2 Effects of HFHS diets supplemented with 1% or 2.5% SBp on insulin and insulin resistance in mice. A: Insulin. B: HOMA-IR. Values were expressed as mean ± SD (n = 8/group). *, **: P<.05 or 01 vs. CTL group; +, ++: P<.05 or .01 vs. HFHS group.

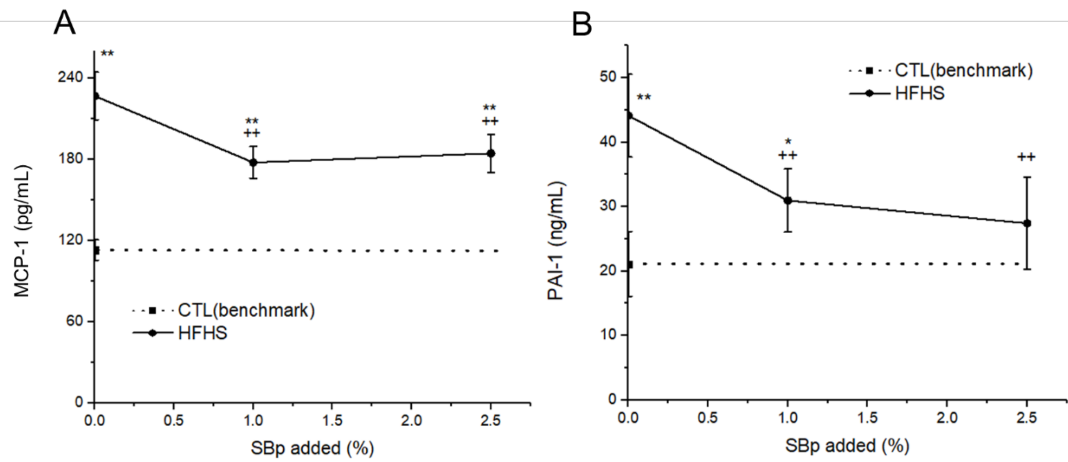

Figure 3-3-3 Levels of inflammatory markers in the plasma of mice receiving the HFHS diet supplemented with 1% or 2.5% SBp. A: MCP-1. B: PAI-1. Values were expressed as mean ± SD (n = 8/group). *, **: P<.05 or 01 vs. CTL group; +, ++: P<.05 or .01 vs. HFHS group.

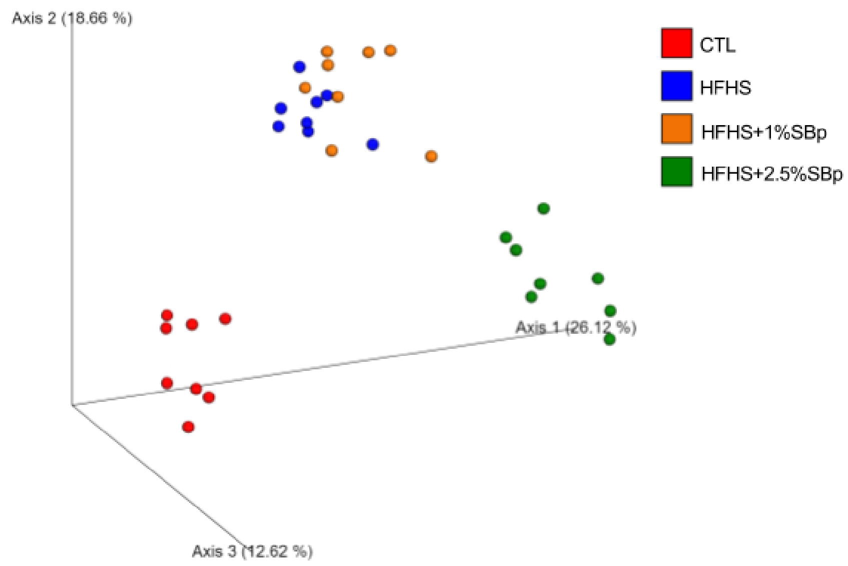

Figure 3-3-4 Effect of the HFHS diet supplemented with 1% or 2.5% SBp on β-diversity in mice. The PCA analysis was based on Bray–Curtis dissimilarities between all sample sets.

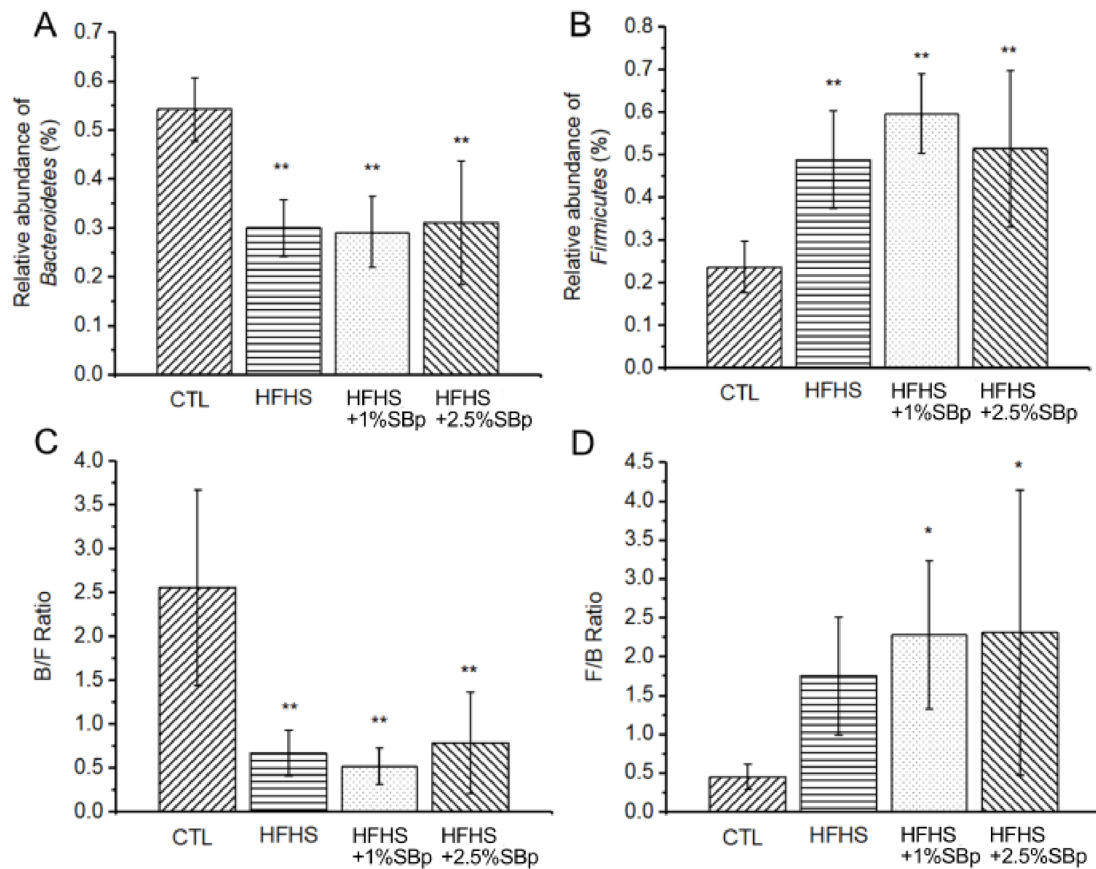

Figure 3-3-5 Effects of HFHS diet supplemented with 1% or 2.5% SBp on the relative abundance of *Bacteroidetes* and *Firmicutes*. A: Relative abundance (%) of *Bacteroidetes* in gut microbial composition. B: Relative abundance (%) of *Firmicutes* in gut microbial composition. C: Ratio of B/F in gut microbiota. D: Ratio of F/B in gut microbiota. Values were expressed as mean ± SD (%) (n = 8/group). *, **: P<.05 or 01 vs. CTL group; +, ++: P<.05 or .01 vs. HFHS group.

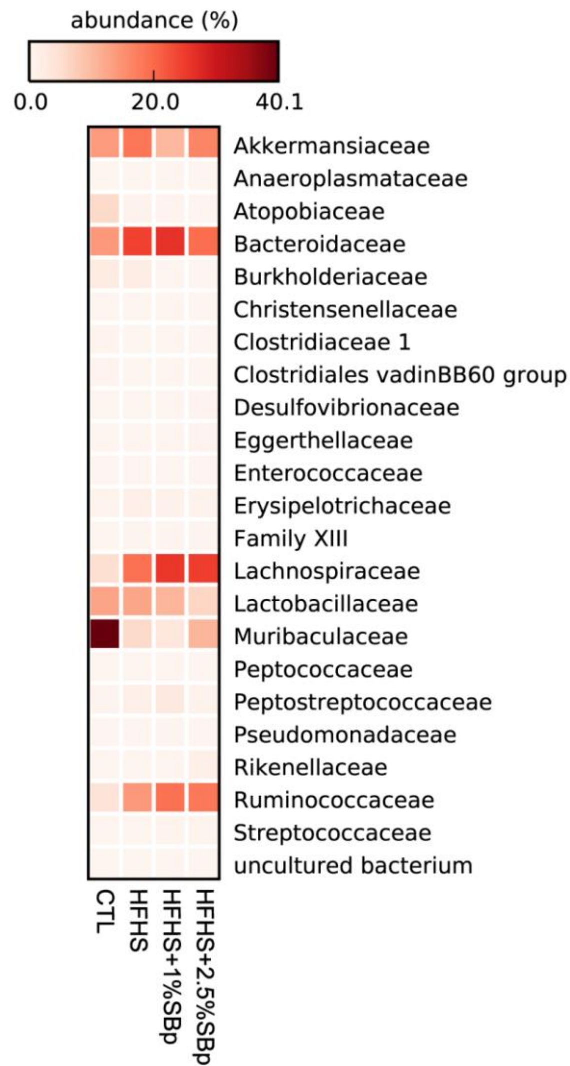

Figure 3-3-6 Effect of HFHS diet supplemented with 1% or 2.5% SBp on the relative abundance of gut microbiota on family level.

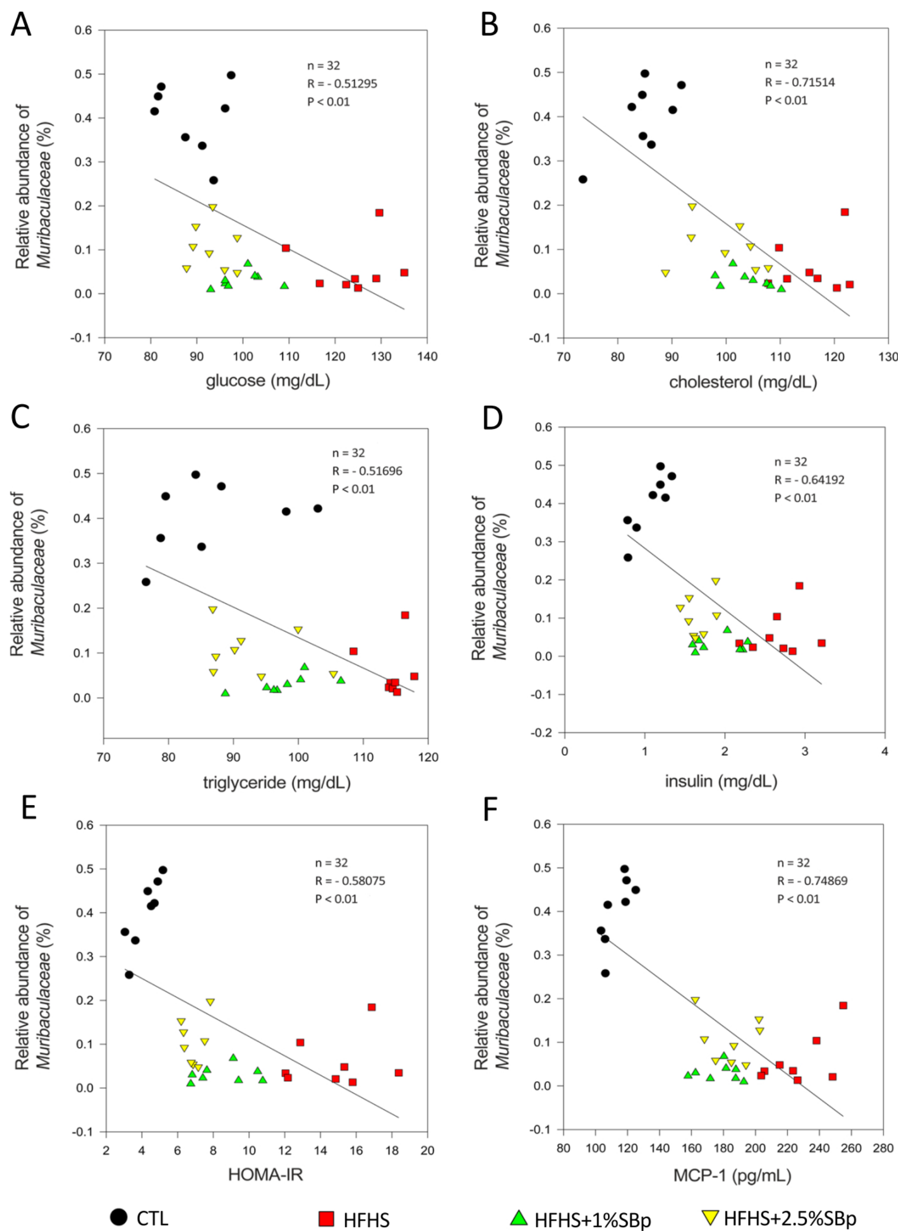

Figure 3-3-7 Linear regression analysis of correlations between the relative abundance of *Muribaculaceae* and metabolic variables or inflammatory markers in plasma of the studied mice. A: Glucose. B: Cholesterol. C: Triglyceride. D: Insulin. E: HOMA-IR, and F: MCP-1 (n=32).

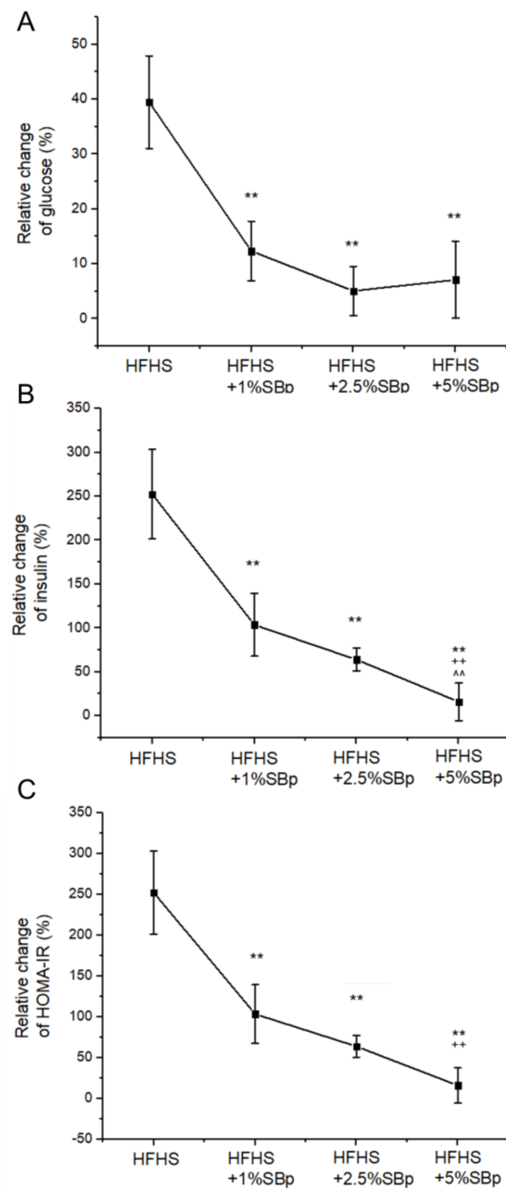

Figure 3-3-8 Comparison of relative changes in glucose, insulin and HOMA-IR of HFHS group, HFHS+1% SBp group, HFHS+2.5% SBp group and HFHS+5% SBp group. Relative changes were calculated by comparison to the control group in the corresponding study part. A: Relative change of glucose B: Relative change of insulin C: Relative change of HOMA. Values were expressed as mean ± SD (n = 8/group). *, **: P<.05 or 01 vs. HFHS group; +, ++: P<.05 or .01 vs. HFHS+1%SBp group; ^, ^^: P<.05 or 01 vs. HFHS+2.5%SBp group.

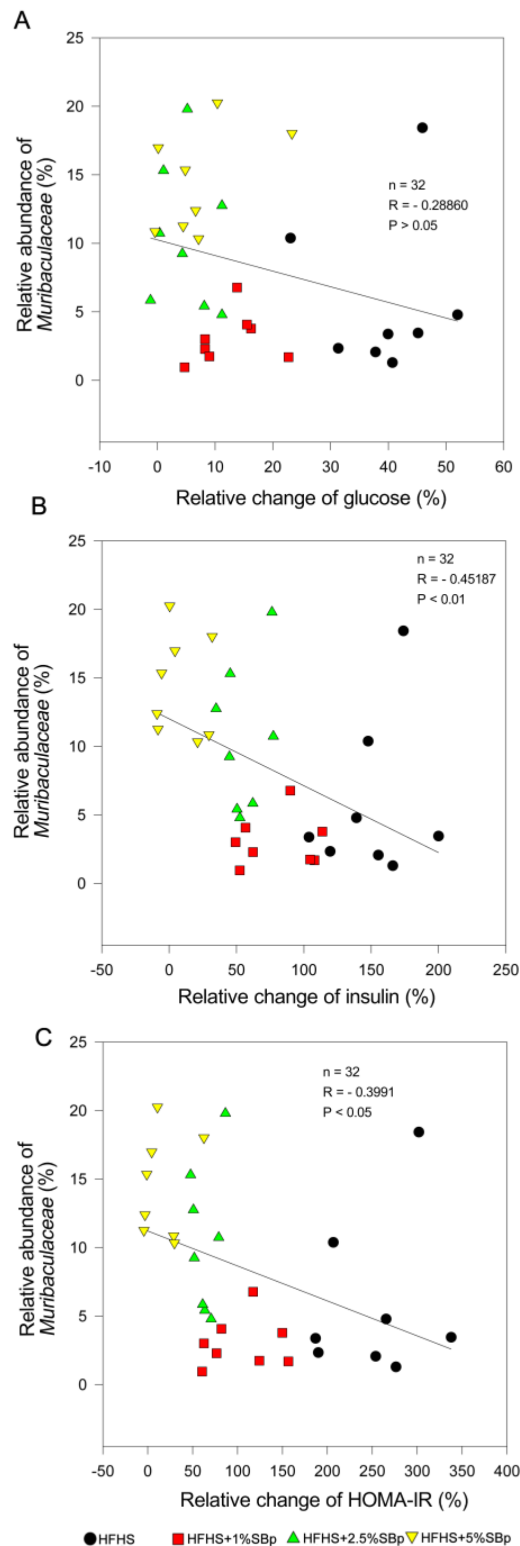

Figure 3-3-9 Linear regression analysis of correlation between the relative abundance of *Muribaculaceae* and relative changes of glucose, insulin and HOMA-IR, respectively. A: Glucose. B: Insulin. C: HOMA-IR.

CHAPTER 4: DISCUSSION

Major novel findings of the studies

This study originally demonstrated that the supplementation of 1-5% of freeze-dried SBp through oral administration effectively reduced the levels of key metabolic markers for T2D, including fasting plasma glucose, insulin resistance, cholesterol and triglycerides in HFHS diet-induced obese and insulin resistant mice. The intake of SBp also attenuated the levels of inflammatory markers in the plasma. Those findings indicate that SBp can improve metabolic and inflammatory status in people with high risks of type 2 diabetes as a functional food.

This is also the first project that explored the impact of Saskatoon berry on gut microbiota. The PCA analysis results demonstrated that the profiles of gut microbiota in mice receiving ≥2.5% of SBp supplemented were well separated from those in mice fed with HFHS diet or control diet. The addition of SBp into HFHS diet significantly altered the compositions of gut microbiota on both phylum and family level. SBp increased the relative abundance of *Bacteroidetes* (B/F ratio) and decreased that of *Firmicutes* (F/B ratio). On family level, SBp increased the relative abundance of *S24-7/Muribaculaceae* in HFHS diet-fed mice. *S24-7/Muribaculaceae* is a family of bacteria with over 600 members commonly found in mice and humans and often associated with increased levels of short chain fatty acids (SCFAs) (Lagkouvardos et al., 2019). Acarbose as an anti-diabetic agent was found to contribute to the increased levels of *Muribaculaceae* and propionate in the mouse gut (Smith et al., 2019). Those findings indicated that the anti-diabetic effect of SBp may be mediated through the modification of bacterial composition and related metabolites in the gut.

C3G is the second most abundant anthocyanin in Saskatoon berry fruits. Previous studies demonstrated that C3G increased the activation of AMP-activated protein kinase, a key modulator of glucose metabolism, in skeletal muscle of mice (Yamashita

et al., 2016). C3G also augmented glucose-induced insulin secretion in pancreatic β-cells and glucose uptake in hepatocytes (Luna-Vital & Gonzalez de Mejia, 2018). Results presented in the part 2 of this thesis demonstrated that supplementation of C3G significantly lowered fasting plasma glucose, lipids, insulin resistance in HFHS diet induced insulin resistant mice, which supported the previous findings on the effects of C3G (Luna-Vital & Gonzalez de Mejia, 2018; Yamashita et al., 2016). Our study revealed that the supplementation of C3G enhanced the abundances of *Bacteroidetes* phylum and *Muribaculaceae* family in HFHS diet-fed mice, which may explain the beneficial effects of Saskatoon berries in glucose metabolism. The inhibitory effects of C3G on glucose, lipids, insulin resistance, inflammation and gut microbiota were comparable to SBp containing similar amount of C3G. The findings suggest that C3G potentially may contribute to the beneficial effects of SBp in HFHS diet-fed mice.

Although previous studies examined the effects of 0.1-20% of SBp on monocyte adhesion (Zhao, Le, et al., 2014), the dose response of SBp on metabolic or microbial effects had not been documented in either animal or human studies. Due to the interest to know whether lower dosages of SBp could still effectively lower glucose and correlate with the disorders in metabolic and gut microbiota, the Part 3 experiment was conducted.

In the Part 3 study, two different concentrations of SBp were used: 1% and 2.5%. Biochemical results showed that supplementation of 1% SBp to HFHS diet had the ability to lower the levels of fasting plasma glucose, cholesterol, triglycerides, insulin resistance and inflammatory markers. The results of PCA demonstrated that the compositions of gut microbiota in mice receiving HFHS+1% SBp were partially separated from HFHS diet-fed mice, which suggested low doses of SBp may only partially alter the gut microbiota. The levels of fasting plasma glucose in mice receiving HFHS+2.5% SBp were also significantly lower than those receiving HFHS diet alone. However, no significant difference in glucose levels was found between HFHS+1%

SBp and HFHS+2.5% SBp group. Moreover, β-activity of gut microbiota in HFHS+2.5% SBp group was separate from that of HFHS group. In addition, analysis of relative changes showed that 5% SBp further inhibited the increase in insulin or HOMA-IR induced by HFHS diet compared to low dosages of SBp. A collection of results suggested that the impact of SBp on metabolism and gut microbiota could be dose-dependent.

Hypoglycemic effects of berries and possible mechanism

Berries and berry products have been considered as healthy food and have gained continuing popularity due to varieties of contained nutrients, low calories and unique flavorful taste. Berries not only are rich in macronutrients such monounsaturated fats and polyunsaturated fats, but also contain more vitamins and minerals, such as vitamin K and manganese, compared to other fruits such as apples. In recent decades, research on the benefits of berry consumption extended from antioxidant property to interaction with glycemia. In spite of having components that may elevate the blood glucose such as glucose and fructose, many berries that have similar nutritional structures as Saskatoon berry were proven to stabilize glucose level.

Previous human study demonstrated that sea buckthorn berry suppressed postprandial hyperglycemia and maintained glucose level in the form of both whole berries and ethanol extracts (Lehtonen et al., 2010). Glucose-stabilizing ability of ginseng berry was found in mice through a glucose tolerance test study in which intake of Panax ginseng berry extract of 150 mg/kg decreased glucose excursion by 46 % compared to control group (Attele et al., 2002). Consecutive administration of American ginseng berry juice of 0.6 mL/kg body weight for 10 days resulted in significant decrease in fasting blood glucose levels, with even a maintained effect observed after termination of administration (Xie et al., 2007).

In the dose-response study, although the plasma level of glucose was significantly

decreased after adding SBp (1%, 2.5% or 5%) to HFHS diet, higher concentrations (2.5% or 5%) of SBp did not cause further significant decrease in glucose level. However, this dose dependency was found in other berries in glucose regulation. Two different doses of American Ginseng Berry Juice, 50 mg/kg body weight and 150 mg/kg body weight, were used in a mouse model, and almost 20% lower level of fasting plasma glucose was found in 150 mg/kg group compared to 50 mg/kg group (Xie et al., 2007). Time dependence study with Chinese juniper berries showed that a single oral administration decreased plasma glucose level gradually with the time of the treatment (Ju et al., 2008). Those findings suggest that optimization of regimen for the hypoglycemic berries is important for an effective glucose control.

Although hypoglycemic effect was found in both Saskatoon berries and many other berries, the key functional ingredients in berries have not been fully uncovered. Since anthocyanins are major phytochemicals contained in many berry fruits, their involvement in glucose uptake and absorption were focused in recent decades. Such studies provided possible mechanism to elucidate the hypoglycemic effect of many berries. For example, study on the effect of delphinidin found that daily oral application of delphinidin for 4 months not only lowered plasma glucose level significantly, but also inhibited sodium-dependent glucose transport in jejunum mucosa (Hidalgo et al., 2014). C3G increased the translocation of glucose transporter-4, a key regulator for glucose uptake and insulin resistance, in skeletal muscle cells in mice (Yamashita et al., 2016). Besides, C3G-rich bayberry fruit extract (CRBFE) was reported to protect pancreatic β cells and upregulate pancreatic duodenal homeobox 1 gene expression responsible for encoding glucose transporter 2 (Sun et al., 2012). The findings imply that glucose-transporters in peripheral tissue are possible targets for anthocyanins to reduce intestine glucose absorption and glucose uptake.

The results of the present study showed that the supplementation of SBp or C3G relieved insulin resistance. The findings from other studies also support the insulin

resistance-alleviating capability of anthocyanin-rich berries. For example, jaboticaba berries, a type of Brazilian berries enriched with anthocyanins and potent antioxidant capacities, revealed that the intake of high-fat diet supplemented with 4% jaboticaba peel significantly reduced insulin resistance (Batista et al., 2017). The mechanism of berries responsible for insulin resistance alleviation is possible through regulating the insulin receptor substrate (IRS). Research found that Ginseng berry extract upregulated IRS with increased insulin sensitivity, suggesting that Ginseng berries may facilitate insulin function through modulation of IRS (Seo et al., 2015).

Although the present study first found the hypoglycemic effect of Saskatoon berries. Saskatoon berry is not the first berry that is reported to have such effect. The findings on the mechanisms from other studies, such as the regulation of glucose transporter and IRS, may help shed light on the mechanisms of the glucose-lowering and insulin resistance-reliving ability of Saskatoon berries.

Hypolipidemic effects of berries and possible mechanism

The present study demonstrated that SBp administration significantly decreased the plasma levels of cholesterol and triglyceride elevated by HFHS diet in mice. The findings on hypolipidemic effect in our study are consistent with a number of previous studies on other berry products. For example, daily consumption of 500mg aronia berry extract decreased fasting plasma total cholesterol by 8% and low-density lipoprotein cholesterol (LDL-C) by 11% (Xie et al., 2017). Bilberry was also proven to significantly reduce LDL-C and increase high-density lipoprotein-cholesterol (HDL-C) in clinical trials (Zhu et al., 2015).

The effect of C3G on plasma lipids in our study was also supported by other studies using either anthocyanin-rich extracts or C3G. For example, both black soybean-derived anthocyanins and black rice extract modulated the lipid profile by increasing HDL-C concentration and decreasing the plasma levels of triglyceride, total cholesterol

and non-HDL cholesterol (Kwon et al., 2007; Xia et al., 2006). Incubation of the whole blood from hypercholesterolemic patients with C3G resulted in statistically significant reduction in cholesterol concentration in erythrocytes (Duchnowicz et al., 2012).

The mechanism of the hypolipidemic effect of SBp was not reported previously. However, based on the studies of other berries, the inhibition of lipid aggregation is a possible mechanism for maintaining normal lipid level. For example, the lipid aggregation-inhibiting ability of berries was demonstrated by a decrease in the rate of platelet aggregation in healthy normolipidemic men after taking sea buckthorn berry oil 5 g per day for 4 weeks, which also suggested the ability of berry consumption to prevent blood clotting (Johansson et al., 2000). Platelet aggregation was also found to be inhibited in studies with other anthocyanin-rich fruits such as bilberry, blueberry and cranberry (Wightman & Heuberger, 2015).

C3G was also found to decrease the plasma level in our study, implying that anthocyanins may play a regulating role in lipid metabolism. Processes such as synthesis, oxidation and transportation of lipids are reported to be improved in other supporting studies with anthocyanin supplementation. For example, significantly reduced activities of lipogenic enzymes cooccurred with decreased plasma cholesterol and triglycerides after administration of 150 mg /kg body weight black rice-derived C3G for 4 weeks (Um et al., 2013). Besides, both decrease in fatty acid synthesis and increase in fatty acid oxidation were found after Mulberry-derived anthocyanins (MAE) were provided (Chang et al., 2013). Fecal bile acid facilitates the elimination of cholesterol from the body. C3G was also found to promote fecal bile acid excretion, implying that the hypocholesterolemic activity of C3G was, at least in part, mediated by activating bile acid excretion (Wang et al., 2012).

In our study, we found the plasma lipid-lowering effect of SBp. Although the hypolipidemic components in SBp are not fully understood, C3G might be one of the

effective compounds in SBp, supported by the evidence showing the ability of C3G to inhibit fatty acid synthesis, facilitate fatty oxidation and promote bile acid excretion.

Inhibition of inflammation and possible mechanisms

Anti-inflammatory effects of supplementation of SBp were detected in the present study characterized by reduced inflammatory markers including TNF-α, MCP-1 and PAI-1. TNF-α, MCP-1 and PAI-1 are produced by vascular endothelial cells and adipocytes under inflammatory stimuli. TNF-α is a key inflammatory cytokine and implicated in the regulation of multiple inflammatory reactions. MCP-1 is a chemokine mediating the chemotactic reaction of monocytes (Fain, 2006). And PAI-1 is a physiological inhibitor for urokinase and tissue plasminogen activator. Recent studies demonstrated that PAI-1 is an inflammatory mediator and actively involved in monocyte adhesion (Zhao et al., 2017; Zhao, Ren, et al., 2014). Besides those three inflammatory markers used in the study, other common inflammatory markers may include interleukin-6 (IL-6) and interleukin-1β (IL-1β).

In addition to Saskatoon berries, the anti-inflammatory effect can be also found in many other berries. For example, research discovered that aronia berry inhibited IL-6 stimulated by IL-6 in murine splenocytes (Martin et al., 2014). Administration of Korean ginseng berry extract (KGBE) inhibited the expression of inflammatory cytokines in primary macrophages, including TNF-α and interleukin-1β (IL-1β) (C. K. Kim et al., 2012). Moreover, European black nightshade-derived compounds also showed its anti-inflammatory ability with significant inhibitory effects on the LPS-induced IL-6 and IL-1β production (Wang et al., 2017). Kiwi berry extracts suppressed the secretion of pro-inflammatory cytokines, including IL-6 and TNF-α in murine macrophage cell line (An et al., 2016).

The supplementation of a major anthocyanin in SBp, C3G, in HFHS diet also reduced the levels of inflammatory markers in mice in the present study, which is comparable

to that in mice fed with SBp containing equal amount of C3G, implying that the anti-inflammatory effect of SBp may be attributed at least partially to the contained anthocyanins. Moreover, the anti-inflammatory effect of anthocyanins has also been observed in other experimental and clinical studies. In a human study, a total of 150 subjects with hypercholesterolemia had decreased levels of plasma cytokine IL-1β after being treated with purified anthocyanin mixture (320 mg/day) for 24 weeks (Zhu et al., 2013). Research with white adipose tissue also showed that serum concentrations of inflammatory cytokines, including TNF-α, IL-6 and MCP-1, were reduced by C3G (Guo et al., 2012). In addition, cell studies on human macrophages found that the black currant extract rich in C3G inhibited the LPS-induced secretion of IL-6 in macrophages (Desjardins et al., 2012).

Two pathways might be involved in the anti-inflammatory effect of anthocyanins: nuclear factor-κB (NF-κB) pathway and mitogen-activated protein kinases (MAPKs) pathway. Both pathways modulate gene expression under varieties of conditions such oxidative stress from reactive oxygen species (ROS). The stimuli that trigger the activation of those pathways contribute to the inflammatory response, and compounds that neutralize this process help inhibit inflammation occurrence. Anthocyanins from blueberry, blackberry and blackcurrant were observed to significantly decrease cellular reactive oxygen species (ROS) levels with a concomitant decrease in mRNA levels of both IL-1β factor and TNF-α (Lee et al., 2014). Sea buckthorn berries were also reported to markedly reduce the expression of both TNF-α and IL-1β by increasing superoxide dismutase (SOD) (Zhang et al., 2017). Cherry juice, containing C3G, cyanidin-3-rutinoside, pelargonidin-3-glucoside and pelargonidin-3-rutinoside, showed a strong antioxidant activity by increasing levels of SOD and decreasing lipid peroxidation in the mice consuming the cherry juice (Šarić et al., 2009).

In the present study, inflammatory markers were also found to be lowered by C3G. Supportive evidence from the other anthocyanin-containing berries suggests that the

anti-inflammatory effect of Saskatoon berries may result from C3G or other phytochemicals with similar capacity.

Modulation of gut microbiota and implications

Gut microbiota plays a key role in host physiology and metabolism. Both whole berry fruits and berry extract play an important part in the modulation of gut microbiota (Etxeberria et al., 2013). Many berry-derived phytochemicals may have poor bioavailability for animal gastrointestinal tract due to lack of specific enzymes for degradation and utilization in animal physiological systems, which, however, raises the question about how berries and their components actually impact on health. Recent research pointed out that the main site for metabolizing the complex polyphenols into smaller phenolic compounds may be through the action of gut microorganisms. Specifically speaking, berry fruits and berry extracts lead to an increase in *Bifidobacterium*, *Lactobacillus* and *Akkermansia*, suggesting that a prebiotic-like effect may exist in berries and their contained compounds (Lavefve et al., 2020).

In fact, berry fruits and gut microbiota may have a mutual effect, since some phytochemicals in berries are utilized by certain bacteria, which might in turn affect the abundance of those bacteria and the profile of gut microbiota. The bioavailability of ellagitannins is an example. As an abundant phytochemical in strawberries, raspberries, and cloudberries, its concentration in the body appears to be dependent on the composition of gut microbiota (Puupponen-Pimiä et al., 2013). Another common type of polyphenol, anthocyanins, was also found to be catabolized by gut microbiota. A study showed that incubation of three single gut microbial strains, *Enterobacter cancerogenous*, *Bifidobacterium dentium* and *Dorea longicatena*, with elderberry-derived anthocyanins caused the degradation of anthocyanins, implying that anthocyanins might be largely transformed into other forms of metabolites in the gut environment (Bresciani et al., 2019).

Similar to our result on *Bacteroidetes* and *Firmicutes*, many studies found similar changes in either the relative abundance or the ratio of those two by berry-derived component-containing diets. A study in mice using 16S rRNA gene sequencing technique revealed that the HF diet-increased *Firmicutes*/*Bacteroidetes* ratio was reduced by honeyberry polyphenols. And bacterial genera belonging to the phylum *Firmicutes*, such as *Staphylococcus*, *Lactobacillus*, *Ruminococcus* and *Oscillospira*, were decreased by the polyphenols (Wu et al., 2018).

Berry extract may also promote the growth of certain bacteria as well as change the overall gut micriobiota profile. In a study with aronia extract, the growth of *Anaerostipes* was found to increase about 10% with *Bacteroides* increased by 193% (Istas et al., 2019). Jaboticaba extract also was found to alter gut microbiota profile by increasing *Lactobacillus*, *Bifidobacterium* and *Enterobacteriaceae* (Silva-Maia et al., 2019). Sea buckthorn berry juice enriched with polyphenols increased the abundances of *Lactobacilli*, *Bacteroides* and *Bifidobacteria* in vitro continuous gut model (Attri & Goel, 2018). Moreover, blueberries altered microbiota composition with an increase in *Gammaproteobacteria* with the normalization of TNF-α and IL-1β in HF diet-fed rats (Lee et al., 2018).

Studies also have been trying to find the common characteristics of those berry-enriched bacteria along with health improvement. Study with Goji berry found that supplementation of this fruit promoted *Lachnospiraceae* and *Ruminococcaceae* family bacteria. Butyryl coenzyme A (CoA) transferase is a key enzyme responsible for butyrate synthesis, which was increased in the fecal samples of Goji berry group, implying the mechanism of berry function may lie in the increase of butyrate-producing bacteria (Kang et al., 2018). Butyrate is one of SCFAs resulting from dietary fiber fermentation by gut microbiota in the colon (Wong et al., 2006). Changes in the profile of gut microbiota co-exist with alteration in the levels of SCFAs. Childhood obesity was found to be associated with an altered gut microbiota characterized by increased

levels of *Firmicutes* and decreased levels of *Bacteroidetes*, which is also associated with the changes in SCFAs (Riva et al., 2017).

Besides butyrate, the other two common SCFAs, acetate and propionate, were also found to be associated with supplementation of berries or berry extract. Bilberry anthocyanin extract was found to cause the changes in SCFAs and intestinal mucosal barrier, which was associated with increases in *Lactobacillus*, *Bacteroides*, *Clostridiaceae 1*, *S24-7* and *Lachnospiraceae,* and decreases in *Verrucomicrobia* and *Euryarchaeota* in rats (Li et al., 2019).

However, compared to correlation evidence, fecal microbiota transplantation (FMT) could be a more direct proof of the role of gut microbiota in disease development. FMT transfers gut microbiota from a donor into the gastrointestinal tract of a recipient in order to achieve certain health benefits (Kim & Gluck, 2019). In a study using FMT, blueberry-derived proanthocyanins and anthocyanins were found to improve insulin resistance, and this findings reoccurred in germ-free mice after receiving FMT, giving a piece of direct evidence that anthocyanin-induced improvements in insulin resistance and glucose level may be at least partially caused by changes in the gut microbiota profile (Morissette et al., 2020).

Although FMT is not a core part of our present study, certain bacteria could be targets for future study. For example, *Muribaculaceae* is a candidate since its relative abundance was increased by SBp and C3G and found to be negatively correlated with low levels of glucose, cholesterol, triglycerides, insulin, and insulin resistance. The family of *Muribaculaceae*, also known as *S24-7*, is a predominate member of the mouse gut microbiome and common family in other mammalian gut environments. *Muribaculaceae* was also reportedly involved in translation and carbohydrate metabolism, as well as correlation with SCFAs (Smith et al., 2019). However, information on *Muribaculaceae* coming from 16S rRNA gene studies or short read

metagenomic studies still remains limited (Lagkouvardos et al., 2019).

Besides *Muribaculaceae*, *Akkermansiaceae* is another possibly beneficial family that belongs to *Verrumicrobia*. For example, *Akkermansiaceae* was reported to be associated with low-protein diet with a increase in the abundance (Lai et al., 2019). In another study, both *Akkermansiaceae* and *Burkholderiaceae* showed negative relationships with triglycerides and LDL-cholesterol, with three SCFAs (acetate, propionate and butyrate) positively associated with *Akkermansiaceae* (Wang et al., 2020). In our study, the abundance of *Akkermansiaceae* in the HFHS group was the lowest and that of the control group was the highest. SBp or C3G supplementation relatively increased *Akkermansiaceae*, which implies that *Akkermansiaceae* may contribute to the health benefits of SBp or C3G.

Akkermansia muciniphila, a species of *Akkermansiaceae* family, has been widely studied in humans. *Akkermansia muciniphila* is a bacterium able to degrade goblet cell-produced mucin, which allows intestinal tract to become an ideal habitat for *Akkermansia muciniphila* to live (Derrien et al., 2004). Many studies found that this bacterium decreased in the gut of patients with obesity or type 2 diabetes (Ellekilde et al., 2014; Le Chatelier et al., 2013). Meanwhile, other studies found that *Akkermansia muciniphila* was shown to have probiotic-like properties and adjust diabetes-related metabolic markers. For example, 4 weeks of oral administration of *Akkermansia muciniphila* resulted in a significant increase of high-density lipoprotein and decrease in the hepatic glycogen (Zhang et al., 2018). Inflammation level was also alleviated after supplementation of *Akkermansia muciniphila*, characterized by decreases in the level of LPS after adding this bacterium to streptozotocin-induced diabetic rats (Zhang et al., 2018).

The present study was not able to identify gut microbiota at species level due to the limitation of 16S-rRNA gene sequencing technique. However, future studies using

metagenomics gene sequencing technique may help determine the involvement of *Akkermansia muciniphila* in the SBp-induced health benefits.

Overall, the mechanism for the anti-diabetic effect of Saskatoon berries is complicated and multidimensional. On one hand, berry fruit-derived components or their metabolites are possible to be utilized by some gut bacteria, which might explain the increase in the abundance of some bacteria such as *Muribaculaceae*. On the other hand, multiple pathways could be involved in the metabolism-regulating effect of Saskatoon berries, and gut microbiota might only contribute to the effect partially, indicating the complicacy of anti-diabetic mechanism of Saskatoon berries.

Conclusion

The present study demonstrated that the intake of SBp reduced HFHS diet-induced hyperglycemia, hyperlipidemia, insulin resistance and inflammation in mice. C3G, as a type of anthocyanin in SBp, has a similar ability to lower the levels of metabolic and inflammatory variables in mice. The beneficial effect of SBp is dose-dependent in the mice treated with 1-5% of SBp supplemented HFHS diet. Alterations in gut microbiota were also detected in the mice treated either SBp or C3G. SBp or C3G supplementation increased *Bacteroidetes/Firmicutes* ratio and abundances of *S24-7/Muribaculaceae* in mouse gut compared to mice fed with HFHS diet alone. The changes of the abundances of gut bacteria, particularly *Muribaculaceae* family bacteria, negatively correlated with the metabolic and inflammatory variables in the mice, showing the potential of Saskatoon berries in protecting against metabolic diseases and contributing to the overall health.

Limitations of the researches in the present study

Although the present study showed an anti-diabetic effect of both SBp and C3G, whether other phytochemicals in Saskatoon berries still have such effect remains unclear. In addition, the present study was only conducted in male mice due to the

purpose of simplifying the variables during the first stage of exploring the beneficial effects of SBp and C3G. Besides, the female and male mice might have different metabolic response to the provided diets in the study. Therefore, those effects of Saskatoon berries will be studied in female animals and women in subsequent studies. Periodical changes in hormones need to be taken into consideration when female individuals are included in the future study as well as the statistical methods for multiple variables (diet and sex) within a group. The profiles of gut microbiota were only assessed using 16S rRNA gene sequencing technique in the present study. Metagenomic analysis could be applied for more information in the future study. To fully understand the anti-diabetic mechanism of Saskatoon berries, the bioavailability and pharmacokinetics may also need to be examined in *in vivo* studies.

Future directions and knowledge translation

The anti-diabetic effect of SBp and C3G is likely regulated via a number of metabolic pathways. Thus, future studies will investigate the possible signal transduction pathways for SBp, C3G and their metabolites, which will help to understand the mechanism of the beneficial effects of Saskatoon berries. Since it is not clear whether the *Muribaculaceae* family is involved in preventing the development of diabetes in humans, FMT using identified *Muribaculaceae* may prove the therapeutic efficacy and help to identify the precise mechanism for the beneficial effects of Saskatoon berries and contained active compounds in the future study.

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