

**Search For Gut Microbiota-Mediated Composition And Influence In Type 2 Diabetes**

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# Search For Gut Microbiota-Mediated Composition And Influence In Type 2 Diabetes

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Type 2 diabetes mellitus (T2DM) has proven to be of utmost importance in clinical care, having been diagnosed in more than 29 million Americans in 2012 ("Statistics about Diabetes," 2012). The microbiome has a strong influence in the development of diabetes, but the relationship is not well understood. **Methods:** This current study utilized diabetic and nondiabetic porcine fecal samples, focusing on bacteria of interest from pre-existing studies to observe bacterial changes in the gut related to type 2 diabetes. We aimed to characterize the microbiota both compositionally and functionally. Relative abundance comparisons and retrospective analysis of the literature allowed a preliminary analysis of the alteration of the microbiota which may contribute to the consequent dysglycemia characteristic of type 2 diabetes. **Results:** Our results revealed, at the genus level, a significant decrease of intestinal bacterium *Roseburia* ( $p < 0.0001$ ) and *Bacteroides* ( $p = 0.018$ ) in the gut microbiome of diabetic compared to the non-diabetic porcine. **Conclusion:** This study revealed preliminary relationships between gut microbiome and diabetes in a porcine model. *Roseburia*, which was decreased in the diabetic microbiome, has been associated with anti-inflammatory mechanisms activated by the short chain fatty acid (SCFA) butyrate. This study found the pig to be a strong model for analysis. Further study may help with understanding potential mechanisms linking SCFA, the microbiome and diabetes. A more comprehensive analysis of taxonomic resolution of the gut microbiome to better define these complex interconnections is needed. A deeper understanding of the impact of this interaction on diabetes could help with the development of more targeted prevention mechanisms for at-risk populations and better management for diabetic patients.

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## Preface

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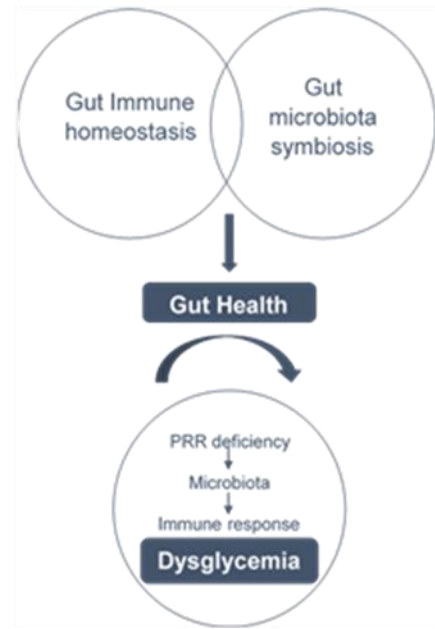


## 1.0 Background and Significance

As of 2014, 422 million people worldwide were diagnosed with a form of diabetes, according to the World Health Organization, and this number is expected to continue to rise annually. It is also reported that, of the estimated 29.1 million people with diabetes in the United States, roughly ninety to ninety-five percent have type 2 diabetes mellitus (T2DM) ("Diabetes," 2014).

Various susceptibility factors, including older age, African American ethnicity, and impaired glucose tolerance have been identified for T2DM. Within the senior population, an estimated 25.9% of people have a form of diabetes. According to the most recent statistical data, individuals above age 65 have a rate of 20.35 diagnosed diabetics per 100 individuals, as compared to a rate of 6.75 diagnosed diabetics per 100 individuals under age 65. The diagnosed rate increased even higher in African Americans, at a rate of 32.63 diagnosed diabetics per 100 individuals ("Diabetes - Rate by Age," 2014). The complexity of this disease may be attributed to its various stages of progression, compensatory mechanisms, and a vast number of predisposing factors (Ussar et al., 2015). T2DM is characterized by insulin resistance in peripheral vascular tissue and decreased insulin production as a result of  $\beta$  cell failure. In early phases of the disease,  $\beta$  cell compensation occurs, during which the cells expand their mass to produce more insulin to combat environmental factors in susceptible individuals. The disease progresses as these compensatory mechanisms are overworked, unable to keep up with the body's demand, and eventually leading to  $\beta$  cell failure (Drouin et al., 2009; Marc Prentki & Christopher J Nolan, 2006)

While the process of glucose metabolism is extremely complex, requiring the effective communication of many metabolic processes to maintain homeostasis, new insight into the role of the gut microbiome as a contributor to disease may link these pathological processes. This interwoven element may be a universal component contributing to the development of metabolic and autoimmune disease. To further investigate the microbiome as a potential link in disease processes, a novel approach must be developed which takes this into account to conceptualize and define the microbial influence in diabetes disease development. The interdependence of the various mechanisms relating many organs with the microbiota of the gut may be of particular interest when studying glucose metabolism in general, most notably during diseased states such as that in diabetes when a dysbiosis of the bacteria is observed .



**Figure 1 Immune and Microbiota overlap in T2DM**

Intestinal gluconeogenesis (IGN) is a metabolic process which regulates energy homeostasis and fasting glucose metabolism through anti-diabetic effects relating to dietary soluble fibers. When functioning, IGN promotes a decrease in food intake and moderates satiety. Mithieux and Gartier-Stein reported dietary soluble fibers only demonstrate an anti-diabetic effect in the body when IGN is functional (Mithieux & Gautier-Stein, 2014). When this applies, soluble fiber is fermented by the gut microbiome into short-chain fatty acids (SCFAs), most significantly propionate and butyrate (De Vadder et al.) Propionate, when unused by IGN, may have detrimental effects on the liver. The expression of IGN is further stimulated through generation of butyrate in the colon (De Vadder et al.; Mithieux & Gautier-

Stein, 2014). When IGN is not properly functioning, metabolic imbalance is evident through increased body weight and loss of glycemic control (De Vadder et al.). An example of the effectiveness of this process is following gastric bypass, where IGN is credited for the rapid improvement of insulin resistance (Mithieux, 2009).

There is growing focus on the impact of the microbiome on the development and progression of T2DM. The microbiome is the underlying hub of metabolism and immune functioning in the human body. The healthy microbiome is distinguished by its vast diversity and unique functional capacity (Tilg & Adolph, 2017). It is home to 100 trillion bacteria and has significant effect on “body weight, bile acid metabolism, proinflammatory activity and insulin resistance, and modulation of gut hormones” (Han & Lin, 2014). The gut microbiome with regard to diabetes disease has yet to be well defined. However, evidence from recent studies has now solidified the understanding that the microbiome bears a role in host metabolism (Wu et al., 2010). Western lifestyle, most significantly diet, has been seen to have significant effects on adiposity, glucose metabolism, oxidative markers, and inflammatory profile (Rodriguez-Castano, Caro-Quintero, Reyes, & Lizcano, 2016). The microbiome has been implicated in major signaling pathways which contribute to the inflammation of diseased states and improvement of the dysbiosis of the microbiome observed in type 2 diabetes has been linked to improved management of blood glucose measures. Microbial organisms of the intestines ferment and digest nutritional byproducts in the body (De Vadder et al.) and are responsible for controlling gut permeability and defensive bacteria which degrade harmful mucoproteins (Han & Lin, 2014). It is hypothesized the role of the microbiome in diabetes contributes to energy metabolism, innate immune system functioning and inflammation, and integrity of the gut barrier (He, Shan, & Song, 2015).

Slight differences in some specific bacterial populations have been noted in previous literature (Wu et al., 2010). Generally, a moderate degree of dysbiosis, or disruption of the normal microbiota, is seen as a result of T2DM. This dysbiosis is characterized by a shift in bacterial composition with increased prevalence of opportunistic pathogenic bacteria and decreased butyrate-producing bacteria (J. Qin et al., 2012).

T2DM is associated with alteration of the gut microbial composition, most significantly at the phylum and class levels of bacterial classification (Larsen et al., 2010). Microbiota can influence the development of metabolic disease through extraction of energy from dietary nutrition, energy homeostasis control via synthesis of peptides in the gut, and fat storage regulation (Cani et al., 2009). A decrease in protective gut barrier microbes may cause oxidative stress and functional alteration leading to metabolic disease (Yassour et al., 2016). Moderate dysbiosis has been observed in the gut microbiome of type 2 diabetics. One specific, notable alteration in the diabetic microbiome showed decrease in the amount of butyrate-producing microbes correlates with the increase in presence of opportunistic pathogens including bacteria which promote oxidative stress in the body. Overall, functional capacity was decreased in the microbiome of these T2DM patients and an inverse relationship between beneficial and harmful bacteria in the gut was observed. This may indicate a beneficial role of butyrate-producing bacteria in preventing metabolic disease (J. Qin et al., 2012). SCFA such as butyrate exhibit anti-inflammatory effects which have been found to improve insulin resistance, glucose reuptake by peripheral tissues, and blood glucose levels (Puddu, Sanguineti, Montecucco, & Viviani, 2014). While this is a notable trend, the role of other microbes and metabolites need to be further studied to better understand the holistic nature of gut metabolism in disease.

Bifidobacterium and Lactobacillus spp have been related to barrier protection against opportunistic pathogens. Bifidobacterium spp is also associated with improved glucose tolerance. The modification of the microbiome in favor of this population may therefore be a therapeutic intervention for the management of T2DM (Cani et al., 2009). Increase in Lactobacillus gasseri and Streptococcus mutans and Escherichia coli indicate development of insulin resistance in obese individuals (Hartstra, Bouter, Backhed, & Nieuwdorp, 2015). Additionally, Firmicutes and Clostridia are reduced in the gut of diabetic patients while Bacteroides, Bacteroidetes, and Betaproteobacteria are enriched in the diabetic microbiome (Larsen et al., 2010). While these findings were significant in their respective studies, it is important to consider the patient-to-patient variability of the microbiome and the vast number of influences on bacterial composition of the gut. Han and Lin described the contradictory results published by many studies in regards to this topic indicating different microbes of interest in diabetes development (Han & Lin, 2014).

The possible consequence of this dysbiotic shift may contribute to upregulated inflammatory signaling pathways leading to the inflammation seen in diabetic patients. One possible hypothesis which has emerged regarding the role of the microbiota on diabetes development is that the microbiota has an underlying endocrine function. This hypothesis relies on the understanding that the microbiota communicates with one another and the various organs of the body to illicit a response from a stimulus. When there is an alteration in the bacteria comprising the gut microbiota, this communication link is disrupted, resulting in dysglycemia. As diabetes is a very complex disease, this component could link the many mechanisms contributing to disease state.

Chronic inflammation has been directly associated with diabetes development and is caused by a compilation of various signaling mechanisms (Wang et al., 2013). The complete

pathology of inflammation as a marker for diabetes development is yet to be fully explained due to the complexity which results from the dysregulation of many homeostatic processes involving several immune markers. Three significant aspects in the development of inflammations leading to diabetes development involve oxidative stress, cytotoxic activity, and a few key immune markers. Key immune markers CRP, which serves as a biomarker of inflammation, is associated with elevated blood glucose and prevalence of diabetes. IL-6 is an inflammatory cytokine which may have direct correlation with CRP. It has been recognized as having been elevated within diabetic individuals (Wang et al., 2013). Understanding these inflammatory pathways as well as the implication of microbial metabolites on these pathways will better enhance the understanding of insulin resistance resulting from chronic inflammation.

Theories have emerged involving the effect microbes have on digestion through fermentation which produces various metabolites and SCFAs. These products then can affect the integrity of the gut barrier. Specifically, an example of this is seen with the protective function of the SCFA butyrate in maintaining optimal gut barrier function. When butyrate is missing, the gut barrier is impaired which results in inflammatory mediators leaking into systemic circulation (Upadhyaya & Banerjee, 2015). Over time, this state of chronic inflammation results in insulin resistance as seen in type 2 diabetes. Evidence has shown a positive correlation in prolonged activation of pro-inflammatory signaling receptors and insulin resistance. The mechanism behind this is related to interference in insulin signaling. Carl de Luca and Jerrold M. Olefsky summarize this in their review on “Inflammation and Insulin Resistance” (de Luca & Olefsky, 2008).

The degree of microbial alteration in the gut is related to varying stages of glucose tolerance, according to Zhang et al. Generally, Fasting Plasma Glucose (FPG) and C-reactive Protein (CRP) correlate with bacterial colonies evident in the development of diabetes. The study

reported 28 operational taxonomic units (OTUs) that were related to the dysbiosis of the diabetic microbiota. Specifically, relative abundance comparisons of bacteria in the gut demonstrate an increased prevalence of Betaproteobacteria and Clostridia across the spectrum of disease development. Streptococcus, on the other hand, decreased in abundance across the spectrum of disease. While the study found clear, statistically significant biomarkers of bacterial alterations across diabetic progression, they noted inconsistency with the bacteria of interest they found as compared with previously published paper, indicating the need for further study (Zhang et al., 2013). Another, more recent study by Egshatyan et al. also reported alterations in bacterial composition of prediabetic patients and further changes in diabetic patients. They describe a relationship between the genera Blautia and Serration. Both are increased in glucose intolerant individuals, while the phylum Verrucomicrobia is decreased in glucose intolerant individuals. These comparisons were within assigned dietary groups, thus limiting the potential confounding variable of the diet when analyzing the microbiota within study populations (Egshatyan et al., 2016). Another study reported differences in genera Ruminococcus, Dialister, Faecalibacterium, Catenibacterium, Streptococcus in subjects with varied stages of glucose tolerance and dietary habits (Ciubotaru, Green, Kukreja, & Barendolts, 2015). As there have been many studies which describe the geographic and population-based specificity of the microbiota, these discrepancies in results might be explained by geographical influences, further demonstrating the need for population-specific therapeutic interventions when attempting to correct the diabetes-related alteration of the gut bacteria.

Karlsson et al. developed a highly accurate diabetes prediction model based on metagenomic profiles of diabetic individuals . This model recognized diabetes-like metabolism based on the metabolism exhibited from the gut microbiome. While it was effective in predicting

disease development, it could only do so in patient populations with similar age and geographic location to the original training dataset (Karlsson et al., 2013). The findings which this study presents highlight the need for individualized nature of the microbiome and specifically implementation through personalized diets intended to modify the gastrointestinal microbiome for patients at risk of developing diabetes (Leulier et al., 2017). Attempts to understand diabetes development prevent disease progression with regards to the gut bacteria or simpler, molecule-based mechanisms is a continual effort in research. Advanced glycation end products (AGEs), which can be produced through food processing techniques, largely impacting the gut directly, may contribute to diabetes development directly through the promotion of pro-inflammatory genes (Kellow & Coughlan, 2015). Vitamin D deficiency has also been related to diabetes development, specifically as it interacts with the gut microbiota, and the significance of this role vitamin D may play in diabetes is evident through studies showing the effect of vitamin D supplementation on enhanced prediabetes management (Barengolts, 2013; Ciubotaru et al., 2015). A final key prediabetic consideration is the effective prediabetes medication Acarbose, whose benefits may be directly related to its ability to modulate the gut bacteria, specifically in the genera *Lactobacillus*, *Dialister*, *Butyricoccus*, *Phascolarctobacterium*, and *Ruminococcus* (Hu, Li, Lv, Wu, & Tong, 2015; Zhang et al., 2017).



## 2.0 Purpose

There is growing focus on the impact of the microbiome on the development and progression of T2DM. Evidence from recent studies has now solidified the understanding that the microbiome bears a role in host metabolism. While it has been well established that the microbiome and gut microbial dysbiosis play a role in type 2 diabetes development, the extent of this impact is not yet well understood. Furthermore, there lacks a complete understanding of the microbial populations of influence in the shift from healthy to diseased. One study deduced that there is a moderate degree of microbial dysbiosis in cases of T2DM (J. Qin et al., 2012). While there are many hypotheses, the specific type of microbe that plays a critical responsibility in the susceptibility to this disease remains vaguely understood and it has not been explored whether the microbial shift presents as a cause or consequence of diabetes. The role of the bacteria in inflammatory pathways and maintaining gut barrier integrity may be important in understanding peripheral insulin resistance related to chronic inflammation which characterizes type 2 diabetes. This research intended to be a pilot study and hypothesis-generating research with real-life data to guide a more comprehensive study.

The purpose of this study was to observe differences in the bacterial composition of the gut and determine the functional implication of these bacterial shifts in the microbiome for diabetic and non-diabetic subjects. Therefore, the specific aim was as follows:

**Specific Aim:** Examine the relative abundance of bacterial populations in diabetic and non-diabetic porcine. Quantify relative prevalence of preselected bacteria of interest related to universal bacteria and compare in diabetic and non-diabetic porcine samples.

## **3.0 Methods**

### **3.1 Design**

Samples collected were discarded fecal samples from diabetes-induced and non-diabetic mini pigs and repurposed for this study. Bacteria were selected for study that have been reported in previous studies to potentially play a role diabetes and that have biological relevance for diabetes. Bacterial DNA was isolated from the fecal samples and normalized for DNA concentration. Genus-specific bacterial DNA was targeted from total bacterial DNA using known bacterial primers which was confirmed with gel electrophoresis. These primers were used for the isolation and quantification of genus-specific bacteria from each of the samples collected. Total bacteria were quantified using a universal primer known to be conserved across most bacterial genera. Bacterial genera were related to the total bacteria in each sample to create a standard for comparison. Literature was then reviewed for functional implications of the selected bacteria and summarized.

### **3.2 Subjects**

Our experiments were conducted using two samples each from three 4-year-old nondiabetic and three 4-year-old diabetes-induced mini pigs. Diabetes was induced with Streptozotocin (STZ) to replicate damage to the pancreas and subsequent impaired insulin production as seen in advanced diabetes. The pigs were also fed a high fat (HF) diet which is

known to lead to inflammation and insulin resistance in diabetes and can result in microbial alterations reflective of type 2 diabetes in humans (Heinritz et al., 2016). The pig serves as an effective model for microbiome studies since it has a similar microbiome to the human. The pig also has a similar digestive tract to humans and are gut fermenters just as humans are making it a strong model for diabetes studies (Heinritz, Mosenthin, & Weiss, 2013).

### **3.3 DNA Extraction**

Bacterial DNA was isolated from the samples using the Qiagen QIAamp DNA Stool Extraction Kit according to manufacturer protocol (QIAGEN, Hilden, Germany). Samples were collected fresh and transferred to Cary Blair Transport Medium for storage at -80°C. Following DNA extraction, DNA concentration was measured using NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer. DNA was stored at a target concentration of 5 ng/μL for each sample.

### **3.4 PCR and Primer Validation**

Lactobacillus, Roseburia, Bacteroides, and Prevotella were selected for study as these bacteria are all genus-level clusters which have been noted in the literature as being altered in prevalence in the diabetic microbiome (Ciubotaru et al., 2015; Karlsson et al., 2013; J. Qin et al., 2012). Total bacteria were quantified by targeting the V4 hypervariable portion of the 16S rDNA subunit which is known to be conserved in most bacterial genera. The primers which were used to isolate the various bacterial populations were chosen from previous papers (see Table 1)

(Hermann-Bank, Skovgaard, Stockmarr, Larsen, & Molbak, 2013; Larsen et al., 2010) and confirmed with PowerUp SYBR Green real-time PCR and gel electrophoresis. Genus-specific and total bacteria were quantified with SYBR Green I Dye qPCR in sample triplicates according to manufacturer protocol. The triplicates for each bacterium in each porcine sample were averaged and outliers were removed.

**Table 1 Bacterial primers**

Genus	PrimerF 5'-3'	PrimerR 3'-5'
Lactobacillus	AGCAGTAGGGAATCTTCCA	CACCGCTACACATGGAG
Roseburia	TACTGCATTGGAAACTGTCTG	CGGCACCGAAGAGCAAT
Bacteroides	AAGGTCCCCCACATTGG	GAGCCGCAAACCTTTCACAA
Prevotella	CACCAAGGCGACGATCA	GGATAACGCCYGGACCT
Universal	TCCTACGGGAGGCAGCAGT	GACTACCAGGGTATCTAATCCTGTT

### 3.5 Bacterial Abundance

Relative abundance calculations were computed to standardize the bacteria in the samples and account for inter-individual differences. This calculation was made by dividing the bacteria of each genus in each sample by the total bacteria in that sample and multiplying by 100. The results for total bacteria quantities and calculations can be found in Appendix A and B.

### **3.6 Statistical Analysis**

Statistical analysis was computed with unpaired t tests with two-tailed p-values. Diabetic and nondiabetic samples were compared for each genus of bacteria under study as well as total bacteria present. Genus specific bacteria were compared by percent abundance relative to total bacteria while total bacteria were compared by relative number of bacteria present in diabetic and nondiabetic samples. Statistical significance was recognized as  $p > .05$ . Outliers recognized by the program were removed from triplicates.

## 4.0 Results

### 4.1 DNA Extraction

Prior to DNA extraction, samples were thawed for one hour prior to analysis. Bacterial DNA was extracted from fecal samples and reconstituted for a target concentration of 5 ng/ $\mu$ L. This target concentration allowed for relative control of inter-individual differences in the mini pigs or mechanical error in technique.

Table 2 DNA Extraction Results

Sample	Extraction 1 DNA Concentration		Extraction 2 DNA Concentration	
	Raw	Reconstituted	Raw	Reconstituted
Non-DM 1	3.9	5.0	4.5	5.6
Non-DM 2	4.3	5.2	5.7	5.1
Non-DM 3	5.1	5.3	6.0	5.4
T2DM 4	8.8	5.8	11.5	5.0
T2DM 5	8.9	4.8	13.2	5.2
T2DM 6	11.3	4.9	10.3	5.5

### 4.2 PCR and Electrophoresis

Bacterial primers accurately identified the specific bacterial genera of interest. Lactobacillus had between 300 and 400 base pairs which is expected as it is known to have 341 base pairs. Roseburia fell between 200 and 300 base pairs and was expected to have 230 base pairs. Bacteroides fell around 300 base pairs as it has 300 base pairs. The universal primer known

to be conserved in most bacteria fell between 400 and 500 base pairs as expected with 466 base pairs.

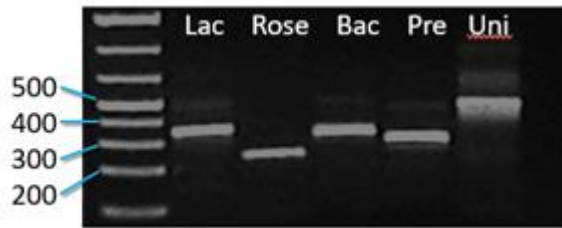


Figure 2 Gel electrophoresis for bacterial primers

### 4.3 Bacterial Abundance

No trend was noted in the total bacteria between diabetic and nondiabetic samples ( $p=0.3582$ ). Similar quantities of total bacteria were found for the diabetic and nondiabetic porcine samples. This is expected as DNA concentration was standardized prior to qPCR.

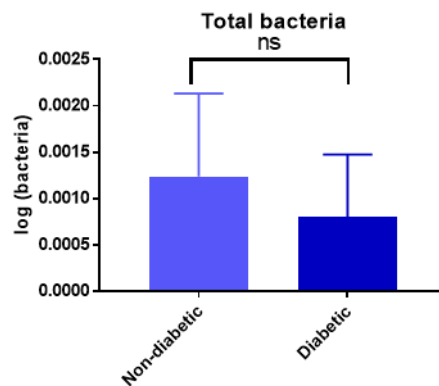


Figure 3 Total Bacteria Comparison

*Roseburia* and *Bacteroides* were significantly decreased in the diabetic microbiome ( $p<0.0001$  and  $p=0.0180$ , respectively). *Lactobacillus*, a generally beneficial bacterial genus,

showed a small increase in the diabetic microbiome and was close to significance ( $p=0.0807$ ).

*Prevotella* showed no apparent trend in either the diabetic or nondiabetic samples ( $p=0.7828$ ).

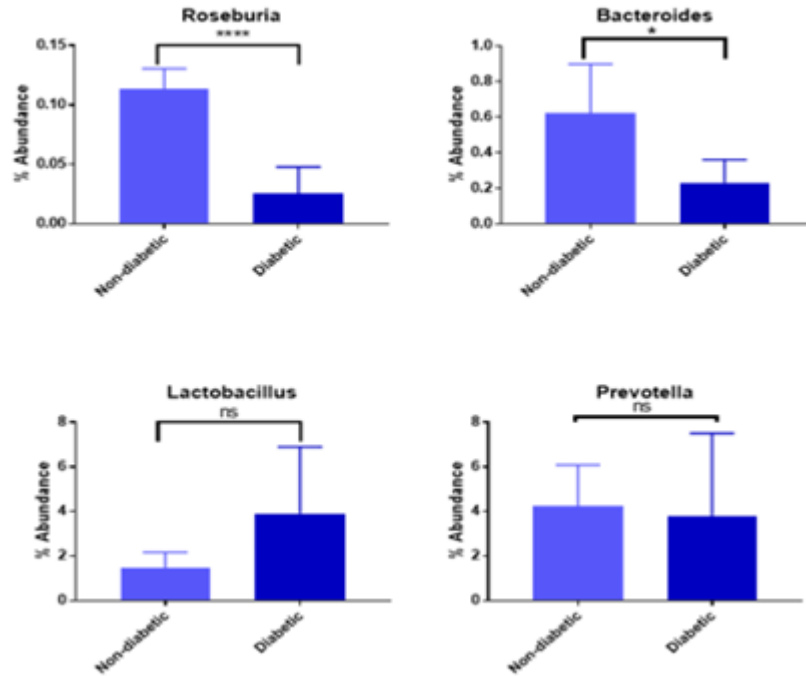


Figure 4 Bacterial Genus Comparisons



## 5.0 Discussion

Our study found significant differences in Roseburia and Bacteroides when comparing the gut microbiome of nondiabetic and diabetic porcine, with decreased prevalence noted in the diabetic group. The decrease in Roseburia in the diabetic microbiome was hypothesized since it is a butyrate-producing bacteria (Ciubotaru et al., 2015; Karlsson et al., 2013; Junjie Qin et al., 2012). Bacteroides has been shown to correlate with increased expression of proinflammatory genes, Vitamin D deficiency, and high serum lipopolysaccharide (LPS). Bacteroides has also been identified in previous studies as increased in the diabetic microbiome (Ciubotaru et al., 2015; Karlsson et al., 2013; J. Qin et al., 2012). However, our results showed an increase in this cluster in the nondiabetic sample. This may indicate species-specific alterations which need to be further investigated in this genus. Lactobacillus has been previously identified as having four species increased in the diabetic microbiome (Karlsson et al., 2013). We note a trend of increased Lactobacillus in the diabetic porcine samples, however no significance was found in this alteration. Interestingly, Finally, there was no significant alteration in the prevalence of Prevotella. While this may indicate the bacteria does not have a significant role in the diabetic microbiome, further study is needed to confirm this.

Total bacteria in the diabetic and nondiabetic samples was not significant, which is not unexpected since the samples were standardized for DNA concentration to accommodate the small sample size. This prevented inter-individual differences in total bacteria of the pigs from confounding results. As a way to manage this confounding influence and better capture differences in the total bacteria, fresh collection and analysis with a set amount of stool could provide a better method of standardization across the samples. This would reintroduce the potential for inter-

individual differences to have an effect so a larger sample size would better capture this. Furthermore, more samples from each of the pigs could also serve to limit sampling error.

Overall, the pig model served as a strong model for this research. Results fairly consistent with previous findings are promising that the pig model was effective at demonstrating microbial alterations in type 2 diabetic subjects. This furthermore supports the evidence that HF diet leads to these microbial alterations. Finally, the method of diabetes induction involving STZ and HF diet seems effective while preserving the microbial populations of the gut for study. It would be interesting to see the effects of the HF diet alone and if diabetes could be induced through microbial alteration without additional external factors. This could lead to better understanding of the changes that occur in the gut and better management of diabetic patients with this regard.

### **5.1 Strengths and Limitations**

The porcine model provided a strong model for the study as supported by the aforementioned similarities with the human digestive system and microbial composition. Furthermore, the pigs from which the samples were collected were aged thereby demonstrating maximum effect on microbial populations. The pigs were also raised in a laboratory environment so there was relative control over external factors which may have been confounding variables had human samples been used. Unfortunately, there were some limitations which could not be overcome. The samples collected were discarded samples and as such, further testing on the pigs including laboratory testing such as HbA1c could not be conducted. Furthermore, while the protocol for diabetes induction has been optimized with the dual-method approach involving the use of STZ and HF diet, this may have some unknown effect on the results obtained. Calculations

of total bacteria may not be considered with high regard as the samples collected were standardized for DNA concentration to account for any mechanical error or inter-individual differences so there may exist some significance in total bacteria quantified between diabetic and non-diabetic microbial populations. Finally, some DNA degradation may have occurred as a result of storage. Samples were collected fresh, transferred to Cary-Blaire preservative and stored at  $-80^{\circ}\text{C}$ . Prior to analysis they were thawed for extraction of DNA from samples. The temperature fluctuation may have caused some minor influence on the samples, however as they only went through this one time, the influence is limited.

## **5.2 Future Directions**

As mentioned, prediabetes is the time period when  $\beta$  cells compensate to elevated nutritional intake before the progression to diabetes during which blood glucose levels are slightly elevated (Drouin et al., 2009; M. Prentki & C. J. Nolan, 2006). While this is a well-recognized phase of the disease and prevention methods are targeted at the prediabetic population, it is understudied in regards to the dysbiosis of the microbiome in diabetes. The hypothesis of a gradual dysbiosis of the microbiome, which would be apparent in this phase of the disease should be studied explicitly in order to develop a more comprehensive understanding of the disease progression. The early recognition of susceptible individuals according to gut permeability and inflammation and microbial intervention to manage this risk may be effective in better managing T2DM before the actual clinical onset of the metabolic disease (Yassour et al., 2016). Additionally, while the risk factors for T2DM have been clearly emphasized and established, they are not explicitly studied in comparison with individuals who do not have the risk factors. This

may give great insight as to microbial populations of interest and reasoning behind why some populations are at greater risk of developing diabetes.

Defining the primary species of diabetic influence may allow for improvement of directed prevention methods (He et al., 2015). Future directions for intervention in the prevention and management of diabetes include the use of fecal microbiota transplant (FMT) therapy and probiotic dietary supplementation (Marchesi et al., 2016). Donor alteration for FMT optimization is of peak interest among these direct management techniques (Olesen, Gurry, & Alm, 2017). While these will likely not transcend the standard approach of diet and exercise, they may provide more direct support to disease management allowing for better glycemic control, particularly in susceptible populations.

## **6.0 Conclusion**

Overall, the findings of this preliminary study revealed trends towards an altered bacterial composition in the gut of diabetic individuals. While most of these findings were consistent with previous findings in the literature, some inconsistencies have been noted. These inconsistencies may be the result of differences in species-specific bacteria within the genera of interest. This research supports the use of the porcine model with induction of diabetes with STZ and HF diet. Future studies could use this same approach and instead of targeting species of interest in the development of diabetes, could use an approach that is discovery based to find additional or even novel species associated with diabetes in this model. Future studies should also analyze the fecal samples of humans with notable risk factors, as noted above, to more pointedly determine whether the shift in the microbiota is a cause or consequence of diabetes development and to better define the shift which occurs.

## Appendix A Experiment 1 Bacterial Abundance

Sample	$2^{(-1 \cdot Ct)}$	%Abundance
1, Lacto	1.22269E-05	0.654784037
1, Rose	2.04398E-06	0.109460965
1, Bac	6.09869E-06	0.32660227
1, Pre	6.67095E-05	3.57248608
2, Lacto	1.84417E-05	1.270744973
2, Rose	1.58906E-06	0.109496237
2, Bac	1.27228E-05	0.876675715
2, Pre	7.0831E-05	4.880687977
3, Lacto	6.31083E-06	0.999483639
3, Rose	6.62632E-07	0.104944941
3, Bac	2.2177E-06	0.351229726
3, Pre	4.1563E-05	6.582580182
4, Lacto	4.01028E-05	2.506773285
4, Rose	2.3078E-07	0.014425797
4, Bac	2.33836E-06	0.146167708
4, Pre	1.32728E-06	0.082966354
5, Lacto	3.80771E-05	9.379730835
5, Rose	2.83188E-07	0.069759162
5, Bac	5.2679E-06	1.297668777
5, Pre	4.41283E-05	10.87035027
6, Lacto	3.80576E-05	2.226797738
6, Rose	2.53424E-07	0.014828154
6, Bac	3.7163E-06	0.217445414
6, Pre	4.1373E-05	2.420789312
1 Universal	0.001867313	
2 Universal	0.00145125	
3 Universal	0.000631409	
4 Universal	0.001599776	
5 Universal	0.000405951	
6 Universal	0.001709072	

## Appendix B Experiment 2 Bacterial Abundance

Sample	$2^{(-1 * Ct)}$	% Abundance
1, Lacto	9.56751E-06	2.688177104
1, Rose	4.41413E-07	0.124023681
1, Bac	3.58881E-06	1.00834653
1, Pre	1.29518E-05	3.639050797
2, Lacto	3.36593E-05	1.288021959
2, Rose	2.38318E-06	0.091195842
2, Bac	1.64042E-05	0.6277302
2, Pre	3.37043E-05	1.289741874
3, Lacto	9.27201E-06	1.794009492
3, Rose	7.2615E-07	0.140500242
3, Bac	2.8187E-06	0.545379933
3, Pre	2.82602E-05	5.467973899
4, Lacto	1.35722E-05	5.321613338
4, Rose	6.3884E-08	0.025048665
4, Bac	1.15614E-06	0.453319625
4, Pre	1.10611E-05	4.337028298
5, Lacto	5.53271E-06	2.52856849
5, Rose	3.92978E-08	0.017959922
5, Bac	4.72324E-07	0.215862397
5, Pre	6.35515E-06	2.90444167
6, Lacto	8.37234E-06	1.397294889
6, Rose	5.18921E-08	0.008660483
6, Bac	7.85932E-07	0.131167408
6, Pre	1.14968E-05	1.918744378
1		
Universal	0.000355911	
2		
Universal	0.002613257	
3		
Universal	0.000516832	
4		
Universal	0.000255039	
5		
Universal	0.000218808	
6		
Universal	0.000599182	

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