

## ORIGINAL ARTICLE

# Combining Lemon and Glycerin may Beneficially Regulate Blood Glucose Levels by Modulating Gut Microbiota in Non-obese Diabetic Mice

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## Abstract

Both dietary lemon and glycerin have shown beneficial effects in diabetic humans and animals. It was hypothesized that there were potential therapeutic advantages of combining both agents in hyperglycemic and healthy mice. In a 6-month study using mature adult male non-obese diabetic (NOD) mice, oral treatment with either lemon or glycerin alone increased blood glucose levels during the third month glucose tolerance test and decreased the activity of the predicted glycolysis/gluconeogenesis pathways when compared to the vehicle control; however, this was no longer observed when lemon and glycerin were combined. Metabolomic analysis indicated that acetate was increased in the fecal samples after treatment with either

glycerin or the combination. A 2-month study was also conducted in both male and female NOD mice and suggested that there were more gut microbiome changes at one month in comparison to six months. In older NOD male mice, treatment with the combination for six months decreased insulin resistance. In both adult male and female C57BL/6 mice, dosing with the combination for two months decreased blood glucose levels, as well as glucose tolerance and insulin resistance. In addition, treatment with the combination decreased body weights in male mice, especially in older NOD males. Overall, these studies suggest that lemon and glycerin in combination may reduce the side effects of individual treatments (e.g., transient hyperglycemia) and have some additional benefits (e.g., weight loss). Microbiome modulation likely contributed to the observed beneficial effects.

**Key Words:** *Gut microbiome; Lemon; Glycerin; Hyperglycemia; Body weight*

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## Introduction

Hyperglycemia, referring to high levels of blood glucose levels in patients with type 1 or type 2 diabetes, occurs when there is an insulin insufficiency or insulin resistance. The price of insulin in United States has gone up approximately 1,200% since 1996 [1]. This increased cost forces many patients to ration the insulin they are able to afford, leading to severe complications including death in some cases. Thus, there is an urgent need to develop preventive/complementary treatments to control hyperglycemia that are low-cost, long-lasting, and non-invasive. Many factors, including food, can help manage hyperglycemia. Previous studies support the health benefits of lemon and glycerin. Citrus extracts have been reported to decrease weight [2]. With a high content of vitamin C and soluble fibers, low glycemic index and other beneficial characteristics, the American Diabetes Association lists lemons as a diabetes superfood to lower blood glucose levels and insulin resistance. The components in lemon, e.g., citric acid and flavonoids, may contribute to the postprandial hyperglycemia-suppressing action [3]. In rats, oral administration of naringenin, a flavonoid in citrus peels, counteracted most unfavorable changes and improved ketoacidosis with type 1 diabetes (T1D) by reducing chronic inflammation and oxidative damage in proteins and lipids that lead to insulin resistance [4-5].

Glycerin occurs naturally in fermented foods and beverages. Because of its dehydrating effect, glycerin is beneficial to patients with recent cerebral infarction by reducing focal cerebral edema [6]. Cerebral edema is the leading cause of death in T1D children presenting with diabetic ketoacidosis [7]. Glycerin has also been therapeutically used for the treatment of increased intracranial pressure and acute stroke.

The ingestion of glycerin in combination with excess fluid can also lead to an increased plasma osmolality, a reduced urine volume and an expanded plasma volume [8]. Glycerin consumption also mimics caloric restriction by shifting metabolism away from glycolysis and towards oxidative phosphorylation. Glycerin supplementation has a number of other beneficial effects, including lifespan extension, improved stress resistance and enhanced locomotory and mitochondria activity in older age classes of *Brachionus manjavacas* rotifers [9]. Glycerin ingestion, however, has a transient hyperglycemic effect [10]. Therefore, it became important to know if lemon extracts could mitigate the potential hyperglycemic effects elicited by glycerin consumption.

Microbiome is the basis of metagenomics that predicts functions affected by changes in the gut microbiota and has the potential to explain the benefits of consuming specific foods. Recent studies have reported gut dysbiosis as a major mechanism in rapid progression of hyperglycemia, with specific variations in different microbial populations [11]. It was hypothesized that lemon and glycerin consumption in combination may mitigate hyperglycemia by modulating the gut microbiota. To determine the mechanisms underlying potential beneficial effects of lemon, glycerin and their combination, the gut microbiome was examined in mice after oral treatment in addition to assessing effects on hyperglycemia. Studies were first conducted in the male adult non-obese diabetic (NOD) mouse, an established T1D model. Further studies were conducted to determine whether lemon and glycerin in combination elicited different effects between sexes and ages in the NOD model. Parallel studies were then conducted in the C57BL/6 mice as a non-diabetic model.

## Materials and Methods

### Animal husbandry and housing

Mature adult NOD and C57/BL/6 mice (3-6 months), as well as middle-aged (10-14 months) and old (18-24 months) NOD mice (Taconic Biosciences) were used in this study. They were housed in individually ventilated cages at the Coverdell Rodent Vivarium of the University of Georgia (UGA). Room temperature was kept at 22-25°C with relative humidity of 50 ± 20%. A 12-h light/dark cycle was maintained, and red light was provided for illumination during the dark cycle. To prevent coprophagy between groups, only mice of same strain with the same treatment were housed together. Irradiated laboratory animal bedding and Bed-r'Nest (The Andersons Inc., Maumee, Ohio) were provided for enrichment. Water and food, e.g. the 5053 PicoLab diet (LabDiet, St. Louis, MO) (Table 1), were provided *ad libitum*. Animals were treated humanely with regard to alleviating suffering. All procedures were conducted under an approved Animal Use Protocol (A2021 01-013-Y3-A7) by the UGA Institutional Animal Care and Use Committee. The PicoLab® Rodent

Diet 20 is a complete life cycle diet formulated using managed formulation, delivering constant nutrition.

### Animal exposure

Initial body weight and blood glucose levels (BGLs) were analyzed using the analysis of variance (ANOVA) to ensure the lack of significant differences among groups before treatment. The dosing solutions of lemon, glycerin, and the lemon and glycerin in combination were obtained from HGG Research LLC (Athens, GA). The ingredients for the combined solution of lemon and glycerin were: lemon extract prepared by diluting lemon juice concentrate that was formulated at a controlled and consistent strength, vegetable glycerin (Kosher US Pharmaceutical Grade), and less than 1% lemon oil. The lemon or glycerin dosing solutions were equivalent to their levels in the combined dosing solution. Water was used as the vehicle control. The manufacturer recommends ~10-30 mL three times daily for an average 62 kg human (0.5-1.5 mL/kg); therefore, 0.05 mL was used to gavage an approximate 30 g mouse (~1.5 mL/kg) daily.

**TABLE 1**  
**Diet ingredients and nutrients (% and g/kg) for the 5053 - PicoLab® Rodent Diet 20.**

	Ingredients			Nutrients	
	% (w/w)	Concentration (g/kg)		% (w/w)	Concentration (g/kg)
Corn	30	300	Carbohydrate	50.5	505
Wheat	17	170	Protein	20	200
Soybean meal	15	150	Fat	4.5	45
Midds	6	60	Fiber	6	60
Fish	4	40	Ash	7	70
Oats	4	40	Moisture	12	120
Alfalfa	3	30			
Misc.	21	210			

Four studies were conducted using NOD and C57BL/6 mice (Table 2). Study 1 was a 6-month study in mature adult male NOD mice: Thirty animals were randomly divided into naïve (NAM), vehicle (VHM), lemon (LEM), glycerin (GYM), and lemon+glycerin (LGM) groups with 6 mice per group. Study 2 was a 2-month study in both mature adult male and female NOD mice: 12 male and female mice were randomly divided into VH and LG groups, respectively. Study 3 was a 6-month study in middle-aged/old male NOD mice (Average 14 months of age at the beginning of the study and 20 months of age at the end of the study): 23 mice were randomly divided into VH and LG groups. Study 4 was a 2-month study in mature adult C57BL/6 mice: 12 male and female mice were randomly divided into VH and LG groups,

respectively. Mice were dosed orally by gavage daily.

### Body weight, measurement of blood glucose levels and diabetic incidence

Body weight and non-fasting BGLs were measured weekly. The Accu-Check Diabetes monitoring kit was used to test BGLs in venous blood samples from a tail nick. Mice were considered diabetic when BGL measurements exceed 250 mg/dL [12]. If two consecutive BGL readings  $\geq 600$  mg/dL were detected, mice were considered severely diabetic and were humanely euthanized using CO<sub>2</sub> asphyxiation. At the end of treatment, organs (spleen, pancreas, GI tract, thymus, liver, lungs, heart and kidneys with adrenals) were collected and weighed during necropsy.

**TABLE 2**  
**Mouse strain, age, sex, and treatments and duration in each study**

Strain	Duration	Age; Sex	Treatment	Study #
NOD	6 months	Adult Male	NAM (6) <sup>a</sup>	1
			VHM (6)	
			LEM (6)	
			GYM (6)	
	2 months	Adult Male	LGM (6)	2
			VH (6)	
6 months	Adult Female	LG (6)	3	
		VH (11)		
C57BL/6	2 months	Old Male	LG (12)	4
			VH (6)	
		Adult Male	LG (6)	
			Adult Female	
			LG (6)	

The number in the parenthesis represents the number of animals in each group. Mice were treated by gavage daily. M: Male Mice; NA: Naive; VH: Vehicle; LE: Lemon; GY: Glycerin; LG: Lemon and Glycerin in Combination.

### Glucose and insulin tolerance tests

Baseline body weights and BGLs were determined before performing glucose and insulin tolerance tests. Glucose tolerance tests (GTTs) were conducted by fasting the mice for 15 h overnight. The mice were then injected (i.p.) with 2 g/kg of glucose (Sigma) and BGLs were measured at 15, 30, 60, and 120 mins post injection. Insulin tolerance tests (ITTs) were conducted after four hours of fasting [13]. BGLs were measured at 15, 30, 60, and 120 mins post injection (i.p.) with 1.5 IU/kg of insulin (Sigma).

### Microbiome bioinformatics analysis

Fecal samples of individual mice from studies 1 and 2 were collected at the 6-month and one-month time points following initial dosing, respectively, immediately after defecation and kept at -20°C. DNA was extracted using QIAmp DNA stool mini kits (Qiagen, Valencia, CA), and normalized for library preparation. The library was sequenced at the Georgia Genomics and Bioinformatics Core at UGA using Illumina Miseq.

The sequence data was de-multiplexed and filtered. Double end sequences were trimmed using BBDuk and merged using Geneious Prime (<https://www.geneious.com>). Quantitative Insights into Microbial Ecology (QIIME) version 1.9.1 [14] was used to pick Operational Taxonomic Unit and conduct alpha and beta diversity analyses. PICRUST (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) was used to predict the potential differentially expressed functional contents [15]. Linear Discriminant Analysis (LDA) Effect Size (LEfSe) was used to identify significantly altered taxonomy and functional contents [16].

### Fecal metabolomics analysis

To prepare samples for nuclear magnetic

resonance (NMR), fecal samples were extracted using a biphasic extraction method [17]. Briefly, fecal matter (80 mg) were added to Eppendorf tubes with a 3.2 mm Bearing, ice-cold methanol (320 L) and ultrapure water (68 L). Samples were homogenized using a tissuelyser (Qiagen, Valencia, CA). Then, ice-cold chloroform (160 L) was added and followed by homogenization. Next, another 160 L of ice-cold chloroform and ultrapure water (160 L) were added and followed by homogenization. Finally, samples were centrifuged, and the upper phase (polar extract) was added to a 96-well plate and dried under vacuum at room temperature. A volume of 240 µL NMR phosphate buffer (0.2 mM sodium 4,4-dimethyl-4-silapentane-1-sulfonic acid-d6 (DSS) and 100 mM phosphate buffer in 99.96% D2O) was added to reconstitute the wells and transferred to 3 mm NMR tubes (SP Wildmad-Labglass, Vineland, NJ). DSS was used as an internal standard. <sup>1</sup>H 1D NOESY spectra for all samples were acquired on a 600 MHz <sup>1</sup>H NMR spectrometer with a cryogenic probe and an autosampler (Agilent, Santa Clara, CA) with 128 scans at 20°C [18].

The metabolites were annotated using Chemomx Profiler (Alberta, Canada). <sup>1</sup>H NMR data analysis was conducted using ACD software (ACD Labs, Ontario, Canada). Variation of chemical shifts across samples was reduced by aligning spectra using SpecAlign (Oxford, UK). The average spectrum of the control class (vehicle) was subtracted from the average spectrum of individual exposure class to produce the difference spectrum. Next, each bin was analyzed for significant differences between the average for the exposed class and the control using the Student's t-test (p-value<0.05). This resulted in a t-test filtered difference spectrum for each exposed class with the y-axis showing a constant scale (relative intensity). Where no significance between

classes was found, differences were set to zero. Thus, positive peaks correspond to significantly increased metabolites upon treatment, whereas negative peaks are from significantly decreased metabolites [17].

### Statistical analyses

Body weight, organ weight, and blood glucose level data were analyzed using JMP Pro 15 (SAS Institute Inc., Cary, NC, 1989–2021). The values were presented as mean  $\pm$  SEM. ANOVA was first performed, followed by unequal variance tests. If the Bartlett test was significant in the unequal variance test, the Wilcoxon test was performed. Otherwise, the Dunnett's test was used for comparison with vehicle as the control. Correlation analyses were carried out with XLSTAT (Addinsoft, New York).

## Results

### Administration of lemon, glycerin, and their combination in mature adult male NOD mice for six months

A 6-month study was conducted to determine the effects of lemon, glycerin, and their combination in mature adult male NOD mice that are predisposed to the development of T1D. All groups treated via gavage had a decrease in body weight compared to the naive (no treatment received) group (Figure 1A). The lemon- or glycerin-treated mice had a lower, but not statistically significant, body weight than those of vehicle-treated mice (Figure 1A). The LG-treated group had the largest decrease in body weight with significant changes observed at two time points (week 1 and 11) when compared to the vehicle group (Figure 1A). There was a significant decrease in the absolute weights of the heart (174 vs. 208 mg) and spleen (74 vs. 82 mg) in LG and glycerin-treated mice, respectively, when compared to those from the VH mice; however, these changes were

no longer significant when the organ weights were normalized to their body weight (data not shown). In contrast, mice dosed with glycerin had a significant increase in the relative heart weight (0.71% vs. 0.62%).

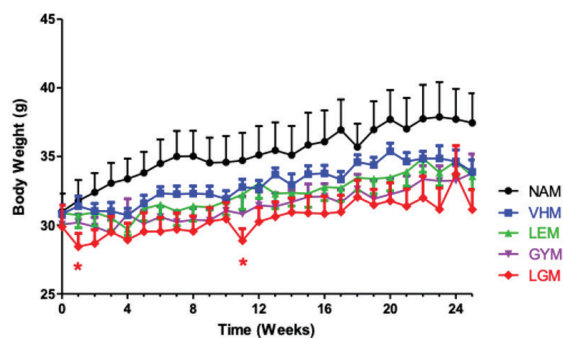
In terms of T1D incidence, there were no statistically significant differences in either treatment groups when compared to vehicle control. At the time of euthanasia, one mouse from each of the VHM, GYM, and LGM group became diabetic (BGL  $\geq$  250 mg/dL). The LEM group, however, remained free of diabetic mice throughout the entire duration of the study (data not shown). When the weekly non-fasting BGLs were compared in non-diabetic mice, the average BGLs in the lemon-treated mice were lower than that of the vehicle group for most time points, with significant differences observed in the 11<sup>th</sup> week. Significant decreases were also found in the glycerin-treated mice when compared to the vehicle mice in the 11<sup>th</sup> week. Mice treated with the combination of lemon and glycerin had significantly lower glucose levels than the mice in the vehicle group at the 11<sup>th</sup> week time point (Figure 1B).

GTTs were conducted to examine how well a standardized amount of glucose was metabolized. The first GTT was performed at the end of the third month of the treatment. The blood glucose levels of all mice peaked at 15 min after glucose was given, with the lemon and the glycerin-treated mice having significantly higher blood glucose levels than the control at 15 mins, and BGLs in glycerin-treated mice remained significantly higher at 30 mins after glucose injection (Figure 1C). Notably, mice treated with the combination of lemon and glycerin exhibited similar responses to glucose challenge as those in the vehicle and naive groups. The second GTT was performed at the end of the sixth month of treatment (Figure 1D). The glycerin-treated mice had

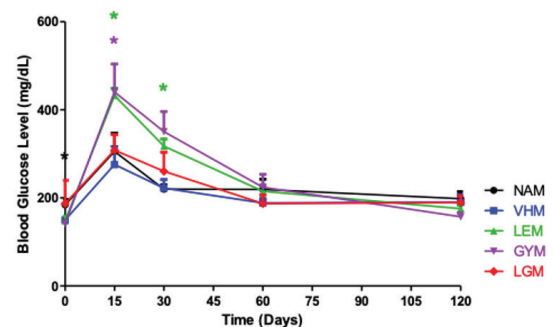
a delayed glucose clearance, and they had significantly higher BGLs at 30 and 60 mins than the lemon-treated mice and at 60 to 120 mins than the lemon+glycerin treated mice. Mice treated with the combination of lemon and glycerin exhibited similar responses as those in the lemon treatment group, which had overall lower BGLs than vehicle and naive mice. These

observations indicated that combining glycerin with lemon mitigated the effects of glycerin on glucose tolerance. An ITT was conducted to assess the effects of lemon, glycerin, and their combination on insulin resistance following treatment for 5 months, and there were no statistical significances being present (data not shown).

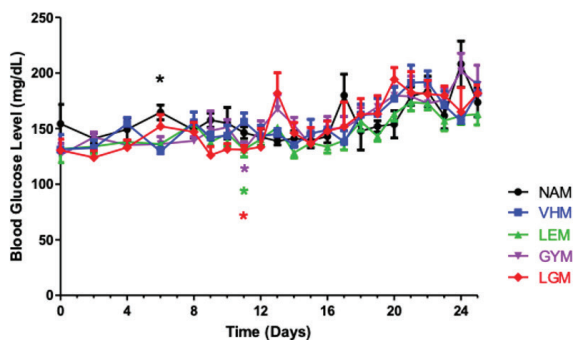
#### A: Body Weight



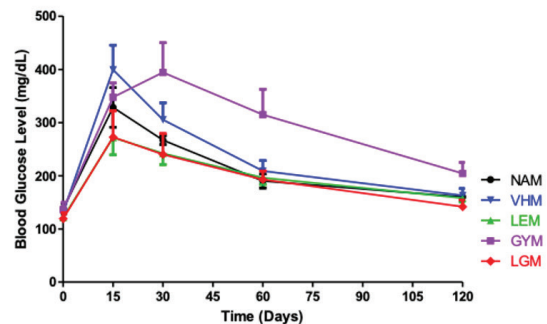
#### C: 3<sup>rd</sup> Month GTT



#### B: Blood Glucose Levels



#### D: 6<sup>th</sup> Month GTT



**Figure 1)** Weekly body weights and blood glucose levels, and the 3<sup>rd</sup> and 6<sup>th</sup> month glucose tolerance tests (GTTs) in non-diabetic adult NOD male mice following daily administration of lemon, glycerin, and their combination for six months. (A) The LGM group had the largest decrease in body weight when compared to the VHM group. (B) Sporadic changes in weekly blood glucose levels were observed in all treatment groups. (C) The LGM group exhibited similar responses as those of the VHM and NAM groups during the 3<sup>rd</sup> month GTT in contrast to the LEM and GYM groups, which had significantly higher blood glucose levels than VHM at 15 min following glucose challenge. (D) During the 6<sup>th</sup> month GTT, the LGM group exhibited similar responses as the LEM group, which had overall lower blood glucose levels than the VHM and NAM groups. \*,  $p < 0.05$  as compared to the VHM group. NAM=Males without any treatments (naïve control), VHM=Males dosed with water; LEM=Males dosed with lemon extract, GYM=Males dosed with glycerin, and LGM=Males dosed with the combination.  $N=5-6$ .

To determine the mechanisms underlying the apparent beneficial effect of the combined treatment of lemon and glycerin, the gut microbiome was evaluated in these mice. The  $\alpha$ -diversity was significantly changed in fecal samples collected from these mice following six-months of daily administration of lemon, glycerin, and their combination ( $p=0.023$ ). Further analysis indicated that the overall significant difference was caused by the treatment of lemon when compared to the combined treatment group, with mice in the lemon group having significantly higher  $\alpha$ -diversity value ( $p=0.018$ ) (Figure 2A). Compared with the vehicle group, mice treated with lemon, glycerin and their combination did not exhibit changes in their  $\alpha$ -diversity. No significance was found in the beta diversity between any of the treatments or control (data not shown). The top two phyla in vehicle mice were the Bacteroidetes (52.8%) and Firmicutes (39.1%). Proteobacteria (2.2%) was significantly decreased in mice treated with the combination of lemon and glycerin when compared to the vehicle control mice and mice treated with only glycerin (Figures 2B and 2D). Both Verrucomicrobia (0.9%) and Cyanobacteria (2.4%) were significantly decreased in both the lemon and combinational treatment groups when compared to the vehicle control (Figures 2B and 2C). Additionally, TM7 (0.4%) was significantly reduced in mice treated with lemon when compared to vehicle control (Figure 2C).

For other taxonomic levels, there was an increase in the *Coriobacteriaceae* family, and decreases in the RF32 and YS2 orders, *Clostridiaceae*, *Peptostreptococcaceae* and *Comamonadaceae* families, *Cloacibacterium* and *Ruminococcus* genera, and *muciniphilia* and *prausnitzii* species in mice treated with the combination of lemon+glycerin when compared to vehicle control (Figure 2E). When mice treated with

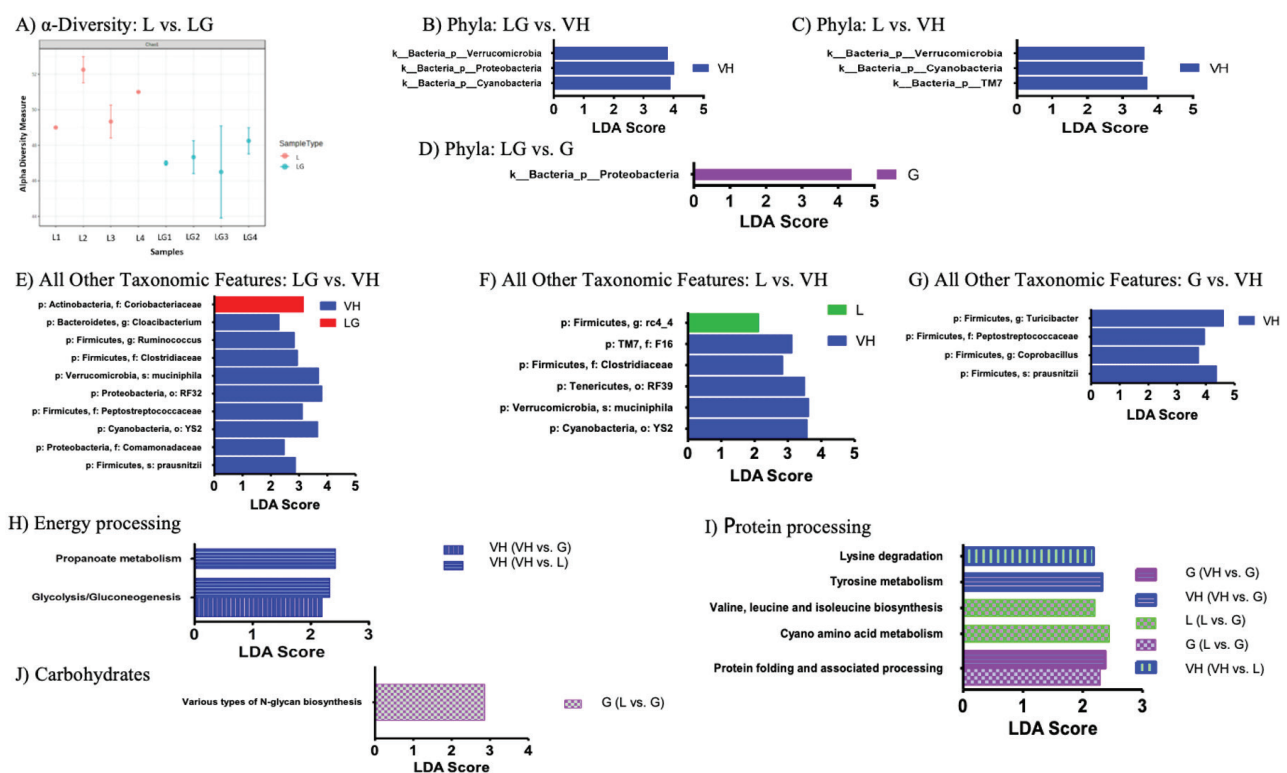
lemon were compared to the vehicle, there was a significant increase in the *rc4-4* genus, along with significant decreases in the RF39 and YS2 orders, *F16* and *Clostridiaceae* families, and the *muciniphila* species (Figure 2F). Mice treated with glycerin were found to have significantly decreased *Peptostreptococcaceae*, *Turicibacter* and *Coprobacillus* genera, and the *prausnitzii* species when compared to the vehicle (Figure 2G). However, glycerin treatment did not induce significant increases in any taxa when compared to the vehicle. Glycerin administration was predicted to decrease glycolysis/gluconeogenesis when compared to the vehicle control group (Figure 2H), but an increase in protein folding and associated processing was predicted when compared to the group that administered only lemon (Figure 2I). Treatment with lemon was predicted to decrease both propanoate metabolism and glycolysis/gluconeogenesis when compared to the vehicle control (Figure 2H). Treatment with glycerin was also predicted to increase various types of N-glycan biosynthesis when compared to the lemon treatment group (Figure 2J).

Fecal metabolites were further evaluated to understand the underlying mechanisms of the changes in the gut microbiome of those mice. Using NMR, many amino acids (alanine, arginine, glutamate, glutamine, glycine, isoleucine, leucine, lysine, methionine, serine, taurine, threonine, tyrosine and valine) were examined. In addition, the following metabolites were also assessed: short chain fatty acids (SCFA: propionate, malonate, butyrate and acetate), sugars (fructose and glucose), intermediate metabolites (succinate and lactate), and many other metabolites (3-hydroxyphenylacetate, 4-hydroxyphenylacetate, acetoin, bataine, choline, creatine, creatine phosphate, dimethylamine, glycerin, homocysteine, methylamine, myo-inositol, o-phosphocholine

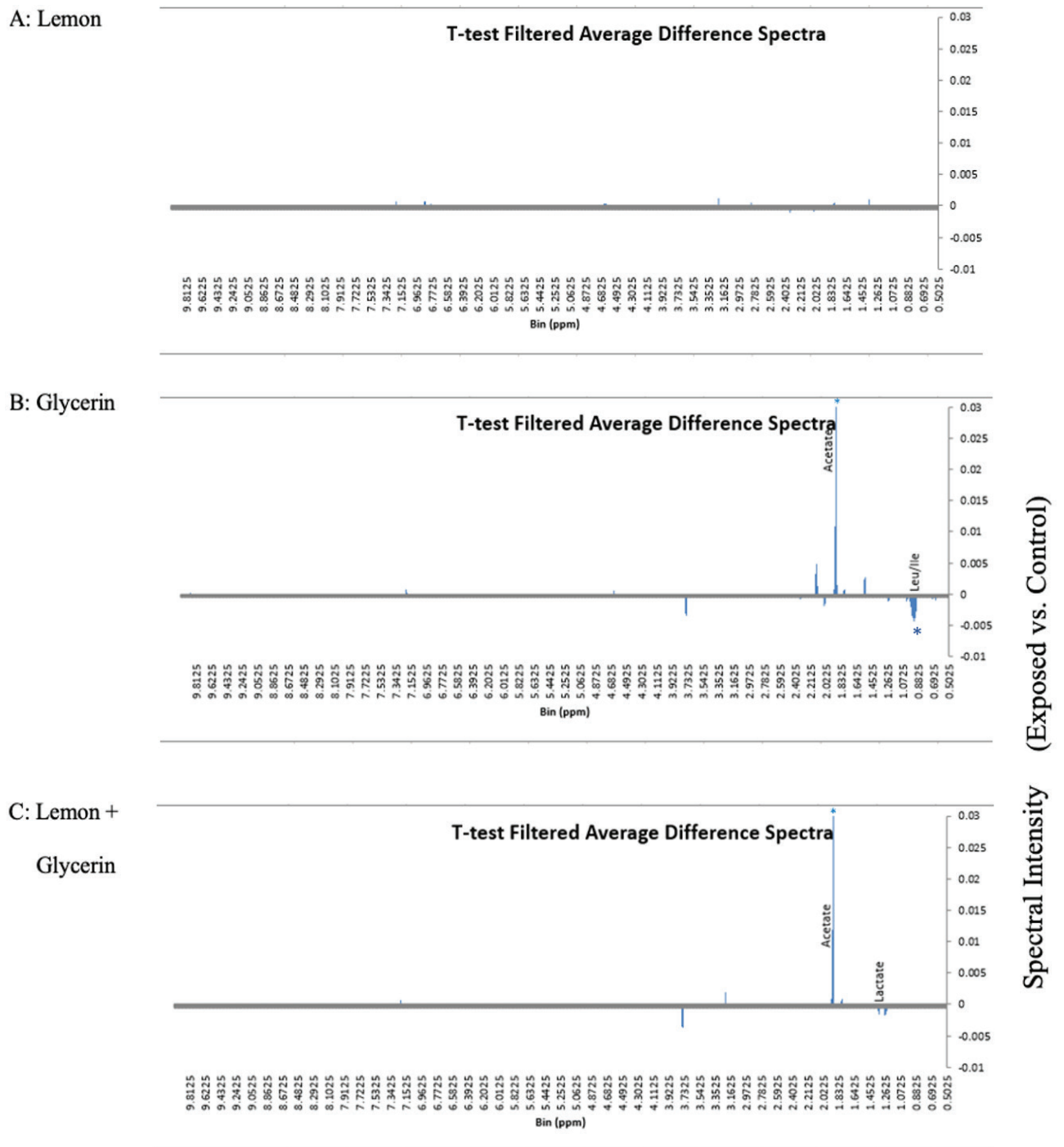


and trimethylamine N-oxide). No significant changes were observed for lemon-treated mice when compared to the vehicle control (Figure 3A). However, significantly higher positive peaks were detected at 1.91 ppm in both the glycerin (Figure 3B) and the LG treatment (Figure 3C) groups when compared to the vehicle control, indicating an increase in acetate, one of the SCFAs. Significantly lower negative peaks occurring in the glycerin treatment group when compared to the control included 0.99 ppm (one

of the peaks for isoleucine: 0.93 ppm, 0.99 ppm, 1.46 ppm, 3.66 ppm) and 0.95 ppm (one of the peaks for leucine: 0.95 ppm, 1.7 ppm, 3.73 ppm), indicating decreased concentrations of these metabolites compared to control samples. Negative peaks for lactate (1.32 ppm, 4.10 ppm) were identified in the group receiving the combination of lemon and glycerin treatment when compared to the control; however, the changes in lactate level were not statistically significant.



**Figure 2)** Gut microbiome in adult NOD male mice following six-months of daily dosing with lemon (L), glycerin (G), and their combination (LG). (A) A significant difference was observed in  $\alpha$ -diversity between the L and LG groups. (B) Phyla including Verrucomicrobia, Proteobacteria and Cyanobacteria were significantly decreased in mice treated with LG when compared to the VH group. (C) Phyla including Verrucomicrobia, Cyanobacteria and TM7 were significantly decreased in mice treated with lemon when compared to the VH group. (D) Phylum Proteobacteria was significantly decreased in mice treated with LG when compared to mice treated with only glycerin. (E) Changes of other taxonomic features in mice treated with LG when compared to the VH group. (F) Changes of other taxonomic features in mice treated with lemon when compared to the VH group. (G) Decreases of other taxonomic features in mice treated with glycerin when compared to the VH group. (H) Decreases of the energy processing-related functional contents in mice treated with only glycerin or lemon when compared to the VH group. (I) Changes of the protein processing-related functional contents when the treatment groups were compared to each other. (J) Increases of the carbohydrate-related functional contents in mice treated with only glycerin when compared to the lemon group. VH=Mice dosed with water. N=4.



**Figure 3)** Changes in fecal metabolites (Spectral Intensity: Exposed vs. Control) in adult NOD male mice following daily dosing with (A) lemon, (B) glycerin, and (C) their combination (LG) for six months by comparing to the vehicle control (water). Significantly higher positive peaks were detected at 1.91 ppm in both the glycerin (B) and the LG treatment (C) groups when compared to the vehicle control, indicating an increase in acetate. Significantly lower negative peaks occurring in the glycerin treatment group when compared to the control, indicating decreased concentrations of isoleucine and leucine.  $N=6$ . \* ,  $p<0.05$  as compared to the vehicle control group.

### Administration of the combination of lemon and glycerin in mature adult male and female NOD mice for two months

Studies using mature adult NOD male and female mice were conducted to evaluate the potential sex-dependent effects of administration of the combination of lemon and glycerin for two months. Male mice weighed significantly less after administration of the combination of lemon and glycerin for six weeks and maintained the significant differences for the remaining time of the study (Figure 4A). However, such reduction in body weight was not observed in the female mice (Figure 4B). The weekly non-fasting BGLs of the male mice treated with the combination of lemon and glycerin had one significant increase at the 4-week time point (Figure 4C). The weekly non-fasting BGLs of the female mice treated with the combination of lemon and glycerin were not significantly different from the control at any time points during the two-month study (Figure 4D). There was one diabetic incidence at the 8-week time point in the control group of the female study (data not shown). In the ITT studies, male mice treated with the combination of lemon and glycerin had a significantly lower baseline fasting blood glucose than the vehicle control (89.8 vs. 114.3 mg/dL). However, there were no other significant differences in either males or females at any of the time points during the two-hour period following the injections of glucose or insulin during the GTT or ITT studies (data not shown).

The gut microbiota was also evaluated in both male and female mature adult NOD mice following administration of the combination of lemon and glycerin for one month as a potential mechanism. The alpha diversity analysis did not reveal statistically significant differences among different groups in either males or females (data not shown). Principal Coordinate Analysis (PCoA) of beta diversity indicated that the first two vectors accounted for most of total

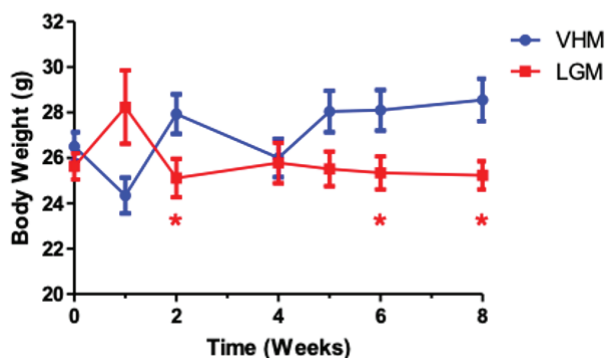
variance, which revealed significant differences in both LG-treated males (Figure 5A) and females (Figure 5B) when compared to the corresponding control group. The top two phyla for the female NOD mice are the Bacteroidetes (76.97%), and the Firmicutes (20.90%). For the male NOD mice, the top three phyla are the Firmicutes (38.07%), the Proteobacteria (15.27%), and the Bacteroidetes (13.09%). Six and three phyla were significantly increased in male and female mice, respectively (Figure 5C, 5D). *Deferribacteres* was increased in both male and female mice following LG treatment, while Proteobacteria and Tenericutes were increased only in females (Figure 5C, 5D). Another five phyla, including Cyanobacteria, TM7, Nitrospirae, Chloroflexi and Gemmatimonadetes, were increased in male mice (Figure 5C). For other taxonomic levels (Figure 5E, 5F), there were significant decreases in the orders *Sediment* and *Streptophyta*, the family *Polyangiaceae*, and the genera *SMB53* and *Lactobacillus* in the male mice. There were also increases in the class *Ellin6529*, the orders *YS2*, *Ellin6067*, *Sphingobacteriales*, *S\_BQ2\_57* and *Pedosphaerales*, the families *Haliangiaceae*, *0319\_6A21*, *Rikenellaceae*, *Bacillaceae*, *Sphingobacteriaceae* and *Xanthomonadaceae*, the genera *Aerococcaceae*, *Prevotella*, *DA101*, *Ruminococcus*, *Sporosarcina*, *Bacteroides*, *Bilophila*, *Hylemonella*, *Jeotalicoccus* and *4\_29*, and the species *schaedleri*, *genosp*, *rhizosphaerae* and *sciuri*. In the female mice, there were decreases in the family *Erysipelotrichaceae*, the genera *Bacteroides* and *Parabacteroides*, and the species *acidifaciens* and *uniformis*. This was accompanied by increases in the orders *Clostridiales*, *RF32* and *RF39*, the family *Ruminococcaceae*, the genera *Allobaculum*, *Biophilina*, *Oscillospira*, *Desulfovibrio*, *Coproccoccus*, *Bacteroides* and *Pseudomonas*, and the species *schaedleri*.

PICRUSt analysis was conducted to predict potential pathways altered due to the microbiome changes. These predicted metagenomic functions

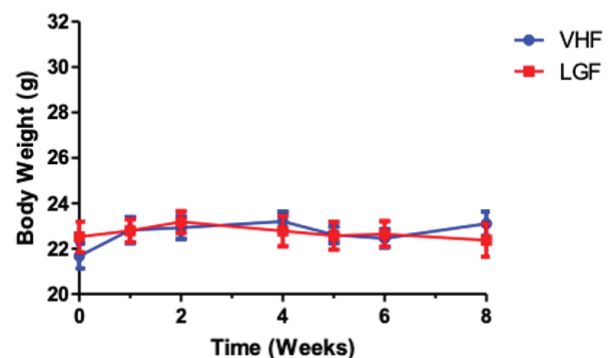
were organized into ten different categories. Of these, three most relevant categories include processes related to carbohydrates (Figure 6A), lipids (Figure 6B) and energy processing (Figure 6C). For carbohydrates, the pentosephosphate pathway was predicted to decrease in males dosed with LG, while the aminosugar and nucleotide sugar metabolisms were predicted to decrease in both LG-treated male and female mice (Figure 6A). In addition, decreases in peptidoglycan biosynthesis, carbon fixation pathways in prokaryotes, other glycan degradation, galactose metabolism and glycosyl transferases were predicted in LG-treated female mice (Figure 6A). For lipids, increases in both fatty acid metabolism and biosynthesis, and a decrease in glycerolipid metabolism were predicted in LG-treated male mice (Figure 6B). In LG-treated females, an increase was predicted in glycerolipid metabolism, while

decreases of fatty acid biosynthesis, lipid biosynthesis proteins, sphingolipid metabolism, glycosphingolipid biosynthesis in both the globoseries and ganglioseries, and adipose signaling pathways were predicted (Figure 6B). For energy processing, glycolysis/gluconeogenesis were predicted to decrease in LG-treated males, which was consistent with those dosed with lemon, as well as those dosed with glycerin for six months (Figure 2H). Pantothenate and CoA biosynthesis, C5-branched dibasic acid metabolism, and energy metabolism on the other hand, were predicted to increase in LG-treated males (Figure 6C). Propanoate and pyruvate metabolism, carbon fixation pathways in prokaryotes, and the citrate/TCA cycle were predicted to decrease, while butanoate metabolism was predicted to increase in LG-treated female mice (Figure 6C).

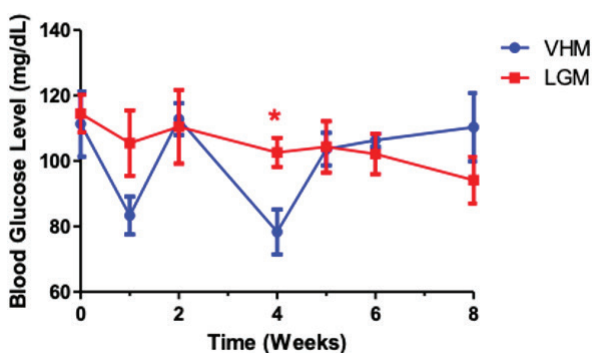
#### A) Body Weight: Males



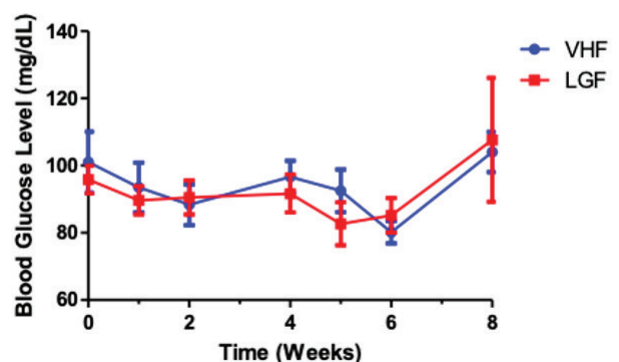
#### B) Body Weight: Females



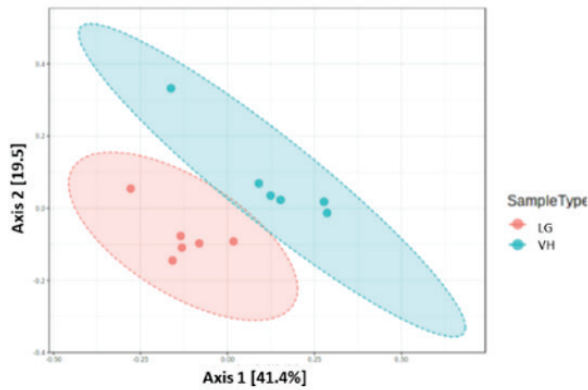
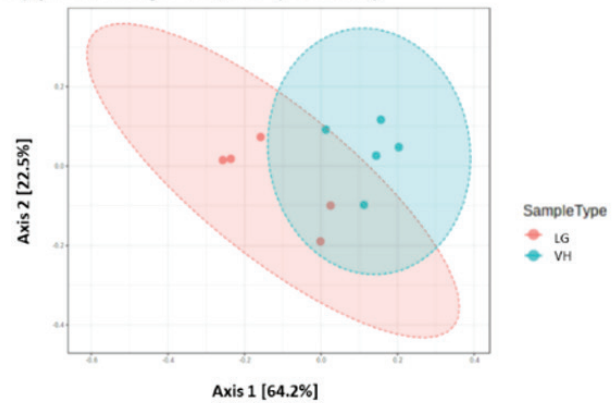
#### C) Blood Glucose: Males



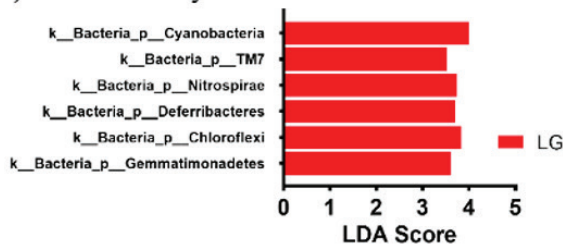
#### D) Blood Glucose: Females



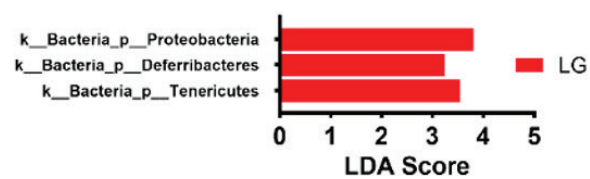
**Figure 4)** Weekly body weights and blood glucose levels in adult male and female NOD mice following daily administration of the combination of lemon and glycerin for two months. (A) The LGM group weighed significantly less after administration of the combination of lemon and glycerin. (B) The LGF group had no changes in their weekly body weights following daily administration of the combination of lemon and glycerin. (C) The LGM group had a significant increase in their weekly non-fasting blood glucose levels only at the 4-week time point. (D) The LGF group had no changes in their weekly non-fasting blood glucose levels. VHM=Males dosed with water, LGM=Males dosed with lemon and glycerin, VHF=Females dosed with water, and LGF=Females dosed with lemon and glycerin. N=6.

A)  $\beta$ -Diversity: VH, LG (Males)B)  $\beta$ -Diversity: VH, LG (Females)

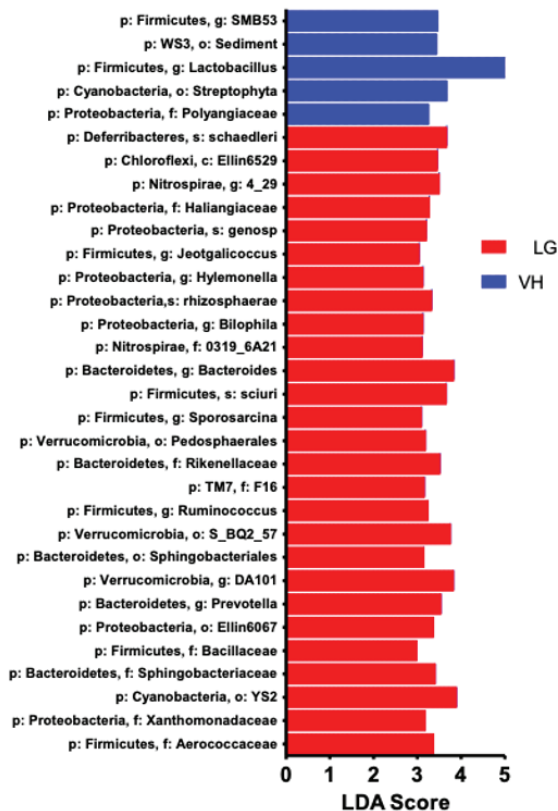
C) Differential Phyla: Males



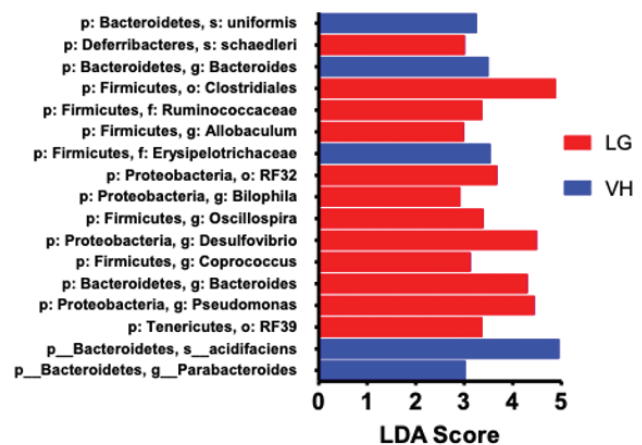
D) Differential Phyla: Females



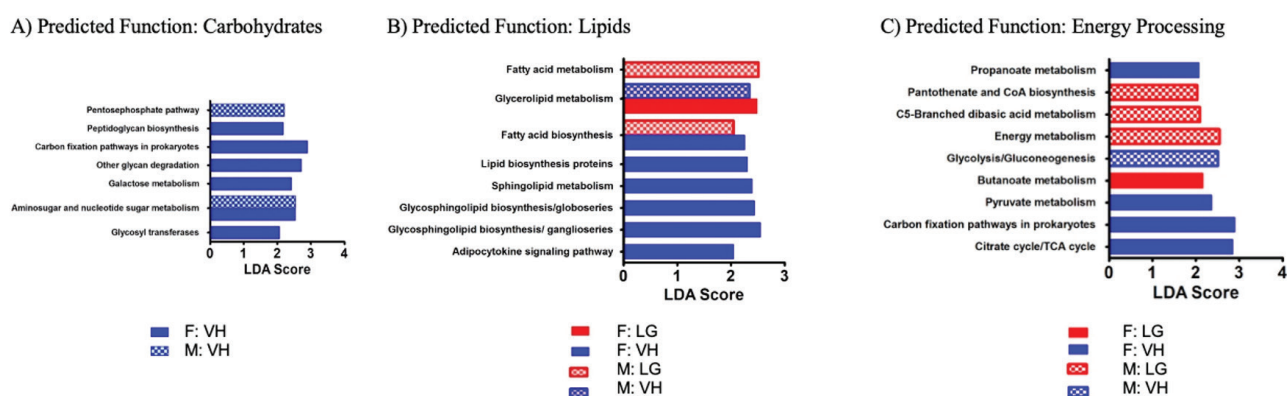
E) All Other Taxonomic Features: Males



F) All Other Taxonomic Features: Females



**Figure 5)** Gut microbiome in adult male and female NOD mice following one-month of daily dosing with the combination of lemon and glycerin (LG). (A) Significant differences in  $\beta$ -diversity in LG-treated males when compared to the control. (B) Significant differences in  $\beta$ -diversity in LG-treated females when compared to the control. (C) Six phyla were significantly increased in male mice treated with LG when compared to the VH group. (D) Three phyla were significantly increased in female mice treated with LG when compared to the VH group. (E) Changes of other taxonomic features in male mice treated with LG when compared to the VH group. (F) Changes of other taxonomic features in female mice treated with LG when compared to the VH group.  $N=6$ .



**Figure 6)** Predicted functional contents in both male and female adult NOD mice following one-month of daily dosing of the combination of lemon and glycerin (LG). (A) Decreases in carbohydrate-related functions in either male or female mice when compared to the control. (B) Changes in lipid-related functions in either male or female mice when compared to the control. (C) Changes in energy processing-related functions in either male or female mice when compared to the control. VH=Mice dosed with water. M=male. F=Female. N=6.

### Administration of the combination of lemon and glycerin in old male NOD mice for 6 months

A sixth month study was conducted in middle-aged/old male NOD mice to determine if age differences had any influences on the effects of the combined treatment of lemon and glycerin. Initially, there were 11 and 12 mice in the vehicle and treatment groups respectively, with three diabetic mice (BGL: 339-357 mg/dL) in the vehicle group and four diabetic mice (BGL: 284-600 mg/dL) in the treatment group. Significant decreases of the weekly body weight in the LG-treated group were observed at all but one time point, e.g., after the first week of dosing (Figure 7A), suggesting that the combined treatment could significantly reduce the body weight of old male NOD mice. While there were significant decreases in the absolute weights of pancreas (278 vs. 327 mg), liver (1997 vs. 2183 mg) and kidneys (536 vs. 639 mg) in these mice, the 6-month of dosing with LG did not significantly alter the weights of any organs after normalization to the body weight (data not shown). Although the average glucose levels of LG-treated mice were lower than those of the vehicle mice, there were only three time points with significant changes, and

the difference became smaller as the mice grew older (Figure 7B).

The GTT after six months of treatment revealed that LG-treated male mice had higher glucose levels than the vehicle mice and required longer times for the BGLs to return from the peak to baseline levels (Figure 7C). In contrast, mice dosed with the combination of lemon and glycerin had an accelerated decrease in glucose during the ITT after six months of treatment (Figure 7D).

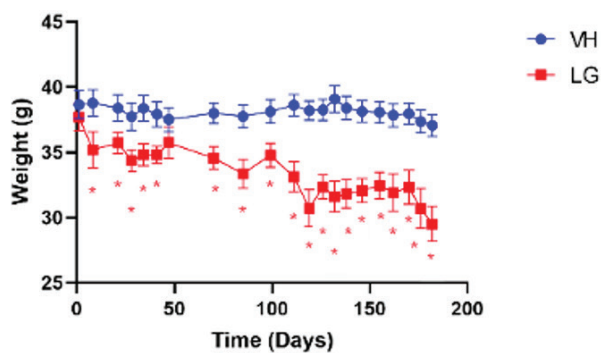
### Administration of the combination of lemon and glycerin in mature adult C57BL/6 mice for 2 months

Studies were conducted in mature adult C57BL/6 male and female mice to examine strain and sex differences in responding to the treatment of lemon and glycerin in combination. While an overall decrease in body weight was observed in both male (Figure 8A) and female (Figure 8B) mice following administration for 2 months, a significant reduction was only observed at one time point in male mice. The absolute weight of livers was significantly decreased in male C57BL/6 mice dosed with LG; however, no significant changes in relative organ weights were observed in either the male

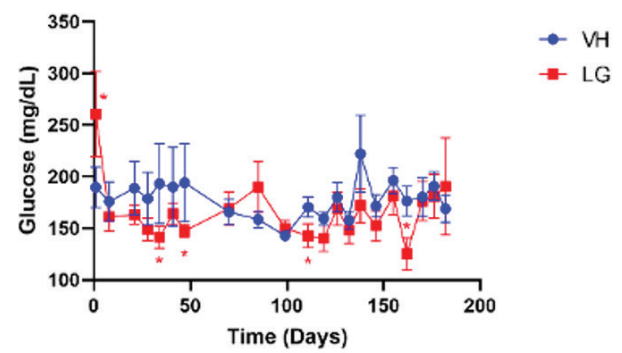
or female C57BL/6 mice (data not shown). The potential impact of combined administration of lemon and glycerin on blood glucose levels was also examined. Both LG-treated male (Figure 8C) and female (Figure 8D) mice had lower blood glucose levels than the vehicle mice, with more significant differences observed in the second month. Both GTT and ITT were further conducted to understand the modulatory effects of the combined treatment on glucose tolerance and insulin resistance, respectively. In the GTT

for both the male (Figure 8E) and female (Figure 8F) mice, the LG treatment elicited lower blood glucose levels with significant changes observed at 15 mins in males and at both 30 and 60 mins in females post glucose injection. For ITT, the LG treatment elicited a lower trend in the blood glucose levels than the control in males (Figure 8G); In contrast, the blood glucose level at the 120 min time point in the LG-treated female mice was significantly lower than the vehicle control (Figure 8H).

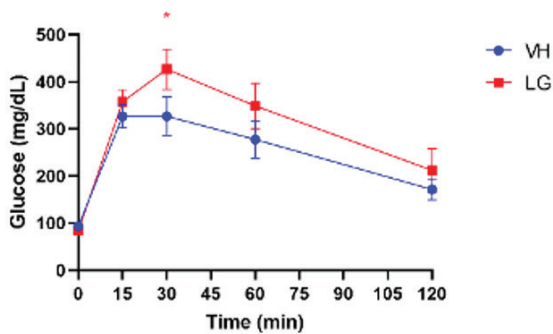
### A) Body Weight



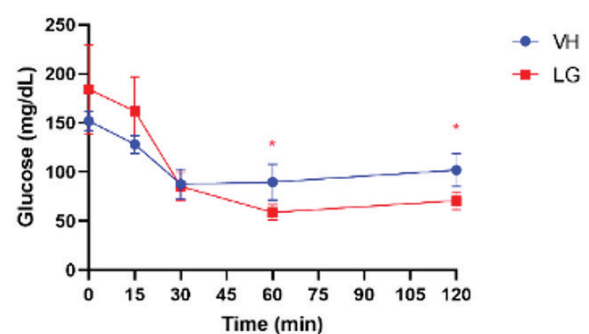
### B) Blood Glucose



### C) The 6<sup>th</sup> Month GTT

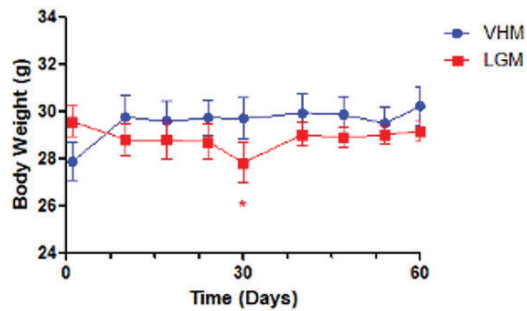


### D) The 6<sup>th</sup> Month ITT

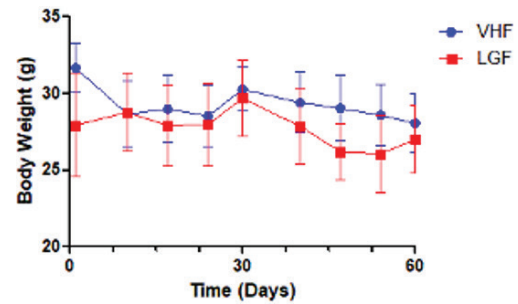


**Figure 7)** Weekly body weights and non-fasting blood glucose levels in old male NOD mice following daily dosing with the combination of lemon and glycerin (LG) for six months. (A) Significant decreases of the weekly body weights in the LG-treated group were observed at all but one time point, e.g., after the first week of dosing. (B) The difference in the weekly blood glucose levels became smaller as the mice grew older. (C) The LG-treated male mice had higher glucose levels than the vehicle mice during the glucose tolerance test. (D) The LG-treated male mice had an accelerated decrease in glucose during the insulin tolerance test. VH=Mice dosed with water. N=11-12.

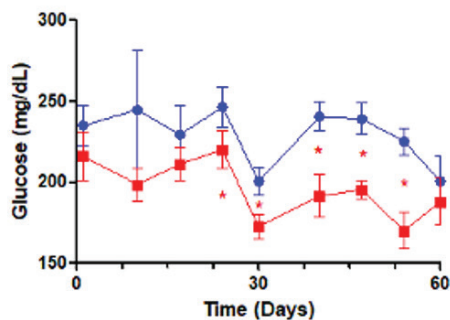
## A) Body Weight: Male C57BL/6



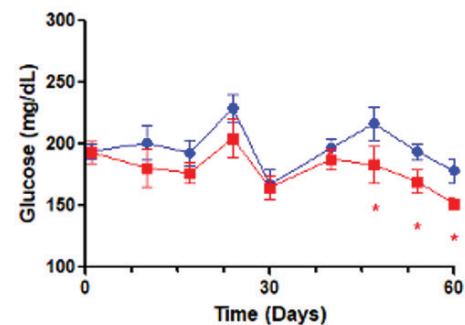
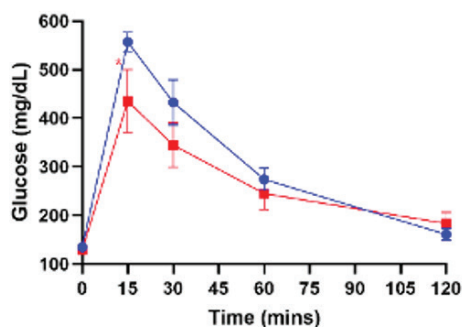
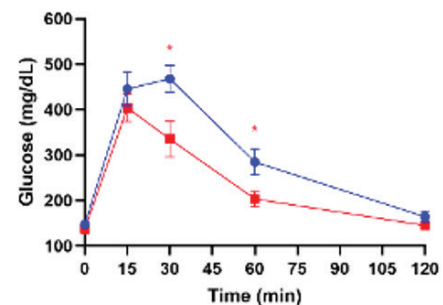
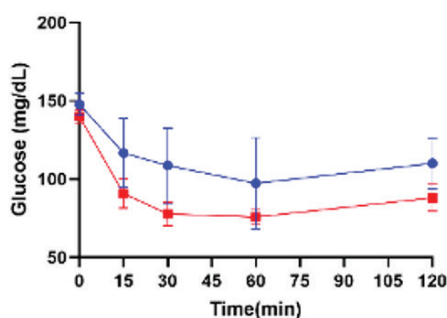
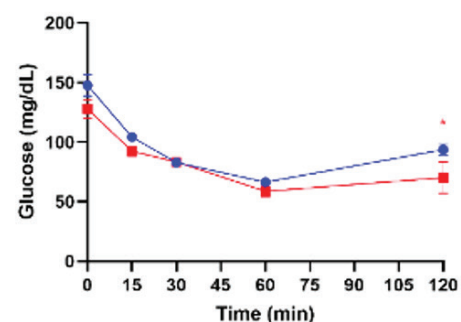
## B) Body Weight: Female C57BL/6



## C) Blood Glucose: Male C57BL/6



## D) Blood Glucose: Female C57BL/6

E) Male C57BL/6: 2<sup>nd</sup> Month GTTF) Female C57BL/6: 2<sup>nd</sup> Month GTTG) Male C57BL/6: 2<sup>nd</sup> Month ITTH) Female C57BL/6: 2<sup>nd</sup> Month ITT

**Figure 8)** Weekly body weights, weekly non-fasting blood glucose levels, the glucose tolerance tests (GTTs) and the insulin tolerance test (ITT) in adult male and female C57BL/6 mice following daily dosing with the combination of lemon and glycerin (LG) for two months. (A) A significant reduction in weekly body weights was only observed at one time point in male mice. (B) No significant changes in weekly body weights were observed in female mice. (C) The LGM group had lower weekly non-fasting blood glucose levels than the VHM group. (D) The LGF group had lower weekly non-fasting blood glucose levels than the VHF group. (E) The LGM group had lower blood glucose levels at 15 min following glucose challenge in the GTT. (F) The LGF group had lower blood glucose levels at both the 30 and 60 min timepoints following glucose challenge in the GTT. (G) The LGM group had a lower trend in the ITT than the VHM group. (H) The LGF group had lower blood glucose levels than the VHF group at 120 min following insulin challenge in the ITT. VHM=Male mice dosed with water; LGM=Males dosed with LG; VHF=Female mice dosed with water; LGF=Females dosed with LG. N=5-6.



## Discussion

To support our hypothesis, we have found apparent health advantages in using lemon and glycerin in combination. In this study, several beneficial effects have been observed following consumption of lemon and glycerin in combination. A consistent finding among the studies was an increased glucose tolerance and/or a decreased insulin resistance following the combined treatment. The 6-month adult male NOD study showed that either lemon or glycerin decreased glucose tolerance after three months of daily treatment; however, such adverse effects were mitigated when taken together (Figure 1C). Both increased glucose tolerance and decreased insulin resistance in male and female adult C57BL/6 mice were observed following the LG treatment for two months (Figure 8E-H). This was further supported by the weekly blood glucose test (Figure 8C, 8D). A decreased insulin resistance also occurred in old male NOD mice after six months of LG treatment (Figure 7D). Glycerin treatment reduced the level of leucine, and such effect was not observed following LG treatment (Figure 3). The biosynthesis of valine, leucine and isoleucine was also predicted to decrease when glycerin was compared to lemon treatment. This is important as leucine supplementation can attenuate macrophage foam-cell formation [19]. Additionally, when leucine is ingested with glucose, it stimulates insulin secretion and lowers blood glucose levels [20]. Glycolysis is the pathway by which glucose degrades into lactate [21]. This pathway was predicted to decrease following treatment with either lemon or glycerin in the 6-month adult male NOD study, and these effects on energy metabolism were obliterated following LG treatment. This observation was consistent with a decrease, albeit not statistically significant, in the fecal lactate level in the LG treatment group.

Diet plays a major role in shaping the gut

microbiota, and a diet yielding high amounts of SCFA (e.g. acetate and butyrate) protected mice against T1D [22]. In this study, we showed that mature adult NOD male mice treated with lemon and glycerin in combination exhibited a distinctive gut microbiota, which increased the levels of fecal acetate. Among the many fecal metabolites analyzed in this study, acetate was the only one that was significantly increased in both the glycerin and LG treatment groups when compared against the control. Acetate could be produced by most of the enteric bacteria such as *Bacteroides spp.*, *Prevotella spp.*, and *Ruminococcus spp.* [23], which were indeed increased following LG treatment for one month in male NOD mice (Figure 5E). It has been reported that acetate feeding led to a marked suppression of the allergic airway disease in mice, a model for human asthma [24]. Acetate is the main SCFA distributed throughout the body with a blood concentration between 50-100 M, while blood butyrate is usually <5 M. Acetate is produced in our body when blood glucose levels are elevated, and it improves  $\beta$ -cell metabolism [25]. Acetate also renders the gut environment unfavorable to pathogenic and detrimental bacteria [26]. Therefore, it is possible that increased acetate level will be one of the underlying mechanisms for the beneficial effects of the combined treatment of lemon and glycerin.

Lemon and glycerin seemed to have a synergistic effect on weight loss. LG treatment decreased body weights, especially in male mice: the 6-month study in mature adult NOD males (Figure 1A), the 2-month study in mature adult NOD males (Figure 4A), the 6-month study in old NOD males (Figure 7A), and the 2-month study in mature adult C57BL/6 males (Figures 8A and 8B). In the 6-month study in mature adult NOD males, the abundance of *Comamonadaceae*, which positively correlated with body weight ( $R=0.59$ ), was significantly

decreased in the LG treatment group. In the same study, the abundance of *RC4-4*, which also positively correlated with body weight ( $R=0.60$ ), was significantly increased in adult NOD male mice after dosing with lemon, and this effect was no longer seen following LG treatment. Previous studies indicated that increased abundances of phyla Verrucomicrobia, Proteobacteria and Cyanobacteria were associated with obesity [27]. Importantly, there were reductions of Verrucomicrobia, Proteobacteria and Cyanobacteria in mature adult male NOD mice following 6-month of LG treatment (Figures 2B and 2E). Moreover, decreased Verrucomicrobia has been associated with protection against dopaminergic neuronal damage loss [28]. Proteobacteria was reported to be more abundant in babies born to mothers with gestational diabetes mellitus [29]. Our study suggested that Proteobacteria were less abundant in the LG-treated male adult NOD mice than the control and glycerin groups after six months of treatment (Figures 2B and 2D). In terms of blood glucose levels, the abundance of family *Clostridiaceae*, which positively correlated ( $R=0.61$ ) with BGLs at the end of the 6-month study in mature adult NOD males, was significantly decreased in both the lemon and LG treatment groups. Interestingly, the abundance of *Clostridiaceae* that negatively correlated with the BGLs at the 15 mins timepoint in the 3<sup>rd</sup> month GTT was decreased by both lemon and LG treatments. However, significantly increased BGLs were observed in the lemon treated mice, but not in the LG treated mice that had similar BGLs as the vehicle and naive mice. It was possible that *Clostridiaceae* had differential effects in regulating the non-fasting glucose levels and glucose tolerance.

The 2-month study in adult NOD mice also supported that the gut microbiome changes were the potential mechanisms underlying the beneficial effects of LG treatment in body weight and blood glucose levels. For example,

the family *Deferribacteres* was enriched in male adult NOD mice treated with LG for one month (Figure 5E). It was also increased when mice were given polyphenol extracts from blueberries, which was interpreted as an anti-obesity effect [30]. In the 2-month male NOD study, there were significant decreases in body weight at the 2<sup>nd</sup>, 6<sup>th</sup>, and 8<sup>th</sup> weeks of treatment, and these decreases in body weight positively correlated with the decreases in the abundance of phylum Streptophyta following LG treatment ( $R=0.60$ ,  $0.66$  and  $0.74$ , respectively). Consistently, a more than two-fold increase in the order Streptophyta was observed in obese adolescents [31].

Moreover, the body weights at the 2<sup>nd</sup> and 6<sup>th</sup> weeks had a negative correlation with the abundance of YS2 ( $R=-0.70$  and  $-0.66$ , respectively), which was increased following LG treatment. Similarly, a negative correlation between obesity and YS2 (Cyanobacteria) has been reported in a Hispanic Community [32]. Additionally, the genera *Sporosarcina* ( $R=-0.65$ ) and *Jeotgalicoccus* ( $R=-0.63$ ), which were increased following LG exposure, were also found to negatively correlate with the body weight at 2 weeks of treatment. The increases in the relative abundances of the genera *Jeotgalicoccus* and *Sporosarcina* are associated with beneficial outcomes in animal models fed high-fat diets [33]. In the 2-month male NOD study, the BGLs at 4 weeks correlated with the abundances of the species *S-BQ2-57* ( $R=0.59$ ), the genera *Lactobacillus* ( $R=-0.61$ ), *Prevotella* ( $R=0.58$ ), 4-29 ( $R=0.67$ ) and *Bacteroides* ( $R=0.61$ ), the family *Sphingobacteriaceae* ( $R=0.58$ ), and the orders Ellin6067 ( $R=0.62$ ) and Sphingobacteriales ( $R=0.61$ ). These effects of LG on body weight and BGLs could be very beneficial in humans, if further confirmed, since the adult obesity rate in the United States was 42.4% in 2017-2018 [34], and the prevalence of obesity has increased at an even faster rate in patients with T1D than the general population

[35].

Glycerin administration was predicted to decrease glycolysis/gluconeogenesis when compared to the vehicle control group (Figure 2H). This may be attributed to the fact that glycerin can provide an alternative energy source. Treatment with lemon was predicted to decrease both propanoate metabolism and glycolysis/gluconeogenesis when compared to the vehicle control (Figure 2H). In agreement with this observation, the citrus fruit extracts hesperidin and naringin have been shown to shift the production of SCFAs away from propionate, the propanoate acidic conjugate, toward acetate when administered to stool samples collected from healthy human volunteers [36]. Further, limonin, a compound found in citrus, could suppress glycolysis [37], while the citrus flavanones hesperetin and naringenin inhibits gluconeogenesis [38]. It has been suggested that over-activation of the pentose phosphate pathway is an important mechanism for the vascular damage associated with hyperglycemia [39]. Interestingly, in the 2-month study in adult NOD mice, the pentose phosphate pathway was predicted to decrease in males dosed with LG. Amino sugar and nucleotide sugar metabolisms are closely related to glycosylation observed in diabetic patients, and excessive glycosylation of some proteins in the insulin signaling pathway can result in decreased phosphorylation, ultimately increasing the flow through the gluconeogenic pathway and decreasing the synthesis of glycogen [40]. In our study, both the amino sugar and nucleotide sugar metabolisms were predicted to decrease in both LG-treated male and female mice. In addition, glycosyl transferases were predicted to decrease in LG-treated female mice. About 90% of insulin-regulated gluconeogenesis occurs in the liver, and gluconeogenesis has been identified as the primary source of glucose production in patients with type 2 diabetes [41].

Importantly, gluconeogenesis was predicted to decrease in LG-treated males.

In this study, less significant effects on body weight were observed in females following LG treatment. Whether and how the combined consumption of lemon and glycerin had a sex-specific effect on body weight, however, has yet to be systematically studied. A previous study indicated that the decreased abundance of phylum Tenericutes was associated with the development of metritis, an inflammatory disease of the uterus involving its muscular membrane [42]. It is interesting to note that Tenericutes were increased only in female mice following one month of LG treatment. On the other hand, genus *Nitrospirae* and *Gemmatimonadetes* were only increased in NOD male mice following one month of LG treatment. *Nitrospirae* and *Gemmatimonadetes* were shown to be anti-tumor and immuno-modulatory in mice treated with the shiitake mushroom *Lentinula edodes*-derived polysaccharides [43]. Lipolysis is an integral part of the glycerolipid/free fatty acid cycle, and obesity is associated with an increase in basal lipolysis but a decrease in catecholamine-stimulated lipolysis [44]. In the 2-month study of adult NOD mice, a decrease in glycerolipid metabolism and increases in both fatty acid metabolism and biosynthesis were predicted in LG-treated male mice, while an increase in glycerolipid metabolism and decreases in fatty acid biosynthesis and adipose signaling pathways were predicted in LG-treated females. Further studies of the differential effects of LG in males and females in relation to gut microbiota-associated biological pathways are warranted.

It should be noted that a seemingly undesired effect was the decreased glucose tolerance in the LG treatment group in the 6-month NOD middle-aged/old male study (Figure 7C). This could be explained by their differences in age, as older age increases the risk of

developing hypoglycemia because of the higher rate of comorbidities such as renal failure, malnutrition, malignant diseases, and dementia [45]. In contrast, the insulin resistance was significantly decreased in these old NOD male mice after 6 months of LG treatment. In addition, two mice with blood glucose levels of 600 mg/dL or higher were included in the LG-treated group at the beginning of the study. Interestingly, one diabetic mouse was able to recover from T1D to become non-diabetic. The other one did not show improvement after two weeks of dosing and was subsequently euthanized for ethical reasons. Thus, the clinical outcome of a decreased glucose tolerance in elderly should be further explored. Additional limitations of the study include lacking detailed biological molecular mechanisms of the impact of modulated gut microbiota by lemon and glycerin in combination on glycemic control. Future studies should focus on, but not limited to, the impacts of such treatment on incretin secretion, bile acid metabolism and adipose tissue regulation. In addition, the role of metabolites produced by altered gut microbiota on hepatic glucose metabolism and insulin sensitivity should be further studied.

### **Conclusions**

Taken together, it is likely that decreased body weight and BGLs following consumptions of lemon and the combination of lemon and glycerin were related to gut microbiota changes. Further, combining lemon and glycerin may reduce the side effects of individual treatments, e.g., a transient hyperglycemia, by modulating gut microbiota in NOD mice. Current lemon and glycerin mixture products are used clinically, in the forms of cough syrups and swabsticks (Medline Industries, Inc) for sore throat and mouth application in hospice. Glycerin is an FDA-approved prescription product for glaucoma therapy. Although additional studies on species and sex differences should be

conducted to fully understand the safety of combining lemon and glycerin for consumption, it is reasonable to conclude that LG could be a potential dietary supplement by modulating microbial diversity and composition to attenuate the symptoms of various health problems that are linked to altered gut microbiota, such as T1D, obesity and aging [46].

### **Acknowledgment**

The authors greatly appreciate Dr. Steven D. Holladay (Department Head, Professor, Department of Veterinary Biomedical Sciences at the University of Georgia) for his critical comments.

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### **Author Contributions**

H.S.X. and T.L.G. designed the study. H.S.X., C.M.M and A.P. performed the animal experiments; Q.T. performed the metabolite assessments. H.S.X. analyzed the data; H.S.X. drafted the manuscript; and T.L.G. and Q.T. edited it. T.L.G. is the guarantor of this work, has full access to all data for animal and cell experiments, and is responsible for the accuracy and integrity of all data.

### **Author Declarations**

Tai L. Guo is the owner of HGG Research LLC, where Lemon Glycerin is manufactured. Other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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