EFFECTS OF ISOFLAVONE GENISTEIN AND DAIDZEIN ON TYPE 1 DIABETES THROUGH IMMUNOMODULATION

by

GUANNAN HUANG

(Under the Direction of Tai L. Guo)

ABSTRACT

Type 1 diabetes (T1D), previously referred to as "juvenile diabetes", is alarmingly increasing in young adults. The associations of high soy consumption and risk of T1D are uncertain and intricate, which may depend on the window of exposures and its interaction with the other dietary intake, as epidemiological evidences are limited. Two soy isoflavones of particular significance are genistein (GEN) and daidzein (DAZ). The soy intake in adult T1D patients was associated with protected renal function, while the mechanisms of isoflavone protection on T1D are largely unknown. The current study showed adult GEN intake decreased the hyperglycemia in adult male non-obese diabetic (NOD) mice and reduced the incidence of T1D in female NOD mice. The immunomodulatory effect of GEN is sex-specific, in that the male adults demonstrated an anti-inflammatory response whereas the adult female demonstrated a pro-inflammatory response, as shown by cytokine/chemokine and antibody profiles. To further reveal the mechanisms, the 16s ribosomal RNA (rRNA) genes from feces were sequenced to reflect the abundance and diversity of gut microbes, and GEN dosing could affect both the diversity and the abundance of gut microbiota (GMB) in males toward an anti-inflammatory response, while affecting the abundance GMB in females toward a pro-inflammatory response. An alteration of urinary metabolomic

profile was also observed in males. As a comparison, in severe combined immunodeficiency (SCID) adult female mice, an alteration of GMB, but no alteration of blood glucose level was found following GEN intake. However, there is evidence that soy milk formula consumption in infants was associated with increased T1D risk. Therefore, we also evaluated the effect of GEN from perinatal exposure. The incidence of T1D in offspring with maternal GEN intake reflected an exacerbation effect in female offspring (in accordance with antibodies, cytokines, chemokines, and splenocyte subpopulations); despite no protection among male offspring with regard to T1D incidence and blood glucose level, an immune-modulation toward anti-inflammatory was found. The GMB in the three-month feces from female offspring showed a clear separation and an increase of pro-inflammatory microbes, but not in one-month feces. DAZ intake could also modulating immune homeostasis by increasing T cell numbers and modulating immune system as well. Taken together, our studies on T1D have identified that isoflavones, especially GEN, have a strong sex-specific and exposure window-specific effect in NOD mice that was associated with GMB-related immunomodulation.

INDEX WORDS: type 1 diabetes, genistein, daidzein, gut microbiota, immune, antibody, cytokine/chemokine

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DEDICATION

To GOD be the glory

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

GENISTEIN, DAIDZEIN, TYPE 1 DIABETES, AND GUT MICROBIOTA[1]

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0. Introduction

Autoimmune disease, to be distinguished from autoimmunity [defined as a failure in the mechanisms of immune self-tolerance (Abbas *et al*., 2014)], is a category of diseases that result from a disordered immune response in which autoantibodies or autoreactive immune cells are produced against one's own tissues. It was previously reviewed that the common mechanisms for autoimmunity include, but not limited to: defects in negative selection of T or B lymphocytes, defects in regulatory T (Treg) cells, inadequate function of inhibitory receptors, and activation of antigen presenting cells (APCs) (Abbas, Lichtman and Pillai, 2014). Autoimmune diseases could be either systemic (such as systemic lupus erythematosus) or local/organ specific [such as type 1 diabetes (T1D) and rheumatoid arthritis], and are usually characterized by progressiveness, self-perpetuation, and great disease burden (Joensen *et al*., 2016). Both genetic [e.g., major histocompatibility complex (MHC) I and II, the genes found to be strongly associated with autoimmune diseases] and environmental factors [e.g., infections (Bach, 2005)], are linked to autoimmune diseases.

T1D, also known as insulin-dependent diabetes or juvenile diabetes, is an autoimmune disease in which insulin-secreting pancreatic β cells are selectively destroyed by immune cells (Badami *et al*., 2011). T1D is alarmingly increasing in US (1.25 million in American children and adults) (American Diabetes Association, 2016), and the incidence is higher in males while women with T1D have a 40% excess risk of death (Huxley *et al*., 2015). In T1D patients, people under the age of 18 are mostly afflicted (Bluestone *et al*., 2010), although adult onsets of T1D are becoming more common (Li *et al*., 2016; Menke *et al*., 2013). It should be noted that, for latent autoimmune diabetes in adults, it is unclear in its mechanism whether it is a late manifestation of T1D, an amalgamation of T1D and type 2 diabetes (T2D), or a completely distinct form of diabetes (Basile

et al., 2014). In terms of prevalence, males are more afflicted (males 1.47 times higher than females) (Diaz-Valencia *et al*., 2015) and the mechanisms might include hormones, genetic predisposition, and epigenetics (Ngo *et al*., 2014).

1. Pathogenesis of T1D

A number of immune cell subsets play important roles in the pathogenesis of T1D, and these cells include T cells, B cells, natural killer (NK) cells, and APCs (Li *et al*., 2014a), although the roles of B cells are less clear (Bluestone, Herold and Eisenbarth, 2010). Badami *et al*. (2011) found that the $CD4^+CD25^+FoxP3^+$ Tregs were significantly reduced in T1D patients, and the $CD4^+CD25^+$ T cells could not be converted to Treg cells. It is widely accepted that destructive autoimmunity in T1D mostly depends on a Th1-dominated autoimmune response (Campbell *et al*., 1991; Katz *et al*., 1995), although a Th2-dominated islet autoimmunity occur after a latent period (Shimada *et al*., 1996). In a recent study, it was shown that the ratio of Th17/Th1 plays a role in β cell destruction and T1D (Baharlou *et al*., 2016). Common markers for T1D include autoantibodies for insulin, glutamic acid decarboxylase 65, islet antigen-2 and zinc transporter 8 (Li *et al*., 2014a), although blood glucose level was often used as a readout.

Genetics

The genetic aspects of T1D are well studied, and it is known that more than 20 different regions are associated with the susceptibility, while 40-50% of human T1D risk can be accounted by human leukocyte antigen (HLA, a cluster of genes in MHC)-DR and -DQ genes, the ones that are mostly studied in terms of T1D risk (Pociot and McDermott, 2002). For example, DRB1*0301/0401 heterozygotes are strongly associated with T1D with an odds ratio of 35 (Abbas *et al*., 2014). It has also been reported that approximately 30% of patients with T1D are

heterozygous for HLA-DQA1*0501–DQB1*0201/DQA1*0301–DQB1*0302 alleles (Pociot and McDermott, 2002).

Environment

For environmental factors, the immunity-diet-microbiota consortium has been paid great attention because the diet and microbiota together modulate the maturity of immune system (Mejia-Leon and de la Barca, 2015). It was reviewed by Kagohashi and Otani (2015) that environmental exposure could destroy pancreatic β cells directly (e.g., attacking β cells) or indirectly (e.g., molecular homology or perturbation of immune system). Diet could regulate the gut microbiota through direct (e.g., providing substrate for bacterial digestion) and indirect (e.g., regulation of intestinal and pancreatic physiology) effects (Mejia-Leon and de la Barca, 2015). As mentioned previously, several cell types (e.g., APCs, B cells, T cells, and macrophages) interplay in the pathogenesis of T1D. One subset of T cells, the Tregs that are differentiated from $CD4^{+}$ cells into $CD4^{+}CD25^{+}Foxp3^{+}$ cells, can be induced by commensal gut microbiota and is related to the short-chain fatty acids (SCFAs) production from gut microbiota (this will be discussed in depth later).

There are several epidemiological studies on the risk factors of T1D during developmental stages. Frederiksen *et al*. (2013) reported that among children less than 5 years of age, early exposure to fruit and late exposure to rice/oat are associated with an increased risk of T1D, while breastfeeding at the time wheat/barley consumption showed a protective effect. In another study with Finnish cohort, it was found that the maternal consumption of butter, low-fat margarines, berries, and coffee was negatively associated with the development of advanced β-cell autoimmunity in the offspring (Virtanen *et al*., 2011). In animal studies, low-glycotoxin

environment (e.g., low advanced glycation end products) during fetal or neonatal exposure was shown to prevent T1D (Peppa *et al*., 2003).

2. Animal models

Non-obese diabetic (NOD) mouse

The T1D in NOD mice shows great similarity to human T1D, in that more than 40 loci discovered in both NOD mouse and human play important roles in mediating T1D susceptibility (Noble and Erlich, 2012). NOD mice develop autoimmune diabetes spontaneously and produce autoantibodies and autoreactive T cells as human T1D (Anderson and Bluestone, 2005). In addition, it was found that NOD mice have a significantly increased incidence of T1D in females (60-70%) than in males (20-30%) by 6 months of age (Anderson and Bluestone, 2005). Also, the pancreatic inflammation was higher in NOD females than in males, as represented by the $CD4^+$ cell number per islet (Young *et al*., 2009). The number of $CD4^+CD25^+Foxp3^+$ Treg cells increase with age in NOD mice (Young *et al*., 2009).

Akita mouse

The Akita mouse is a monogenic strain with a spontaneous mutation in the insulin 2 gene. The reduced β cell mass and insulin secretion are found in the mice, leading to hyperglycemia, hypoinsulinemia, polydipsia, and polyuria by 3-4 weeks of age. In contrast to NOD mouse, the Akita males have a higher incidence of T1D than the females. Studies have shown that both the Akita mice and human T1D patients lack a critical component of endoplasmic reticulum response, to which the β cells are vulnerable with (Sonenberg and Newgard, 2001).

Streptozotocin-induced mouse model

Streptozotocin (STZ), a drug commonly used to treat cancer, is able to destroy pancreatic β cells and induce T1D. Several STZ-induced T1D mouse models have been previously validated, such as B6C3F1 (Guo *et al*., 2014) and C57BL/6 mouse (Li *et al*., 2014c).

Rat models of T1D

The common rat models used to study T1D include Biobreeding diabetes-prone (BB-DP) rat (Mordes *et al*., 2004), LEW.1AR1-iddm rat, and Komeda rat (Jorns *et al*., 2014). BB-DP rats, one of the most widely used diabetic rat models, have a similar T1D incidence (~90% between 8 to 16 weeks of age) in males and females (Al-Awar *et al*., 2016). The rats have a reduction of $CD4^+$ T cells, and a near absence of $CD8^+$ T cells. Following initial manifestation of the disease (e.g., the increased expression of interferon-α), the pancreatic islets are infiltrated by macrophages, NK cells, T cells and B cells, leading to a progression of T1D (Al-Awar *et al*., 2016). BB rats are amenable to study the interactions of genetics and environment that may be critical for disease expression in humans; genetic analysis of the BB-DP rat has been accelerated, e.g., Ian4L1, cblb and Iddm4 are now known to play major roles in rat autoimmunity, while data generated in the BB-DP rat have correctly predicted the outcome of several human diabetes prevention trials, notably the failure of nicotinamide and low dose parenteral and oral insulin therapies (Mordes *et al*., 2004). In addition, neonatal exposure has been performed successfully in BB rats (Neu *et al*., 2005). Unless treated with exogenous insulin, hyperglycemic BB-DP rats quickly progress to fatal diabetic ketoacidosis.

3. Soy food

Soy food contains large amount of isoflavones. Soy products are of particular interest since they are an intrinsic part of Asian cuisine (such as soymilk and tofu), and that they are widely used

in vegetarian meals due to its high (about 40%) protein contents (Liu, 2012). As reported by the United Nation Food and Agriculture Organization in the beginning of 20th centaury, the European and North American soy protein consumption is less than 1 g/day per capita, while the consumption is 8.7g, 6.2-9.6g, and 3.4 g in Japanese, Koreans, and Chinese, respectively (Barrett, 2006). The intake of soy largely depends on location, as it was reported that the intake among Chinese Americans (4 g/d) was 9-fold less compared with their counterparts from China (36 g/d). In addition, the perinatal consumption cannot be neglected, as soy formula takes up 25% of infant formula in the US (Cao *et al*., 2009), partly due to its higher content in calcium and phosphorus than cow milk (Bhatia *et al*., 2008). A cross-sectional study found that in infants consuming soy-based formula, the urinary genistein (GEN) concentration was 500 times higher than in those consuming milk-based formula (Cao *et al*., 2009).

There are several guidelines available for soy consumption. The U.S. Food and Drug Administration Code of Federal Regulation Title 21 suggests that the intake of 25 g soy protein per day, as part of a diet low in saturated fat and cholesterol, could reduce the risk of heart disease. The Chinese government recommends consuming a minimum of 50 g of soy foods daily to meet nutrient needs (Liu *et al*., 2004). The WHO does not have a threshold for soy intake, however, it listed soybean as a good source of folic acid.

Epidemiological studies on soy food

In terms of T1D and soy, among the limited epidemiological studies, one study showed that for infants aged 4-6 and 7-12 months, soy milk formula consumption was associated with a two-fold increase of T1D risk in China (Strotmeyer *et al*., 2004), while information on other exposure windows (e.g., prenatal, juvenile, and adult) is largely unknown. Another epidemiological study has also shown that twice as many diabetic children consumed soy-based

formula in infancy as compared to control children (Fort *et al*., 1986; Strotmeyer *et al*., 2004). In T1D patients, however, dietary intake of soy products showed a protective effect on renal function and lowered cholesterol (Stephenson *et al*., 2001).

The main concern of soy consumption is the effect on reproductive health with multiple health outcomes during developmental stages. In a large retrospective cohort study, soy-based formula consumption through infancy was associated with heavier menstrual bleeding and greater menstrual discomfort in young adulthood (Strom *et al*., 2001). In a longitudinal study, early soy-fed girls have a median age of 12.4 years at menarche, compared to 12.8 years in non-soy-fed girls (Adgent *et al*., 2012). The same research group found that among girls aged 42 months, early-life soy consumption was associated with less female-typical play behavior, while the same trend was not found in boys (Adgent *et al*., 2011). In a prospective cohort study, however, the formula types were not associated with benign breast diseases in young women (Berkey *et al*., 2015).

Isoflavone genistein

The two major chemical classes of phytoestrogens found in diets are lignans (enterodiol and enterolactone) and isoflavones [GEN, daidzein (DAZ), and glycitei] (Bhathena and Velasquez, 2002). The content of GEN and DAZ in common soy food is described in **Table 1.1**. Isoflavones exerts estrogen-like effects in the soy. For GEN, it shares structural similarity with 17β-Estradiol (as illustrated in **Figure 1.1.**). The content of GEN could vary a lot in soy food, based on the food processing. The highest content was found in roasted soybeans, reaching 1,425 μg/g (Wang and Murphy, 1994), and GEN accounts for about 50% of total isoflavone, and is the most dominant isoflavone. Isoflavone has a relative weak affinity to both estrogen receptors (ERs) α and β, while the affinity to ER-β is 20-30 fold higher than to ER-α (Mueller *et al*., 2004). Despite the low affinity, the serum isoflavone level can reach up to micromolar concentration following an intake

of soy meal (Cassidy *et al*., 2006). Combinatorial isoflavone exposure was found to inhibit the induction of interferon-γ (IFN-γ) $CD4^+$ T cells by lipopolysaccharide-dendritic cell in mucosal immune system (Wei *et al*., 2012).

The GEN component will be the major chemical discussed in this review. GEN is the active form of genistin (the form existing primarily in isoflavone), which is hydrolyzed in the intestine (Bhathena and Velasquez, 2002). As a major endocrine disruptor (ED; a compound that competes with endogenous estrogen to bind to ER), GEN could either function as ER agonist (when the estrogen concentration in the body is low) or ER antagonist (when the estrogen concentration in the body is high) (Mueller *et al*., 2004). It should be noticed, however, that the beneficial effect of soy food can be attributed to other isoflavone subclasses (Bhathena and Velasquez, 2002); for example, soy isoflavone glycitein exerts a protective effect on oxidative stress in *Caenorhabditis elegans* (Gutierrez-Zepeda *et al*., 2005). In addition, one study found that soy, not GEN, altered the body weight in the pregnant mouse and puberty onset in female offspring (Cao *et al*., 2015).

In general, GEN exposure showed a protective effect in adults for diseases such as cancer and cardiovascular diseases in certain animals or stains (Guo *et al*., 2007); however, GEN's effects on the immune related diseases varied, as GEN effects being tissue specific and dependent on various factors (e.g., specific cofactors, ER levels, levels of certain intracellular kinases, and estrogen levels) (Yang *et al*., 2015). To emphasize, the effect of GEN on immune system can be largely sex-specific and age-specific, which would be the focus for this research.

Adult exposure to GEN has been shown to associate with a reduced risk of diabetes in animals. GEN is reported to have anti-T2D function due to its protective effect on pancreatic β cells (Gilbert and Liu, 2013). There have been multiple lines of evidence showing the association between GEN intake and T1D protection. GEN was known for its anti-inflammatory functions and

was shown to attenuate the cell-mediated immune responses (Verdrengh *et al*., 2003). From a mechanistic view, GEN reduces inflammation by decreasing interleukin (IL)-6 and monocyte chemoattractant protein (MCP)-1 levels while down-regulating a number of genes involved in inflammation, as shown in *in vitro* study (Sergent *et al*., 2010), and sex can play an important role in the development of T1D.

In adult male mice, our research group found out that B6C3F1 males on the National Toxicology Program (NTP) standard diet, GEN treatment decreased blood glucose significantly on days 33 and 82 following STZ administration to induce diabetes, while the same trend was not observed in mice fed with high fat diet (Guo *et al*., 2014). It was also found that GEN exposure affected both T cells and B cells, while modulation on neutrophils may be more related to the anti-diabetic and diabetogenic potentials (Guo *et al*., 2014). In another study investigating reno-protective effect of GEN on C57BL/6 males, GEN reduced blood glucose level significantly, but it showed no protective effect on plasma insulin level (Elmarakby *et al*., 2011).

In adult female mice, GEN intake by oral gavage reduced the incidence and modulate the onset of T1D in NOD females fed on soy- and alfalfa-free diet, while the same effect was not observed in NOD mice on NTP-2000 diet (Guo *et al*., 2015). In addition, it was suggested that GEN can potentially prevent the onset of diabetes, elevate plasma glucose, and alter the hepatic glucose by increasing insulin secretion and preserving pancreatic β cells in NOD females (Choi *et al*., 2008).

In animal models, perinatal exposure to GEN was found to act through immune-modulation. The perinatal exposure is of concern, as phytoestrogens have also been detected in amniotic fluid (Foster *et al*., 2002), suggesting that *in utero* exposure also occurs. Also, the metabolic and/or excretion rates of GEN are different between mother and fetus, and once GEN is

transferred to the fetus, it is eliminated much more slowly from fetal tissues as compared to maternal elimination (Todaka *et al*., 2005). In our previous study, among B6C3F1 mice, GEN lead to an increased T-cell activity in the first-litter females and second-litter males, as represented by a decreased $CD4^+CD25^+$ regulatory T cells, which indicates an increased immune response (Guo *et al*., 2005). In another study, developmental GEN exposure induced the alteration of number and subpopulation in splenic T cells, as well as number and activity of NK cells in C57BL/6 mice (Guo *et al*., 2006). Similarly, perinatal exposure to GEN from gestational day 7 to postnatal day 64 induced an increase in splenic B cells, macrophages, T cells (total, helper, and cytotoxic subsets) in Sprague-Dawley rats (Guo *et al*., 2002).

GEN could also have sex-specific effect through non-immune-associated pathways. It was found in C57BL/6 mice, GEN exposure was associated with an earlier puberty in females, as indicated by decreased age for vaginal opening and accelerated development in mammary gland (Li *et al*., 2014b), as well as altered expression of epithelial-specific genes but not ER genes (Li *et al*., 2014b). In addition, it was found that in male rats, the exposure of GEN stimulated the proliferation of testicular Leydig cells from lactational exposure (Napier *et al*., 2014), Leydig cells differentiation from perinatal exposure (Sherrill *et al*., 2010), and androgen secretion by testicular Leydig cells from perinatal exposure (Akingbemi *et al*., 2007), suggesting a disrupted gonadal development. In addition, following perinatal exposure to GEN in rats, the males and females had a down-regulated and up-regulated progesterone receptors, respectively (Takagi *et al*., 2005). In C57BL/6 mice (Wisniewski *et al*., 2005) and rats (Wisniewski *et al*., 2003), perinatal GEN exposure leads to a persistent demasculinization in males, as represented by less aggressive behaviors. Compared to mouse models, one study found chronic isoflavone consumption had no adverse effect on reproductive patterns among male rabbits (Cardoso and Bao, 2007).

Isoflavone daidzein (DAZ)

The structure of DAZ is depicted in **Figure 1.1**. The effect of DAZ on T1D is less studied; however, DAZ can mitigate lipopolysaccharide-triggered immune responses represented by decreased neutrophil, tumor necrosis factor (TNF)-α, macrophages, and NF-κB markers in male rats (Feng *et al*., 2015). In a randomized intervention among menopausal women, equol (a major metabolite from DAZ) production is associated with an increased anti-inflammatory response (van der Velpen *et al*., 2014); the possible mechanisms include decreased inflammatory cytokines (Nicastro *et al*., 2013), modulation of dendritic cells and major histocompatibility complex molecules (Wei *et al*., 2012), and increase in insulin level (Choi *et al*., 2008). DAZ exerts similar protective effect on hyperglycemia and glucose tolerance as GEN (Choi *et al*., 2008).

<u>4. Gut Microbiota and T1D</u>

Development of gut microbiota in human

The human intestine hosts approximately 100 trillion bacteria (at least 1000 species) (Hooper and Macpherson, 2010). Gut microbiota could be determined by different factors throughout life. In fetus, early-life gut bacterial colonization could play an important role in metabolic tissue development in determining risk for autoimmune diseases (Kozyrskyj and Sloboda, 2016). This is because intestinal immune system development, such as the maturation of intestinal mucosa, Peyer's Patch, isolated lymphoid follicles and mesenteric lymph nodes, starts from 11 weeks of gestation (Mejia-Leon and de la Barca, 2015).

The bacterial DNA could transfer from mother to fetus through amniotic fluid, umbilical cord (Kozyrskyj and Sloboda, 2016), and placenta (Romano-Keeler and Weitkamp, 2015). Evidences showed that probiotic use (*Lactobacillus rhamnosus* or *Bifidobacterium lactis*) during pregnancy altered infant immune responses (Prescott *et al*., 2008). After birth, the mode of

delivery, antibiotic use, as well as infant formula, could all help shape the infant microbiota (Mejia-Leon and de la Barca, 2015; Mueller *et al.*, 2015; Romano-Keeler and Weitkamp, 2015) and further modulate the immune system. For example, the composition of gut microbiota is closely related to maternal skin. Plant-based dietary intake, compared to dairy-based food, demonstrated a distinct gut microbiota profile (Smith-Brown *et al.*, 2016). The infant formula consumption, compared to the breast feeding, was found to be detrimental to neonatal immune system development (Innis, 2007). Overtime, food can play a major role in shaping the gut microbiota; as reviewed by Johnson *et al.* (Johnson *et al.*, 2017), in some studies, animal-based diet led to the increased abundance of *Bacteroides* and *Alistipes*, while it was found in other studies that the *Bacteroides* increased with the fiber intake. Antibiotics, which is often used to battle bacterial infection and colitis, could induce an attenuated mucus layer and reduced goblet function, suggesting a weakened mucosal barrier (Wlodarska *et al.*, 2011).

Epidemiological evidence of gut microbiota and T1D

Two major phyla, *Bacteroides* and *Firmicutes*, made up most of the human gut microbiota. After seven years of age, the ratio between *Bacteroides* and *Firmicutes* are relatively stable, while a disturbance might be contributing to metabolic syndrome such as obesity and T1D. There have been a multitude of epidemiological studies bridging microbiota and T1D. An international research group investigated the gut microbiota among the newborn infants from the Finnish Type 1 Diabetes Prediction and Prevention Study, and found that two species, *Bacteroides Dorei* and *Bacteroides Vulgatus*, were higher in infants who later developed T1D, and the abundance was in coincidence with the intake of solid food (Davis-Richardson *et al.*, 2014). The same group looked at the metagenomic profile from stool samples from four pairs of matched case-control subjects, and observed elevated *Actinobacteria, Bacteroidetes, Proteobacteria* and reduced *Firmicutes,*

Fusobacteria, Tenericutes, Verrucomicrobial in T1D cases (Brown *et al*., 2011). In addition, it was found that when the subjects were approaching the toddler age, those who will be T1D-free in the future had a healthier and more stable gut microbiota, while those will develop T1D had a less diverse and stable gut microbiota (Giongo *et al*., 2011). On the contrary, a European team analyzed the fecal samples from a European case-control study and observed an increased microbial diversity among T1D children older than 2.9 years (de Goffau *et al*., 2014). In a Mexican case-control study, it was noticed that in T1D cases, the dispersion in the structure of gut microbiota was higher, whereas no difference in α-diversity was found among controls, T1D at onset, and T1D under treatment (Esther Mejia-Leon *et al*., 2014). Genus *Bacteroides* showed a higher abundance in cases, while *Prevotella* showed a higher abundance in control (Esther Mejia-Leon *et al*., 2014). Another finding is that age might be a major determinant for certain microbial species, since children older than 2.9 years was found to have a higher fraction of *Clostridium* clusters IV and XIVa than younger children (Esther Mejia-Leon *et al*., 2014).

Animal study in gut microbiota and T1D

In lab animals, the research on the association between gut microbiota and T1D largely focused on the presence of xenobiotics, exposure window, and gut leakiness (Schwartz *et al*., 2007). Patterson *et al*. (2015) investigated the gut microbiota change on male Sprague-Dawley rats with STZ-induced T1D and found a reduced α-diversity after exposure. STZ-induced T1D also induced β-diversity over time, while perturbing the *Bacteroidetes/Firmicutes* balance. The proportion of *Bacteroidetes* was significantly increased, while the *Firmicutes* was significantly decreased over 4-week study period. On the other hand, a Japanese study did not find the difference in diabetes incidence between the specific pathogen free mice (SPF) and germ-free mice, while the insulitis was accentuated in germ-free mice (Alam *et al*., 2011). The significant discrepancy of

previous studies on how gut microbiota could affect T1D could be attributed to genetic drift in colonies (Alam *et al*., 2011). In addition, the gut microbiota alteration could lead to the hormone level change, which further leads to the T1D incidence change (Markle *et al*., 2013).

Germ-free animals are widely used to establish an association between gut microbiota and T1D by depleting the commensal bacteria; however, the price might be prohibitive for a lot of labs. In that sense, antibiotics are used as a substitute since a majority of gut microbiota can be eliminated as confirmed by polymerase chain reaction (PCR). In human, no effect was found so far between T1D and antibiotics use (Gulden *et al*., 2015). With animal models, Brugman *et al*. (2006) treated Bio-Breeding diabetes-prone (BB-DP) rats with antibiotics [targeting both Gram positive (G^+) and negative (G^-) bacteria] in the drinking water and found that the T1D was completely prevented. Almost at the same time, Schwartz *et al*. (2007) fed the NOD mice with doxycycline (a broad-spectrum antibiotic)-containing regular rodent diet and confirmed that the incidence of T1D was significantly lower than those not fed with doxycycline. In another study treating both NOD pups and adult NOD pups with vancomycin, an antibiotic depleting both the majority of G^+ and $G^–$ bacteria, and the principal component analysis profile showed a significant separation among the groups compared to control (Hansen *et al*., 2012). In addition, antibiotic treatment was found to have a protective effect on T1D for both neonatal and adult exposure in terms of blood glucose and insulitis score (Hansen *et al*., 2012). Another study selectively depleted G^- bacteria with antibiotics in NOD mice during prenatal period and found similar protective effect and that the effect largely depended on exposure window (pregnant, born, and wean) and transferable to new host (Hu *et al*., 2015). As reported by the same research group that depletion of G^+ bacteria led to an accelerated T1D in NOD mice, it was concluded that increased G^+/G^- ratio was associated with a reduced risk of T1D (Hu *et al*., 2015). On the contrary, a recent study found

that oral fecal transplantation between NOD mice and non-obese resistant mice induced T1D in the latter, and that antibiotics use in NOD mice accelerated T1D occurrence, accompanied by increased Th1 response and reduced Th17 response (Brown *et al*., 2016).

For antibiotic depletion of gut microbiota, it should be noticed that the interdependence is found for different microbial taxa; for example, G^- microbial could be effectively depleted by vancomycin that mainly targets G^+ microbial (Ubeda *et al*., 2010).

Gut microbiota and immunity

One possible mechanism of gut microbiota modulation on T1D is depicted in **Figure 1.2**. Besides the normal function of digesting dietary nutrients such as polysaccharides, intestinal bacteria play an important role in fighting against pathogenic infections (Hooper and Macpherson, 2010). For example, the innate and adaptive immune responses against *Toxoplasma gondii* can be induced by gut commensal bacteria (Benson *et al*., 2009). At the same time, to remain the homeostasis, the immunological barriers function to minimize the contact of intestinal bacteria with the epithelial cells, to get rid of pathological bacteria which tend to penetrate intestinal issues (both innate and adaptive immunity, and cells involved include $CD4^+$ T cells, $CD8^+$ T cells, NK cells and such), and to minimize exposing resident bacteria to systemic immune system (Hooper and Macpherson, 2010). In terms of T1D, the alteration of gut microbiota was different depending on future T1D status, and the gut microbiota profile was further associated with change in immune markers such as $Foxp3^+$ regulatory T cells, $CD11b^+$ dendritic cells, and IFN- γ production (Krych *et al*., 2015). On the other side, the immune system can modulate the gut microbiota as well. it was found that the innate and adaptive immune system could coordinate antibodies (IgA) against microbiota and thus impact the microbial community (Kubinak *et al*., 2015). In addition, the

presence/absence of IL-10 could modulate both the structure of gut microbiota, and the metabolism of arsenic by gut microbiota, as was found in IL-10 knockout mice (Lu *et al*., 2014).

$CD4^+CD25^+Foxp3^+$ Tregs, which suppress the inflammation, are essential in maintaining the gut homeostasis and its relationship with gut microbiota and SCFA are well studied. A study using both germ-free mice and SPF mice found an absence of Treg cells in the gut and mesenteric and pancreatic lymph nodes, while significantly increased Treg cells were detected in islet infiltrates, explaining the innocuousness of insulitis in germ-free mice (Alam *et al*., 2011) and suggesting that microbiota is important in maintaining the immune function of gut. In another study, the induction of commensal *Bacteroides fragilis* promoted the conversion from $CD4^+$ T cells into $CD4^+Foxp3^+$ Tregs (Round and Mazmanian, 2010). Antibiotics were also suggested to have an effect on Tregs. Hu and colleagues (2015) applied a maternal antibiotics model and found increased subset of Tregs in the spleen, mesenteric lymph nodes, and pancreatic lymph nodes in the offspring with maternal antibiotics exposure. A Japanese study found out that the production of SCFA, especially butyrate, was positively associated with the number of Tregs in the colon (Furusawa *et al*., 2013). The polysaccharide A produced by certain bacterial strains such as *Bacteriodes fragilis* is able to induce the production of $Foxp3^+$ Tregs (Romano-Keeler and Weitkamp, 2015).

Soy effect on gut microbiota

The GEN effect on the gut microbiota was not well studied yet. In an epidemiological study among children, it was found that the *Bifidobacteria*, *Bacteroides*, and *Clostridia* were significantly reduced following soy consumption (Hoey *et al*., 2004). However, the change of microbial diversity was not reported in this study. It was reviewed by Huang *et al*. (2016) that soy consumption could increase *Lactobacillus* (Cheng *et al*., 2005; Clavel *et al*., 2005),

Bifidobacterium (Cheng *et al*., 2005; Clavel *et al*., 2005), and *Bacteroides* (Fernandez-Raudales *et al*., 2012), while the changes were comparable to lab animals (Wang *et al*., 2013). It was surprising that the similar results were observed despite a range of dose, soy products, and animal species were used (Huang *et al*., 2016). In a fermentation study using human feces, isoflavone induced an increase of *Bifidobacterium spp*. and *Lactobacillus spp*. (Steer *et al*., 2003), and both of the species were found to have a protective effect on T1D (Brown *et al*., 2011).

5. SCFAs and T1D

SCFAs are considered to be end products of the microbial fermentation of macronutrients, in which the majority are polysaccharides not digestible by human alone and polysaccharide lysates (Qin *et al*., 2010). The gut *Bacteroidetes* participated in providing the host with energy, and this process manufactures SCFAs that supply up to 10% calories to the hosts (Johnson *et al*., 2017). The most common SCFAs are butyrate, propionate, acetate, and such. It was reported that the proportion of the major SCFAs could vary greatly; however, butyrate, acetate, and propionate in the gut make up the majority of the SCFAs in the gut (Davis-Richardson and Triplett, 2015). SCFAs, especially butyrate, are essential to intestinal health since butyrate is the major energy source for colonic cells and has anti-inflammatory properties (Brown *et al*., 2011) and immunomodulatory effects on intestinal macrophages (Lin and Zhang, 2017), and it was found that butyrate could inhibit the expression of pro-inflammatory cytokines such as TNF-α (Lin and Zhang, 2017). SCFAs produced by microbiota stimulate epithelial metabolism, deplete intracellular oxygen, and thus enhance the epithelial barrier function (Kelly *et al*., 2015). SCFAs can be directly anti-inflammatory through the activation of peroxisome proliferator-activated receptor (PPAR)-γ, the suppression of NF-κB pathway, the inhibition of histone deacetylases (Lopez *et al*., 2014), and the activation of G protein coupled receptors (e.g., GPR41, GPR43, and

GPR109a) (Thorburn *et al*., 2014). Also, it was suggested by Chassaing *et al*. (2015) that SCFAs directly inhibit the intestinal atrophy and induce microbiota-mediated intestinal inflammation. More importantly, SCFAs, especially propionate and butyrate, are essential for Treg accumulation and differentiation (Becattini *et al*., 2016). Except for the immune modulatory effects, SCFAs could also regulate intestinal environments by reinforcing the intestinal epithelial cell barrier (Lin and Zhang, 2017).

The association between SCFAs produced by gut microbiota and T1D remains a gap. Brown *et al*. (2016) found that susceptibility to T1D correlated well with reduced fecal SCFAs by gas chromatography analysis. In an earlier study, however, the production of butyrate was found to be associated with increased tight conjunction and thus healthy gut, while other SCFAs (propionate, acetate, and succinate) were linked with less mucin synthesis, leaky gut, and autoimmunity such as T1D (Brown *et al*., 2011). The interaction between SCFAs and microbiota is intrigue: certain strain of commensal bacterial (e.g., *Bifidobacteria* can ward off pathogens through production of acetate and thus help maintain the intestinal health (Fukuda *et al*., 2011). To date, no studies have been conducted to investigate if oral exposure to GEN could affect the SCFAs content or not. In addition, it should be mentioned that during the fermentation of soybeans, gut microbiota could produce metabolites other than SCFAs that could regulate Th1/Th2 response (Lin and Zhang, 2017), which is associated with the development of T1D.

Conclusion

Starting from prenatal exposure to adult exposure, individuals can be exposed to isoflavone compounds such as GEN and DAZ, which in turn can modulate the risk of autoimmune diseases such as T1D. Interestingly, the immunological responses and consequently T1D risk largely depends on exposure windows, and previous evidences including epidemiological studies and

animal models have not fully elucidated the mechanisms yet. Gut microbiota and epigenetics are two hot areas in environmental exposures; for gut microbiota, extensive research has enabled us to develop probiotics to reverse the disease status. The case for soy isoflavone GEN and DAZ in modulating autoimmune diseases can serve as a model for assessing the health effect of other polyphenolic compounds.

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Table 1. 1. The genistein, daidzein, and total isoflavone content in common soy food

Food	Per serving	Genistein (mg)	Daidzein (mg)	Isoflavone (mg)
Soybeans, boiled	4 oz	6	6	12
Soybeans, dry, roasted	1 oz	15	19	37
Soy protein concentrate, aqueous washed	3.5 oz	56	43	102
Miso	4 oz	34	22	59
Tempeh	1 oz	5	7	12
Tofu	1 oz	4	3	7
Meatless hot dog	1 hotdog	6	3	11
Soymilk	8 oz	17	12	30

Source: http://lpi.oregonstate.edu/infocenter/phytochemicals/soyiso/index.html#source

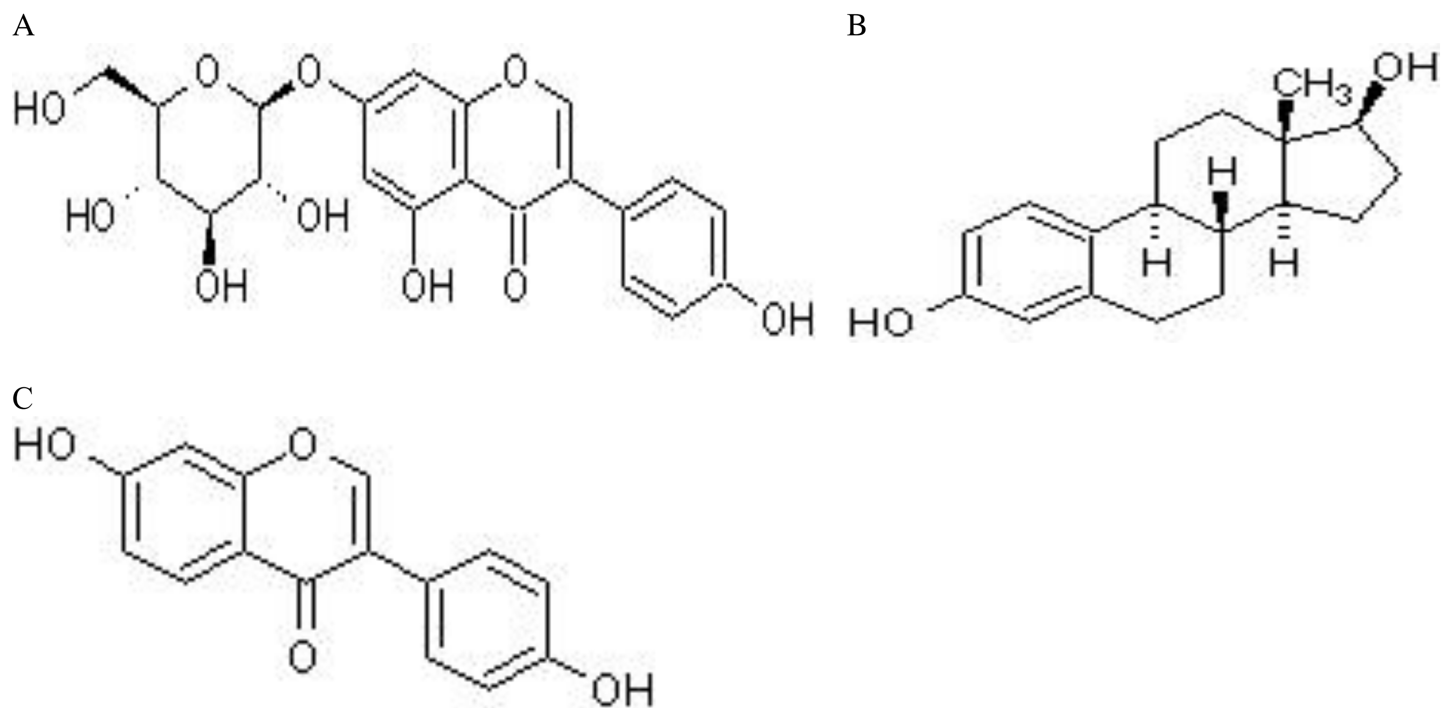

Figure 1. 1. The chemical structure of (A) genistein, (B) 17β-Estradiol, and (C) daidzein.

Images achieved on Abcam on October 2017.

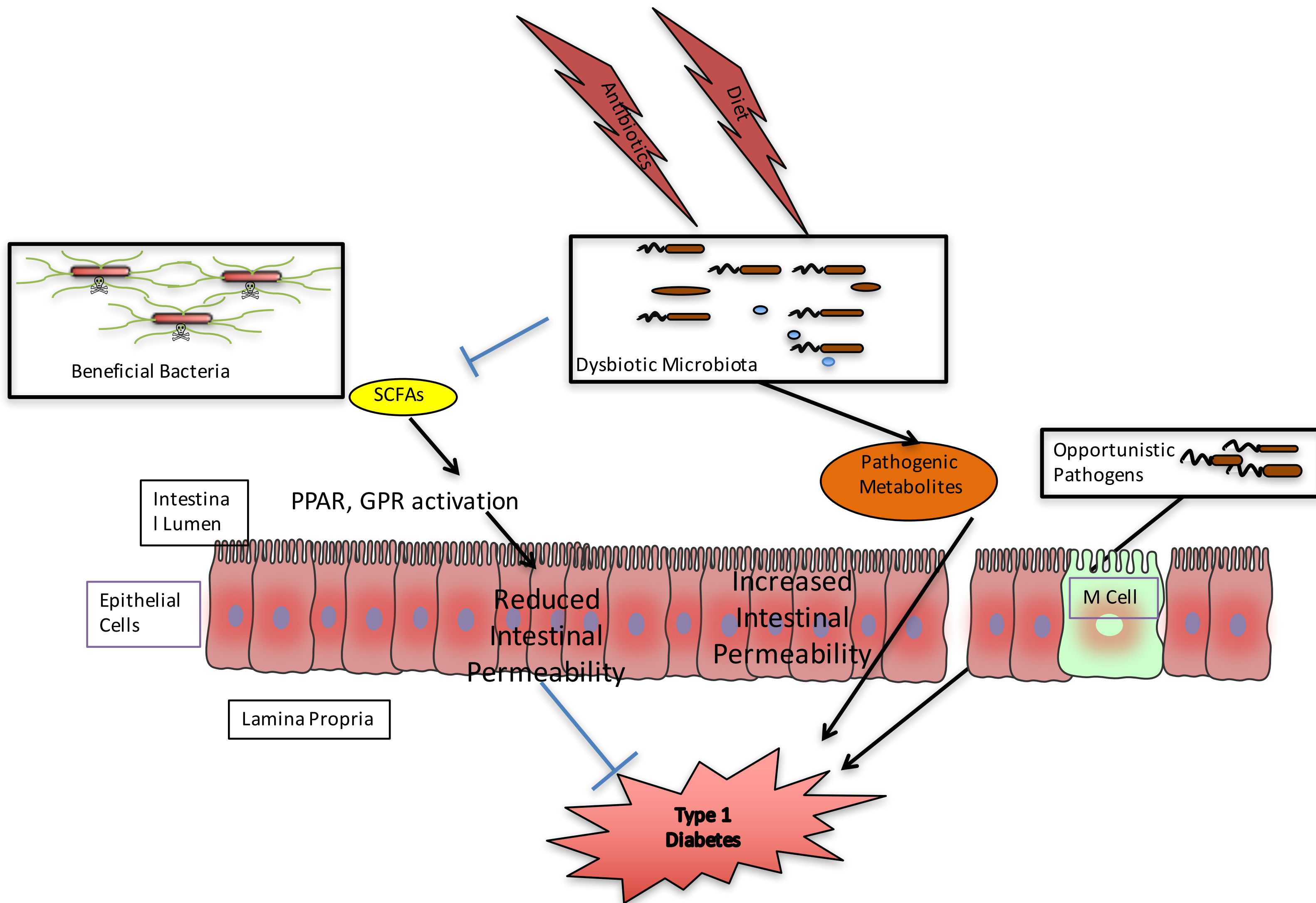

Figure 1. 2. One possible mechanism for the interaction of type 1 diabetes, gut integrity, gut microbiota, and metabolites.

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CHAPTER 2

GENISTEIN PREVENTION OF HYPERGLYCEMIA AND IMPROVEMENT OF GLUCOSE TOLERANCE IN ADULT NON-OBESE DIABETIC MICE ARE ASSOCIATED WITH ALTERATIONS OF GUT MICROBIOTA AND IMMUNE HOMEOSTASIS[2]

[2] Huang, G., Xu, J., Lefever, D. E., Glenn, T. C., Nagy, T., & Guo, T. L. Accepted by Toxicology and Applied Pharmacology. Reprinted here with permission of publisher.

Abstract

Although studies have linked soy phytoestrogen 4,7,4-trihydroxyisoflavone genistein (GEN) to reduced type 1 diabetes (T1D) risk, the mechanism of dietary GEN on T1D remains unknown. In our studies, adult non-obese diabetic (NOD) mouse model was employed to investigate the effects of GEN exposure on blood glucose level (BGL), glucose tolerance, gut microbiota, and immune responses. Adult male and female NOD mice were fed with either soy-based or casein-based diet and received GEN at 20 mg/kg body weight by gavage daily. The BGL and immune responses (represented by serum antibodies, cytokines and chemokines, and histopathology) were monitored, while the fecal gut microbiota was sequenced for 16S ribosomal RNA to reveal any alterations in gut microbial communities. A significantly reduced BGL was found in NOD males fed with soy-based diet on day 98 after initial dosing, and an improved glucose tolerance was observed on both diets. In addition, an anti-inflammatory response (suggested by reduced IgG_{2b} and cytokine/chemokine levels, and alterations in the microbial taxonomy) was accompanied by an altered β-diversity in gut microbial species. Among the NOD females exposed to GEN, a later onset of T1D was observed. However, the profiles of gut microbiota, antibodies and cytokines/chemokines were all indicative of pro-inflammation. This study demonstrated an association among GEN exposure, gut microbiota alteration, and immune homeostasis in NOD males. Although the mechanisms underlying the protective effects of GEN in NOD mice need to be explored further, the current study suggested a GEN-induced sex-specific effect in inflammatory status and gut microbiota.

Introduction

Type 1 diabetes (T1D) is an autoimmune disease in which insulin-secreting pancreatic β cells are selectively destroyed by self-reactive immune responses that are dictated by pro-inflammatory cytokines (Badami *et al.*, 2011; Li *et al.*, 2014; Ye *et al.*, 2015). Isoflavones in soy exert weak estrogen-like (i.e., phytoestrogenic) effects, and the major components in the isoflavone family are genistein (GEN), daidzein, and glycitein. GEN is reported to have anti-diabetic functions, especially in type 2 diabetes (Gilbert and Liu, 2013), and this notion is supported by a number of epidemiological studies (Ding *et al.*, 2016; Nanri *et al.*, 2010). In contrast, the associations of high soy consumption and risk of T1D are uncertain and intricate, which may depend on the window of exposures and its interaction with the other dietary intake, as epidemiological evidences are limited (Strotmeyer *et al.*, 2004). The effect of GEN on T1D and glucose regulation has not been fully recognized. However, there have been multiple lines of evidence indicating an association between GEN intake and T1D. GEN administration by gavage reduced the incidence and delayed the onset of T1D in female non-obese diabetic (NOD) adult mouse (Choi *et al.*, 2008; Guo *et al.*, 2015), a strain that spontaneously develops insulitis and shares many similarities with human T1D (Anderson and Bluestone, 2005). In addition, our previous studies have shown that GEN decreased blood glucose levels (BGL) in streptozotocin (STZ) - treated male B6C3F1 mice (Guo *et al.*, 2014), which was due to, at least in part, immunomodulation (e.g., decreased Gr-1^{+}Mac-3^{-} neutrophils). In the current study, the animal model we have used is the NOD mouse. NOD females have a T1D incidence rate of 60-80% with an earlier onset than males (Guo *et al*., 2014).

Due to recent advances in molecular biology and DNA sequencing techniques, the gut microbiota has been increasingly subjected to extensive studies. Evidence from both human and

animal studies suggest that T1D is originated in the gut, which houses 70% of the body's immune system (Vighi *et al.*, 2008), and is associated with a profound dysbiosis, favoring pro-inflammatory microbial communities. The sex-specific autoimmune disease could also be attributed to differences in gut microbiota (Yurkovetskiy *et al.*, 2013), which prompted us to study if GEN modulation of T1D was mediated by microbiota. As GEN demonstrates a protective effect on T1D in both males (Guo *et al*., 2014; Zimmermann *et al.*, 2012) and females (Choi *et al*., 2008; Guo *et al*., 2015), we hypothesized that the exposure to GEN in adulthood could: (a) reduce the BGL and insulitis score; (b) lead to an alternation in gut microbiota; and (c) alter the inflammatory status. In addition, sex-specific effect was also expected in the modulation of gut microbiota and immune responses by the phytoestrogen GEN. Our study focused on the association between GEN exposure and BGL from a microbiota perspective and could be applied as a strategy to evaluate the preventive effects of nutritional products on autoimmune diseases.

Methods

Animal husbandry

NOD mice were initially purchased from Taconic Biosciences (Hudson, NY), and a breeding colony has been maintained in our lab. The mice were housed in the Central Animal Facility located in the College of Veterinary Medicine at University of Georgia (UGA) and kept in standard plastic cages with irradiated laboratory animal bedding (The Andersons company, Maumee, OH), with each cage housing 4-5 mice. The mice were separated from the dams on postnatal day (PND) 21 and were used in this study after reaching sexual maturity (PND 55-60). The cages were maintained at temperature ranged 22–25°C, and relative humidity 50 ± 20 with 12-h light cycles (7:00–19:00). The cages were changed on a bi-weekly basis, or they were changed when the cages became wet because T1D was associated with sanitary conditions. They

had free access to water supply from the animal room, and animal procedures were conducted under an animal protocol approved by the UGA Institutional Animal Care and Use Committee (IACUC). The mice were treated humanely, and efforts were made to relieve the suffering (e.g., to avoid excessive handling to lessen the stress).

We have previously found that GEN modulated the immune responses (e.g., IgE production) more when mice were maintained on the soy-based diet than on the soy-free diet (Guo *et al.*, 2005). Therefore, an initial study was performed using a soy-based diet. A total of forty adult NOD mice (20 males and 20 females) were used for the soy-based diet study. For each sex, the mice were randomized into vehicle control (VH; 25 mM sodium carbonate) and GEN groups according to body weight (BW) and BGL. Starting from PND 55-60, the mice in the GEN groups were gavaged daily with 0.1 ml/10g BW GEN dissolved and sonicated in VH (2 mg GEN/mL) (Guo *et al.*, 2014), while the VH groups received the same volume of VH daily with 18 G gavage needles. The mice were fed with the soy-based PicoLab Rodent Diet 20 (LabDiet, St. Louis, MO) for 5 months, and the nutrients were described in **Table 2.1**. In addition, the soy-free diet was used to rule out the dietary isoflavone effect. A total of 8 adult NOD males and 10 adult NOD females were equally randomized into VH and GEN groups and fed with a soy-free diet for 7 months, and they received the same dosing regimen as above. The Verified Casein Diet 10 IF, the phytoestrogen-free 5K96 diet (TestDiet, St. Louis, MO; **Table 2.1.**), is based on the NIH-31 formula, except that casein replaces the protein contributed by soy and alfalfa, and soy oil is replaced by corn oil, to preclude other phytoestrogens that might be present. The 5K96 diet was assayed for GEN and daidzein after hydrolysis of conjugates. The concentrations of both GEN and daidzein were found to be below the limit of detection (0.5 ppm) (Guo *et al.*, 2002). The protein, carbohydrates, fat, and total calories are comparable between soy-based and soy-free diets.

To compare with NOD mouse strain, a total of 30 severely combined immunodeficiency (SCID) female adults were included in the study and randomly divided into two groups (VH and GEN). The same dosing regime was applied, and they were fed with regular phytoestrogen-containing diet and then switched onto phytoestrogen-free diet. T1D was induced by injecting streptozotocin *i.p.* four times along the study.

Body weight, organ weight, and blood glucose measurement

The BW and BGL were monitored every 1-2 wks. Non-fasting BGL was measured directly in small samples of venous blood (tail nick) using Accu-Chek Diabetes monitoring kit (Roche Diagnostics, Indianapolis, IN) (Guo *et al*., 2014). T1D was defined as a BGL higher or equal than 250 mg/dL (Guo *et al*., 2014). The mice were euthanized by carbon dioxide (CO_2) inhalation after a BGL of 600 mg/dL was detected in 2 consecutive weeks or at the end of study. Individual organs were collected and weighed during necropsy.

Glucose Tolerance Test (GTT)

GTT, a measurement for the metabolic function of NOD mice, was performed as described (Cui *et al.*, 2015). The test was conducted 5 months after initial GEN dosing. The mice were fasted for 15 h (Ayala *et al.*, 2010) followed by glucose injection (2 g/kg BW; *i.p.*). The fasting BW and BGLs, together with BGLs at 15, 30, 60, and 120 min after glucose injection, were measured.

Antibody and insulin measurement by enzyme-linked immunosorbent assay (ELISA)

At the end of each study (8 months for the soy-based diet and 7 months for the soy-free diet), the mice were euthanized by CO_2 inhalation. After euthanasia, the terminal blood was collected by cardiac puncture, centrifuged for serum, and stored at -20 °C. The levels of serum IgG subclasses (including IgG_1, IgG_{2a}, IgG_{2b}) and IgM were measured using ELISA kits (eBioscience, San Diego, CA). Briefly, capture antibodies (primary antibodies, 1:1000 v/v) were

coated on 96-well microtiter plates and incubated overnight at 4°C. Capture antibodies used in this study were purified goat anti-mouse IgG_1, IgG_{2a}, IgG_{2b} and IgM, respectively. After washing with phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBST), the wells were blocked with 5% (w/v) milk power (prepared in PBS) at room temperature (RT) for 2 h. After washing with PBST, the diluted samples were added to the microplates and incubated for 2 h at RT. After washing with PBST, horseradish peroxidase (HRP)-conjugated detection antibodies (secondary antibodies) were added to each well and incubated for 1 h at RT. After washing with PBST, the substrate solution (citrate acid tablet and 2,2'-azino-bis, ABTS, Sigma, in PBS) was added into the microtiter plates and incubated for 45 min at RT. The optical density (OD) was read using a microplate fluorescence reader (Synergy 4 Hybrid Microplate Reader, BioTek, Winooski, VT) at a wavelength of 405 nm. Alternatively, the 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Sigma) was used, and the reaction was stopped by adding 2N sulfuric acid (100 µL/well). The OD was read at a wavelength of 450 nm. The serum insulin levels were measured with the Ultra Sensitive Mouse Insulin ELISA Kit (Crystal Chem, Inc., Downers Grove, IL) following manufacture protocol.

Cytokine/chemokine measurement

The cytokine/chemokine levels in the terminal sera were measured using Multiplex Mouse Cytokine/chemokine Magnetic Bead Panel Kit 96 Well Plate Assay (Cat. No. MCYTOMAG-70K, EMD Millipore, Billerica, MA). These cytokine/chemokines include granulocyte-colony stimulating factor (G-CSF), EOTAXIN, granulocyte macrophage colony-stimulating factor (GM-CSF), interferon (IFN)- γ, interleukin (IL)-1α, IL-1β, IL-2, IL-4, IL-3, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12p40, IL-12p70, leukemia inhibitory factor (LIF), IL-13, LIX (C-X-C motif chemokine 5 or CXCL5), IL-15, IL-17, IFN-γ-induced protein 10 (IP-10), keratinocyte chemoattractant (KC),

monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein (MIP)-1α, MIP-1β, macrophage colony-stimulating factor (M-CSF), MIP-2, MIG (C-X-C motif chemokine 9 or CXCL9), RANTES (C-C motif ligand 5 or CCL5), vascular endothelial growth factor (VEGF), tumor necrosis factor (TNF)-α. Plates were run on a Bio-Plex MAGPIX™ Multiplex Reader with Bio-Plex Manager™ MP Software (Luminex, Austin, TX). Each cytokine/chemokine concentration was calculated as pg/ml.

Histopathology

The pancreas from NOD males and females were collected, mounted on a cassette, and stored in 10% formalin for histological analysis. Hematoxylin and eosin (H&E) staining was performed and the slides were reviewed in a blinded manner by a board-certified veterinary pathologist for any pathological changes (Score 0 = no insulitis, Score 1 = peri-islet insulitis, Score 2 = intermediate insulitis, Score 3 = intra-islet insulitis, and Score 4 = complete islet insulitis).

DNA extraction for gut microbiota analysis

The feces were collected from individual mice 4 months after initial GEN dosing, transferred to 500 μL Eppendorf tubes and kept in a -20 °C freezer. For each animal, a total of 4-5 fecal pellets (weighed from 80-120 mg) were used to ensure proper homogenization and DNA concentration. On the day of DNA extraction, the fecal samples were thawed on ice, and the DNA was extracted using QIAamp DNA stool mini kits (Qiagen, Valencia, CA) following manufacture protocols. Briefly, the fecal pellets were homogenized by vortex, and lysed with proteinase K to digest protein. The bacterial DNA was then bound to silica membrane, washed in ethanol to remove residual protein and salt, and eluted in Tris buffer. Typically, 15–60 μg DNA was yielded in each sample.

Library preparation and sequencing

On a polymerase chain reaction (PCR) plate, the extracted DNA was normalized to 20 ng/µL at Georgia Genomic Facility (GGF). The locus-specific primers (forward: 16S_341_F, and reverse: 16S_785_R) were used to target the V3-V4 region of 16S rRNA (**Table 2.2.**). The Read 1 and Read 2 sequencing primers were Illumina-specific iTru_R1_5'_fusion and iTru_R2_5'_fusion. In addition, a total of 20 internal tags ranging from 5 nucleotides (NTs) to 8 NTs long were used; 8 of them were the forward fusion primers and 12 the reverse fusion primers (**Table 2.2.**) (Glenn *et al.*, 2016). Following the preparation of Taggi-matrix library, the PCR mix (Kapa Biosystems, Inc., Boston, MA) was added, together with forward fusion primers (including the Read 1 sequencing primer, Read 1 tag, and forward locus-specific primer) and reverse fusion primers (including the Read 2 sequencing primer, Read 2 tag, and reverse locus-specific primer). Different samples were distinguished by 20 tags (**Table 2.2.**). The first round PCR was conducted with 25 cycles. The PCR amplicon was run on a 1.5% agarose gel to check the integrity of the PCR bands at 550 bp. Then, the PCR amplicon aliquot (10 µL per DNA sample) was purified with Speedbeads and quantified on Qubit, and the second round PCR was performed using Illumina iTru5 primer and iTru7 primer. The PCR products were purified with Speedbead, run on the 5% agarose gel, quantified, and sequenced on Illumina Miseq (Illumina Inc., San Diego, US) with 500 cycles, resulting in pair-end 250 bases to target 25,000 reads per sample.

Bioinformatic analysis

The bioinformatic analysis was performed as described (Lefever *et al.*, 2016). Briefly, the Read 1 and Read 2 sequence files from each sample were first merged, demultiplexed (a process that removes the internal tag and primers), and filtered with Phred Quality Score of 20, allowing for an error rate of 1 in 100. The subsequent analysis was performed on Quantitative Insights Into Microbial Ecology (QIIME) version 1.7.0, a pipeline that worked with high-throughput 16S rRNA

sequencing data (Caporaso *et al.*, 2010). The *pick_de_novo_otus.py* workflow was applied to pick Operational Taxonomic Units (OTU), align the representative sequence set, assign taxonomy, and build a phylogenetic tree and OTU table at the time (Caporaso *et al.*, 2010). Subsequent *core_diversity_analysis.py* workflow was applied to determine α (within sample) and β (between samples) diversity after the OTU table was rarefied to a depth 80% of the minimum read per sample. The α diversity metrics used were phylogenetic diversity (PD) representing the genetic diversity and Shannon index representing both richness and evenness. The β diversity metrics used were unweighted and weighted Unifrac (a distance metric that takes account the abundance of each sample), and the principle coordinate analysis (PCoA) was conducted to visualize the phylogenetic distance between different samples with unweighted and weighted Unifrac. Further, the jackknifed β diversity analysis was performed to account for the uncertainty of the data at a depth 80% of the minimum read per sample, and it was repeated 999 times. The Linear Discriminant Analysis (LDA) Effect Size (LEfSe) analysis was performed to identify the taxa that were significant between groups on the website: http://huttenhower.sph.harvard.edu/galaxy, and the differential features identified at both the order and the genus levels. The LEfSe analysis was performed under the following conditions: (a) the α value for the factorial Kruskal–Wallis test and pairwise Wilcoxon test among classes was <0.05, and (b) the threshold on the logarithmic LDA score for discriminative features was >2.0. A critical value of 0.05 was applied throughout the bioinformatic analysis.

Metabolomics

The metabolic profiling following GEN dosing among NOD males was performed with urine samples. For urine collection, approximately 200 uL of urine was collected from each mouse

at the designated time point. The urine was collected with a micropippett on a plastic wrap or petri dish when scruffed and stored immediately in -20 °C to prevent any bacterial degradation.

About 150 uL of the sample is used during each run. Briefly, the samples were lyophilized for 8 h the night before and stored in 4 °C overnight. On the day of analysis, the samples were reconstituted with 230 uL of NMR phosphate buffer (200 mM in 99.8% D_2O to yield the final phosphate concentration of 100 mM, with 20 uM 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) added as an internal standard). The phosphate buffer was added to minimize the alteration in pH. The samples were vortexed and centrifuged at 3000 g for 10 min at 4 °C and transferred to 5 mm standard NMR tubes. An additional quality control (QC) sample was prepared by pooling from different samples. Subsequently, the samples were run on Varian 600 MHz ^{1}H NMR spectrometers (Varian, Palo Alto, California) at US EPA (Athens, GA). The parameters were set at a number of transient (nt) of 128 and variable temperature of 20 °C.

The data output from NMR were processed with MestReNOVA (MNOVA) (Willcott, 2009). The data were first zero-filled, line broadened at 0.3Hz, Fourier transformed, and a multiplication of first points by 0.5. Phase correction was done manually, and baseline correction full auto (Bernstein polynomials), and the spectra were calibrated with the lactate doublet (with chemical shifts at δ 1.326/1.314 ppm). The next step involved chemical shift calibration and signal alignment by imposing the spectra from different samples and aligned in different areas. This was to ensure that metabolites have the same chemical shift across all samples. The spectra between δ4.5 to 5.1 ppm and between δ 5.0 to 6.0 were removed to avoid the effect of water and urea resonance, respectively. The aligned spectra were binned to segment into small areas by integrating the spectral peak area, normalized, exported to excel spreadsheet and checked for normalization.

The exported spectra were then processed through a web-based metabolomics tool, MetaboAnalyst 3.0 (Xia and Wishart, 2016). The samples were normalized by sum and scaled by Pareto scaling. One unsupervised multivariate analysis, Principle Component Analysis (PCA), and two supervised multivariate analyses, Partial Least Squares - Discriminant Analysis (PLS-DA), and Sparse Projections to Latent Structures Discriminant Analysis (SPLS-DA) (Teng, 2012) were performed. The PCA was used to check for outliers (none was found), while generating a 95% confidence interval ellipse with Hotelling's T^2 test. To check the model validity, a permutation of 1000 was run, and the R^2 (how the model explains the data) and Q^2 (the predictability of the model) were compared to identify the best model. We then used Student's t-test to compare each spectral bin assuming unequal variance between VH and GEN groups. In addition, a hierarchical clustering tree was constructed.

Statistical analysis

Dunnett's test was used to compare the means among treatment groups when the equal variance assumption was met; otherwise, and Wilcoxon test was performed to compare the means. Likelihood ratio test was used to compare the diabetes incidence, and Student's t-test was used to compare the abundance of taxa between treatment groups. JMP Pro 11 (SAS Inc., Cary, NC), R 3.3.1 (R Core Team, 2013), and GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA) were used for statistical analysis and data visualization. In addition, non-parametric t-test was used to test the statistical significance for α diversity, and Analysis of Similarities (ANOSIM) for β diversity.

In addition, the linear correlational analysis was performed within each pair: BGL in males or days to develop hyperglycemia in females, and variables that were found to be significant

between GEN and VH. The Pearson' correlation was performed to measure the strength of correlation, and the p-value, altogether with a 95% confidence interval, were provided.

Results

Blood glucose measurements and glucose tolerance test in males and females

We first determined the effect of GEN on BGL when the mice were maintained on soy-based diet. As depicted in **Figure 2.1**, GEN treatment in males produced a decrease in BGL with a significant change observed on day 98 following initial dosing (**Figure 2.1A**). The protective effect of GEN was further supported by GTT results in which the fasting BGL and 120-min BGL in GEN-treated males were significantly lower than the control, showing an improved metabolic function (**Figure 2.1B**). No males developed T1D during the study according to the criteria defined in the Method section. In females, a numerical ($P > 0.05$) decrease in BGL (**Figure 2.1D**) and an improvement in the glucose tolerance ($P > 0.05$; data not shown) were observed; however, GEN exposure significantly delayed the onset of T1D (**Figure 2.1E**) within three months of treatment. Taken together, the results showed that GEN decreased BGL in NOD males and delayed T1D onset in NOD females.

For male NOD mice fed with soy-free diet, no difference in BGL was observed throughout the study (data not shown); however, the GTT showed a similar protective effect in GEN-exposed males as those fed with the soy-based diet (**Figure 2.1C**). In our previous study, adult GEN exposure delayed the T1D onset in female NOD mice when fed the soy-free (Guo *et al*., 2015). In this study, a similar numerical decrease ($P > 0.05$) in BGL was observed in female mice on soy-free diet (**Figure 2.1F**) as those fed with soy-based diet. Taken together, adult GEN exposure produced a protective effect in both male and female NOD adults independent of diet. In

comparison, the BW and BGL in SCID female adults were not altered throughout the study, with gut microbiota alteration observed at genus level (**Supplemental Figure 2.1**).

GEN alteration of gut microbiota in males and females

The gut microbial taxonomy was firstly characterized to determine if GEN exposure altered the compositional structure of gut microbiota. **Figure 2.2A** shows the identified gut bacterial strains assigned at the phylum level from 16S rRNA sequencing in NOD males, with each color representing an individual bacterial phylum. Phylum *Bacteroidetes* represented 58.6% of the total bacteria in the VH group; while in GEN group, it represented 67.8% of the total taxa. *Firmicutes/ Bacteroides* (F/B) ratio was decreased following GEN treatment; however, it did not reach the level of statistical significance. Both the PD whole tree and chao1, indexes of α diversity that reflected the genetic diversity of the communities under study (Lozupone and Knight, 2008), were not significantly altered by GEN treatment (data not shown). In addition, GEN treatment did not show a clear pattern on the unweighted Unifrac, an index of β diversity (data not shown). However, when taking difference in abundance into account using weighted Unifrac (**Figure 2.2B**), the gut bacteria communities in the VH and GEN groups were well separated, with 55.1% and 25.32% variation explained by principal component (PC) 1 and PC2, respectively. The weighted Unifrac result is supported by ANOSIM ($P < 0.05$ with 999 permutation), which suggested the difference in the gut microbiota induced by GEN exposure was readily observable and well differentiated. Moreover, when the microbial taxa at the genus level were compared, GEN treatment increased *Prevotella*, and decreased *Alistipes* and *Blautia* in terms of relative abundance (**Figure 2.2C**), suggesting a protection against T1D (Brown *et al.*, 2011; Murri *et al.*, 2013; Qi *et al.*, 2016).

Among NOD females, the abundance of taxa in individual animals at the phylum level was not significantly altered. Phylum *Bacteroidetes* represented 68.5% of the total bacteria in VH

group, while it represented 79.0% of the total bacteria in GEN group. The taxonomy at the phylum level did not exhibit a significant change (**Figure 2.3A**). Neither Unweighted Unifrac (data not shown) nor weighted Unifrac (**Figure 2.3B**) showed a clear separation by treatment ($P > 0.05$). However, three taxa at the order level (**Figure 2.3C**) and six at the genus level (**Figure 2.3D**) were significantly increased following GEN treatment, suggesting a pro-inflammatory effect (Furet *et al.*, 2010; Qi *et al.*, 2016; Schwab *et al.*, 2014; Yang *et al.*, 2015). In addition, we have found that two diets used in this study had a differential effect (indicated by α-diversity, β-diversity, and taxonomy) on gut microbiota in VH females, which is in agreement with previous literatures (Ravussin *et al.*, 2012).

Serum antibody levels in males and females maintained on soy-based diet

As a reflection of alterations in immune homeostasis, serum antibody levels were measured using ELISA (**Figure 2.4**). GEN exposure significantly decreased the IgG_{2b} production (**Figure 2.4B**) among NOD males while the levels of IgG_{2a}, IgM and IgG_1 remained unchanged (**Figure 2.4A, C, D**). Among females, an elevated IgG_{2a} level (**Figure 2.4E**) was observed following GEN exposure, while no differences were found for IgG_{2b}, IgM, and IgG_1 (**Figure 2.4F-H**). Serum IgG_{2a} levels in females was positively associated with days to develop hyperglycemia (BGL ≥ 250 mg/dL).

Serum cytokine/chemokine levels in males and females maintained on soy-based diet

To further determine the systemic alterations in immunological response, we measured the levels of cytokines/chemokines in NOD males and females. Among the 32 cytokines/chemokines (**Table 2.3.**), those that had significant changes following GEN treatment are as follows. GEN induced a decrease in GM-CSF (24.4%), IFN-γ (22.9%), IL-5 (63.7%), IL-10 (54.7%), and MCP-1 (72.2%) among males, suggesting an overall anti-inflammatory effect. However, an increased

level in GM-CSF (2.2-fold), IL-1α (2.2-fold), LIX (2.2-fold), and MIP-2 (1.6-fold), G-CSF (1.8-fold), IL-1β (2-fold) and TNF-α (1.5-fold) was found among females following GEN treatment, suggesting a pro-inflammatory effect. Taken together, the serum levels of cytokines/chemokines suggested that GEN had a differential effect on the immune system in NOD males and females.

Body weight and pancreatic histopathology

Body weight was not changed for both NOD males and females on soy-based diet throughout the study (data not shown). No difference was found for the terminal body and organ weights except for an increased liver weight at 9.2% (1079.2 g in VH vs. 1178.6 g in GEN) following GEN exposure among GEN females. In mice fed with soy-free diet, no significant changes in body weight and organ weights were found at euthanasia (data not shown). With regards to the pathological changes, 25% of males exposed to VH showed severe insulitis (with an insulitis score of 3+) whereas none of the males exposed to GEN showed severe insulitis ($P > 0.05$). The representative images of insulitis were presented in **Figure 2.5**. Among females, 20% of the mice in both VH and GEN group showed severely insulitis (data not shown). In addition, no difference in serum insulin level was observed (data not shown).

Correlational analysis

Figure 6 illustrates the relationships between pairs of the variables that were significant by Pearson's correlation. In NOD males, the increase in BGL is associated with increased levels of GM-CSF (**Figure 2.6A**), IFN-γ (**Figure 2.6B**), *Alistipes* (at the genus level, **Figure 2.6C**), and a decreased *Provetella* (at the genus level, **Figure 2.6D**). In females, no correlation was found between hyperglycemia development and antibody/cytokine/chemokine levels, whereas two OTUs at the genus level, *Enterocccus* (**Figure 2.6E**) and *Escherichia* (**Figure 2.6F**), were negatively associated with days to develop hyperglycemia (BGL ≥ 250 mg/dL) in females.

Urinary metabolomics reveals an alteration of global metabolites following GEN dosing among NOD males

Although we did not specifically identify the metabolites, using the metabolomics study with NMR, we are able to observe an excellent separation of the VH and GEN dosed animals using the first two components of PCA (**Figure 2.7A**), PLS-DA (**Figure 2.7B**), sPLS-DA (**Figure 2.7C**), which is supported by the hierarchical clustering tree (**Figure 2.7D**).

Discussion

The prevalence of T1D, previously referred to as "juvenile diabetes", is alarmingly increasing in the US (1.25 million in American children and adults as in the year of 2012) (American Diabetes Association, 2016). In comparison, the low incidence of T1D in Asian countries coincides with the high consumption of soy-based diet, with the intake of soy food at a level of 36 g/d (Hilakivi-Clarke *et al.*, 2010). Our previous studies showed that GEN treatment induced a decreased BGL in STZ - treated adult male B6C3F1 mice (Guo *et al*., 2014) and a delayed onset of T1D in female NOD mice (Guo *et al*., 2015). Gut microbiota has a "cross-talk" with mucosal immunity (Purchiaroni *et al.*, 2013), and is closely associated with T1D risk (de Goffau *et al*., 2013; Dietert, 2014; Giongo *et al.*, 2011). To our knowledge, our study is the first to evaluate the GEN effect on gut microbiota *in vivo* and relates it to immunological changes and T1D occurrence.

In our previous studies with different diets, we have found that NOD mice maintained on the soy-free diet developed T1D faster (data not shown). In our male NOD mice, the development of T1D was slower (0% by 6 months of age) than the rate reported in literature (20 to 30%), which might be due to the protective effect of soy in the soy-based diet, with an isoflavone level range of 237-655 ppm (**Table 2.1.**). In this study, it is assumed that the food intake of a 25 g mouse is 2 g;

thus, the dietary intake of isoflavone is approximately 18.96-52.4 mg/kg BW. We used blood glucose measurement as the major readout in males. We were able to make a precise measurement of non-fasting BGL as we have kept the measuring time consistent (around noon on the day of measurement) as well as fasting BGL as we followed the 15-h fasting protocol (Ayala *et al*., 2010). In addition to a decreased BGL, we observed an increased glucose tolerance among NOD males following GEN exposure, which is in agreement with our previous studies in STZ - treated male B6C3F1 mice (Guo *et al*., 2015) and has been confirmed by others (Weigt *et al.*, 2015). The immune system is key to T1D development in terms of GEN modulation since we did not observe difference in BGL alteration in SCID mouse.

T1D was found to closely associate with the diversity and abundance of taxa in gut microbiota in both human epidemiological studies (Mejía-León *et al.*, 2014) and animal studies (Hansen *et al.*, 2012; Hu *et al.*, 2015; Patterson *et al.*, 2015). Although no alteration in β diversity was observed between sexes in our study (data not shown), a significantly different gut microbial taxonomy between male and female NOD mice was noted (data not shown), which suggested the sex effect on T1D might be due to gut microbiota (Markle *et al.*, 2013). Patterson *et al*. (2015) investigated the gut microbiota changes in STZ-induced T1D in male Sprague-Dawley rats, and found a reduced α-diversity and F/B ratio while a change in β diversity. In our study with male NOD mice, we found that GEN exposure had a significant effect on β diversity when accounting for the abundance of taxa (e.g., weighted Unifrac), although the effect on F/B ratio was not significant. In addition, the increased *Prevotella* as well as decreased *Alistipes* and *Blautia* at the genus level following GEN exposure in NOD males were all in accordance with a reduced T1D risk and an anti-inflammatory status (Brown *et al*., 2011; Murri *et al*., 2013; Qi *et al*., 2016). The gut microbiota might function as a modulator on T1D by the metabolites, and we need to example

on specific metabolites to support this. Further evidence that adult GEN exposure induced anti-inflammation in NOD males included a reduced IgG_{2b} level (Ichikawa *et al.*, 1999) and the cytokine/chemokine profiles with reduced GM-CSF, IFN-γ, IL-5, and MCP-1 levels. MCP-1 was found to be higher in T1D cases (Ismail *et al.*, 2016). As an immune modulatory cytokine, GM-CSF could exacerbate autoimmune diseases in inflammatory microenvironments (Bhattacharya *et al.*, 2015). IFN-γ induces pancreatic β cell apoptosis through STAT1-mediated Bim protein activation and exacerbates T1D (Barthson *et al.*, 2011).

In spite of the protective effect of GEN on T1D among females, increased *Erysipelotrichaceae* (Schwab *et al*., 2014), and decreased *Escherichia* (Furet *et al*., 2010), *Lachnoslira* (Qi *et al.*, 2016), *Firmicutes* (other genus) and *Enterococcus* (Yang *et al*., 2015) at the genus level suggested a pro-inflammatory status in our GEN-treated NOD females. At the order level, increased *Erysipetotrichalae* (Schwab *et al*., 2014) and *Clostridia* (other order), and decreased *Firmicutes* (other order) also indicated an exacerbating effect of T1D. The notion that GEN is pro-inflammatory in females is further supported by increased levels of cytokines/chemokines in GEN-treated NOD females when compared to the control, including GM-CSF, IL-1α, LIX, and MIP-2, G-CSF, IL-1β, and TNF-α (Barthson *et al*, 2011; Lewis *et al.*, 2011; Navarro-Gonzalez and Mora-Fernandez, 2008; Schirmer *et al.*, 2016). It was suggested that sex has a strong effect on IL-1β, while gut microbiota has more effect on TNF-α (Schirmer *et al.*, 2016). In fact, GEN also showed a pro-inflammatory response among female B6C3F1 mice in our previous studies (Guo *et al.*, 2007; Guo *et al.*, 2001). Therefore, GEN exposure led to a protection on T1D among NOD females, which could not be explained by the pro-inflammatory status as represented by gut microbiota, antibody and cytokine/chemokine profiles.

The protective effect of GEN on T1D in NOD females could be due to direct inhibition of insulitis, increased insulin receptor expression (Bhattacharya *et al.*, 2015) or up-regulation of hepatic glucose-6-phosphate dehydrogenase (G6PD) activities (Choi *et al.*, 2008). However, these effects were observed in both females (Choi *et al.*, 2008) and males (Zimmermann *et al*, 2012). It has been reported that T1D is gender-specific, and phytoestrogens like GEN can exert estrogen-like effects (Barrett, 2006; Gilbert and Liu, 2013). There is evidence that estrogen receptor (ER) agonist (such as phytoestrogen) could reduce the risk of diabetes in animals (Liu *et al.*, 2013). Thus, GEN might function as an ER agonist in female NOD mice in which the estrogen level is relatively low by preserving the pancreatic β cells (Choi *et al.*, 2008; Fu *et al.*, 2010; Gilbert and Liu, 2013) and regulating insulin-secreting pathways (Liu *et al.*, 2006). In addition, it was possible that a decreased *Enterobacteriaceae* at the order level (Soyucen *et al.*, 2014) and elevated IgG_{2a} could have a protective effect on T1D (Todd *et al.*, 1998) in GEN-treated NOD females, as IgG_{2a} antibodies might non-specifically delay the onset of diabetes in female NOD mice (Todd *et al.*, 1998).

With regards to glucose regulation, Weigt *et al*. (2015) showed that GEN improved glucose tolerance in female rats using a GEN dosage that was twice as ours. In our current study with NOD females, GEN exposure at 20 mg/kg produced a later onset of T1D but had no significant effects on glucose tolerance. This amount of GEN in a mouse is equivalent to a human clinical trial dose (approximately 100 mg/day) in terms of milligram/square meter of body surface that usually gives more accurate interspecies extrapolation (Hooper and Macpherson, 2010). Interestingly, no glucose tolerance improvement was found among Chinese women who used a dose of 50 mg GEN daily for 6 months in an epidemiological study (Ye *et al.*, 2015). It is possible that adult GEN

exposure at physiologically relevant doses can decrease T1D incidence but not BGL in females; however, further epidemiological studies are needed to confirm this.

This study also has some limitations. First, the implicated causal relationship in the immunity-microbiota-T1D was not studied, and thus it is important to carry out approaches involving fecal transfer, immune reconstitution in SCID mice, and antibiotic treatment to deplete microbiota or germ-free mice in the future. Secondly, only one time point along the course of T1D development was shown in the study, which limited our ability to study the sequential events of immunological alteration and microbiota changes with the progression of T1D. In addition, with the use of soy-based diet, it was difficult to exclude the confounding effects of daidzein and glycitein, as their average concentration is 175.1 ppm and 37.8 ppm, respectively, compared to 191.9 ppm for GEN concentration. Therefore, a mixture study by combining GEN with daidzein and/or glycitein will help further elucidate the underlying mechanisms of action.

Overall, our studies suggested that the GEN exposure is associated with alterations in both gut microbiota and immune responses. An intricate interaction between microbiota and immunity, especially mucosal immunity, has been suggested in multiple studies through production of mucin, antimicrobial peptides, and cytokines (Hooper and Macpherson, 2010). In our study, GEN-mediated gut microbiota alterations, together with immunological changes, were pro-inflammatory in NOD females and anti-inflammatory in NOD males. Further investigations should be carried out to determine the causal relationship among the estrogen-microbiota-immune consortium, and if GEN protection of T1D is through microbiota-mediated immunomodulation. This study will serve as a model to study exposure to natural products and prevention of autoimmune diseases.

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Table 2. 1. The major nutrients composition in the diets

Diet	Amount by weight				Calories (kcal/g)
	Protein (%)	Carbohydrates (%)	Fat (%)	Isoflavone (ppm)	
PicoLab Rodent Diet 20 (soy-based)	24.7	62.1	13.2	237-655	3.41
5K96 Verified Casein Diet	22.1	66.6	11.3	<10	3.44

Note: All numbers are obtained from the manufacturer's protocols. Websites
http://www.labdiet.com/cs/groups/lolweb/@labdiet/documents/web_content/mdrf/mdi4/~edisp/ducm04_028427.pdf
http://www.labdiet.com/cs/groups/lolweb/@labdiet/documents/web_content/mdrf/mdi4/~edisp/ducm04_028436.pdf

Table 2. 2. The primers and tags used in sequencing

Primers			
16S_341_F	CCTACGGGNGGCWGCAG		
16S_785_R	GACTACHVGGGTATCTAATCC		
iTru_R1_5'_fusion	ACACTCTTTCCCTACACGACGCTCTTCCGATCT		
iTru_R2_5'_fusion	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT		
Tags			
1	GGTAC	11	ccACGTC
2	cAACAC	12	ttcTCAGC
3	atCGGTT	13	CTAGG
4	tcgGTCAA	14	tGCTTA
5	AAGCG	15	gcGAAGT
6	gCCACA	16	aatCCTAT
7	ctGGATG	17	ATCTG
8	tgaTTGAC	18	gAGACT
9	AGGAA	19	cgATTCC
10	gAGTGG	20	tctCAATC

Table 2. 3. The cytokine/chemokine levels measured by Multiplex Mouse Cytokine/chemokine Magnetic Bead Panel Kit 96 Well Plate Assay in NOD male and female sera

	G-CSF	EOTAXIN	GM-CSF	IFN- γ	IL-1α	IL-1β	IL-2	IL-4
VHF	131.95±28.09	288.22±71.23	10.17±4.05	5.59±2.39	76.99±22.33	5.02±1.46	10.63±4.94	n.d.
GEF	242.24±26.27*	437.94±66.63	22.08±3.79*	11.58±2.24	168.89±24.32*	10.18±1.37*	19.68±4.62	n.d.
VHM	163.15±32.98	676.00±51.06	24.58±5.88	8.17±2.00	239.96±27.22	14.70±2.25	n.d.	n.d.
GEM	195.36±36.88	565.61±57.09	6.00±6.57*	1.87±2.20*	222.21±30.43	11.40±2.52	n.d.	n.d.

	IL-3	IL-5	IL-6	IL-7	IL-9	IL-10	IL-12p40	IL-12p70
VHF	n.d.	8.23±2.32	4.14±2.44	16.90±5.30	243.98±64.56	3.41±0.41	n.d.	3.72±0.82
GEF	n.d.	9.34±2.17	8.00±2.29	7.51±8.39	330.15±60.39	4.15±0.39	n.d.	4.27±0.76
VHM	n.d.	7.01±0.61	13.08±3.88	n.d.	356.53±59.57	6.07±1.13	n.d.	4.67±0.87
GEM	n.d.	4.46±0.68*	3.17±4.33	n.d.	176.75±66.60	3.32±1.27**	n.d.	2.92±0.98

	LIF	IL-13	LIX	IL-15	IL-17	IP-10	KC	MCP-1
VHF	n.d.	10.75±3.07	914.39±330.69	352.19±98.54	n.d.	227.06±36.51	67.57±17.99	18.64±3.62
GEF	n.d.	19.36±2.87	2014.00±309.33*	51.96±92.17	n.d.	239.84±34.15	101.03±16.83	24.80±3.39
VHM	n.d.	22.59±4.83	1722.36±223.59	66.90±61.97	n.d.	220.87±18.00	108.84±18.35	26.43±2.65
GEM	n.d.	12.09±0.81	1107.63±249.98	198.54±69.28	n.d.	189.77±20.13	80.87±20.51	19.08±2.97*

	MIP-1α	MIP-1β	M-CSF	MIP-2	MIG	RANTES	VEGF	TNF-α
VHF	97.75±35.84	22.62±6.23	n.d.	79.04±10.40	224.64±44.65	7.52±0.63	3.96±1.36	2.88±0.42
GEF	165.01±33.53	41.03±5.84	n.d.	125.41±9.73*	147.07±41.76	8.06±0.59	6.34±1.22	4.37±0.40*
VHM	131.70±22.89	36.03±6.29	5.65±0.85	129.76±5.05	263.06±30.66	11.41±1.28	4.79±0.61	5.09±0.38
GEM	64.72±25.59	20.21±7.04	7.29±0.95	116.15±5.65	265.15±34.28	10.10±1.43	3.74±0.69	4.09±0.42

Note: All values represent mean ± S.E. (pg/ml). IL = interleukin, IP-10 = interferon γ-induced protein 10, G-CSF = granulocyte-colony stimulating factor, GM-CSF = granulocyte macrophage colony-stimulating factor, LIX = C-X-C motif chemokine 5, KC = keratinocyte chemoattractant, MCP-1 = monocyte chemoattractant protein-1, MIP-1α = macrophage inflammatory protein 1-α, MIP-1 β = macrophage inflammatory protein 1- β, MIG = C-X-C motif chemokine 9, RANTES = C-C motif ligand 5, VEGF = vascular endothelial growth factor, TNF-α = tumor necrosis factor-α, LIF = leukemia inhibitory factor, M-CSF = macrophage colony-stimulating factor, n.d. = not detected, VHM = NOD males dosed with vehicle (VH, N = 10); VHF = NOD females dosed with VH (N = 8); GEM = NOD males dosed with genistein (GEN, N = 10); and GEF = NOD females dosed with GEN (N = 9). *, P<0.05, **, P<0.01.

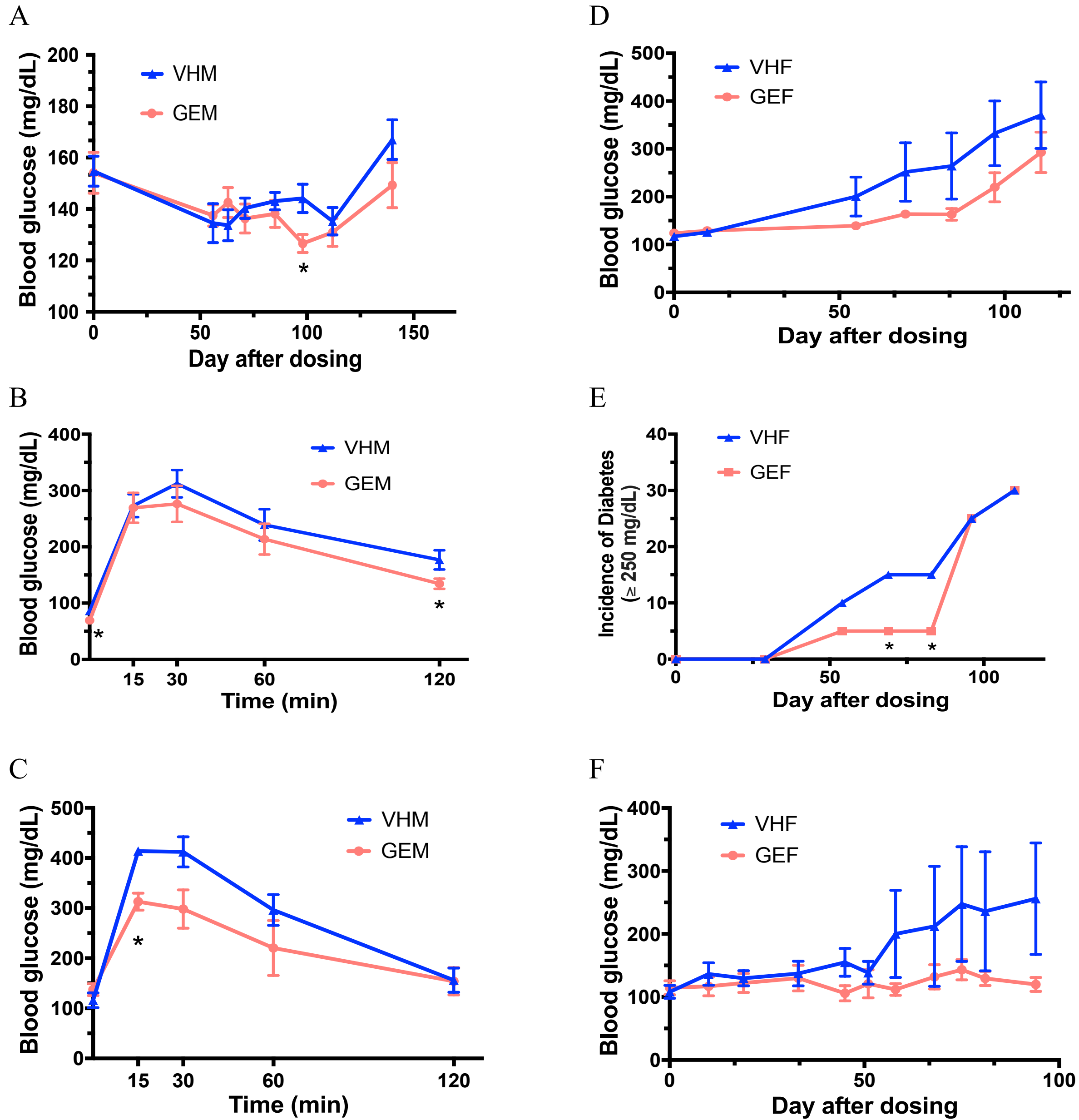


Figure 2. 1. Time course studies for the blood glucose levels and glucose tolerance test (GTT) in NOD mice. (A) Blood glucose levels (BGLs ± SE) in NOD males (N = 10/group) on PicoLab diet; (B) GTT for NOD males at day 128 after initial dosing on PicoLab diet (N = 10/group); (C) GTT for NOD males on 5K96 diet prior to euthanasia (N = 4/group); (D) BGL (± SE) in NOD females on PicoLab diet (N = 10/group); (E) The incidence of T1D in NOD females on PicoLab diet (N=10/group). A BGL ≥ 250 mg/dL was considered T1D. (F) BGL (± SE) in NOD females on 5K96 diet (N = 5/group). *, $p < 0.05$. VHM = NOD males dosed with vehicle (VH); VHF = NOD females dosed with VH; GEM = NOD males dosed with genistein (GEN); and GEF = NOD females dosed with GEN.

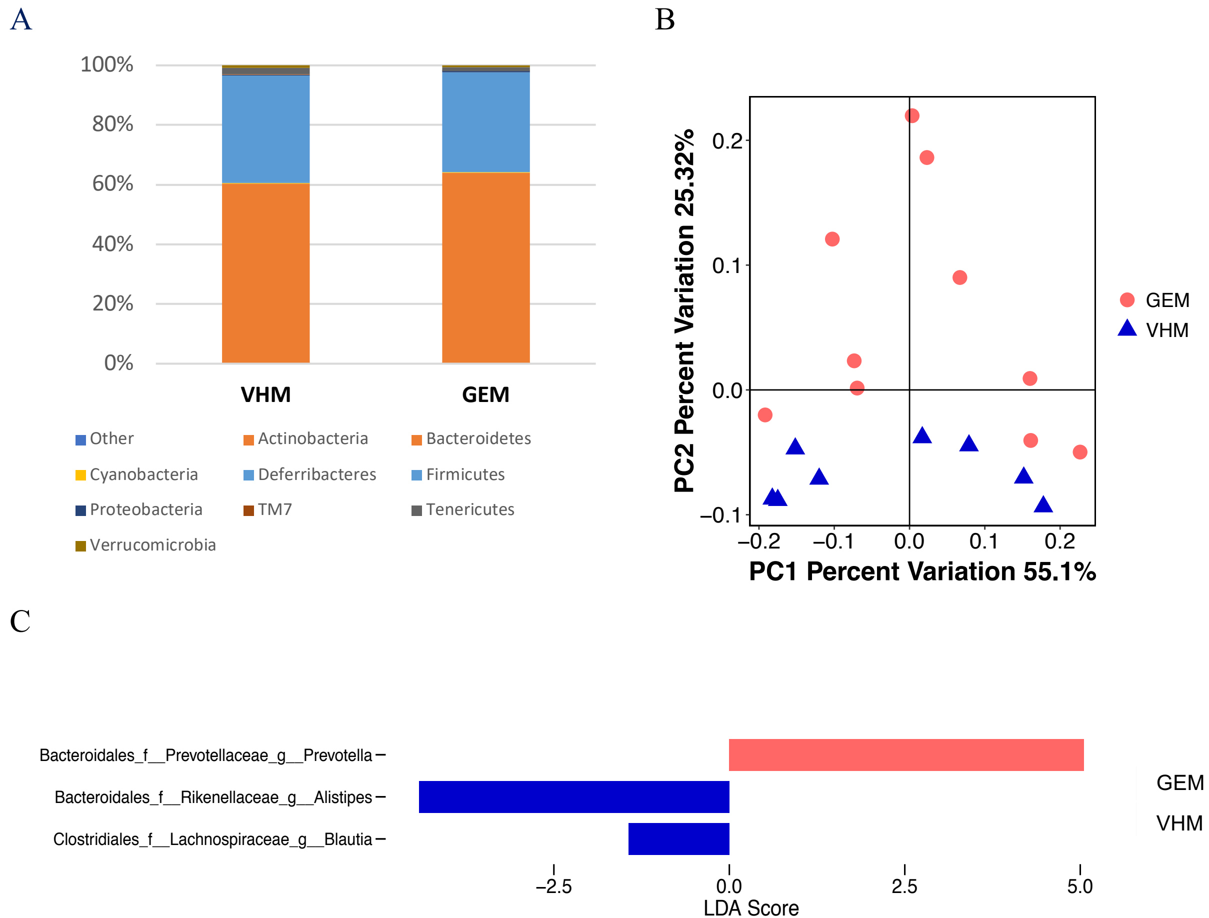


Figure 2. 2. The composition of gut microbiota based on 16S rRNA sequencing in NOD males on PicoLab diet treated with genistein (GEM, N = 10) or vehicle (VHM, N = 8). (A) The taxonomy of the gut microbiota at the phylum level when the individual animal data were combined for analysis according to the treatment. (B) The β-diversity based on the weighted Unifrac index. Each of the symbols (closed circles and triangles) represents one animal and is illustrated by Principal Coordinate Component (PCoA), and the primary principal component (PC1) and secondary principal component (PC2) are shown. Eigenvalues of PCoA represented by the distance matrix can be interpreted in terms of percentage of total variability (x-axis and y-axis). The distance between two symbols suggests dissimilarity between the two samples. Weighted Unifrac takes into account the differences in abundance of taxa between samples. (C) Linear Discriminant Analysis Effective Size (LEfse) results at the genus level between VHM and GEM groups. LEfse is used to elucidate the differences in bacterial taxa.

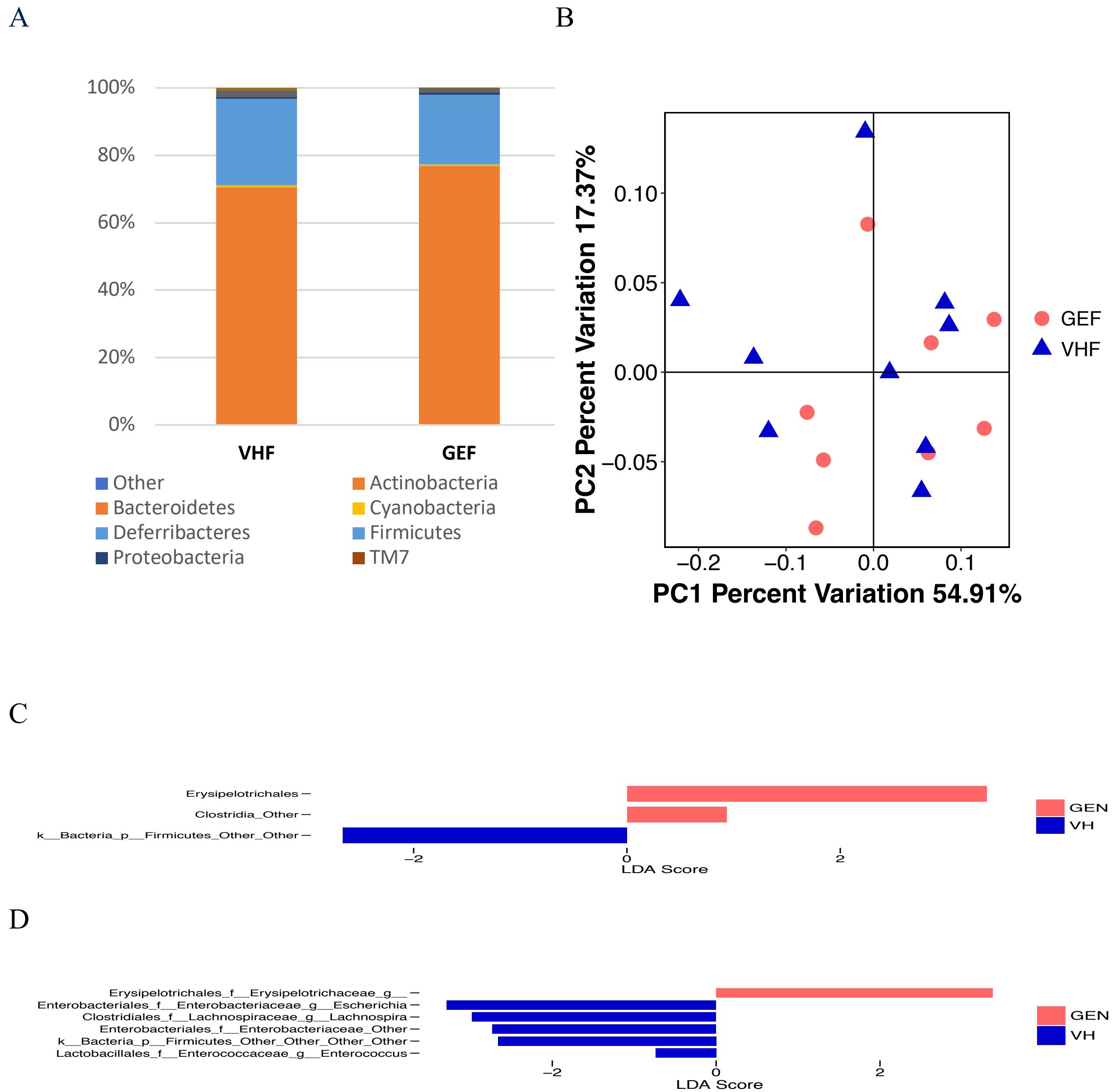


Figure 2. 3. The composition of gut microbiota based on 16S rRNA sequencing in GEN-treated (GEF, N = 8) and VH-treated (VHF, N = 9) NOD females on the PicoLab diet. (A) The taxonomy of the gut microbiota at the phylum level when the individual animal data were combined for analysis according to the treatment. (B) The β-diversity based on the weighted Unifrac index. (C) LEfse results between VHF and GEF groups at the order level. (D) LEfse results between VHF and GEF groups at the genus level.

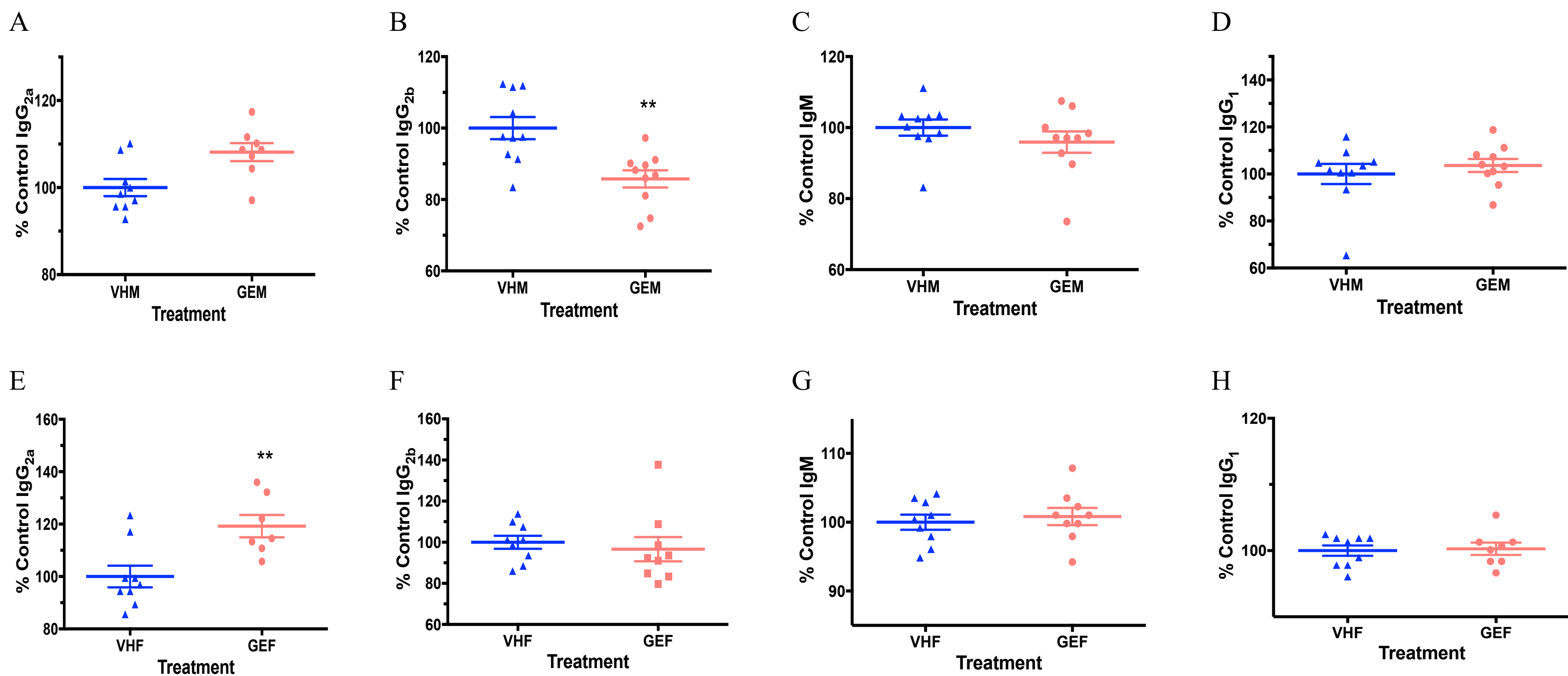


Figure 2. 4. The serum antibody levels in NOD males (A-D) and females (E-H) on PicoLab diet. IgG_{2a} (A), IgG_{2b} (B), IgM (C), and IgG_1 (D) levels in the sera of NOD males were measured at dilutions of 1:50, 1:50, 1:500, and 1:5000 (v/v), respectively, by ELISA. IgG_{2a} (E), IgG_{2b} (F), IgM (G), and IgG_1 (H) levels in the sera of NOD females were determined at dilutions of 1:50, 1:50, 1:500, and 1:5000 (v/v), respectively. The sera were obtained when the mice were euthanized. *, $p<0.05$, and **, $p<0.01$. Each of the serum antibody concentration was calculated as the percent control. VHM = NOD males dosed with vehicle (VH, N = 10); VHF = NOD females dosed with VH (N = 8); GEM = NOD males dosed with GEN (N = 10); and GEF = NOD females dosed with GEN (N = 9).

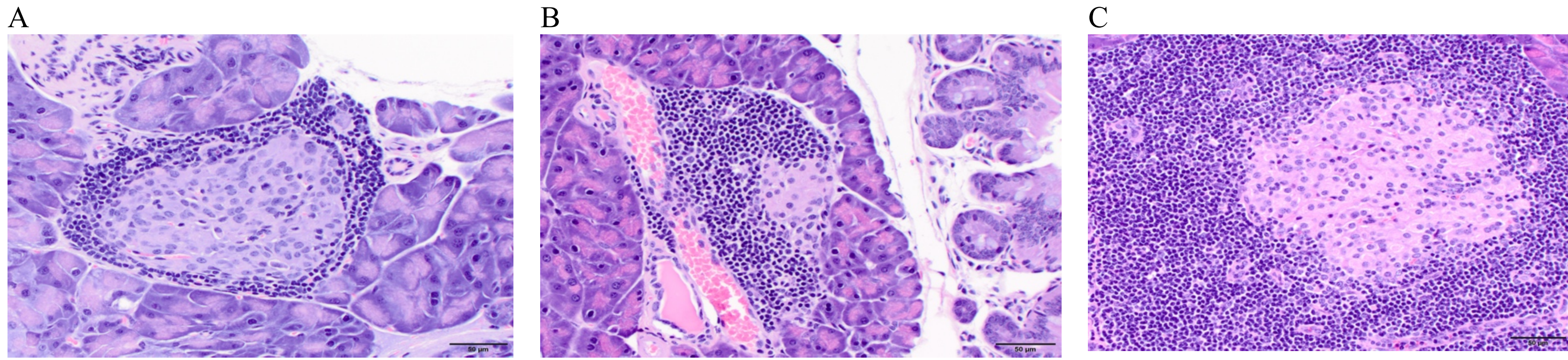


Figure 2. 5. Representative images of insulitis in NOD mice. (A) Female mouse dosed with vehicle control with an insulitis score of 1+; (B) Male mouse dosed with genistein with an insulitis score of 2+; and (C) Male mouse dosed with vehicle control with an insulitis score of 3+.

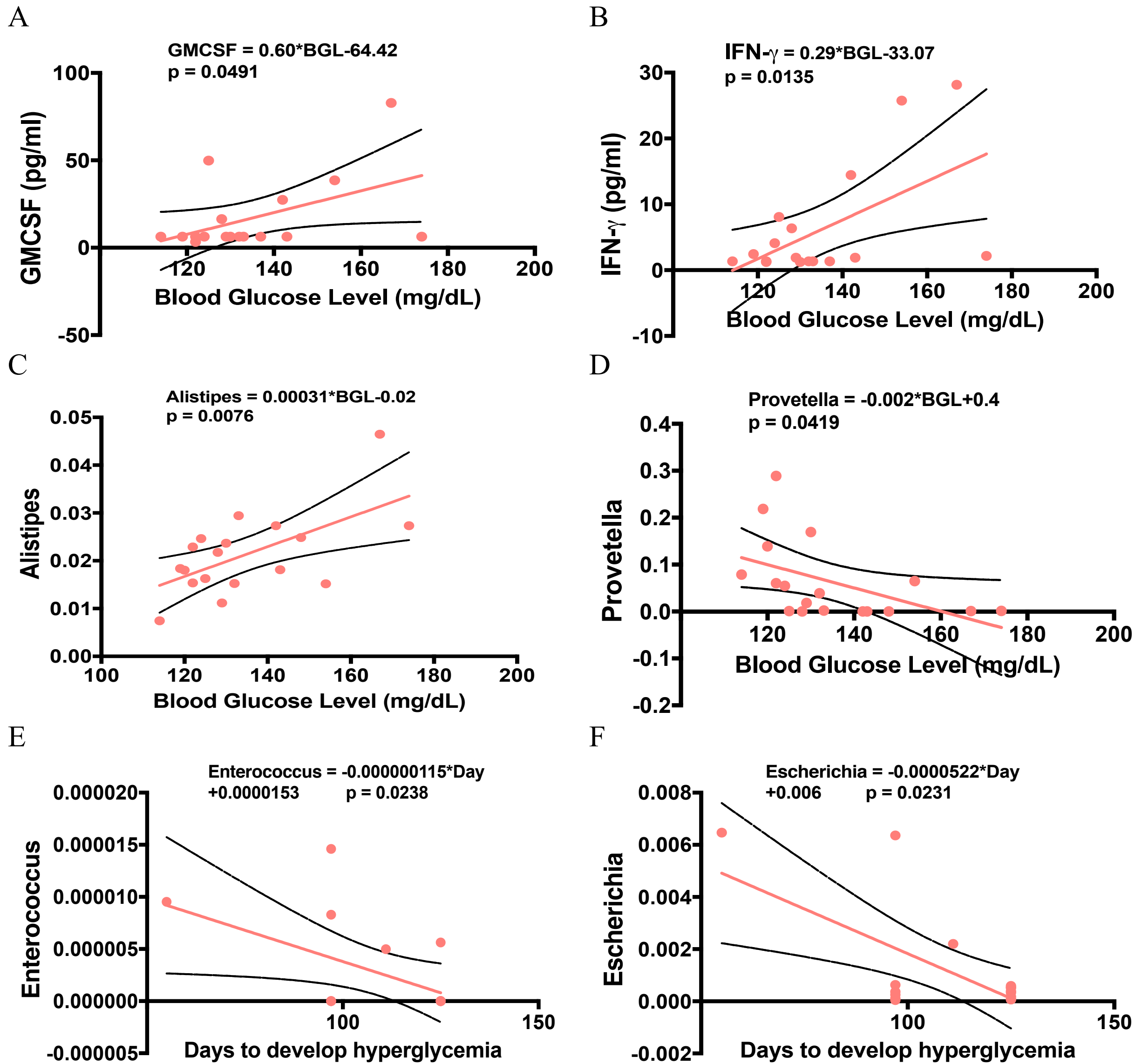


Figure 2. 6. Correlational analysis. (A) Serum GM-CSF and blood glucose levels in males at Day 98 after initial dose, (B) Serum IFN-γ and blood glucose levels in males. (C) The relative abundance of *Alistipes* and blood glucose levels in males. (D) The relative abundance of *Provetella* and blood glucose levels in males. (E) The relative abundance of *Enterocccus* and days to develop hyperglycemia in females. (F) The relative abundance of *Escherichia* and and days to develop hyperglycemia in females. Hyperglycemia was defined as a blood glucose level higher or equal than 250 mg/dL. The dotted lines represent 95% confidence interval.

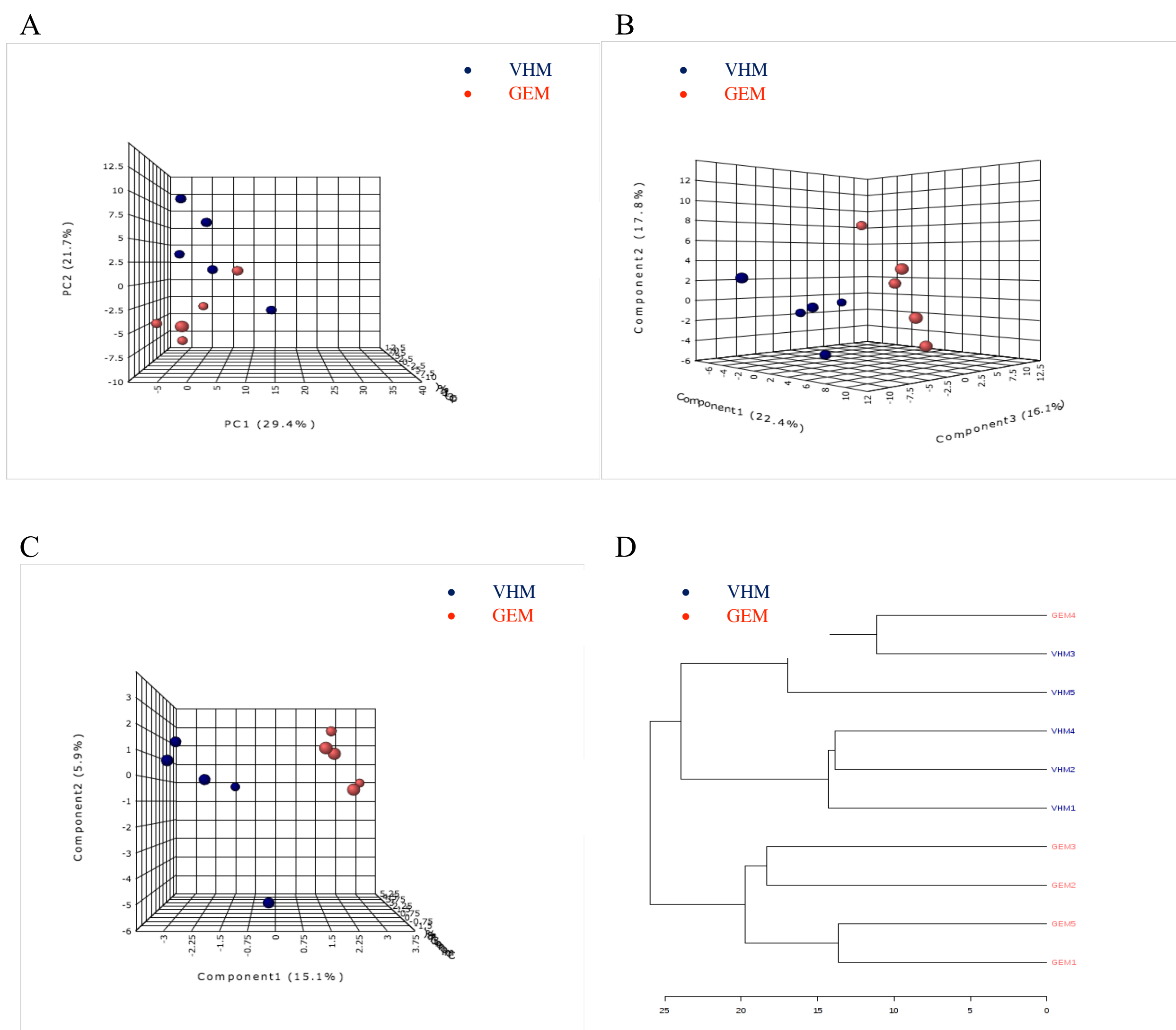
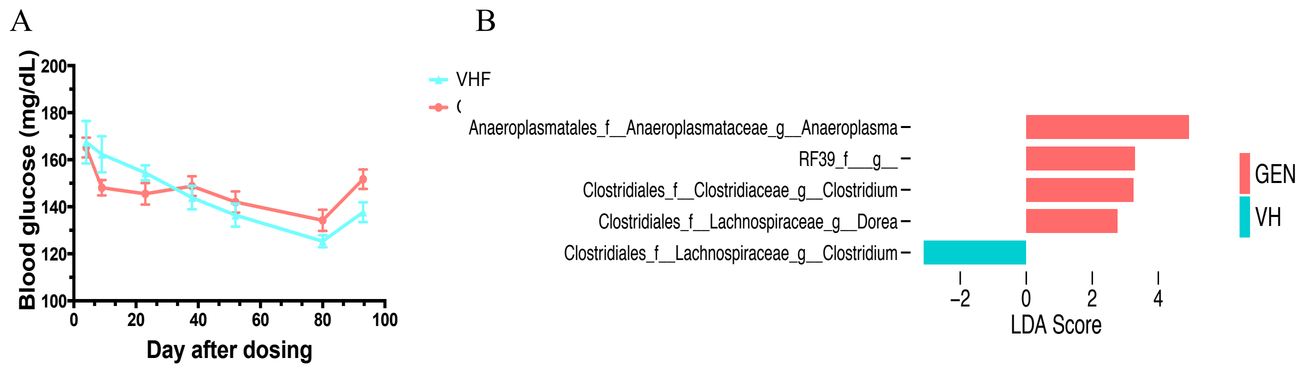


Figure 2. 7. Urine metabolomics represented by (A) Principle Component Analysis (PCA), (B) Partial Least Squares - Discriminant Analysis (PLS-DA), (C) Sparse Projections to Latent Structures Discriminant Analysis (SPLS-DA), and (D) hierarchical clustering tree between genistein and vehicle control treatments.



Supplemental Figure 2. 1. (A) The blood glucose for female SCID female mice receiving a daily dose of 20 mg/kg GEN dissolved in 25 mM Na_2CO_3 (VH) by gavage. To induce high blood glucose, streptozotocin (STZ), a chemical that destructs pancreatic β cells, was injected *i.p.* four times during their lifespan. (B) Linear discriminant analysis Effect Size showing taxa at genus level that were differentially expressed. VHF = females dosed with vehicle, and GEF = females dosed with genistein. N=15 in each group.

CHAPTER 3

EXACERBATION OF TYPE 1 DIABETES IN PERINATALLY GENISTEIN EXPOSED FEMALE NON-OBESE DIABETIC (NOD) MOUSE IS ASSOCIATED WITH ALTERATIONS OF GUT MICROBIOTA AND IMMUNE HOMEOSTASIS[3]

[3] HUANG, G., XU, J., CAI., D., CHEN, S-Y., & GUO, T. Accepted by Toxicological Sciences. Reprinted here with permission of publisher.

Abstract

Despite various hypothesized benefits of dietary isoflavone genistein (GEN) deriving from soy-based products, many questions surrounding GEN's immunotoxic effects, especially during perinatal exposure, have yet to be answered. In the current study, we exposed NOD offspring to GEN *per oral* at a physiological dose (20 mg/kg body weight) from embryonic day 7 to postnatal day (PND) 21. In female offspring, perinatal GEN dosing significantly increased the incidence of type 1 diabetes (T1D) at PND 113 (24% vs. 3%), 120 (24% vs. 6%), 147 (39% vs. 16%) and 162 (45% vs. 23%), and the exacerbation was associated with decreased serum levels of interleukin (IL)-10, IgG_{2a} and IgM. In male offspring dosed with GEN, the incidence of T1D was numerically decreased, and a decrease in serum IgG_1 was also observed. Flow cytometric analysis in females suggested an increased pro-inflammatory splenic $CD5^+CD24^-$ and $CD4^-CD8^+$ cell counts, while both %T cells and $\%CD4^+$ T cells were significantly decreased in males, suggesting a potential anti-inflammatory effect. Gut microbiota (GMB) analysis indicated that fecal microbiota from PND 90 female offspring exhibited an increased level of *Enterobacteriales* (suggesting a pro-inflammatory response), while the similar changes were not found in PND 30 females. Moreover, RNA sequencing showed that intestinal α-defensin expression was down-regulated in GEN-treated females, supporting a pro-inflammatory response. However, perinatal GEN administration perturbed GMB toward an anti-inflammatory response in PND 90 males. Taken together, a strong sex-specific effect was found in the unique perinatal GEN exposure window, and the TID exacerbation in NOD females was associated with GMB-related immunomodulatory mechanisms.

Introduction

Among children in the U.S., the prevalence of type 1 diabetes (T1D) is increasing from 1.48 to 2.32 per 1,000 during 2002-2013 (Li *et al.*, 2016), and several hypotheses have been

brought into attention, such as pathogen, vitamin D deficiency, and food (Egro, 2013). Soy products are of particular importance since they are an intrinsic part of Asian cuisine, and the market of soy-based formula is increasing in the U.S. (about 20% of the formula market), partly due to its higher content in calcium and phosphorus than cow milk (Bhatia, Greer, & Comm, 2008). A cross-sectional study found that the urinary genistein (GEN) concentration in infants consuming soy-based formula was 500 times higher than in those consuming milk-based formula (Cao *et al*., 2009). Isoflavones in soy exert estrogen-like effects, and one major soy isoflavone is GEN (Choi *et al*., 2008). Adult GEN dosing is reported to have anti-diabetic function on both T1D (Guo *et al*., 2014) and type 2 diabetes (Gilbert & Liu, 2013) due to its immunomodulatory properties and protective effects on pancreatic β-cells in murine models. However, the effects of GEN on immune regulation, and the onset of T1D and glucose regulation during perinatal stages have not been fully understood. Perinatal GEN treatment was found to have immunomodulatory effect in animal studies. For example, our group has reported changes in immune response (such as increased cytotoxic T cells activity and decreased regulatory T cells) following perinatal GEN dosing in female B6C3F1 mice, and the response depends largely on sex, exposure duration, as well as litter order (Guo *et al*., 2005). In another study with C57BL/6 mice, perinatal GEN dosing induced an increase in the number of splenic T cell subpopulations, as well as number and activity of natural killer cells in both male and female offspring, showing a pro-inflammatory response (Guo *et al*., 2006).

The association of high soy consumption during perinatal period and risk of T1D is uncertain and intricate. Among the limited epidemiological reports, one study showed that for infants aged 4-6 and 7-12 months, soy milk formula consumption was associated with a two-fold increase of T1D risk in China (Strotmeyer *et al*., 2004), while information on other exposure

windows is largely unknown. Another epidemiological study suggested twice as many diabetic children consumed soy-based formula in infancy as healthy children (Fort *et al*., 1986). However, the mechanism underlying soy intake effect on T1D in perinatal stages is largely unknown, and studies on the gender effect are lacking.

In the fetus, early-life gut bacterial colonization plays an important role in metabolic tissue development and in influencing the risk of autoimmune diseases (Kozyrskyj & Sloboda, 2016) because intestinal immune system development starts as early as 11 weeks of gestation in humans (Mejia-Leon & de la Barca, 2015). Bacterial DNA can transfer from mother to fetus through amniotic fluid, umbilical cord (Kozyrskyj & Sloboda, 2016), and placenta (Romano-Keeler & Weitkamp, 2015). There are evidences to show that modulation of gut microbiota (GMB) by probiotics (*Lactobacillus rhamnosus* or *Bifidobacterium lactis*) during pregnancy alters infant immune responses (Prescott *et al*., 2008). The mode of delivery, antibiotic use after birth and infant formula consumption could all help shape the infant microbiota (Mejia-Leon & de la Barca, 2015; Mueller *et al*., 2015; Romano-Keeler & Weitkamp, 2015) and further modulate the immune system. In mice, the colonization of thymus starts from day 10-11 of gestation (Holladay & Smialowicz, 2000). To understand the effect of perinatal GEN intake on T1D, which represent a major deficit in our present knowledge, we investigated the potential links between GEN at the physiological level (20 mg/kg body weight) and increased risk of developing T1D in non-obese diabetic (NOD) mice, a model that closely resemble human T1D (Anderson & Bluestone, 2005), with regards to different exposure windows (adult vs perinatal) and sex from perspectives of immunomodulation, GMB and urinary metabolome.

Methods

Perinatal study

A total of 24 time-mated (male: female = 1:1) NOD female mice with normal blood glucose level (BGL) were used and randomized into vehicle (VH) and GEN groups (12 per group) based on body weight and baseline BGL. From gestational day (GD) 7, the mice in VH group received a 0.1 ml/10g BW sodium carbonate (Na_2CO_3, 25 mM) daily because of GEN's low water solubility, and the mice in GEN group were gavaged with 0.1 ml/10g BW GEN dissolved and sonicated in VH (2 mg GEN/mL (Guo *et al*., 2014) *per oral* till postnatal day (PND) 21). Thus, the perinatal dosing included both *in utero* and lactational exposures. The daily dosing was performed with 18 G gavage needles. After parturition, the pups were housed in the same cage with dams with one litter per cage. As the pups on 5K96 diet were usually smaller than the ones on regular diet, they were separated from the dams around PND 28, and housed up to five same-sex littermates per cage. Each litter was treated as an experimental unit. Animal husbandry was included in the Supplemental Material.

In the second study, a total of 8 NOD females with normal BGL were randomized into VH (N=4) and GEN (N=4) groups, mated (male: female = 1:2), and received the same dosing regimen as above. These 8 females gave birth to 8 litters. This separate study was conducted for GMB analysis, transcriptomic analysis and to verify previous results. Each litter was treated as an experimental unit. All animal procedures in the lab were approved by the UGA Institutional Animal Care and Use Committee (IACUC).

Body weight (BW) and blood glucose level (BGL) measurement

The BW and BGL were monitored every week for the NOD offspring. The non-fasting BGL was measured using a blood glucose meter (Bayer Contour, Ascensia Diabetes Care,

Parsippany, NJ) with a small sample of venous blood sample. T1D was defined as a BGL higher than or equal to 250 mg/dL (13.9 mmol/L) (Guo *et al*., 2014). Mice with BGL of 600 mg/dL or higher (33.3 mmol/L) in two consecutive weeks or at the end of the study were euthanized with carbon dioxide (CO_2) inhalation. Individual organs were collected and weighted during necropsy. In addition, glucose and insulin tolerance tests were performed (in Supplemental Material).

Serum antibody measurement

The enzyme-linked immunosorbent assay (ELISA) was performed to detect the total antibody level (IgG_{2a}, IgG_{2b}, IgM, IgG_1) in mouse sera according to the reported procedures (Guo *et al*., 2014). Briefly, the plates were added with blocking solution (5% milk powder in PBS with Tween 20) for 1 h and washed with PBST (Tween 20 in PBS). The diluted serum was then added in the plates and incubated in room temperature for 2 h. Then the secondary antibody was added and incubated for 1 hour. Substrate TMB was added at 100 μL/well and the plate was read at the wavelength of 405 nm. Serum insulin and anti-insulin antibody were also measured and listed in the Supplemental Material.

Serum cytokine and chemokine

A total of 9 cytokines (IFN-γ, IL-1α, IL-1β, IL-2, IL-6, IL-10, IL-12p40, IL-12p70, TNF-α) and 1 chemokine (MCP-1) were measured using Luminex Mouse Cytokine/Chemokine Magnetic Bead Panel (EMD Millipore Corporation, Billerica, MA, USA) following manufacturer's protocols. Sera and beads were added, and the plates were incubated at 4 °C overnight on a shaker. Then detection antibody was added and followed by streptandin-phycacrythrin. Plates were run on a Bio-Plex MAGPIXTM Multiplex Reader with Bio-Plex ManagerTM MP Software (Luminex, Austin, TX). Each biomarker concentration was calculated as pg/ml.

Flow cytometry

The spleen was subject to flow cytometric analysis following mashing between two slides with frosted ends (Guo *et al*., 2006). For male offspring, different cell surface markers were labeled with a cocktail of monoclonal antibodies conjugated with a fluorescent molecule (Phycoerythrin onjugated antibody for CD4, Peridinin ChlorophyII Protein Complex (PerCP) for CD8, fluorescein isothiocyanate (FITC) for IgM, and PerCP for CD3), for visualization. For female offspring (Huang *et al*., 2017), the fluorochrome-labeled antibody cocktails for cell staining were: APC-H7 and V450 (for CD4 and CD8), PerCP-Cy5-5 and PE-CF594 (for total T cells and B cells), and FITC and PE (for CD24 and CD5). All antibodies were obtained from BD PharMingen (San Diego, CA). Isotype-matched irrelevant antibodies were used as controls. Following the addition of the reagents, the cells were incubated at 4 °C in the dark for 30 min. The cells were washed, and enumeration performed on a Becton Dickinson LSRII Flow Cytometer in which log fluorescence intensity was read and a forward scatter threshold high enough to eliminate red blood cells. Ten thousand cells were counted for each sample.

16s rRNA taxonomy and diversity

The DNA extraction and sequencing were included in Supplemental Materials. Bioinformatics analysis was performed as previously described with fecal DNA amplicons (Huang *et al*., 2017; Lefever *et al*., 2016). In brief, Read 1 and Read 2 sequence files were merged with Mr_Demuxy 1.2.0 (available from https://pypi.python.org/pypi/Mr_Demuxy), a python program designed for demultiplexing sequences with dual index, allowing a maximum difference of 15. The *multiple_split_libraries.py* script was then run with a default Phred offset and a Phred quality threshold of 20, allowing for 1% incorrect base call probability. The fasta file then was analyzed with Quantitative Insights into Microbial Ecology (QIIME) version 1.7.0 (Caporaso *et al*., 2010).

The workflows included *pick_de_novo_otus.py*, which picked operational taxonomic units (OTUs) based on 97% similarity, assigned taxonomy by uclust, aligned representative sequences with PyNAST, built a phylogenetic tree, and then made the OTU table. The sequences were rarified at a depth of 6,000 reads, then α-diversity and β-diversity were computed with *core_diversity_analysis.py*. The metrics used for α-diversity include PD whole tree, observed OTUs, and chao1. β-diversity was represented by both phylogenetic-based (unweighted Unifrac and weighted Unifrac) and non-phylogenetic-based (Jaccard distance and Bray-Curtis distance) (Caporaso *et al*., 2010). In addition, linear discriminant analysis effect size (LEfSe) was applied to identify the most biologically informative microbial features differentiating between two treatment groups (Segata *et al*., 2011) on the website: http://huttenhower.sph.harvard.edu/galaxy. For Illumina output, a total of 140,479 reads were obtained from PND 30 female fecal samples with a mean of 419.8 nucleotides (NTs) per sample; after filtering, 122,899 reads left. For PND 90 female fecal samples, a total of 136,001 reads were obtained with a mean of 417.0 NTs per sample; after filtering, 122,781 reads still left. Among PND 90 male fecal samples, a total of 538,291 reads were obtained and after filtering, 446,814 reads left. The Illumina Miseq provides excellent quality with 90% reads reaching a Phred Quality Score > 30.

RNA-sequencing (RNA-seq)

The ileum tissues from NOD female offspring were harvested upon euthanasia at the end of study (PND 206), emptied for intestinal content, transferred to liquid nitrogen and later -80°C freezer for storage. RNA was extracted using RNeasy Mini Kit (QIAGEN), and the extracted RNA was measured for concentration, quality (as indicate by A260/260 and A260/230), and integrity (RNA integrity number greater than 6). The RNA was then submitted to Genewiz (South Plainfield, NJ) for quality control, DNAase removal, library preparation, and sequencing with

Illumina Hi-seq (San Diego, CA), which yielded 2X150 pair-end reads (total = 372,034,607 with a mean quality score of 37.48). The subsequent steps were performed in Georgia Advanced Computing Resource Center (GACRC): First, the data passed the quality control via FastQC; then the low-quality reads and adapters were trimmed by Trimmomatic (Bolger, Lohse, & Usadel, 2014), with >90% pair-end reads survived. The reads were aligned with the mouse reference genome mm10, available at https://genome.ucsc.edu/cgi-bin/hgGateway?db=mm10) using Tophat 2.1.1 (Trapnell, Pachter, & Salzberg, 2009) allowing for a minimum intron length of 20 and a maximum intron length of 500,000. Subsequently the mapped reads were searched for differentially expressed genes (DEGs) in transcript expression using Cuffdiff (Trapnell *et al*., 2010). The DEGs were identified with a q value less than 0.05.

Quantitative real-time PCR (qRT-PCR) for mRNA expression

To confirm RNA-seq results that there were actual changes in α-defensin mRNA alteration, qRT-PCR was used. RNA was extracted as described in RNA-seq section, and RNA isolates were reverse transcribed to cDNA with the iScript™ cDNA synthesis kit (Bio-Rad). The cDNA obtained was used for real-time PCR analysis. The qRT-PCR assays were performed in 1 μL reactions containing 5 μL SYBR Green I Master and 1 μL gene-specific primers (described in **Supplemental Table 3.1.**). Samples were analyzed in technical triplicate using a Stratagene Mx3005 qPCR thermocycler (Agilent Technologies, La Jolla, CA).

Histology and urinary metabolites

The histology of pancreas was evaluated by a board-certified veterinary pathologist (Dr. Nagy). The details of histologic analysis and urinary metabolic studies using NMR were described in the Supplemental Material.

Statistical Analysis

Student's t-test (two-tail) was used for comparisons with VH as reference group when the equal variance assumption was met; otherwise, Wilcoxon test was performed. Likelihood ratio was performed for comparing diabetes incidence between groups. In LEfSe analysis, Kruskal-Wallis and Wilcoxon tests were used for logarithmic LDA scores >2.0 with a critical value of 0.05. JMP Pro 12 (SAS Inc., Cary, NC), GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA), and R (3.3.1) (R CoreTeam, 2013) were used for statistical analysis and data visualization.

Results

T1D incidence was increased in female, but not male offspring following perinatal GEN dosing

Female offspring perinatally dosed with GEN (from GD7 to PND 21) had a significant earlier onset of T1D (**Figure 3.1A**), and the incidence of T1D in GEN-treated group was twice as much as that in VH group from PND 100 to PND 175; significant increases in the incidence of T1D were observed at PND 113 (24% vs. 3%), 120 (24% vs. 6%), 147 (39% vs. 16%), and 162 (45% vs. 23%), respectively. The incidence of severe T1D (BGL ≥ 400 mg/dL) was also increased at PND 113 (33% vs. 4%), 120 (33% vs. 8%), 125 (38% vs. 12%), 147 (54% vs. 20%), 162 (63% vs. 28%), and 168 (67% vs. 36%), respectively (**Supplemental Figure 3.1**). In addition to T1D incidence, perinatal GEN treatment exacerbated BGL in non-diabetic female offspring on PND 64, 203, and 274, respectively (**Figure 3.1B**). However, the T1D incidence in males (**Figure 3.1C**) and the BGL in non-diabetic males (**Figure 3.1D**) were not significantly affected by the GEN treatment. Taken together, administration of GEN during early postnatal development caused a detrimental effect in female offspring (both the onset of T1D and the BGL), but not in the male offspring.

Perinatal GEN dosing elicited a pro-inflammatory response in females while an anti-inflammatory response in males

Serum antibody levels were altered following perinatal GEN dosing: Among the four antibodies measured (IgG_1, IgG_{2a}, IgG_{2b}, and IgM), IgG_{2a} was decreased by 5.7% ($P < 0.05$, **Figure 3.2A**), and IgM was decreased by 16.4% ($P < 0.01$, **Figure 3.2C**) in female offspring. Among male offspring, serum IgG_1 level was reduced by 13.6% ($P < 0.05$, **Figure 3.2H**). No significant alterations in other antibodies were observed (**Figure 3.2**).

Flow cytometric analysis of splenic immune cells also demonstrated a sex-specific effect following perinatal GEN dosing (**Figure 3.3** and **Figure 3.4**). For female offspring, the percentage of $CD5^+CD24^-$ splenic subpopulation was significantly reduced (**Figure 3.3A**), which was accompanied by an increase in $CD5^-CD24^+$ subpopulation (**Figure 3.3B**). In addition, an increase in total $CD4^-CD8^+$ subpopulation (representing cytotoxic T cells; **Figure 3.3D**) was observed. The alterations of total T cells did not achieve statistical significances ($P > 0.05$; data not shown). Splenic T cell and its subpopulation were also analyzed in male offspring for comparison. Following perinatal GEN dosing, both the percentages of $CD3^+IgM^-$ (representing total T cells, **Figure 3.4A**) and $CD4^+CD8^-$ cells (representing helper T cells, **Figure 3.4C**) were significantly reduced, while the $CD4^-CD8^+$ subpopulation was not altered (data not shown). B cell population and its subtypes did not show a difference (as represented by CD5CD24, data not shown).

Among the 9 cytokines (IFN-γ, IL-1α, IL-1β, IL-2, IL-6, IL-10, IL-12p40, IL-12p70, TNF-α) and 1 chemokine (MCP-1) analyzed in this study, there was no significant difference in most cytokines/chemokines except for serum IL-10, which was noticeably reduced (by 8.9%) in female offspring following perinatal GEN dosing (**Table 3.1.**). However, no significant changes were observed in male offspring in either the cytokines or chemokine measured (**Table 3.1.**).

Gut microbiota was perturbed toward a pro-inflammatory response among female offspring, but toward an anti-inflammatory response among male offspring at PND 90

Fecal samples from both PND 30 and PND 90 female offspring, and from PND 90 male offspring were used for 16s rRNA sequencing. At the phylum level, the ratio of *Bacteroides/Firmicutes* was numerically decreased ($P > 0.05$) following GEN (GEF) dosing compared to VH (VHF) at PND 30 in female offspring (2.63 in GEF and 2.70 in VHF; **Supplemental Figure 3.2A**). No alteration in both α diversity and taxonomy at either order or genus level was observed (data not shown). Furthermore, principal coordinate analysis (PCoA) did not show a significant difference in the bacterial community composition at PND 30 between GEN and VH-treated females with both unweighted Unifrac metric (data not shown) and weighted Unifrac (**Figure 3.5A**).

At PND 90, the *Bacteroides/Firmicutes* ratio was non-significantly decreased from 2.38 in VHF to 1.47 in GEF in female offspring (GEF vs VHF at PND 90: $P = 0.088$ by Wilcoxon test; **Supplemental Figure 3.2B**), indicating that the taxonomy at phylum level was not significantly affected by GEN treatment within first three months. In addition, α-diversity remained unchanged following perinatal GEN dosing in PND 90 females ($P > 0.05$, data not shown). However, at PND 90, GEN-treated mice could be well separated by weighted Unifrac, a phylogenetic-based metric that takes bacterial abundance into account (**Figure 3.5B**, $P = 0.045$ by non-parametric test). In addition, *Enterobacterials* at the order level (**Figure 3.5C**) and *Enterobacteriaceae* at the genus level (not shown) were significantly increased following perinatal GEN dosing at PND 90 (LDA score > 2.0, $P < 0.05$ by non-parametric test). The non-phylogenetic-based metrics did not show a difference (data not shown).

In male offspring, taxonomy at phylum level (**Figure 3.6A**), unweighted (data not shown) and weighted Unifrac (**Figure 3.6B**) did not show a difference at PND 90, while an increased *genus Parabacteroides* was found in males perinatally dosed with GEN (**Figure 3.6C**). At order level, no alteration in taxonomy was observed, and the non-phylogenetic-based metrics did not show a difference (data not shown).

RNA-seq identified down-regulation of multiple defensing subclasses and other inflammatory markers

To explore further the mechanism of pro-inflammatory immune response following perinatal GEN dosing in female offspring, a transcriptomic study was conducted, and a total of 124 out of 35,607 genes were identified with a *P*-value less than 0.05 (in **Supplemental Table 3.2.**). Significantly up-regulated and down-regulated genes from RNA-seq were shown in **Supplemental Table 3.2**. Noticeably and consistently, perinatal GEN dosing in NOD female offspring down-regulated *Defa17*, *Defa24*, *Defa29* and *Defa30*, which were confirmed by examining their mRNA levels using quantitative PCR analyses ($P = 0.01$, **Figure 3.7A, B**).

Perinatal GEN dosing impacts the urinary metabolomics profile

NMR-based analysis of urine metabolites was conducted to determine if the GMB perturbation could affect the global metabolic fingerprinting. To provide an overview of our dataset and identify outlier samples, we first performed principle component analysis. It showed that data points corresponding to GEN and VH treatment on each individual animal was differentiable from each other (**Figure 3.8A-B**). Further, another supervised multi-variate analysis, Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) that provides higher capability to distinguish variability between and within groups, revealed a clear separation between controls and treatment groups (**Figure 3.8C-D**). The OPLS-DA models were

characterized by a faithful representation of the data (R^2Y = 98.9% for males and 97.5% for females) and an excellent cumulative confidence criterion of prediction (Q^2 = 78.4% for males and 69.6% for females). In addition, the perinatal GEN treatment effects were significant for both male ($F_{6,6} = 4.53$, $P = 0.044$) and female ($F_{8,9} = 3.22$, $P = 0.050$).

Discussion

T1D, a devastating and expensive organ-specific autoimmune disease, is increasing in incidence in US adults (once mainly in juveniles) with 40% excess risk of death in women (Li *et al.*, 2016, Huxley *et al.*, 2015). Environmental exposure (e.g., endocrine disruptors) and genetic predisposition may be the underlying causes (Jobling *et al.*, 2013). Soy milk formula consumption during infancy was associated with a two-fold higher risk of T1D (Fort *et al.*, 1986; Strotmeyer *et al.*, 2004) and a significant increase in use of asthma or allergy drugs in young women (Strom *et al.*, 2001). Our studies have identified unique windows of T1D susceptibility in female NOD mice for GEN: perinatal GEN dosing (from GD 7 to PND 21) accelerated the onset of T1D. In contrast, similar detrimental effect was not found in males, and adult GEN dosing are protective in both sexes (Huang *et al.*, 2017). Although the mechanisms underlying a fetal basis of adult T1D are currently unclear, sex differences in immunoregulation in early life (Bao *et al.*, 2002) and GMB-driven estrogen metabolism (Markle *et al.*, 2013) might be responsible for our observations that female offspring dosed with GEN perinatally showed an exacerbation of T1D. The dose selected in this study is physiologically relevant because soy formula can provide a 4-month-old infant with approximately 6 to 9 mg/kg of isoflavones (Irvine *et al.*, 1998). The dose of 20-mg/kg GEN in mice is much lower than a clinical human supplement dose (around 100 mg/day (Djuric *et al.*, 2001)) in terms of milligrams per square meter of body surface, which often gives more accurate interspecies extrapolation (Hodgson & Levi, 2010). The serum level of GEN (1.4-7.5 μM) in

rodents that have been fed a 1,000 ppm GEN-containing diet (about 80-mg/kg BW) was equivalent to that in men who received 100-mg GEN/day (Bhandari *et al*., 2003; Djuric *et al*., 2001; Yellayi *et al*., 2002).

Evidences in both humans and animal models increasingly suggest that T1D is originated in the gut (70% of the body's immune system dwells here) and associated with a profound dysbiosis during the perinatal period and in adulthood, favoring pro-inflammatory microbial communities (Kostic *et al*., 2015; Markle *et al*., 2013; Vaarala, Atkinson, & Neu, 2008). In NOD mice, differences in sex hormones and GMB underlie its sexual dimorphism of T1D (Yurkovetskiy *et al*., 2013). Thus, an intricate interplay between of perinatal GEN intake, GMB, immune dysregulation, and sex hormone imbalance (Gourdy *et al*., 2016) may play critical roles in the T1D exacerbation. Our current study revealed that perinatal GEN dosing perturbed the GMB toward a pro-inflammatory response in females at PND 90, but not at PND 30, in addition to altering the β-diversity. In males, however, perinatal GEN dosing perturbed the GMB toward an anti-inflammatory response at PND 90. Our study is the first to identify the unique window of effect for GMB alteration. For inflammatory status following perinatal GEN dosing, the increase in *Enterobacterials* at the order level and *Enterobacteriaceae* at the genus level in females were associated with a pro-inflammatory status (Ellis *et al*., 2011), while an increased *genus Parabacteroides* was found in males, indicating a modulating effect on immune system and possibly regulatory T cells (Li *et al*., 2016). To date, the only study investigating GMB alteration induced by perinatal endocrine disruptor exposure found bisphenol A perturbed β-diversity in rabbits on PND 42 (Reddivari *et al*., 2017).

With regards to antibody and cytokine alterations, there was evidence that IgG_{2a} demonstrated an anti-inflammatory effect and thus protected female NOD mice from T1D (Todd

et al., 1998). In our studies, we observed a decrease in IgG_{2a} in NOD females following perinatal GEN dosing, suggesting a pro-inflammatory status. Moreover, the pro-inflammatory response in female offspring perinatally dosed with GEN was supported by a decreased IgM level, as IgM has been shown to protect against autoimmune diseases (Hampe, 2012), and lack of IgM was shown to correlate with increases in pathogenic IgG (Hampe, 2012). This is in agreement with a reduced IL-10 level in females perinatally dosed with GEN, as IL-10 producing B cells suppress inflammation in various mouse models of autoimmune diseases (Hampe, 2012), and it is possible that the IL-10 producing B cells are $CD5^+$ as discussed later (Yanaba *et al*., 2009). In addition, IL-10-deficient mice experience severe T1D accompanied with an increase in Th1 cytokines (Tian *et al*., 2001). In contrast, the decrease in IgG_1 among males perinatally dosed with GEN implies a decreased Th2 response. However, for this current study, no statistical significances in the insulin level, insulin autoantibody level and histopathology were observed (data not shown), possibly due to the fact that the sera and tissue samples were collected at the end of study when most animals developed T1D.

For splenic cell subpopulation, CD24 is a marker for mature T cell, while CD5 is a common marker for regulatory B cells (Yanaba *et al*., 2009). In recent studies, it was found that $CD5^+$ cells were negatively associated with pro-inflammatory status in autoimmune disease (Baglaenko *et al*., 2015). In our study, a decrease in $CD5^+CD24^-$ and an increase in $CD5^-CD24^+$ splenic cells were identified in females following perinatal dosing with GEN, and consequently, a weakened regulation of autoimmune responses was observed as reflected by the exacerbation of T1D. This is in agreement with our finding that IL-10 was decreased in GEN exposed female offspring as the IL-10 producing regulatory B cell subset with a unique $CD1d^{hi}CD5^+$ phenotype controls T cell-dependent inflammatory responses (Vighi *et al*., 2008). In contrast, among male offspring, the

decreases of both helper T cell and total T cell populations would suggest an anti-inflammatory status, and thus were associated with a protective effect against T1D.

In infants, diets play a major role in the alteration of GMB and metabolites (Del Chierico *et al*., 2015). In the current study, perinatal GEN exposure in female offspring led to an alteration in GMB at PND 90. Importantly, the down-regulated mRNA expression of defensins, an antimicrobial peptide that is an essential component of intestinal innate immunity (Coretti *et al*., 2017), has been observed in these mice. Interestingly, the production of antimicrobial peptides is decreased in NOD mouse compared to their mutated strain with lower susceptibility (Mullaney *et al*., 2018). Defensin can stabilize the GMB (Coretti *et al*., 2017, Salzman, 2010) while the reduction of α-defensin is associated with gut dysbiosis (Wehkamp *et al*., 2005). Among the few studies investigating ileum α-defensin mRNA expression, NOD mice showed a reduced expression as compared to the non-obese diabetic-resistant mice (Daft, Wolf, & Lorenz, 2011). The anti-inflammatory effect of defensin was associated with an increased IL-10 production in humans (Kanda *et al*., 2011). Consistently, a decreased IL-10 level in female offspring perinatally exposed to GEN was observed in our study, which might explain the pro-inflammatory response following perinatal GEN dosing in females. Moreover, IL-10 deficiency leads to an activation of immune system (Sellon *et al*., 1998) and dysbiosis, which can be restored by certain bacterial strains (e.g., *Lactobacillus spp*.) (Madsen *et al*., 1999). The down-regulated expression of defensin in intestinal tissues in female offspring perinatally exposed to GEN might be related to the epidemiological evidence that soy-based formula induced a higher T1D risk than milk-based formula (Strotmeyer *et al*., 2004). After birth, the infant formula consumption, compared to breast feeding, was found to be detrimental to neonatal immune system development (Innis, 2007), as

breast milk can provide defensins that enables infants to ward off environmental pathogens (Walker & Iyengar, 2014).

Our previous studies have identified that the immunomodulatory effect of adult GEN treatment toward protection against T1D in NOD mice is associated with the alteration of GMB (Guo *et al*., 2015) and metabolomics (unpublished data). In this study, perinatal GEN exposure also had a great impact on untargeted urinary metabolomics profile, although the targeted metabolites [e.g., short chain fatty acids (SCFAs)] were not identified in the current study. The hypothesized mechanism in perinatal GEN modulation of T1D in female NOD offspring is depicted in **Figure 9**. Despite some epidemiological and animal studies suggest a potential detrimental effect of perinatal soy consumption on autoimmune diseases such as T1D, this current study is the first to: (1) reveal the susceptible window of exposure, (2) discover the sex dimorphic effect, (3) use the "omics" technique to explore the gut microbiota-immune-transcriptomics consortium of T1D development, and provide solid data for future risk assessment of infant diet on increasing autoimmune diseases. This study can also serve as a model of studying immune-related diseases for other environmental chemicals (such as dietary supplement and endocrine disruptors) and contribute to the discovery of novel biomarkers for early disease detection.

However, this study also has some limitations. For instance, the harvest of terminal blood and organs has forbidden us to detect a histopathological alteration of disease progression, and that we are not able to identify individual metabolites from bio-samples. More time points in sample collection would enable a better trajectory of biomarkers (such as GMB) and could be included in future studies. In addition, the causal effect of GEN-induced mRNA expression, gut microbiota, and intestinal immunological changes are not examined and require future investigation.

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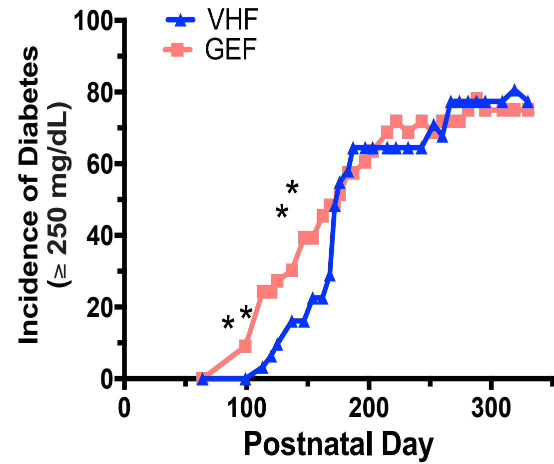
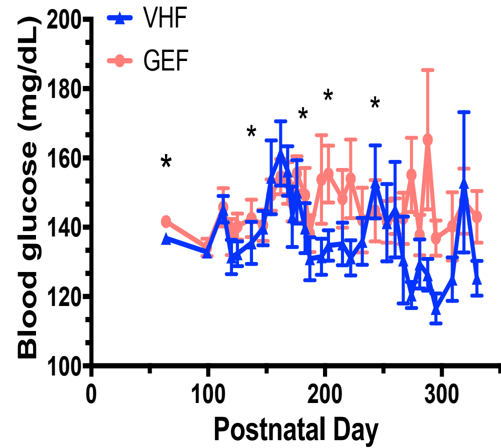
	IFN- γ	IL-1 α	IL-1 β	IL-2	IL-6
VHF	6.21±0.81	82.34±2.60	33.48±1.21	22.23±1.71	22.52±1.13
GEF	5.15±0.25	78.66±6.23	31.38±0.00	21.90±1.09	22.74±2.85
VHM	5.22±0.73	88.85±7.47	31.34±0.83	21.70±1.39	22.92±1.84
GEM	5.35±0.60	79.10±2.41	31.71±0.33	21.24±0.83	21.87±0.82
	MCP-1	TNF- α	IL-10	IL-12p40	IL-12p70
VHF	79.96±1.27	26.67±3.52	14.46±0.50	22.23±1.71	22.52±1.13
GEF	76.86±1.34	25.92±0.63	13.17±0.36*	21.90±1.09	22.74±2.85
VHM	79.13±2.21	26.11±0.51	13.76±0.35	21.7±1.39	22.92±1.84
GEM	26.47±0.53	26.47±0.53	14.45±0.49	21.24±0.83	21.87±0.82

Table 3. 1. The cytokine/chemokine levels measured by Multiplex Mouse Cytokine/chemokine Magnetic Bead Panel Kit 96 Well Plate Assay in sera of NOD male and female offspring.

Note: All values represent mean ± S.E. (pg/ml). IL = interleukin, MCP-1 = monocyte chemoattractant protein-1, TNF-α = tumor necrosis factor-α, VHM = NOD males dosed with vehicle (N = 10); VHF = NOD females dosed with vehicle (N = 17); GEM = NOD males dosed with genistein (N = 16); and GEF = NOD females dosed with genistein (N = 8). *, $P < 0.05$.





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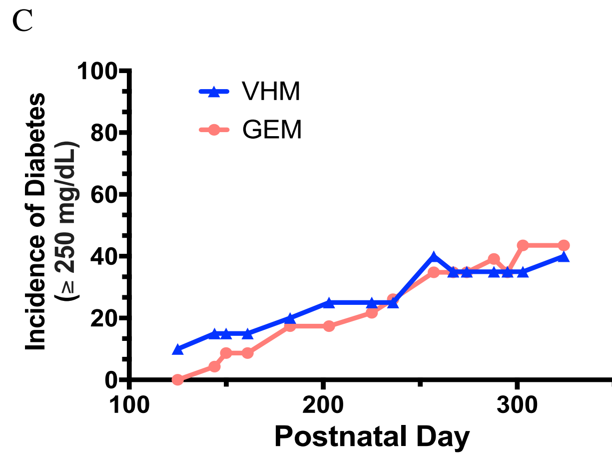

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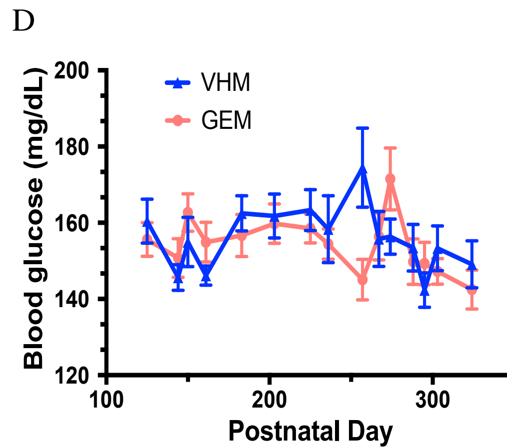


Figure 3. 1. Perinatal GEN treatment increased T1D incidence in female, but not in male, offspring. (A) The incidence of T1D (BGL ≥ 250 mg/dL) among female offspring perinatally exposed to GEN; (B) The BGL for non-diabetic female offspring; (C) The incidence of T1D among male offspring; and (D) The BGL for non-diabetic male offspring. VHF = females exposed to vehicle control (N = 36), GEF = females exposed to GEN (N = 33), VHM = males exposed to vehicle control (N = 32), GEM = males exposed to GEN (N = 35). * indicates $P < 0.05$. BGL = blood glucose level, GEN = genistein, T1D = type 1 diabetes

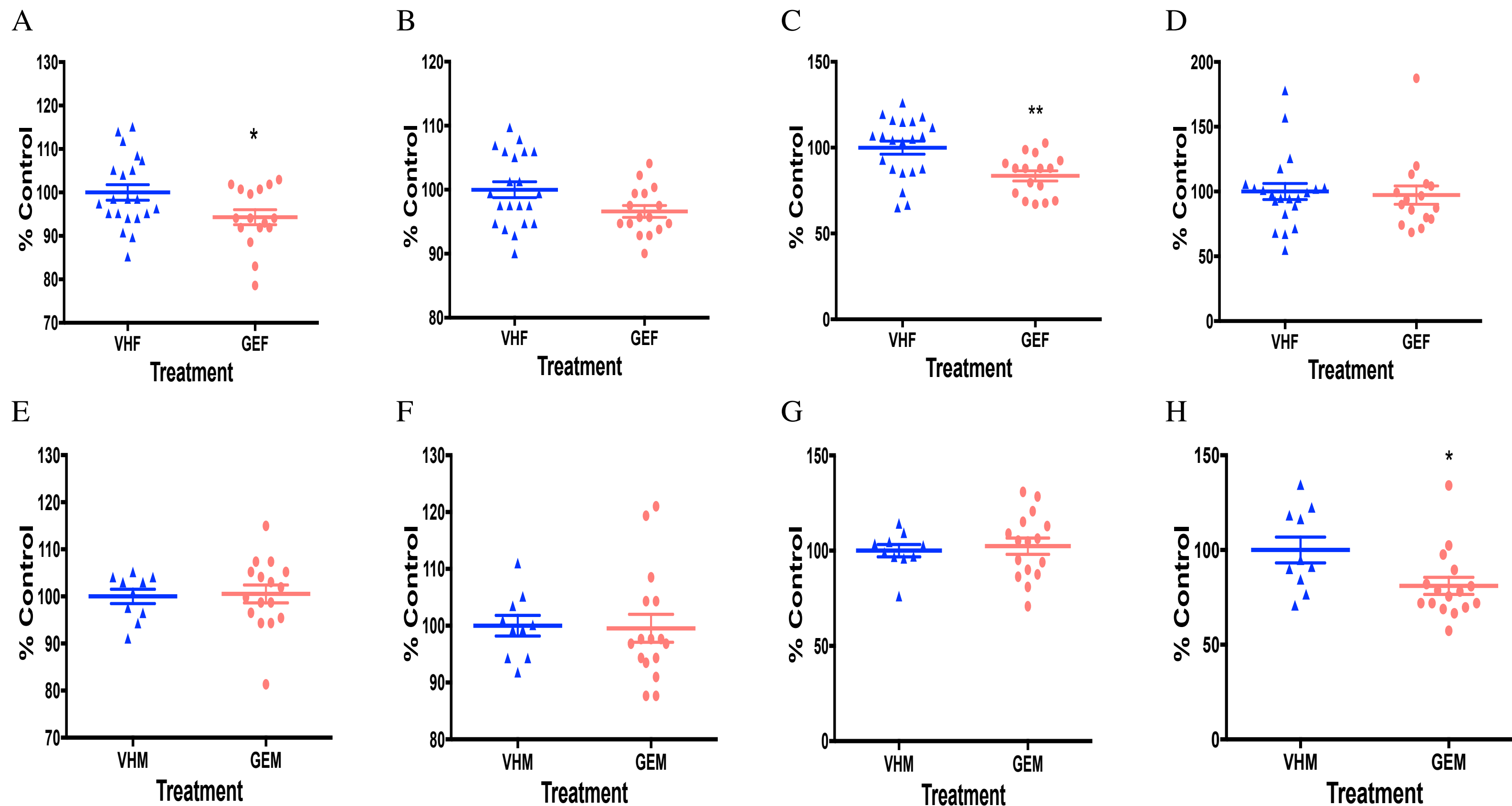


Figure 3. 2. The serum antibody levels in NOD female offspring (A-D) and male offspring (E-H) on 5K96 diet. (A, E) IgG_{2a}, (B, F) IgG_{2b}, (C, G) IgM, and (D, H) IgG_1 levels in the sera of NOD female and male offspring were measured at dilutions of 1:50, 1:50, 1:500, and 1:5000 (v/v), respectively, following titration by serial dilution using ELISA. The sera were obtained when the mice were euthanized. *, p<0.05, and **, p<0.01. Each of the serum antibody concentration was calculated as the percent control. VHF = females exposed to vehicle control (N = 21), GEF = females exposed to GEN (N = 16), VHM = males exposed to vehicle control (N = 10), GEM = males exposed to GEN (N = 16). GEN = genistein. ELISA = enzyme-linked immunosorbent assay.

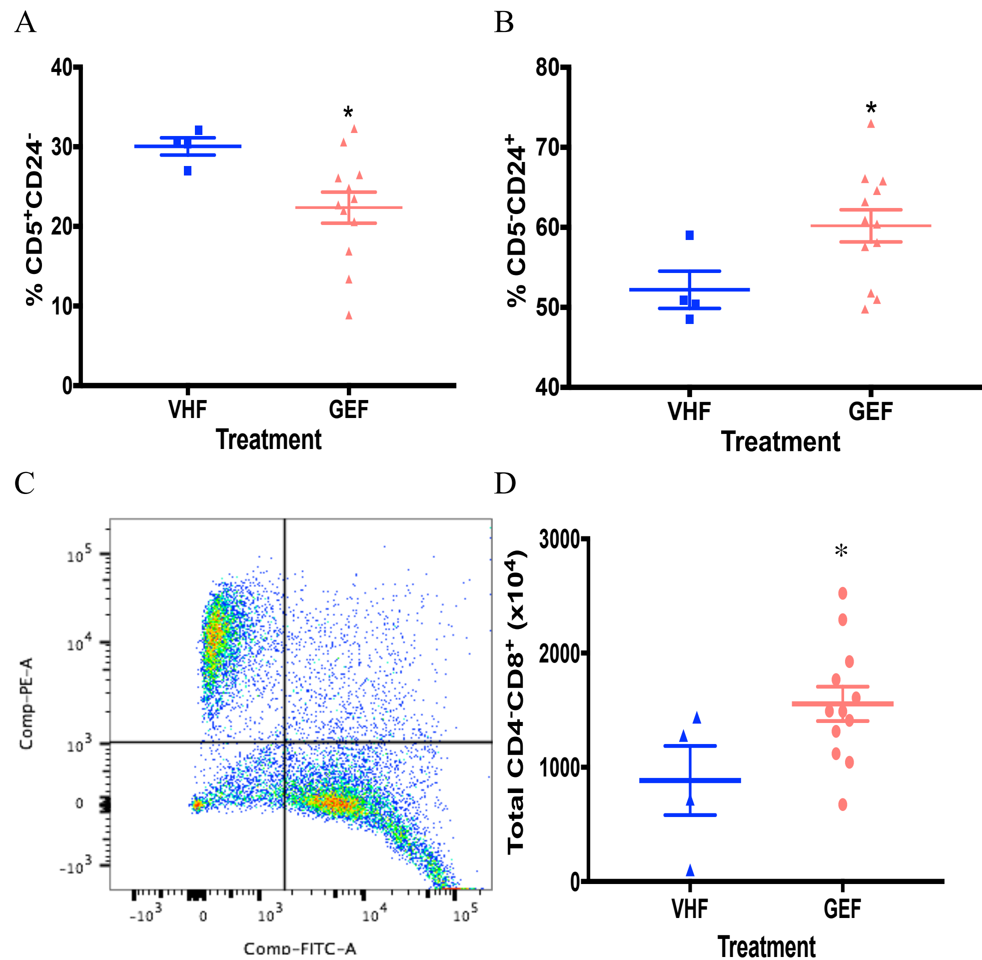


Figure 3. 3. GEN treatment caused alteration in subpopulations of splenic immune cells in female offspring on 5K96 diet. (A) The percentage of splenic $CD5^{+}CD24^{-}$. (B) The percentage of splenic $CD5^{-}CD24^{+}$. (C) The gating strategy for CD5CD24 markers. X-axis represents CD24, and y-axis represents CD5. (D) The total number of splenic $CD4^{-}CD8^{+}$ cells. VHF = female exposed to vehicle control (N = 4), GEF = females exposed to genistein (N = 12). * indicates $P < 0.05$.

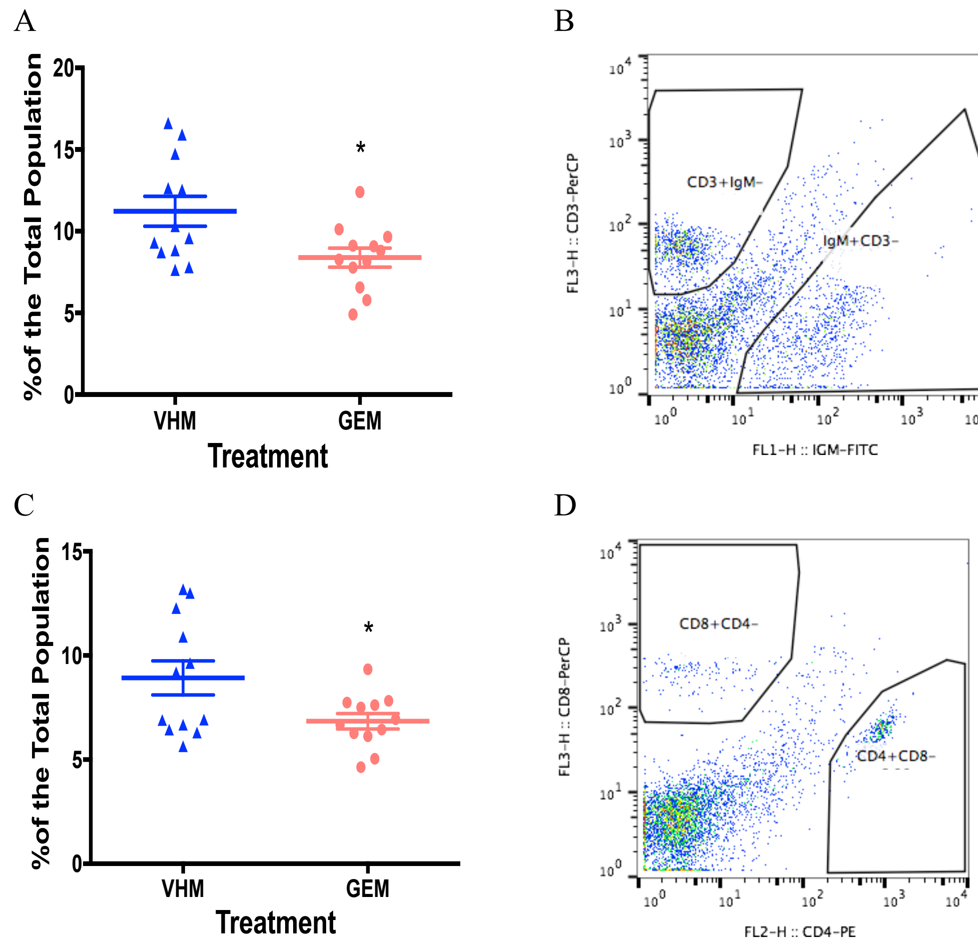


Figure 3. 4. Flow cytometric analysis of splenic T cell subpopulation in male offspring on 5K96 diet. (A) The percentage of T cell population; (B) The gating strategy for total T cell population; (C) The percentage of helper T cell population; and (D) The gating strategy for helper T cell population. VHM = males exposed to vehicle control (N = 12), GEM = males exposed to genistein (N = 12). * indicates $P < 0.05$.

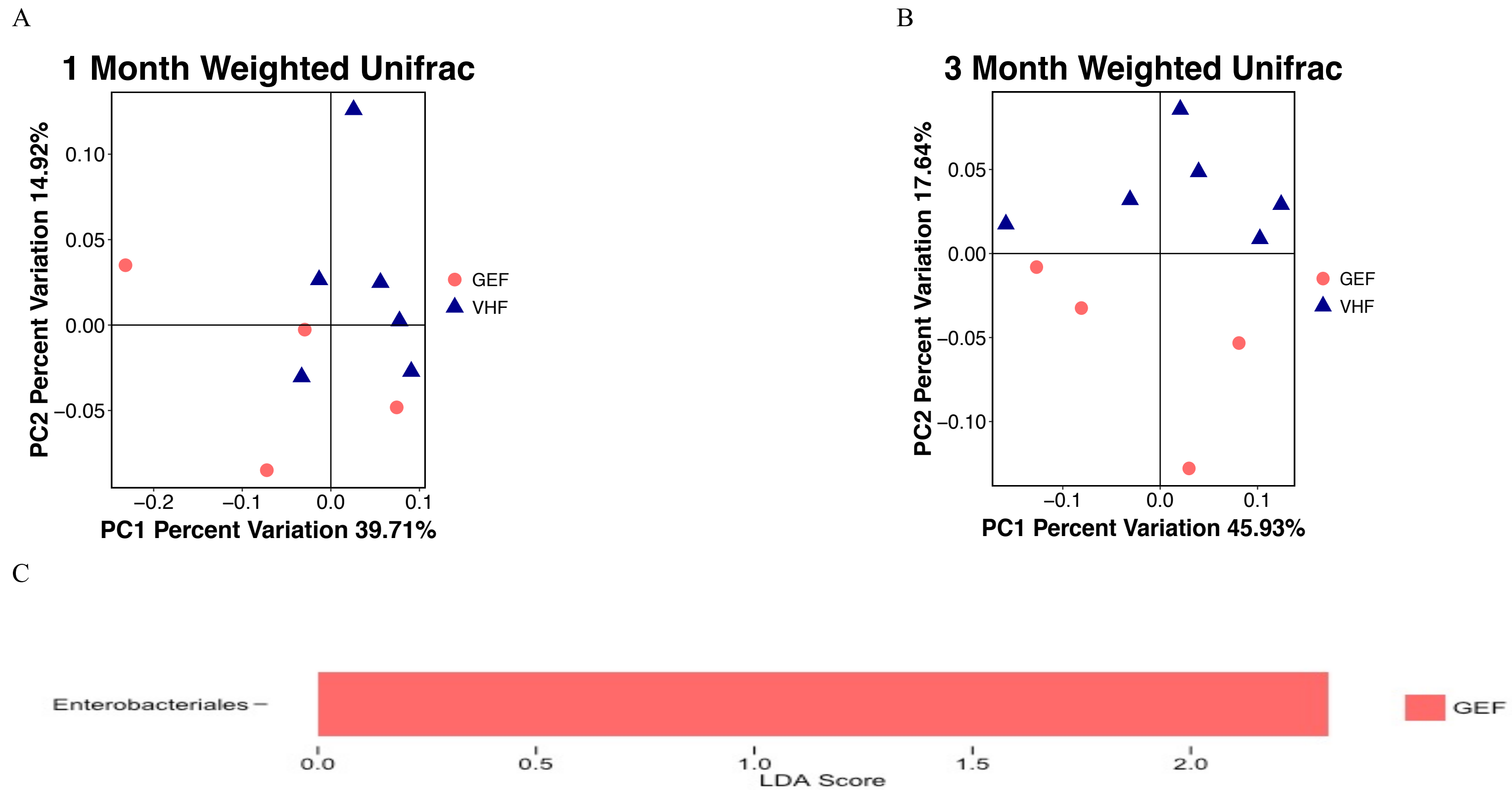


Figure 3. 5. The composition of gut microbiota based on 16S ribosomal RNA sequencing in NOD female offspring on 5K96 diet treated with genistein (GEF, N = 4) or vehicle (VHF, N = 6). The β-diversity based on the weighted Unifrac index at (A) postnatal day (PND) 30 and (B) PND 90. Each of the symbols (closed circles and triangles) represents one animal and is illustrated by Principal Coordinate Component (PCoA), and the primary principal component (PC1) and secondary principal component (PC2) are shown. (C) Linear discriminant analysis Effect Size (LefSe) showing the microbial species with differential abundance at the order level at PND 90.

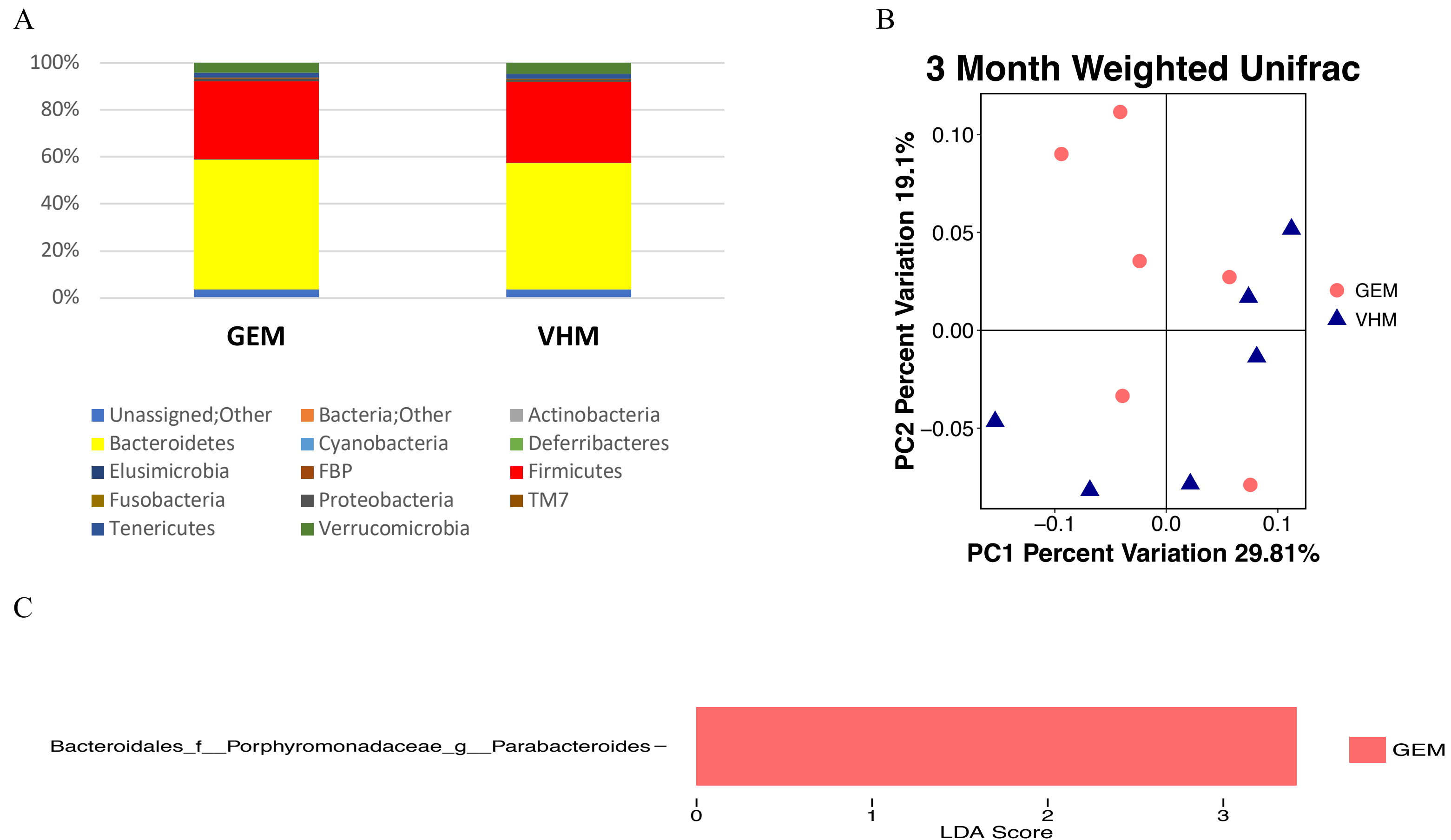


Figure 3. 6. The composition of gut microbiota based on 16S ribosomal RNA sequencing in NOD male offspring at postnatal day (PND) 90 on 5K96 diet. (A) The taxonomy of the gut microbiota at the phylum level when the individual animal data were combined for analysis according to the treatment. (B) The β-diversity based on the weighted Unifrac index. Each of the symbols (closed circles and triangles) represents one animal and is illustrated by Principal Coordinate Component (PCoA), and the primary principal component (PC1) and secondary principal component (PC2) are shown. (C) Linear discriminant analysis Effect Size (LefSe) showing *Parabacteroides* with differential abundance at the order at the genus level. VHM = males exposed to VH (N=6), GEM = males exposed to GEN (N=6).

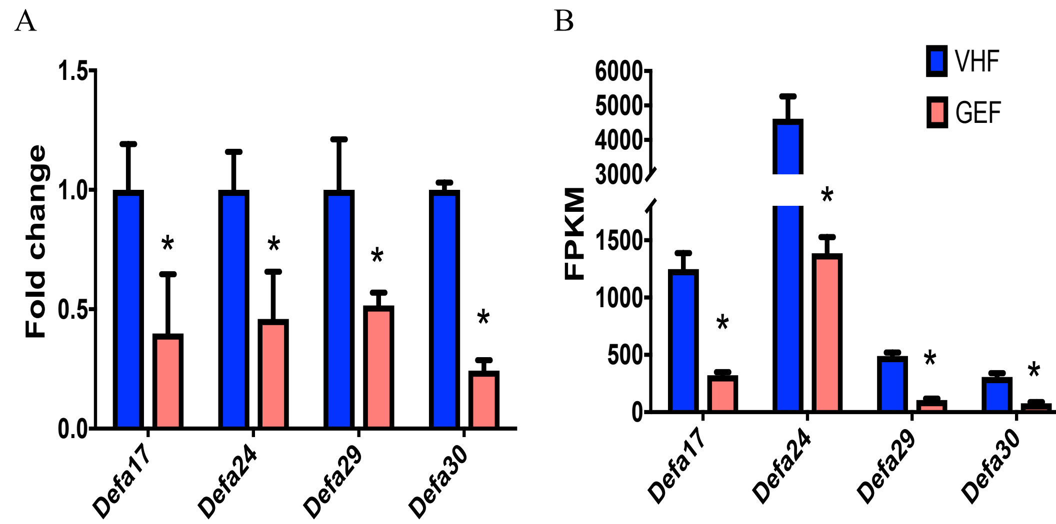


Figure 3. 7. The expression of mRNA transcripts. (A) qRT-PCR (N = 4/group), and (B) RNA-seq. Error bar = standard error of mean. * indicates $P < 0.05$. FPKM = fragment per kilobase of transcript per million mapped reads.

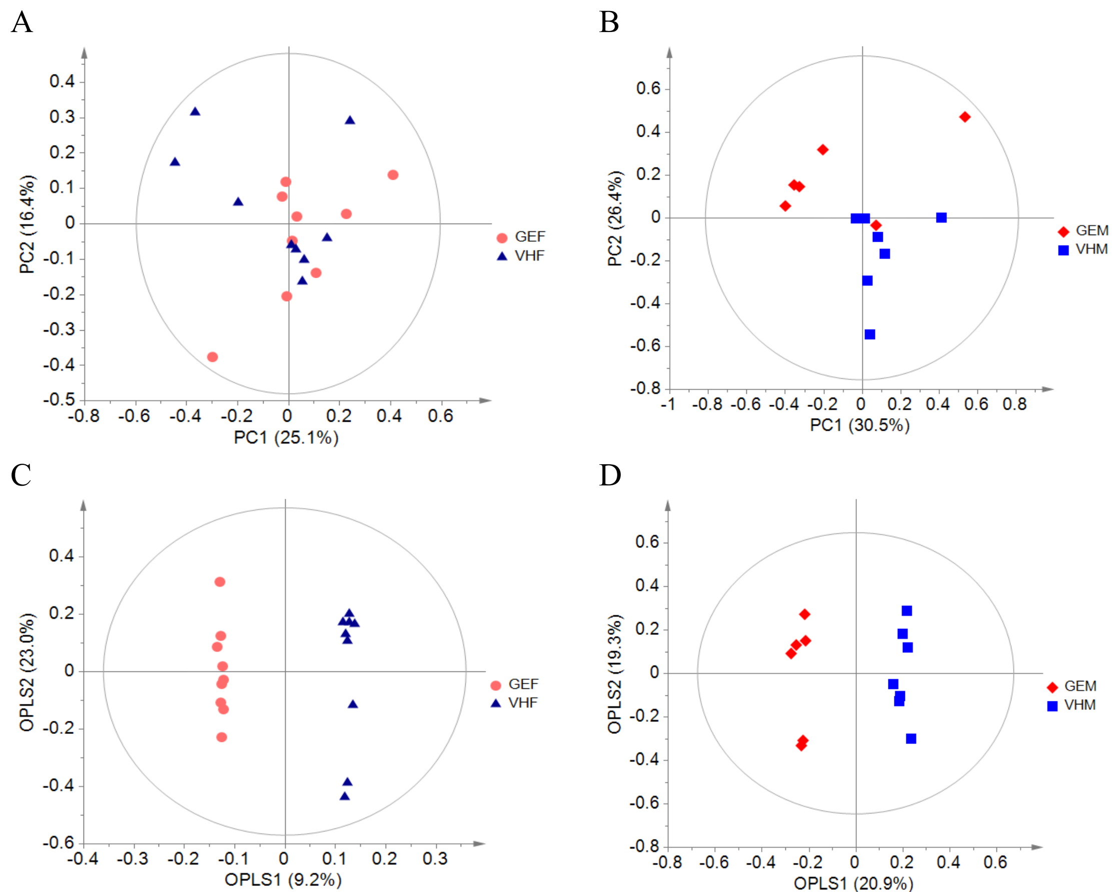


Figure 3. 8. Urinary metabolomics from PND 60. (A-C) females (B-D) males perinatally exposed to genistein using principal component analysis (A-B) and Orthogonal Projections to Latent Structures Discriminant Analysis (C-D). VHF = female exposed to vehicle control (N = 9), GEF = females exposed to genistein (N = 9), VHM = males exposed to vehicle control (N = 7), GEM = males exposed to genistein (N = 6). For males, $R^2Y = 98.9\%$, $Q^2 = 78.4\%$, $F_{6,6} = 4.53$, $P = 0.044$. For females, $R^2Y = 97.5\%$, $Q^2 = 69.6\%$, $F_{8,9} = 3.22$, $P = 0.050$.

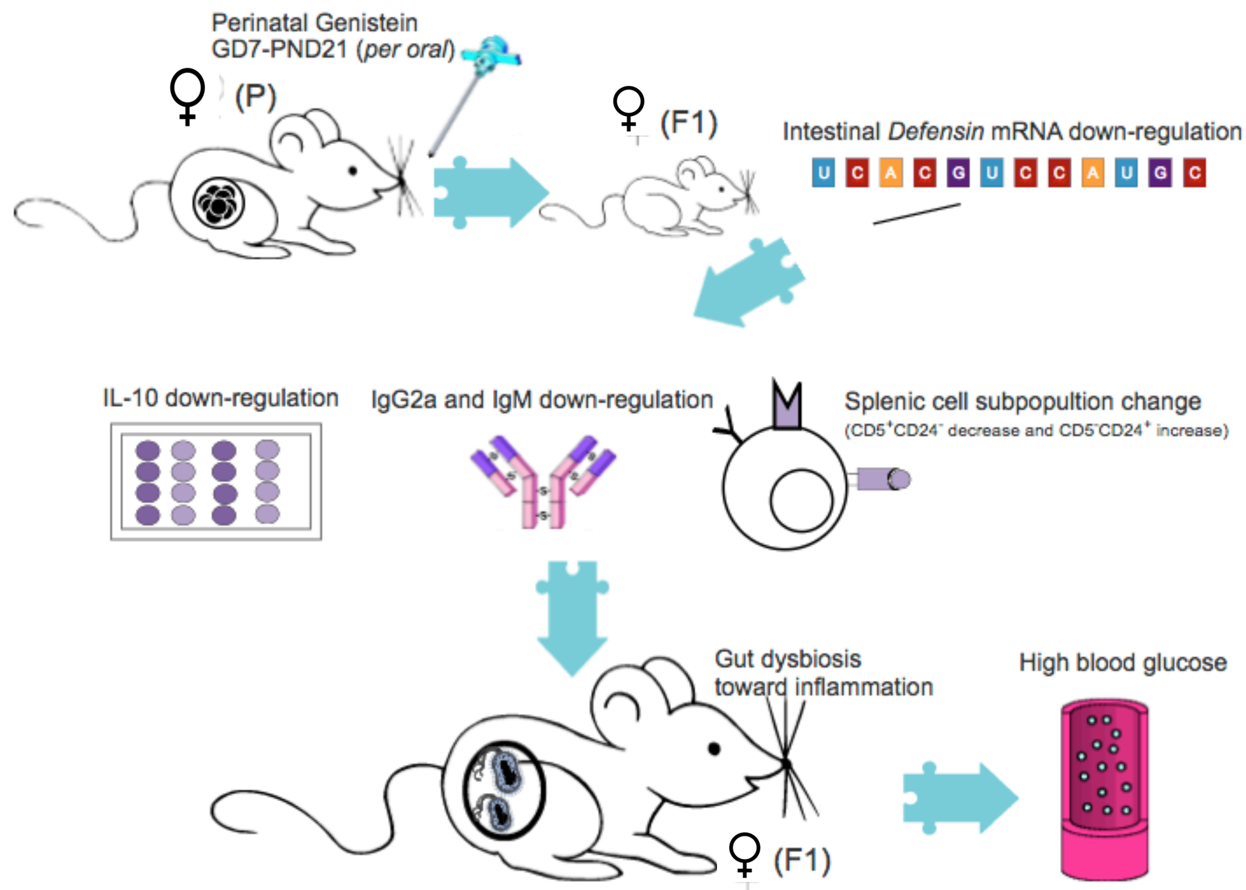


Figure 3. 9. The hypothesized mechanism for increased type 1 diabetes incidence in females that are perinatally exposed with genistein

Supplemental Methods

Animal husbandry

The NOD mice were initially obtained from Taconic Biosciences (Hudson, NY), and has been maintained in our lab by breeding. The mice were kept in Central Animal Facility, College of Veterinary Medicine at University of Georgia (UGA) and housed in standard plastic cages from which only an undetectable of bisphenol A leached out with irradiated laboratory animal bedding (The Andersons company, Maumee, Ohio), with each cage housing 4-5 mice. The animal room was maintained in the range of 22–25 °C in temperature, 50 ± 10 in relative humidity with 12/12 h light/dark cycle. Mice were fed with 5K96 Rodent Diet (TestDiet, St. Louis, MO, a casein-based, soy and alfalfa free rodent diet) and had access to tap water *ad libitum*.

Glucose tolerance test (GTT) and insulin tolerance test (ITT)

For GTT, the mice were fasted for 15 hours followed by glucose injection (1g/kg) intraperitoneally (*i.p.*). The fasting BW and BGL, altogether with BGL at 15 min, 30 min, 60 min, and 120 min after injection, were measured. In addition, the ITT was performed on mice without fasting. The mice were injected with insulin (1.5 IU/kg BW) *i.p.*, and the BGL at 15 min, 30 min, 60 min, and 120 min were measured.

Serum insulin and serum insulin antibody (IAA)

The serum insulin was measured by mouse insulin ELISA kit (Millipore Sigma), and the absorption at 630 nm (A630) was subtracted from A450 within 30 minutes. It has been reported that IAA peaks between 8 and 16 weeks in NOD mice and declines afterwards. Thus, the sera were sampled from the NOD offspring 12 weeks after birth. The measurement of IAA was performed. Briefly, polyethylene ELISA plates were coated with 0.8 mg/ml bovine insulin (SIGMA I0516) overnight at 4 °C, blocked with 5% milk powder in PBS with Tween 20 for 1 h, and washed with

PBST. The diluted serum was added to the plates and incubated at room temperature for 2 h. Then the goat anti–mouse IgG second antibody (Southern Biotechnology Associates, at 1: 1,000 dilution) was added 100 µL/well. Substrate TMB was added at 100 µL/well and the plate was read at a wavelength of 405 nm.

Histopathology

Histological analysis was performed on pancreas from females. During necropsy, the pancreas was collected from each mouse, placed in a cassette, fixed in 10% neutral-buffered formalin for 48 hours, transferred to 70% ethanol, and then processed for paraffin embedding. Paraffin blocks were sectioned at 4 µm thickness, tissue sections were mounted on positively charged glass slides and stained with hematoxylin and eosin (H&E). The slides were scored 0-3 (0 = no insulitis, 1 = peri-islet insulitis, 2 = insulitis with still recognizable islet cells, 3 = insulitis with complete destructions of islets) by a board-certified veterinary pathologist.

DNA extraction, library preparation and sequencing for gut microbiota analysis

The fecal samples from the females on PND 30 and PND 90 were stored in -20 ° C freezer. Briefly, DNA was extracted from fecal samples using QIAamp DNA stool mini kits (Qiagen, Valencia, CA). Approximately 120 mg fecal samples were used, and the subsequent steps were performed following the kit manual. The DNA product was normalized to 20 ng/µL. Locus-specific primers (forward: 16S_341_F, and reverse: 16S_785_R) were used to target the V3-V4 region of 16S rRNA, and Illumina-specific sequencing primers were used together with 20 internal tags (8 forward fusion primers and 12 reverse fusion primers) ranging from 5 nucleotides (NTs) to 8 NTs long 25 (described in **Table 1.**). After 25 cycles of PCR with locus-specific primers, the amplicon aliquot (10 µL per DNA sample) was purified with Speedbeads and quantified with Qubit. The second round PCR was run using Illumina iTru5 primer and iTru7 primer, and the

products were sequenced on Illumina Miseq for pair-end (PE)150 sequencing (Illumina Inc., San Diego, US).

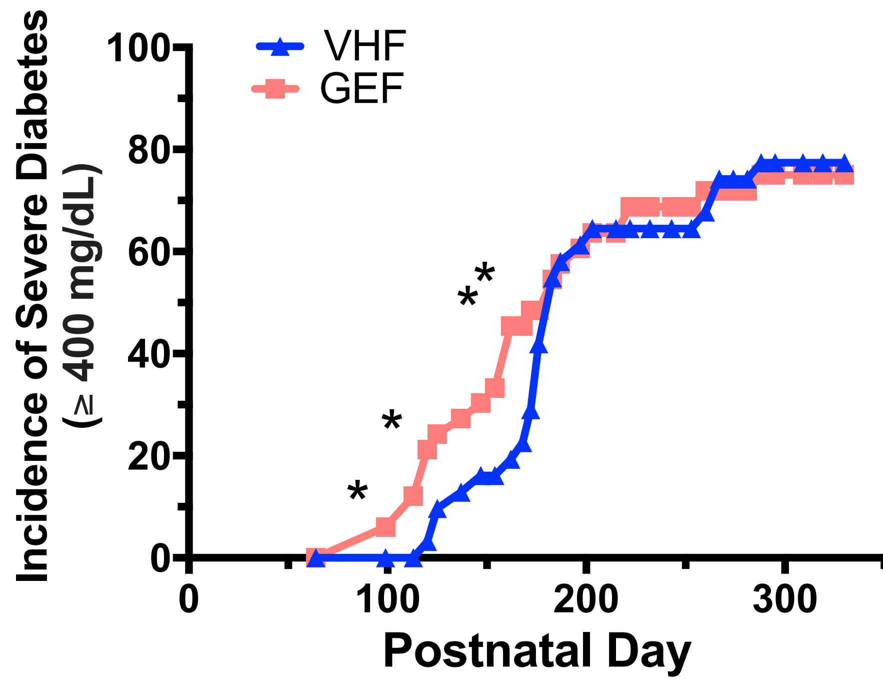
Urinary metabolites

Approximately 200 uL of urine was collected from each mouse at PND 60 with a micropipette on a plastic wrap or petri dish when the mice were scruffed and stored immediately in -20 °C to prevent any bacterial degradation. A total of 50 uL urine from each mouse (with 1% sodium azide, to prevent bacterial degradation) was used during each run. Briefly, the samples were lyophilized for 8 h and then stored at 4 °C overnight. On the day of analysis, the samples were reconstituted with 230 uL of NMR phosphate buffer (pH 7.4, in 99.8% D_2O, Cambridge Isotope Laboratories) to yield the final phosphate concentration of 100 mM, with 200 uM 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) added as an internal standard). The phosphate buffer was added to minimize the alteration in pH. The samples were vortexed and centrifuged at 3000 g for 10 min at 4 °C and transferred into 3 mm NMR tubes. An additional quality control (QC) sample was prepared by pooling from different samples. Subsequently, the samples were run on an Agilent 600 MHz ^{1}H NMR spectrometer (Agilent, Santa Clara, California) with 128 transients at 20 °C.

The NMR data were processed with ACD software (ACD Labs, Ontario, Canada). The data were zero-filled, line broadened at 0.3Hz and Fourier transformed, and then phase-corrected. The spectra baseline was corrected with 3rd-order Bernstein polynomials, and chemical shifts were calibrated with the DSS at 0 ppm. All spectra were aligned using SpecAlign (Oxford, UK). This step was used to reduce the variation of chemical shifts across samples. The spectra regions between 4.5 to 5.1 ppm and between 5.0 to 6.0 ppm were removed to avoid the effect of water and urea resonance, respectively. The aligned spectra were then binned to 0.005-ppm segments by

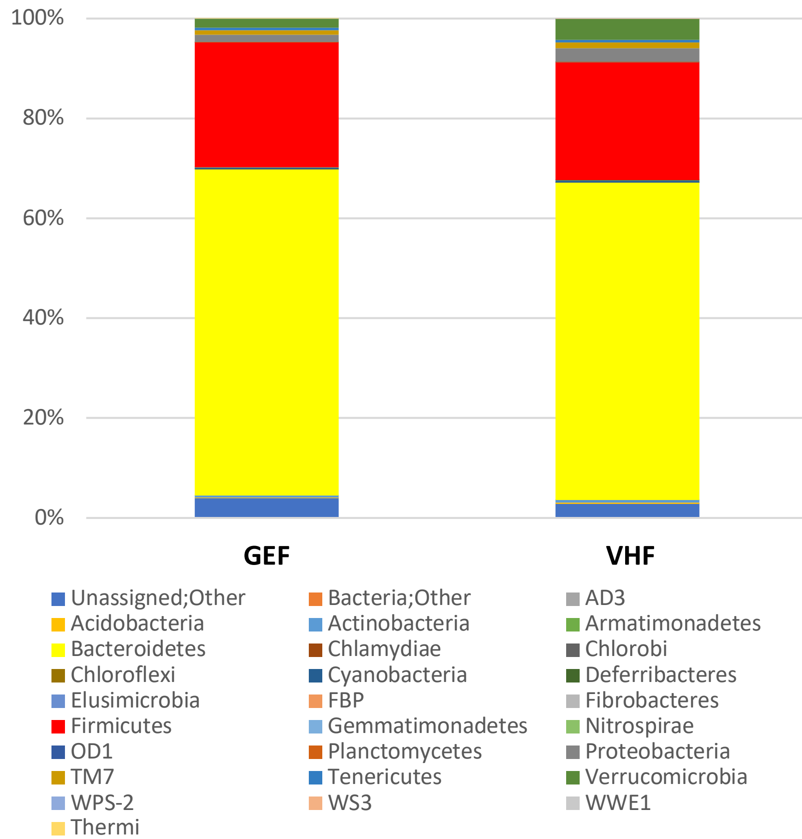
integrating the spectral peak area, normalized, and exported to excel spreadsheet for statistical analysis. Alternatively, the FID files were processed with ACD software (ACD/labs, Ontario, Canada). All NMR data were normalized, mean centered and pareto scaled. Each peak was picked and aligned with SpecAlign (Oxford, UK).

The processed data were analyzed with SIMCA (version 13, Umetrics, Sweden). The binned NMR data were mean centered and pareto-scaled. Auto-fit function was used to avoid overfitting. Unsupervised multivariate analysis (principal component analysis, PCA), and supervised multivariate analyses, (partial least squares discriminant analysis, PLS_DA, and orthogonal projections to latent structures discriminant analysis, OPLS-DA) were performed. The PCA was used to check for outliers, while generating a 95% confidence interval ellipse with Hotelling's T^2 test. To check the model validity, a permutation of 200 was run, and the R^2 (how the model explains the data) and Q^2 (the predictability of the model) were compared to identify the best model. Student's t-test was then used to compare each spectral bin assuming unequal variance between VH and GEN groups. Outliers were defined as those points that fell out of the 95% confidence interval of a Hotelling's T^2 test. No outlier was identified. OPLS-DA models were validated using permutation testing by randomizing the order of Y variable for 200 models.

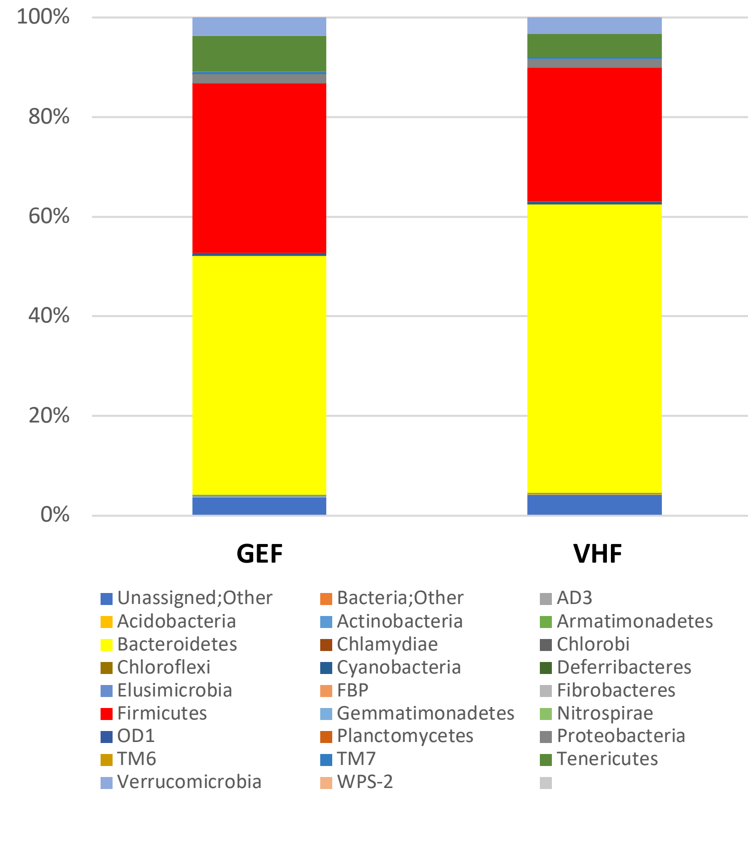


Supplemental Figure 3. 1. The incidence of severely diabetic following perinatal GEN treatment.

A



B



Supplemental Figure 3. 2. At phylum level, the taxonomy of gut microbiota based on 16S ribosomal RNA sequencing in NOD female offspring. (A) postnatal day (PND) 30 (B) PND 90 on 5K96 diet treated with genistein (GEF, N = 4) or vehicle (VHF, N = 6).

Supplemental Tables

Supplemental Table 3. 1. The primers used for quantitative reverse transcript PCR (qRT-PCR).

Gene name	Primer sequences	qPCR annealing, ° C
Defa17	F: GACCAGGCTGTGTCTGTCTC	57.5
	R: TGGTCTCCATGTTCAGCGAC	57.2
Defa24	F: CCAGAAGGCGCTTCTCTTCA	57.3
	R: AAGTGGTCATCAGGCACCAG	57.4
Defa29	F: ACTAGTCCTCCTCTCTGCCC	57.7
	R: GAGCCTTCTGTGCCTCCAAA	57.6
Defa30	F: TGTCGCTGAACATGGAGACC	57.2
	R: AAGTGGTCATCAGGCACCAG	57.4

Supplemental Table 3. 2. Significantly up-regulated and down-regulated genes from RNA-seq in female offspring perinatally exposed to genistein compared to vehicle control.

Gene symbol	Gene ID	log 2 ratio	q value	Definition
AY761184	NM_001007582	-2.11441	0.0102992	cDNA sequence AY761184
Defa24	NM_001024225	-1.73508	0.0102992	defensin, alpha, 24
Gm5431	NM_001024230	1.35184	0.0102992	predicted gene 5431
N/A	NM_001030294	-1.28764	0.0102992	not sufficient support
Bank1	NM_001033350	-1.60569	0.0361865	B cell scaffold protein with ankyrin repeats 1
Garem1	NM_001033445	0.798356	0.0176171	GRB2 associated regulator of MAPK1 subtype 1
Nipal1	NM_001081205	0.791728	0.0176171	NIPA-like domain containing 1
Ifit1bl1	NM_001101605	1.63654	0.0102992	interferon induced protein with tetratricpeptide repeats 1B like 1
Tmc5	NM_001105252	0.853937	0.022315	transmembrane channel-like gene family 5
Ltbp4	NM_001113549	-1.28923	0.0102992	latent transforming growth factor beta binding protein 4
Lepr	NM_001122899	-1.30225	0.0403978	leptin receptor variant 3

Wdfy4	NM_001146022	-0.841602	0.0321851	WD repeat and FYVE domain containing 4
Igsf10	NM_001162884	-1.33092	0.0485891	immunoglobulin superfamily, member 10
Apol7a	NM_001164640	1.04891	0.0102992	apolipoprotein L 7a
Defa17	NM_001167790	-1.95673	0.0102992	defensin, alpha, 17
Col6a5	NM_001167923	-1.16702	0.0102992	collagen, type VI, alpha 5
Defa30	NM_001177485	-1.97413	0.0102992	defensin, alpha, 30
Mptx2	NM_001205011	-1.6567	0.0321851	mucosal pentraxin 2
Col12a1	NM_001290308	-0.871489	0.022315	collagen, type XII, alpha 1
Inpp4a	NM_001290797	2.04244	0.0102992	inositol polyphosphate-4-phosphatase, type I, transcript variant 3
Bgn	NM_007542	-1.20535	0.0102992	biglycan
Car3	NM_007606	3.2081	0.0273245	carbonic anhydrase 3
Cd53	NM_007651	-1.05839	0.0403978	CD53 antigen

Cybb	NM_007807	-1.22007	0.0102992	cytochrome b-245, beta polypeptide
Defa29	NM_007844	-2.20345	0.0102992	defensin, alpha, 29
Fbn1	NM_007993	-0.740123	0.0403978	fibrillin 1
Fgf15	NM_008003	5.49562	0.022315	fibroblast growth factor 15
Igfbp3	NM_008343	-0.737991	0.0485891	insulin-like growth factor binding protein 3
Lama2	NM_008481	-0.9848	0.0102992	laminin, alpha 2
Lamb1	NM_008482	-0.912378	0.0102992	laminin B1
Mep1a	NM_008585	1.22059	0.022315	meprin 1 alpha
Npas2	NM_008719	1.35989	0.0102992	neuronal PAS domain protein 2
Reg1	NM_009042	-2.64511	0.0102992	regenerating islet-derived 1
Scd1	NM_009127	0.933542	0.0102992	stearoyl-Coenzyme A desaturase 1
Cfh	NM_009888	-1.15579	0.022315	complement component factor h

Coro1a	NM_009898	-0.900659	0.0321851	coronin, actin binding protein 1A, transcript variant 2
Col15a1	NM_009928	-1.03449	0.0403978	collagen, type XV, alpha 1
Col3a1	NM_009930	-0.883844	0.0102992	collagen, type III, alpha 1
Col6a1	NM_009933	-0.760807	0.022315	collagen, type VI, alpha 1
Edn1	NM_010104	1.34323	0.022315	endothelin 1
Fkbp5	NM_010220	1.04431	0.0102992	FK506 binding protein 5
Icam1	NM_010493	-1.38725	0.04463	intercellular adhesion molecule 1
Itga4	NM_010576	-0.97369	0.0102992	integrin alpha 4
Itln1	NM_010584	-1.18899	0.0102992	intelectin 1 (galactofuranose binding)
Jun	NM_010591	0.842368	0.0102992	jun proto-oncogene
Klf4	NM_010637	0.845473	0.0102992	Kruppel-like factor 4 (gut)
Mpeg1	NM_010821	-0.932477	0.0102992	macrophage expressed gene 1

Msn	NM_010833	-0.815778	0.0273245	moesin
Nos2	NM_010927	1.57779	0.0102992	nitric oxide synthase 2, inducible, transcript variant 1
Uhrf1	NM_010931	-1.87181	0.0361865	ubiquitin-like, containing PHD and RING finger domains, transcript variant 1
Reg3b	NM_011036	1.37548	0.0321851	regenerating islet-derived 3 beta
Pdgfra	NM_011058	-0.72807	0.022315	platelet derived growth factor receptor, alpha polypeptide, transcript variant 2
Pnliprp2	NM_011128	-0.830884	0.0102992	pancreatic lipase-related protein 2
Ccl11	NM_011330	-2.44936	0.0273245	chemokine (C-C motif) ligand 11
Slc10a2	NM_011388	1.98858	0.0102992	solute carrier family 10, member 2
Slc22a5	NM_011396	0.886993	0.0102992	solute carrier family 22 (organic cation transporter), member 5
Slc34a2	NM_011402	2.55551	0.0102992	solute carrier family 34 (sodium phosphate), member 2
Tfrc	NM_011638	-0.800891	0.0273245	transferrin receptor (Tfrc), transcript variant 1
Vip	NM_011702	-1.48359	0.0102992	vasoactive intestinal polypeptide
Vnn1	NM_011704	0.930924	0.0176171	vanin 1

Zfp36	NM_01 1756	0.7 86497	0.01 02992	zinc finger protein 36
Dusp1	NM_01 3642	1.1 384	0.01 02992	dual specificity phosphatase 1
Sfrp1	NM_01 3834	- 1.0859 6	0.01 76171	secreted frizzled-related protein 1
Map3k6	NM_01 6693	1.1 0051	0.01 02992	mitogen-activated protein kinase kinase kinase 6
Lyz2	NM_01 7372	- 0.9463 92	0.01 02992	lysozyme 2
Gsdme	NM_01 8769	0.8 86322	0.01 02992	gasdermin E
Calcrl	NM_01 8782	- 0.9314 83	0.04 85891	calcitonin receptor-like
Npff	NM_01 8787	N/A	0.01 76171	neuropeptide FF-amide peptide precursor
Hhip	NM_02 0259	- 1.2338 2	0.01 02992	Hedgehog-interacting protein
P2ry4	NM_02 0621	1.0 4193	0.01 02992	pyrimidinergic receptor P2Y, G-protein coupled, 4
Adamdec1	NM_02 1475	- 1.3975 5	0.01 02992	ADAM-like, decysin 1
Scube1	NM_02 2723	- 1.0823 6	0.04 463	signal peptide, CUB domain, EGF-like 1, transcript variant 2
Svep1	NM_02 2814	- 0.9944 44	0.01 76171	von Willebrand factor type A, EGF and pentraxin domain containing 1

Clps	NM_025469	-1.03645	0.0102992	colipase, pancreatic, transcript variant 1
Mptx1	NM_025470	-1.30752	0.0102992	mucosal pentraxin 1
Tmigd1	NM_025655	2.07198	0.0102992	transmembrane and immunoglobulin domain containing 1
Mgat4c	NM_026243	1.62162	0.0102992	MGAT4 family, member C
Reg4	NM_026328	-1.09907	0.0102992	regenerating islet-derived family, member 4
Fbxo32	NM_026346	0.881507	0.0102992	F-box protein 32
Nudt4	NM_027722	0.86814	0.0176171	nudix (nucleoside diphosphate linked moiety X)-type motif 4, transcript variant 2
Ceacam20	NM_027839	1.41444	0.0102992	carcinoembryonic antigen-related cell adhesion molecule 20 (Ceacam20), mRNA
Cnn3	NM_028044	-0.864982	0.022315	calponin 3, acidic
Ppp1r14d	NM_028104	0.883567	0.0102992	protein phosphatase 1, regulatory (inhibitor) subunit 14D, transcript variant 2
Nuak2	NM_028778	0.863469	0.0102992	NUAK family, SNF1-like kinase, transcript variant B
Hist1h1a	NM_030609	-0.818787	0.022315	histone cluster 1, H1a
Plvap	NM_032398	-0.941702	0.0102992	plasmalemma vesicle associated protein

Dock2	NM_033374	-1.17636	0.0102992	dedicator of cyto-kinesis 2
Aldh1a3	NM_053080	-0.982099	0.0176171	aldehyde dehydrogenase family 1, subfamily A3
Plpp3	NM_080555	-0.768833	0.0273245	phospholipid phosphatase 3
Xpnpep2	NM_133213	0.891597	0.04463	X-prolyl aminopeptidase 2, membrane-bound, transcript variant 1
Slc5a8	NM_145423	1.13288	0.0102992	solute carrier family 5 (iodide transporter), member 8
Mall	NM_145532	0.714215	0.0361865	T cell differentiation protein-like
Slc51a	NM_145932	0.877686	0.0102992	solute carrier family 51, alpha subunit
Pdlim2	NM_145978	1.13221	0.0321851	PDZ and LIM domain 2, transcript variant 1
Jchain	NM_152839	-1.18684	0.0102992	immunoglobulin joining chain
Abca8a	NM_153145	-1.0249	0.022315	ATP-binding cassette, sub-family A (ABC1), member 8a
Nckap1l	NM_153505	-1.18212	0.0102992	NCK associated protein 1 like
Clec2e	NM_153506	0.835216	0.0273245	C-type lectin domain family 2, member e
4930539E08Rik	NM_172450	1.27513	0.0102992	RIKEN cDNA 4930539E08 gene
Slc17a5	NM_172773	0.727252	0.0361865	solute carrier family 17 (anion/sugar transporter), member 5, transcript variant 1

Suox	NM_173733	1.05811	0.0273245	sulfite oxidase
Pgm5	NM_175013	0.729279	0.022315	phosphoglucomutase 5
Tprn	NM_175286	0.765103	0.0176171	taperin
Ston2	NM_175367	0.689766	0.0361865	stonin 2
Aldh4a1	NM_175438	0.776806	0.022315	aldehyde dehydrogenase 4 family, member A1
Hist1h2bk	NM_175665	-0.856937	0.0485891	histone cluster 1, H2bk
Hist2h2bb	NM_175666	-0.888666	0.0102992	histone cluster 2, H2bb
Dab1	NM_177259	0.911958	0.0102992	disabled 1, transcript variant 2
Ang4	NM_177544	-1.84589	0.0102992	angiogenin, ribonuclease A family, member 4
Duox2	NM_177610	1.16472	0.0102992	dual oxidase 2
Prex1	NM_177782	-1.20249	0.0102992	phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor 1
Insig2	NM_178082	0.950947	0.0176171	insulin induced gene 2, transcript variant 2
Hist1h2ak	NM_178183	-0.78315	0.0273245	histone cluster 1, H2ak

Hist1h2bl	NM_178199	-0.715184	0.0403978	histone cluster 1, H2bl
Hist1h2bm	NM_178200	-1.10686	0.0102992	histone cluster 1, H2bm
Hist1h3b	NM_178203	-1.28406	0.0102992	histone cluster 1, H3b
Hist1h3h	NM_178206	-0.953665	0.0176171	histone cluster 1, H3h
Hist1h4k	NM_178211	-0.776717	0.0361865	histone cluster 1, H4k
Lrrc8d	NM_178701	1.06683	0.022315	leucine rich repeat containing 8D, transcript variant 2
Tmem117	NM_178789	1.18147	0.0102992	transmembrane protein 117
Slc51b	NM_178933	1.24766	0.0102992	solute carrier family 51, beta subunit
Tmem140	NM_197986	0.945959	0.0361865	transmembrane protein 140
Ccdc120	NM_207202	0.78646	0.04463	coiled-coil domain containing 120
BC064078	NR_015455	-1.12736	0.0321851	cDNA sequence BC064078

Note: a negative log2 ratio indicates a down-regulation of mRNA by genistein exposure. N/A = not available.

CHAPTER 4

IMMUNOMODULATION OF ISOFLAVONE DAIDZEIN IN NON-OBESE DIABETIC (NOD) AND B6C3F1 MOUSE MODELS[4]

[4] Huang, Guannan. To be submitted to Toxicology and Applied Pharmacology, August, 2018.

Introduction

Type 1 diabetes (T1D) is increasing in a rapid rate in US (1.25 million in American children and adults, American Diabetes Association, 2016), and the incidence is higher in males while women with T1D have a 40% excess risk of death from all causes compared to men with T1D (Huxley *et al*., 2015). In T1D patients, people under the age of 18 are mostly afflicted (Bluestone, Herold, & Eisenbarth, 2010), although adult onsets of T1D are becoming increasingly common (Li *et al*., 2016; Menke *et al*., 2013). Diet has been speculated to modulate T1D progression, and polyphenolic compounds, which are naturally occurring compounds found in fruits and vegetables, are of interest with regards to the pathogenesis of T1D. Phytoestrogen, a class of polyphenolic compounds that exerts weak estrogenic effect, includes lignans (enterodiol and enterolactone) and isoflavones [daidzein (DAZ), genistein (GEN), and glycitei] (Bhathena & Velasquez, 2002).

DAZ (Choi *et al*., 2008) in soy isoflavones is found in equal amount as GEN (Ding *et al*., 2016; Gilbert & Liu, 2013; Nanri *et al*., 2010); however, the compound is less studied. DAZ can be metabolized into dihydrodaidzein, equol, or O-desmethylangolensin (ODMA). Equol has a high affinity to the ERs. ODMA and equol are associated with obesity (Frankenfeld *et al*., 2014; Miller *et al*., 2017) and cardiometabolic risk (Hazim *et al*., 2016; Reverri *et al*., 2017). For example, in human, ODMA non-producer phenotype is found to positively associate with obesity (Miller *et al*., 2017). It is also reviewed that DAZ can mitigate the adverse health effect induced by GEN (Yang *et al*., 2012).

There are multiple studies examining the health effect of DAZ. One case-control study demonstrated that high serum DAZ level was associated with a reduced ovarian cancer risk compared to low DAZ level among Japanese women (Otokozawa *et al*., 2015). In one systematic literature review, high DAZ intake was associated with a 34% reduced risk of breast cancer, while

the same association was not found for equol (Rienks, Barbaresko, & Nothlings, 2017). The same study summarized that the risk for type 2 diabetes (T2D) was reduced by 19% following high amount of DAZ intake. Similarly, in a nested case-control among US women, urinary DAZ level correlated with a protective effect against T2D (odds ratio = 0.71, 95% CI 0.55-0.93, Ding *et al*., 2015). From a mechanistic standpoint, the protective effect of DAZ could be related to the inhibitory effect on α-glucosidase and α-amylase (Park *et al*., 2013), promotion of AMPK phosphorylation and GLUT4 translocation (Cheong *et al*., 2014), inhibition of glucose uptake in adipocytes (Claussnitzer *et al*., 2011), enhancing the epithelial function and reducing oxidative stress (Roghani *et al*., 2013), increase of PPAR-γ transcription (Cho *et al*., 2010), upregulation of ER- β (Xu *et al*., 2009), increase of the insulin/glucagon ratio and regulation of hepatic lipid regulating enzymes (such as hepatic fatty acid synthase) (Park *et al*., 2013), and enhancing IL-17 gene expression (Kojima *et al*., 2015).

To date, only one study evaluated the effect of DAZ on T1D (Choi *et al*., 2008), and concluded that DAZ exerted a similar protective effect on hyperglycemia and glucose tolerance compared to GEN. With regards to autoimmune effect, equol reduced the inflammatory response and down-regulated IL-6 mRNA expression in mouse rheumatioid arthritis model (Lin *et al*., 2016). In another mouse study, low dose DAZ administration (20 mg/kg) did not affect experimental allergic encephalomyelitis (EAE) development while high dose DAZ (300 mg/kg) alleviated EAE symptoms by modulating T cell responses (Jahromi *et al*., 2014). Here in this study, we applied both the non-obese diabetic (NOD) mouse and B6C3F1 mouse as complementary models to study the immunomodulatory effect of DAZ.

Method

NOD mouse model

24 adult male mice (12/group) and 20 adult female mice (10/group) were used in this study after postnatal day (PND) 56. They were randomized into VH and DAZ groups based on body weight (BW) and baseline blood glucose levels (BGLs). The DAZ dose used in this study were 20 mg/kg BW because for GEN, we were able to detect a difference in BGL at that dose in different murine diabetic models (Guo *et al*., 2015; Huang *et al*., 2017), and it was reported that DAZ has similar effect for BGL modulation (Choi *et al*., 2008). Mice in DAZ groups were dosed with 0.1 ml DAZ solution/10g BW (2 mg/mL DAZ dissolved in 25mM Na_2CO_3 by sonication) while the VH groups be dosed with the same volume of Na_2CO_3 daily. A micropipette was used for dosing to minimize the stress caused by gavage. The mice were fed with a purified phytoestrogen-free diet (Research Diets, Inc., New Brunswick, NJ) throughout the study.

BW, insulin, BGL measurement, glucose tolerance test (GTT), and insulin tolerance test (ITT)

Our previous publication (Huang *et al*., 2017) described the measurement of BW and BGL. GTT and ITT were performed by injecting the mice with glucose (*i.p*.; 2 g/kg BW) or insulin (*i.p*.,1.5 IU/kg BW), and measuring subsequent BGL at 0, 15, 30, 60 and 120 minutes following initial injection.

Enzyme-linked immunosorbent assay (ELISA)

We performed ELISA to detect the total antibody level (IgG_{2a}, IgG_{2b}, IgM, IgG_1) in mouse sera following (Guo *et al.*, 2014; Huang *et al.*, 2017). The plates coated with primary antibody were blocked (5% milk powder in PBS with Tween 20), added with primary antibodies, and incubated at room temperature for 2 h. Then the secondary antibody was added and incubated for

1 hour. Substrate TMB and sulfuric acid (2N) was added at 100 μL/well, and the plate was read at the wavelength of 450 nm.

B6C3F1 model

B6C3F1 mouse is the offspring of a cross between C57BL/6J females (B6) and C3H/HeJ males (C3). The female B6C3F1 mice reaching adulthood were used for the study. The female mice (3-4 weeks with initial weight of 21-28g) were purchased from Taconic. They were randomized into naïve (NA), VH and DAZ groups (N = 8 for each group) based on body weight (BW) and baseline blood glucose levels (BGLs). The dosing regime was same as NOD mice.

Late apoptosis

The apoptosis measurement was performed following our previous publication (Guo *et al*., 2014). Thymocytes (1×10^6 cells/ml) were stained for 15 min with 40 nM 3,3′-dihexyloxacarbocyanine ($DiOC_6(3)$; Life Technologies) and 2 μM hydroethidine (HE, Life Technologies) for assessing mitochondrial transmembrane potential ($\Delta\Psi m$) and reactive oxygen species (ROS) generation. Following excitation at 488 nm (250 mW), emission was monitored through a 530/30 nm bandpass filter for DiOC6(3) and 575/26 nm bandpass filter for HE, and then logarithmic amplification was used to detect the fluorescence. The late apoptosis cell population was represented by $DiOC_6(3)^-$ HE^+ cells.

Flow cytometry

In NOD males and females, the splenocytes were collected upon euthanasia and the flow cytometric analysis was performed following our previous publication (Huang *et al*., 2017). The antibody cocktails were CD4-CD8-CD25, B220-CD40L, CD40-CD44, CD5-CD24. In B6C3F1 females, the antibodies include IgM-CD3, CD4-CD8-CD25, NK1.1-CD3, Gr-1-Mac3.

Statistical analysis

Dunnett's test was used to compare the means among treatment groups when the equal variance assumption was met; otherwise, and Wilcoxon test was performed to compare the means. Likelihood ratio test was used to compare the diabetes incidence, and Student's t-test was used to compare the abundance of taxa between treatment groups. JMP Pro 11 (SAS Inc., Cary, NC) and GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA) were used for statistical analysis and data visualization.

Results

BGL and tolerance tests did not show a strong effect in DAZ-exposed NOD mice

DAZ exposure led to a reduction of BW in NOD males (**Figure 4.1A**), not in females (**Figure 4.1B**). The blood glucose level, together with glucose tolerance test and insulin tolerance test, did not reflect a strong effect on T1D protection in both sexes (**Figure 4.1C-D, Figure 4.2**).

DAZ exposure induced an increased level of serum IgG_{2b} in NOD females and IgG_1 in NOD males

Figure 4.3. demonstrates the alteration of serum antibodies following DAZ exposure. DAZ dosing led to a significant increase in IgG_{2b} in females ($P = 0.0211$, **Figure 4.3B**) and an increase in IgG_1 in males ($P = 0.0166$, **Figure 4.3H**). The other antibodies were not significantly altered by DAZ dosing (**Figure 4.3A, C, D, E, F, G, H**).

DAZ increased $CD8^+CD25^+$ splenocytes in NOD females

Flow cytometric analysis demonstrated an increase in percentage of $CD8^+CD25^+$ cells (which are possibly regulatory T cells (Churlaud *et al*., 2015), **Figure 4.4.**) in spleens collected from NOD females following DAZ dosing ($P = 0.0268$). In addition, DAZ dosing increased $CD4^-CD25^+$% (data not shown), $CD8^+CD25^+$% (**Figure 4.4.**, $P = 0.0268$), and $B220^+CD40L^+$ intensity

in NOD females (data not shown). While for other splenocyte subtypes from NOD females, no differences were observed (data not shown).

DAZ reduced the late apoptosis in B6C3F1 females

As shown in **Figure 4.5.**, DAZ dosing induced a decrease in percentage of late apoptotic cells ($P = 0.0092$), while no difference in early apoptotic cells, live cells, and necrotic cells were observed (data not shown).

DAZ altered the splenocyte subpopulation in B6C3F1 females

Figure 4.6. demonstrated the splenocyte population of B cells, T cells, and neutrophils following DAZ dosing. The percentage of total B cells (represented by $CD3^{-}IgM^{+}$) was reduced (**Figure 4.6A.**, $P = 0.0388$), while the total T cells were increased (**Figure 4.6B**, $P = 0.0004$). CD4-CD8-CD25 markers revealed that both the helper T cell population (**Figure 4.6C**, $P = 0.038$) and cytotoxic T cell population (**Figure 4.6D**, $P < 0.0001$) were increased. In addition, the splenic percentage of neutrophils was also significantly reduced ($P = 0.0063$).

Discussion

Soy-based food is of interest to study not only because it is an intrinsic part of Asian cuisine, but also that it takes up 25% of the infant formula market (Cao *et al*., 2009). DAZ and GEN are the major isoflavones in soy-based food, and they exert estrogen-like effects. In most soy product (such as boiled soybean and tofu, **Table 1.1.**), DAZ content is equivalent to GEN. In general, the majority of studies focused on DAZ metabolites (ODMA and equol) following bacterial metabolism because of the particular protective effect against obesity; however, the parent compound is not well studied (partly due to its low solubility and low bioavailability). In our previous study, GEN exposure reduced the blood glucose level in NOD males and improved the glucose tolerance, while delaying the onset of T1D among NOD females (Huang *et al*., 2017). It

was previous reported by (Choi *et al*., 2008) that DAZ (added at 0.2 g/kg diet) demonstrated a similar protective effect against T1D (for both blood glucose level and glucose tolerance) as GEN. It is calculated that the dose used would be similar to our dose at 20 mg/kg BW, which is a physiological dose of isoflavone exposure (Guo *et al*., 2014). However, in the current study, we did not observe the improvement in blood glucose and tolerance although the same animal model was used. This may be due to different route of exposure (gavage vs food intake, as for food intake, animals are exposed to a mix of compounds). In the near future, to improve the bioavailability of DAZ and to enhance the potential beneficial effect, we may consider the use of DAZ metabolites, which will require an in-depth understanding of host-microbiota interaction and a thorough investigation on the mode of action following DAZ exposure in both sexes.

In male rats, DAZ was reported to mitigate lipopolysaccharide-triggered immune responses represented by decreased neutrophil, tumor necrosis factor (TNF)-α, macrophages, and NF-κB markers (Feng, Sun, & Li, 2015), suggesting an anti-inflammatory response. This is agreement with the findings by Yum *et al*. (2011) that DAZ exposure suppressed pro-inflammatory cytokine such as IL-12p40, IL-6 and TNF-α. Further, DAZ has a suppressive effect on dendritic cells as well (Yum *et al*., 2011).

With regards to antibodies, Fan *et al*. (2018) observed an increase in IgG following exposure to DAZ at 20 mg/kg BW in chicken breeders. This finding is supported by Liu *et al*. (2014) that both 300 mg/kg and 400 mg/kg DAZ increased serum IgG in lactating cows. We observed an increase in IgG_{2b} in NOD females and IgG_1 in NOD males. The same alteration was not found in GEN-exposed NOD adults, as the males demonstrated a decreased IgG_{2b}, not IgG_1, while IgG level was not changed (Huang *et al*., 2017). In contrast, combinatorial exposure of GEN and DAZ in female BALB/c mice demonstrated a decreased IgG_1 for mucosal immune response

(Wei *et al*., 2012). The function of IgG_{2b} is usually vague because it can be either Th1 or Th2 antibody. The increase of IgG_1 suggests a stronger Th2 response in NOD males, which is contradictory to the anti-inflammatory response reported in other studies. A numeric decrease in female IgG_1, which corresponds to a decreased Th2 response, is in agreement with a decreased B cell population.

For flow cytometric and apoptotic cell analyses, one study that exposed chicken breeders to the same DAZ dose as ours (20 mg/kg BW) observed an increase of B cells and the ratio of CD4/CD8 cells (not total T cell population) in a 21-day treatment but not in the 42-day treatment (Fan *et al*., 2018). In addition, a down-regulation of gene expression related to lymphocyte apoptosis was found (Fan et al., 2018). Our finding suggests a reduced total B cell population (which might associate with the weakened Th2 response) in females, while the T cell populations (total, cytotoxic and helper T cells) were significantly increased, which was inconsistent with Fan *et al*. (2018). Interestingly, the $CD8^+CD25^+$ population (possibly Treg cells) was greatly increased. In Chapter 3, the exacerbation of T1D was associated with an increased total T cell and helper T cell population. In this study, the lack of T1D exacerbation in the presence of T cell increase might be due to the up-regulated Treg cells in addition to the decreased B cells, which are essential in maintaining the immune homeostasis against pro-inflammatory responses. It was widely accepted that, late apoptosis (with compromised cell membranes), with delayed clearance by phagocytes, can lead to autoimmunity (Patel *et al*., 2006), and this current study showed a reduced late aoptotic cell population. The effect of neutrophils on T1D has been brought to attention in recent years, and it is widely accepted that the reactive oxygen species and cytokine produced by neutrophils can destruct the pancreatic cells and lead to the perpetuation of T1D (Huang *et al*., 2016).

In this study, we used two complementary models (NOD and B6C3F1). The use of NOD is mainly to assess the anti-diabetic effect as the mice resemble human T1D with regards to insulitis development and to investigate the immunological alterations following DAZ exposure. The use of B6C3F1 female model is to confirm the findings in NOD female model, and the cell population is further studied with different cell markers. In conclusion, DAZ dosing reduced the B cell population as well as IgG_1 production, while increased the T cell population in females. It has been speculated by Hampe (2012) that T1D development is initiated by B cell and followed by T cell response. The lack of protection or exacerbation of T1D can be explained reduced B cell population and increased regulatory T cells, accompanied by increased T cells in NOD mouse model, and revealed a completely different mechanism compared to GEN exposure in adults with both sexes.

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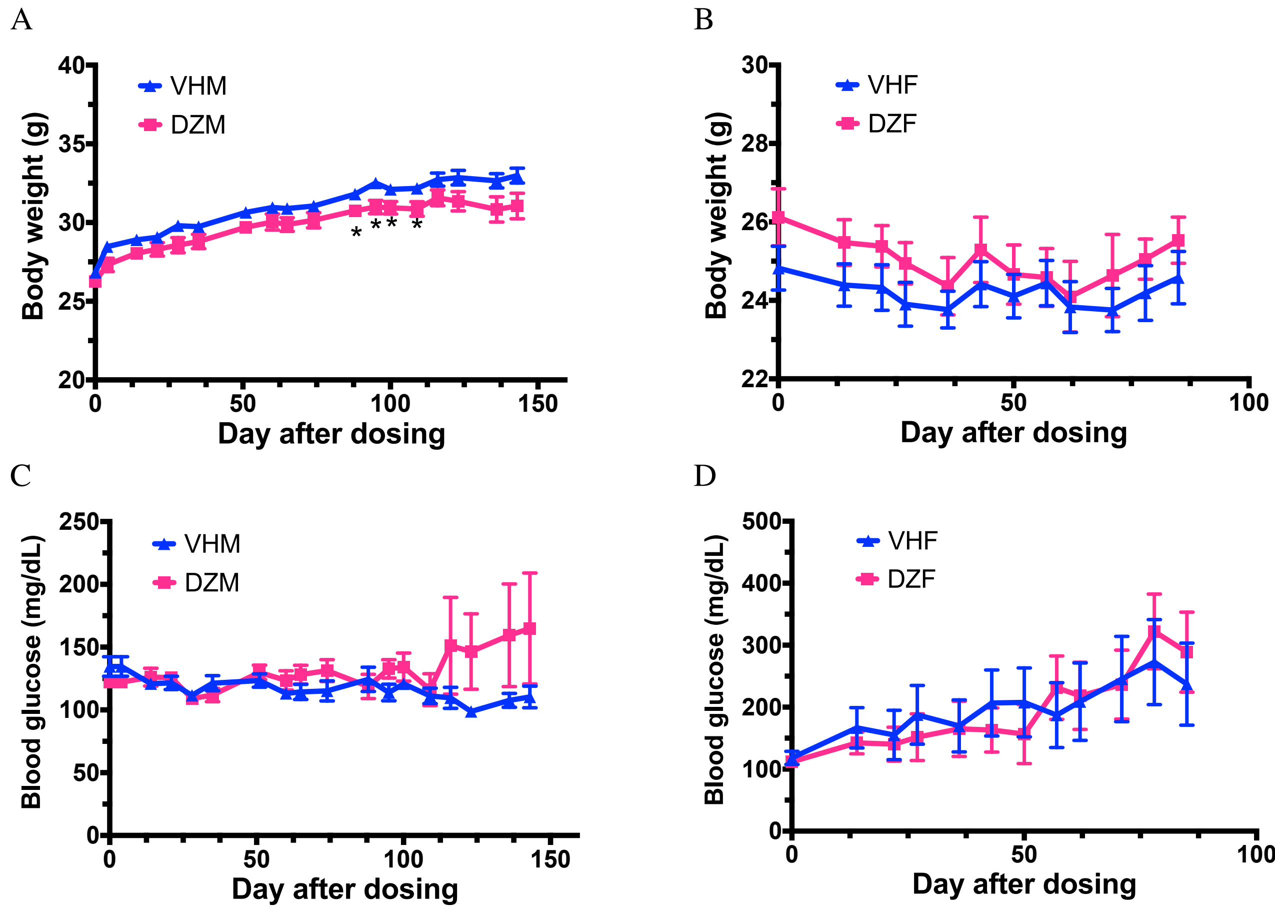

Figure 4. 1. The body weight (BW) change of (A) NOD males and (B) NOD females, and the blood glucose level (BGL) in (C) NOD males and (D) in females. VHM = males dosed with vehicle (N=6), DZM = males dosed with daidzein (N=6), VHF = females dosed with vehicle (N=5), and DZF = females dosed with daidzein (N=5).

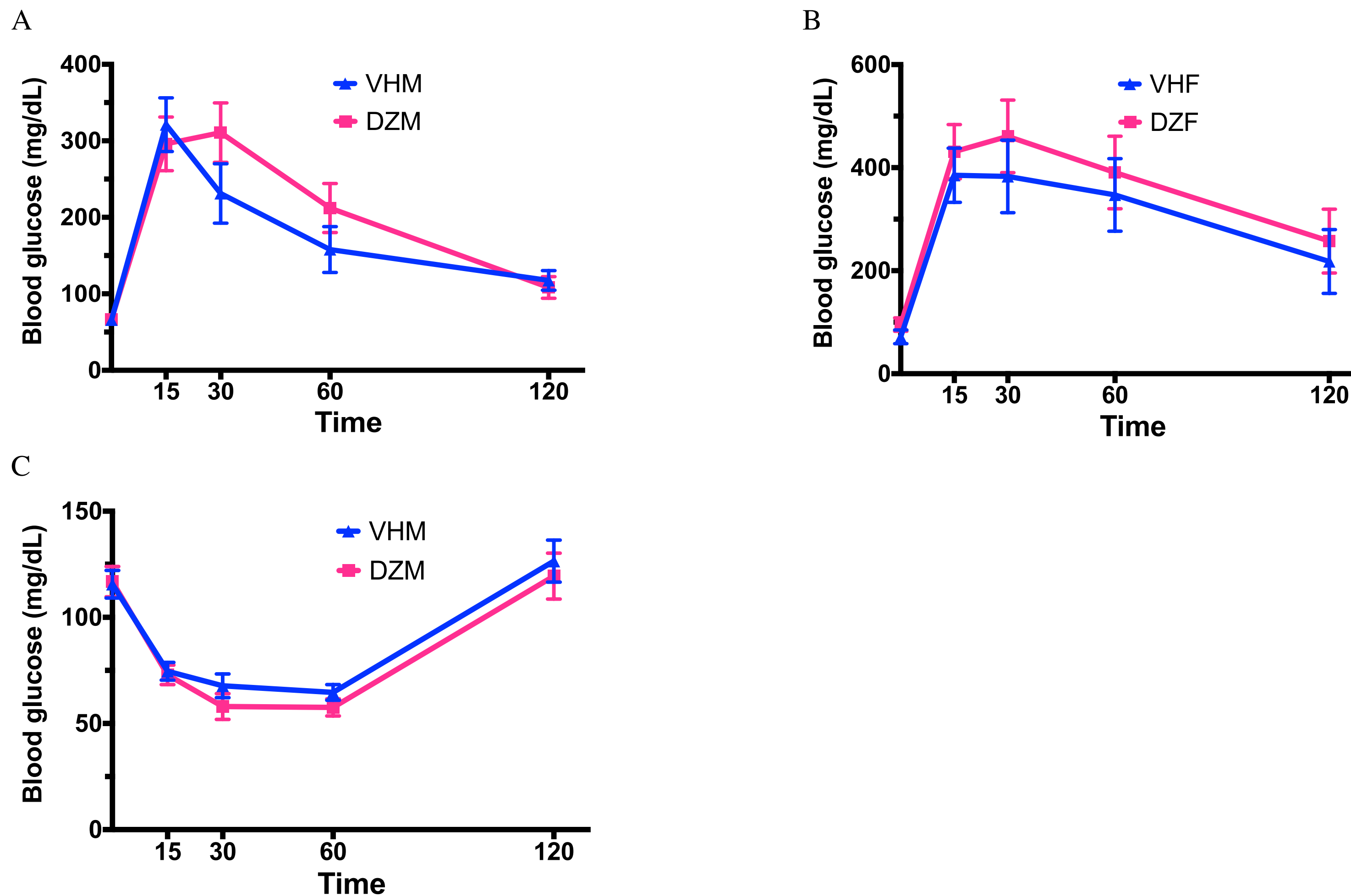

Figure 4. 2. The glucose tolerance test (GTT) in (A) NOD males and (B) in NOD females, and (C) the insulin tolerance test (ITT) in NOD males. VHM = males dosed with vehicle (N=6), DZM = males dosed with daidzein (N=6), VHF = females dosed with vehicle (N=5), and DZF = females dosed with daidzein (N=5).

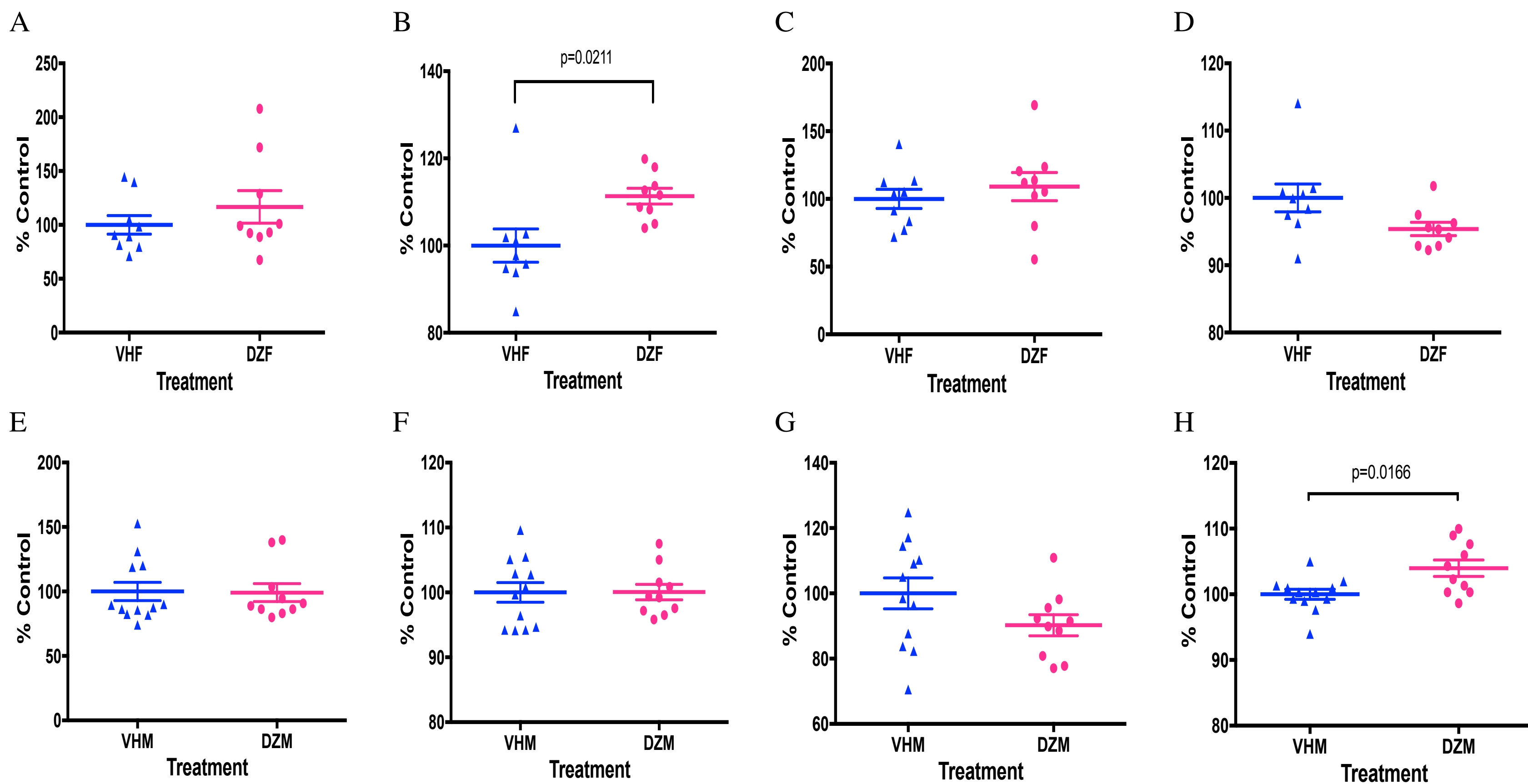

Figure 4. 3. The serum (A) IgG_{2a}, (B) IgG_{2b}, (C) IgM, (D) IgG_1 in NOD females and (E) IgG_{2a}, (F) IgG_{2b}, (G) IgM, (H) IgG_1 in NOD males. VHM = males dosed with vehicle (N=12), DZM = males dosed with daidzein (N=10), VHF = females dosed with vehicle (N=9), and DZF = females dosed with daidzein (N=9).

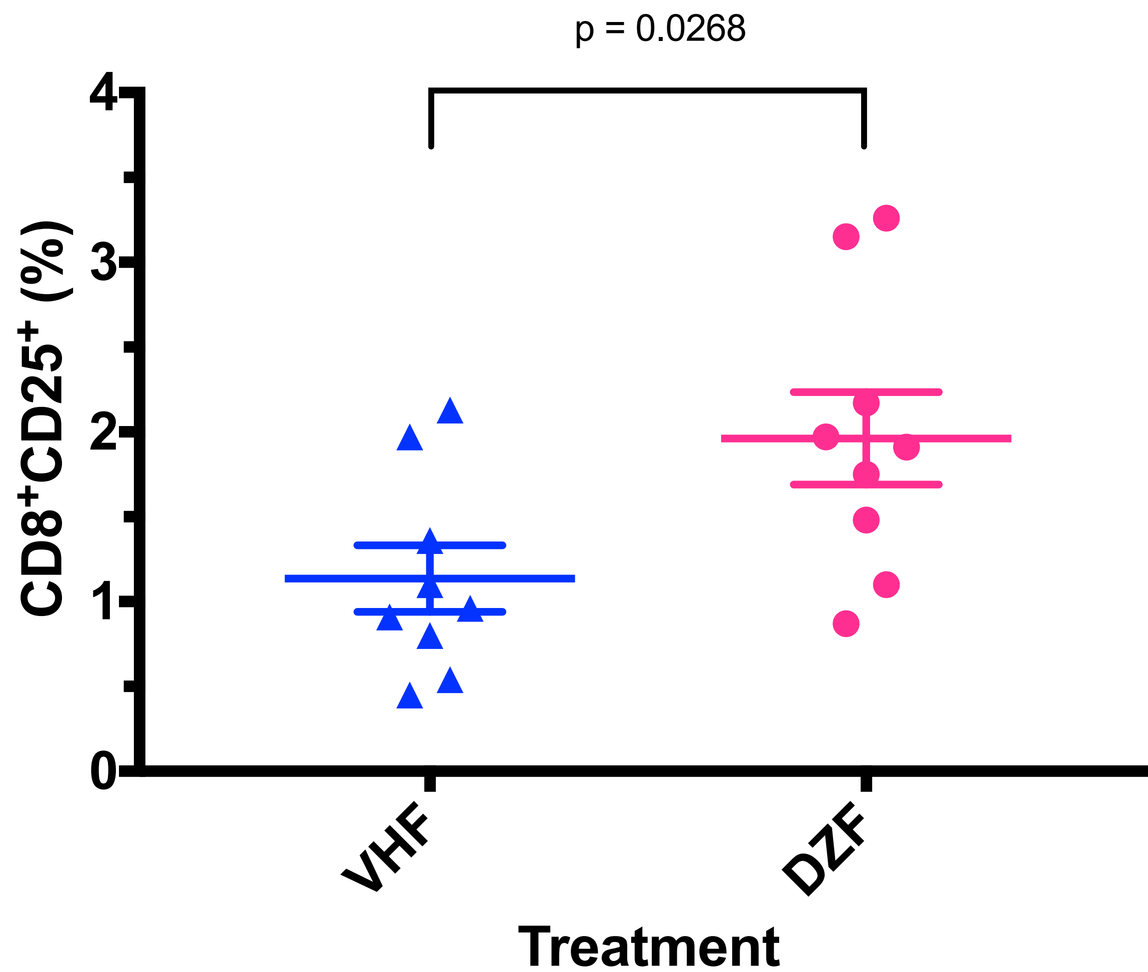

Figure 4. 4. Percentage of $CD8^{+}CD25^{+}$ splenocytes following daidzein treatment among NOD females. VHF = females exposed to vehicle (N=9), DZF=females exposed to daidzein (N=9).

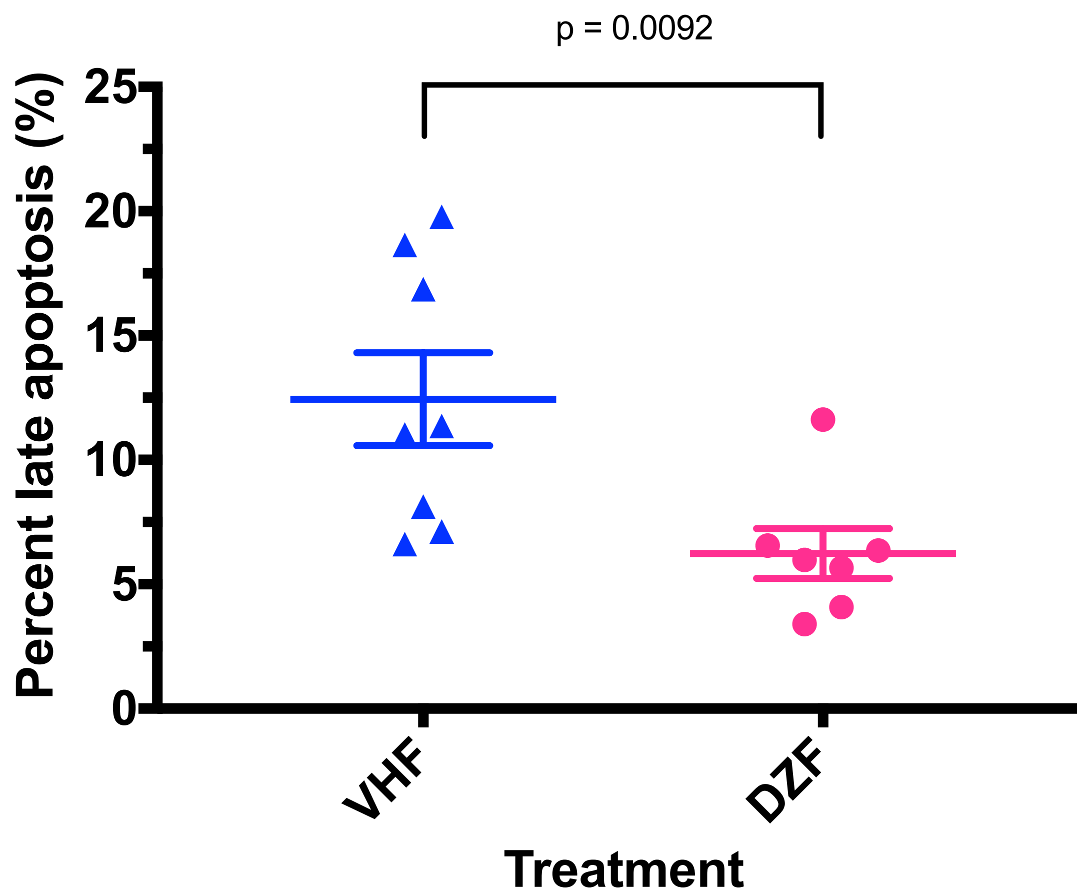

Figure 4. 5. Late apoptosis (%) measured in B6C3F1 adult females dosed with daidzein only. VHF = females exposed to vehicle (N=8), DZF=females exposed to daidzein (N=7).

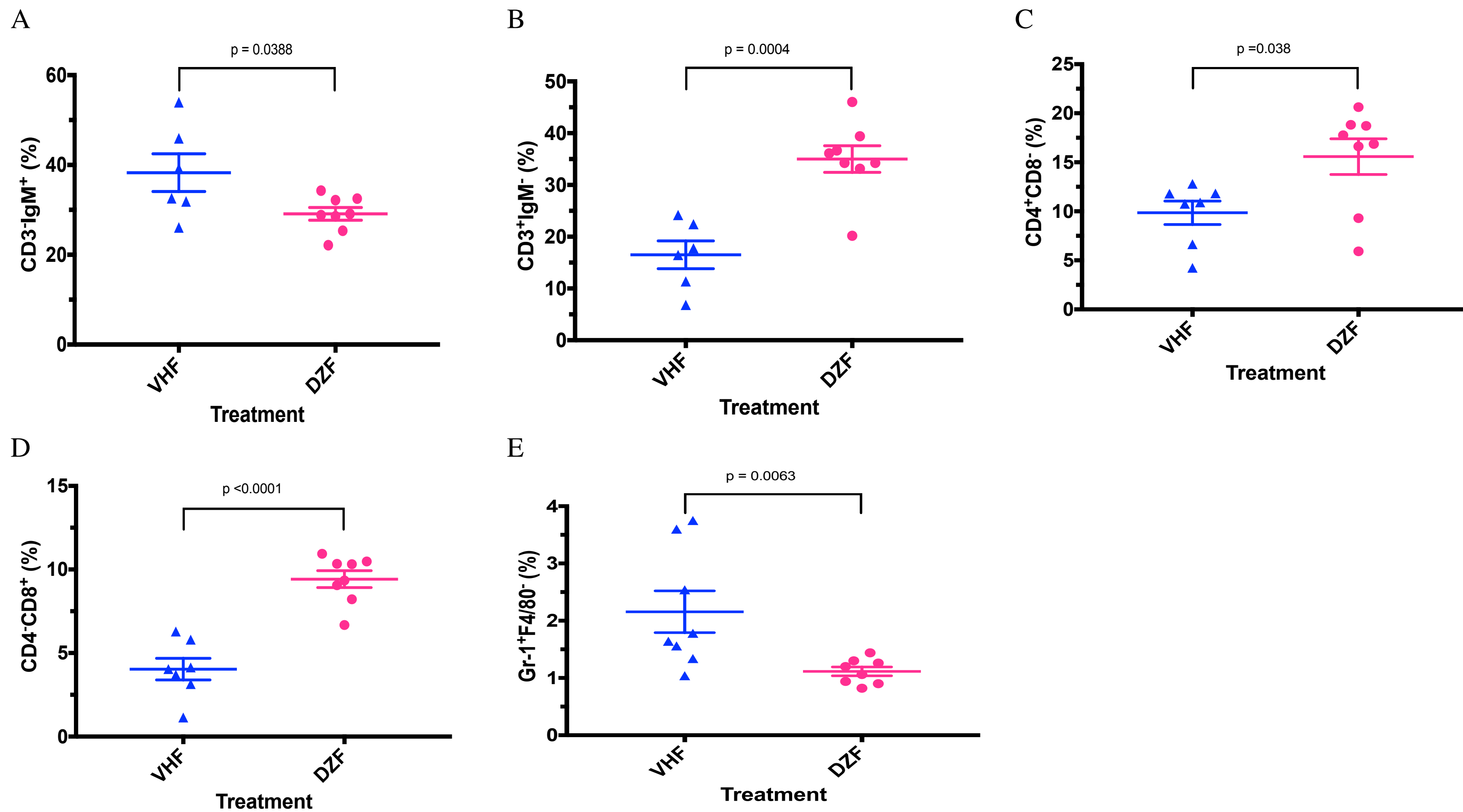

Figure 4. 6. Percentage of (A) $CD3^{-}IgM^{+}$, (B) $CD3^{+}IgM^{-}$, (C) $CD4^{+}CD8^{-}$, (D) $CD4^{-}CD8^{+}$, and (E) $Gr\text{-}1^{+}F4/80^{-}$ splenocytes following daidzein treatment among NOD females. VHF = females exposed to vehicle (N=7), DZF=females exposed to daidzein (N=8).

CHAPTER 5

CONCLUSION

The prevalence of type 1 diabetes (T1D) is increasing at an annual rate of 3% (DIAMOND Project Group, 2006) with an estimated male: female ratio of 1.47 (Diaz-Valencia *et al*., 2015). The incidence of T1D varies in different parts of the world, with high figures in the Nordic countries (~30 cases/year/100,000 individuals) and low figures in the countries of East Asia such as China and Japan with ~1 case/year/100,000 individuals (Frisk *et al*., 2008; Kitts *et al*., 1997). These geographical differences in T1D incidence may be related to food consumption (Frisk *et al*., 2008), as large quantities of soy products are widely used in traditional Asian diets (with intake of soy food at a level of 36 g/d) (Hilakivi-Clarke, Andrade, & Helferich, 2010). Genistein (GEN) and daidzei (DAZ) are natural prebiotics found in some plants with soybeans being one of the most abundant sources, while GEN (Ding *et al*., 2016; Gilbert & Liu, 2013; Nanri *et al*., 2010) and DAZ (Choi *et al*., 2008) possess anti-diabetic properties. Our studies have consistently found that adult exposure to GEN at physiological doses [e.g., 2-20 mg/kg body weight (BW) by gavage] protected both sexes from T1D in various murine diabetic models using a gut microbiota (GMB)-related immunomodulation (Guo *et al*., 2015; Guo *et al*., 2014; Huang *et al*., 2017). Literature is largely missing with regards to the mechanism of isoflavone effect on T1D and immune responses for both sexes at different exposure windows.

In chapter 2, we dosed both male and female non-obese diabetic (NOD) adult mice with 20 mg/kg BW GEN for over 100 days. GEN reduced the blood glucose level (BGLs) for males

while delayed the onset for females. The antibodies, cytokine alterations and splenocyte subclasses, reflected an anti-inflammatory response in males and pro-inflammatory responses in females. 16s ribosomal RNA (rRNA) suggested an altered beta-diversity for males, not for females. In addition, the taxonomy alteration at both order and genus level, in accordance with immunological change, showed an anti-inflammatory response in males and pro-inflammatory responses in females.

In chapter 3, we evaluated the male and female offspring for T1D incidence, immunological response, GMB and metabolites. The NOD dams were dosed with GEN (20 mg/kg BW) and the offspring were monitored for over 200 days. The females showed a higher incidence of T1D, and the BGL for non-diabetic mice were increased following perinatal GEN exposure. The same trends were not found the males. Interestingly, the results of antibodies, cytokines (IL-10), and flow cytometry were consistent, showing an anti-inflammatory response in males and pro-inflammatory responses in females (which is consistent with chapter 2 also). The GMB from female offspring were analyzed at PND 30 and PND 90; PND 30 fecal samples did not show any alteration for α, β diversity as well as taxonomy; while PND 90 fecal samples exhibited an alteration in β diversity and taxonomy at both order and genus level, corresponding to a pro-inflammatory response. In contrast, GMB from male offspring at PND 90 demonstrated an anti-inflammatory response. Urinary metabolites at PND 60 were well separated by perinatal GEN treatment. To further explore the mechanism of GEN exacerbation of T1D in females, ileum mRNA by both RNA-seq and qRT-PCR reflected a significant reduction of α-defensin by GEN treatment, which can contribute to the dysbiosis of the gut.

In chapter 4, two complementary models, B6C3F1 and NOD, were used to study the effect of DAZ exposure in both adult females and males. Contrary to the findings for GEN, no alterations

in BGL and/or glucose tolerance were found following adult exposure in both sexes. Interesting, an increased T cell and neutrophil population, accompanied by a decreased B cell population, was observed. However, this did not translate into a phenotypic effect.

In this dissertation, T1D incidence change, blood glucose level, glucose tolerance, cytokine/chemokine profile, antibody levels, gut microbiota, gene expression, untargeted metabolites, splenocyte subpopulation all served as major outcomes for studying the effect of isoflavones on the development of T1D. However, this study was mainly hypothesis-generating instead of hypothesis testing, and the causation was lacking (e.g., the relationship between GEN-immune-gut microbiota). Mechanistic approaches, such as the use of germ-free or antibiotic animal model, fecal transplant together with thymus transplant, should be applied to reveal the causation of exposure-disease paradigm.

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