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COMMENSAL MICROBIOTA AND ITS IMPACT ON HEALTH AND AUTOIMMUNE DIABETES

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A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham, in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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2013

COMMENSAL MICROBIOTA AND ITS IMPACT ON HEALTH AND AUTOIMMUNE DIABETES

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CELLULAR AND MOLECULAR BIOLOGY

ABSTRACT

Environmental exposures such as diet, use of antibiotics, and lifestyle have significant impact on the health of an individual. One mechanism that acts as a major pathway in these environmental exposures is the role intestinal commensal microbiota play in both metabolic and immunological responses. Metabolic pathways are altered through changes in metabolites provided by commensal microbiota; alterations in microbial make-up can have drastic impacts on metabolic function. Similarly, the development and maturation of the immune system is dependent on the intestinal microbes to induce tolerance and act as both an immune modulator as well as a barrier against pathogens.

We have examined the importance of microbial diversity and the role specific microbes play in metabolic diseases such as obesity and onset of type 2 diabetes (T2D). Alterations in the ratio of commensal bacteria belonging to the phyla *Bacteroidetes* or *Firmicutes* is one of the largest contributing factors in metabolic disease. *Firmicutes* tend to have a higher degree of diversity in metabolic genes, allowing for a more efficient use of all dietary antigens compared to *Bacteroides*. Interestingly, individuals who are obese

or who are diagnosed with T2D have a higher number of *Firmicutes* compared to lean individuals.

In the development of immunological diseases, the commensal make-up is equally important. We hypothesized that alterations in commensal make-up can have serious consequences in the development of auto-immune Type 1 diabetes (T1D). We tested this hypothesis by placing NOD/ShiLtJ mice on either neutral (NOD N, pH ~7.0) or acidified (NOD A pH~ 3.2) H_2O and monitoring the composition of the fecal commensal microbiota and incidence of T1D. NOD N mice had an increased incidence of T1D by 30wks of age; exhibiting decreased *Firmicutes* and increases in *Bacteroides*, *Actinobacteria*, and *Proteobacteria*. These alterations in microbiota were seen as early as 2-weeks of age. NOD N mice had decreased levels of FoxP3 expression in CD4+FoxP3+ cells, and decreased CD4+IL17+ cells, and a lower ratio of IL17/IFNγ CD4+ T-cells. Our data indicates that changes in diet alter the commensal microbial environment, the presence of protective Th17 and Treg cells, and incidence of T1D.

Keywords: Microbiota, Diabetes, Obesity, Autoimmunity, Th17

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TABLE OF CONTENTS

Page

ABSTRACT.....ii

ACKNOWLEDGMENTS.....iv

LIST OF TABLES.....vii

LIST OF FIGURES.....viii

CHAPTER

INTRODUCTION.....1

GUT MICROBIOTA AND OBESITY.....15

ACIDIC BEVERAGES ALTER THE COMMENSAL MICROBIOTA AND DECREASE THE RISK OF TYPE 1 DIABETES IN THE NOD MOUSE MODEL.....41

DISCUSSION.....89

GENERAL LIST OF REFERENCES.....100

APPENDIX: ANIMAL USE APPROVAL FORM.....107

LIST OF TABLES

Table *page*

ACIDIC BEVERAGES ALTER THE COMMENSAL MICROBIOTA AND DECREASE THE RISK OF TYPE 1 DIABETES IN THE NOD MOUSE MODEL

1 Relative Abundance of Bacterial Phyla in the Fecal Microbiome of NOD-A and NOD-N Mice Generated from Pyrosequencing Data 71

S1 Summary Statistics for the Pyrosequencing Data of the Fecal Microbiome of NOD A and NODN Mice 72

S2 Relative Abundances of Bacterial Genera in the Fecal Microbiome of NOD A and NOD N Mice Generated From Pyrosequencing Data 73

S3 Genera Removed From Analysis With a Total Population Below 0.1% 76

S4 Genera Removed From PLS-DA Analysis With a Variable Influence of Projection Value Less Than 0.3.....77

LIST OF FIGURES

Figure *Page*

ACIDIC BEVERAGES ALTER THE COMMENSAL MICROBIOTA AND DECREASE THE RISK OF TYPE 1 DIABETES IN THE NOD MOUSE MODEL

1 NOD Mice on Neutral Drinking Water Have an Increased Incidence of T1D and Alterations in the Diversity of Their Fecal Microbiota. 57

2 Impact of Neutral of Acidified Drinking Water on the Fecal Microbiota Composition of NOD-N and NOD-A mice 59

3 qRT-PCR Analysis of Bacteria Copy Numbers in Feces of NOD Mice Shows a Significant Dysbiosis in Mice on Neutral Drinking Water. 61

4 NOD Mice on Neutral Drinking Water Have Similar Percentages of $CD4^{+}FoxP3^{+}$ Tregs, but Decreased Expression Levels of FoxP3. 63

5 NOD Mice on Neutral Drinking Water Have Decreased Production of IL17 by $CD4^{+}$ T Cells. 64

S1 The $[H^{+}]$ Concentration in the GI Tract of NOD A Mice is Greater Than in NOD N Mice. 66

S2 Flow Cytometric Analysis of Splenocytes and Small Intestine (SI) and Colon (Co) Lamina Propria (LP) Lymphocytes From NOD A and NOD N Females at 2 and 8-10 Weeks of Age. 67

S3 Coefficient Plots of the Taxa Analysed by PLS-DA Found in 454 Pyrosequencing.....69

CHAPTER 1

INTRODUCTION

Bacteria have always played a role in human existence, sometimes as pathogens, evading our immune system and causing bodily harm, and other times, acting as symbiots, providing important services in return for a stable environment in which to thrive. This symbiotic relationship has evolved over time, allowing humans to commonly recognize these symbiotic creatures as non-threats. This allows these bacteria to live with us without activating the immune system. In return, many of these microbiota act to protect us, out-competing pathogens and providing us with important nutrients and products we cannot produce ourselves. Because they no longer are seen as "non-self" some have come to regard this population of beneficial bacteria as "the other self", suggesting that these populations act as an organ to the host and is therefore seen as "self"(1). The populations of bacteria that work with us as symbionts are given the term "commensal microbiota"(2). The diversity of the commensal populations is extensive. Unlike other "self" structures, resident microbiota can be altered within 24-48 hours when exposed to dietary changes (3). This makes creating a unified, comprehensive picture of the commensal populations very challenging. Different bacteria become prevalent in different species, or even within the same species depending on health, diet, and geographic location of the host (4; 5). The dynamic within the commensal populations have become so that some populations cannot thrive without the presence of

another specific population. It is so intricate, that many have come to regard the commensal bacterial populations within the GI tract as its own ecosystem; one that we call "The Microbiome"(3). The microbiome is the totality of microbes (bacteria, viruses, yeasts etc.), their genetic elements, and interaction with the GI tract (6).

As with any ecosystem, there exists compartments, or pockets, within the system where specific organisms tend to reside. The GI tract is no different. Different microbes tend to colonize only specific areas of the GI tract, whether it is the stomach, small intestine, cecum, or large intestine (colon). Even within tissues, there exists a stratum. The small intestine is comprised of three main compartments; the most proximal (closest to the stomach), the duodenum (approx. 5cm in the mouse, or 25-38cm in humans) where most chemical digestion takes place. The jejunum, or mid-gut (approx. 2.5m in humans) is specialized for the absorption of carbohydrates and proteins (7). Though much digestion takes place within the duodenum and jejunum, microbiota within the small intestine primarily reside within the ileum, leaving the duodenum and jejunum only sparsely colonized (7; 8). The remaining commensal populations that take up residence within the GI tract can be found in the cecum and large intestine (colon) (2). The cecum and colon are home to a large number of bacteria, especially aerobic (oxygen requiring), which is unique because the overwhelming majority of microbes comprising the gut flora are anaerobic (does not require oxygen)(4). The bacteria within the GI tract are responsible for the breakdown of many compounds our digestive systems are unable to naturally digest (9).

While it has been reported that there are over 1000 species of bacteria within the gut of an individual, a vast majority of bacteria reside within four primary phyla (3; 10;

11). *Firmicutes* on average are the most abundant phyla represented in the gut (3). *Firmicutes* are a highly diverse (genetically and phenotypically) group of bacteria that can claim over half of the commensal species that reside in the gut (12). *Bacteroidetes* is the second most abundant phyla, with a majority of their population belonging to the *Bacteroidales* and claims on average 20-30% of the species found in the GI tract (3; 12; 13). The last two phyla found in large numbers *Proteobacteria*, and *Actinobacteria* which fluctuate in density and make up approximately 5-10% of commensal populations (14). Together, these four phyla represent over 90% of the bacteria that have been discovered in the GI tract. During late digestion, the host is unable to utilize all of the calories, nutrients, and materials that are ingested (15). Commensal populations are able to utilize these materials for their own metabolism, and as a byproduct, produce materials that are important to the metabolism of the host (15). A perfect example is butyrate, a short chain fatty acid that is produced as a byproduct of bacterial fermentation in the gut. Butyrate is a primary source of energy for colonocytes, the epithelial cells that line the colon (5). Without adequate supplies of butyrate, the colonic epithelium would be unable to function properly. Such processes are vital in the maturation, and maintenance of the host. Specific groups of microbiota are better geared toward certain tasks compared to others (9). *Firmicutes* for instance, specifically *Clostridia*, on average, have a higher number of metabolic genes and enzymes (16). The larger diversity in metabolic pathways allows the microbiota to utilize a more diverse selection of molecules as food sources. In turn, this also will ultimately provide a higher number of usable caloric substrates to the host. The wide range of abilities commensal populations display have

lead researchers to look into what kind of effects microbial composition plays in their host's metabolic cycles and if these microbes play any role in disease.

The microbiome is currently under scrutiny as being (along with genetic predisposition), the leading environmental factor that influences the incidence and development of a multitude of diseases, a perfect example of which is inflammatory bowel disease (IBD) (17). IBD is caused by unregulated activation of pro-inflammatory pathways in the gut and mucosal associated lymphoid tissue (GALT and MALT respectively)(18; 19). The GALT is a system of lymphoid structures that reside specifically along the GI tract (tonsils, Peyer's patches, lamina propria, etc.) while MALT contains all lymphoid tissues that are resident along mucosal surfaces (gut, nasal, and bronchial) (20). As the GI tract is the most active site of interaction with external antigens, the gut lymphoid system requires a high degree of regulation in order to maintain balance between inflammation and tolerance (2). When tolerance in disrupted and IBD begins, it commonly manifests in the form of ulcerative colitis (UC) or Crohn's disease (CD) (21). Ulcerative colitis is found strictly within the large bowel (colon), creates ulcers (open sores) within the colon, and bloody stool (22). In contrast, Crohn's disease is not limited to the colon and can cause inflammation throughout the entire GI tract (21). While similar, these are very distinct diseases and have very unique immunological and pathological characteristics.

Despite their differences, both diseases can be ameliorated with antibiotics (23). This has lead researchers to believe that the commensal microbiome is acting as one of the primary antagonists. As the commensal microbiota is considered by many to be another form of "self-antigen" and should not act as an antagonist of the immune system,

both ulcerative colitis and Crohn's disease have been labeled as autoimmune disorders. Autoimmune disorders, or autoimmune diseases, can be defined as a situation where the body fails to properly distinguish self from non-self antigen, causing the immune system to attack the body.

The incidences of autoimmune diseases have exponentially increased over the past 60 years (24-26). Environmental antigens (Ag) and commensal microbiota have been linked, in both protective and pathogenic manners, to a number of autoimmune diseases including multiple sclerosis, rheumatoid arthritis, and Type 1 Diabetes (T1D) (27-30). In the 1980s Dr. David Strachan surmised that the increased incidence of allergies in developed countries was directly correlated with a distinct lack of environmental interaction. It was hypothesized that as developed countries used more soaps, antibiotics, and stressed sanitized environments, the interactions with antigens that were required to develop tolerant immune systems would diminish (31). This would lead to populations of individuals who were sensitive to innocuous compounds. Dr. Strachan labeled this the "Hygiene Hypothesis" (31). As the gastrointestinal tract is the primary site of contact with external antigen, it was not long before the question of what role the GI tract and commensal microbiota had in the development of the immune system arose (32; 33). It was later discovered just how important commensal microbiota are to the proper development of the immune system (2). Gnotobiotic, or Germ-Free (GF) mice, which are completely microbially devoid, have been used to discover the roles microbes play in immunity. GF mice have significantly diminished secondary lymphoid tissues compared to conventional (normal bacterially colonized) mice (34; 35). This suggests that the microbiome is indispensable to the proper development of the immune system.

The necessity of the microbiome in immune development expands out past the gut and mucosal associated lymphoid tissues (GALT and MALT respectively), and can have wider implications effecting systemic immune responses (36). It is commonly accepted that bacteria in the GI tract alone can reach 10^{14} colony forming units (CFU), which ranges anywhere between 10 and 100 times the number of eukaryotic cells in an average human (37). The importance of the microbiome in immune development cannot be stressed enough. Since the time of birth, an infant is colonized by a number of bacterial groups; this early acquisition is vital in the postnatal mucosal and systemic immune development and will impact lifelong immunity and health.

There has been a lot of research looking into the functions of specific commensal bacteria in the development of the immune system since the inception of the hygiene hypothesis. The research has been primarily focused on looking at antibody and T helper differentiation in response to specific microbes. Recent discoveries include a bacterium belonging to the group *Clostridia* Cluster IV, named Segmented Filamentous Bacteria (SFB) (38). These microbes are found in low numbers in the ileum of mice as a commensal population. What makes SFB unique is its ability to propagate Th17 populations (39). Research from the Littman laboratory displayed that the same mouse strain from either Jackson laboratories (Ann Harbor, ME) or Taconic (Hudson, NY), two of the largest producers of inbred mouse strains, produced substantially different levels of IL17 in the gut and systemically. Taconic mice, which possessed SFB, produced significantly greater levels of IL17 compared to mice from Jackson laboratories. This was later verified when conventionally raised or germ-free mice were inoculated with SFB and substantial increases in IL17 were witnessed (39; 40). SFB is not the only

commensal able to induce specific responses. *Lactobacillus* and *Clostridia* (non-pathogenic species), common *Firmicutes* commensal groups, are able to act in the induction of IL17 expression and T regulatory cell expansion (41; 42). *Lactobacillus* has also been described to be able to act as an immune modulator, acting as a buffer between mucosal immunity and other microbes, often been correlated with decreased pathology in a multitude of diseases (43; 44). Similarly, *Bacteroides* can induce both T helper 2 and T regulatory cells (43; 45). The balance between specific bacterial groups appears to be a major player in the regulation of the mucosal and systemic immune response.

Since its inception in 1989, the hygiene hypothesis has come to be widely accepted and also been shown to have a number of implications in autoimmune diseases as well. After which, many researchers began looking into the correlations between gut microbiota, immune development, and incidence of auto-immunity in developed countries. The correlation was made that increases in auto-immunity, like allergies, exponentially increased with wide-spread use of antibiotics and the practice of "cleaner" lifestyles (46-48). It's been shown that expansion of T cells in the absence of infection or "immune insufficiency" can increase the incidence of auto-immunity (24; 49). Indeed the old saying "Idle hands are the devil's workshop" rings true as it appears that without adequate stimuli during immune development, the immune system is unable to mature properly, resulting in a distorted balance between pro-inflammatory and regulatory responses.

One of the current hot topics in autoimmunity is the correlation between commensal microbiota and Type 1 diabetes mellitus (T1D) (30; 47; 50; 51). T1D is defined as an autoimmune disease, characterized by the immune mediated destruction of

insulin producing β-cells in the pancreas. Often called juvenile diabetes, the onset of T1D often occurs relatively early in life (as late as early 20's in humans) (52). The destruction of the insulin producing β cells causes a shortage of insulin, an invaluable enzyme in the regulation of blood glucose and metabolic homeostasis in the body. It is responsible for the homeostasis of blood glucose levels and controls the synthesis or break-down of glycogen, an intermediate between free glucose and long-term energy storage. The loss of insulin regulation leads to the inability of the body to control their blood glucose and ultimately their ability to control their own metabolism (34; 53). There are long-term health issues associated with T1D including neuropathy (nerve damage), retinopathy (eye damage), nephropathy (kidney damage), and a host of other complications that are tied to the bodies inability to regulate its blood glucose. Uncontrolled blood glucose can cause an imbalance in any number of pathways and lead to organ failure and death if not controlled. When an individual loses the ability to regulate blood glucose, it means that over 85% of the insulin producing β cells have been destroyed (54; 55). Because this is the first clinical symptom of disease, it makes it nearly impossible to detect the disease before the destruction has already occurred. This is why current research is focused on preventative measures against the disease. Individuals are predisposed to T1D through polymorphisms in a host of important gene loci. These polymorphisms allow for the rise of auto-reactive T cells through poor negative selection of T cells in the thymus, as well as production of antibodies from B cells that are specific to pancreatic antigens. The major histocompatibility class II (MHC-II) haplotype is another genetic pre-disposition in T1D. Cells expressing the MHC-II $H2^{G7}$ haplotype are thought to promote autoimmunity through its affinity for self-antigen (56). Under

healthy conditions the autoimmune response would be intercepted and quelled by T-regulatory T cells (Tregs), $CD4^+$ T cells that regulate and modulate pro-inflammatory responses (57). Characterized by the production of anti-inflammatory cytokine Interleukin 10 (IL10), and transcription factor Forkhead box P3 (FoxP3), Tregs prevent the expansion of pro-inflammatory T effector cells and work to prevent uncontrolled inflammation. In autoimmunity, it appears that Tregs are unable to control the pro-inflammatory response(57).

Animal models are available in the study of T1D. The two most common autoimmune models are the non-obese diabetic (NOD) mouse and the bio-breeding diabetes prone rat (BB-DP) (54; 58). Both of these murine models develop spontaneous T1D and mimic many of the genetic polymorphisms and environmental sensitivities witnessed in human disease including increased insulitis (infiltration of lymphocytes into the pancreatic islets) (54; 59). In the NOD mouse, "onset of disease" characterized by the inability to control blood glucose (blood and urine glucose increases), can occur between 13 and 25wks of age. The incidence of T1D in NOD mice may range anywhere from ~60 -90% of females and ~30-60% of males in a colony (1; 54; 55; 60). Similar to human disease, the NOD mouse has shown sensitivity to gluten-high diets which are highly correlated with celiac disease. Celiac disease is can be described as a food allergy to gluten, a protein found in foods derived from cereal grains (61; 62). The similarities between the NOD mouse and human disease allows for a very accurate portrayal in the pathogenesis of T1D from both a phenotypic and immunological standpoint.

T1D accounts for 10-15% of all cases of diabetes and the current treatment is for exogenous addition of insulin and close monitoring of an individual's diet and blood

glucose levels. Transplantation of pancreatic islets from a healthy donor is also an option; however, patients then require long term immune suppression to prevent the immune system from destroying the new islets, which causes its own unique set of problems. Currently the scientific community is split between researching preventative measures against the disease, and a cure for patients with existing T1D. Research into the preventative measures against T1D has been focused on changes in diet, childhood infection, and alterations in commensal bacteria.

The incidence of T1D has a strong correlation with commensal populations. Patients and animal models with T1D exhibit altered microbial composition compared to healthy controls (25; 63; 64). Increases in groups of *Firmicutes*, namely *Lactobacillus*, and *Clostridia* clusters IV, and XIV have been shown to have a substantial impact on the incidence of T1D in experimental mouse models (64-66). Decreased *Firmicutes* and increased *Bacteroidetes* is also seen in human patients with T1D (25). In humans, it is difficult to say with certainty that it is the commensals impacting the incidence of T1D and not the disease influencing the microbiota. However, using experimental mouse models such as the non-obese diabetic (NOD) mouse, or the bio-breeding diabetes-prone rat (BB-DP), we can add exogenous bacteria into the GI tract to witness if changes in disease are affected by commensals.

Commensal bacteria are able to influence more than localized mucosal immunity, the microbial influence is systemic and is important in the immune system as a whole (67; 68). This is important to think about in T1D since the damage is being done in the pancreas and not the GI tract. However, there is the potential for the systemic effects of commensals to alter disease. The pancreas is directly linked to the pancreatic lymph

nodes (PLN), which are considered staging grounds for immune responses. These PLNs are the destination of the lymphatics draining from the GI tract, specifically the transverse (middle) section of the colon (69). It is highly likely that immune cells and signals originating from the GI tract drain directly to the PLNs, directly inflammatory and regulatory responses. This would suggest that alterations in commensal microbiota can have direct consequences on immune responses occurring in the PLN.

It has been shown by numerous groups that by experimentally inoculating mice with different groups of *Firmicutes*, they are able to significantly decrease the incidence of T1D in the murine models. One of the potent bacteria found to inhibit diabetes is *Lactobacillus johnsonii* a common commensal bacterium (42; 66). Inoculation with *L. johnsonii* inhibits disease and causes Th17 proliferation (42). Similarly, a recent studied showed that segregation of NOD mice that were positive for the commensal segmented filamentous bacteria (SFB,) displayed a drastic decrease in the incidence of diabetes and increased Th17 populations compared to those mice that were SFB negative (65). Germ-free NOD mice have been shown to have an increased incidence of T1D and diminished Th17 populations compared to specific pathogen free (SPF) NOD mice (34). This is in concordance with the hygiene hypothesis, suggesting that the expansion of commensal microbiota is required in governing both the mucosal and systemic immune response. However, there are also a number of reports displaying mono-colonized (germ-free mice inoculated with a single species or strain of microbe) NOD mice are more protected from T1D onset than SPF NOD mice (70). This has begun to shape the idea that specific groups of microbes, and not general commensal diversity, have important specific functions in immune development and disease progression.

The role of Th17 cells in T1D and autoimmunity in general has been hotly debated. There is significant evidence to argue both a protective and pathogenic tendency in T1D (65; 71). T1D is traditionally thought of as a classic T helper 1 (Th1) pro-inflammatory mediated disease, along with an auto-reactive cytotoxic $CD8^+$ T cells response (72). Th17 cells are considered to be pro-inflammatory T effector cells, so it would be expected that these pro-inflammatory cells would be activated during a pro-inflammatory immune response like what is seen in T1D.

Th17 cells are a recently described $CD4^+$ T effector cell lineage. These cells are characterized by the production of Interleukin 17 (IL17) and RAR-related orphan receptor gamma (RORγt). Th17 populations are derived from naïve T cells after exposure from tumor growth factors beta (TGFβ) and IL6, and other specific cytokines have been shown to have potent proliferative effects on Th17 populations, such as interleukin 1-beta (IL1β) (73). Alternative mechanisms of Th17 differentiation have been shown through activation by either IL21 or IL23. The Th17 cell populations produce primarily IL17, IL22, and IL21 in response to microbial and fungal pathogens (28). Th17 cells are found at the highest concentration in the gut (74). Th17 and Tregs are thought to be evolutionarily related as both cell types require TGFβ in their differentiation from naïve T helper cells (75).

Th17 cells are somewhat unique compared to the other classical T helper responses like Th1 and Th2 cells. Th1 cells, like Th17, produce a highly pro-inflammatory response defined by production of tumor necrosis factor alpha (TNFα) and Interferon gamma (IFNγ). Th1 cells are characterized by the production of these pro-inflammatory cytokines in addition to the expression of the transcription factor T-box

transcription factor TBX21 (Tbet). Th1 cells are T helper cells that primarily mediate macrophage and $CD8^+$ cytotoxic T cells responses in infection (18; 76). Th2 cells are polarized to produce a classical "humoral" response, activating B cells and antibody production. Th2 cells are characterized by the production of Interleukin 4 and Trans-acting T cell-specific transcription factor GATA-3 (GATA-3) (77). Th17 responses are unique when compared to Th1 and Th2 because Th17s are able to directly attack their targets without the required recruitment of other cells. This is not to say that Th17 populations act alone, Th17s are able to recruit neutrophils and granulocytes to assist in the immune response, as well as allow epithelial cells to produce antimicrobial peptides (20; 78-81). These unique abilities of Th17 cells are most likely due to the natural specificity for gut pathogens and the proximity of Th17 to the mucosal epithelia.

Because of their specificity to be induced by pathogens, Th17s are considered to be highly pro-inflammatory. Th17s have also been shown to play key roles in a number of autoimmune diseases such as multiple sclerosis, Crohn's disease, and rheumatoid arthritis as a highly pathogenic cell subset (27; 28; 72; 73; 82). In arthritis, Th17 cells act in a highly aggressive, pro-inflammatory manner, homing into the sights of arthritic inflammation and expanding upon the damage already caused, though the mechanisms governing this reaction is not well understood (27). As previously stated though, the role of Th17 in T1D is not clear. There is research that suggests Th17s can behave differently depending on how and when the cells were activated. There are publications displaying increased IL17 levels in patients with T1D, while others show that IL17 production in diabetic animals and humans is significantly decreased (42; 71; 83-85). The discrepancy in the research of Th17s in T1D makes it a topic worthy of future studies and a focus in

disease pathogenesis. In order to understand how microbes influence the Th17 populations and thus disease onset, we must design a model to look at how and when commensals are initially altered in individuals who are predisposed to T1D.

T1D differs greatly compared to the much more common Type 2 Diabetes (T2D)(a metabolic disease), in that T2D is when an individual becomes desensitized to insulin. This is not based on the inflammatory response from the immune system (86). The desensitization to insulin is thought to occur through unhealthy lifestyle practices (high sugar and high fat diets, and lack of exercise are common factors) (37; 53). One thing both T1D and T2D have in common, is that they are both influenced through commensal populations. Obese individuals and Type 2 diabetics have altered commensal microbiota compared to healthy lean individuals. In order to combat the rising obesity epidemic in the United States, it will be important to understand how microbiota are influencing obesity and if alterations in commensal flora could assist in recovery from obesity.

GUT MICROBIOTA AND OBESITY

by

KYLE J. WOLF, ROBIN G. LORENZ

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ABSTRACT

The current obesity epidemic clearly has many causes, including the impact of our modern world on both our diet and our lifestyle/physical activity. Although many interventions have been recommended, the prevalence of obesity continues to rise and has forced a re-evaluation of the potential interventions that could have an impact. In recent years it has been definitively shown that microbiota in the gastrointestinal tract are altered in obese individuals. Recent data provide a potential mechanistic understanding of the interactions between microbiota and obesity and allow potential new interventions to the control of obesity to be proposed.

INTRODUCTION

There is currently an epidemic of obesity occurring in the United States, with the most recent study showing a prevalence of 32.2% among adult men and 35.5% among adult women [1]. Significant factors in this epidemic are our diets, which are increasingly high in carbohydrates and fats, and our lack of physical activity [2]. Although critical, these factors clearly are not the whole story; in 2004, Bäckhed et al. [3] proposed an additional mechanism that implicated gastrointestinal (GI) microbiota.

The resident population of microbiota is an essential part of the development and maturity of the host intestinal track and immune system and has therefore come to be considered by some a virtual organ known as the microbiome [4]. The gut microbiome is the totality of microbes (bacteria, viruses, etc.), their genetic elements (genomes), and environmental interactions within the GI track. This microbiome contains over 10 times more organisms than the number of cells in a human body, but unlike other organs its composition is somewhat unstable. The resident populations of bacteria can be altered within 24 h of a dietary change; therefore, obtaining a unified picture of the microbiome can be a challenging proposition [5].

The involvement of the gut microbiota in the obesity epidemic was first suggested by the fact that adult germ- free (GF) (ie, bacteria-free) C57BL/6 mice had a 60% in-crease in body fat content when they were conventionalized (ie, colonized) with cecal microbiota from a healthy, normal C57BL/6 mouse [3]. The mechanism for this increase in body fat content was hypothesized to include the fact that the microbiota would have the ability to regulate energy harvest from food components and therefore alter energy

storage in the host. Since that original publication in 2004, there have now been 138 primary data publications and 60 reviews that are found by a PubMed search for obesity and microbiota. These publications have led to the proposal of three unique mechanisms through which microbiota may impact host obesity, and these are discussed in this review.

EXPERIMENTAL APPROACHES TO THE STUDY OF THE MICROBIOME

The study of the gut microbiome is unique among organ systems, as the microbiome can be shed and replenished and there is the unique opportunity to study this "organ" over long periods of time by obtaining fecal samples from a single individual. This type of analysis has led to the concept of "enterotypes" of the gut microbiome and recent data from 22 individuals have indicated a limited number of host-microbial symbiotic states that might respond differently to diets [6]. However, data from fecal samples have to be interpreted with caution, as several groups have indicated that fecal microbiota communities differ from mucosal- associated bacteria in the GI tract [7, 8]. As the techniques to study, measure, and modify the microbiome are some- what unique to the field and sometimes are not within the usual repertoire of skills other biologists would utilize, we have detailed some experimental approaches in this review.

Bacterial Culture and Identification

Bacterial culture and identification have been extensively used to identify pathogenic or residential bacterial compo- nents of feces or tissue [9]. This method utilizes long-standing phenotypic identification practices such as motility, shape, colony structure, and sugar/metabolite utilization. However, many species remain undefined because there currently is no known method to culture these groups outside of the intestinal tract, and for this reason more advanced methods have been developed using nucleotide amplification.

Fluorescence in Situ Hybridization

Fluorescence in situ hybridization (microscopy-FISH) has historically been utilized to identify bacteria present in tis- sue sections without nucleic acid purification. Briefly, radioactive or fluorescent-tagged nucleic acid-based probes targeting 16S ribosomal RNA are used to permeate pre- served histologic samples and allow for visualization of specific organisms [10]. This procedure has the advantage of precise localization of the bacteria, but does not give quantitative results. A newer method that combines FISH with flow-cytometry (FCM-FISH) no longer allows for tis- sue localization, but when combined with DNA stains is a rapid, reliable, and quantitative method for the analysis of mixed bacterial samples in feces [11].

Quantitative Real-Time Polymerase Chain Reaction

Quantitative real-time polymerase chain reaction (qRT- PCR) is a second method for enumerating the numbers of bacteria present in feces (or tissue samples), but it relies on nucleic acid extraction from the samples. qRT-PCR has very high sensitivity and reproducibility and is very rapid to perform [12]. As with FISH, specific microorganisms are detected based on sequence-specific probes, but only organisms with known sequences can be quantified.

Denaturing Gradient Gel Electrophoresis and 454Pyrosequencing

There are two nucleic acid-based methods that can identify unknown and non-culturable organisms. Denaturing gradient gel electrophoresis (DGGE) is a method of

creating a physical picture of bacterial diversity through a two-dimensional (2D) denaturing gel. DNA is amplified and separated on the 2D gel, where the amplified products migrate according to G:C content and are visualized as unique bands on the gel [13]. Bacteria can be identified through a combination of purification of DNA from the gel and Sanger sequencing methods [14]. Although Sanger sequencing methods can be used to identify numerous bacterial sequences in GI samples, the new high-throughput pyrosequencing technology offers a more rapid and cost-effective method for total microbiome analysis. 454 Pyrosequencing is a method that differs from traditional sequencing in that it does not measure chain termination, but instead relies on the detection of pyrophosphate release upon nucleotide incorporation. This method has now been combined with a novel barcoding approach, which allows simultaneous sequencing of multiple individual samples [15, 16].

Metatranscriptomic Approach and Nuclear Magnetic Resonance

The use of these rapid and extensive sequencing techniques has revealed the enormous diversity of the GI microbiota and its rapidly changing nature [5, 17]. Therefore, recent studies have combined these methods with bacterial gene expression analysis. This metatranscriptomic approach has identified a “core microbiome” at the gene expression, rather than at the organismal lineage, that is associated with obesity [17, 18]. A second method to look at the function of this “core microbiome” is via metabolomics. Nuclear magnetic resonance (NMR) can be used to measure very small molecules, such as individual amino acids, carbohydrates, and lipids/fatty acids. By utilizing the unique magnetic properties from each molecule, NMR measures the

magnetic radiation from a sample and is able to measure hundreds of molecules. This is optimal when attempting to measure small molecules from either serum or even feces [19]. Using this type of technique, microbial metabolites generated during colonic fermentation of food stuffs can be determined and their subsequent impact on blood and tissue metabolites determined [20–22].

Germ-Free Models

The concept of altering commensal populations to enhance the health of humans has been long studied, but has only recently been utilized for manipulations of the obese phenotype. Through the use of mouse models, we are able to extract information about how each individual group of bacteria contributes to the microbiome and to the host. GF models are mice or rats that are completely bacteria free. These mice are optimal as negative controls and also invaluable as a "clean source" when looking to mono-colonize an individual with single bacteria to understand how they impact the host [23–25]. One of the landmark experiments indicating the role of the microbiota in obesity utilized GF mice, which were colonized with an "obese microbiota" or a "lean microbiota." The "obese microbiota" transfer resulted in mice with a greater increase in total body fat and clearly identified the gut microbiota as a contributing factor in the obesity story [26]. Mice that are colonized with a specific known bacteria are termed gnotobiotic (or "known life") and can help us understand the role of specific bacteria in inflammation and disease course [24].

MECHANISMS LINKING MICROBIOTA AND OBESITY

The earliest observations indicated that mouse models of obesity (ie, the ob/ob mouse) had an alteration in the overall proportions of two major divisions of bacteria. Normal humans and mice have 60% to 80% Firmicutes (which are primarily nonculturable, butyrate-producing Clostridium cluster XIVa) and 20% Bacteroidetes (Cytophaga-Flavobacterium-Bacteroides) [27]. However, the obese mouse model (ob/ob) had a 50% reduction in Bacteroidetes and an increase in Firmicutes [27, 28]. A similar decrease in Bacteroidetes and increase in Firmicutes is also seen when C57BL/6 mice are fed a high-fat (HF) diet [28, 29, 30•, 31, 32•]. The reciprocal result is seen in caloric reduction studies [30•]. To determine if these alterations in microbiota contributed to HF diet-induced obesity and insulin resistance, several groups have now fed a HF diet to GF mice. Two groups used GF C57BL/6 mice and both determined that GF animals were protected against both obesity and insulin resistance after HF diet, therefore implying that gut microbiota clearly influence the effects of diet on the host [33, 34]. However, a third group utilized C3H mice and concluded that the absence of intestinal microbiota did not protect mice from diet-induced obesity [35]. Although the reasons for this difference in results are not known, one possibility is that some strains of C3H mice are resistant to the gram-negative bacterial product, lipopolysaccharide (LPS). As increased levels of and response to systemic LPS have been proposed as one of the potential mechanisms of microbiota-influenced obesity (see below), if these mice cannot respond to LPS, then this might explain the discrepancy.

Altered Energy Intake

The resident bacteria within the GI tract are responsible for a significant portion of our energy intake, allowing us access to energy sources that may have otherwise been indigestible. The Firmicutes that are increased in obese mice and humans have been shown to be more adept at breaking down otherwise indigestible carbohydrates and converting them into absorbable energy products [5, 17, 36, 37]. If the microbiota were to shift between lean and obese individuals, it would seem likely that this change would affect the efficiency of energy production/absorption in the GI tract and may either facilitate or inhibit progression toward obesity. When analyzed via gene chips, it was observed that bacteria from obese individuals have increased expression in gene sets specific to motility, transcription, and saccharide metabolism [26].

Taking all of this into account, you can begin to piece together a picture of the path toward obesity. Westernized diets push the commensal populations toward a Firmicutes friendly environment, ending with an overall increase in Clostridia populations. The increased Clostridia populations, acting as more efficient carbohydrate metabolizers, extract greater energy from the caloric intake, allowing for higher energy utilization. That extra energy, if not spent, will ultimately be stored as fat deposits. To better understand the disposition obese individuals have toward increased energy consumption, colonic/cecal health was examined as well as GI metabolites. Upon examination, cecal contents of both mouse and human studies revealed that obese individuals had significantly increased levels of short-chain fatty acids (SCFAs) [36, 38]. SCFAs such as acetate, propionate, and butyrate were in greater abundance in obese individuals. SCFAs are common byproducts of carbohydrate metabolism [39]. It should

not be surprising that most SCFAs (specifically butyrate) producing bacteria belong to Clostridia cluster XIVa and IV [40]. Concentrations of SCFAs were measured in lean and obese mice via NMR. Overall, SCFAs were increased in the urine of obese mice compared with lean [41]. Although acetate has been primarily researched as a factor in high cholesterol, butyrate is highly regarded as an integral component to colonic health [42, 43].

Increased Fatty Acid Metabolism

One of the first publications that implicated the gut microbiota as an environmental factor that regulated fat storage observed that GF C57BL/6 mice conventionalized with normal microbiota had a suppressed expression of intestinal fasting-induced adipose factor/angiopoietin-like protein 4 (Fiaf/Angptl4) [3]. Fiaf/Angptl4 is a target of the nuclear receptor PPAR-α in the liver, but is also expressed in white adipose tissue, skeletal muscle, and intestine [44]. One function of Fiaf/Angptl4 appears to be its ability to raise plasma triglycerides via its ability to inhibit lipoprotein lipase activity. Through the use of Fiaf knockout mice it was established that the suppression of Fiaf/Angptl4 is essential for the microbiota-induced deposition of triglycerides in adipocytes seen after conventionalization of GF mice [3, 33]. It has also recently been shown that the Chinese supplement Rhizoma coptidis can lower body adipose weight and that one potential mechanism for this finding is an inhibition of gut bacterial growth and a subsequent increase in Fiaf/Angptl4 expression in the intestine [45].

Microbiota-Associated Inflammation

For more than 15 years, it has been clear that adipose tissue in obese models has an elevated expression of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α). This has been reported for multiple rodent models of obesity, including the diabetes (db/db), obese (ob/ob), and tubby (tub/tub) mice and the Zucker (fa/fa) rat, as well as obese female patients [46, 47]. This TNF-α is primarily made by adipose tissue macrophages and it mediates insulin resistance through its ability to decrease the tyrosine kinase activity of the insulin receptor [48, 49]. Diets known to induce obesity and insulin resistance, such as the HF diet, can increase expression of TNF-α [50]. However, the induction of obesity and insulin resistance are ameliorated if mice are deficient in either TNF-α or the TNF-αR [51, 52].

But why does a HF diet and/or obesity lead to a chronic inflammatory state? Initially, the hypothesis was that increased nutritional fatty acids could lead to activation of the toll-like receptors (specifically TLR4) and subsequent inflammation [53]. However, as discussed above, a HF diet shifts the intestinal microbiome very quickly to a decrease in Bacteroidetes and an increase in both Firmicutes and Proteobacteria [5, 29]. One proposal is that this alteration in intestinal microbiota could lead to increased activation of TLR4, and therefore be partially responsible for the chronic inflammatory state seen in obese individuals.

To address this question, Cani et al. [54] initially asked if a HF diet would increase plasma concentrations of LPS, a TLR4 ligand made by gram-negative bacteria. This low level of LPS in the plasma has been termed “metabolic endotoxemia.” The data indicated that a HF diet in C57BL/6 mice did increase levels of plasma LPS and that

direct infusion of LPS mimicked the physiologic effects of a HF diet [54]. Moreover, the effects of the HF diet were ameliorated in mice lacking a component of the TLR4 receptor complex—CD14. This same group went on to implicate intestinal bacteria in the increased plasma concentrations of LPS through the use of oral broad-spectrum antibiotics, which significantly decreased the levels of intestinal microbiota and the levels of plasma LPS [55]. Additionally, the administration of a prebiotic (oligofructose) resulted in an increase in gram-positive intestinal bacteria (including Bificobacteria) and a decrease in plasma LPS [56].

These observations allow the consideration that plasma LPS might be a biomarker of the status of obesity-prone individuals or the impact of therapeutic probiotics on obesity-associated intestinal microbiota. Several recent studies indicate that the answer may be yes. The first study investigated serum LPS activity in more than 7000 subjects with a 10-year follow-up. This study concluded that both previously diagnosed diabetic patients, as well as patients with newly diagnosed diabetes (incident diabetes) had higher LPS levels than nondiabetes individuals [57]. In addition, therapeutics such as oral probiotics (Lactobacillus casei), when given to mice with diet-induced obesity, can improve not only insulin resistance, but can also reduce plasma levels of LPS-binding protein (a marker of endotoxemia) [58].

This involvement of TLR activation was confirmed in a Sprague-Dawley rat model fed a HF diet, which can exhibit either an obesity-prone or an obesity-resistant phenotype. All the obesity-prone rats, but none of the obesity-resistant rats, had increased TLR4 activation [37]. Additional support comes from an experiment utilizing gnotobiotic and conventional Swiss Webster mice, which demonstrated that conventionally raised

mice on the HF diet had increased hepatic levels of the inflammatory marker serum amyloid A, but that this effect of the HF diet was ameliorated in MyD88-deficient mice (MyD88 is a component of the TLR signaling pathway) [59]. Although TLR4 has been the receptor most implicated in this mechanism, it has also been recently shown that mice lacking TLR5 have metabolic syndrome [60]. This is at least in part due to an altered intestinal microbiota, as transfer of the microbiota from a TLR5-deficient mouse to a wild-type gnotobiotic mouse conferred metabolic syndrome to the recipients [60]. Intriguingly, a recent study on insects has also demonstrated a metabolic syndrome that is induced by a protozoan intestinal infection [61].

The mechanism for this increased plasma LPS from intestinal microbiota is probably increased intestinal permeability. C57BL/6 mice fed a HF diet have increased permeability to small molecules, such as FITC-dextran and also have decreased or altered expression of the tight junctional proteins occludin and zonulin-1 [55]. Similar findings were seen in HF diet-raised obesity-prone Sprague-Dawley rats, but not in obesity-resistant rats [37]. The impact of intestinal microbiota on permeability has recently been shown to involve glucagon-like peptide-2 (GLP-2) [62]. If ob/ob mice are given a GLP-2 agonist, then intestinal permeability is lowered, and tight-junction integrity and the systemic inflammatory phenotype is improved. As GLP-2 has receptors not only in the intestine but also in the brain, it is an intriguing possibility that there is a gut-brain axis that might potentially link intestinal microbiota to feeding behaviors [63]. Intracerebroventricular infusion of GLP-2 can inhibit food intake and, consequently, alterations in intestinal microbiota may have long-term effects on the gut-brain axis and body weight homeostasis [64].

ESTABLISHMENT OF THE MICROBIOME

It appears clear that the microbiota can impact energy metabolism and be associated with obesity and metabolic endotoxemia. If so, then the questions arise of: How do we acquire our microbiota? What is known to influence the microbiota present? Can we modify our microbiota in a predetermined fashion? Many studies have shown that the initial bacterial colonization of the intestine is at birth, primarily from the mother and/or other caregivers [65, 66]. However, newer work has now focused on the impact of microbiota on weight gain during pregnancy and on whether this impacts the subsequent weight of the child later in life.

The majority of this work has been published by a group from Finland, who have primarily utilized the techniques of FCM-FISH and qRT-PCR to show that if a mother was overweight before pregnancy, then she had significant increases in the numbers of fecal Bacteroides-Prevotella group (FCM-FISH) and Staphylococcus aureus (qRT-PCR) from the first to the third trimester [67]. The impact of this alteration in maternal microbiota on the microbiota of 1- and 6-month-old infant stool samples indicated that the infant fecal microbial composition was influenced by the maternal weight gain during pregnancy and by maternal body mass index (BMI) during early pregnancy [68]. Six-month-old infants from mothers with a BMI greater than 25 kg/m2 had more fecal Clostridium histolyticum (FCM-FISH) and Clostridium leptum (qRT-PCR), but less Bifidobacterium genus (qPCR). However, in contrast to the findings in the mothers, the levels of Bacteroides-Prevotella decreased in 6-month old infants from mothers with high BMI or who had excessive weight gain. In support of this decrease in Bacteroides-

Prevotella in offspring of overweight mothers, the offspring of rats fed a HF diet also had fewer Bacteroides-Prevotella in the jejunum [69]. Additionally, rats that were exposed to pre-weaning overnutrition also had lower numbers of Bacteroides-Prevotella [70].

To determine if this altered microbiotal composition actually has any correlation with weight in children, this same group of children was followed until age 7 years [71]. None of the bacterial groups found to be significant in overweight mothers or their offspring were correlated with increased weight gain in childhood; however, increased levels of S. aureus during infancy did correlate with a child being overweight at age 7 years. A second study also investigated whether factors known to alter intestinal microbiota have an effect on body weight at age 7 years [72]. Factors investigated included delivery mode, maternal prepregnancy BMI, and early exposure to antibiotics (<6 months of age). Children from mothers with a high pre-pregnancy BMI were more likely to have overweight children at age 7 years; however, this study did not correlate these findings with the intestinal microbiota composition.

Although these studies appear to indicate that our microbiota is established very early in life, there are also studies that indicate that microbiota can be manipulated by various weight loss techniques. In adolescents subjected to an obesity treatment program including both calorie restriction and increased physical activity, there was an increase in the Bacteroides-Prevotella group and a decrease in Clostridium spp [73, 74]. In adults who have undergone Roux-en-Y gastric bypass the Bacteroides-Prevotella and Escherichia coli species increased 3 months after surgery, whereas lactic acid bacteria (including Lactobacillus/Leuconostoc/Pedicoccus group and Bifidobacterium genus) decreased [75, 76].

If weight loss is associated with altered microbiota and if the obesity phenotype can be transferred by fecal microbiota, then it could be proposed that a bacteria might exist that could induce a lean phenotype. This concept has been most extensively tested through the administration of probiotics. Probiotics are live microorganisms that are thought to be beneficial to the host. The most common types are lactic acid bacteria and bifidobacteria and are often found in yogurt or dietary supplements. Studies using Lactobacillus rhamnosus GG, Lactobacillus plantarum strain 14, Lactobacillus paracasei ssp paracasei F19, and Bifidobacterium breve B-3 all demonstrated that probiotic intervention appears to have a beneficial effect on obesity [77–80]. The probiotics appear to work by reducing mean adipocyte size, inhibiting lipoprotein lipase, and improving insulin sensitivity [77–79].

CONCLUSIONS

Obese individuals and models all show a propensity for a dysbiosis that includes an increased ratio of Firmicutes:Bacteroidetes. This alteration in the proportion of bacteria in the lumen of the GI track affects not only the ability of the microbiome to generate energy sources from indigestible carbohydrates, but also the deposition of triglycerides in adipocytes. This altered bacteria also appears to have an increased exposure to the host immune system due to a leaky intestinal barrier and induces a constant state of chronic inflammation. This impact of the microbiota on obesity has led to multiple preliminary studies on the use of "good" probiotic bacteria to alter the obese phenotype. These studies have all shown that probiotic intervention has a beneficial effect and may lead to novel interventions for overweight or obese human patients.

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ACIDIC BEVERAGES ALTER THE COMMENSAL MICROBIOTA AND DECREASE THE RISK OF TYPE 1 DIABETES IN THE NOD MOUSE MODEL

by

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ABSTRACT

Infant formula and breastfeeding are environmental factors that influence the acidity of newborn diets and the subsequent incidence of Type 1 Diabetes (T1D). To determine if altering the intestinal microbiome is one mechanism through which these factors play a role in T1D, we placed NOD/ShiJt mice on neutral (N) or acidified H_2O and monitored the impact on microbial composition and T1D incidence. NOD-N mice had increased development of T1D, while exhibiting a decrease in Firmicutes and an increase in Bacteroidetes, Actinobacteria, and Proteobacteria as early as 2-weeks of age. NOD-N mice also had decreased Foxp3 and IL17 expression. Our data clearly indicates that slight changes in diet alter the intestinal microbiome, the presence of protective Th17 and Treg cells, and the incidence of T1D. This data suggests that early dietary manipulation of intestinal microbiota may be a novel mechanism to delay T1D onset in genetically pre-disposed individuals.

INTRODUCTION

The non-obese diabetic (NOD) mouse model of Type 1 Diabetes (T1D) shares genetic and environmental pre-dispositions with human patients (1; 2). Current evidence indicates that limited exposure to breastfeeding is one of these environmental risk factors (3). Dietary and environmental factors can modify the intestinal microbiota; however, whether it is actually an environmentally-induced dysbiosis that leads to changes in T1D incidence is not well defined (4-8). Understanding the potential pathogenesis of dietary-associated dysbiosis may be a promising avenue to help prevent T1D (9-11).

The intestinal microbiota in murine T1D models and human patients is altered in comparison to non-diabetics (12-14). This dysbiosis may impact the presence of interleukin-17 (IL17) producing T-helper cells (Th17) and regulatory T cells (Tregs), as the development of these cells is heavily reliant on the microbial composition (15-19). Previous publications are in disagreement on whether Th17s are protective or pathogenic in T1D; however, both animals and patients with T1D display imbalances between Th17 and Treg responses (8; 17; 20-24). The connection between the microbiota, Th17s, and Tregs in T1D patients is not well defined. Although T1D is a disease of the pancreas, mucosal immune responses to dietary and bacterial antigens may impact the pancreas as areas of the transverse colon drain directly into the pancreatic lymph nodes (PLNs) (25-28).

Breastfed infants have a lower gut pH (acidified environment) and a higher lactic acid concentration when compared to infants fed cow's milk or formula (29). We hypothesized that this acidified environment shapes the intestinal microbiome,

consequently modifying the mucosal and systemic immune responses, and ultimately impacting the incidence of T1D. NOD/ShiJt (NOD) mice from The Jackson Laboratory (Bar Harbor, ME) are routinely maintained on water acidified with hydrochloric acid (pH 2.8-3.1) to prevent the growth of microorganisms. Mice maintained on acidified H_2O live longer lives and gain weight slower than mice maintained on neutral (pH ~7.0) H_2O, but this has not been reported to have any significant affect on immune function (30-32). Therefore, to test our hypothesis, we switched NOD breeding pairs from acidified to neutral water, and subsequently studied the microbiome of their offspring, their mucosal immune responses, and their incidence of T1D. As breastfed babies have a decreased risk of development of T1D later in life (33), this experiment offered the unique opportunity to determine if the intake of liquids that impact the acidity of the gut environment could alter not only mucosal microbiota and immune responses, but also a systemic autoimmune disease such as T1D.

RESULTS

NOD-N mice have a higher incidence of T1D

Female NOD mice on neutral (NOD-N) and acidified (NOD-A) H_2O were followed for 30wks to ascertain the incidence of diabetes. NOD-N mice displayed a significantly higher incidence of T1D. Only 11% of NOD-N mice remained T1D free at 30wks of age compared to 46% of NOD-A mice (Figure 1a). NOD-N mice at 20wks displayed increased insulitis compared to NOD-A mice (Figure 1b). Following a similar trend, NOD-N males also had a greater incidence of T1D at 30wks (55% n=9) compared to NOD-A males (36% n=11). It has recently been reported that caging, often influences differences in microbiota (34). Therefore, to confirm that changes in the incidence of T1D seen in Figure 1 were not caused by a caging/founder effect, litters from multiple breeding pairs on acidified H_2O were split at weaning, raising half of each litter on either acidified H_2O, or switching them onto neutral H_2O (NOD-AtoN). NOD-AtoN mice displayed a 77% incidence of diabetes, a result that is intermediate between mice raised on neutral H_2O (89%) and those raised on acidified H_2O (58%) (Figure 1a). This demonstrates that the water pH is directly correlated with T1D incidence; however, as the change in incidence was not complete, it also implies that the timing of the switch may also be critical, as these NOD-AtoN mice were exposed to the altered water source after weaning (instead of from birth).

NOD-N mice have a higher GI pH and fewer Firmicutes

To determine if the acidity of drinking water can actually alter the gastrointestinal (GI) luminal environment, the pH of the GI tract of 5wk old female NOD-A and NOD-N mice was measured. NOD-N mice displayed a nearly 2-fold decreased concentration of H^+ ions and thereby, a significantly higher pH in the duodenum, jejunum, cecum, and colon compared to NOD-A mice (Supplemental Figure 1). Potential alterations in microbial diversity associated with this altered pH was initially analyzed via Denaturing Gradient Gel Electrophoresis (DGGE). 10wk NOD-N mice demonstrated dramatic shifts in microbial communities compared to NOD-A mice (Figure 1c). NOD-A and NOD-N mice only share 27% band similarity compared to the 60.7% and 80.8% banding similarity (respectively) shared within each group (Figure 1d). NOD-AtoN and NOD-N mice share a higher degree of similarity (53.7%) compared to NOD-A mice. Unique bands were removed and sequenced (Figure 1c). Sequenced bands were discovered to belong to the groups of bacteria *Lactobacillus*, *Bacteroides*, and *Clostridia* cluster XIV. Although not quantitative, it is apparent that there are decreases in populations of both *Lactobacillus* and *Clostridia* populations in NOD-N mice.

In order to quantitate these results, fecal DNA from 10wk female NOD-A (5) and NOD-N (5) mice from 2 different litters/mothers per group was purified and analyzed via 454 pyrosequencing (Supplementary Table1). NOD-A mice, although non-significant, had numerically higher richness and diversity. Distinct grouping of the treatments based on sequencing information was shown via partial-least-squared discriminant analysis (PLS-DA) in Figure 2a. The goodness of fit and predictive value of the model was tested using R^2 (0.97) and Q^2 (0.85), respectively. Genera most characteristic of NOD-A and NOD-N mice were shown in Figure 2b with significantly associated genera highlighted

per treatment. NOD-N mice displayed significantly decreased levels of Firmicutes, the phylum in which *Lactobacillus* and *Clostridia* genera belong. Less than 41% of the reads in NOD-N fecal DNA belonged to the phylum Firmicutes while Firmicutes in NOD-A samples were responsible for 70% of reads (Table 1). NOD-N mice were shown to have increased populations of the phylum Bacteroidetes which contains the genus *Bacteroides*. 31% of the reads from NOD-N fecal DNA belonged to Bacteroidetes compared to only 23% in the NOD-A fecal DNA. In agreement with previous studies, phyla Actinobacteria and Proteobacteria were higher in the NOD-N than NOD-A mice (6.57% vs 1.71%). The shifts observed in the microbiome of pre-diabetic NOD-N mice were similar to previously described shifts in diabetic patients(35). Additional data showing differences between NOD-A and NOD-N mice can be found in Supplementary Tables 2, 3 and 4.

To evaluate if the differences in bacterial populations observed at 10wks between NOD-N and NOD-A mice were maintained throughout the lifespan of the mouse, fecal DNA from NOD-N and NOD-A mice was subjected to qRT-PCR specific for individual groups of bacteria. The presence of *Lactobacillus sp.* and *Clostridia coccoides* (both Firmicutes), and *Bacteroides sp.* were investigated, as well a determination of total bacterial DNA to identify if there was a change in bacterial burden. Time points were chosen to represent pre-insulitis (5wks), post-insulitis, but before overt diabetes (10-13wk), and advanced progression of disease (16-20wk). There was little difference in the total bacterial populations between NOD-N and NOD-A mice across time (Figure 3a). However, both *Lactobacillus sp.* and *C. coccoides* were significantly decreased at multiple ages in NOD-N mice compared to NOD-A (Figure 3a). Conversely, the NOD-N

feces had increased populations of *Bacteroides sp.* by 13wks (Figure 3a). In the NOD-AtoN mice described previously, no significant differences were seen in the total bacterial numbers, but *Lactobacillus sp.* and *C. coccoides* from NOD-AtoN mice were significantly lower than their NOD-A counterparts and mirrored observations from NOD-N mice raised from birth on neutral water. *Bacteroides* populations were much more similar to NOD-A mice and were significantly lower than the NOD-N mice at 13wks and 16wks (Figure 3a). Do to the observation that the NOD-AtoN mice did not completely mirror the T1D susceptibility of the NOD-N mice, we studied 2wk neonatal mice to determine if there was a microbial dysbiosis early in life. At 2wks, there is a significant dysbiosis in NOD-N mice with >1000 fold decrease in *C. coccoides* and >50% increase in *Bacteroides sp.* (Figure 3b).

The level of Foxp3 expression is decreased in NOD-N Tregs

There is increasing evidence that autoimmune diseases, including T1D, are controlled by $CD4^+Foxp3^+$ Tregs (36). However, our results indicated that there was no discernible difference in the percentage or total number of $CD4^+Foxp3^+$ lymphocytes isolated from the spleen (Sp) or small intestinal (SI) and colonic (Co) lamina propria (LP) of 2wk NOD-A and NOD-N mice or in the Sp and Co LP of 8-10wk NOD-A and NOD-N mice (Figure 4a, Supplemental Figure 2e). A significant increase in the percentage and total number of $Foxp3^+CD4^+$ Tregs in the SI LP of NOD-N mice was seen at 8-10wks (Figure 4a, Supplemental Figure 2e). Recent data implicates that the level of Foxp3 expression in Tregs reflects their functional status, with increased expression being directly associated with increased regulatory function (37). Our data demonstrates that at both 2wks and 8-10wks, Foxp3 expression in $CD4^+$ splenocytes and SI LP lymphocytes

is significantly decreased (Figure 4b). This indicates that in NOD-N mice, regardless of whether there is a similar (Sp) or higher (SI LP) number of Tregs, these cells should have decreased functional activity due to their lower Foxp3 expression. This conclusion is supported by the observation that NOD-A mice have an increase in colonic gene expression of the regulatory cytokine IL10 from 2-20wks, while there is no change in NOD-N mice (data not shown).

NOD N mice have decreased expression of intracellular IL17 in $CD4^+$ T-cells

Several recent publications have demonstrated that components of the commensal microbiota, such as the common Firmicutes species, *Lactobacillus* and *Clostridia*, are able to induce IL17 expression and Treg cell expansion (15; 17). However, the role of IL17 in autoimmunity and T1D is controversial, with reports showing both protective and pathogenic roles (17; 19; 38; 39). To elucidate the effects of the dysbiosis in NOD-N mice, the SI and Co LP and Sp from 2wk and 8-10wk NOD-A and N mice were analyzed via flow cytometry for the presence of $CD4^+$ lymphocytes producing either IFN□ or IL17. The only significant difference in the percentage or total number of $CD4^+$ cells was an increase in the NOD-N SI LP at 2wks of age (Figure 5a and Supplemental Figure 2b). However, significant decreases were seen in the percentage of $CD4^+IL17^+$ cells within the Sp, SI and Co LP in 2wk NOD-N mice compared to NOD-A (Figure 5b). A decrease in absolute numbers of $CD4^+IL17^+$ cells was also seen in the spleen and Co LP of NOD-N mice (Supplemental Figure 2c). By 8-10wks, decreased levels of $CD4^+IL17^+$ T cells were only seen in the SI LP of the NOD-N mice. There was no significant difference in the mean fluorescent intensity (MFI) of the IL17 expression (data not

shown). When the prototypical Th1 cytokine, IFN□, was analyzed, the only significant change was an increase in $CD4^+IFN\square^+$ cells in the NOD-N Sp at 2wks (Figure 5c). To determine the relationship between the Th17 and Th1 populations, we calculated the ratio of cells expressing IL17 to those expressing IFN□. This analysis clearly demonstrates that NOD-N mice have dramatically fewer $CD4^+$ T cells expressing IL17 (Figure 5d). This altered ratio appears to be due to an increased percentage of $CD4^+$ cells from NOD-A mice producing IL17 at 2wks. By 8-10wks, there is no difference in the ratio of IL17/IFN□ between the NOD-N and NOD-A mice.

DISCUSSION

Microbial colonization of our gastrointestinal tract begins at birth, is primarily derived from maternal transmission, and profoundly impacts the development of the mucosal immune system (34; 40-42). The composition of this microbiota is shaped by the newborn diet, with breastfed babies having a more stable microbiome characterized by lower (more acidic) pH and higher lactic acid concentrations (29; 43). As breastfed babies have a lower incidence of T1D, we designed a set of experiments to determine if this alteration in T1D risk was secondary to changes in the microbiota and its subsequent impact on immunity. Our data demonstrates the increased incidence of T1D in NOD-N mice is directly correlated with the changes in commensal microbiota caused by the shift from acidified to neutral H_2O. This shift in microbiota is best characterized by a decrease in Firmicutes (including *Lactobacillus sp.* and *Clostridia sp.*) and increases in *Bacteroides sp*. prior to disease initiation.

There are thought to be two distinct phases of disease progression in NOD mice (44; 45). Phase one occurs at 3-4wks of age and consists of a peri-insulitis, while the second stage (8-12wks) involves progression to invasive insulitis and destruction of β-cells. Our data indicates that the dysbiosis in 2wk old mice caused by changing the gut pH (secondary to altered water sources for the dam) occurs prior to this first phase and implicates dysbiosis as a initial disease trigger. This idea is reinforced by the observation that the incidence of T1D can be shifted by switching NOD mice from acidified to neutral water at weaning, but that the shift is not complete. Our data also support the conclusion that there are environmental exposures that occur early in life that play an

important role in the risk of development of T1D, as it appears that the changes in the *Lactobacillus sp., C. coccoides,* and *Bacteroides sp.* early in life are causing an imbalance in the mucosal immune system leading to increased susceptibility to T1D. Other publications have demonstrated that expansion of *Bacteroides sp.* and decreases in *Clostridia sp.* result in decreased colonic health and increased epithelial leakage, which lead to mucosal and systemic inflammation (46; 47).

Others have shown that alterations in commensal microbial populations are strongly correlated with changes within the immune system (18). Our data indicate that 2wk old NOD-N mice have a large decrease in $IL17^{+}CD4^{+}$ cells, when compared to NOD-A mice. These findings suggest that prior to the first phase of disease, Th17 populations confer protection against T1D. This is consistent with multiple studies that have concluded that increased Th17 cells, induced by either various Firmicutes (including segmented filamentous bacteria (SFB) and *Lactobacillus johnsonii)* or by adjuvant immunotherapy, can delay the onset of T1D (17; 19; 38). However, other studies in diabetic patients have shown that children with new onset T1D have higher numbers of Th17 cells (24; 48). In addition, inhibition of IL17 after the initial phase of peri-insulitis (by either antibodies or diet change) has been shown to protect mice from T1D (8; 39). An explanation for these apparent contradictory reports may be that the reports on 'protective' Th17 cells were from models where these cells were present prior to the onset of T1D, while the 'pathogenic" Th17 were described in patients with diagnosed disease. This observation leads to the innovative paradigm that Th17 cells initially play a protective role, being induced by Firmicute colonization and contributing to the gastrointestinal epithelial barrier. However, after the stabilization of the microbiota and

the subsequent formation of the adult immune system, then high levels of Th17 cells can contribute to disease. It has been proposed that there are two different types of Th17 cells (classical and alternative) that are differentially developed in the presence of either TGF□ plus IL6 (classical/nonpathogenic) or IL6, IL1□, and IL23 (alternative/pathogenic) (49). The colons of NOD-N mice show higher expression of the genes for both IL1□ and IL6 at 2 weeks of age (compared to 2 week NOD-A colons, data not shown), which could indicate that even the small number of Th17 cells present may be of the alternative/pathogenic type, instead of the classical/nonpathogenic type. This sub-classification of Th17 cells will need to be further explored in order to completely determine their role in disease.

The mechanism through which classical Th17 cells can protect NOD mice from T1D is unknown. Th17 cells can up-regulate intestinal epithelial barrier function, as well as helping to promote effective contact-dependent suppression by Treg cells (50-52). While we did not witness any changes in frequency of Tregs between the NOD populations, we did find a significant decrease in the expression of Foxp3 in NOD-N Tregs. Lower levels of Foxp3 expression have been correlated with decreased function of Tregs in other models and this potential decreased in function may also contribute to disease in the NOD model, as functional defects in Tregs have also been described in patients with T1D (24).

We have noticed that NOD mice raised on acidified H_2O in our facility have a lower incidence of diabetes compared to data from Jackson laboratories. This is probably secondary to microbiota differences between facilities. As there has been no previous

complete sequencing of the microbiota of the NOD mouse, we are currently unable to compare the microbiota of our mice with other colonies. A recent publication demonstrated that natural colonization of NOD mice with SFB, caused attenuated disease progression/onset in mice; however, our NOD mice were tested and found to be SFB negative (19).

The potent changes seen in the incidence of T1D in NOD mice induced by something as simple as the pH of the water they drink, clearly strengthens the hypothesis that subtle alterations within the GI microbiota early in development can have a significant impact on disease. It appears that the largest impacting factor in disease comes from the population shift between Firmicutes and Bacteroidetes within the GI tract and the subsequent induction of protective Th17 and Treg cells. Our research would suggest that while changes in microbiota initiated at weaning can alter the incidence of disease, the protective effect is truncated compared to changes made at or before 2wks of age. Therefore, it may be necessary to expose children to protective bacteria prior to or immediately at the time of birth by giving pregnant mothers bacterotherapy. Infants whose mothers were taking probiotics before birth are colonized with the probiotic strains for at least 6 months after birth (40; 53). This could prove to be a novel and effective method in delivering protective bacteria to infants who may carry genetic susceptibility for diseases like T1D.

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AUTHOR CONTRIBUTIONS

KJ.W. jointly conceived the study with R.G.L., performed all experiments, and prepared the initial draft of the manuscript; E.K. and R.H. did the 454 sequencing, analyzed the sequencing data, and helped prepare the manuscript; S.M.T. completed lamina propria preparations on 2wk animals; R.G.L. analyzed the data and edited the manuscript.

FIGURES

Figure 1

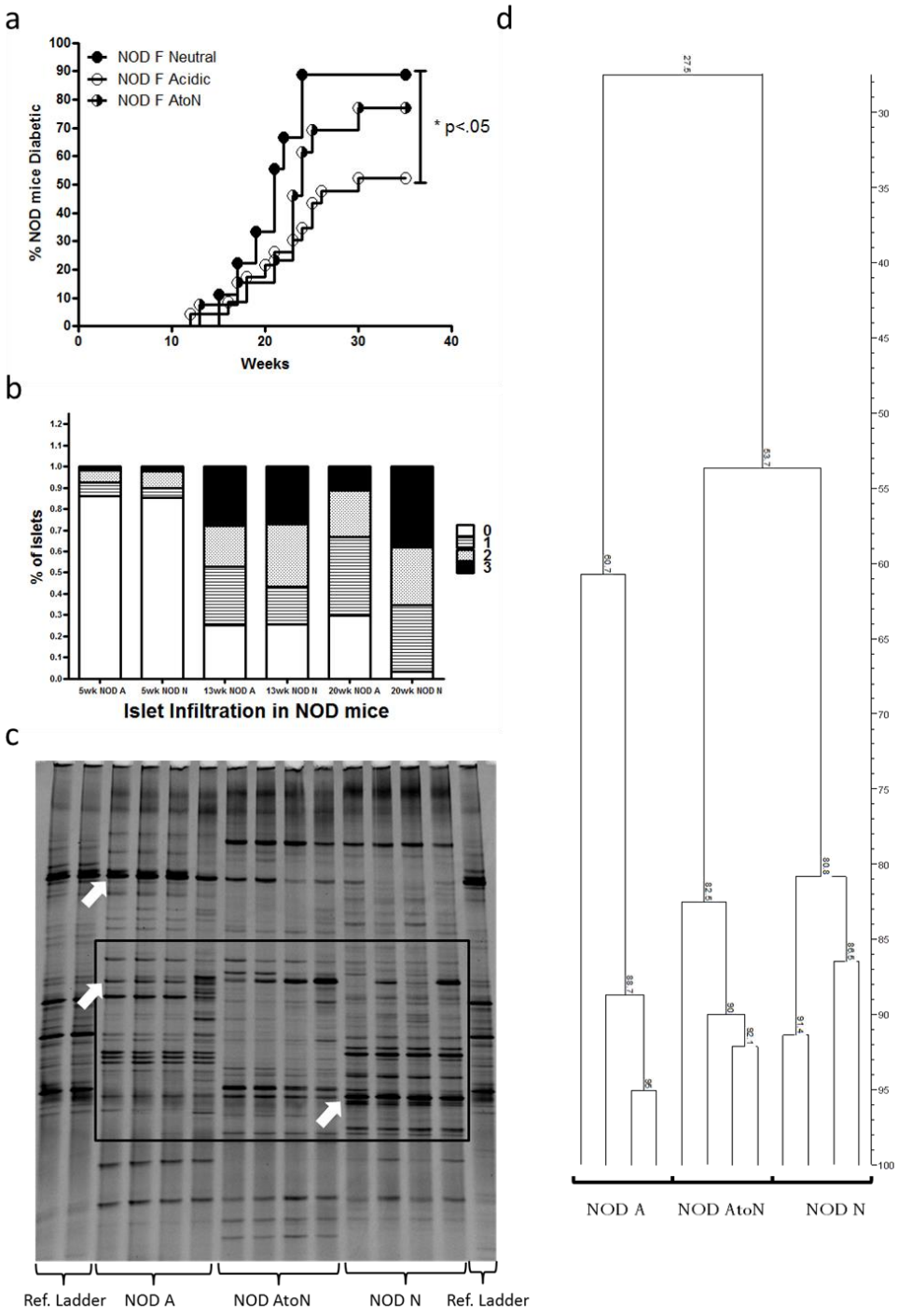

Figure. 1. NOD mice on neutral drinking water have an increased incidence of T1D and alterations in the diversity of their fecal microbiota. a) Incidence of T1D in NOD female mice on either neutral water (NOD-N, open circles, n=9), acidified water (NOD-A, filled circles, n=23) or water switched from acidified to neutral at weaning (NOD-AtoN, half-filled circles n=13). Data represents 3 individual experiments. Significance was determined by the Mantel-Cox Test. b) Analysis of lymphocytic infiltrate and islet destruction in NOD mice on neutral or acidified water. 0= no infiltration, 1= peri-insulitis, 2= <50% of islet infiltrated, 3= >50% of islet infiltrated. A minimum of 30 islets were counted per group with at least 7 mice from each group counted. c) A representative DGGE analysis of banding patterns from 10wk female NOD-A (left), NOD-AtoN (middle) or NOD-N (right) mice. *Top arrow* points out a band identified by sequencing as *Lactobacillus johnsonii*. *Middle arrow* points out a band identified as *Clostridia* Cluster XIV species. *Bottom arrow* points out a band identified as *Bacteroides sp*. d) Banding similarity analysis of the representative samples from 10wk NOD-A, NOD-AtoN and N mice indicates that their microbiota share a 27.5% similarity. NOD-AtoN and NOD-N mice share a higher degree of similarity (53.7%) compared to NOD-A mice. Banding analysis was conducted using the Pearson correlation analysis.

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Figure 2

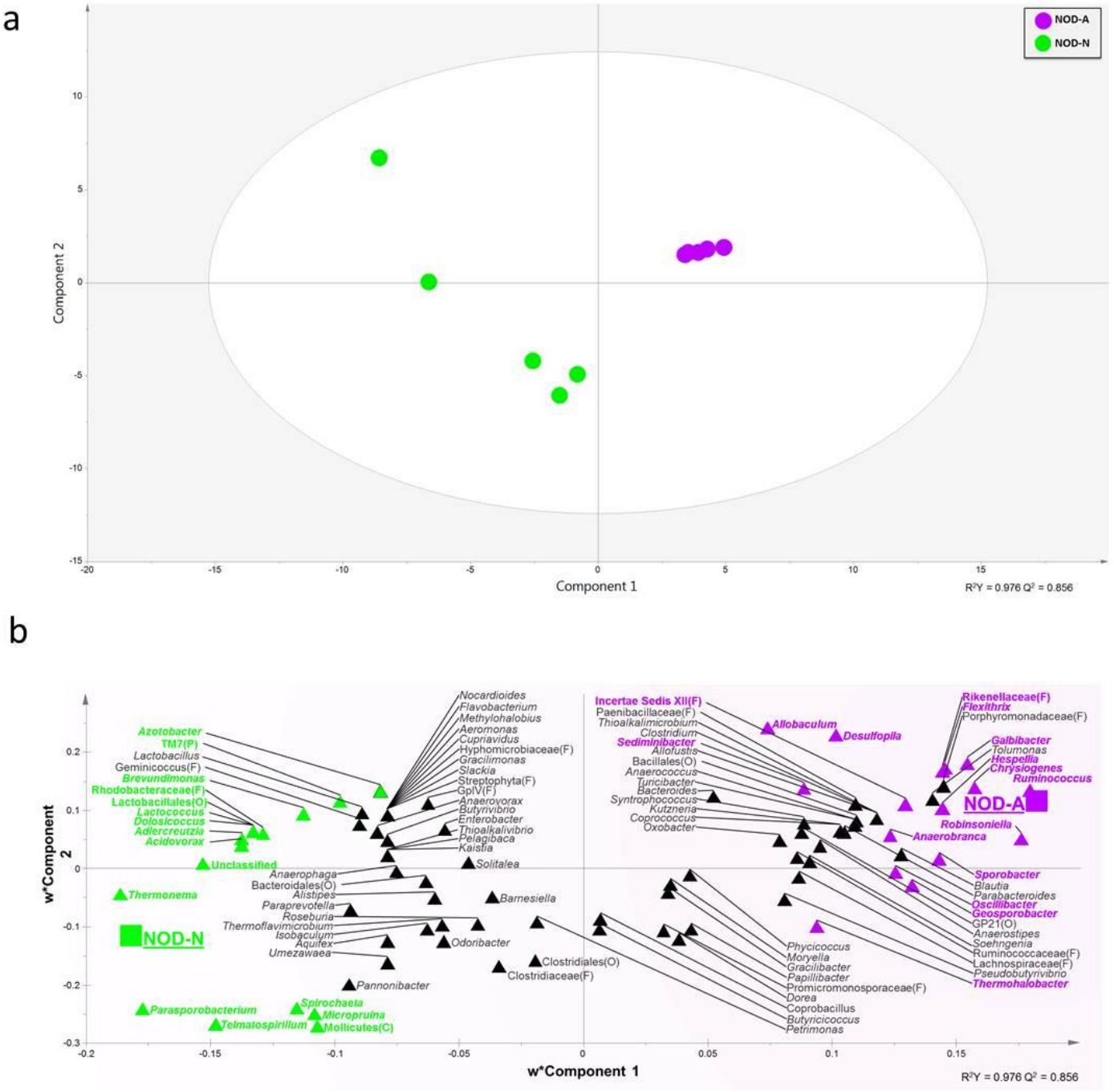

a
NOD-A
NOD-N
Component 2
Component 1
R²Y = 0.976 Q² = 0.856
b
w*Component 2
w*Component 1
R²Y = 0.976 Q² = 0.856
Nocardioides
Flavobacterium
Methylohalobius
Aeromonas
Cupriavidus
Hyphomicrobiaceae(F)
Gracilimonas
Slackia
Streptophyta(F)
GpIV(F)
Anaerovorax
Butyrivibrio
Enterobacter
Thioalkalivibrio
Pelagibaca
Kaistia
Solitalea
Barnesiella
Odoribacter
Clostridiales(O)
Clostridiaceae(F)
Pannonibacter
Azotobacter
TM7(P)
Lactobacillus
Geminicoccus(F)
Brevundimonas
Rhodobacteraceae(F)
Lactobacillales(O)
Lactococcus
Dolosicoccus
Adlercreutzia
Acidovorax
Unclassified
Thermonema
NOD-N
Anaerophaga
Bacteroidales(O)
Alistipes
Paraprevotella
Roseburia
Thermoflavimicrobium
Isobaculum
Aquifex
Umezawaea
Parasporobacterium
Telmatospirillum
Spirochaeta
Micropruina
Mollicutes(C)
Incertae Sedis XII(F)
Paenibacillaceae(F)
Thioalkalimicrobium
Clostridium
Sediminibacter
Allofustis
Bacillales(O)
Anaerococcus
Turicibacter
Bacteroides
Syntrophococcus
Kutzneria
Coprococcus
Oxobacter
Allobaculum
Desulfopila
Rikenellaceae(F)
Flexithrix
Porphyromonadaceae(F)
Galbibacter
Tolumonas
Hespellia
Chryslogenes
Ruminococcus
NOD-A
Robinsoniella
Anaerobranca
Sporobacter
Blautia
Parabacteroides
Oscillibacter
Geosporobacter
GP21(O)
Anaerostipes
Soehngenia
Ruminococcaceae(F)
Lachnospiraceae(F)
Pseudobutyrivibrio
Thermohalobacter
Phycicoccus
Moryella
Gracilibacter
Papillibacter
Promicromonosporaceae(F)
Dorea
Coprobacillus
Butyricicoccus
Petrimonas

Figure. 2. Impact of neutral or acidified drinking water on the fecal microbiota composition of NOD-N and NOD-A mice. Pyrosequencing data were subjected to partial least squares discriminant analysis (PLS-DA). a) Score scatter plot representing individual animals from each treatment, grouped based on the composition of fecal microbiota. The R^2 and Q^2 of the model were 0.97 and 0.85, respectively. b) Bacterial taxa plotted using weighted PLS component 1 and 2. Genera in the plot closer to either treatment are more strongly associated to it. Genera found to significantly contribute to the model prediction are shown in green (NOD-N) and magenta (NOD-A). When a sequence could not be classified to the genus level, the closest level of classification was given, preceded by F (family), O (order), C (class), or P (phylum).

Figure 3

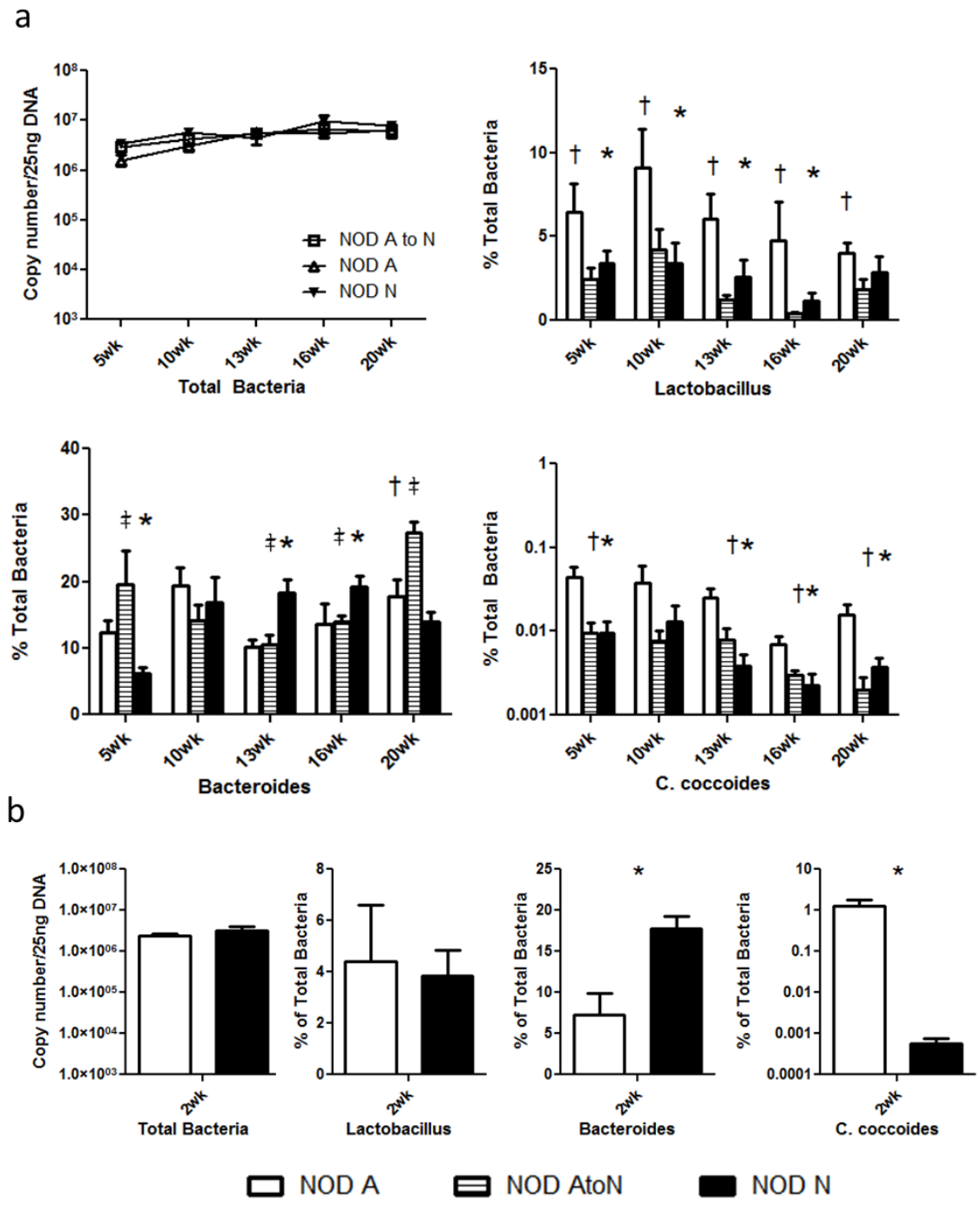

a
Copy number/25ng DNA
NOD A to N
NOD A
NOD N
5wk
10wk
13wk
16wk
20wk
Total Bacteria
% Total Bacteria
Lactobacillus
Bacteroides
C. coccoides
b
Copy number/25ng DNA
2wk
Total Bacteria
% of Total Bacteria
Lactobacillus
Bacteroides
C. coccoides
NOD A
NOD AtoN
NOD N

Figure. 3 qRT-PCR analysis of bacteria copy numbers in feces of NOD mice shows a significant dysbiosis in mice on neutral drinking water. a) qRT-PCR analysis of the total copy numbers of NOD mice on acidified, acidified to neutral, or neutral water (upper left). Bacterial populations of *Lactobacillus sp.* (upper right), *Bacteroides sp.* (lower left), and *C. coccoides* (lower right) in the feces of female NOD mice on acidified (NOD-A, white, n=8-10), acidified to neutral (NOD-AtoN, striped, n=5), or neutral (NOD-N, black, n=8-10) water. b) Analysis of fecal microbial populations between 2wk NOD-A and NOD-N mice comparing numbers of total bacteria, *Lactobacillus sp.*, *Bacteroides sp.*, and *C. coccoides* (NOD-A n=4, NOD-N n=5). Significance was determined using Welches' t-test at $p<0.05$. Significance is indicated by *- NOD-A vs NOD-N, †- NOD-A vs NOD-AtoN, and ‡- NOD-N vs NOD-AtoN.

Figure 4

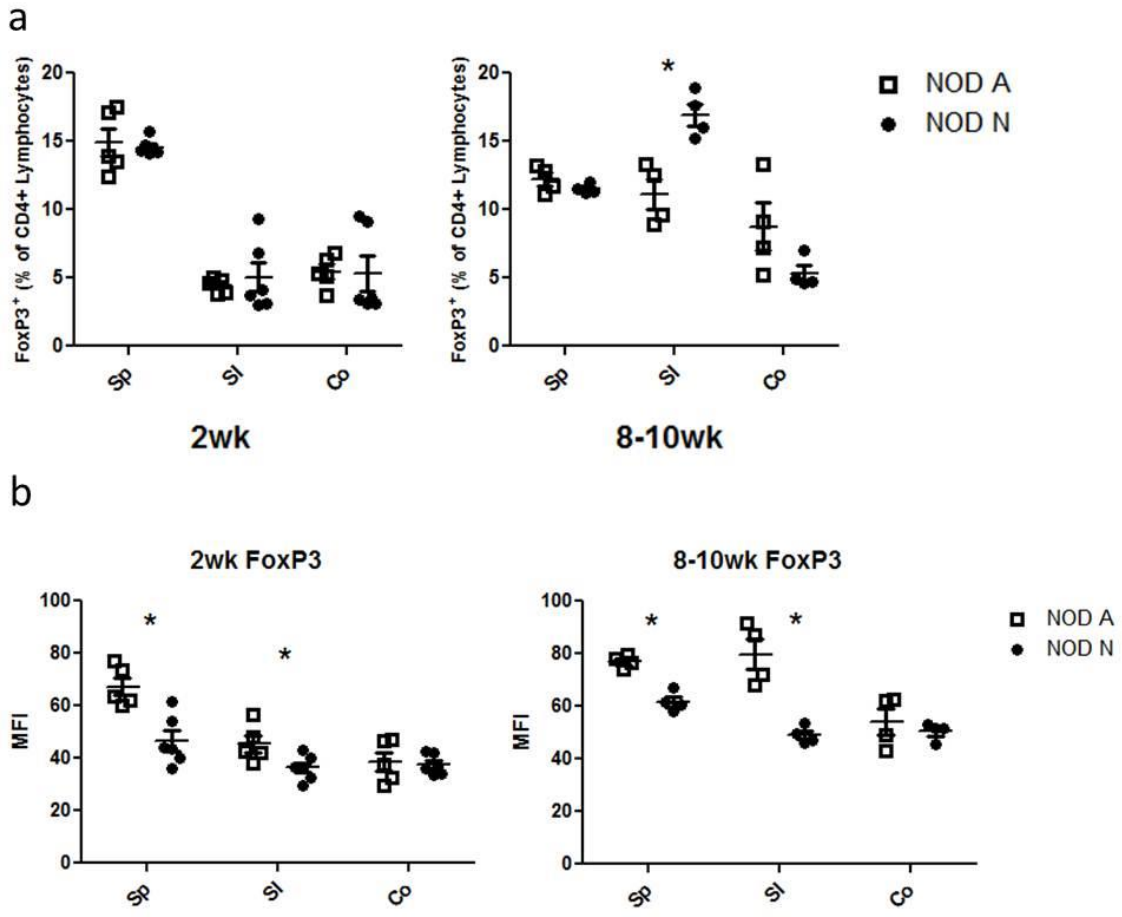


Figure 4. NOD mice on neutral drinking water have similar percentages of CD4$^+$Foxp3$^+$ Tregs, but decreased expression levels of Foxp3. a) The percentage of CD4$^+$Foxp3$^+$ Tregs in splenocytes (Sp) and small intestinal (SI) and colonic (Co) lamina propria (LP) lymphocytes from NOD-A and NOD-N mice at 2w weeks (NOD-A n=5, NOD-N=6, left panel) and 8-10 weeks (NOD-A n=4, NOD-N=4, right panel) of age. b) Mean fluorescent intensity (MFI) of Foxp3 expression in 2wk (left panel) and 8-10wk (right panel) NOD-A and N mice. Significance was determined using Welches' t-test at $p<0.5$.

Figure 5

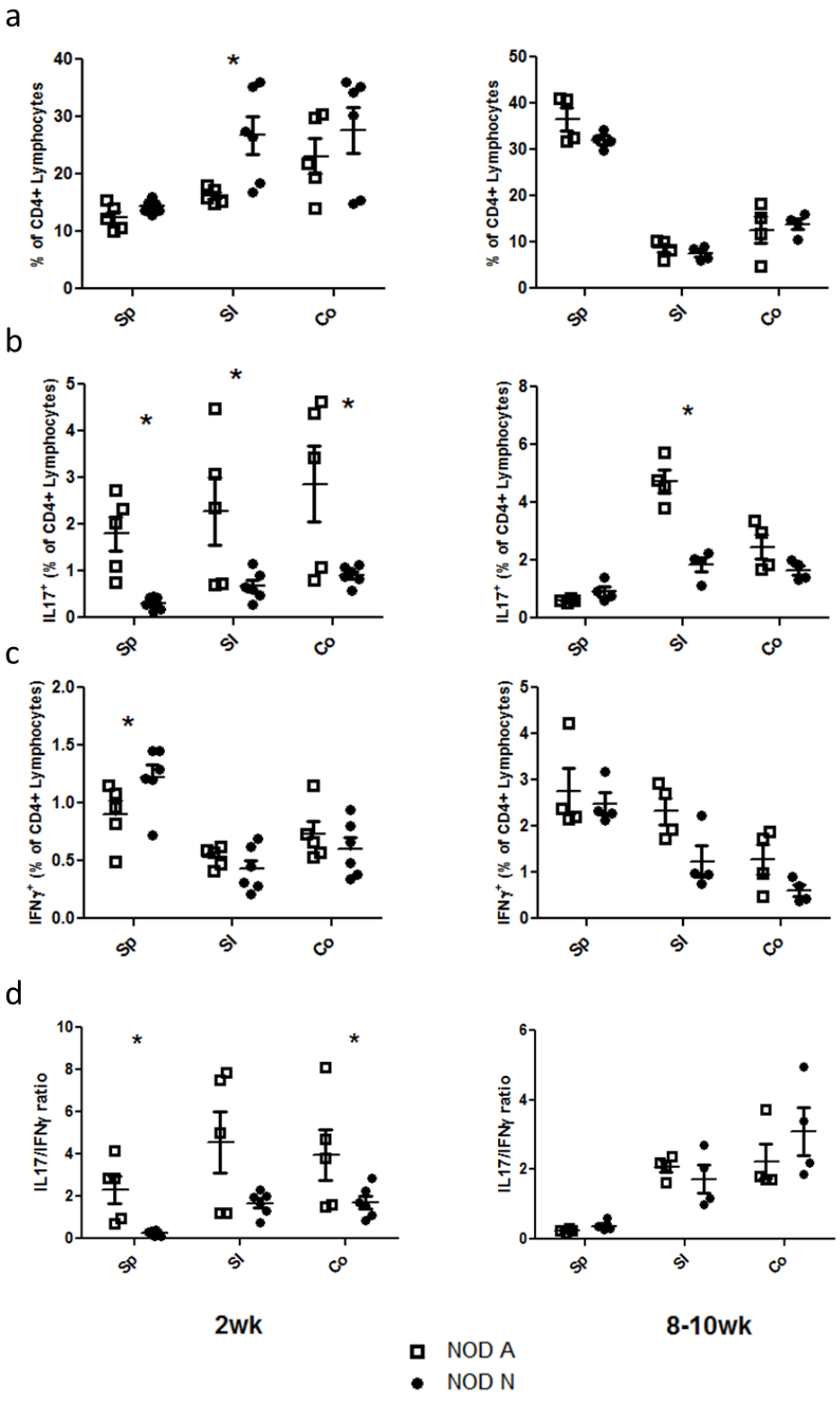
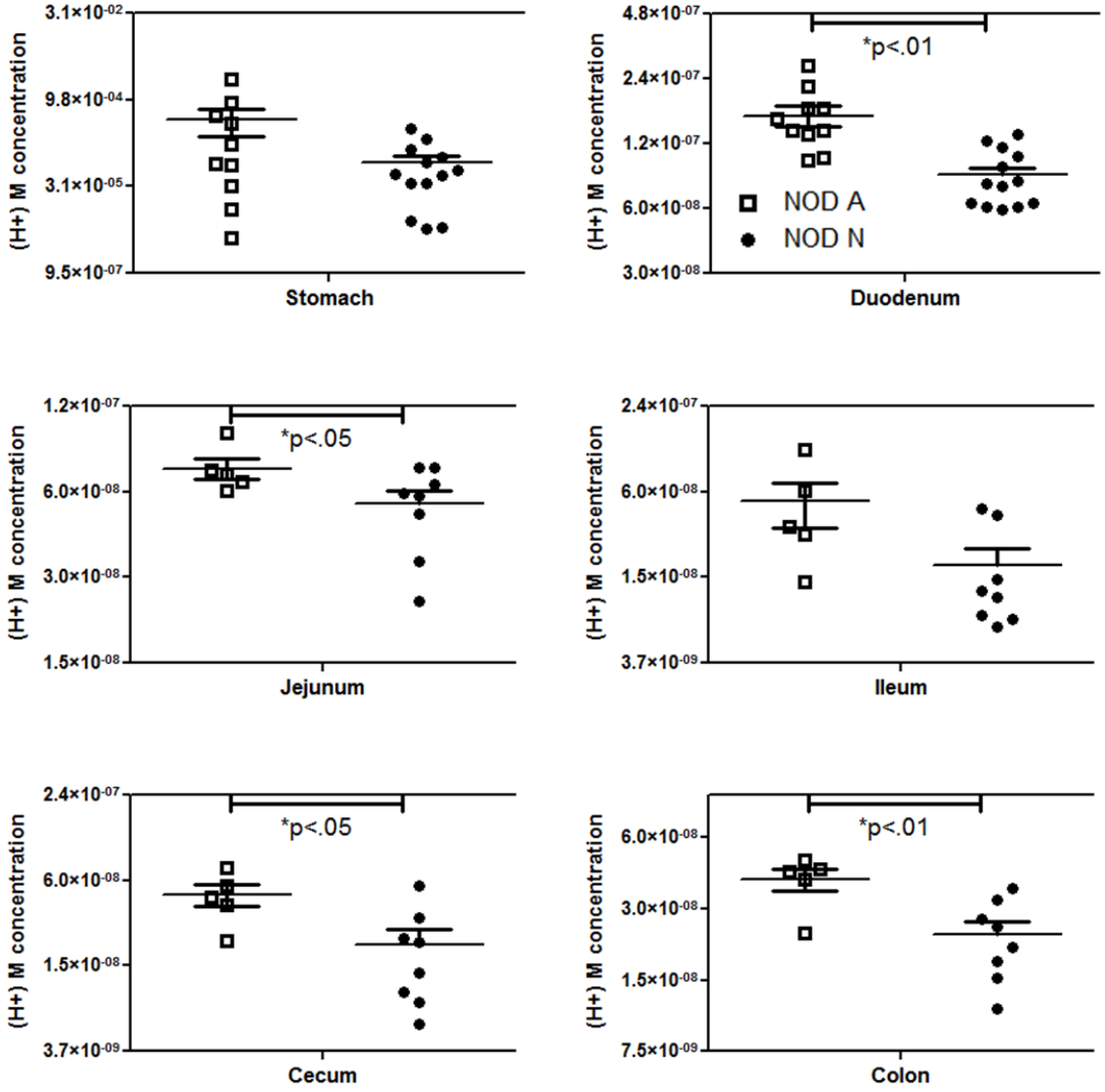

a
b
c
d
% of CD4+ Lymphocytes
IL17+ (% of CD4+ Lymphocytes)
IFNγ+ (% of CD4+ Lymphocytes)
IL17/IFNγ ratio
Sp
Sl
Co
2wk
8-10wk
NOD A
NOD N

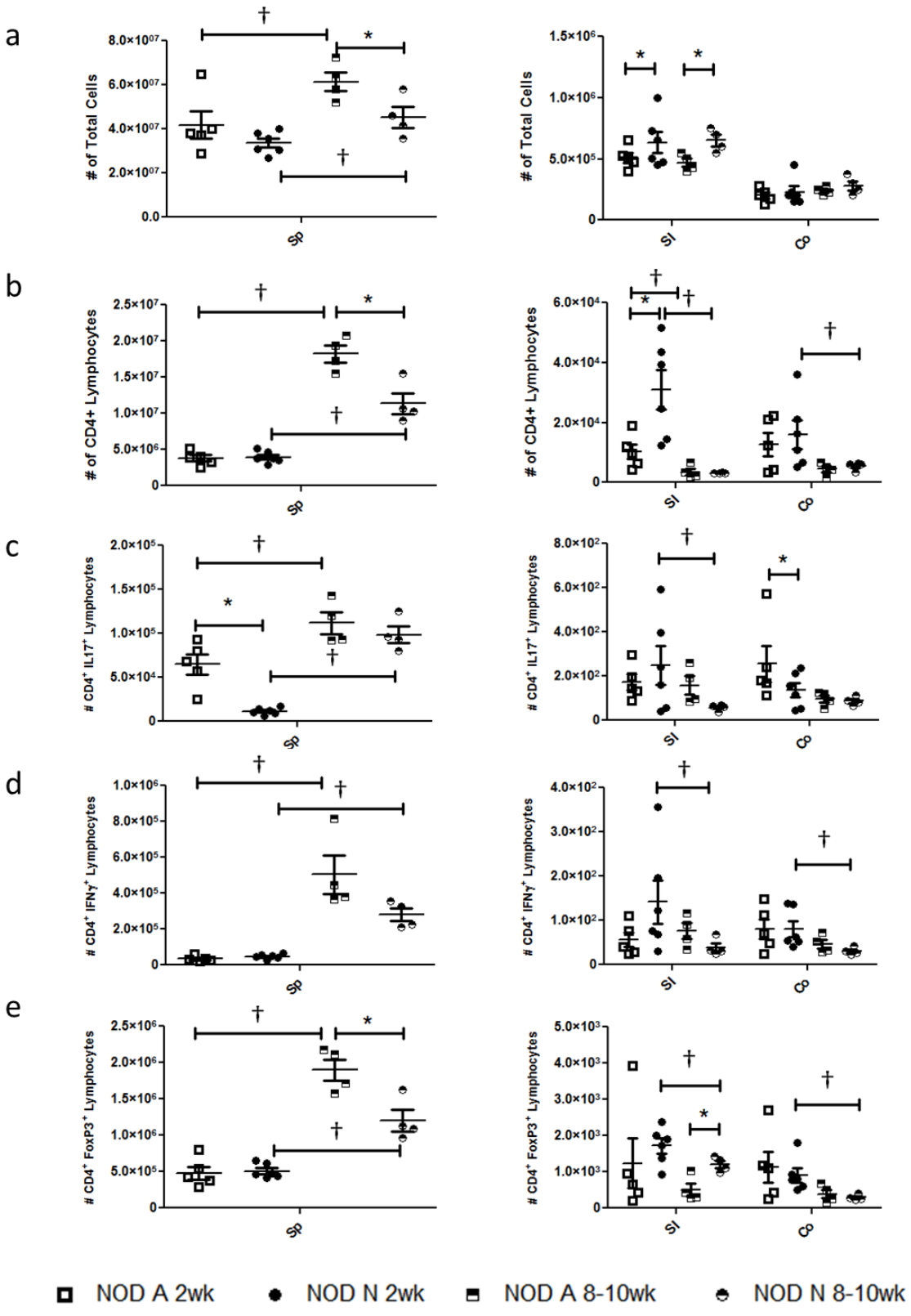
Figure 5. NOD mice on neutral drinking water have decreased production of IL17 by $CD4^+$ T cells. a) The percentage of $CD4^+$ splenocytes and lymphocytes from the small intestine (SI) and colonic (Co) lamina propria (LP) of 2 week (left panel) and 8-10 week (right panel) old NOD-A and NOD-N mice. b,c) Percentages of $CD4^+$ lymphocytes that are $IL17^+$ (b), or $IFN\gamma^+$ (c). d) Comparison of the ratio of $CD4^+IL17^+$ and $CD4^+IFN\gamma^+$ cells in 2wk (left panel) and 8-10wk (right panel) NOD-A and NOD-N mice. 2wk (NOD-A n=5, NOD-N=6). 8-10wk (NOD-A n=4, NOD-N=4). Significance was determined using Welches' t-test at $p<0.5$.

Supplementary Figure 1



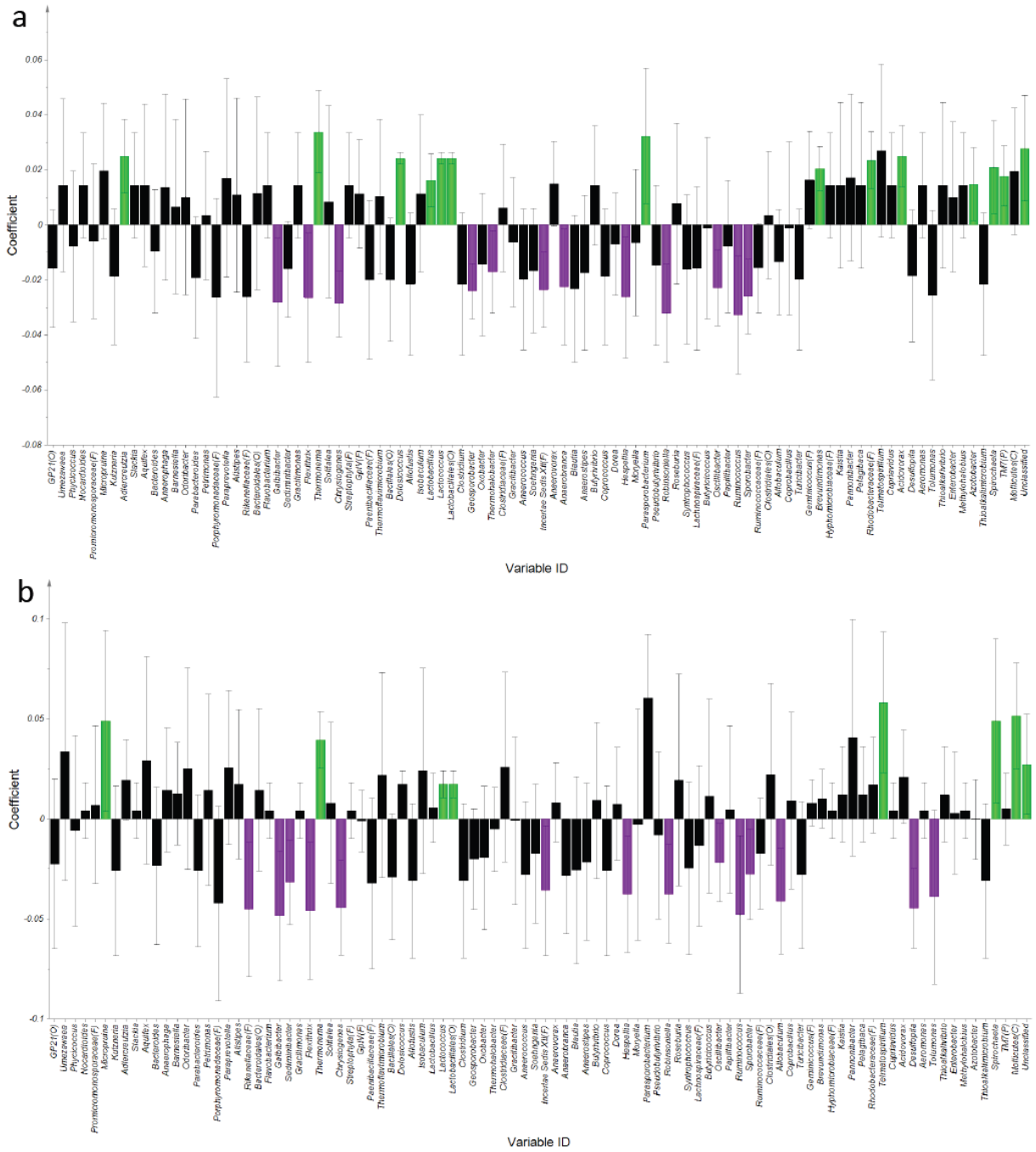
Supplemental Figure 1. The [H^+] concentration in the GI tract of NOD A mice is greater than in NOD N mice. The pH of 1mL water wash through the stomach, small, and large intestinal compartments was put to the power of 10 to calculate the H+ concentration. Mice were all female and 5wks of age. Significance was determined using the Welches' t-test at $p<0.05$. n=5-13

Supplementary Figure 2



Supplemental Figure 2. Flow cytometric analysis of splenocytes and small intestine (SI) and colonic (Co) lamina propria (LP) lymphocytes from NOD A and NOD N females at 2 and 8-10 weeks of age. 2 week mice were pooled together in groups of 2. The number of total lymphocytes (a), total $CD4^+$ lymphocytes (b), $CD4^+$ lymphocytes that were $IL17^+$ (c), $IFN\gamma^+$ (d), or $FoxP3^+$ (e) were calculated from the spleen (Sp, left column) or the LP of the SI and Co (right column) from 2wk NOD A (n=5) and NOD N (n=6) mice as well as 8-10wk NOD A (n=4) and NOD N (n=4) mice. Significance was determined using the Welches' t-test at $p<0.05$. *-significance between NOD A and N mice, †- significance between 2wk and 8-10wk animals of the same group.

Supplemental Figure 3



Supplemental Figure 3. Coefficient plots of the taxa analysed by PLS-DA found in 454 pyrosequencing. Bacteria that significantly contribute to the model are highlighted in the treatment color they are associated with: green for NOD-N and magenta for NOD-A. Plots are based on coefficients determined from statistically determined model component 1 (a) and component 2 (b).

TABLES

Table 1

Table 1: Relative abundances of bacterial phyla in the fecal microbiome of NOD-A and NOD-N mice generated from the pyrosequencing data

Phyla[2]	Percentages of sequences in:		SEM	*P*-value[2]
	NOD-A	NOD-N		
	----------above 1% of population----------			
Actinobacteria	1.71	6.57	1.20	0.02[G]
Bacteroidetes	22.78	26.73	4.58	0.56[G]
Firmicutes	68.55	50.63	5.27	0.04[G]
Proteobacteria	4.08	10.25	1.17	0.007[P]
	----------between 0.1 and 1% of population----------			
Acidobacteria	0.53	0.21	0.20	0.29[G]
Aquificae	1.13E-6	0.93	0.22	0.98[P]
Chrysiogenetes	0.73	0.10	0.18	0.03[G]
Cyanobacteria	0.08	0.86	0.27	0.19[P]
Nitrospira	0.08	0.05	0.11	0.87[N]
Planctomycetes	0.08	0.05	0.11	0.87[N]
Spirochaetes	1.13E-6	0.26	0.11	0.98[P]
Tenericutes	0.56	1.05	0.24	0.19[G]
TM7	0.09	0.61	0.29	0.24[G]
Verrucomicrobia	0.47	0.58	0.23	0.74[G]

[1] Unclassified bacteria accounted for 0.26% of NOD-A and 1.21% of NOD-N sequences.

[2] Method of analysis denoted by [G] (Gaussian), [N] (Negative Binomial), and [P] (Poisson).

Supplemental Table 1

Supplementary Table 1: Summary statistics for the pyrosequencing data of the fecal microbiome of NOD A and NODN mice

Treatment	Mean results for indicated variable						
	Average Sequences per Sample	Number of OTUs[1]	Coverage (%)	Richness		Diversity	
				Chao1	ACE	Shannon	Simpson
NOD-A	1337.60	178.20	93.46	311.58	308.70	3.68	0.08
NOD-N	1572.20	156.00	95.32	267.53	384.66	3.21	0.17
SEM	-	33.87	1.00	63.29	72.91	0.42	0.07
P-value	-	0.66	0.22	0.64	0.48	0.45	0.28

[1] OTU = operational taxonomic unit.

Supplementary Table 2: Relative abundances of bacterial genera in the fecal microbiome of NOD A and NOD N mice generated from pyrosequencing data

Phylum	Family, Genus[3]	Percentages of sequences in:[1,2]		SEM	*P*-value[4]
		NOD-A	NOD-N		
		above 1% of population			
Actinobacteria	Coriobacteriaceae, *Adlercreutzia*	0.53[B]	4.02[A]	1.23	0.079[G]
Bacteroidetes	Bacteroidaceae, *Bacteroides*	2.16	1.61	0.60	0.54[G]
Bacteroidetes	Porphyromonadaceae, *Barnesiella*	8.57	10.05	2.37	0.67[G]
Bacteroidetes	Porphyromonadaceae, *Petrimonas*	1.19	1.32	0.43	0.83[G]
Bacteroidetes	Porphyromonadaceae, Unclassified	1.98[A]	0.98[B]	0.32	0.060[G]
Bacteroidetes	Rikenellaceae, *Alistipes*	3.66	4.62	0.92	0.48[G]
Bacteroidetes	Flammeovirgaceae, *Thermonema*	0.80[b]	2.87[a]	0.41	0.0069[G]
Firmicutes	Lactobacillaceae, *Lactobacillus*	1.85	3.00	0.71	0.28[G]
Firmicutes	Clostridiaceae, *Geosporobacter*	2.84[A]	1.44[B]	0.52	0.092[G]
Firmicutes	Lachnospiraceae, *Dorea*	2.14	1.84	0.47	0.65[G]
Firmicutes	Lachnospiraceae, *Hespellia*	3.99[A]	1.86[B]	0.69	0.061[G]
Firmicutes	Lachnospiraceae, *Parasporobacterium*	1.40[b]	3.95[a]	0.56	0.013[G]
Firmicutes	Lachnospiraceae, *Robinsoniella*	10.15[a]	4.61[b]	1.24	0.014[G]
Firmicutes	Lachnospiraceae, *Sporobacterium*	4.05	4.56	1.19	0.77[G]
Firmicutes	Lachnospiraceae, *Syntrophococcus*	2.20	1.57	0.39	0.29[G]
Firmicutes	Lachnospiraceae, Unclassified	7.56	6.23	0.84	0.30[G]
Firmicutes	Ruminococcaceae, *Oscillibacter*	5.06	2.49	1.03	0.11[G]
Firmicutes	Ruminococcaceae, Sporobacter	4.34[A]	1.94[B]	0.79	0.064[G]
Firmicutes	Ruminococcaceae, Unclassified	3.65	2.62	0.66	0.30[G]
Firmicutes	Clostridiales[O], Unclassified	2.12	2.42	0.90	0.82[G]
Firmicutes	Erysipelotrichaceae, *Allobaculum*	2.65	1.72	0.66	0.35[P]
Proteobacteria	Rhodospirillaceae, *Telmatospirillum*	0.35[B]	1.76[A]	0.44	0.054[G]
		between 0.1 and 1% of population			
Acidobacteria	Gp21[O], Unclassified	0.53	0.21	0.20	0.29[G]
Actinobacteria	Actinosynnemataceae, *Umezawaea*	1.13E-6	0.21	0.10	0.98[P]
Actinobacteria	Intrasporangiaceae, *Phycicoccus*	0.18	0.08	0.16	0.67[P]
Actinobacteria	Nocardioidaceae, *Nocardioides*	1.13E-6	0.39	0.14	0.98[P]
Actinobacteria	Promicromonosporaceae, Unclassified	0.47	0.37	0.19	0.70[G]
Actinobacteria	Propionibacteriaceae, *Micropruina*	0.08	0.92	0.41	0.19[G]
Actinobacteria	Pseudonocardiaceae, *Kutzneria*	0.26	0.08	0.10	0.21[G]
Actinobacteria	Actinomycetales[O], Unclassified	0.10	0.10	0.14	0.97[P]
Actinobacteria	Coriobacteriaceae, *Slackia*	1.13E-6	0.39	0.14	0.98[P]
Aquificae	Aquificaceae, *Aquifex*	1.13E-6	0.93	0.22	0.98[P]
Bacteroidetes	Marinilabiaceae, *Anaerophaga*	0.10	0.32	0.20	0.49[P]
Bacteroidetes	Porphyromonadaceae, *Odoribacter*	0.57	0.81	0.25	0.51[G]
Bacteroidetes	Porphyromonadaceae, *Parabacteroides*	0.53	0.18	0.17	0.20[G]
Bacteroidetes	Prevotellaceae, *Paraprevotella*	0.55	1.45	0.52	0.26[G]
Bacteroidetes	Rikenellaceae, Unclassified	0.45[A]	0.00[B]	0.14	0.061[G]
Bacteroidetes	Bacteroidales[O], Unclassified	0.44	0.82	0.34	0.46[G]
Bacteroidetes	Flavobacteriaceae, *Flavobacterium*	1.13E-6	0.39	0.14	0.98[P]
Bacteroidetes	Flavobacteriaceae, *Galbibacter*	0.26[a]	0.00[b]	0.08	0.041[G]
Bacteroidetes	Flavobacteriaceae, *Sediminibacter*	0.65	0.32	0.20	0.29[G]
Bacteroidetes	Chitinophagaceae, *Gracilimonas*	1.13E-6	0.39	0.14	0.98[P]
Bacteroidetes	Flammeovirgaceae, *Flexithrix*	0.35[A]	0.00[B]	0.11	0.058[G]
Bacteroidetes	Sphingobacteriaceae, *Solitalea*	0.26	0.42	0.20	0.59[G]
Chrysiogenetes	Chrysiogenaceae, *Chrysiogenes*	0.73[a]	0.10[b]	0.18	0.036[G]

Cyanobacteria	Streptophyta, Unclassified	1.13E-6	0.39	0.14	0.98[P]
Cyanobacteria	GpIV, Unclassified	0.08	0.39	0.20	0.39[P]
Firmicutes	Paenibacillaceae, *Cohnella*	0.20	0.22	0.20	0.97[P]
Firmicutes	Paenibacillaceae, Unclassified	0.27	1.13E-6	0.12	0.98[P]
Firmicutes	Thermoactinomycetaceae,Thermoflavimicrobium	0.09	0.31	0.19	0.48[P]
Firmicutes	Unclassified, Unclassified	0.27	0.06	0.17	0.48[N]
Firmicutes	Aerococcaceae, *Dolosicoccus*	1.13E-6	0.71	0.19	0.98[P]
Firmicutes	Carnobacteriaceae, *Allofustis*	0.39	1.13E-6	0.14	0.98[P]
Firmicutes	Carnobacteriaceae, *Isobaculum*	0.09	0.41	0.21	0.38[P]
Firmicutes	Streptococcaceae, *Lactococcus*	1.13E-6	0.71	0.19	0.98[P]
Firmicutes	Lactobacillales[O], Unclassified	1.13E-6	0.71	0.19	0.98[P]
Firmicutes	Clostridiaceae, *Clostridium*	0.39	1.13E-6	0.14	0.98[P]
Firmicutes	Clostridiaceae, *Oxobacter*	0.20	1.13E-6	0.10	0.98[P]
Firmicutes	Clostridiaceae, *Thermohalobacter*	1.01	0.65	0.21	0.26[G]
Firmicutes	Clostridiaceae, Unclassified	0.26	0.37	0.18	0.69[G]
Firmicutes	Gracilibacteraceae, *Gracilibacter*	0.28	0.18	0.21	0.77[P]
Firmicutes	Incertae Sedis XI, *Anaerococcus*	0.59	1.13E-6	0.17	0.98[P]
Firmicutes	Incertae Sedis XI, *Soehngenia*	0.80	0.37	0.26	0.27[G]
Firmicutes	Incertae Sedis XII, Unclassified	0.80	0.21	0.23	0.10[G]
Firmicutes	Incertae Sedis XIII, *Anaerovorax*	0.17	0.60	0.29	0.32[G]
Firmicutes	Incertae Sedis XIV, *Anaerobranca*	0.53	0.18	0.14	0.12[G]
Firmicutes	Incertae Sedis XIV, *Blautia*	1.11	0.45	0.26	0.11[G]
Firmicutes	Lachnospiraceae, *Anaerostipes*	0.59	0.08	0.29	0.25[G]
Firmicutes	Lachnospiraceae, *Butyrivibrio*	0.81	1.16	0.25	0.35[G]
Firmicutes	Lachnospiraceae, *Coprococcus*	0.26	0.08	0.10	0.21[G]
Firmicutes	Lachnospiraceae, *Moryella*	0.43	0.31	0.20	0.68[G]
Firmicutes	Lachnospiraceae, *Pseudobutyrivibrio*	0.85	0.47	0.26	0.33[G]
Firmicutes	Lachnospiraceae, *Roseburia*	0.35	0.62	0.37	0.62[G]
Firmicutes	Ruminococcaceae, *Butyricicoccus*	0.20	0.18	0.20	0.95[P]
Firmicutes	Ruminococcaceae, *Papillibacter*	0.98	0.76	0.30	0.61[G]
Firmicutes	Ruminococcaceae, *Ruminococcus*	0.65	0.10	0.25	0.25[P]
Firmicutes	Erysipelotrichaceae, *Coprobacillus*	0.20	0.18	0.20	0.95[P]
Firmicutes	Erysipelotrichaceae, *Turicibacter*	0.59	1.13E-6	0.17	0.98[P]
Proteobacteria	Geminicoccus, Unclassified	1.13E-6	0.89	0.21	0.98[P]
Proteobacteria	Caulobacteraceae, *Brevundimonas*	1.13E-6	0.61	0.17	0.98[P]
Proteobacteria	Hyphomonadaceae, *Henriciella*	0.25	0.21	0.21	0.88[P]
Proteobacteria	Hyphomicrobiaceae, Unclassified	1.13E-6	0.39	0.14	0.98[P]
Proteobacteria	Rhizobiaceae, *Kaistia*	1.13E-6	0.22	0.10	0.98[P]
Proteobacteria	Rhodobacteraceae, *Pannonibacter*	1.13E-6	0.50	0.16	0.98[P]
Proteobacteria	Rhodobacteraceae, *Pelagibaca*	1.13E-6	0.22	0.10	0.98[P]
Proteobacteria	Rhodobacteraceae, Unclassified	0.00	0.67	0.26	0.10[G]
Proteobacteria	Alcaligenaceae, *Parasutterella*	0.70	0.61	0.25	0.80[G]
Proteobacteria	Burkholderiaceae, *Cupriavidus*	1.13E-6	0.39	0.14	0.98[P]
Proteobacteria	Comamonadaceae, *Acidovorax*	0.09[B]	0.79[A]	0.25	0.079[G]
Proteobacteria	Desulfobulbaceae, *Desulfopila*	1.10	0.39	0.37	0.21[G]
Proteobacteria	Aeromonadaceae, *Aeromonas*	1.13E-6	0.39	0.14	0.98[P]
Proteobacteria	Aeromonadaceae, *Tolumonas*	0.83[A]	0.08[B]	0.25	0.070[G]
Proteobacteria	Ectothiorhodospiraceae, *Thioalkalivibrio*	1.13E-6	0.22	0.10	0.98[P]
Proteobacteria	Enterobacteriaceae, *Enterobacter*	0.20	0.50	0.26	0.47[P]
Proteobacteria	Methylococcaceae, *Methylohalobius*	1.13E-6	0.39	0.14	0.98[P]
Proteobacteria	Pseudomonadaceae, *Azotobacter*	0.18	0.61	0.29	0.33[G]
Proteobacteria	Piscirickettsiaceae, *Thioalkalimicrobium*	0.39	1.13E-6	0.14	0.98[P]
Spirochaetes	Spirochaetaceae, *Spirochaeta*	1.13E-6	0.26	0.11	0.98[P]
Tenericutes	Mollicutes[C], Unclassified	0.56	1.05	0.24	0.19[G]
TM7	Unclassified, Unclassified	0.09	0.61	0.29	0.24[G]
Verrucomicrobia	Verrucomicrobiaceae, *Akkermansia*	0.47	0.42	0.30	0.92[P]

[1] Statistically different ($P<0.05$) denoted by ^{a,b,c}.
[2] Statistically different ($P<0.10$) denoted by ^{A,B,C}.
[3] When Family and *Genus* are both Unclassified, ^{C} signifies Class, and ^{O} signifies Order.
[4] Method of analysis denoted by ^{G} (Gaussian), ^{N} (Negative Binomial), and ^{P} (Poisson).
[5] Unclassified bacteria accounted for 0.26% of NOD-A sequences and 1.21% of NOD-N sequences.

Supplemental Table 3

Supplementary Table 3: Genera[1] removed from analysis with a total population below 0.1%

Acetanaerobacterium	*Filifactor*	*Lutibacter*	Planctomycetaceae[F]
Arcobacter	Flammeovirgaceae[F]	*Lutimonas*	*Proteiniborus*
Bacteroidetes[C]	*Fusibacter*	*Microvirga*	*Roseibaca*
Chromatiaceae[F]	*Guggenheimella*	Nitrospiraceae[F]	*Streptococcus*
Cyanobacteria[C]	*Helcococcus*	*Paralactobacillus*	*Thermacetogenium*
Desulfotomaculum	*Klugiella*	Peptococcaceae[F]	*Veillonella*
Enterococcus	*Lawsonia*	*Persicirhabdus*	*Vulcanibacillus*

[1] When *Genus* is Unclassified, [C] signifies Class, [F] signifies Family, and [O] signifies Order.

Supplemental Table 4

Supplementary Table 4: Genera[1] removed from PLS-DA analysis with a Variable Influence on Projection value less than 0.3.

Actinomycetales[O]	*Cohnella*	*Sporobacterium*
Henriciella	*Parasutterella*	*Akkermansia*

[1] When *Genus* is Unclassified, [O] signifies Order.

METHODS

Mice: NOD/ShiJt mice originally obtained from Jackson Laboratory (Bar Harbor, Maine) were bred and maintained under specific pathogen free (SPF) conditions in Thoren Isolator racks (Hazelton, PA) under positive pressure and were fed autoclaved NIH-31 rodent diet (Harlan Teklan, Madison, WI), and sterile water at libitum. Original animals were acclimatized to our facility 2 weeks prior to mating. Water in the animal research building (RSB), is from the Birmingham city water supply and is chlorinated and autoclaved. Original breeding pairs were split between neutral (pH ~7, NOD-N) and acidified H_2O (pH ~3.2, NOD-A) and all pups born from these breeding pairs and thereafter were maintained on their specific water source. Acidified H_2O is comprised of 1mL of 1N HCl per 500mL of H_2O (pH~3.2). A minimum of 2 sets of founder mice, originally ordered from The Jackson Laboratory (Bar Harbor, Maine), have been used to create each mouse population to ensure any changes we witness are not the result of a founder effect (34)

Incidence of T1D: NOD mice on either water source were evaluated from 9-10wks of age until 30wks of age for onset of T1D. Blood glucose from a tail bleed was taken weekly via OneTouch© Blood Glucose Meter (Greenwood Village, CO). T1D was defined as two weekly adjacent readings of over 200 mg/dL, or a single reading over 400 mg/dL. All experiments were approved by the UAB Institutional Care and Use Committee.

Pancreas Histology: Pancreatic tissue was removed from diabetic animals and placed in formalin for >24hrs. Tissue was washed in 70% EtOH and imbedded in HistoGel

(Richard-Allan Scientific, Kalamazoo, MI). Tissue was cut into 5µm sections and stained with standard H&E for histologic examination.

pH of Intestinal Compartments: NOD-N or NOD-A mice were fasted for 4hrs. Mice were sacrificed and the stomach, duodenum, jejunum, ileum, cecum, and colon were washed individually with 1mL of filtered deionized water each. Particulate matter was removed and pH of the contents was measured via Corning Pinnacle 540 pH meter (Corning, NY). pH was converted to H^+ concentration through the equation $pH=-\log_{10}(H^+)$.

Denaturing Gradient Gel Electrophoresis (DGGE): Fecal contents, collected weekly from NOD mice, were stored at -20°C until further use. Fecal pellets were weighed and then Phenol:Chloroform extracted as previously described in order to extract bacterial DNA (41; 54). DNA was quantified using NanoDrop 1000 (NanoDrop, Wilmington, DE). DNA was diluted to 150ng/ul and underwent polymerase chain reaction (PCR) using 16S universal bacterial primers 341GC -5' CGCCCGCCGCGCGCGGCGGGCGGGGGGGGGCACGGGGGGCCTACGGGAGGCAGCAG 3'
and 534R -5' ATTACCGCGGCTGCTGG 3' (Sigma, St. Louis, MO). PCR was performed using TaKaRa ExTaq HotStart Taq Polymerase kit (Fisher, Pittsburg, PA). Thermal profile was set at 95°C for 5 min, 95°C for 1 min, 65°C for 45 secs (decreasing 0.5°C per cycle), 72°C for 1min, repeat for 19 additional cycles; 95°C for 1 min, 55°C for 45 secs, 72°C for 1 min, repeat for 9 additional cycles; final extension of 72°C for 5min. Polyacrylamide gels were produced and samples ran as previously described (54).

Briefly, PCR samples were diluted with gel loading dye and loaded onto the 60/35% gradient gels. Gels were loaded onto a Bio-Rad Dcode system (Bio-Rad Laboratories, Hercules, CA) and ran overnight at 58°C and 58V in 7L 1X TAE solution. Gels were stained with ethidium bromide and imaged and analyzed via Bio-Rad ChemiDoc XRS and Image Lab Software (Bio-Rad Laboratories, Hercules, CA). Bands of interest were removed and DNA extracted was subjected to another round of PCR with the same primers as described previously only tagged with M13 vector tails. DNA was sequenced by the UAB Heflin Genetics Center. Bacterial species were identified by sequence pairing through the NCBI BLAST database. Taxonomic specification was determined with a 75% homology to sequences within the BLAST database. Band similarity was analyzed and calculated using the GelComparII program (Applied Maths Inc., Austin Texas).

454 pyrosequencing: Pyrosequencing was performed on genomic DNA samples using the bacterial tag-encoded GS FLX-Titanium amplicon with primers 28f (5'-GAGTTTGATCNTGGCTCAG-3') and 519r (5'-GTNTTACNGCGGCKGCTG-3')(55). Sequences were processed with the *mothur* software package (56). Briefly, barcodes and primers were depleted and sequences with an average quality score of less than 30 were removed from the dataset. Sequences shorter than 250 base pairs, containing ambiguous base-pair designation or greater than 8 homopolymers were also removed to maintain sequencing quality and aligned to the V1-V3 region of bacterial 16S RNA gene using the SILVA reference alignment as a template. Chimeric sequences were removed using the UCHIME algorithm (57). Sequences were assigned taxonomically using the SILVA

database, a distance matrix was created with a threshold of 0.15 and was use to cluster remaining sequences into operational taxonomic units (OTU) using the average neighbour grouping method with a cutoff of 95% sequence similarity. Finally, OTUs were classified into consensus taxonomies. Data quality was checked using α-diversity analysis. To estimate richness, Chao1 and abundance based coverage estimation (ACE) indices were used. Diversity was estimated using both Shannon and Simpson indices. Rarefaction curves were also generated to estimate sequencing quality and coverage.

Bacterial Quantitative Real Time PCR: 25ng of fecal extracted DNA was subject to quantitative real-time PCR. Briefly, 12.5ul SYBR© Green (Clontech), 0.05ul of both 20um forward and reverse primers, and 25ng DNA were added per well, sterile H_2O was used to bring wells to 25ul total volume. Samples were compared to a standard curve specific to the target bacteria starting at 1x 10^8 copy numbers and serially diluted to 1x 10^1 copy numbers. Thermal profiles for the reaction is 95°C for 10min, 95°C for 15 sec, 56°C for 18sec, 45°C for 45 seconds, repeated for 44 additional cycles. Extension temperature varies depending on bacterial specific primers of either total bacteria (58), *Lactobacillus* (59), *Clostridia* (59) or *Bacteroides* (59). Bacterial specific primers were purchased from Invitrogen (Carlsbad, CA).

Lamina Propria Preparation: Large and small bowel were removed from female 2wk and 8-10 week old NOD-A or NOD-N mice and were digested in order to extract lamina propria lymphocytes (41). 2 NOD pups were pooled for each sample due to the small size and number of cells collected from 2wk pups. Briefly, GI tissue was open

longitudinally and cleared of fecal debris. Large and small intestine were handled separately, digested in HBSS media + 5mM EDTA and filtered to remove epithelial cells. Tissue was minced and further digested by HBSS media + collagenase IV (Sigma Aldrich, St. Louis, MO), and the resulting solution was filtered through a 100μm filter and collected. Cells were washed and re-suspended in 40% Percoll (Fisher, Pittsburg, PA) and layered onto 70% Percoll before centrifugation. The 40/70% Percoll interface containing the lymphocytes was collected and stored overnight at 4°C to allow cells to recover cell surface molecules.

Lymphocyte Activation and Flow Cytometry: For identification of IL17 and IFNγ producing cells, lymphocytes were activated with 100 ng/mL phorbol myristate acetate (PMA) (Sigma, St. Louis, MO), 1 ug/mL ionomycin, and 0.7ul/mL Golgistop (BD Biosciences, San Jose, CA) in R-10P media (RPMI 1640 (Mediatech, Manassas, VA), 10% Fetal Calf Serum (Thermo Scientific, Rockford, IL), 1% penicillin/streptomycin, 0.1% 2 β-mercaptoethanol, and 1% Glutamax (Fisher, Pittsburg, PA) for 5hr at 37°C. Staining was performed as previously described (60). Briefly, the FcR were blocked via αCD16/32 (Biolegend, San Diego, CA) and CD4-APC was used as a cell surface marker for Th1/Th17 lymphocytes. Permeabilization of cells allowed for intracellular staining of IL-17-PE, IFN□-FITC (Th17/Th1) and Foxp3-APC before fixation and FACS analysis on both Tregs and Teff cells (Biolegend, San Diego, CA). Cell surface antibodies listed as CD4-FITC and CD25-PE were used as Treg markers.

Graphic and Statistical Analysis: Graphs were made using GraphPad Prism 5 (San Diego, CA). Significance was performed for quantitative bacterial copy number via

Welch's t-test. Significance for the incidence of T1D was calculated using the Mantel-Cox test. Sequence data for each sample was converted into percentage data at the phylum and the genus level, tested for normality using PROC Univariate, and analyzed using PROC Mixed in SAS (SAS Institute Inc, NC). Data that was not normally distributed was treated with PROC GLIMMIX through either Poisson or negative binomial distributions; with the Pearson chi-square / degrees of freedom ratio being applied to determine goodness of fit for each non-normal distribution method. In order to evaluate further significant differences between the two treatments, partial least-squared discriminant analysis (PLS-DA) was used within the SIMCA P+ 13.0 software package (Umetrics, Umea, Sweden). Y variables were used to describe the two treatments, NOD-A and NOD-N, while X variables were used to represent the bacterial genera. The number of significant components was determined using R^2 and Q^2 values. Variable influence on projection value (VIP) was determined for each genus, and any with a VIP value below 0.3 was removed from the model. Score scatter plots and loading scatter plots were generated, and genera significantly associated with either treatment was determined by the PLS-regression coefficients and their plots (Supplementary Fig. 3).

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CHAPTER 4

DISCUSSION

The potent changes we witnessed in the incidence of T1D in NOD mice depending on something as simple as the pH of the water they drink only strengthens the idea that subtle alterations within the GI tract and exposure to external environmental antigens can have a significant impact on disease. It appears the largest impacting factor in disease that is influenced by microbiota, comes from the populations shift between populations of *Firmicutes* and *Bacteroidetes* within the GI tract. Both have been shown to have effects that are unique to their bacterial groups, or even specific to a species, as with the case of the SFB *Firmicutes* in the ileum (39). SFB alone can cause surprisingly large increases in Th17 populations and influence pathogenicity in a number of diseases in both protective and pathogenic manners (27; 65). It would not be surprising if other small, specific groups have similar roles in other aspects of the immune system. It is imperative that future studies focus on how it is that such a small number of organisms are able to elicit such profound changes. Those discoveries will allow for the potential research into commensal microbiota based medicines, treatments, and vaccinations to begin.

We initially saw that changes within the microbiota between NOD A and NOD N mice in our studies could occur prior the "initial onset" of disease; being after initial infiltration of lymphocytes into the pancreas but before loss of insulin production and

control over blood glucose levels. This time in the NOD mouse is between 5 and 16wks of age. Environmental determinants such as commensal microbiota and infection are thought to have their most potent effects at two distinct time points in the life cycle of the NOD mouse; the first is very early in life, at approximately two weeks of age. At 2wks, the mouse pancreas undergoes "remodeling" and a massive wave of apoptosis ensues, as well as receiving exposure to commensal microbiota during early immune development (86). This is especially important when working with the NOD mouse. NOD mice have previously been shown to have increased intestinal permeability (87). Increased permeability, often described as a "leaky gut", allows increased microbial and dietary debris across the epithelial barrier. A leaky gut can create abnormal inflammatory responses due to the large influx of microbial and dietary antigen (88). A leaky gut can be caused by a number of genetic and environmental factors. Deficiencies in tight junction proteins can weaken barrier integrity, while pathogens and food allergies can cause inflammation which would further disrupt the epithelial layer (30; 89; 90).

The remodeling of the pancreas is thought to be important as data suggests that mice pre-disposed to T1D do not effectively dispose of apoptotic debris which is later seen by the immune system. Interactions with commensal microbiota at 2wks of age will have the greatest effect on the immune system as it shapes immune development in the infants (91). The trend we witnessed in 2wk pups between the *Firmicutes* and *Bacteroides* remained the same at older time points; the variations in the numbers were varied and the microbial composition between mother and pups were drastically different. There is data showing that microbiota is passed down from mother to pup during the suckling phase (92). Our data shows that the microbiota of mothers and 2wk old pups are

considerably different (data not shown), though it is very likely the change we witness is due to the fact that pups are only ingesting milk and the dietary difference between milk and chow influences microbial diversity which has also been seen in other reports as well (40; 92-94).

The second time point thought to be vital in disease development is at 7-8 wks of age. During this time, lymphocytes are actively infiltrating the pancreas and initial destruction of the insulin producing pancreatic β cells begins (83). Commensal microbiota during this time may have an indispensable role in controlling the auto-immune response through homeostatic expansion of Th17 cells or induction of T regulatory cells (83). Our research would suggest that while changes in microbiota at 4-5wks of age can alter the incidence of disease, the protective effect is truncated compared to changes made at or before 2wks of age. This would suggest both time points are important, but changes at the later time point alone are not sufficient. It may even be possible to pre-dispose children to protective bacteria immediately at the time of birth by giving pregnant mothers probiotics. Infants whose mothers were taking probiotics before birth were colonized with the probiotic strains for at least 6 months after birth (94). This could prove to be an effective method in delivering bacteria shown to be protective to infants who may carry genetic susceptibility for diseases like T1D.

The data collected in our studies examining the $CD4^+$ T cell repertoire is consistent with other data displaying Th17s protective effect in the prevention of T1D in the NOD mouse model (83). The increase in Th17 populations in the somewhat diabetes resistant NOD A mice compared to the highly susceptible NOD N mice at 2wks of age reinforces our hypothesis that the protective effects of Th17 cells are the most important

early in life. By 8-10wks of age, the difference in Th17 populations is muted between the NOD A and NOD N populations, yet there incidence in T1D is significantly shifted. We are also able to infer this early protection through the observation that NOD A mice switched to neutral water at weaning (4-5wks of age) were unable to fully recover the NOD N disease incidence. A future study using foster mothers to switch suckling pups from acidified to neutral H_2O would go help solidify this observation.

The changes we witnessed in the make-up of commensal microbiota in the GI tract focused on 2 large groups of bacteria, the *Firmicutes* and *Bacteroidetes*. Together, these two phyla are responsible for approximately 75-80% of all the bacteria found in the gut. In *Firmicutes*, not only in our studies, but also other published data, show that a few very specific groups are most likely responsible for a majority of the protective affects commensal microbiota have in T1D (42). *Lactobacillus* and *Clostridia* Clusters XIV and IV are important in the regulation of disease in T1D (42; 65). Numerous studies display *Lactobacillus* having protective affects in almost any disease thought to be influenced by commensal microbiota (42; 95-98). Recent publications have shown both *Lactobacillus* is able to influence T helper populations, often skewing away from Th1 mediated responses and eliciting a more Th17 dominated response, or simply suppressing or modulating the response in general (42; 95; 98). It is still not understood how or why *Lactobacillus* species elicit such potent immune modulatory affects, but it is obvious that *Lactobacillus* will play a key role in future studies for microbial based medicine.

It is difficult to ascertain the role of *Bacteroidetes* in disease. Our data, along with others have shown that *Bacteroidetes*, specifically the large group *Bacteroides*, are increased in T1D patients (25). However, it is not clear if *Bacteroides* is

pathogenic in disease, or, if the increase is *Bacteroides* is simply is response to the loss of the protective *Firmicutes* in diabetic animals and patients. There is some data available looking at the roles of other prominent phyla of commensal microbiota. *Proteobacteria,* like *Bacteroides,* is increased in diabetic animals, though research on *Proteobacteria* in diabetes is focused on pathogenic bacteria such as *Pseudomonas, Helicobacter,* and *Burkholderia* (99). It is also not clear why genetically pre-disposed individuals to T1D naturally have altered commensal microbiota compared to healthy individuals. It is important to research the microbe:host interaction in order to better understand how the genetic pre-disposition to T1D may cause abnormal commensal selection and what can be done in order to modify predisposed individuals commensal flora in order to prevent disease.

Perhaps one of the most fascinating aspects when studying the microbe:host interaction is the diversity in how the host responds to the same microbial signal. Bi-products from microbial metabolisms such as short-chain fatty acids (SCFAs) have a variety of effects depending on the cell type that is exposed. Colonocytes utilize SCFAs as a primary source of energy, while most exposure of immune cells to butyrate, a common SCFA, induces cell cycle arrest and apoptosis (5; 9; 16). While all bacterial produce SCFAs (primarily butyrate, acetate, and propionate), the dominate producers of SCFAs within the gut belong to the *Firmicutes* phyla. Within the phyla *Firmicutes,* there are large groups of bacteria, specifically *Clostridia*, which produce a majority of the SCFAs within the gut (9). The modulatory and health benefits of SCFAs is a currently a hot rising topic of research as a method of naturally modifying the mucosal immune

system. This reiterates how important it is for future research to uncover the mechanisms and pathways that impact the host immune system. The use of newer technologies like mass spectrometry and nuclear magnetic resonance (NMR) can be utilized to measure the levels of important metabolites and this could provide us a better understanding in the sensing and utilization of bacteria and bacterial bi-products and disease and health.

It is worth noting that the roles of commensals are vastly different when comparing type 1 and type 2 diabetes (T2D). As a metabolic disease, the regulation of T2D is influenced more by the dietary intake and microbial bi-products than by the influence the microbiota have on the immune system (12). Despite their seemingly protective attributes in T1D, *Firmicutes*, specifically *Clostridia* and butyrate producers, appear to play an enabling role in the onset of T2D (53). The higher proportions of metabolic enzymes and genes associated with *Clostridia* allows for a greater amount of the diet to be broken down to usable energy by the host (12; 37). The increased caloric intake promotes weight gain and eventually glucose/insulin imbalance leading to development of T2D (85). It has been shown that obese individuals have higher proportions of *Firmicutes* compared to healthy individuals and are more efficient at absorbing calories due to the ability of the microbiota to breakdown a greater portion of the individual's diet (15; 53). Current research looking into human fecal bacterial transplants has yielded interesting results. When obese individual's commensal populations are changed to mimic that of healthy individuals, they experience increased weight loss compared to individuals who microbiota were not altered (37). These steps in identifying the microbial roles in metabolic disease will lead to future developments

into treatments by combining altering commensal microbiota, diet change, and healthy exercise regiments.

The focus of my research over the past years has attempted to take many of these variables into account when examining the role of commensal microbiota in T1D. However, the field of mucosal immunology and the study of the impact of commensal microbiota on the immune system lays largely unexplored. Mechanisms and pathways utilized in the cross talk between immune cells and bacteria are still vague at best. If we want to ever understand the answers to these questions, the next big push in research must focus on these interactions. New methods must be developed to effectively tease out why specific microbes are able to instill long term tolerogenic responses. While we currently understand which specific groups of bacteria are responsible in certain changes in immunity, we have yet to uncover the methods/mechanisms that are used. Is it related to homeostatic expansion of T cells? Do T cells lose their pro-inflammatory potential after long-term low-level exposure to antigen? How do specific groups of bacteria modify the response from the innate immune system via pattern recognition receptors? These are important questions that will eventually bring about a new understanding of the interactions between microbes and immunity. This will have the potential to create the bridge that will connect experimental and bedside medicine which is currently lacking in much of mucosal immunology.

The more we understand about how the commensal microbiota influence the mucosal and systemic immune system, the further we will advance in understanding the roles different immune cells play in disease. Our data currently suggests that not all $CD4^+$ T effector cells are pathogenic in T1D. At least early in life, our data would

suggest Th17 cells behave in a protective manner. The increased number of Th17s in young NOD A mice compared to NOD N lends credit to previously published data supporting the hypothesis that Th17 cells are protective. However, there are still many questions that remain to be answered, and data showing Th17 cell's pathogenicity in diabetes cannot be discounted. More work has to go into ascertaining the Th17s role in T1D, before, and during disease. There may even be a link between the role of Th17 cells in T1D and the role Tregs have in attempting to control the autoimmune response (75). It is still unclear whether it is the T effectors (Teff) or Tregs that are responsible for the breakdown of tolerance in autoimmunity. Currently, our data shows little change in the number of Tregs the spleen and lamina propria between NOD mice on acidified or neutral water; however we have yet to measure the Tregs capacity for immune suppression between NOD A and NOD N mice. Is it that there are deficiencies in the Tregs that obstructs their ability to prevent the autoimmune inflammatory response? Or, is it that Teff cells have gained resistance to suppression by Tregs in some capacity? Currently there has been data that have displayed Treg deficiencies as well as T effector's resistance to suppression in the NOD mouse model, however these observations have never been seen at the same time (49; 57; 78; 100) Criss-cross studies, looking at proliferative and suppressive abilities of Teff and Tregs respectively between diabetic prone and resistant animals or diabetic and healthy individuals would take large steps in answering this question.

Published data displays that not only the adaptive, but also the innate immune system has abnormal activity in T1D. Increased microbial sensing molecules such as toll-like receptors (TLR) are up-regulated in diabetic prone animals (101; 102).

Similarly, both dendritic cell and macrophage populations are shifted to more inflammation inducing phenotypes in T1D (83). This could skew T effector repertoires and promote Th1 proliferation even in the absence of strong stimuli. TLRs signal downstream primarily through the myeloid differentiation primary response gene 88 (MyD88), a potent inducer of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) which promotes inflammation through the induction of Tumor necrosis factor alpha (TNFα) (76; 103). MyD88$^{-/-}$ NOD mice are protected from T1D through a defining mechanism involving the lack of NF-κB activation by TLRs and disease incidence has not been established (63). The absence of MyD88 also is able to manipulate commensal populations to an extent, just as the commensal diversity is able to alter TLR expressive and MyD88 activation. As an example, specific species of *Lactobacillus* and *Bacteroides* have been shown to modulate a host of TLR specific signaling responses which can ultimately lead to modulation of the immune system through the down-regulation of antimicrobial agents such as α defensins (104-106). The more knowledge we gain in the pathogenesis of T1D, the most it becomes obvious that in order to fully understand T1D, immunologists, pathologists, and microbiologists are going to have to work together in order to bridge the gaps in understanding of how the innate and adaptive immune systems are responding to commensal organisms in diabetic prone models/individuals.

The field of T1D has advanced significantly over the past few decades. The lifespan and quality of life for patients with T1D have improved significantly. However, the progress made in working toward a cure or prevention of diabetes in humans has been slow. Research into curing patients with existing T1D using inbred murine models rarely

displays translational success to human disease in T1D. Data on the pathogenesis of T1D varies greatly between experimental models and human disease; or even between different sets of human patients. Differences in genetic polymorphisms resulting from different combinations of diabetogenic loci may lead to a diversity of potential susceptibility and resistance to disease in humans. Because of this, it is difficult to determine the extent that environmental determinants such as diet, microbes, and exposure to pathogens, influence disease course in T1D. It is convenient to use genetically inbred murine models to examine T1D in an experimental setting since all mice are generally genetically identical. The use of animal models in the study of disease has highlighted many of the key genetic susceptibilities and provided invaluable insight into disease pathogenesis. However, human diabetic patients are not genetically identical. The number of genetic loci being uncovered as having disease potential in diabetes is numerous, and continues to rise. The different repertoires of genetic predisposition to disease make it difficult for us to target specific treatments to T1D. I believe in order to cure T1D; it will soon no longer be acceptable to think of diabetes as a single disease. Instead, we will have to start seeing T1D as a microcosm of very similar diseases that produce the same phenotype. However, in order to prevent T1D, I believe the use of commensals and other environmental determinants will be more effective in protecting the genetically diverse human population. This belief is fortified by the observation that in human studies, the changes in the bacterial groups within *Firmicutes* and *Bacterodetes* appear to remain constant, regardless of the genetic diversity of the human patient populations (25; 33; 107).

Future studies that rise from this research will eventually identify single organisms that produce the most beneficial responses. It will be determined what happens during the interaction between the bacterium and the mucosal barrier that elicits such an important response. From there, we will be able to develop a method to induce these responses in children who may carry genetic susceptibility to T1D and influence their immune system away from autoimmunity and toward a tolerogenic response.

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APPENDIX: Animal Use Approval Form

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF RENEWAL

DATE: July 16, 2012

TO: ROBINNA GAIL LORENZ, Ph.D.
SHEL-602 2182
FAX: (205) 934-1875

FROM: Judith A. Kapp
Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: Title: The Gastrointestinal Ecosystem and Risk of Diabetes Development
Sponsor: Juvenile Diabetes Foundation
Animal Project Number: 120808577

As of August 10, 2012, the animal use proposed in the above referenced application is renewed. The University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) approves the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Mice	A	240
Mice	B	480
Mice	C	90

Animal use must be renewed by August 9, 2013. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 120808577 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7692.

Institutional Animal Care and Use Committee
CH19 Suite 403
933 19th Street South
205.934.7692
FAX 205.934.1188

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