Abstract

Accumulating evidence supports the idea that intestinal microbes are involved in the pathogenesis of type 1 diabetes (T1D) through the gut-pancreas nexus. While it has been hypothesized that a “leaky” gut could facilitate exposure to microbial antigens and accelerate diabetes onset, the role of the microbiota and their effect on intestinal inflammation and barrier function have not been well studied. Our aim was to determine if the intestinal microbiota in the non-obese diabetic (NOD) mouse model plays a causal role in diabetes onset through a leaky and inflamed gut. To examine the effect of the intestinal microbiota on the onset of T1D we manipulated gut microbes by: 1) fecal transplantation between diabetic susceptible (NOD) and resistant (NOR) mice and 2) antibiotic and probiotic treatment of NOD mice. We monitored diabetes onset, determined microbial community structure using high throughput sequencing, examined intestinal inflammation by qPCR, barrier function using FITC-dextran, and quantified testosterone and insulin auto-antibodies in the sera. We found that fecal transplantation of NOD microbes induced insulitis in the NOR host supporting the idea that the microbiome from NOD mice is “diabetogenic” or diabetes-inducing. Furthermore, peri-natal antibiotic exposure resulted in accelerated diabetes onset in NOD mice accompanied by the production of insulin auto-antibodies, independent of a leaky gut. Neither transplantation of microbes from NOR mice nor probiotics (VSL3) were able to reverse diabetes progression in NOD mice. Antibiotic treatment resulted in lower diversity and decreased levels of several commensal microbes. The microbiota of vancomycin-treated NOD mice were enriched in pathobionts like Enterobacteriaceae and depleted of beneficial microbes like segmented filamentous bacteria (SFB). This
corresponded with increased colonic inflammation, the loss of Th17 associated cytokines and decreased testosterone. Despite accelerated diabetes, neomycin-treated NOD mice contained SFB and had similar Th17-associated cytokines and serum testosterone levels as untreated NODs, indicating that SFB alone is not sufficient to protect against the effects of the diabetogenic microbiome. We conclude that NOD mice harbor gut microbes that induce diabetes which cannot be overcome by the presence of protective microbes like SFB or addition of probiotics and that antibiotics accelerate diabetes by augmenting the diabetogenic microbiome.
Preface

Animal experiments were approved by UBC animal care committee (Animal care #: A11-0290) and performed at the Child and Family Research Institute (CFRI) in Vancouver, BC. Animal experiments were performed primarily by Caixia Ma, with assistance from Ben Dai and I under the supervision of Drs. Deanna Gibson (UBC Okanagan, Kelowna), Jan Dutz (CFRI & UBC, Vancouver) and Bruce Vallance (CFRI & UBC, Vancouver). I contributed to experimental design for the antibiotic experiments and conducted subsequent tissue analysis and cell culture experiments at the UBC Okanagan campus. Dr. Carol Chan performed the testosterone assay, pyrosequencing was performed by the Laboratory for Advanced Genomic Analysis (Vancouver, BC) and the insulin auto-antibody assay was performed by the Barbara Davis Center for Diabetes (Aurora, CO, USA). Part of this work was presented as a student talk at the Canadian Digestive Disease Week (CDDW) conference in Montreal (February 2012), and as a poster at the Banff Inflammation Workshop in Banff, AB (January 2013) and the Keystone Symposia: The Gut Microbiome in Taos, NM, USA (February 2013). I wrote this thesis with suggestions and guidance from Dr. Deanna Gibson and revisions from my committee members Drs. Joyce Boon, Miranda Hart and Sanjoy Ghosh. Editorial assistance was provided by Andreas Peters.
Publications:

Much of the research presented in this thesis is included in the following publication:


Concepts and background information from this thesis are presented in part in the following manuscripts, review paper and book chapter. These publications are in bold where cited in text:


Review Paper:


Book Chapter:

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List of Symbols and Abbreviations

°C  Degree (Celsius)
APC  Antigen presenting cell
BB  Biobreeding
BSA  Bovine serum albumin
Ccr7  Chemokine receptor type 7
CD4  Cluster of differentiation 4
CD8  Cluster of differentiation 8
CTL  Cytotoxic T lymphocyte
Cxcl9  Chemokines (C-X-C motif) ligand 9
DC  Dendritic cell
EPEC  Enteropathogenic Escherichia coli
FISH  Fluorescence in situ hybridization
FITC  Fluorescein isothiocyanate
g  Grams
GALT  Gastrointestinal associated lymphatic tissue
GF  Germ free
GI  Gastrointestinal
H2O  Water
HLA  Human leukocyte antigen
HTS  High throughput sequencing
IFN  Interferon
l  Litre
MHC  Major histocompatibility complex
NOD  Non-obese diabetic
NOR  Non-obese resistant
PBS  Phosphate buffered saline
PCO  Principle coordinates
PCR  Polymerase chain reaction
PLN  Pancreatic lymph nodes
RELM  Resistin-like molecule
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>SFB</td>
<td>Segmented filamentous bacteria</td>
</tr>
<tr>
<td>SPF</td>
<td>Specific pathogen free</td>
</tr>
<tr>
<td>T1D</td>
<td>Type 1 diabetes</td>
</tr>
<tr>
<td>Tc</td>
<td>Cytotoxic T cell</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TEER</td>
<td>Trans-epithelial electrical resistance</td>
</tr>
<tr>
<td>Th1</td>
<td>T-helper 1</td>
</tr>
<tr>
<td>Th17</td>
<td>T-helper 17</td>
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<tr>
<td>Th2</td>
<td>T-helper 2</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<tr>
<td>α</td>
<td>Alpha</td>
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<td>β</td>
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<td>γ</td>
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Chapter 1: Introduction

1.1 Type 1 diabetes

Type 1 diabetes (T1D) is an autoimmune disease characterized by destruction of insulin-secreting beta cells in the islets of Langerhans in the pancreas. The destruction of beta cells results in a complete lack of insulin, which prevents the uptake of glucose from the blood into cells with insulin-dependent glucose transporters. In T1D, while glucose levels in the blood are elevated, cells that depend on insulin for glucose transport are unable to access glucose which compromises their ability to function. The importance of insulin in regulating blood glucose was recognized by Banting and Best in 1922 [1]; prior to this discovery T1D resulted in wasting and death within ~1.4 years of diagnosis [2]. T1D is now managed with exogenous sources of insulin although it remains difficult to regulate insulin/glucose balance which can cause damage to blood vessels leading to morbidity and early mortality in those who suffer from the disease. T1D incidence is rising 3-5% annually in Canada [3], with similar increases in other ‘western’ countries [4]. According to US statistics, the current prevalence of 2.13/1000 will double to 5.2/1000 by 2050 should current trends continue [5].

The etiology of T1D is not fully understood although there is a genetic component to the disease. Susceptibility loci that are associated with increased risk of developing the disease have been identified, most of which are related to immune cell function. The human leukocyte antigen (HLA) region is the most strongly associated region in T1D. It encodes the major histocompatibility complex (MHC) in humans, which is involved in T cell activation. Regions that encode interleukin-2 receptor α, protein tyrosine phosphatase, non-receptor type 22 and cytotoxic T lymphocyte antigen 4 show weaker, yet significant association (reviewed in [6]). Although there is an established genetic link, the observed
increase in incidence suggests that the environment is playing a role in disease pathogenesis. In support of this, concordance levels between monozygotic twins are as low as 13% [7] and migration studies reveal that incidence is a result of location rather than genetic background (reviewed in [8]). The most pronounced increase in incidence is observed in children 0-4 years of age [9, 10], which indicates that early life exposures may be critical in shaping disease progression.

Clinical T1D research is challenging because of the long asymptomatic period prior to complete destruction of beta cells and the fact that procedures to sample pancreatic tissue are invasive. Animal models have emerged as valuable tools to study the disease pathogenesis. Two of the most commonly used animal models of spontaneous T1D are the non-obese diabetic (NOD) mouse and the Biobreeding diabetes prone (BB-DP or BB) rat. The NOD mouse and BB rat share genetic susceptibility loci with human T1D: both have loci within the MHC genes that are the primary associated susceptibility regions [11, 12]. Furthermore, progression to diabetes is preceded by insulitis, or inflammation in the islets, which is similar to the progression of diabetes in human T1D. In NOD mice, invasive insulitis is present at ~10 weeks and overt diabetes between 14 and 30 weeks of age [13]. BB rats typically develop insulitis between 5-10 weeks, with overt diabetes following within 2-3 weeks [14]. Both NOD mice and BB rats have patterns of cytokine expression and immune cell infiltration into the pancreatic islets similar to what is seen in human T1D [15]. It should be noted, however, that there are some differences in how T1D progresses in these animals models compared to humans. In NOD mice, there is a unique pattern of peri-insulitis, or infiltration around the beta cell, and there is a considerable sex bias in NOD mice; incidence rates in female are 60-80% and 20-30% in males [12]. Although in general,
females are more susceptible to autoimmune conditions [16], a sex bias is not noted in human T1D or the BB rat [17]. As well, in the BB rat, T cell lymphopenia (abnormally low levels of T cells) occurs and is required for T1D to develop [18]; this is not observed in human T1D. Despite the differences in certain aspects of diabetes pathogenesis in NOD mice and BB rats, the similarity in genetic susceptibility and organ specific autoimmunity make them the best available models. Since NOD mice were used in this study, a more detailed review of their immunology is summarized below.

1.2 Immunology of T1D

In order for autoimmune destruction of beta cells to occur, the immune response must be initiated by a stimulus and a pro-inflammatory environment maintained. Many of the identified disease susceptibility loci in T1D are located in regions that affect immune cell function, although how this translates to autoimmunity is not well defined. It is possible that deregulation occurs at the level of antigen recognition and initiation of the inflammatory response, or in the cells responsible for propagating or regulating inflammation. Below is a summary of what is currently known.

Initiation of the immune response

Antigen presenting cells (APCs) are involved in initiating inflammatory responses by sensing antigens throughout the body and presenting them to other cells of the immune system. Dendritic cells (DCs) and macrophages are the two main types of APCs. Elimination of DCs prevents initiation of T1D in NOD mice [19] while the role of macrophages in T1D is less clear. Studies have shown that elimination of macrophages has
no effect [19], while others indicate that macrophages are critical for activation of diabetogenic T cells [20].

B cells are part of the antibody-mediated branch of the immune system and develop a B cell receptor (antibody) which binds a specific antigen. B cells propagate when they come into contact with their antigen, present it to T cells that possess a T cell receptor (TCR) that specifically recognizes the antigen/MHC complex, and then differentiate into plasma B cells or memory B cells in the presence of specific cytokines like IL-4. Plasma B cells secrete antibodies which augment the immune responses to the specific antigen, while memory B cells reside in the lymphatic fluid and facilitate a rapid response if a subsequent exposure to the same antigen occurs. Dysfunctional B cell production prevents diabetes in NOD mice suggesting that they are critical cells in the initiation of autoimmunity leading to T1D [21, 22].

Generally antibodies are directed towards foreign or pathogenic antigens and facilitate immune responses to aid in their destruction. If they are directed toward antigens on the body’s own cells, they are called auto-antibodies. Certain auto-antibodies react with proteins in the pancreatic islets including those directed to insulin, glutamic acid decarboxylase, insulinoma-associated antigen 2, and zinc-transporter-8. The auto-antibodies are often present in T1D patients prior to clinical onset and their presence in the sera is strongly associated with disease. In genetically at-risk individuals, 69.7% with at least two islet auto-antibodies developed T1D, compared to only 14.5% with a single islet autoantibody and 0.4% of those with no islet auto-antibodies [23]. Although auto-antibodies themselves do not cause damage, they can draw cytotoxic immune responses towards the
islet antigens. Since islet auto-antibodies are present prior to the clinical presentation of the disease, it brings into question the origin of the auto-antibodies.

Propagation of the immune response

T cells originate from the same lymphoid precursors as B cells but their maturation occurs in the thymus and their expansion is restricted by the MHC. The MHC is a group of genes that code for cell surface molecules that are required by T cells to recognize antigens. As mentioned, the main susceptibility loci associated with T1D are in the genes that code for the MHC. MHC I is found on all nucleated cells, whereas MHC II is found only on antigen presenting cells. Immature thymocytes have surface glycoproteins CD4 and CD8, as well as a T cell receptor (TCR) that recognizes specific antigen/MHC complexes. If the antigen is presented with MHC I, the CD8 co-receptor binds, and it becomes a mature CD8+ T cell, or cytotoxic T cell (Tₐ). If the antigen is presented by MHC II, the CD4 co-receptor binds and it becomes a mature CD4+ T cell, or T helper cell (Tₜ). While only APCs have MHC II, all cells have MHC I, so CD8+ T cells can be generated by antigens presented by non-immune cells (virus infected or altered self cells), or by immune cells, termed cross-presentation. Dendritic cells are of particular importance in presenting exogenous antigens through MHC I to generate Tₐ to exogenous antigens [24], which could play a key role in T1D progression.

T helper subsets secrete cytokines that facilitate many immune responses including activation of Tₐ cells into cytotoxic T lymphocytes (CTLs). These CTLs function as killer cells that destroy all MHC class I restricted cells (all nucleated cells) including pancreatic beta cells. Several T cells that react with beta cell antigens have been identified. In humans, this includes T cells that react with GAD65, insulin and IA-2, and in NOD mice GAD65, IGRP and insulin (reviewed in [25]). Complete depletion of T cells inhibits diabetes in the
NOD mouse [26] and transfer of CD4+ and CD8+ cells together (but not independently) initiates diabetes indicating that both types are required for the autoimmune response [27].

CD4+ T cells are termed T helper (T\textsubscript{h}) cells because they facilitate many functions of the immune response by direct cell binding and cytokine release. Based on the cytokine environment, CD4+ T cells differentiate into T\textsubscript{h} subsets (T\textsubscript{h}1, T\textsubscript{h}2 and T\textsubscript{h}17) or regulatory T cells (T\textsubscript{reg}) as discussed below (reviewed in [28]). T\textsubscript{h}1 cells function primarily against intracellular bacteria and protozoa. They activate Tc and macrophages through their effector cytokine IFN-\gamma. T\textsubscript{h}2 cells function primarily against helminthes. They activate B cells and other innate immune cells including mast cells, basophils and eosinophils through their effector cytokines IL-4 and IL-5. Under physiological conditions, there is a balance between T\textsubscript{h}1 and T\textsubscript{h}2 cell populations and disruption of this balance can result in disease. T1D is classified as a T\textsubscript{h}1-mediated disease since destructive insulitis is associated with higher levels of IFN-\gamma and lower levels of IL-4[29]. It has been shown that helminth infection, which shifts the Th\textsubscript{1}/Th\textsubscript{2} balance in favour of T\textsubscript{h}2 cells is protective in NOD mice [30].

Another main subset of T helper cells is the T\textsubscript{h}17 cells. T\textsubscript{h}17 cells differentiate in the presence of TGF-\beta and their main effector cytokine is IL-17A. They are believed to play a key role in autoimmune diseases such as arthritis and psoriasis, however their role in T1D remains unclear (reviewed in [31]). In new-onset T1D patients, there is an increase in IL-17 producing cells [32] and there is elevated IL-17 expression in the colon of NOD mice [33]. Silencing IL-17 does not protect NOD mice from diabetes [34], yet the consistent association of T\textsubscript{h}17 cells with T1D suggests that they have some role in disease progression. One mechanism by which T\textsubscript{h}17 cells may contribute to T1D is by converting to T\textsubscript{h}1 cells. This is supported by data showing that transfer of T\textsubscript{h}17 cells does not lead to diabetes in
NOD mice, but in NOD mice that do not generate B and T cells on their own (NOD.scid), T\(_h\)17 cells take on a T\(_h\)1 phenotype and rapid T1D is induced[35]. Another proposed mechanism for T\(_h\)17 cells is to shift the balance of T\(_{reg}\)/T\(_{eff}\) cells in favour of T\(_{eff}\) cells, which is reported in BB rats[36]. In contrast, microbe-induced T\(_h\)17 responses have been shown to protect NOD mice from T1D. Segmented Filamentous Bacteria (SFB) selectively colonize the terminal ileum of laboratory mice and are potent inducers of T\(_h\)17 responses [37]. It has been shown that colonization with SFB positively correlates with protection from diabetes, with the only difference in T cell subsets being that there are increased T\(_h\)17 cells in the small intestinal lamina propria [38].

**Immune Regulation**

The effect of pro-inflammatory and cytotoxic immune cells is minimized by regulatory cells of the immune system. Many individuals who are genetically susceptible to T1D do not develop the disease and T cells that are auto-reactive to islet antigens are present in the unaffected population [39]. This indicates that dysfunctional regulation of the immune system is involved in the development of autoimmunity.

Differentiation of regulatory cell populations is promoted by the cytokine environment, notably TGF-\(\beta\) and IL-10, which are mainly produced by T\(_{reg}\) cells. T\(_{reg}\) cells are characterized by the expression of forkhead box transcription factor (Foxp3) and are key cells in maintaining peripheral tolerance. Elimination of the T\(_{reg}\) cells causes almost complete penetration of diabetes in BDC2.5 TCR NOD mice which normally have only 10-20% diabetes [40]. In addition, T1D patients have fewer or less effective T\(_{reg}\) cells [41]. T\(_{regs}\) are known to be regulated by the microbiota [42] and a key function of T\(_{regs}\) is to maintain the balance of T\(_h\)1 and T\(_h\)2 responses. Precisely how they protect from diabetes is
not well understood although a few proposed mechanisms include production of anti-inflammatory cytokines (including TGF-β and IL-10), killing of APCs and competition for IL-2 (reviewed in [43]).

Dendritic cells (DCs) play a role in tolerance and regulation in addition to acting as APCs. Depletion of DC in NOD mice prevents the autoimmune response [19], but depleting this cell type after an immune response has been primed accelerates diabetes [19]. This points to a dual role for DCs in initiation and regulation of the immune response. In NOD mice, the plasmocytoid DCs have been proposed to switch from pro-inflammatory (IFN-γ secreting) early in disease to regulatory (indoleamine 2, 3-dioxygenase (IDO) secreting) as T cells accumulate and disease progresses [44]. The switch from IFN-γ to IDO promotes T_{reg} cell populations [45] and regulatory DCs are also proposed to prevent the activation of naïve CD8+ T cells, and to deplete memory T cells [46].

In summary, CD8+ T cells, CD4+ T cells, B cells and macrophages are all found in infiltrated islets of T1D patients[47]. Dysfunctional antigen recognition through DC and production of auto-antibodies by B cells appear be involved in disease initiation while both CD4+ and CD8+ T cells are required for the propagation of the autoimmune response. The T_{h1}/T_{h2} balance is shifted towards T_{h1}, although the role of T_{h17} remains unclear. Dysfunctional regulation through T_{reg} or tolerogenic DCs may also be playing a role in T1D progression. Many aspects of the immune response appear to be important in the autoimmunity leading to T1D. Ultimately, whether the dysfunction leading to autoimmunity occurs in its initiation, propagation and/or regulation of the immune response is not known.
1.3 T1D and the gastrointestinal tract

Predominant hypotheses for the increased incidence of T1D include: viral infections, vitamin D deficiency, infant nutrition (particularly the early introduction of cow’s milk) and the hygiene hypothesis (reviewed in [48]). In the past few years, compelling evidence that diet, microbiota and enterovirus infection can alter disease progression supports the idea that the gastrointestinal (GI) tract is central to disease pathogenesis (reviewed in [49]).

1.3.1 T1D and diet

The increase in T1D correlates with a lifestyle shift that has impacted both maternal and infant diet. The study of diet in T1D has provided evidence that various dietary antigens are pro- or anti-diabetogenic, some of which are reviewed in Table 1. The two food components that have been studied most extensively in the context of T1D are cow’s milk protein exposure relating to breast-feeding practices, and gluten because of its pro-inflammatory effect in the gut. Breast feeding is known to support maturation of the gut epithelium and immune system [50] and early introduction of cow’s milk may interfere with these processes. Cow’s milk proteins are also more complex than breast milk proteins and the introduction of foreign proteins may be contributing to autoimmune: a clinical trial in at-risk individuals found that supplementing breast feeding with a hydrolyzed casein formula decreased diabetes incidence compared to supplementation with a cow’s milk formula [51]. Gluten peptides stimulate pro-inflammatory responses in small intestinal biopsies from T1D patients [52] and some T1D patients exhibit enhanced T_h1 and T_h17 cell reactivity to wheat [53] which suggests that gluten promotes gut inflammation and may contribute to autoimmunity.
Table 1: Dietary components that influence T1D progression

<table>
<thead>
<tr>
<th>Component</th>
<th>Administered</th>
<th>Control</th>
<th>Model</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diets that promote T1D</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wheat-germ</td>
<td>Weaning</td>
<td>Wheat germ free</td>
<td>NOD mice</td>
<td>[54]</td>
</tr>
<tr>
<td>Early cereal exposure</td>
<td>Age of exposure to cereals reported</td>
<td>Age, HLA, family history, caloric intake, omega-6 intake-matched controls</td>
<td>At risk children (clinical)</td>
<td>[55]</td>
</tr>
<tr>
<td><strong>Diets that delay or prevent T1D</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soy-based and lactose-free</td>
<td>Weaning</td>
<td>Normal chow</td>
<td>NOD mice</td>
<td>[33]</td>
</tr>
<tr>
<td>Hydrolyzed casein</td>
<td>Weaning</td>
<td>Non-hydrolyzed casein</td>
<td>NOD mice</td>
<td>[56]</td>
</tr>
<tr>
<td>Gluten-free</td>
<td>Weaning</td>
<td>Normal chow</td>
<td>NOD mice</td>
<td>[57]</td>
</tr>
<tr>
<td>Low (8%) protein (maternal)</td>
<td>Conception to weaning</td>
<td>Normal chow (20% protein)</td>
<td>NOD mice</td>
<td>[58]</td>
</tr>
<tr>
<td>Low n6/n3 fatty acid ratio (maternal)</td>
<td>Conception to weaning</td>
<td>Normal chow</td>
<td>NOD mice</td>
<td>[59]</td>
</tr>
<tr>
<td>Omega-3 fatty acids</td>
<td>Reported dietary intake from 1-~6 years</td>
<td>Age, HLA, family history, caloric intake, omega-6 intake-matched controls</td>
<td>At risk children (clinical)</td>
<td>[60]</td>
</tr>
</tbody>
</table>

Despite the associations between diet and T1D, the mechanisms by which dietary antigens direct T1D progression are unclear. It may involve direct deregulation of the intestinal immunity or the intestinal barrier or it could be exerting an effect through the microbiota. A soy-based and lactose-free diet was shown to be anti-diabetogenic in NOD mice by regulating Th17 responses [33], while a hydrolyzed casein diet was shown to improve barrier function leading to protection in the BB rat [61]. Diet is known to influence the composition of the intestinal microbiota, which affects the progression of several diseases (reviewed in [62]). It has been shown that a gluten free diet decreases diabetes in NOD mice and is associated with reduced numbers of bacteria in the cecum [63]. A gluten free diet also causes changes in the composition of the fecal microbiota [64]. Diet-induced changes in the microbiota may represent an interesting angle to be further explored in the context of T1D.
1.3.2 Leaky Gut and T1D

The GI tract has a surface area greater than 200m$^2$, making it the largest surface in the body that is exposed to the external environment. The epithelial barrier is a dynamic membrane that regulates interactions with the external environment including dietary and bacterial antigens. The integrity of the barrier is maintained by tight junctions between the epithelial cells that are regulated by tight junction proteins (TJPs) including occludin [65], members of the claudin family [66], and junctional adhesion molecules (JAMs) [67]. Under physiological conditions, constant communication occurs between contents of the gut and the systemic immune system. Specialized epithelial cells, M (or microfold) cells, transport antigens across the barrier and expose them to cells of the immune system on the basolateral side. Also, circulating dendritic cells pass between epithelial cells and sample antigens in the lumen. By these mechanisms, the immune system develops tolerance to commensal antigens. A dysfunctional epithelial barrier, known as a “leaky gut”, causes increased exposure of luminal antigens that may disrupt homeostasis and lead to deregulated immunity.

There is an established link between inflammation and a leaky gut. Incubating monolayers of gut epithelial cells with pro-inflammatory cytokines TNF-α [68] and IFN-γ [69] results in a loss of trans-epithelial resistance through the tight junction proteins JAM-1, claudin-1 and claudin-4 [70].

T1D patients have leaky guts prior to clinical onset of the disease with barrier dysfunction also observed in relatives of T1D patients [71, 72] suggesting that a leaky gut is not simply a result of disease. Increased gut permeability is observed in NOD mice and BB rats prior to diabetes onset [73, 74] and improving the intestinal barrier in BB rats protects
them from T1D [75]. Furthermore, disrupting the epithelial barrier with an enteric pathogen accelerates insulitis in NOD mice while a mutant strain that fails to disrupt the barrier does not accelerate insulitis [74]. Increasing the function of the intestinal barrier has been shown to have no effect on T1D incidence in NOD mice indicating that subsequent improvement of barrier function is not sufficient to reverse the predisposition to diabetes [76]. In summary, a leaky gut is associated with T1D; however its importance is debated in the scientific community since a causative effect has not yet been established.

1.3.3 Intestinal inflammation and T1D

The gut represents a critical interface in regulating intestinal immunity and systemic immunity. However, the link between the inflammatory status in the intestine and diabetes progression is not fully understood (reviewed in [77]). The GI tract contains a rich source of antigens from commensal and pathogenic microbes as well as ingested food. In the gut, circulating APCs sample antigens from the lumen of the GI tract and activate circulating T cells. T cells differentiate into different subsets based on the cytokine environment, which is influenced by both innate immune cells and bacterial stimulation of toll-like receptors on epithelial cells. Through these mechanisms, normal development of the gut immune system results in tolerance to commensal bacteria while pathogenic microbes are destroyed. It is known that T cells in the pancreatic lymph nodes (PLN) are activated by antigens from the GI tract and the peritoneal cavity [78]. In young NOD mice, the majority of diabetogenic T cells are harboured in the gut associated lymphatic tissue (GALT) suggesting that this is the site where these T cells are primed to become auto-reactive [79]. A breakdown in the
recognition of microbial antigens or over-active responses may result in inappropriate
activation of the immune system leading to autoimmunity.

1.4 The intestinal microbiota in T1D

1.4.1 The intestinal microbiota

An estimated $10^{14}$ bacterial cells associated with the human organism colonize all
areas exposed to the external environment. The intestines are the most heavily colonized area
with bacterial density increasing along the length of the GI tract to the ileum ($10^7$ cells/gm)
and colon ($10^{12}$ cells/gm) [80]. This collection of microbes is termed the intestinal
microbiota or the intestinal microbiome if also considering the bacterial genetic material
[81]. The majority of the microbes in the intestine are from two phyla, Firmicutes and
Bacteroidetes, with Actinobacteria, Verrucomicrobia, Proteobacteria, Ternicutes and
Cyanobacteria present in smaller amounts [82]. At the phylum level, most human microbiota
are similar, but there is considerable variation between individuals at lower taxonomic levels:
there are ~1100 different species in each individual gut but only ~160 are common across all
individuals [83]. Colonization of the GI tract occurs in three major steps: delivery,
breastfeeding and weaning [84]. During colonization, the microbial composition fluctuates
but increases in density and diversity until approximately 2-4 years of age after which it has
been proposed to remain relatively stable into adulthood [85]. The process by which an
individual microbial fingerprint develops is not fully defined, but vertical transmission of
microbes from the parents as well as maternal and infant nutrition and antibiotic exposure
play a role (reviewed in [86]). Early influences on the intestinal microbiota composition
include method of delivery and infant nutrition. Infants born vaginally acquire the mother’s
vaginal and intestinal flora including Bacteroides, Bifidobacterium, Lactobacillus, and Escherichia coli, whereas those born by caesarean section have increased levels of skin associated bacteria including Staphylococcus spp. [87]. The microbiota acquired based on method of delivery persist into childhood [88]. Breast fed infants have higher levels of Bifidobacteria spp. while formula fed infants have higher levels of Bacteroides, Clostridium coccoides and Lactobacillus spp. [89]. Interestingly, these factors that influence colonization are also associated with altered incidence of T1D. Children born by Cesarean section have a higher T1D incidence than those born by vaginal birth [90] and breast fed children have a decreased incidence of T1D when compared to formula fed infants [91].

The intestinal microbiota that each individual develops is now known to play a pivotal role in structure of the GI tract, host nutrition and metabolism, as well as immune system development in the intestines and throughout the body (reviewed in [92]). The importance of microbes is often defined through studies using germ free (GF) mice that are devoid of microbes. These mice are born through cesarean section and maintained in sterile conditions and serve as models to test the effects of colonization with whole microbiota or individual species. GF mice have slower cell turnover and cell regeneration which results in shortened villi [93]. The function of the intestinal barrier is also affected by microbial colonization with certain Lactobacillus spp. shown to improve barrier integrity [94]. The importance of microbes in nutrition was noted since GF mice require considerably more calories to maintain the same body weight as colonized mice [95]. Two ways that bacteria contribute to host nutrition is by degrading oligosaccharides that cannot otherwise be digested and by facilitating uptake of nutrients [95].
In addition to aiding in digestion and uptake of nutrients, the GI tract is a major immunological organ. The intestinal mucosal immune system is comprised of gastrointestinal lymphoid tissue (GALT) including the Peyer’s patches in the small intestine, lymphoid aggregates in the colon and the lamina propria (a thin layer of connective tissue beneath the epithelium which contains capillaries and lymphatic vessels) throughout the gut. GF mice have reduced lymphatic tissue [96], decreased numbers and function of intraepithelial lymphocytes [97], a decrease in sIgA secretion [98] and a lack of expansion of CD4+ T-cell populations [99].

The GALT contains the largest number of immune cells in the mammalian body, which must remain tolerant to commensal microbial and food antigens but responsive to pathogens. This relationship is generally maintained, however dysbiosis (alterations in the composition of the normal microbiota) can lead to disease in the GI tract and throughout the body [62]. Dysbiosis is associated with inflammatory bowel diseases, celiac disease and irritable bowel syndrome as well as obesity, type I and type II diabetes, autism and multiple sclerosis in both humans [100] and animal models [101]. Considerable research has focused on elucidating causative effects and mechanisms by which the intestinal microbiota contributes to these diseases.

1.4.2 T1D and the intestinal microbiota

The intestinal microbiota has been shown to be altered in T1D both clinically and in animal models. The diabetic microbiota is characterized by decreased diversity and a decreased Firmicutes: Bacteroidetes ratio, as well as fewer Lactobacillus, Bifidobacteria, Prevotella and Roseburia, and more Bacteroides and Clostridium species [102-104]. Current
studies that characterize the microbiota in T1D and their specific findings are outlined in Table 2.

**Table 2: Type 1 diabetes-associated microbiota in clinical studies and rodent models**

<table>
<thead>
<tr>
<th>T1D-associated microbes</th>
<th>Model</th>
<th>Control</th>
<th>Year</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>↓ <em>Bifidobacteria</em> and <em>Roseburia</em> ↑ <em>Clostridia</em> and <em>Bacteroides</em></td>
<td>Clinical: At-risk children prior to onset with at least 2 auto-Ab (n=18)</td>
<td>Early feeding history, HLA, age and sex matched auto-Ab negative children (n=18)</td>
<td>2013</td>
<td>[105]</td>
</tr>
<tr>
<td>↓ Firmicutes: Bacteroidetes ↓ <em>Lactobacillus</em>, <em>Bifidobacteria</em>, &amp; <em>Prevotella</em> ↑ <em>Clostridium</em>, <em>Bacteroides</em> &amp; <em>Veillonella</em></td>
<td>Children with T1D (n = 16)</td>
<td>Children without T1D (n=16)</td>
<td>2013</td>
<td>[102]</td>
</tr>
<tr>
<td>↓ Firmicutes: Bacteroidetes ratio ↓ <em>Lactobacillaceae</em>, <em>Porphyromaadaceae</em> and <em>Rickenellaceae</em></td>
<td>NOD Female</td>
<td>NOD MyD88 KO Female (non-diabetic)</td>
<td>2008</td>
<td>[103]</td>
</tr>
<tr>
<td>↓ Diversity ↓ <em>Lactobacillus</em> &amp; <em>Bifidobacteria</em> spp.</td>
<td>BB-DP rat</td>
<td>BB-DR rat</td>
<td>2009</td>
<td>[104]</td>
</tr>
<tr>
<td>↓ Diversity ↓ Firmicutes: Bacteroidetes</td>
<td>Genetically susceptible infants who develop T1D (n=4)</td>
<td>Genetically susceptible infants who do not develop T1D (n=4)</td>
<td>2010</td>
<td>[106]</td>
</tr>
</tbody>
</table>

Observational studies show that the gut microbiota is altered in T1D, whether it is a cause or an effect of the disease is not fully defined. It was observed that penetrance of diabetes in NOD mouse colonies varied depending on the facility where they were housed [107]; indeed diabetes incidence in female NOD mice ranges from 60-100% depending on...
facility [108]. GF mice that are born and maintained without microbes have a more consistent incidence of T1D (95-100%) regardless of facility [108].

GF models have provided some insight into a causal relationship between the microbiota and T1D progression. An initial study that established a connection between the microbiota and T1D used NOD mice that lacked MyD88, an innate signaling molecule that relays signals from bacteria on the epithelial cell surface. MyD88 KO NOD mice were protected from T1D, while GF MyD88 KO NODs developed robust diabetes [103]. Subsequent studies in GF NOD mice have shown that microbiota colonization delays insulitis [109], and mono-colonization with *B. cereus* [110] and Segmented Filamentous Bacteria (SFB) [38] delays diabetes in female NOD mice. The NOD model has been studied in the context of gender biased autoimmunity as male mice have a slower progression and lower incidence of diabetes than female NODs. Recent evidence suggests that this may be connected to the microbiota since the gender bias is reduced under GF conditions [111]. Transplantation of mature male microbiota into 4 week old female NODs increases serum testosterone correlated with diabetes protection [111]. Another study reveals that colonization with SFB or a microbe isolated from male NOD microbiota (SECS) protects GF NOD females from diabetes whereas a combination of *Lactobacillus* and *Bifidobacteria* species (VSL3) does not [108]. While the previous study identified a role for testosterone, this study does not show a correlation between protection from diabetes and the induction of testosterone. Thus it is unclear if testosterone is a mechanism by which the microbiota influences the gender bias in NOD mice.
Manipulating the microbiota with probiotics or with antibiotics are potential mechanisms to assess how changing the microbiota affects diabetes progression. Probiotics are loosely defined as live microorganisms that are claimed to have health benefits to the host. Common probiotics are members of the Lactobacillaceae and Bifidobacteriaceae families and several of these have been tested in T1D models. It was determined that post-weaning administration of *Lactobacillus casei* is protective in NOD mice [112], and that a particular strain of *Lactobacillus johnsonii* (N6.2) but not *Lactobacillus reuteri* is protective in BB rats[113]. VSL3 (a combination of *Lactobacillus, Bifidobacteria, and Streptococcus* spp.) has been found to delay T1D in NOD mice when administered post-weaning [114]. Yet no differences in diabetes onset was observed when GF NODs were colonized with VSL3 [108]. Antibiotics kill certain bacteria by targeting aspects of bacterial anatomy and physiology that are different from eukaryotic cells, including the bacterial cell wall and ribosomes. It has been reported that post weaning treatment with a combination of antibiotics (sulfatrim and colistine sulphate) delays diabetes in BB rats [115] and a study in a virus-induced model of T1D found that treating rats with the Sulfatrim also delays diabetes [116]. Hansen *et al.* found that the antibiotic vancomycin delays onset of diabetes if administered in the first 4 weeks of life and this is associated with overgrowth of the Gram-negative commensal *Akkermansia muciniphila* [117]. A list of studies in GF mice and those using probiotics and antibiotics are listed in table 3 below.
### Table 3: Germ-free, antibiotic and probiotic studies in T1D models.

<table>
<thead>
<tr>
<th>Key Result</th>
<th>Model</th>
<th>Year</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Germ Free Studies</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPF colonization delays diabetes</td>
<td>MyD88 KO NOD mice</td>
<td>2008</td>
<td>[103]</td>
</tr>
<tr>
<td>SPF colonization delays insulitis but diabetes progression is similar to GF</td>
<td>NOD mice</td>
<td>2011</td>
<td>[109]</td>
</tr>
<tr>
<td>Mono-colonization with SFB delays</td>
<td>NOD mice</td>
<td>2011</td>
<td>[38]</td>
</tr>
<tr>
<td>Mono-colonization with <em>B. cereus</em> delays</td>
<td>NOD mice</td>
<td>2012</td>
<td>[110]</td>
</tr>
<tr>
<td>No gender bias in GF mice. Colonization with mature male microbes delays</td>
<td>NOD mice</td>
<td>2013</td>
<td>[111]</td>
</tr>
<tr>
<td>No gender bias in GF mice. Colonization with SFB and SECS but not VSL3 protects</td>
<td>NOD mice</td>
<td>2013</td>
<td>[108]</td>
</tr>
<tr>
<td><strong>Probiotic Studies</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post-weaning <em>Lactobacillus casei</em> delays</td>
<td>NOD mice</td>
<td>1997</td>
<td>[112]</td>
</tr>
<tr>
<td>Post weaning VSL3 (<em>Lactobacillus</em> and <em>Bifidobacteria</em> spp.) delays</td>
<td>NOD mice</td>
<td>2005</td>
<td>[114]</td>
</tr>
<tr>
<td>Post weaning <em>Lactobacillus johnsonii</em> but not <em>Lactobacillus reuteri</em> delays</td>
<td>BB rat</td>
<td>2010</td>
<td>[113]</td>
</tr>
<tr>
<td><strong>Antibiotic Studies</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post weaning sulfamethoxazole, trimethoprim and colistine sulphate delays diabetes</td>
<td>BB rats</td>
<td>2006</td>
<td>[115]</td>
</tr>
<tr>
<td>Vancomycin treatment (0-4 weeks) delays but late life (8+ weeks) does not significantly delay</td>
<td>NOD mice</td>
<td>2012</td>
<td>[117]</td>
</tr>
<tr>
<td>Antibiotic treatment (Sulfatrim, trimethoprim + sulfamethoxalone) prevents</td>
<td>Kilham rat virus (KRV) induced</td>
<td>2012</td>
<td>[116]</td>
</tr>
</tbody>
</table>

#### 1.4.3 Techniques to study the intestinal microbiota

Traditional approaches to studying bacteria do not transfer well to the study of the intestinal microbiota because the majority (>80%) of gut microbes are anaerobic which makes them difficult to grow in the laboratory setting [82]. As a result, techniques have been developed to study the microbiota at the molecular level, primarily using the 16S ribosomal RNA subunit (16S rRNA) gene. This gene codes for a component of bacterial ribosomes and is widely accepted as a method to define bacterial communities taxonomically. It is used
because the gene is vertically transmitted through generations, it is an appropriate size (~1500 base pairs) and it has regions which are conserved by ‘all’ bacteria, and variable regions which vary amongst lower taxonomic levels down to species [118]. Designing probes or primers to conserved regions allows detection of many different bacteria whereas those designed to variable region may detect a single species. As whole or partial sequences for 16S rRNA genes are generated for bacteria, they are added to databases such as Greengenes or NCBI’s 16S ribosomal RNA database. Many techniques to study the microbiota make use of 16S genes including high throughput sequencing, quantitative PCR and fluorescence in situ hybridization. These techniques will be briefly discussed as they have been utilized in these studies.

16S sequencing/high throughput sequencing

Bacteria can be identified by sequencing the 16S rRNA gene and comparing it to 16S rRNA databases. In the study of microbial communities, sequencing of the whole 16S gene has largely been replaced by sequencing of an amplified region of the 16S gene. Because of the conserved and variable regions in the 16S gene, primers are designed in the conserved regions to pick up most bacterial diversity, but span one or two highly variable regions so that when they are sequenced lower taxonomic levels can be defined. High throughput sequencing makes use of molecular identifier (MID) tags or barcodes, which are incorporated into the primers. Each sample is amplified with a primer that has a different MID barcode, and then the amplicons are pooled together and sequenced. The sequences are then grouped based on the MID and compared to 16S gene databases to determine which bacteria are represented. Each group of sequences represents the structure of the microbial community in
each of the samples. A flow chart describing this process is shown in Figure 1. High throughput sequencing has revolutionized how we evaluate the microbiota and is largely considered the gold standard for inferring community structure. It allows for fairly accurate taxonomic resolution and has a much higher throughput than whole gene sequencing. The downside of high throughput sequencing is that it is a more expensive technique and extensive bioinformatics are required for analysis.

**Figure 1: Workflow for amplicon sequencing of a microbial community.** In sample preparation, DNA from microbial samples is amplified with universal bacteria primers targeting conserved regions of the 16S rRNA gene and flanking variable regions. Each sample is amplified with a primer that has a different MID barcode (an identifying sequence of DNA) included. Following PCR amplification, the band of interest is gel extracted to
remove contaminants and the concentration of each sample is standardized prior to pooling. emPCR and sequencing is performed which yields a pool of sequenced amplicons. In data analysis, the pool of sequences is split up by sample according to the specific MID barcode used in the amplification step. The sequenced amplicons are compared to databases of known 16S rRNA sequences to construct community profiles for each sample.

*Quantitative polymerase chain reaction (qPCR)*

In qPCR, DNA is extracted from the microbiota samples and then amplified with specific primers. Primers designed in variable regions of the 16S gene will pick up a phylogenetic group of interest while those designed in a conserved region pick up all bacteria. SYBR green dye intercalates with double stranded DNA and is incorporated into the PCR reaction. Following each cycle of PCR, the relative fluorescence units (RFU) of SYBR are measured, which indicates the amount of double stranded DNA and thus the number of amplicons. The number of PCR cycles it takes for the RFU to reach a threshold level (cT) indicates the abundance of the microbial DNA in the starting material. The cT for a microbe or group of microbes of interest is normalized to the cT for all bacteria to control for the amount of total bacteria present in each sample. The relative abundance of the specific microbe examined is then compared to a chosen control and the relative quantity can be compared between samples. Some disadvantages of qPCR are that it is necessary to know what you are looking for and thus any unknown or novel species will not be detected. It is also not possible to infer community structure since only one microbe or group of microbes is probed for at any one time. It can also be difficult to design primers for higher taxonomic levels (genus, order, phyla), since the primers must fall within a region that is conserved by only the desired group and ideally results in an amplicon between 75 and 200 base pairs. The
main advantage of qPCR is that it is very sensitive and will pick up even low abundance microbes. It is also relatively quick, inexpensive and analysis of data is relatively simple.

*Fluorescence in situ hybridization (FISH)*

FISH employs fluorescent DNA probes that are targeted to sections of the 16S rRNA genome. The microbial DNA is fixed in formalin to prevent degradation. A fluorescent probe directed to a conserved region of the 16S gene would hybridize to all bacteria and a different colored probe to variable regions may target phylogenetic groups from phyla to species. This can be used to view microbiota samples under high magnification and determine the percentage of a given bacteria resulting in a rudimentary picture of community structure. Formalin-fixed and paraffin embedded sections of gut tissue can also be stained to determine the localization of bacteria relative to the epithelial surface. In determining the bacterial community structure, FISH is outdated. This is because each bacterial group must be stained individually and similar to qPCR, novel bacteria will not be identified due to probes being specific to certain bacterial groups. Rare species may not be well enough represented to be quantified using this technique, and furthermore, the method is labour intensive and susceptible to human error as nuclei must be manually counted. However, FISH is the only technique where bacteria are visualized making it valuable to gain insight into bacterial localization within the GI tract.
Molecular techniques allow us to study microbes without having to culture them, and as such, have advanced our knowledge of the intestinal microbiota considerably. It should be noted, however, that molecular techniques have some general pitfalls. Since the DNA is analyzed, both live and dead bacteria will be included and it is not possible to determine if the bacteria are playing a physiological role. Also, since bacteria have variable anatomical and physiological features, DNA may be extracted more readily from some bacteria than others. The number of copies of 16s rRNA is variable between bacteria which can also affect how they are represented in the sample. Furthermore, any techniques that employ PCR amplification are susceptible to PCR bias or overrepresentation of the DNA that is randomly amplified first.
Chapter 2: Overall rationale, hypothesis and objectives

Incidence of T1D is accelerating faster than genetics alone can account for, implicating environmental factors in initiating or accelerating autoimmune responses that lead to beta cell destruction. The intestines harbour a complex community of microbes, the composition of which is shaped by a combination of genetic factors and early life exposures. Intestinal microbiota is altered in T1D patients and T1D animal models with recent evidence suggesting it is involved in directing disease progression. **We hypothesized that NOD mice harbour a microbiota that can induce diabetes through a leaky and inflamed gut.**

To study this hypothesis, two experiments were performed using the NOD mouse model of T1D. In the first experiment, microbiota was transplanted between diabetes susceptible NOD mice and mice resistant to T1D (non-obese resistant, NOR). In the second experiment the microbiota of NOD mice was disrupted using two antibiotics that target either predominantly Gram-negative or Gram-positive bacteria. Diabetes progression was assessed as well as microbiota composition, intestinal barrier function, and intestinal inflammatory profile. Finally, we treated NOD mice with the probiotic VSL3 to determine if we could prevent T1D with beneficial microbes.

Specifically, we had the following objectives:

**Objective 1:** Examine the causal relationship of the intestinal microbiota in T1D onset and progression.

**Objective 2:** Profile the microbiota associated with diabetes susceptible and resistant mice

**Objective 3:** Measure intestinal inflammation and barrier function in diabetes susceptible and resistant mice
Chapter 3: Methods

3.1 Animal Care

Non-obese diabetic (NOD) and non-obese resistant (NOR) mice (Jackson Laboratory, Bar Harbour, ME, USA) were housed and bred under specific pathogen free conditions at Child and Family Research Institute (Vancouver, BC, Canada). Mice were maintained in temperature controlled (22 ± 2 °C) animal facility with 12 hour light/dark cycle. Animals received a sterile chow diet (Laboratory Rodent Diet 5001, Purina Mills, St. Louis, Missouri) and filtered UV sterilized water *ad libitum* throughout the experiments. Only female mice were used for experiments except where indicated to be male. *Animal experiments were performed at CFRI, Vancouver, BC. Establishment and maintenance of colonies was performed by Caixia Ma, a technician at the Vallance Lab.*

3.2 Tissue Collection

Mice were anaesthetized with isofluorane and blood was collected via cardiac puncture. Blood was stored overnight at 4°C and then spun at 16,000g for 15 minutes to separate sera. Mice were euthanized by cervical dislocation and pancreas, cecum, ileum and distal colon segments were collected and fixed in 10% formalin for histological analysis. Pancreas, ileum and colon segments were stored in RNAlater (Qiagen) at -20°C for RNA analysis, and ileum and colon segments were homogenized in PBS and stored at -80°C for microbial analysis. *Tissue collection was performed by Caixia Ma with assistance from Ben Dai and me on a few occasions.*
3.3 Fecal Microbiota Transfer

Fecal transplantations were performed as previously described [101]. NOD and NOR mice were treated with antibiotics (500mg/L vancomycin, 1g/L neomycin, 1g/L ampicillin and 1g/L metronidazole) in drinking water from 4-8 weeks. Stool from donor mice (100mg in 1.2mL PBS) was orally gavaged into recipient mice 3 times between 8-10 weeks creating “chimeric” mice (H = host, M = microbiota): NOR\(^H\)+NOR\(^M\), NOR\(^H\)+NOD\(^M\), NOD\(^H\)+NOD\(^M\), and NOD\(^H\)+NOR\(^M\). *This experiment was performed by Caixia Ma at CFRI. Two experiments were performed and I assisted with euthanization and tissue collection at 11 weeks of the second experiment.*

3.4 Antibiotic and VSL3 Treatment

For peri-natal treatment, dams were administered antibiotics in drinking water (1g/L neomycin, 0.5g/L vancomycin or 1.25g/L VSL3) immediately following birth of the pups. Antibiotics were continually administered to the dams throughout weaning and then directly to the pups post-weaning until onset of diabetes. For post-weaning treatment, antibiotics were administered to the pups in drinking water post weaning (21 days). Blood glucose levels were monitored weekly in the tail vein and mice were euthanized following 2 consecutive readings of >14mmol/L. All remaining mice were euthanized at 22 weeks of age. *These experiments were performed by Caixia Ma at CFRI. Ben Dai and I assisted with glucose monitoring and euthanization throughout the experiment and Caixia and I performed the final euthanization and tissue collection at the end of the experiment.*
3.5 Insulitis scoring

Following excision, sections of pancreatic tissue were fixed in 10% formalin overnight and then placed in 70% alcohol until processing. Tissues were processed by the CFRI histology group and two 5μm sections were cut and placed onto slides which were stained with haematoxylin and eosin. Slides were visualized by light microscopy and each islet was given a score ranging from 0 to 3 (0= no immune cell infiltration, 1= peri-insulitis, 2= infiltration <50% islet, and 3 = severe infiltration > 50% of the islet). Graphs represent the percentage of islets that have insulitis (score 2 or 3). These tissues were prepared by CFRI histology group and insulitis was assessed by Caixia Ma and Justin Chan, Vallance Lab, CFRI, Vancouver. I performed analysis of the data.

3.6 Insulin autoantibody determination

At time of euthanization (11-22 weeks), blood was collected via cardiac puncture, stored overnight then sera separated by centrifugation (16,000g for 15 minutes at 4°C). Samples were stored at -80°C until use. Autoantibody analysis was performed at the Barbara Davis Centre for Diabetes in Aurora, Colorado, USA.

3.7 Quantitative polymerase chain reaction

3.7.1 RNA extraction

Following excision, tissues were immersed in RNAlater (Qiagen: 76106) and stored at -20°C until extraction. RNA was extracted using RNNeasy Fibrous Tissue Mini Kit (Qiagen: 74704) following the manufacturer’s instructions. A ~5mm section of colon or ileum tissue was combined with 300uL RLT buffer and 3uL β-mercaptoethanol (Sigma) in a 2mL
homogenization tube. Tissues were homogenized with a 5mm stainless steel bead using a Retsch mixer mill M400 (Retsch, Germany) set at 30 hertz for 2 x 2 minutes. The homogenized sample was placed in a new 1.5mL MCT and 590μL nuclease-free H$_2$O is added followed by 10μL of proteinase K. The sample is incubated with proteinase K for 10 minutes at 55°C to allow for digestion of proteins. The sample is then centrifuged at 10,000g for 3 minutes to collect cell debris and proteins. The supernatant was removed and combined with 450μL 100% ethanol in a new 1.5mL MCT to precipitate nucleic acids. The sample was then applied to a silicone column, washed once with wash buffer (RW1, Qiagen) and incubated with DNaseI for 10 minutes at 25°C. The column was washed once with wash buffer (RW1), twice with wash buffer (RW2) and then RNA eluted with 50μL of RNase free H$_2$O. RNA concentration was determined spectrophotometrically (nanodrop) and 1ug of total RNA was converted to cDNA using iScript cDNA synthesis kit (Bio-Rad) then diluted 10 times in nuclease-free H$_2$O.

3.7.2 DNA extraction

At time of excision, gut segments were homogenized in 1mL PBS by steel bead mill (Retch, Germany) for 2 x 2 minutes at 30Hz. Bacterial DNA was extracted using QIAmp DNA Stool Mini Kit (Qiagen: 51504) using 200μL of homogenized sample with 1.4mL lysis buffer (ASL). Sample was homogenized with ASL buffer in a 2mL round bottom tube with a 5mm steel bead using a bead mill (Retch) for 2 x 2 minutes at 30Hz. Sample was then placed in 95°C water bath for 10 minutes to lyse bacteria. Sample was vortexed (15s) then centrifuged at full speed for 1 minute to pellet the stool particles. All of the supernatant was transferred into a new 2mL MCT and an InhibitEX Tablet (Qiagen) was added, mixed
thoroughly with the sample then incubated for 1 minute at 25°C. The sample was
centrifuged at full speed for 3 minutes to pellet the Inhibitex tablet and bound inhibitors. The
supernatant was placed in a new MCT and centrifuged for 3 minutes. All of the supernatant
was then combined with 15μL proteinase K with 200μL of AL buffer (Qiagen) and incubated
at 70°C to lyse protein contamination. 200μL of 100% ethanol was added to the lysate,
mixed, and applied to a silicone membrane in a column (Qiagen). The membrane was
washed twice with buffers (AW1/AW2, Qiagen) then eluted with 200μL nuclease-free H₂O.
Concentration was determined spectrophotometrically (nanodrop) and standardized to
40ng/μL.

3.7.3 Quantitative PCR

1μL of host cDNA or 40ng bacterial DNA was combined with 0.2μL 10μM forward
primer, 0.2μL 10μM reverse primer, 5μL Ssofast Evagreen Supermix (Biorad) and sterile
H₂O to 10μL. Beta actin and 18S rRNA were used as reference genes for relative expression
of host mRNA and eubacteria was used as a reference for relative abundance of bacteria.
Primer sequences, annealing temperatures and sources of the sequences are shown in table 1.
Primer efficiencies were determined using a standard curve with a dilution factor of 3.78
where the slope of the line is used to calculate efficiency as follows:

\[ E = 10^{-1/slope} \]

\[ \%\ Efficiency = (E - 1) \times 100 \]

Primers were tested in silico against the NCBI database for mus musculus for specificity to
intended targets and a single product was observed in melt peak analysis. Duplicate reactions
were performed and expression was calculated using the relative quantity method as described in the Biorad CF96X manual. For a given gene of interest (GOI) with a control sample and an experimental sample:

\[
Relative \, Quantity_{\text{sample (GOI)}} = E^{(C_{q(\text{control})} - C_{q(\text{sample})})}
\]

E = efficiency of the gene of interest  
\(C_{q(\text{control})}\) = Average \(C_q\) of control sample  
\(C_{q(\text{sample})}\) = Average \(C_q\) for the experimental sample  
GOI = gene of interest

\[
Normalized \, Expression_{\text{sample (GOI)}} = \frac{RQ_{\text{sample (GOI)}}}{RQ_{\text{sample (18S rRNA)}} \times RQ_{\text{sample (\beta-actin)}}}
\]

Using the relative quantity (RQ) of the sample for the gene of interest (GOI) as well as reference genes (18S rRNA and Beta-Actin). qPCR was performed on a CFX96 Touch Real Time Detection System (Biorad) and CFX manager V3.0 software was used for analysis. Efficiencies, annealing temperatures and original sources for primer sequences are shown in Table 4.
Table 4: Sequences, annealing temperatures, efficiencies and sources of primers used in this study.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (F) and reverse (R) primers in 5’-3’ orientation</th>
<th>Anneal. Temp</th>
<th>% Efficiency</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primer Sets</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mouse mRNA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td>F – CAGCTTCTTTGCAAGCTCCTT R – CTTTCCATGTCGTCCAGT</td>
<td>58</td>
<td>90.6</td>
<td>[119]</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>F – CGGCTACCACATCCAAAGGAA R – GCTGGAATTACCAGGGCT</td>
<td>58</td>
<td>97.9</td>
<td>[120]</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>F – TCAAGTGGCATAGATGTGAAGAA R – TGCCCTCTGAGATTTTCATG</td>
<td>60</td>
<td>103.6</td>
<td>[121]</td>
</tr>
<tr>
<td>TNF-α</td>
<td>F – CCACACGGTCTCTCTGTCTAC R – AGGTGCTTGGCCATAGAAGCT</td>
<td>59</td>
<td>103.9</td>
<td>[122]</td>
</tr>
<tr>
<td>Cxcl9</td>
<td>F – CAGAACCTCCCACGTAGCTTTC R – GCTCTGAAGATGGGATCAAGTTAATA</td>
<td>58</td>
<td>99.3</td>
<td>[123]</td>
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<tr>
<td>Claudin-1</td>
<td>F – CTGGAAGATGATGAGGTGACAAG G R – CCACTTATGCAGCAGACCTGAA</td>
<td>58</td>
<td>80.5</td>
<td>[124]</td>
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<tr>
<td>Ccr7</td>
<td>F – CACGCTGAGATGCTACCTGG R – CACTCTGGCCACCTTGGA</td>
<td>58</td>
<td>116.2</td>
<td>[125]</td>
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<tr>
<td>Foxp3</td>
<td>F – GGCCCCTCTCCAGGACAGA R – GTGATCATGGCTGGTTTGT</td>
<td>58</td>
<td></td>
<td>[109]</td>
</tr>
<tr>
<td>IL-17f</td>
<td>F – TGCTACTTGTAGTGGAGAC R – AATGCCCTTGGCTTTTGA</td>
<td>58</td>
<td>85.0</td>
<td></td>
</tr>
<tr>
<td>IL-23a</td>
<td>F – TGGCATCGAGAAACTGTGAGAAG R – TCAGTCTGTAGTGCTTTGTA</td>
<td>58</td>
<td>106.8</td>
<td>[109]</td>
</tr>
<tr>
<td>IL-17A</td>
<td>F – TCCCTCTGTGATCTGGGAAAG R – CTGACCCCTTGGGAAGG</td>
<td>58</td>
<td>101.6</td>
<td>[109]</td>
</tr>
<tr>
<td>IL-1β</td>
<td>F – AGCTCTCTTGTGCAAGTGT G R – CCTCTTGGCCATCTTTGAGG</td>
<td>58</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RELMβ</td>
<td>F – ATGGGTGTCGACTGGATGTGCTT G R – AGCCATGGCAGTGAGGAG TG</td>
<td>58</td>
<td></td>
<td>[126]</td>
</tr>
<tr>
<td>TGFβ</td>
<td>F – AGGGCGCTTTGCTATGGGTG R – TGGCCACAGCAGCAGTGT</td>
<td>58</td>
<td>98.3</td>
<td>[109]</td>
</tr>
<tr>
<td>IL-10</td>
<td>F – AGGGCCCTTCTGTGCTATGGGTG R – TGGCCACAGCAGCAGTGT</td>
<td>58</td>
<td>97.7</td>
<td>[129]</td>
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<tr>
<td><strong>Bacterial DNA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eubacteria</td>
<td>F – CGGTGAATACGCCTCGCCG R - TACGACTACCTTGTTACGAC</td>
<td>60</td>
<td>111.5</td>
<td>[127]</td>
</tr>
<tr>
<td>SFB</td>
<td>F – CCGAGCATTGATGGTTAATTTC R - GCCTCTCTGGCTAAAGTGCT</td>
<td>55</td>
<td>92.2</td>
<td>[128]</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>F – GTGCCAGCMGCCGCGGTAA R - GCCTCAAGGGCACAACCTCAGGA</td>
<td>60</td>
<td>140.0</td>
<td>[129]</td>
</tr>
<tr>
<td>A.muciniphila</td>
<td>F - CCTTGCCAGGTGGCTTCAGAT R - CAGCAGCTGAAAGGTGGGGGA</td>
<td>60</td>
<td>107.2</td>
<td>[130]</td>
</tr>
</tbody>
</table>
Table 4 (cont.): Sequences, annealing temperatures, efficiencies and sources of primers used in this study.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (F) and reverse (R) primers in 5’-3’ orientation</th>
<th>Anneal. Temp</th>
<th>% Efficiency</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eubacteria (FISH probe)</td>
<td>5’-GCTGCCTCCCGTAGGAG-3’</td>
<td>N/A</td>
<td>N/A</td>
<td>[131]</td>
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<tr>
<td>SFB (FISH probe)</td>
<td>5’-GGGTACTTATTGCGTTTGCGACGGCAC-3’</td>
<td>N/A</td>
<td>N/A</td>
<td>[132]</td>
</tr>
</tbody>
</table>

* Denotes primers designed using primerBLAST (NCBI). Primer fragments were designed to cross exon junctions, fragments size between 75 and 200, and melting temperature within 3 degrees of 58°C. Primers were checked against the mouse genome and were required to have at least 2 mismatches within the last 5 base pairs at the 3’ end of any other mouse transcripts.

3.8 High throughput sequencing

3.8.1 Sample preparation

Following DNA extraction, bacterial DNA was amplified using eubacterial primers 341_F and 805_R [133] which flank the V3 & V4 variable regions of the 16S gene [134]. Each primer contained a sample specific barcode and primer linker sequences for Roche 454 technology [135] (Table 5). PCRs were performed in duplicate 25μL reactions (12.5μL Ecotaq master mix (Lucigen: 30033-1), 2.5μL 10μM forward primer, 2.5μL 10μM reverse primer, 50-200ng DNA and sterile filtered nuclease free H₂O to 25μL with no template controls performed for each sample/forward primer combination. Samples were run on a 1% agarose gel in TAE buffer (50X TAE: 242g Tris base, 571mL glacial acetic acid, 14.6g EDTA in 1L H₂O), for 30 minutes at 80V. Duplicates were then extracted together using a
QIAquick gel extraction kit (Qiagen: 28704). Concentration of each sample was normalized to 1.25ng/μL using Sequalprep (Invitrogen: A10510-01) and samples were pooled prior for sequencing. Samples were sequenced at the Laboratory for Advanced Genomic Analysis in Vancouver, BC using Roche 454 FLX+ technology.

Table 5: List of primers used for 454 pyrosequencing

<table>
<thead>
<tr>
<th>MID</th>
<th>ROCHE LIB-A ADAPTOR LINKER SEQUENCE MID 341F (TSS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1 (MID1)</td>
<td>CGTATCGCCTCCCTCGCCATCAGACGAGTCGTCTACGGGNGGCW GCAG</td>
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<tr>
<td>N2 (MID2)</td>
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<tr>
<td>N3 (MID3)</td>
<td>CGTATCGCCTCCCTCGCCATCAGACACCTCCCTACGGGNGGCW GCAG</td>
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<td>N4 (MID4)</td>
<td>CGTATCGCCTCCCTCGCCATCAGACACTGACCTTACGGGNGGCW GCAG</td>
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<td>N5 (MID5)</td>
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<tr>
<td>N6 (MID6)</td>
<td>CGTATCGCCTCCCTCGCCATCAGACACTGACCTTACGGGNGGCW GCAG</td>
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<tr>
<td>N7 (MID7)</td>
<td>CGTATCGCCTCCCTCGCCATCAGACACTGACCTTACGGGNGGCW GCAG</td>
</tr>
<tr>
<td>N8 (MID8)</td>
<td>CGTATCGCCTCCCTCGCCATCAGACACTGACCTTACGGGNGGCW GCAG</td>
</tr>
<tr>
<td>N9 (MID10)</td>
<td>CGTATCGCCTCCCTCGCCATCAGACACTGACCTTACGGGNGGCW GCAG</td>
</tr>
<tr>
<td>N10 (MID11)</td>
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<td>N11 (MID13)</td>
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<td>N12 (MID14)</td>
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<tr>
<td>N13 (MID15)</td>
<td>CGTATCGCCTCCCTCGCCATCAGACACTGACCTTACGGGNGGCW GCAG</td>
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<td>N14 (MID16)</td>
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<tr>
<td>N15 (MID17)</td>
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<td>N16 (MID18)</td>
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<td>N17 (MID19)</td>
<td>CGTATCGCCTCCCTCGCCATCAGACACTGACCTTACGGGNGGCW GCAG</td>
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Table 5 (cont.): List of primers used for 454 pyrosequencing

<table>
<thead>
<tr>
<th>Primers Used</th>
<th>ROCHE LIB-A ADAPTOR LINKER SEQUENCE MID 341F (TSS)</th>
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<tr>
<td>N18 (MID20) 341F</td>
<td>CGTATCGCCTCCCCCTCGCCCATCAGACGACTACAGCCTACGGGNGGCGW GCAG</td>
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<td>N19 (MID21) 341F</td>
<td>CGTATCGCCTCCCCCTCGCCCATCAGCGTAGACTACCGCTACTACGGGNGGCGW GCAG</td>
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<tr>
<td>N20 (MID22) 341F</td>
<td>CGTATCGCCTCCCCCTCGCCCATCAGTGACTCCTACGGGNGGCGW GCAG</td>
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<td>N21 (MID23) 341F</td>
<td>CGTATCGCCTCCCCCTCGCCCATCAGTCGACTACGGGNGGCGW GCAG</td>
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<td>N22 (MID24) 341F</td>
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<td>N25 (MID27) 341F</td>
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<td>N26 (MID28) 341F</td>
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<td>N27 (MID29) 341F</td>
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<td>N28 (MID30) 341F</td>
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<td>N30 (MID32) 341F</td>
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<td>N31 (MID33) 341F</td>
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<td>N32 (MID34) 341F</td>
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<tr>
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<td>N35 (MID37) 341F</td>
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<td>N37 (MID39) 341F</td>
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<td>N38 (MID40) 341F</td>
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Table 5 (cont.): List of primers used for 454 pyrosequencing

<table>
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<tr>
<th>N39 (MID41) 341F</th>
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<tr>
<td>N40 (MID42) 341F</td>
<td>CGTATCGCCTCCCTCGCCCATCAGTCGATCACGTCCCTACGGGNGGCWG</td>
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</table>

3.8.2 Sequence Analysis

Analysis of sequencing was performed using the quantitative insights into microbial ecology (QIIME) pipeline [136]. A total of 406,120 sequences between 200 and 600 base pairs in length with a minimum quality score of 25 were returned from the sequencing facility. Singletons were included in the analysis, no primer mismatches were allowed and homopolymer repeated >6 were excluded. Chimera detection (usearch61[137]) resulted in the removal of 42,066 sequences. OTUs were picked using usearch61 with a 97% similarity threshold and a representative set of sequences was aligned using MUSCLE [138] with the ribosomal database project (RDP) 97% similarity OTUs as a template. Taxonomy was assigned using RDP from the Greengenes database based on 97% similarity [139]. The sequence for Candidatus arthromitus (SFB) was added manually to the database. OTU tables rarefied to 923 and 230 sequences were used in subsequent parts of the analysis.

3.8.3 Statistical Analysis of Sequencing

For principal coordinate plots, OTU tables (rarefied to 923 sequences for NOR/NOD comparisons and 230 for antibiotics) were fourth root transformed using PRIMER-E software. To determine the level of similarity between samples, the Bray-Curtis dissimilarity metric was used [140]. This metric was chosen since it accounts for abundance of species as well as the number of shared species in determining similarity of samples and it also negates
the problem of double negatives of some similarity metrics [141]. Principle coordinate plots were constructed from the Bray Curtis resemblance matrices and shown representing the two axes that explain the most variation in the samples. Pairwise tests were performed between each group and significance was determined using the PERMANOVA add-on to the PRIMER-E software. PERMANOVA determines if groups are different from each other by comparing the dataset to a set number of random permutations of the data [142]. The effects of the treatments were determined based on that maximum number of permutations up to 9999.

Alpha diversity within the samples was determined using the Chao1 statistic that uses the actual number of observed OTUs and projects diversity by accounting for the number of singletons and doubletons in a sample [143]. The formula is as follows:

\[
S_{\text{Chao1}} = S_{\text{obs}} + \frac{n_1^2}{2n_2}
\]

Where \(S_{\text{chao1}} = \text{Chao1 diversity}, S_{\text{obs}} = \text{the number of observed OTUs, } n_1 = \text{the number of singletons in the sample and } n_2 = \text{the number of doubletons in the sample. Differences between groups were determined by one-way ANOVA with Tukey’s post hoc test.}

Differences in abundance of taxa within groups were determined by ANOVA with false discovery rate (FDR) correction to control for multiple hypothesis testing from a single set of data. Where \(p < 0.1\) for FDR-corrected ANOVA, Dunn’s multiple comparisons post-hoc test was performed to determine if groups were significantly different.
3.9 Fluorescence in situ hybridization

Formalin fixed and paraffin embedded ileum and cecum sections were deparaffinized with xylenes and rehydrated through an ethanol gradient to water. An Alexa-fluor488 labelled probe for eubacteria and an Alexa-fluor594 labelled probe for SFB (Table 4) at a concentration of 2.5ng/uL in hybridization solution (52.6g NaCl, 12.2g Trizma base, 300mL formamide, 1g SDS in 1L, pH 7.2) was incubated with the tissues at 37°C overnight. Slides were washed for 15 minutes in hybridization solution then 15 minutes in wash buffer (52.6g NaCl, 12.1 Tris base in 1L, pH 7.2) then placed in dH₂O. Slides were mounted with fluoroshield with DAPI (Sigma) and visualized with an Olympus FV1000 confocal microscope (Facility for Environmental and Biological Imaging, UBC, Okanagan campus). Specificity of SFB sequence was checked by alignment with NCBI 16S ribosomal RNA sequences (Bacteria and Archae) database. SFB from three species had 100% coverage (e-value: 8 e-9) (Candidatus Arthromitus sp.SFB-mouse-Japan, Candidatus Arthromitus SFB-mouse-Yit, Candidatus Arthromitus sp. SFB-rat-Yit) and the next closest hits were members of the Clostridaceae family with 88% coverage (5e -7).

3.10 Testosterone Assay

Blood was collected from mice by cardiac puncture and stored overnight at 4°C. Sera was separated by centrifugation (16,000g for 15 minutes at 4°C), then stored at -80°C until use. Testosterone was quantified using testosterone EIA detection kit (Cayman Chemicals, Cat #: 582701). 20μL of serum sample was combined with 180μL EIA buffer in a clean glass vial. 400μL diethyl ether was added and the sample was vortexed. Once the sample had separated, the ether layer (upper layer) was transferred into a clean test tube. The ether was
evaporated at 30°C under a gentle stream of nitrogen, then the sample was dissolved in 200uL EIA buffer. EIA protocol was followed as per manufacturer’s instructions and concentration determined based on a standard curve from 3.9 pg/mL to 5ng/mL using software provided by Cayman Chemicals. This protocol was performed by Dr. Carol Chan in the Gibson lab at UBC Okanagan.

3.11 FITC-dextran barrier function assay

FITC-dextran assays were performed as previously described [74]. Mice were orally gavaged 150uL of 80g/L FITC dextran (Sigma: FD4). Four hours post-gavage, blood was collected by cardiac puncture and added to 50uL acid-citrate dextrose buffer (20mM citric acid, 110mM sodium citrate, 5mM dextrose). Serum was separated by centrifugation at 4°C for 12 minutes at 16,000g. The RFU of the samples undiluted and diluted 1:10 in PBS were measured at excitation 485nm/ emission 530nm and compared to standards of known FITC-concentration (440, 220, 110, 55, 27.4, 13.75, 6.875, and 1 ng/uL) to determine the concentration in the sera. These assays were performed at CFRI (Vancouver, BC) by Caixia Ma (fecal transplant experiment) and Ben Dai (antibiotic experiment) and Ben and I together (VSL3 experiment). I performed the data analysis.

3.12 Claudin-1 staining with horseradish peroxidase (HRP) and 3,3’–Diaminobenzidine (DAB)

Paraffin embedded ileum sections on microscope slides were washed with xylenes and rehydrated through an ethanol gradient to water. Antigen retrieval was performed with trypsin (1mg/mL in dH2O) for 30 minutes at 37°C, washed 2 x 3 minutes in PBS then
blocked with 5% bovine serum albumin (BSA) in PBS for 20 minutes at 25°C. Without washing, tissues were incubated at 4°C overnight with goat-anti claudin-1 antibody (Cat #: sc-17658, Santa Cruz Biotechnology) at a concentration of 1/50 in 5% BSA. Tissues were washed (2x3 minutes in PBS) then incubated with 0.3% H₂O₂ in PBS for 15 minutes at RT. Tissues were washed (2x3 minutes in PBS) and then incubated with a HRP conjugated anti-goat secondary (1/200 in BSA) for 1 hour at RT. Tissues were washed (3x5 minutes in PBS) the incubated with DAB substrate mix (Sigma cat #: D0426) for 5 minutes at 25°C. Tissues were rinsed for 5 minutes under running deionized water then counterstained using haematoxylin (5 seconds in Mayer’s haematoxylin (Thermo Scientific cat #: TA-060-MH), rinse with tap water, then 0.1% sodium bicarbonate for 45 seconds). Slides were dehydrated through an ethanol gradient (70%-100%), cleared with xylenes, and then mounted with toluene based mounting media (Shurmount Cat #LC). Slides were visualized on an Olympus IX81 microscope with Metamorph software.

3.13 Caco-2 cell culture and TEER measurements

Caco-2 cells, obtained from American Type Culture Collection, were grown at 37°C and 5% CO₂ in DMEM with 4.5 g/l d-glucose, 1× nonessential amino acids, L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), and 20% fetal bovine serum (Sigma). For TEER experiments, cells were seeded at 5,000 cells per 12-well insert (Grenier BioOne: 665641) and media was changed every ~3 days. Resistance was measured using an EVOM2 epithelial voltohmeter (WPI: EVOM2) with STX2 chopstick electrodes. Cells were grown until they reached resistance >1000ohms (~14 days) when experiments were performed. Resistance was measured then media was replaced with antibiotic free media and 100μg of
luminal content from the ileum of freshly euthanized animals was added to the apical surface of the monolayer. TEER was measured after 2 hours and graphed as a percentage of initial TEER.

### 3.14 Immunofluorescence staining of Caco-2 monolayers

Cells were grown on Thincert membranes (Grenier-Bioone) until resistance was >1000ohms. At time of staining, media was removed from the insert and staining performed directly on the insert. Cells were washed gently with DPBS then fixed in 10% neutral buffered formalin for 7 minutes. Cells were washed (2 x 3 minutes) with DPBS then permeabilized with 0.5% triton-X 100 in PBS for 10 minutes. Cells were washed (2 x 2 minutes) with DPBS then blocked with 5% BSA in PBS at 25°C for 20 minutes. Cells were incubated with rabbit anti-E-Cadherin antibody (Cat #: 3195S, New England Biolabs) at a concentration of 1/100 overnight at 4°C. Cells were washed twice with DPBS then incubated with Alexa-fluor 488 labelled anti-rabbit secondary for 1 hour at 25°C. Cells were washed twice with DPBS then incubated with a mouse anti-claudin-1 antibody (sc-166338, Santa Cruz Biotechnology) at a concentration of 1/50 for 1 hour at 25°C. Cells were washed twice in PBS then incubated with Alexa-fluor 594 labelled anti-mouse secondary antibody for 1 hour at 25°C. Membranes were then cut out of the tissue culture inserts, placed onto glass slides and mounted with Fluoroshield with DAPI (Sigma: F6182). Monolayers were visualized using an Olympus IX81 microscope with Metamorph software.
3.15 Statistical analysis

The number of mice in each experiment and the statistical tests used are indicated in the figure legends for each experiment. Graphing and statistical analysis was performed using Graphpad Prism and PRIMER-E software.

Chapter 4: Results

4.1 Diabetes susceptible and diabetes resistance mice have different intestinal microbiota

The intestinal microbiota in the colon and ileum of diabetes susceptible (NOD) and diabetes resistant (NOR) mice was profiled by 454 pyrosequencing. We found that in both regions of the gut, NOR and NOD mice harbour distinct microbial communities (Figure 2A). Although at the phyla level the microbiota was similar (Figure 2B), at lower taxonomic levels we found several differences. In the colon, NOD mice had fewer members of the Alphaproteobacteria class, no Roseburia or Bifodobacteria spp. and fewer Oscillospira spp. compared to NOR. There were also fewer Bacteroides acidifaciens and Ruminococcus gnavus. Microbes increased in the colon of NOD mice include Desulfovibrio and Prevotella spp. (Figure 2C). In the ileum, the NOD microbiota was lower in Lactobacillaceae and had increases in Anaeroplasma spp. and Desulfovibrio spp. (Figure 2C). In general, the NOR microbiota is marked by more beneficial microbes whereas the NOD microbiota has more potentially pathogenic microbes, termed pathobionts.
Figure 2: NOR and NOR mice have different microbiota in the colon and ileum. Microbial communities were assessed by 454 pyrosequencing. Principal coordinates analysis was performed on Bray-Curtis similarities from fourth root transformed OTU tables with 230 sequences per sample. The microbiota in NOR and NOD mice is different in the colon (p(perm) = 0.0085) and ileum (p(perm) = 0.0442) (A). At the phyla level, NOR and NOD microbiota is similar (B) yet the abundance of certain taxa is different in NOD vs NOR in the
colon (C) and ileum (D). Abundance values represent the percentage of each taxon per 923 sequences per sample. *<0.05 and ** <0.01 by unpaired t-test. N = 4-8 per group (PCO) and n= 3-5 per group (abundance).

4.2 NOD microbiota initiates diabetes progression in diabetes resistant mice

To determine if the microbiota is involved in directing diabetes progression, we performed a microbial transplant experiment between NOR and NOD mice. Mice were treated with antibiotics then feces from donor mice were transferred into the antibiotic-treated recipient mice. This created four groups of ‘chimeric’ mice (H = host, M = microbiota): NOD\textsuperscript{H}+NOD\textsuperscript{M}, NOD\textsuperscript{H}+NOR\textsuperscript{M}, NOR\textsuperscript{H}+NOR\textsuperscript{M} and NOR\textsuperscript{H}+NOD\textsuperscript{M} (Figure 3A). Mice were euthanized at 11 weeks and diabetes progression was measured by assessing pancreatic islets for inflammation (insulitis). We found that NOD microbiota initiates insulitis in NOR mice although NOR microbiota did not protect NOD mice from insulitis (Figure 3B & C). This indicates that NOD microbiota contains either microbes or other molecules that are diabetogenic and leads to initiation of insulitis in the pancreas.
Figure 3: Mice susceptible to diabetes harbour a microbiota that initiates insulitis in diabetes resistant mice. Fecal transplantation between resistant (NOR) and susceptible (NOD) mice was performed as described (A). Tissues were collected at 11 weeks and cross-sections of pancreas tissues were stained with H&E and scored by two blinded observers. The percentage of islets with insulitis (score of 2 or 3) was recorded (B) and representative pictures showing islet infiltration (C); scale bar = 100μm. N = 10-12 per group. **<0.01 by unpaired t-test.

4.3 Peri-natal antibiotics accelerates diabetes in NOD mice

Since NOD microbiota could initiate insulitis in NOR mice, our next experiment assessed how disrupting the microbiota with antibiotics would affect diabetes onset. We chose vancomycin since it predominately targets Gram-positive microbes, and neomycin to target Gram-negative microbes. Neither are absorbed across the intestinal wall. Antibiotics were administered to NOD dams in drinking water either just before or immediately
following birth of the pups and continued throughout weaning and then to the pups drinking water post-weaning until they developed diabetes. We found that despite the differences in target bacteria, both vancomycin and neomycin accelerated diabetes in NOD mice (Figure 4A). We also noted a higher glucose reading at sacrifice in the antibiotic treated mice compared to the controls (Figure 4B) and the presence of insulin autoantibodies (Figure 4C). In a different cohort of mice treated with vancomycin from weaning at 4 weeks of age as opposed to peri-natal treatment, vancomycin did not have the effect of accelerating diabetes compared to controls (Figure 4D). To determine if probiotics could protect the NOD mice from diabetes, we treated mice with VSL3 (a combination of *Lactobacillus*, *Bifidobacteria* and *Streptococcus* spp.) and found there was no significant difference in diabetes onset (Figure 3E). This indicates that disruption of the microbiota through antibiotics early in life augments a diabetogenic microbiome leading to accelerated development of diabetes but that treatment with protective microbes cannot overcome disease onset.
Figure 4: Peri-natal, but not post-weaning, antibiotic exposure accelerated diabetes in NOD mice. VSL3 does not protect from diabetes. Groups of NOD mice were treated with vancomycin, neomycin or VSL3 peri-nataly (to dams within 2 days post-partum) and continued in drinking water until diabetes onset (A-C, E) or in drinking water post weaning (D). Mice were euthanized following 2 consecutive daily blood glucose readings of >14mmol/L. Blood glucose was measured in the tail vein the same week that the mouse was
euthanized (B). Insulin auto-Ab was measured in sera at the time of euthanization (C). *<0.05 and **<0.01 by one-way ANOVA with Tukey’s post-hoc or Mantel-Cox for survival statistics vs untreated NOD mice. N.S. = no significance.

4.4 Dysbiosis with antibiotic-induced diabetes

To determine which microbes are associated with antibiotic-induced diabetes, the microbial populations in the ileum and colon of antibiotic treated mice were profiled by 454 pyrosequencing. We found that in both the ileum and colon, there was lower diversity with antibiotic treatment, although only significantly lower in the vancomycin treated mice (Figure 5A). Vancomycin and neomycin also resulted in distinct microbial communities in both the colon and ileum (Figure 5B) although there was considerable variation within groups (Figure 5C).
Figure 5: Microbiota in the colon and ileum of NOD mice treated with vancomycin and neomycin. Communities were profiled in the colon and ileum of control NOD mice and those treated with vancomycin and neomycin by 454 pyrosequencing. Chao1 statistic used to estimate diversity within a sample (A). Principle coordinate analysis was performed on Bray Curtis similarities calculated on fourth root transformed abundance data (B). The taxa
present in a sample are displayed in a heat map of log transformed percent abundances for each taxon. Smaller numerical values (lighter band) indicate a more abundant microbe (C). Analysis is based on 290 sequences per sample. *<0.05, **<0.01 and ***<0.001 based on one way ANOVA with Tukey’s post hoc (A, colon, n=4-8) or by unpaired t-test between NOD and NOD+vancomycin (A, ileum, since n=2 for neomycin) and by PERMANOVA based on the maximum number of permutations to 9999 (B).

We found that there was considerable variation in individual microbial fingerprints between mice with the same genetic background and treatment (5C). However, we found that several microbes were lost with both vancomycin and neomycin treatment, concurrent with augmentation of different microbes in the vancomycin and neomycin treated mice. In the colon, both antibiotics resulted in lower levels of several genera including Anaerostipes, Oridobacter and Prevotella as well as the species Bacteroides acidifaciens and Ruminococcus gnatus (Table 6.1). Neomycin treatment resulted in an overgrowth of Cyanobacteria, the Rikennellaceae family as well as Blautia and Coprococcus species (Table 6.2). In the vancomycin treated mice, there is increased abundance of Enterobacteriaceae, Anaeroplasma spp., and Akkermansia muciniphila (Table 6.3).
Table 6: Proposed protective and diabetogenic microbes in the colon with antibiotic-induced diabetes. Numbers represent the average % of sequences that each taxon represents from 290 sequences. FDR corrected ANOVA values are reported and significance indicated between groups based on Tukey’s post hoc multiple comparison test where *<0.05, **<0.01 and ***<0.001 vs untreated NOD control. N = 5 for each group.

<table>
<thead>
<tr>
<th>6.1 Protective in NODs: decreased with both vancomycin and neomycin</th>
<th>NOD</th>
<th>NOD+Vancomycin</th>
<th>NOD+Neomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FDR Corrected ANOVA Mean</td>
<td>SEM</td>
<td>Mean</td>
</tr>
<tr>
<td>Oribacter spp.</td>
<td>5.99E⁻⁹⁴</td>
<td>0.3448</td>
<td>0</td>
</tr>
<tr>
<td>Prevotella spp.</td>
<td>0.001944</td>
<td>4.621</td>
<td>0.9667</td>
</tr>
<tr>
<td>Bacteroides acidifaciens</td>
<td>&gt;1.0</td>
<td>0.06897</td>
<td>0.06897</td>
</tr>
<tr>
<td>Lactobacillus johnsonii</td>
<td>&gt;1.0</td>
<td>0.1379</td>
<td>0.1379</td>
</tr>
<tr>
<td>Ruminococcus gnatus</td>
<td>0.244</td>
<td>0.8276</td>
<td>0.2801</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>6.2 Pathobionts with neomycin: increased only with neomycin treatment</th>
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<th>NOD+Vancomycin</th>
<th>NOD+Neomycin</th>
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<tbody>
<tr>
<td></td>
<td>FDR Corrected ANOVA Mean</td>
<td>SEM</td>
<td>Mean</td>
</tr>
<tr>
<td>Cyanobacteria</td>
<td>0.07</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Rikenellaceae</td>
<td>0.00014</td>
<td>2</td>
<td>0.6669</td>
</tr>
<tr>
<td>Blautia spp.</td>
<td>&gt;1.0</td>
<td>0</td>
<td>0</td>
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<tr>
<th>6.3 Pathobionts with vancomycin: increased with vancomycin</th>
<th>NOD</th>
<th>NOD+Vancomycin</th>
<th>NOD+Neomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FDR Corrected ANOVA Mean</td>
<td>SEM</td>
<td>Mean</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>1.04E⁻⁵</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Anaeroplasma spp.</td>
<td>0.011</td>
<td>0.4828</td>
<td>0.2069</td>
</tr>
<tr>
<td>Akkermansia muciniphila</td>
<td>1.29E⁻⁵</td>
<td>3.517</td>
<td>3.346</td>
</tr>
</tbody>
</table>
Changes in microbes in the ileum were similar to changes in the colon, however some different microbes were present and some in higher or lower abundance. Although not statistically significant, the ileum of both antibiotic treatments resulted in lower levels of unclassified Lactobacillaceae, as well as reduced abundance of several genera including \textit{Prevotella, Aldercreutzia, Desulfovibrio, Lactococcus, Oscillospira,} and \textit{Turibacter} and depleted \textit{Ruminococcus gnavus} (Table 7.1). In the neomycin treated mice there are increased members of the Christenellaceae and Rikenelaceae families and the genera \textit{Allobaculum} and \textit{Coprococcus} (Table 7.2). Vanomycin treatment results in an increase in the abundance of Enterobacteriaceae and \textit{Sutterella} species as well as \textit{Akkermansia muciniphila} (Table 7.3).
Table 7: Proposed protective and diabetogenic microbes in the ileum with antibiotic-induced diabetes. Numbers represent the average % of sequences that each taxon represents from 290 sequences. Unpaired t-tests were performed between NOD and NOD+vanc and significance indicated vs. control NOD where *<0.05, **<0.01 and ***<0.001. Number of mice per group are as follows: NOD (8), NOD+Vanc (5) and NOD+Neo (2).

7.1 Protective in NODs: decreased with both vancomycin and neomycin

<table>
<thead>
<tr>
<th>Taxon</th>
<th>NOD</th>
<th>NOD + Vancomycin</th>
<th>NOD + Neomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
</tr>
<tr>
<td>Lactobacillaceae</td>
<td>3.103</td>
<td>1.608</td>
<td>0.06897</td>
</tr>
<tr>
<td>Prevotella spp.</td>
<td>0.3017</td>
<td>0.1777</td>
<td>0</td>
</tr>
<tr>
<td>Desulfovibrio spp.</td>
<td>3.319</td>
<td>1.426</td>
<td>0</td>
</tr>
<tr>
<td>Oscillospira spp.</td>
<td>0.8190</td>
<td>0.4014</td>
<td>0</td>
</tr>
<tr>
<td>Turibacter spp.</td>
<td>1.078</td>
<td>0.6059</td>
<td>0.06897</td>
</tr>
<tr>
<td>Ruminococcus gnavus</td>
<td>0.3017</td>
<td>0.1653</td>
<td>0</td>
</tr>
</tbody>
</table>

7.2 Pathobionts with neomycin: increased only with neomycin treatment

<table>
<thead>
<tr>
<th>Taxon</th>
<th>NOD</th>
<th>NOD + Vancomycin</th>
<th>NOD + Neomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
</tr>
<tr>
<td>Christenellaceae</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Rikenellaceae</td>
<td>0.2155</td>
<td>0.09071</td>
<td>0.06897</td>
</tr>
<tr>
<td>Allobaculum spp.</td>
<td>0.08621</td>
<td>0.05644</td>
<td>0</td>
</tr>
<tr>
<td>Coprococcus spp.</td>
<td>0.1724</td>
<td>0.09216</td>
<td>0</td>
</tr>
</tbody>
</table>

7.3 Pathobionts with vancomycin: increased with vancomycin

<table>
<thead>
<tr>
<th>Taxon</th>
<th>NOD</th>
<th>NOD + Vancomycin</th>
<th>NOD + Neomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>1.336</td>
<td>0.8826</td>
<td>47.31 ***</td>
</tr>
<tr>
<td>Anaeroplasma spp.</td>
<td>0.2586</td>
<td>0.1420</td>
<td>1.379</td>
</tr>
<tr>
<td>Sutterella spp.</td>
<td>0.9052</td>
<td>0.3254</td>
<td>4.414 **</td>
</tr>
<tr>
<td>Akkermansia muciniphila</td>
<td>2.241</td>
<td>0.8497</td>
<td>35.52 ***</td>
</tr>
</tbody>
</table>
Although neomycin and vancomycin treatment disrupts the microbiota in different ways, both antibiotics result in the loss of commensal microbes. In each treatment there is overgrowth of different microbes that may have pathogenic properties (pathobionts). The loss of commensals combined with the overgrowth of pathobionts augments the diabetogenic microbiome that results in accelerated diabetes in NOD mice.

4.5 Comparison of qPCR with abundance data from pyrosequencing sequencing

We determined the abundance of some microbes through qPCR and found similar trends in the qPCR and sequencing data (Figure 6). This validates the idea that both the qPCR and 454 sequencing approaches give rise to similar data.
Figure 6: qPCR validates 454 pyrosequencing results. Percentage abundance of sequences in the pool of sequences from 454 sequencing (A) and relative abundance determined by qPCR with specific primer relative to total bacteria determined with a universal primer set (B). n=3-5 per group for both sequencing and qPCR data (excluding neomycin ileum, n=2). *<0.05, **<0.01 and ***<0.001 by 1 way ANOVA with Tukey’s post-hoc test. Statistics could not be performed on the sequencing data from the neomycin ileum samples due to low samples size.

4.6 Intestinal inflammatory profile with antibiotic induced diabetes

The intestinal microbiota is known to regulate inflammation in the gut; however the importance of this in the context of T1D is not understood. The inflammatory profile in the colon and ileum of mice treated with neomycin and vancomycin was assessed by measuring transcript levels of inflammatory markers. In the colon, vancomycin treatment resulted in an
increase in the chemokines RELM-β and Cxcl9 although there was no change in many other inflammatory markers (Ccr7, IFN-γ, TNF-α, IL-1β and IL-2) (Figure 7). In the ileum, there was a general decrease in inflammatory markers with vancomycin treatment with significant decreases in IFN-γ and TNF-α lower levels of IL-1β and IL-2 in both antibiotic treatments (Figure 7A). Th17-associated cytokines were unchanged in the colon, and while IL-17f and IL-23a were decreased in all diabetes susceptible mice, IL17a was only decreased with vancomycin treatment (Figure 7B). In the ileum, but not the colon, TGF-β expression was lower with vancomycin treatment as was Foxp3, and IL-10 in all diabetes susceptible mice compared to NORs (Figure 7C).
Figure 7: Inflammatory profile in the colon and ileum of mice with antibiotic induced diabetes. Expression of inflammatory mediators in the colon (black bars) and ileum (white bars) was determined by qPCR with specific primers relative to expression of reference genes β-Actin and 18S rRNA. N=6-16 mice per group. *<0.05, **<0.01 and ***<0.001 by one way ANOVA with Tukey’s post hoc test.
4.7 Segmented filamentous bacteria, testosterone and IL-17A

One species of bacteria, Candidatus Arthromitus, known as Segmented Filamentous Bacteria (SFB), has been extensively studied due to its close interaction with the epithelium in the terminal ileum and its strong induction of Th17 immune responses [144]. Colonization of GF NOD mice with SFB protects them from the onset of diabetes and is associated with increased Th17 cell populations in the gut [38]. We determined the abundance levels of SFB in the colon and ileum using qPCR. We found that SFB are more abundant in the ileum and are depleted with vancomycin treatment (Figure 8A). Since SFB were still present in the neomycin-treated NODs despite accelerated diabetes, we wanted to determine if the SFB were in contact with the intestinal epithelium in these mice (Figure 8B). We visualized the SFB by using FISH on paraffin embedded sections of the ileum. As expected, there were no visible SFB present in the vancomycin-treated mice but SFB were in close contact with neomycin treated NODs similar to NOR and NOD. To determine if the SFB in contact with the epithelium and were inducing their typical IL-17 responses we correlated the presence of SFB to IL-17A and found that abundance of SFB is positively correlated with IL17A expression (Figure 8C). Finally, since testosterone is believed to be protective in the NOD model and is induced by SFB [108], we quantified testosterone in the sera of the NOD mice treated with antibiotics and while there was lower testosterone in vancomycin treated NODs, no significant differences were observed (Figure 8D). These results show that while SFB and its associated responses are lost in vancomycin-induced diabetes, neomycin maintains SFB and the responses suggesting SFB are not enough to overcome the diabetogenic effects of the microbiome.
Figure 8: SFB are most abundance in the ileum where they correlate with IL-17A expression. Abundance of SFB in the colon and ileum was determined by qPCR as a proportion of total bacteria determined by a eubacteria primer set (A). Localization of SFB to the intestinal epithelium was determined using FISH with probes specific for SFB (Alexa-fluor 594, red) and Eubacteria (Alexa-fluor 488, green). Pictures are representative of 4-6 tissues. Abundance of SFB was found to correlate with expression of IL-17A mRNA with a Pearson’s correlation coefficient of 0.9374 (C). Testosterone was quantified in the sera by enzyme immunoassay in 11 week and diabetic mice and graphed relative to age-matched controls (D). n= 4-5 (SFB qPCR), and 14-15 (Testosterone EIA). Scale bar = 50μm (20x images) and 16.67μm (60X images).
4.8 Intestinal Barrier Function in Antibiotic-Induced Diabetes

4.8.1 Microbiota Controls the Function of the Intestinal Barrier in the NOD model

A leaky intestinal barrier has been implicated in diabetes clinically and NOD mice have been shown to have dysfunctional barriers when compared to NOR mice [74]. To determine if the function of the intestinal barrier is regulated by the microbiota, we assessed the function of the intestinal barrier in the mice that received microbiota transplantation. We found that NOR microbiota improves the function of the intestinal barrier in NOD mice and the NOD microbiota increases dysfunction in NOR mice (Figure 9A). This reveals that the microbiota controls the integrity of the intestinal barrier.

4.8.2 Antibiotic treatment decreases intestinal barrier dysfunction in NOD mice

To address the importance of the intestinal barrier in T1D pathogenesis, we measured the function of the intestinal barrier in the antibiotic treated mice. We found that despite having accelerated diabetes, both vancomycin and neomycin treated mice had improved barrier function as evident by the decreased permeability of FITC into the sera (Figure 9B). This suggests that a leaky gut is not required for the acceleration of diabetes.

4.8.3 Claudin-1 expression does not correlate with intestinal barrier function in antibiotic-induced diabetes.

Claudin-1 is a tight junction protein that is involved in maintaining the function of the intestinal barrier. Expression of claudin-1 is decreased in the ileum of diabetes susceptible
BB rats and has been shown to be modulated by *Lactobacillus johnsonii* N6.2, which is protective in the BB rat model [113]. Since *L. johnsonii* is decreased with antibiotic treatment, we assessed the colon and ileum tissues for claudin-1 mRNA transcripts. We found that expression of claudin-1 was not different in the colon, but expression in the ileum was found to be lower in all NOD groups compared to NORs (Figure 9C). This indicates that Claudin-1 may be involved in the intrinsic barrier dysfunction present in NOD mice as compared to NOR mice, however claudin-1 is not involved in the improved barrier function observed with antibiotic treatment. We also stained the ileum of NOR, NOD and vancomycin-treated NOD mice using diaminobenzidine (DAB) staining and found that it was quite variable and no quantifiable differences in staining intensity or patterns of localization of claudin-1 protein could be determined (9D).
Figure 9: Microbiota controls the function of the intestinal barrier and antibiotics reduce barrier dysfunction. FITC-dextran was measured in the sera 4-hours post oral gavage in 11 week old mice following fecal transplant (A) or peri-natal antibiotic treatment (B). Expression of claudin-1 mRNA in the colon (black bars) and ileum (white bars) relative to housekeeping genes β-Actin and 18S rRNA (C) and representative pictures of DAB staining of claudin-1 protein in NOR, NOD and vancomycin-treated NOD mice (D). Scale bars = 50μm (20X images) and 16.67μm (60X images). N= 6-16 for mRNA and 3-7 for FITC studies. *p<0.05, **p<0.01 and ***p<0.001 by unpaired t-test for comparisons between two group and one-way ANOVA with Tukey’s post hoc test for comparisons between >2 groups.
4.8.4 Ileum microbiota from antibiotic-treated NOD mice protects the epithelial barrier from EPEC induced dysfunction \textit{in vitro}.

To further investigate the effects of the microbiota on the intestinal barrier, luminal content from freshly euthanized mice was added to monolayers of Caco-2 cells which model the epithelial layer of the intestinal wall (Figure 10A). Caco-2 monolayers were grown until trans-epithelial electrical resistance (TEER) reached >1000ohms [145] and formation of tight junctions between cells were formed noted by expression of tight junction proteins between cells (Figure 10B/C). When microbiota was added, we found that NOD microbiota induced a similar loss of resistance as compared to resistance loss of antibiotic treated NOD mice (Figure 10D). Enteropathogenic \textit{E. coli} was added to the monolayers to induce a baseline barrier dysfunction. Under these conditions, the addition of NOD microbes resulted in a loss of resistance in the epithelial layer while antibiotic treated NOD microbiota induced less resistance loss (Figure 10D). These results show that \textit{in vivo} and \textit{in vitro}, antibiotic treated NOD microbiota improves the function of the intestinal barrier. The \textit{in vitro} data suggests that this effect may be limited to a situation where baseline dysfunction is present, or possibly that EPEC interacts differently with antibiotic treated microbiota.
Figure 10: Microbes from antibiotic treated mice protect against EPEC induced barrier dysfunction in vitro. Caco-2 monolayers were grown in cell culture inserts (A) until a resistance of >1000 ohms was reached (B) and tight junctions had formed between cells (C). 100mg of luminal microbiota from freshly euthanized mice was added to caco-2 monolayers and trans-epithelial electrical resistance (TEER) was measured after 2 hours with and without a consistent amount of EPEC. TEER values represent the percentage of the starting TEER. N = 4 (without EPEC) or 1 (with EPEC) performed in duplicate. Statistical analysis could not be performed due to low sample size of the EPEC replicates. Scale bar = 16.67μm, Nuclei (DAPI, blue), E-cadherin (Alexa-594, red) and Claudin-1 (Alexa-488, green).
Chapter 5: Discussion

The autoimmune destruction of beta cells and subsequent loss of endogenous insulin is a result of the deregulation of the initiation, propagation and/or regulation of immune responses by unknown stimuli. In this thesis, we explored the diabetogenic properties of the microbiota in T1D by fecal transplantation and antibiotic treatment the NOD mouse. We found that guts of NOD mice harbour different microbial communities than diabetes resistant (NOR) mice. When transplanted into NOR mice, NOD microbiota induced insulitis indicating that it is diabetogenic, or diabetes inducing. Furthermore, peri-natal, but not post-natal, antibiotic treatment (both vancomycin and neomycin) accelerated diabetes, while neither transfer of microbes from NOR mice nor probiotic VSL3 could reverse diabetes progression in NOD mice. Antibiotic treatment resulted in a microbiota that was lower in diversity and had fewer commensal microbes as well as an increase in potential pathobionts. While intestinal inflammation and serum testosterone may be regulated by the microbiota, they do not dictate diabetes progression. We found that the function of the intestinal barrier is controlled by the microbiota yet the diabetogenic microbiota accelerates diabetes independent of a leaky gut.

Diabetes susceptible (NOD) and diabetes resistant (NOR) mice were found to have different microbial communities. In general, the NOD microbiota had fewer beneficial microbes and more pathobionts. The microbial communities are variable along the length of the GI tract with the colon having the most diversely and densely colonized area, while the ileum is less densely colonized with a different microbial community [146]. In the colon, the NOD microbiota is marked by a lack of Bifidobacterium spp., which has been reported in
T1D patients [102, 105] and the BB rat [104]. *Bifidobacterium* spp. are common probiotics believed to have several beneficial effects in the gut (reviewed in [147]). *Roseburia* species are also absent in the NOD colon microbiota; the absence of which has been reported in children susceptible to T1D [105]. *Roseburia* are generally regarded as beneficial bacteria because they have enzymes which can produce butyrate, a main energy source for colonocytes [148]. In particular, two species of bacteria *Ruminococcus gnavus* and *Bacteroides acidifaciens* are less abundant in antibiotic treated NOD mice. *R. gnavus* contains an enzyme that produces ursodeoxycholic acid [149], a bile acid which is protective in colitis models [150]. Furthermore, a taurine conjugate of this bile acid has recently been shown to protect NOD mice from T1D by reversing the unfolded protein response [151]. *B. acidifaciens* has been shown to induce IgA in the colon [152] which may be an important protective function.

The ileum microbiota in NOD mice has a similar loss of *Bifidobacterium* species as reported in the colon, and in addition has fewer members of the Lactobacillaceae family, particularly *Lactobacillus johnsonii*. *Lactobacillus* species are also common probiotics, with low abundance of *Lactobacillus* species having been noted in several human and rodent T1D studies [102-104]. Several studies have shown that *L. johnsonii* is protective in the BB rat by inducing a Th17 bias, lowering intestinal inflammation and increasing expression of tight junction protein, Claudin-1 [113, 153]. The NOD microbiota in the ileum is also marked by higher abundance of Enterobacteriaceae, which is associated with intestinal inflammation [154].
The diabetogenic properties of the NOD microbiota were assessed by transplanting NOD microbiota into diabetes resistant NOR mice after they had been treated with antibiotics. NOD microbiota induced insulitis in the NOR mice, supporting the idea that the microbiota contribute to diabetes onset. We next attempted to remove the diabetogenic microbes by treating groups of mice with the antibiotics vancomycin and neomycin, which target different bacterial groups. Vancomycin inhibits late stage cell wall synthesis in bacteria, which makes Gram positive bacteria more susceptible due to their thicker cell walls. Neomycin targets Gram negative microbes as it disrupts protein synthesis in the 30S ribosomal subunit of bacteria, which makes thin walled Gram negative bacteria more susceptible to this antibiotic. We found that perinatal administration (to the dams at birth and continued to the pups post-weaning) of both vancomycin and neomycin accelerated diabetes. Accelerated diabetes was associated with a higher blood glucose reading at euthanization and insulin auto-antibodies in the sera. This indicates that antibiotics were not eliminating diabetogenic microbes, but instead were depleting beneficial microbes and allowing for augmentation of microbes that may be pathobionts.

The microbiota is established during the first ~2 years in humans which equates to ~4 weeks in mice, around the time of weaning. Establishment of the microbiota during this initial colonization phase may be essential in shaping the microbial fingerprint that persists in the gut and as such, dictating susceptibility to disease in later life (reviewed in [155]).

Associated with this, the age at which GF mice are exposed to microbes affects patterns of inflammation in the gut and systemically [156], and exposure to a more diverse microbiota peri-nataly is required for immune regulation [157]. We found that in a different cohort of mice that were treated with vancomycin post-nataly (after weaning), diabetes was not
accelerated compared to controls. Furthermore, we found that transplantation of NOR microbes post weaning was unable to protect NOD mice from diabetes. Since NOD microbiota was depleted in *Bifidobacterium* and *Lactobacillus* spp., we treated a group of NOD mice with the probiotic VSL3 (a combination of *Lactobacillus*, *Bifidobacteria* and *Streptococcus* species) peri-nataly, but it did not protect NOD mice from diabetes. This suggests that the diabetogenic microbiome is established early in life, and it cannot be reversed with beneficial microbes and augmentation must occur during colonization to accentuate the diabetogenic effects.

The diabetogenic microbiota augmented by antibiotics was found to be lower in diversity, increased in pathobionts and decreased in beneficial microbes. Several microbes were depleted including the Lactobacillaceae family in the ileum, and *Prevotella* spp., *Bacteroides acidificiens*, *Ruminococcus gnavus* and *Lactobacillus johnsonii* in both the colon and ileum. As mentioned previously, the Lactobacillaceae family are generally regarded as beneficial bacteria, *B. acidificiens* and *R. gnavus* have potentially protective effects in the gut and *L. johnsonii* is protective in the BB rat model of T1D. In addition to those already mentioned, lower numbers of *Prevotella* species have been noted in children with T1D [102]. With vancomycin and neomycin treatments, several microbes that contribute to gut health are depleted or fewer in number.

The functions of microbes identified as being higher in abundance with antibiotic-induced T1D are less clear. The neomycin treated mice have increased abundance of Rikennellaceae in the colon and ileum, higher Cyanobacteria and *Blautia* species in the colon and increased Christenellaceae, *Coprococcus* and *Allobaculum* species in the ileum. Little is
known about the discreet functions of these microbes, making their role in accelerated T1D difficult to discern. Vancomycin treated mice have a considerable increase in abundance of the Enterobacteriaceae family as well as *Akkermansia muciniphila* in the colon and ileum. In the colon, an increase in *Anaeroplasma* species was found and an increase in *Suterella* species in the ileum. Although little is known about the function of *Anaeroplasma* or *Suterella*, Enterobacteriaceae are associated with intestinal inflammation [154] and *A. muciniphila* promotes gene expression of inflammatory pathways including T cell receptor signaling, leukocyte extravasation and antigen presentation [158]. As such, these microbes may be involved in promoting intestinal immune responses.

We found that vancomycin-induced diabetes is associated with elevated expression of the pro-inflammatory markers RELM-β and Cxcl9 in the colon. Cxcl9 is an IFN-γ induced chemokine involved in T cell recruitment in the early stages of autoimmune disorders [159] and RELMβ promotes differentiation of Th1 cell subsets [160]. The ileum, unlike the colon, had lower expression of pro-inflammatory cytokines (RELM-β, Cxcl9, IFN-γ and TNF-α) with vancomycin induced diabetes. Inflammation in the colon may be explained by the increased abundance of Enterobacteriaceae, however, the increase in Enterobacteriaceae is also found in the ileum where there are decreased inflammatory cytokines. So while expression of inflammatory markers are changed with antibiotic treatments and differences are observed in the colon and ileum, no patterns emerge that correlate with accelerated diabetes.

While the immune responses leading T1D are believed to be a Th1-biased, the role of Th17 cells has been subject to debate [31]. Th17 cells are pathogenic in other autoimmune
diseases and there is elevated systemic Th17 cells in T1D patients [161] and NOD mice have elevated expression of IL17 in colon tissue [162]. It is not known what triggers Th17 cells generation in humans, but in mice Th17 expansion in the ileum correlates to the abundance of Segmented Filamentous Bacteria (SFB) [37]. SFB colonization was shown to be protective in NOD mice and associated with expansion of Th17 cells in the ileum [38]. IL17a, expressed by Th17 cells, was depleted with vancomycin treatment, which correlates with depletion of SFB as previously reported [38]. Other Th17 associated cytokines include IL17f, which is released by Th17 cells, and IL23a, which promotes Th17 cell differentiation. There are no significant differences between NOD and antibiotic treated NOD mice, but there is a higher expression of IL17f and IL23a in the ileum of NOR mice. This may indicate a breakdown in regulation of Th17 cell populations in NOD mice compared to NORs.

Regulatory cell types are promoted by transforming growth factor (TGF) β and secrete IL-10. We find that in the ileum, vancomycin treated NOD mice have lower TGF-β expression and all NOD mice have lower IL-10 expression compared to NOR. This may support the idea of dysfunctional regulatory cells in T1D, but it does not appear to be modulated by antibiotic treatment. Overall, despite the changes in microbiota, no consistent changes are observed in the intestinal tissues with both neomycin and vancomycin induced diabetes. This may indicate that intestinal inflammation does not simply correlate with accelerated diabetes. Additionally, we assessed the inflammatory status of the whole tissue and the gut has several cell types including epithelial cells, endothelial cells, macrophages, dendritic cells, B cells and several T cell subsets. It is possible that there are differences in certain types of cells that we were not able to capture with this technique.
Compared to female NOD mice, male NOD mice have delayed onset of diabetes. Testosterone is believed that testosterone plays a protective role in this delay, which is supported by evidence that castration of male NOD mice accelerates diabetes [163], and androgen treatment protects females NODs from T1D [164]. Transplant of mature male NOD microbiota into young female NODs has been found to protect them from T1D while increasing serum testosterone levels [111] and recent evidence shows that SFB may be mediating this protection [108]. In order to determine if testosterone was playing a role in antibiotic-induced diabetes, we assessed the levels of testosterone the in sera of vancomycin and neomycin treated mice and found no significant differences between groups (although vancomycin had a trend of lower levels). Overall, depletion of SFB by vancomycin is associated with lower protective Th17 responses and lower serum testosterone concentration. This may contribute to the accelerated diabetes with vancomycin treatment. However, the loss of SFB and associated responses (Th17 and testosterone) are not observed in the neomycin treated group that also has accelerated diabetes. This points to the multifaceted nature of T1D pathogenesis since not a single mechanism can explain the antibiotic induced diabetes.

An impaired intestinal barrier is proposed to contribute to T1D by increasing exposure of antigens in the gut with leaky guts having been implicated with T1D onset in clinically and in animal models [72, 74]. Disruption of the gut barrier with an enteric pathogen increases the numbers of diabetogenic T cells and accelerates insulitis [74], although subsequent improvement of the barrier does not protect NOD mice from T1D [76]. We find that microbiota regulate the function of the barrier in the NOD model since NOR microbes improve barrier function when transplanted into NOD mice and NOD microbiota...
increases barrier dysfunction when transplanted into NOR mice. Although the increased
dysfunction of the intestinal barrier correlates with initiation of insulitis, we find that
subsequent improvement of the barrier does not reverse the genetic predisposition to T1D.
Microbiota transplants were not started until 4 weeks of age, making it is possible that the
diabetogenic microbes had already initiated early stages of insulitis, as insulitis can begin as
early as 3-4 weeks in NOD mice [12]. We also found that, despite accelerated diabetes,
antibiotic treatment improved the function of the intestinal barrier with both vancomycin and
neomycin treatment, resulting in less dysfunction that in untreated NOD mice. Based on this
evidence it appears that the diabetogenic microbiome is accelerating diabetes independent of
disrupting the intestinal barrier.

A few studies similar to ours have found results that do not agree with some of our
findings. For example, post-weaning administration of VSL3 was shown to protect NOD
mice from diabetes [114] although a more recent publication noted no difference in diabetes
onset when VSL3 was administered to GF mice [108]. In contrast to our study, Hansen et al
found that administration of vancomycin from birth until 4 weeks delays diabetes in NOD
mice [117]. Although there were similar global changes in microbes with vancomycin
treatment, it appears that their mice had a different starting microbiota marked by a higher
Firmicutes to Bacteroidetes ratio, and their mice did not contain SFB. Given the profound
impact that SFB have on intestinal immunity, the difference in SFB status may be a factor in
the different phenotypes observed in the two studies. Furthermore, in Hansen et al.’s study,
antibiotics were discontinued at 4 weeks indicating that prolonged and continuous antibiotic
exposure may have different effects on the diabetogenic microbiome in NOD mice. Both
studies found, however, that slower diabetes progression was associated with increased
intestinal inflammation. It is interesting that increased inflammation in the ileum negatively correlates with accelerated diabetes in both studies, which may point to protective inflammatory responses.

The origin of the mice and location of study should also be considered, since it is known that these factors can influence the composition of the microbiota and could change responses to antibiotic treatment. Our mice were obtained from Jackson Laboratory (Maine, USA) and bred at our facility in Vancouver, whereas Hansen et al obtained their mice from Taconic Farms (Lille Skensved, Denmark) and bred them in their facility in Copenhagen, Denmark. Mice from different vendors have been shown to contain different microbiota, which alters their immune responses [144] and could influence how their microbiota reacts to antibiotic treatment. Evidence of the role of the microbiota in dictating the physiology of the organism is growing, and this highlights the importance of considering the indigenous microbiota when comparing studies in different facilities and with mice from different vendors.

**Conclusion**

Our evidence suggests that NOD mice harbour a microbiome that facilitates autoimmune destruction of beta cells. The diabetogenic microbiome initiates insulitis in genetically resistant hosts and, in our facility the diabetogenic microbiome is augmented with both vancomycin and neomycin treatment leading to accelerated diabetes in NOD mice. Post-weaning administration of vancomycin does not accelerate diabetes, indicating a critical time period during which the diabetogenic microbiome is established. Furthermore, protective microbes from genetically resistant mice or administration of probiotics (VSL3)
cannot overcome the diabetogenic properties of the NOD microbiome. The relationship between intestinal microbes and T1D pathogenesis is complex and multifaceted. It appears that neither the intestinal inflammatory profile nor serum testosterone are critical factors in determining T1D progression. We find, however, that antibiotic induced T1D is independent of a leaky gut. Our study highlights a critical role for the microbiota in T1D progression and contributes to the current perception that early microbial exposures have long-term consequences for the physiology of the host. It brings into question the early life use of antibiotics and questions the effectiveness of probiotics in outcompeting resident microbes.
References


[27] Phillips JM, Parish NM, Raine T, et al. (2009) Type 1 diabetes development requires both CD4+ and CD8+ T cells and can be reversed by non-depleting antibodies targeting both T cell populations. Rev Diabet Stud 6: 97-103


Yurkovetskiy L, Burrows M, Khan AA, et al. (2013) Gender bias in autoimmunity is influenced by microbiota. Immunity 39: 400-412


[121] Eckmann L, Fierer J, Kagnoff MF (1996) Genetically resistant (Ityr) and susceptible (Itys) congenic mouse strains show similar cytokine responses following infection with Salmonella dublin. J Immunol 156: 2894-2900


[152] Yanagibashi T, Hosono A, Oyama A, et al. (2013) IgA production in the large intestine is modulated by a different mechanism than in the small intestine: Bacteroides acidifaciens promotes IgA production in the large intestine by inducing germinal center formation and increasing the number of IgA+ B cells. Immunobiology 218: 645-651


[162] Alam C, Valkonen S, Palagani V, Jalava J, Eerola E, Hanninen A Inflammatory tendencies and overproduction of IL-17 in the colon of young NOD mice are counteracted with diet change. Diabetes 59: 2237-2246
