The Impact of Microbiome Alterations in the NOD Mouse Model of Type 1 Diabetes

by

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A thesis submitted in conformity with the requirements for the degree of MSc.
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Abstract

Epidemiological and experimental data suggest Type 1 Diabetes (T1D) and gut inflammatory disorders share genetic and environmental risk factors. Genetics and microbiome composition differentiate T1D-prone NOD mice and T1D-resistant NOR mice. In the first part of this study, we examined the possible protective effects of NOR-derived microbiome transplant in NOD mice. We report, despite quantifiable, durable changes in the gut microbiome of NOD recipients of NOR-derived microbiome transplant compared to NOD and NOR controls, these mice were not protected from T1D.

Studies have also suggested that probiotic bacteria can have protective effects in T1D and gut inflammatory disorders. In the second part of this study, we examined the possible protective effects of treatment with probiotic microbes in the NOD mice. We report Protecflor probiotic-treated NOD mice showed quantifiable, durable changes in gut microbiome composition, and were protected from autoimmune infiltration of the islets compared to vehicle-treated controls.
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Chapter 1
Introduction
1 Introduction

1.1 Introduction to T1D

Type 1 Diabetes (T1D) is an autoimmune disease which is characterized by the T cell mediated destruction of insulin producing β cells in the pancreas, leading to loss of blood glucose control (reviewed in [1]). The current treatment for T1D is exogenous administration of insulin and frequent monitoring of blood glucose levels [1]. Despite treatment, T1D patients suffer chronic hyperglycemic fluctuations resulting in significant comorbidities, including increased risk of cardiovascular disease, retinopathy, nephropathy, and neuropathy [2]. Due to these comorbidities, the global health burden of T1D is high, and is on the rise.

Global incidence of T1D has been rising over past 50 years [3]. Recent estimates in Canada suggest an annual rate of 20 to 26 new cases per 100,000 children aged 0 to 14 years, among the highest reported in the world [4]. The global rate is reported to be increasing by 232% per year [5]. Clinical management of T1D has not changed significantly over the last several decades. This contrast highlights the need for further research into the genetic/environmental interactions which underlie the etiology of this disease.

1.2 Genetic Risk Factors

T1D is a complex disease involving multiple genes and epigenetic environmental modifiers. Although ~90% of new T1D cases occur in individuals without family history of T1D, the disease is known to be hereditary [6]. Family/pedigree studies have demonstrated that first degree relatives of T1D patients have a 5% to 7% lifetime risk of developing T1D, as compared to 0.4% risk in the general population [7]. The higher concordance rates in monozygotic twins than in dizygotic twins further suggest genetic factors play a role in this disease [8]. Over the past decades, multiple approaches have been used to identify loci associated with risk of T1D. Over 40 T1D-associated loci have been identified, and causal variants have been identified in a number of them [9].

The strongest genetic contribution to risk is MHC class II haplotype (HLA). high-risk alleles at this locus account for approximately half of the increased genetic risk relative to the general population [10]. Other putatively causal single nucleotide polymorphisms (SNPs) have been found in genes with immunological functions: PTPN22 [11], IL2RA [12], CTLA4 [13], IFIHI [14].
Despite the success of genome-wide association studies (GWAS) in identifying T1D susceptibility loci, most of these SNPs display low effect sizes on the disease phenotype. It has been estimated that all non-HLA SNPs identified to date to account for only 10.9% of T1D variance observed[15]. This gap between the observed heritability of T1D and explanatory power of known genetic factors is known as the “missing heritability.” This missing heritability may be explained by multiple common alleles of weak effect, gene-gene, and/or gene-environment interactions. Recent studies have suggested that the rare allele hypothesis is unsupported for T1D[16].

1.2.1 Genetic link to inflammatory diseases of the gut

A number of T1D associated genetic loci are shared with inflammatory diseases of the gut, in particular Crohn’s and celiac disease. ZNF365, MST1, ERBB3, and PTPN22 have been identified as a common set of susceptibility genes in both Crohn’s disease and T1D[17]. T1D and celiac disease share the some susceptible alleles of HLA-DQB1. Additionally, IL2, RGS1, TAGAP and IL18RAP have been reported as susceptibility genes for both T1D and celiac disease[18]. T1D patients are also at a higher risk of developing celiac disease compared to the general population. Anti-tissue transglutaminase antibodies, a marker for celiac disease, are present in ~10% of children and ~2% of adults with T1D[19]. This connection suggests underlying commonalities in the genetic and environmental factors which cause T1D and autoimmune diseases of the gut.

1.3 Environmental modifiers

While there is strong evidence that genetics plays an important role in T1D, several pieces of evidence point to the importance of environmental factors in T1D. Relatively weak concordance between the incidence of T1D in monozygotic (“identical”) twins indicates involvement of stochastic or environmental factors in the etiology of the disease. Studies have placed this concordance rate for T1D between 30-80%[20]–[22]. Meta-analyses of T1D cohort studies over the last 44 decades have also shown decreasing age at onset, as well as accelerated pathogenesis, as measured by loss of beta cell mass[23]. Within a genetically stable population, rapid changes in the development of the disease are not expected, and point to changing environmental factors.

The wide geographical distribution in T1D incidence also suggests gene-environment interactions underlie the disease. Overall age-adjusted incidence of type 1 diabetes ranges from
0.1/100,000 per year in China and Venezuela to 36.8/100,000 per year in Sardinia and 36.5/100,000 in Finland[4]. Some studies have noted that this corresponds roughly to a “north-south” gradient globally[4]. Other studies on geographic distribution have observed a correlation between T1D incidence and latitude, both globally, and within countries; an Australian study, found that T1D was three times prevalent in southerly region than in northerly regions[24]. Data from migrant populations suggest that these geographical tendencies are not entirely dependent on genetic factors. Immigrants tend to adopt the T1D incidence in their destination country and have rates of T1D which approach those of the indigenous population[25], [26]. Furthermore, adjacent countries with similar genetic backgrounds can often have divergent T1D incidences; a six-fold difference in T1D incidence is observed at the eastern Finland-Russian border[27]. These data highlight the need to investigate environmental modifiers in T1D in a controlled system.

1.4 NOD Model of T1D Genetics & Environment

Two rodent models of T1D, the Bio-Breeding (BB) Rat[28] and the Non-Obese Diabetic (NOD) mouse model[29], have enabled studies of the genetics and gene-environment interactions that affect T1D incidence and pathogenesis. The NOD mouse is an inbred mouse strain with a high incidence of spontaneous T1D. Unlike in humans, T1D in the NOD displays a marked sex bias, with ~80% of females and ~30% of males becoming diabetic at 6 months of age[30]. The disease displays many of the known features of T1D in humans. Like in humans, T1D in the NOD mouse is polygenic, with over 30 Insulin-Dependent Diabetes (Idd) loci identified[31]. Furthermore, the MHC haplotype contributes the highest genetic risk in both humans[10] and NOD mice[32]. The NOD mouse model also shares a number of other susceptibility loci and immune pathways with the human disease. T1D in NOD mice also displays much of the immunopathology seen in human T1D: auto-antibody production[33] and APC-mediated lymphocyte infiltration of the islets, termed insulitis[34].

To narrow the study of these genetic susceptibility loci in the NOD model, our lab has compared NOD mice to the closely-related Non-Obese Resistant (NOR) mice, an inbred strain with genetic material derived from NOD and bk/s strains[35]. NOR shares 88% of the NOD genome by descent, and differs from NOD at only 4 of the 20 Idd loci identified; Idd4, Idd5, Idd9, Idd13[36][37][38, p. 13][39]. However, NOR is completely resistant to T1D, although it exhibits
some of the inflammatory pre-clinical traits which precede T1D in NOD mice and humans. Using NOD background congenic strains harboring NOR intervals, our lab has previously mapped early T cell recruitment events in the initiation of insulitis to Idd5 and Idd13, and found these loci to regulate a T cell-dependent progression from a benign to a destructive stage of insulitis[40]. Work in our lab and others has also identified candidate genes in the four loci which differ between NOD and NOR. In conclusion, these two strains provide a system to study the genetics of T1D in a model through the use of inter-crossing and genetic mapping.

1.4.1 Environmental modifiers in NOD

Like in the human disease, there is evidence of environmental modifiers of T1D in NOD mice. Meta-studies compiling natural history and other data from NOD colonies in multiple facilities have noted a variable diabetes rate, ranging from 50%-80% T1D incidence in female NOD mice[30]. It has been reported that T1D incidence is inversely correlated with reports of infectious disease in colonies[30]. Furthermore, generations of NOD mice re-derived from conventional conditions to SPF showed a twofold increase in T1D incidence[41]. These data suggest a role for microbial exposure in modulating T1D incidence. This is further supported by studies showing deliberate infection with multiple organisms, including *Schistosoma mansoni*[42], *Mycobacterium avium*[43], *Trichinella spiralis*[44], *Heligmosomoides polygyrus*[45] and *Salmonella typhimurium*[46], protects NOD mice from diabetes. Interperitonial injection of zymosan, a fungal cell wall component, has also been found to be protective in the NOD model[47]. The potential mechanisms responsible for these protective effects include T helper cell type 2 (T\textsubscript{h2}) skewing, regulatory T cell (T\textsubscript{reg}) and natural killer T (NKT) cell activity, and modulation of antigen presenting cells (APCs) towards a diabetes-protective phenotype.

In addition to systemic antigen exposure, recent studies have also shown that the intestinal microbiota and the innate immune system in NOD mice can influence the development of T1D. NOD mice genetically deficient in MyD88 were protected from T1D and resulted in altered gut microbiota[48]. Transfer of NOD.MyD88\textsuperscript{-/-}-derived gut microbiota into germ-free NOD mice protected against T1D in these mice[48]. Oral exposure of NOD mice to a number of non-infectious bacterial strains has also been shown to be protective. The probiotic mixture VSL#3; a mixture of *Bifidobacterium* and *Lactobacillus* species and *Streptococcus salivarius subsp.*
thermophiles was found to be protective of SPF-colonized NOD mice if given by oral gavage three times a week from 4 to 32 weeks of age[49].

Changes in the intestinal microbiota of mice have also been found to induce hormonal and metabolic changes leading to protection against T1D. In the NOD model, T1D incidence exhibits a distinct sex bias, with ~80% of females and ~30% of males becoming diabetic at 6 months of age[30]. This bias is colonization dependent, as the sex bias is absent in germ-free NOD mice. Work in our lab showed that the transfer of male NOD-derived microbiota into weanling female NOD mice by oral gavage significantly protected them against T1D, and induced changes in testosterone levels and the metabolomics profile of treated mice[50].

These data suggest that changes in the gut microbiota of NOD mice may be correlated with protection, either by direct interaction with the immune system, or by modifying other physiological factors with known influence on T1D incidence. These and other data lead to the idea that microbial exposure may be an important environmental factor in auto-immune diseases in both mice and humans.

1.5 The Hygiene Hypothesis

Epidemiological meta-studies on infectious and autoimmune diseases have shown a dramatic rise in the incidence of T1D and other autoimmune diseases, and a dramatic decline of infectious diseases[41]. This decline is mostly attributable to advances in medicine and sanitation; antibiotics, improved hygiene have helped to manage infectious disease in the developed world. Epidemiological evidence suggests that the increased incidence of autoimmune diseases is linked to improved socio-economic status, but does not identify a specific environmental modifier. This raises the hypothesis that there exists a connection between the rise in autoimmune diseases and the decline in infectious diseases, called the hygiene hypothesis.

The hygiene hypothesis posits that advances of medicine and sanitation have altered the microbial environment to which humans are exposed, and that this altered environment has led to an increase in auto-immune and auto-inflammatory diseases[51]. Correlations have been found between the increasing prevalence and severity of asthma among children in developed nations and improved sanitation and widespread antibiotic use[52]. Recent studies suggest there is a ‘critical window’ in the first 6 months of life; during which immunological developments in
infancy may be influenced by the commensal microbial community might alter allergic sensitization later in life[53]. It has been suggested that rising autoimmune disease incidence may reflect immune responses which were evolved to survive infection, but are now maladaptive in light of the reduced immunological challenge due improved public health. Alternatively, alterations to our microbiota due to decreased exposure to microbes have resulted in the loss of immunological beneficial microbiota. The hygiene hypothesis raises some interesting possibilities, but more work is needed to identify mechanisms linking microbial exposures to immune mediated disease.

1.6 The Gut Microbiome

The microbiome is the collection of microbes which live as a complex community/ecosystem in or on a host organism. This collection of microbes consists of hundreds of species and strains, each with their own complex metabolism and products. Commensal (non-pathogenic) microbes on all colonized body surfaces out-number human cells 10:1, while the human gut alone harbors roughly 1.5 kg of bacteria, with a metagenome of over 3 million genes[54].

The gut microbiome of an individual develops soon after birth. Before birth, the gut is an essentially sterile environment. Soon after birth, the mammalian neonate’s gut is colonized by mainly maternally-derived bacteria; vaginal bacteria for vaginally delivered babies or skin-associated bacteria following cesarean section deliveries[55]. After a brief period of instability, phylogenetic diversity increases over time. The microbiome of children begins to transition to more similar to the adult state starting at 0.6-1 year of age[56]. In a stable gut microbiome, ~1,000 species are represented, over two thirds of which are members of two phyla, Bacteroidetes and Firmicutes[57].

The microbiome influences multiple aspects of host physiology, including nutrition, metabolism and immunity. The presence and composition of the gut microbiome has a profound influence on the development and function of the immune system as a whole. The development of the gut-associated immune system is altered in the absence of the microbiome; in germ-free mice, GALT and Peyer’s patches are deficient in lymphocytes, and IgA production is essentially abrogated[58].
Furthermore, a number of bacterial groups have been identified as immunomodulatory in mice. Bacteroides fragilis, a human commensal, induced systemic Th1 responses in mice mediated by a surface polysaccharide, polysaccharide-A (PSA)[59]. Segmented filamentous bacteria (SFB) were found to be associated with the abundance of lamina propria Th17 cells in the gut of mice, and were found to be sufficient to generate Th17 cells in germ-free mice mono-colonized with SFB[60]. Although SFB have not been identified in humans, it may be that other bacterial strains play similar roles in humans. Clostridium species belonging to clusters IV and XIVA were found to induce differentiation of inducible Foxp3+ and IL-10+ Tregs in the mouse colonic lamina propria[61]. 17 strains of human-derived Clostridium species belonging to clusters IV, XIVA, and XVIII (recently reclassified as the class Erysipelotrichi) were found to induce Treg expansion and functional potency by upregulation of IL-10 and ICOS in mono-colonized mice. It is clear that some commensals can modulate immune cell subsets in the host, in both humans and mice.

Other bacterial strains have also been found to be associated with effects in models of autoimmunity. SFB have been found to co-segregate with T1D protection in the NOD mouse[62]. In another study, SFB was also found to drive autoimmune arthritis in germ-free mice via promotion of the Th17 subset[63]. Faecalibacterium prausnitzii was found to be associated with a lower risk of postoperative recurrence of ileal CD in Crohn’s patients and reduced the severity of TNBS-induced colitis in a murine experimental model of colitis[64]. It is clear that the gut microbiome and alterations in intestinal microbiota can influence incidence in rodent models of T1D, and other models of autoimmune disease.

1.7 Tools and methods for investigating microbiome composition

The detection and quantification of microbes in the gut has been integral to our growing understanding of the effects of both single bacterial strains and broad community structure of host physiology. Current technologies for investigating microbiome composition are largely dependent on sequence variation amongst bacteria in highly conserved genes. The 16S rRNA gene is the most commonly used gene as it is conserved across most prokaryotes[65]. Pan-prokaryotic multiple alignments of 16S rRNA gene sequences reveal regions of high interspecies conservation, and regions of hypervariability[66]. Sequence variation in these hypervariable regions is proportional to phylogenetic distance and therefore can be used to phylogenetically classify bacterial sequences.
By designing degenerate oligonucleotide primers complementary to taxa-specific consensus sequences within the hypervariable regions, PCR amplification of sequences only from templates derived from specific bacterial taxa is possible. In a quantitative PCR (qPCR) reaction, this specific amplification can be used to quantify 16S rRNA copy numbers from specific taxa, providing taxa-specific bacterial quantification, even from a complex mixture.

Conversely, libraries of bacterial 16S rDNA representative of the community variation can be generated using degenerate PCR primers complementary to conserved regions, amplifying into hypervariable regions. Combined with high-throughput DNA sequencing, sequence variation in these hypervariable regions can be used to quantify and phylogenetically classify bacteria in complex community. Sequences are classified into operational taxonomical units (OTUs), which represent the lowest level of taxa reliably assignable to each sequence. Data from high-throughput 16S sequencing can be further analyzed using multivariate analysis techniques, which are mainly aimed at reducing the complexity of large multidimensional datasets.

One of the main methods of dimensional reduction is Principal Components Analysis (PCA). This is done by finding a set of orthogonal linear combinations of the original variables, called principal components, which account for the greatest variance in a dataset. The number of significant principal components is restricted to those which represent most of the variances, thereby representing the microbial composition of a gut community with a greatly reduced number of variables. By looking at the component variables and their weightings, called loadings, which make up each principal component, factors which influence the composition of a microbiome can be inferred. The distance in a PCA plot between two microbiomes can indicate degree of compositional similarity, and clustering analysis can be used to further group microbiomes by similarity.

Ecological community metrics can be used for further comparison of microbiome 16S sequencing data. The two main metrics use are community richness and diversity. More qualitatively similar communities often have similar richness and diversity. Richness is an estimate of the true total number of OTUs in a sample, regardless of the distribution of sequences across these OTUs. The Chao1 statistic is a measure of sample richness [67] which estimates the true total number of OTUs in a sample based on the number of singletons (OTUs observed once in the sample), and doubletons (OTUs observed twice in the sample) observed [67].
The second most common community metric used is diversity. This statistic depends on the number of OTU observed in a sample, and the evenness of the distribution of individual samples across OTU categories[68]. Shannon’s H statistic is a commonly used measure of the diversity or the predictability of a given OTU in a sample[69]. A higher Shannon’s H value indicates higher entropy or “surprise” in a sample; the next OTU to be called is harder to predict [68].

1.8 Previous Work

Previously in our lab, we performed 16S sequencing on female NOD and NOR mice (Figure 3-1). Our data shows there are quantifiable differences between the gut microbiota of NOD and NOR mice, most notably in the 5 taxonomical groupings: Bacteroidetes, Clostridia IV, Ruminococcus, Clostridia XIVa, and Helicobacter (Table 3-1). Given their shared environmental exposures under controlled, laboratory conditions, these differences suggest that there are genetic factors which influence the composition of the gut microbiota. Previously, our lab also showed the transfer of male-derived microbiome into recipient NOD females resulted in a durable alteration in the recipient microbiome, alterations in metabolomics profiles in serum, and a protective effect on T1D in recipient females[50]. These results suggest differences in microbiome composition can affect the risk of T1D and other autoimmune diseases in the host. Together, these data lead to the hypothesis that these differentially abundant bacterial groups in NOD and NOR mice may contribute to the protection of NOR mice from T1D, and that the transfer of these organisms to NOD mice may result in durable changes to the gut microbiome composition, and afford protection against T1D.

1.9 Project Rationale

1.9.1 The effect of NOR-derived microbiome transfer into NOD on microbiome composition and T1D incidence

The NOD mouse is an inbred mouse strain with a high incidence of spontaneous T1D. NOR mouse is a strain closely related to the NOD, but is protected by the genetic contributions from the C57BLKS/J strain. In the NOD model, T1D incidence exhibits a distinct sex bias, with ~80% of females and ~30% of males becoming diabetic at 6 months of age[30]. This bias is colonization dependent, as the sex bias is absent in germ-free NOD mice. Work in our lab showed that the transfer of male NOD-derived microbiota into weanling female NOD mice by
oral gavage significantly protected them against T1D, and induced changes in the microbiome composition, testosterone levels and metabolomics profile of treated mice[50].

We hypothesized that the T1D protection afforded by genetic contributions from the C57BLKS/J strain in the NOR strain might also be linked to microbiome composition. Previous work in our lab found 5 bacterial taxa (Bacteroidetes, Clostridia IV, Ruminococcus, Clostridia XIVa, and helicobacter) that were differentially abundant between NOD and NOR animals. Other studies have suggested that members of these taxa may have immunomodulatory effects. The first goal of my work was to investigate the possible protective effects of NOR-derived microbiome transplant in the NOD model of T1D.

### 1.9.2 The effect of Protecflor probiotic treatment on NOD microbiome composition and insulitis severity

T1D and gut inflammatory disorders have been found to share genetic risk factors, and can occur as co-morbidities suggesting shared genetic and environmental factors in T1D and autoimmune diseases of the gut. Studies in ulcerative colitis, inflammatory bowel syndrome and T1D have suggested that changes in the gut microbiome associated with treatment with probiotic bacteria such as *Lactobacillus* and *Bifidobacterium* can have protective effects in these diseases. Probiotics are generally derived from fermented food products or the gut microbial communities of ruminants. They are generally cultured organisms and relatively well-characterized compared to most of the strains in complex microbial communities such as the gut.

Studies in ulcerative colitis, inflammatory bowel syndrome and T1D have suggested that specific probiotic bacterial strains can have protective effects in these diseases. For example, *Lactobacillus helveticus* R0052 and *Lactobacillus rhamnosus* R0011 have been shown to reduce clinical symptoms in open label experiments on human adults with inflammatory bowel syndrome[61], and to reduce epithelial injury due to *Escherichia coli* O157:H7- and O127:H6-infection in an in vitro gut epithelial cell co-culture system [70]. *Bifidobacterium longum* R0175, given in combination with inulin, a plant polysaccharide, was found to improve self-reported quality of life, symptoms of blood and mucous in stool, and to reduce relapse rate in a small pediatric study of ulcerative colitis (UC) [71].
We hypothesized that continuous treatment with Protecflor probiotic, a formulation containing *Lactobacillus helveticus* R0052, *Lactobacillus rhamnosus* R0011, and *Bifidobacterium longum* R0175 and the yeast *Saccharomyces cerevisiae boulardii*, would impact the gut microbial composition of diabetes-prone NOD mice, and consequently, have an effect on the progression of islet autoimmunity.
Chapter 2
Materials and Methods
2 Materials and Methods

2.1 Mice

NOD/Jsd (NOD) and NOR/Jsd (NOR) Specific Pathogen Free (SPF) mice used in this study were maintained at The Hospital for Sick Children (Toronto), housed in sterilized static caging. The animals received standard mouse diet (LabDiet #5015, PMI Nutrition International) and autoclaved water. All staff wore autoclaved gowns, caps, masks, shoe covers, and sterile gloves. Animal handling and cage changes were done under HEPA filtered air. The health status was determined by weekly exposure of CD-1 sentinels to soiled bedding from the cages in the room. Quarterly serological testing of sentinels confirmed the mice were negative for: Mouse Hepatitis Virus, Minute Virus of Mice, Mouse Parvovirus, Murine Norovirus, Sendai Virus, Theiler’s Murine Encephalomyelitis, Reovirus and for endo- and ectoparasites. The results of sentinel testing indicated presence of Murine Norovirus and Helicobacter sp. in the room. In addition, live animals were subjected to additional, annual comprehensive necropsy, histopathology, bacteriology and parasitology testing. All experiments were carried out in accordance with the Institute animal utilization protocols and Canadian Council on Animal Care (CCAC) guidelines.

2.2 Design and Validation of Taxa-Specific qPCR

16S rRNA gene sequences belonging to the target taxa were retrieved from the RDP database[72] and aligned in PRIMROSE[73]. Primer sequences were then found in consensus regions, with a target theoretical Tm of 60C. These preliminary candidates were validated in silico by performing ProbeMatch searches on the RDP database for spurious amplicons (matches to 16S rDNA sequences classified out of the target taxa).

In vitro validation of primer candidates which passed in silico validation was performed by the following pipeline: the candidate oligonucleotide primer sequences were sent for synthesis (IDT). PCR (see below) was performed on template DNA (see below for bacterial DNA extraction) derived from female NOD caecal contents. PCR products were run on 2% agarose gel electrophoresis, and visualized using ethidium bromide staining and UV trans-illumination. Approximate molecular weight/amplicon lengths were determined by comparison with a concurrently run 100bp DNA molecular weight ladder (FroggaBio). Bands at the predicted amplicon sizes (Table 3-1) were excised using a razor blade and PCR products were extracted
using the QIAquick Gel Extraction Kit (Qiagen) according to manufacturer’s instructions. PCR amplicons were cloned into pCR2.1 plasmids using TOPO TA-cloning according to manufacturer’s instructions (Life Technologies) and transformed into chemically-competent DH5α E. coli for cloning and selection. Transformed bacteria were plated on LB agar containing 100 µg/mL ampicillin and 40 µg/mL x-Gal for blue-white selection. Individual colonies were picked and grown in LB broth.

Plasmid DNA was extracted using QIAprep Spin Miniprep Kit (Qiagen) according to manufacturer’s instructions, and sent for Sanger sequencing by TCAG using M13F and M13R primers. At least 20 clones for each primer set were Sanger sequenced. Sequences were pre-processed to remove pCR2.1 sequences in MEGA4[74] and taxonomically classified by the web-interface RDP classifier[75] using RDP 16S rRNA training set 9 and a confidence threshold of 80% for family-level taxonomical assignment, and 50% for genus-level assignment[76] to validate specificity of designed primers to the target taxa.

2.3 PCR

PCR amplification was conducted in polypropylene 12-cap strips on GeneAmp PCR system 9700 (Applied Biosystems). Each 25-µl reaction contained the following: 2.5 µL of ThermoPol reaction buffer (New England bioLabs), 0.5 µL of 10 mM dNTPs, 0.5 µL of each primer (200 µg/mL; IDT), 0.125 µL Taq DNA polymerase (5000 U/mL), 10 ng template DNA and H2O to make up the volume to 25 µL. PCR conditions were 30s at 95°C, followed by 35 cycles of 95°C for 15s, 60 s at the annealing temperature, and 1 min at 68°C.

PCR products were run on 2% agarose gel electrophoresis, and visualized using ethidium bromide staining and UV trans-illumination. Approximate molecular weight/amplicon lengths were determined by comparison with a concurrently run DNA molecular weight ladder (FroggaBio).

2.4 qPCR

qPCR assays were conducted in polypropylene 96-well plates on an ABI Prism 7900HT (Applied Biosystems). Each 25-µl reaction contained the following: 12.5 µL of PowerSYBRGreen PCR Master Mix (Applied Biosystems), 0.5 µL of each primer (200 µg/mL;
IDT), 10 ng template DNA and H2O to make up the volume to 25 µL. PCR conditions were 10 min at 95°C, followed by 40 cycles of 95°C for 15s, 60 s at the annealing temperature.

Each plate included triplicate reactions per DNA sample and the appropriate set of standards and DNA-negative controls. Melting curve analysis of the PCR products was conducted following each assay to confirm that the fluorescence signal originated from specific PCR products and not from primer-dimers or other artifacts.

2.5 Oral gavage transfer of the gut microbiome

Weanling NOD females (aged 22-26 days) were gavaged with diluted caecal contents from adult (10-12 weeks) female NOR mice, according to previously published methods[50] with slight modifications. Briefly, the cecum of one adult microbiome NOR donor was dissected, opened along its length, the contents transferred to a sterile tube, and resuspended in 50 volumes of sterile water. 250 µL of this suspension was given to each recipient by oral gavage using a 24G round tip gavage needle. Recipients were rested for 24 hours, and this procedure was repeated once.

2.6 T1D assessment

Blood and/or urine glucose levels were measured in females biweekly. Animals were classified as diabetic when blood glucose exceeded 16mmol/L or urine glucose exceeded 250mg/dL. Statistical analysis on T1D life table data was performed using the Log-rank (Mantel-Cox) test, on Prism 5.0b (GraphPad Software, San Diego California USA)

2.7 Treatment protocol for Protecflor probiotics

A commercially available probiotic mixture consisting of Lactobacillus rhamnosus strain R0011, L. acidophilus strain R0052, Bifidobacterium longum R0175 and the yeast Saccharomyces cerevisiae boulardii (Protecflor) and equivalent microbe-free vehicle was provided by Institut Rosell-Lallemand. Weanling NOD females (aged 22-26 days) were gavaged with 1x10⁹ CFU of Protecflor probiotic suspended in water, or the equivalent weight of Protecflor vehicle according to previously published methods[50] with slight modifications. Briefly, 0.017 g of Protecflor probiotic or vehicle was suspended in 1 mL of distilled water. 250 µL of this suspension was
given to each recipient by oral gavage using a 24G round tip gavage needle. Recipients were rested for 24 hours, and this procedure was repeated once.

To maintain treatment of probiotic recipients post-gavage, Protecflor probiotic or vehicle was administered by suspension in drinking water. Briefly, 0.691g or 2 x 108 CFU/mL of probiotic formulation or vehicle was suspended in 200 mL of sterile drinking water and provided as the sole drinking water source for treated mice. Treated water was changed every 48 hours. Water consumption was measured and recorded to calculate an approximate per day CFU dose, and to ensure normal water consumption.

2.8 Insulitis assessment
Pancreata were dissected and immediately immersed in OCT media (Tissue-Tek, Torrance, CA), frozen in -20°C 2-methylbutane, and stored at –70°C. Preparation of frozen sections was performed with a Leica CM 3050 Cryostat (Leica Canada). To maximize analysis of independent islet infiltrates, three 5-µm sections were cut at least 400 µm apart. Pancreatic sections were stained with Mayer’s hematoxylin and eosin Y (H+E, Sigma) to visualize leukocyte infiltration. Assessment of insulitis severity in pancreatic sections was performed as previously described by our group [50][36][37]. Briefly, islets were graded according to the following criteria: 0, no visible infiltrates; 1, peri-insulitis as indicated by peri-vascular and peri-islet infiltrates; 2, <25% of the islet interior occluded by leukocytes displaying invasive infiltrates; 3, >25% but <50% of the islet interior invaded by leukocytes; or 4, invasive insulitis involving 50%-100% of the islet field. Statistical analysis on insulitis severity data was performed using the χ2 test, on Prism 5.0b (GraphPad Software, San Diego California USA.

2.9 Bacterial 16s rDNA PCR library construction
Bacterial profiles were analyzed by broad-range PCR of 16S rRNA genes and phylogenetic sequence analysis. DNA was extracted using the UltraClean fecal DNA kit (MoBio, Inc). Amplicons of the 16S rRNA gene were generated via broad-range PCR (30 cycles) using barcoded primers [77] modified with sequences required for either 454 pyrosequencing (~500 b.p. V1V3 region; primers 27FYM+3 and 515R) [78], or Illumina sequencing-by-synthesis (~250 bp V4 region; primers 538F and 806R) [79]. PCR yields were normalized using a SequalPrepTM kit (Invitrogen, Carlsbad, CA), pooled, lyophilized, and gel purified, as previously
described [80]. Pyrosequencing was performed at The Center for Applied Genomics at the Hospital for Sick Children on a 454/Roche Life Sciences GS-FLX instrument using Titanium chemistry (Roche Life Sciences, Indianapolis, IN). Illumina paired-end sequencing was performed at the University of Colorado, Denver by collaborator Dr. Dan Frank’s laboratory on a MiSeq sequencing platform using the 500-cycle MiSeq Reagent Kit version 2.

2.10 454 pyrosequencing of NOD and NOR microbiome samples

Pyrosequences were sorted into libraries by barcode and quality filtered using bartab[77]. All pyrosequences were screened for nucleotide quality (bases at 5’ and 3’ ends with mean Q <20 over a 10 n.t. window were discarded), ambiguous bases (sequences with >1 N were discarded), and minimum length (sequences <200 n.t. were discarded). Infernal [81] and ChimeraSlayer [82] were used to screen for bacterial 16S rRNA sequences as described previously[83]. Sequences were classified by parsimony insertion into the Silva108 guide tree after alignment with SINA using the ARB software package[84][85][86]. Sequences were then assigned the taxonomies of the clades into which they were inserted, using the ARB export node display setup (NDS) function. Operational taxonomic units (OTUs) were determined by clustering sequences with identical taxonomic assignments.

2.11 Illumina paired-end sequencing and analysis

Illumina Miseq paired-end sequences were sorted by sample via barcodes in the paired reads with a python script. The sorted paired reads were assembled using phrap[87][88][89]. Assembled sequence ends were trimmed over a moving window of 5 nucleotides until average quality met or exceeded 20. Trimmed sequences with more than 2 ambiguities or shorter than 200 nt were discarded. Potential chimeras identified with Uchime [90] using the Schloss Silva reference sequences [91] were removed from subsequent analyses. The remaining 4,578,881 sequences were aligned and classified with SINA (1.2.11)(47) using the 244,077 bacterial sequences in Silva 111NR (55) as reference configured to yield the Silva taxonomy. Sequences with >97% similarity (ie., genus level) were grouped into OTUs. Before analysis, OTUs representing less than 0.005% of all reads (228 reads) were discarded.

Ecological statistics (such as taxa observed, Good's Coverage estimator, Morisita-Horn index[92], Chao1 statistic[67], Shannon’s H statistic[69] and Wilcoxon signed-rank test),
Manhattan plots and heatmaps were prepared with the Explicet software package[93]. Chao1 statistic, Shannon’s H statistic indices were estimated through bootstrap resampling (1000 replicates) of the OTU distributions obtained from each specimen. Collector’s curves were prepared in Explicet. All ecological statistics were generated at a sample rarefaction of 40795 reads, representing the sample with the lowest reads sequenced. All 16S amplicon libraries were sequenced to >95% coverage. Individual microbial groups that differed in prevalence or abundance between treatment groups were identified by Wilcoxon rank-sum test. Because of the exploratory nature of this study, p-values were not corrected for multiple tests.

2.12 Multivariate data analysis

2.12.1 PCA

Multivariate data analysis (principal components analysis and clustering analysis) was performed using Ginkgo[94]. Principal components analysis was performed using Ginkgo [94]. Relative abundance data were standardized by centered-log transformation, then PCA performed on the resulting correlation matrix. The number of principal components to display as significant was determined by the “inclusive elbow” method in which the significance cutoff is set at the next component past the largest difference in variance explained. PCA displays were then generated.

2.12.2 Clustering Analysis

A K-medians/fuzzy C-medians clustering was performed using Ginkgo. K-medians/fuzzy C-medians clustering was performed with randomized seeding and 2-5 starting clusters. Clustering was evaluated by Dunn’s Coefficient; with 2 starting clusters yielding the best clustering (Dunn’s Coefficient = 0.86180). A single linkage hierarchical agglomerative method using Bray-Curtis dissimilarity matrix[95] was used independently used to corroborate the K-medians/fuzzy C-medians clustering.

2.12.3 Statistical Analysis

Spearman rank correlation tests were performed in Prism5.0b to evaluate associations between two variables. Fisher’s exact tests and χ² tests were performed in Prism 5.0b for comparisons of categorical data. A P-value of <0.05 was considered significant. False discovery rate corrections were not performed and P-values are uncorrected.
Chapter 3
The effect of NOR-derived microbiome transfer into NOD on microbiome composition and T1D incidence
3 The effect of NOR-derived microbiome transfer into NOD on microbiome composition and T1D incidence

3.1 Background and Rationale

Recent studies have shown that the intestinal microbiota and the innate immune system in NOD mice can influence the development of T1D. NOD mice genetically deficient in MyD88 were protected from T1D and 16S sequencing showed MyD88 deficiency resulted in altered gut microbiota[1]. Transfer of NOD.MyD88\(^{-/-}\)-derived gut microbiota into germ-free NOD mice protected against T1D in these mice[1]. Changes in the intestinal microbiota of mice have also been found to induce hormonal and metabolic changes leading to protection against T1D. Previous work in our lab by Markle et al. [2] showed that the transfer of male NOD-derived microbiota into weanling female NOD mice by oral gavage significantly protected them against T1D, and induced changes in testosterone levels and the metabolomics profile of treated mice.

These data suggest that changes in the gut microbiota of NOD mice may be correlated with protection, either by direct interaction with the immune system, or by modifying other physiological factors with known influence on T1D incidence. These and other data lead to the idea that microbial exposure may be an important environmental factor in auto-immune diseases in both mice and humans.

Previously in our lab, we performed 16S sequencing on female NOD and NOR mice (Figure 3-1). Our data shows there are quantifiable differences between the gut microbiota of NOD and NOR mice, most notably in the 5 taxonomical groupings: Bacteroidetes, Clostridia IV, Ruminococcus, Clostridia XIVa, and Helicobacter (Table 3-1). Given their shared environmental exposures under controlled, laboratory conditions, these differences suggest that there are genetic factors which influence the composition of the gut microbiota. These results, suggest that these differentially abundant groups in NOD and NOR mice may contribute to the protection of NOR mice from T1D, and that the transfer of these organisms to NOD mice may afford protection.

These preliminary data suggest that, similar to our observations in NOD males vs. females, the NOR female caecal microbiota may also transfer protection to NOD females. Therefore, we established a system to test whether transfer of caecal contents from NOR into NOD animals
provokes a change in the recipient’s microbiota and whether such changes were associated with a T1D-protective effect on recipient NOD mice.

3.2 Results

3.2.1 Design and Validation of Taxa-Specific qPCR

To assess the impact of these caecal microbe transfers, we developed an assay to assess alterations to the recipient microbiome. We expected the changes in the recipient microbiomes to include differentially abundant taxa we had detected in the NOD vs. NOR female 16S rDNA HTS analysis. Therefore, taxa-specific qPCR was chosen to quantify these bacterial groups as it allowed greater sample throughput, and was cheaper than high-throughput sequencing.

qPCR amplification assays were designed to provide specificity for the targeted taxa differentially abundant in NOD vs. NOR females. Related bacterial species display sequence similarity in some regions of the 16S rRNA gene[3]. We selected 5 bacterial taxa (Bacteroidetes, Clostridia IV, Ruminococcus, Clostridia XIVa, and Helicobacter) based on their differential abundance in NOD and NOR mice (Table 3-1). We designed primers by aligning of the 16S rRNA gene from sequenced members of the target taxa (Table 3-1) as assigned in the RDP database[4], then locating regions of the 16S rRNA sequences that were conserved only within targeted taxa using PRIMROSE[5]. These taxa-specific qPCR primers were then tested in silico by performing Probe Match searches using the RDP web interface against RDP 16S rDNA database entries [4]. Primers which matched sequences in RDP from non-target taxa were redesigned.

Once a set of primers which passed in silico testing were found, we used these primers to produce amplicons from NOD and NOR caecal extract DNA by PCR. We then cloned and separately sequenced 20 amplicons by Sanger sequencing and identified the amplified sequences BLASTing against available reference bacterial genome sequences. Any primers which produced amplicons identified as members of a non-target taxa were discarded and redesigned. The final primer sets, which passed both stages of specificity testing are shown in Table 3-2.

The next requirement for the qPCR assay was to ensure consistent quantification and normalization between assay runs. The selected primers were used in qPCR reactions with NOD caecum-derived bacterial DNA as template. Serial dilutions of NOD caecal flush-derived DNA
were quantified by qPCR using each of the primer sets. The copy number of each template type, determined by a standard curve, was plotted against the dilution of the template DNA (Figure 3-2a). All primer sets showed linearity between 25ng/µL and 25pg/µL of template DNA (R2 > 0.99). To test their reproducibility, these assays were then repeated over 3 separate days, and found to display no significant day-effects (p=0.48). These results demonstrated that the qPCR assays provided consistent quantification over multiple experimental replications. PCR amplicon copy numbers, determined by a standard curves, were plotted against average threshold cycle (Ct) values to compare amplification efficiencies (Figure 3-2b). The amplification efficiency in qPCR assays for each of the 5 taxa varied by ≤5%. The consistency in amplification efficiencies suggested that quantification between assays would be comparable without inter-assay normalization.

3.2.2 Comparison of Abundance of Caecal Bacterial Taxa in NOD Mice from Weaning to Adulthood

The gut microbiome is in a dynamic state of flux between weaning and adulthood in both mice and humans[6][7]. In humans, multiple successions of dominant microbial groups occur until the gut microbiome stabilizes at ~1-2 years of age [8]. Schloss et al. found that in C57BL/6 mice, the microbiome was found to from a less stable state 10 days post-weaning to a more stable state 15 days post-weaning[9]. We reasoned that developmental changes in the mouse microbiome would provide a source of variation to test our taxa-specific qPCR assays.

To test if the taxa-specific qPCR assay panel could detect changes in the microbiome from weaning to adulthood, DNA from caecal contents from female NOD mice ages 24-55d was extracted and assayed by qPCR (Figure 3-3). We were able to detect fluctuations in the qPCR profiles of the gut microbiome across this age range for the 5 target taxa. This result suggested that the taxa-specific qPCR assays had the dynamic range and sensitivity to detect the variation in the gut microbiome of mice, as represented by the changes between weaning and adulthood. qPCR profiles of the gut microbiome showed the most fluctuation between 20-42 days of age, but changes became less pronounced after 42 days. This observation suggests that by 42 days, the gut microbiome begins to stabilize and resemble the meta-stable adult state[7].
3.2.3 Comparison of abundance of Caecal Bacterial Taxa in NOD and NOR Mice

Previous 16S sequencing experiments had identified differentially abundant bacterial taxa in the caecum of NOD and NOR mice (Figure 3-1). We hypothesized that these taxonomic groups might contribute to the T1D protection observed in NOR compared to NOD mice. Moreover, we proposed that the transfer of caecal contents from female NOR into female NOD animals (NOR→NOD) might impart a protective effect on the recipients. We expected that changes in the microbiome of NOR→NOD mice would include those differentially abundant taxa detected in of 16S rRNA HTS study (Figure 3-1 and Table 3-1). Therefore we used the qPCR assays to quantify these differentially abundant taxa in DNA extracted from caecal samples from unmanipulated NOD and NOR mice (Figure 3-4).

We observed differences between NOD and NOR in the Bacteroidetes, Clostridia IV, and Helicobacter groups (p = 0.0015, 0.0019, 0.0288, respectively) (Figure 3-4a). We observed a difference trending towards significance in the Ruminococcus group (p = 0.0606) (Figure 3-4a). The relative abundance of these taxa as quantified by qPCR is consistent with the results from the 16S sequencing experiment, although exact proportional difference between mouse strains for each taxa differed between the two types of analysis platforms (Table 3-3). These results suggested that taxa-specific qPCR could reliably differentiate between NOD and NOR mouse gut microbiome samples.

We also observed some intra-mouse strain variation of bacterial taxa, for example in the Clostridia IV and Ruminococcus groups (Figure 3-4b). We hypothesized that this variation might reflect cage to cage variation in housing environment resulting in intra-strain variation in the microbiome. To examine this potential source of variation, we segregated the qPCR results by cage for these two taxa (Figure 3-4b). We observed the no clear clustering of cage/littermates into groups of similar relative abundance. We observed that relative abundance of taxa was actually highly variable within cages. Overall, these data suggests that residence in different cages did not explain the intra-genotype variation detected in these taxonomic groups.

We noted that the variance in abundance in NOD and NOR samples seemed to differ in some of the 5 taxa quantified. We hypothesized that this variation might reflect differences in intra-strain homogeneity in the gut microbiome. To examine these potential differences in variance, we log-
transformed all the data then performed a Brown–Forsythe test[10][11] to compare the sample variance in each taxa between NOD and NOR samples. We found that the variance of abundance in Clostridia IV, Helicobacter, and Ruminococcus was higher between NOR animals compared to NOD (p = 0.00077, 0.0045, 0.0063 respectively). These results suggest that there is greater intra-strain differences in the NOR gut microbiome compared to NOD.

3.2.4 Effects of NOR-derived Caecal microbiota on NOD recipients

Prior experiments in our lab had shown that the transfer of caecal contents derived from adult male into 21 day old female NOD mice altered the microbiome of the recipients and induced protection against T1D[2]. Gavage transfer-induced changes in the recipient’s microbiome were found to persist 100 days after gavage treatment. These results suggested that the transfer of caecal contents derived from an adult donor mouse could alter the microbiome of the recipients.

We hypothesized that we would be able to detect persistent changes in female NOD recipients of female NOR caecal contents using taxa-specific qPCR. The qPCR assay panel was used to quantify selected taxa in DNA extracted from caecal samples from 60 day old un-manipulated female NOD or NOR mice, and from 60 day old NOD females gavaged at weaning with adult NOR female microbiota (Figure 3-5). These data showed differences between untreated NOD and NOR→NOD mice in the Clostridia IV and Clostridia XIVa groups (p = 0.008 for both groups) (Figure 3-5), suggesting that the gavage treatment produced changes in the recipient gut microbiome detectable 40 days post-gavage, at 60 days of age. We also observed differences between NOR and NOR-gavaged NOD mice in the Clostridia IV, Clostridia XIVa, and Helicobacter groups (p = 0.0079, 0.0119, 0.0424 respectively). These data suggested that female NOR→NOD caecal gavage altered the recipient’s microbiome and that these alterations persisted for at least 40 days post-gavage.

Both the 16S rDNA HTS (Figure 3-1) and the qPCR assays documented differences in the composition of the sex and age-matched NOD and NOR microbiota. We hypothesized that altering microbiome composition by oral gavage of the T1D-resistant NOR in T1D-susceptible NOD recipients might impact the incidence of T1D in the recipient NOD mice. To test this hypothesis, a cohort (n=30) of weanling (21d) female NOD mice were gavaged with diluted material from the caecum of adult female NOR mice as we described for the NOD male →female transfers[2]. The NOD recipient mice were then monitored for the development of T1D
We observed no difference in the average age of onset, or incidence of T1D in the NOR→NOD vs. unmanipulated NOD females survival from T1D. This observation suggests that, although the oral gavage introduction of NOR females microbiota caused detectable alterations in the microbiome of the recipient NOD mice, these alterations did not have a detectable effect on the development of T1D, in stark contrast to the female NOD recipients of male microbiome transfer previously reported[2].

3.3 Summary

We present this set of experiments as our effort to validate qPCR as viable method for the detection and quantification of members of specific bacterial taxa, and the application of such an assay panel to probe the effect of the microbiome on T1D development in the NOD mouse model.

By in silico and in vitro methods, we designed and validated the specificity of a set of taxa-specific qPCR primers targeted to the quantification of 5 bacterial taxa found previously to be differentially abundance between NOD and NOR mice. We applied our panel of assays to two experimental settings to provide a proof of concept. We first applied our panel of assays to tracking changes in the composition of the NOD gut microbiome from weaning to adulthood. We found that changes in the gut microbiome from weaning to adulthood could be detected and quantified by taxa-specific qPCR, and a major shift in the microbiome composition was observed between 25d and 41d female NOD mice. Secondly, we applied the qPCR panel to quantifying bacterial taxa in unmanipulated NOD and NOR mice. We observed differences in the microbiome composition of NOD and NOR mice which corroborated previous results in our lab. Bacteroidetes and Helicobacter were enriched in NOR animals and Clostridia IV, and Ruminococcus were enriched in NOD animals.

Having shown that our panel of qPCR assays could quantify the targeted bacterial taxa in NOD and NOR mice, and that these results were in agreement with previous sequencing results, we then applied the qPCR panel to quantifying bacterial taxa in unmanipulated NOD, NOD recipients of NOR caecal material by gavage (NOR-gavaged NOD), and NOR mice. We found significant differences between untreated NOD, NOR-gavaged NOD mice, and NOR mice. These differences suggest that the gavage treatment altered microbiomes of treated NOD mice,
and that the resulting microbiome of recipient mice is not identical to that of the donor, nor is it clearly partially composed of NOD and NOR contributions.

To test the effect of transferring NOR-derived microbiota into recipient weanling female NOD mice on T1D, we assessed T1D incidence in NOD mice treated with NOR-derived microbiota by gavage. We found that NOR-derived microbiota treatment was not protective against T1D.
Figure 3-1: 16S rDNA sequencing of female NOD and NOR caecal flush microbiome. 16S rDNA PCR libraries were generated from caecal flush extracted from 84 day female NOD and NOR mice. Libraries were pyrosequenced and the ratio NOD/NOR for each OTU count was calculated. These data were plotted as bars mapped onto a taxonomical tree to define phylogenetic relationships between OTUs. The dotted line indicates a ratio of 1. Graphic and data analysis provided by Dan Frank.
Figure 3-2: Quality analysis and validation for bacterial taxa-specific qPCR assays. (A): Evaluating linearity and technical consistency of qPCR assays over serial dilutions. DNA was extracted from samples of caecal contents from adult female NOD and NOR mice. DNA concentration was determined by Nanodrop (Thermo Fisher Scientific Inc), and a serial dilution (1 - 1/10000) 50% NOD-NOR admixture template DNA was prepared. Taxa-specific 16S qPCR primers (see Table 3-2) were used to amplify partial 16S sequences for quantification by SYBRGreen. Raw data were then normalized using a parallel reaction quantifying total bacteria, and repeated using the same template on three separate days. Results from 3 days are plotted adjacent. (B): Evaluating efficiency of qPCR assays over serial dilutions. Template dilution was plotted against $C_{threshold}$ to determine amplification efficiency by slope. Template DNA serial dilutions and qPCR was performed as in (A). Points on the graphs represent the average of three technical replicates. Lines represent lines of best fit for each primer set.
Figure 3-3: qPCR quantification of selected bacterial taxa in female NOD mice, ages 25-55. Female NOD mice were aged to 25, 33, 41 and 55d. qPCR assays were performed as described above. Points on the graph represent the average of three biological replicates, each biological replicate represents an average of three technical replicates.
Figure 3-4: qPCR quantification of selected bacterial taxa in female adult NOD and NOR mice. 

(A): Comparison of selected bacterial taxa in NOD and NOR. Caecal DNA from adult female NOD and NOR mice was evaluated by taxa-specific qPCR primers and quantified by SYBRGreen. Resulting taxa-specific bacterial counts were normalized to qPCR reactions quantifying total bacterial counts. Filled circles indicate NOD samples, empty circles indicate NOR samples. The Mann-Whitney U test was used to calculate the P-values presented between NOD and NOR samples. (B): Segregation of NOD and NOR data from (A) by cage in Clostridia IV and Ruminococcus. Each cage is represented by a color; symbols of the same color within a strain represent cagemates; data shown are the same as that displayed in (A). Points on the graph represent the average of three technical replicates; the data presented represent results from 18 mice.
Figure 3-5: qPCR quantification of selected bacterial taxa in female adult NOD, NOR-gavaged NOD and NOR mice. Female NOD weanlings were gavaged with caecal bacteria from adult NOR females and aged to 60 days. qPCR assays were performed as before. Points on the graph represent the average of three technical replicates; the data presented represent results from 5 mice per genotype and condition of treatment. Filled circles indicate NOD samples, empty NOR samples, and half-filled indicate NOD treated by NOR-gavage. The Mann-Whitney U test was used to calculate the P-values presented between the comparisons indicated.
Figure 3-6: T1D incidence in female NOD pup recipients with female NOR-derived intestinal microbiome. Female NOD weanlings were gavaged with caecal bacteria from adult NOR females. T1D was assessed in NOR→NOD female recipients (grey line, n = 30) and compared to historical unmanipulated NOD females (black line, n = 59). T1D survival curves were compared by Log-rank test (p = 0.2541).
<table>
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<th>Sequence Abundance</th>
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<td>31.25%</td>
</tr>
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</table>

Table 3-1: Taxonomic groups/OTUs with differential abundance selected from NOD vs. NOR 16S HTS data
<table>
<thead>
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<th>Rev. Primer</th>
<th>Amplicon Length</th>
</tr>
</thead>
<tbody>
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<td>CCACTGTGGGGGACCTTC</td>
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</tr>
<tr>
<td>(Phylum)</td>
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<td>C</td>
<td></td>
</tr>
<tr>
<td><em>Clostridia IV</em></td>
<td>TTACACAATAAGTWATC</td>
<td>ACCTTCCTCCGTGGTTGTC</td>
<td>314</td>
</tr>
<tr>
<td>(“Family”)</td>
<td>CACCTGG</td>
<td>AAC</td>
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<tr>
<td><em>Clostridia XIVa</em></td>
<td>CGGTACCTGACTAAGAAGC</td>
<td>AGTTTYATTCTTGCGAAGCG</td>
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</tr>
<tr>
<td>(“Family”)</td>
<td>GC</td>
<td>G</td>
<td></td>
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<tr>
<td><em>Helicobacter</em></td>
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<td>CGGAGTTAGCCGGTGCTTATT</td>
<td>220</td>
</tr>
<tr>
<td>(Genus)</td>
<td>ATC</td>
<td>TATT</td>
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</tr>
<tr>
<td><em>Ruminococcus</em></td>
<td>GGCGGCYTRCTGGGCTT</td>
<td>CCAGGTGGATWACTTATTGTGTAA</td>
<td>157</td>
</tr>
<tr>
<td>(Genus)</td>
<td>T</td>
<td>TAA</td>
<td></td>
</tr>
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</table>

Table 3-2: Bacterial 16S taxa specific qPCR primers. Bacterial taxa were selected based on their differential abundance in NOD and NOR mice (Figure 3-1)

Figure 3-1: 16S rDNA sequencing of female NOD and NOR caecal flush microbiome. 16S rDNA PCR libraries were generated from caecal flush extracted from 84 day female NOD and NOR mice. Libraries were pyrosequenced and the ratio NOD/NOR for each OTU count was calculated. These data were plotted as bars mapped onto a taxonomical tree to define phylogenetic relationships between OTUs. The dotted line indicates a ratio of 1. Graphic and data analysis provided by Dan Frank.

16S rRNA genes from sequenced members of the target taxa from the RDP database were aligned conserved regions were found using PRIMROSE[73]. These taxa-specific qPCR primers were then tested in silico by performing Probe Match searches and in vitro by producing and sequencing amplicons from NOD and NOR caecal extract DNA.
<table>
<thead>
<tr>
<th>Taxonomy</th>
<th>NOD/NOR ratio</th>
</tr>
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<tbody>
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<td>Pyrosequencing</td>
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<td><em>Bacteroidetes</em> (Phylum)</td>
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</tr>
<tr>
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</tr>
<tr>
<td><em>Helicobacter</em> (Genus)</td>
<td>0.516</td>
</tr>
</tbody>
</table>

Table 3-3: qPCR quantification of bacterial taxa. The abundance of each taxa in NOD and NOR samples was extracted and the ratio between the abundance in NOD and NOR was calculated.
Chapter 4
The effect of Protecflor probiotic treatment on NOD microbiome composition and insulitis severity
4 The effect of Protecflor probiotic treatment on NOD microbiome composition and insulitis severity

4.1 Background and Rationale

T1D and gut inflammatory disorders have been found to share genetic risk factors, and can occur as co-morbidities. For example, ZNF365, MST1, ERBB3, and PTPN22 have been identified as a common susceptibility genes in Crohn’s disease and T1D[17]. T1D and celiac disease share susceptibility alleles of HLA-DQB1, IL2, RGS1, TAGAP and IL18RAP[18]. Moreover, T1D patients display a higher risk of developing celiac disease compared to the general population[96]. Anti-tissue transglutaminase antibodies, a marker for celiac disease, are present in ~10% of children and ~2% of adults with T1D[19]. These comorbidities suggest shared genetic and environmental factors in T1D and autoimmune diseases of the gut.

Studies in ulcerative colitis, inflammatory bowel syndrome and T1D have suggested that probiotic bacteria can have protective effects in these diseases. For example, Lactobacillus helveticus R0052 and Lactobacillus rhamnosus R0011 have been shown to reduce clinical symptoms in open label experiments on human adults with inflammatory bowel syndrome[61], and to reduce epithelial injury due to Escherichia coli O157:H7- and O127:H6- infection in an in vitro gut epithelial cell co-culture system [70]. Bifidobacterium longum R0175, given in combination with inulin, a plant polysaccharide, was found to improve self-reported quality of life, symptoms of blood and mucous in stool, and to reduce relapse rate in a small pediatric study of ulcerative colitis (UC) [71]. Oral administration of probiotic bacterial strains was reported to protect NOD mice from T1D. The probiotic mixture VSL#3 (Bifidobacterium, Lactobacillus species and Streptococcus salivarius subsp. Thermophiles) was given by oral gavage three times a week from 4 to 32 weeks of age, reduced T1D incidence in NOD mice [49]. However, repeated gavages performed in this study increase the risk of systemic exposure of microbes to the immune system, by abrasion of the esophageal wall, or aspiration of the gavage material. It is well documented by multiple studies that bacterial infection or systemic exposure is protective against T1D in NOD mice. Studies showing deliberate infection with multiple organisms, including Schistosoma mansoni[42], Mycobacterium avium[43], Trichinella spiralis[44], Heligmosomoides polygyrus[45] and Salmonella typhimurium[46], can induce protection in NOD mice. Interperitonial injection of zymosan, a fungal cell wall component, has also been
found to be protective in the NOD model[47]. Another study also suggested that treatment with probiotic strains of *Lactobacillus* and *Bifidobacterium* can be protective in T1D, with the caveat that systemic immune exposure of the mice may have been a confounding factor in their protocol [49].

Collectively, these studies suggest that long-term treatment with probiotic strains of *Lactobacillus* and *Bifidobacterium* may induce alterations in the gut microbiome and/or produce anti-inflammatory effects through other mechanisms. We hypothesized that continuous treatment with Protecflor probiotic, a formulation containing *Lactobacillus helveticus* R0052, *Lactobacillus rhamnosus* R0011, and *Bifidobacterium longum* R0175 and the yeast *Saccharomyces cerevisiae boulardii*, would impact the gut microbial composition of diabetes-prone NOD mice, and consequently, have an effect on the progression of islet autoimmunity. Therefore, we established a system to test whether the treatment of NOD animals with Protecflor altered the recipient’s gut microbiota and whether such changes were associated with an islet autoimmunity-protective effect on treated NOD mice. To address concerns of systemic immune exposure to protective organisms, we designed a treatment protocol to minimize gavage procedures, and maintained continuous Protecflor probiotic dosing by addition to drinking water.

### 4.2 Results

#### 4.2.1 Detection of Protecflor Probiotic organisms by qPCR

Previous studies of the Protecflor probiotic strains have shown that *Lactobacillus helveticus* R0052 was adherent to *in vitro* gut epithelial cell cultures[97], but there was no evidence that Protecflor probiotic strains could colonize humans or mice. The related organism, *Lactobacillus casei* subsp. *rhamnosus* Lcr35, ceased to be found in the gut or feces of mice orally fed these organisms within 3 days after cessation of treatment [98]. *Saccharomyces cerevisiae boulardii* did not colonize or adhere to gut epithelial cell cultures [99]. To test for the retention of Protecflor organisms during our NOD mouse treatment protocol, we developed a qPCR assay to quantify the Protecflor organisms. qPCR primers were designed to the *Bifidobacterium longum* R0175 *rmlB* gene and an extrachromosomal plasmid pIR52 in *Lactobacillus helveticus* R0052. These primers (Table 4-1) were highly specific for the respective type strains, and were selected using the full genome sequence of each strain conveyed to us by our collaborators at Lallemand.
To evaluate consistency of quantification between qPCR runs, the primers were first used to derive a set of standards with Protecflor-derived bacterial DNA as the template. Serial dilutions of Protecflor-derived DNA were quantified by qPCR using each of the primer sets. The copy number of each template type was determined by a standard curve, and plotted against the dilution of the template DNA (Figure 4-1). Both primer sets showed linearity between 25ng and 25pg of template DNA ($R^2 > 0.99$). To evaluate reproducibility, the assays were repeated on 3 separate days, and found to display no significant day-effects (p=0.53). These results demonstrated that the two qPCR assays reliably provided consistent quantification over multiple experimental replications.

To test the ability of these primers to detect Protecflor strains in the feces of mice, we adopted a treatment protocol developed by Philip Sherman’s group for the treatment of rats with probiotics in drinking water[100] to treatment of NOD mice. We recently demonstrated that the gut microbiome of weanling age mice could be altered by oral gavage with complex commensal microbes[50]. Therefore weanling (21d old) female NOD mice were gavaged twice with $1 \times 10^9$ CFU Protecflor probiotic separated by a 24h period. The mice were then given free access to drinking water containing $2 \times 10^8$ CFU/mL of Protecflor over 15 weeks. Water consumption was monitored in treated cages to estimate dosing. To quantify the exposure of treated mice to the Protecflor organisms, *Bifidobacterium longum* R0175 and *Lactobacillus helveticus* R0052 were quantified using strain-specific qPCR on DNA extracted from fecal samples that were collected weekly from each cage. Our standard curve quantifying Protecflor-derived template detected product at 25pg/µL template DNA (Figure 4-1) corresponding to a lower detection limit of ~5000 CFU of Protecflor microbes/g fecal material. Despite this assay sensitivity, we failed detected either strain in feces, suggesting that if these organisms were present in the feces, their concentration was below this limit. One possible explanation for these finding was rapid gastrointestinal transit and clearance of these non-colonizing organisms.

4.2.2 Gastrointestinal Transit times of Protecflor Probiotic organisms in the NOD model

There is no reported evidence that Protecflor probiotic strains can colonize the human or mouse intestinal tract. *Saccharomyces cerevisiae boulardii* were found to fall below detectable levels in 2-5 d in humans treated with a single 1g oral dose[99]. *Lactobacillus helveticus* R0052 to be adherent to *in vitro* gut epithelial cell cultures, but there was no *in vivo* evidence for colonization.
in this study[97]. One explanation for these findings was rapid gastrointestinal transit and clearance of these non-colonizing organisms. In mice, a wide range of gastrointestinal transit times in mice have been reported, depending on the methodology and mouse strain. GI transit times of 154 ± 24 minutes in BL/6 mice were observed using a contrast MRI-based measurement method[101]. In contrast, a GI transit time of 6h was reported in 129SvEv mice using a radio-labeled charcoal pellet[102]. One study in NOD mice observed that a bolus consisting of 10% charcoal in 5% Arabic gum aqueous suspension had transited 60% of the small intestine 20 minutes after feeding.

To establish the gastrointestinal transit time for our studies, NOD mice were gavaged with Protecflor probiotic and rested for 1, 2, 3 or 4 hours. At each time point, cohorts of mice were sacrificed and the GI tract removed and the contents of these organs were recovered. The GI tract of each animal was divided into stomach, small intestine, and large intestine + caecum, and their contents recovered by lavage with sterile saline. Bacterial DNA was extracted from each lavage sample and amplified by qPCR using R0052 and R0175 strain-specific primers (Figure 4-2). DNA from the Protecflor organisms was evident in each GI tract section obtained from mice sacrificed 1, 2, and 3 hours after gavage, but was no longer detectable 4 hours post-gavage. Protecflor organism DNA copy number declined in all GI tract sections over the 4 hour time course. (Figure 4-2Error! Reference source not found.) These observations suggested that these probiotic organisms transit rapidly through the GI tract and were below the limit of detection for the qPCR assay within 4 hours after oral introduction.

### 4.2.3 Evaluating the Effects of Protecflor Probiotic on Insulitis Severity in NOD model

Oral administration of probiotic strains can be protective in a number of GI inflammatory disorders[61][71], and studies have shown genetic and epidemiological data connecting T1D and GI inflammatory disorders [17][18] [96] [19] (See Background and Rationale). Given this evidence, we hypothesized that the exposure to Protecflor probiotic would have an effect on the anti-islet autoimmunity in NOD mice and evaluated islet inflammation severity (insulitis), a T1D phenotype, as a primary endpoint in a longitudinal treatment study. To test this hypothesis, weanling (21d) female NOD mice (n=8 each treatment group) were gavaged twice, 24 hours apart, with 1x10⁹ CFU Protecflor probiotic or a vehicle (the carbohydrate-rich vehicle in which Protecflor is prepared). The mice were then given free access to drinking water containing with 2
x $10^8$ CFU/mL probiotic or an equivalent concentration of vehicle material for an additional 12 weeks. At 15 weeks of age, pancreata were harvested from Protecflor and vehicle treated mice, fixed, sectioned and stained for histological evaluation. Quantitative insulitis assessment was done double-blinded using a well-defined scoring method [36][37][50] (Figure 4-3a). The distribution of insulitis scores was markedly distinct in Protecflor and vehicle-treated mice (Chi-squared test, Bonferroni-corrected $\alpha = 0.0071$, $p < 0.0001$). These data suggested that Protecflor was protective against insulitis in the NOD model. Insulitis in vehicle-treated animals did not differ from age and sex-matched untreated historical controls from our mouse colony (Figure 4-3a) (Chi-squared test, Bonferroni-corrected $\alpha = 0.0071$, $p = 0.035$).

The impact of Protecflor was also evident when insulitis severity scores were segregated into two groups: healthy/peri-insulitis (scores 0-1) and infiltrating insulitis (scores 2-4) (Figure 4-3b; Chi-squared test, $p < 0.0001$). This observation suggested that exposure to the Protecflor probiotic organisms beginning in early life is protective against immune infiltration of the islets, an obligate step in the progression toward T1D in the NOD model.

### 4.2.4 The Effects of Protecflor Probiotic treatment on the gut microbiome in NOD mice

Animal studies in both agricultural and laboratory settings have shown that treatments with exogenous microbes can be associated with changes in the microbiome. Administration of a probiotic containing *lactobacilli*, *bifidobacteria*, *enterococci*, and *pediococci* improved weight gain in broiler chickens, which was associated with an increase in *Bifidobacterium* spp., *Lactobacilli*, and Gram-positive cocci[103]. An increase in the diversity of gut lactobacilli was observed in fecal as well as intestinal tissue samples in mice treated with *L. casei* and *L. plantarum*. Our lab reported that weanling female NOD mice gavaged with caecal contents from adult male NOD mice were protected from T1D, and that this protection was associated changes in testosterone levels provoked by changes in the microbiome[50]. In humans, studies correlating effects of microbial treatment with changes in the gut microbiome composition are currently few in number. Kajander et al reported treatment with a multispecies probiotic supplement (*Lactobacillus rhamnosus* GG, *L. rhamnosus* Lc705, *Propionibacterium freudenreichii* ssp. *shermanii* JS and *Bifidobacterium animalis* ssp. *lactis* Bb12) was found to improve clinical scores of treated IBS patients, and was also associated with stabilization of the gut microbiome composition[104]. We hypothesized that the Protecflor organisms induced a change in gut
microbiome of NOD mice, and that these alterations were linked to the insulitis protection we observed.

To characterize potential changes in the microbiome resulting from Proteclor treatment, we repeated the oral gavage followed by drinking water exposure protocol in cohorts of NOD females. DNA was extracted from caecal flush samples from Proteclor- and vehicle-treated mice at 14 weeks of age, and fecal samples from Proteclor-treated and vehicle-treated cages was prepared at 4, 6, 10, and 14 weeks of age. 16S rDNA libraries were prepared used bar coded primers and sequenced on the Illumina MiSeq platform as previously published [50][50][105]. OTUs (operational taxonomical units) were called for the sequences using RDP Classifier[75]. The number of sequence reads per sample is shown in Table 4-2.

4.2.4.1 Differential abundance of bacterial taxa in Proteclor and vehicle treated NOD mice

To determine if the 16S rDNA sequencing provided sufficiently deep coverage, a Good’s Coverage statistic was calculated for the OTUs from each sample. These data were plotted as a collector’s curve for each library (Figure 4-4). Good’s coverage approaching 100% suggests that the sequences were representative samples of the populations they represented, and that additional sequencing was unlikely to uncover new OTUs. For each 16S library, the sequencing was found to provide ≥99.97% coverage of the actual sample.

The samples were segregated based upon biogeography (fecal or caecal flush), and the top 20 most abundant taxa for each sample were plotted as stacked bars (Figure 4-5). Fecal samples were dominated by Bacteroidales S24-7, division TM-7 and Lachnospiraceae, while caecal samples were dominated by Bacteroidales S24-7, Lachnospiraceae, and Bacteroides. In this display, we found no obvious relationship between Proteclor and vehicle-treated mice. The similar taxonomic diversity between Proteclor and vehicle-treated groups suggested that the probiotics did not cause gross alterations in the microbial biodiversity.

To determine whether specific OTUs were altered in abundance by Proteclor treatment, the Wilcoxon signed-rank test was used and –Log(p-value) was plotted for each OTU using a Manhattan plot. OTUs with –Log(pValue) above ~1.30, corresponded to a p-value < 0.05 and were categorized as differentially abundant between Proteclor and vehicle treated samples. We
observed enrichment of the genera *Allobaculum*, *Candidatus-Arthromitus*, *Roseburia*, and the order *Clostridiales* in caecal flush and fecal samples from Protecflor-treated animals compared to controls, and depletion of the genera *Helicobacter*, *Parabacteroides* and the order *Bacteroidales*, in Protecflor-treated vs. controls (Figure 4-6). Despite sustained treatment with Protecflor *Lactobacillus*, *Bifidobacterium*, and *Saccharomyces* species, we did not observe differential abundance of any of these taxa supporting our prior evidence that the Protecflor organisms did not colonize and transited rapidly through the mouse GI tract. However, Protecflor treatment was associated with observable changes in the microbiome composition of treated mice.

4.2.4.2 β-diversity analysis of 16S rDNA sequencing data

To compare the overall community structure of the microbiomes found in both Protecflor and vehicle-treated mice, the Morisita-Horn distance was calculated between caecal samples, and plotted as a heatmap. Visual comparison of the combined heatmap values was then used to segregate all samples into similar clusters (Figure 4-7). We observed 2 clusters within the set of samples. Clustering occurred independent of treatment type.

4.2.4.3 Principal components analysis of 16S rDNA sequencing data

To further analyze the relationship between the microbiome composition in Protecflor and vehicle-treated mice and insulitis severity, we performed PCA (principal components analysis) on the sequencing dataset. PCA is a multivariate analysis technique which reduces the number of dimensions/variables in higher dimensional data. This is done by finding a set of orthogonal linear combinations of the original variables, called principal components, which account for the greatest variance in a dataset. This can potentially reveal hidden structure in the data. We hypothesized that differences in the gut microbiomes of Protecflor and vehicle-treated mice would be represented by these principal components, and these could be correlated to insulitis severity.

Using Ginkgo, a multivariate statistical analysis software suite [94], we performed principal components analysis (PCA) on the sequencing data from caecal flush from both Protecflor and vehicle-treated mice. After inspection of scree plots for the PCA (Figure 4-8), we used the first two principal components (PC) that together explained 53.47% of the total variance (Figure 4-9). The object loadings for PC1 were ordered from least to greatest (Table 4-3). We found the
positive loadings were populated with Order Clostridales, including the families \textit{Ruminococcaceae} and \textit{Lachnospiraceae}. The negative loadings were populated with Phylum \textit{Proteobacteria} and Order \textit{Bacteroidales}. The object loadings for PC2 were ordered from least to greatest (Table 4-4). We found the positive loadings were populated with family \textit{Erysipelotrichaceae}, Order Clostridales, specifically from the \textit{Lachnospiraceae} family, and Order Bacteroidales, including the \textit{Rikenellaceae} and \textit{Porphyromonadaceae} families. The negative loadings were populated with phylum Cyanobacteria, and Order Clostridales, including the \textit{Veillonellaceae} and \textit{Lachnospiraceae} families, and Candidatus-Arthromitus (SFB).

We noticed clustering of the caecal samples into 2 distinct groups along PC1 (Figure 4-9). To examine this possible relationship using independent statistical models, we performed unsupervised clustering analysis then mapped the clusters on to the PCA components plot (Figure 4-10). We found 2 clusters: Types 1, 2 that were primarily differentiated by PC1. Samples from Protecflor-treated animals were not more closely related by PC1 or PC2 than were vehicle-treated animals, even within the same cluster. These results suggested that the cluster segregation was not related to Protecflor treatment, so we examined other potential correlations.

4.2.4.4 Correlation of principal components analysis with insulitis

Given the relationship between Protecflor and protection from insulitis in our previous results (Figure 4-3), we examined the relationship between change in the microbiome composition and insulitis severity. We performed PCA on the sequencing data from all caecal flush samples, segregated into Protecflor and vehicle-treated groups, then plotted PC1 or PC2 against overall insulitis scores for each mouse. In Protecflor-treated mice, we found positive loading in PC1 was correlated with protection against insulitis ($r^2 = 0.7408$) (Figure 4-11a), while PC2 was not related to insulitis ($r^2 = 0.00986$). These results suggested that Protecflor treatment may alter microbiome composition and that this alteration is associated with protection against autoimmune insulitis. In vehicle-treated mice, positive loading in PC2 was correlated with protection against insulitis ($r^2 = 0.7965$) (Figure 4-11b), while PC1 was not related to insulitis ($r^2 = 0.00181$). Taken together, these data suggested that the microbiome changes associated with lower insulitis progression in vehicle-treated mice were distinct from those associated with insulitis protection in Protecflor treated animals.
4.2.4.5 Correlation of caecal bacterial community richness with principal components analysis and insulitis

Inflammatory phenotypes in GI inflammatory diseases are associated with dysbiosis characterized by altered microbiome composition and lower gut microbial species richness[106][107][108]. The Chao1 statistic is a measure of sample richness [67] which estimates the true total number of OTUs in a sample based on the number of singletons (OTUs observed once in the sample), and doubletons (OTUs observed twice in the sample) observed [67]. To compare the richness of samples and correlated these measures with microbiome composition, we calculated the Chao1 statistic for the samples, then plotted them against PC1 and PC2 (Figure 4-12a). We found that positive PC1 loading was correlated with overall greater community richness. The linear regression of the Chao1 statistic had an $r^2$ value of 0.6687. An F Test performed on the linear regression showed that slope is non-zero ($p = 0.0012$). In contrast, PC2 was not correlated with species richness (Figure 4-12a). An F Test performed on the linear regression shows that the slope does not differ from zero ($p = 0.9021$). Comparing the two clusters differentiated by PC1, Type 1 samples are overall less rich than Type 2 (Mann Whitney test, $p = 0.0025$), with a median Chao1 statistic of 34.15 compared to 51.26 for Type 2 (Figure 4-12b). These results suggest that Type 2 samples represent a more diverse “enterotype” then type 1 samples.

We then asked whether the difference in community richness was correlated with insulitis severity. Samples were segregated into Protecflor and vehicle-treated groups, a Chao1 statistic for community richness[31] was computed for the samples in each group, and plotted against the average insulitis score for each mouse (Figure 4-13). In Protecflor-treated mice, the Chao1 statistic correlated with protection against insulitis (Figure 4-13; $r^2$ value of 0.8988). An F Test performed on the linear regression showed that the slope was non-zero ($p = 0.0040$). In contrast, the Chao1 statistics were not related to insulitis severity in vehicle-treated mice (Figure 4-13), where an F Test performed on the linear regression showed the slope was not different from zero ($p = 0.8434$). These data suggest that community richness was correlated with decreased insulitis severity in Protecflor-treated, but not vehicle-treated mice.
4.2.4.6 Correlation of caecal bacterial diversity with principal components analysis and insulitis

GI inflammatory diseases are associated with dysbiosis characterized by lower diversity, richness and altered microbiome composition[106][107][108]. Shannon’s H statistic is measure of the predictability of a given OTU in a sample[69]. A higher Shannon’s H value indicate higher entropy or “surprise” in a sample; the next OTU to be called is harder to predict [68]. The statistic depends on the number of OTU observed in a sample, and the evenness of the distribution of individual samples across OTU categories and is used to estimate microbial diversity [68]. To compare the species diversity of samples and correlate these measures with microbiome composition, samples were segregated into Protecflor and vehicle-treated groups, a Shannon’s H statistic calculated for each sample, and plotted against the PC1 and PC2 loadings for each sample (Figure 4-14). In vehicle-treated mice, positive PC2 loading was correlated with overall greater species diversity ($r^2 = 0.8491$). In contrast, PC1 was not correlated with species diversity ($r^2 = 0.00917$), and an F Test performed on the linear regression suggested that the slope did not differ from zero ($p = 0.8434$). In contrast, in Protecflor-treated mice, neither PC1 nor PC2 were correlated with species diversity ($r^2 = 0.1330, 0.2138$ for PC1 and PC2, respectively), and an F Test performed on the linear regression suggested that these slopes did not differ from zero ($p = 0.4772, 0.8568$ for PC1 and PC2, respectively). These results suggest that although Protecflor and vehicle-treated mice can exhibit microbiome communities with similar loadings on PC2, Protecflor treatment is associated with loss of correlation between PC2 and community diversity.

To correlate species diversity in samples with insulitis severity, we segregated Protecflor and vehicle-treated groups, calculated the Shannon’s H statistic for each group, then plotted them against insulitis scores for each mouse (Figure 4-15). In vehicle-treated mice, Shannon’s H statistic (species diversity) was correlated with lower insulitis severity (Figure 4-15a). The linear regression of Shannon’s H statistic with insulitis displayed an $r^2$ value of 0.8491, and an F Test of the linear regression suggested a non-zero slope ($p = 0.0090$). In contrast, in Protecflor-treated mice, Shannon’s H statistic was not related to insulitis (Figure 4-15b) and a F Test on the linear regression shows that the slope does not differ from zero ($p = 0.3559$). These results suggest that, in contrast to community richness, community diversity is correlated with decreased insulitis severity in vehicle-treated, but not Protecflor-treated mice.
4.3 Summary

We present this set of experiments as our effort to probe effects of Protecflor probiotic treatment on the gut microbiome, and on its effect on T1D development in the NOD mouse model.

By in silico and in vitro methods, we designed and validated the specificity of a set of probiotic strain-specific qPCR primers targeted to the quantification of Protecflor strains. Using these primers, we measured the GI transit time of the Protecflor strain in NOD mice gavaged with Protecflor. We found the probiotic strains rapidly transited the GI tract and could not be detected after 4 hours post-gavage, suggesting Protecflor consisted of strains which did not colonize the murine gut.

We modified our previously published gavage treatment protocol and tested the effect of Protecflor probiotic on autoimmune insulitis in female NOD mice. We found that Protecflor treatment was protective against insulitis and that Protecflor-treated mice showed less immune infiltration of the islets compared to vehicle-treated mice. We used Illumina high-throughput sequencing of the bacterial 16S rDNA gene to quantify changes in the composition of the gut microbiome associated with Protecflor treatment. Sequencing results showed changes in 7 bacterial taxa in Protecflor-treated animals.

We applied principal components analysis to our sequencing data in order to reduce the dimensionality of the data, reveal hidden structure and correlate changes in the microbiome with insulitis. We found that 53.47% of the total variance could be explained by 2 principal components, PC1 and PC2. The principal components did not differentiate Protecflor and vehicle-treated mice, but were correlated with insulitis severity in each group. Specifically, positive loading on PC1 was correlated with decreased insulitis in Protecflor-treated mice, but not vehicle-treated mice, while, in contrast, positive loading on PC2 was correlated with decreased insulitis in vehicle-treated mice, but not Protecflor-treated mice. These data suggest changes in the bacterial community composition can be correlated to protection against islet autoimmunity in NOD mice.

We applied ecological richness and diversity metrics to the sequencing samples in order to correlate changes in the community structure with changes in the microbiome OTU composition and insulitis. We found community richness and diversity were correlated with protection against
insulitis, suggesting that changes in overall community structure can be correlated to protection against islet autoimmunity in NOD mice.
Figure 4-1: Quality analysis and validation for Protecflor-specific qPCR assays. (A): Linearity and technical consistency of Protecflor qPCR assays. DNA was extracted from samples of the Protecflor formulation. DNA concentrations were determined by Nanodrop (Thermo Fisher Scientific Inc.), and a serial dilutions (from 1 - 1/10000) of template DNA were prepared. Protecflor-specific qPCR primers (see Table 4-1) were used to PCR amplify these sequences which were quantified by SYBRGreen. Raw data were then normalized compared to a PCR reaction quantifying total bacteria, and repeated using the same template on three separate days. Results from the 3 days are displayed. (B): Efficiency of Protecflor qPCR assays. Template dilution was plotted against $C_{\text{threshold}}$ to determine amplification efficiency reported as the slope. Template DNA serial dilutions and qPCR were performed as in (A). Points on the graphs represent the average of three technical replicates. Lines represent lines of best fit for each PCR primer set.
Figure 4-2: In vivo transit time for Protecflor probiotic strains. Twelve mice were gavaged with 200uL Protecflor suspension (2 x 10^8 CFU/mL) then sacrificed 1, 2, 3 or 4 hours post-gavage. The GI tract was dissected into stomach, small intestine, and caecum + large intestine. Contents of each compartment were flushed and DNA was extracted from the resulting material. R0052-plIR52 copy numbers were quantified by qPCR as described above. Data shown is the average of 3 biological replicates each performed with 3 technical replicates.
Figure 4-3: Insulitis outcomes in female NOD pups with Protecflor probiotic. Female NOD weanlings were either gavaged with and subsequently, had their drinking water was treated with either Protecflor probiotic or Protecflor vehicle. Treated drinking water was changed every 2 days. Insulitis severity was assessed by established protocols (see Methods) in female mice either treated with Protecflor probiotic or Protecflor vehicle at 93-98 days of age (n=8 per treatment group). 1 Protecflor-treated and 2 vehicle-treated mice became diabetic before the treatment cutoff and were excluded. Islet histology and scoring contributed to by Steve Mortin-Toth (A): Insulitis scores differ significantly between Protecflor and vehicle-treated mice. Insulitis score distributions were compared by Chi-squared test (p<0.0001). (B): Protecflor treatment affects pathogenesis transition from peri-insulitis to invasive insulinitis. Islets were segregated into Score 0-1 and Score 2-4 categories. Insulitis score distributions were compared by Chi-squared test (p<0.0001).
Figure 4-4: Quality analysis of Illumina 16S rDNA sequencing of Protecflor and vehicle-treated mice. The Good’s Coverage statistic was calculated for each OTU for 40 samples of increasing size using multiple random samplings of the sequencing data. Data was plotted as a collector’s curve showing the increase in coverage as sample size increases (see Methods) for each 16S rDNA PCR library. Dark line indicates actual size the sequencing sample with fewest reads.
Figure 4-5: Top 20 caecal and fecal microbial taxa in Protecflor and vehicle-treated mice. The top 20 represented taxa from all samples were plotted as stacked bars as a percentage of all counts from each library. Samples are segregated by: sample type; Protecflor treatment; Cage/mouse number; and age at sampling. Sequencing and data quality assurance was done by Dan Frank’s Lab.
Figure 4-6: Manhattan plot comparing enriched or depleted OTUs in Protecflor-treated vs vehicle-treated animals. The relative abundance of each OTU was compared by the Wilcoxon signed-rank test, and –Log(p-value) was plotted for each OTU in the comparison in a Manhattan plot. OTUs with –Log(pValue) above ~1.30 (indicated by dark line), corresponding to a p-value less than 0.05 were putatively categorized as those showing differential abundance.
Figure 4-7: Morisita-Horn distance heatmap of Protecflor-treated vs vehicle-treated caecal samples.

Morisita-Horn distance was calculated between each sample and plotted as a heatmap. Samples were ordered, grouping samples with similar compositions (high Morisita-Horn similarity). Black/blue indicates similar compositions, and red indicates dissimilar compositions.
Figure 4-8: Scree plots of PCA comparing all bacterial OTUs across caecal flush samples from 
**Protecflor and vehicle-treated animals.** Scree plots were generated from the Eigenvalues of the 
principal components.
Figure 4-9: PCA comparing all bacterial OTUs across caecal flush samples from Protecflor and vehicle-treated animals. PCA was performed on the OTU abundance data from sequencing caecal flush samples from Protecflor and vehicle-treated NOD mice. The samples were separated by principal components PC1 and PC2, which together explained 53.47% of the total variance between samples. Protecflor treatment data was mapped onto the PCA components plot. Red symbols represent samples from Protecflor treated mice, blue symbols vehicle-treated.
Figure 4-10: Unsupervised clustering analysis of caecal flush samples from Protecflor and vehicle-treated animals. A K-medians/fuzzy C-medians clustering algorithm (Dunn’s Coefficient = 0.86180) and a hierarchical agglomerative method were both used to define clusters across fecal and caecal flush samples from Protecflor and vehicle-treated animals. Clustering data was mapped onto the PCA components plot. Blue symbols represent samples placed in group 1, red symbols group 2.
Figure 4-11: Correlation of principal components with insulitis protection in Protecflor and vehicle-treated mice. PCA was performed on the sequencing data from all caecal flush samples. Samples were segregated into Protecflor and vehicle-treated groups, then PC1 or PC2 was plotted against overall per mouse insulitis. Red symbols represent samples from Protecflor-treated mice; blue symbols vehicle-treated mice. (A): positive loading in PC1 was correlated with protection against insulitis in Protecflor-treated mice, but not vehicle-treated mice. Pearson's r and $r^2$ was calculated for linear regression of each sample group to determine correlation between insulitis severity and PC1 ($r^2 = 0.7408, 0.00986$ for Protecflor and vehicle-treated mice, respectively). An F Test was performed on the linear regression of each sample group to determine if the slope was non-zero. ($p = 0.0278, 0.8515$ for Protecflor and vehicle-treated mice, respectively). (B): positive loading in PC2 was correlated with protection against insulitis in vehicle-treated mice, but not Protecflor-treated mice. Pearson's r and $r^2$ was calculated for linear regression of each sample group to determine correlation between insulitis severity and PC1 ($r^2 = 0.00181, 0.7965$ for Protecflor and vehicle-treated mice, respectively). An F Test was performed on the linear regression of each sample group to determine if the slope was non-zero. ($p = 0.9362, 0.0167$ for Protecflor and vehicle-treated mice, respectively).
**Figure 4-12: Correlation of principal components with bacterial community richness.** (A): positive loading in PC1, but not PC2 was correlated with species richness. PCA was performed on the sequencing data from all caecal flush samples. The Chao1 statistic was calculated for the samples, then plotted against PC1 and PC2. Red symbols represent samples plotted against PC1, black symbols PC2. Pearson's r and $r^2$ was calculated for each linear regression to determine correlation between species richness and PC1 and PC2 ($r^2 = 0.6687$, $0.00158$ for PC1 and PC2, respectively) An F Test was performed on the linear regression of each sample group to determine of the slope was non-zero. ($p = 0.0012$, $0.9021$ for PC1 and PC2, respectively). (B): Type 1 samples are overall less species rich than Type 2 samples. These samples were segregated based upon PC1 into Type 1 and Type 2 samples. Chao1 statistics were calculated and are plotted for each group. The two sample groups were compared by the Mann Whitney test ($p = 0.0025$).
Figure 4-13: Correlation of bacterial community richness with insulitis protection in Protecflor-treated mice. Samples segregated into Protecflor and vehicle-treated groups, the Chao1 statistic was calculated for the samples in each group, and then plotted against overall per mouse insulitis. Red symbols represent samples from Protecflor-treated mice; blue symbols vehicle-treated mice. Pearson’s r and $r^2$ was calculated for linear regression of each sample group to determine correlation between Chao1 statistic insulitis severity ($r^2 = 0.8988$, 0.0110 for Protecflor and vehicle-treated mice, respectively). An F Test was performed on the linear regression of each sample group to determine if the slope was non-zero. (p = 0.0040, 0.8434 for Protecflor and vehicle-treated mice, respectively).
Figure 4-14: Correlation of bacterial community diversity with principal components in Protecflor-treated mice. Samples segregated into Protecflor and vehicle-treated groups, Shannon’s H statistic was calculated for the samples in each group, then plotted against PC1 and PC2. Red symbols represent samples from Protecflor-treated mice, blue symbols vehicle-treated mice. (A): PC1 was not correlated with species diversity in either Protecflor-treated or vehicle-treated mice. Pearson’s r and $r^2$ was calculated for linear regression of each sample group to determine correlation between Shannon’s H statistic and PC1 ($r^2 = 0.1330, 0.00917$ for Protecflor and vehicle-treated mice, respectively). An F Test was performed on the linear regression of each sample group to determine of the slope was non-zero. ($p = 0.4772, 0.8568$ for Protecflor and vehicle-treated mice, respectively). (B): positive loading in PC2 was correlated with greater species diversity in vehicle-treated mice, but not Protecflor-treated mice. Pearson’s r and $r^2$ was calculated for linear regression of each sample group to determine correlation between insulitis severity and PC2 ($r^2 = 0.2138, 0.8491$ for Protecflor and vehicle-treated mice, respectively) An F Test was performed on the linear regression of each sample group to determine of the slope was non-zero. ($p = 0.3559, 0.0090$ for Protecflor and vehicle-treated mice, respectively).

Figure 4-15: Correlation of bacterial community diversity with insulitis protection in Protecflor-treated mice. Samples segregated into Protecflor and vehicle-treated groups, Shannon’s H statistic was calculated for the samples in each group, then plotted against overall per mouse insulitis. Red symbols represent samples from Protecflor-treated mice; blue symbols vehicle-treated mice. Pearson’s $r$ and $r^2$ was calculated for linear regression of each sample group to determine correlation between Shannon’s H statistic and insulitis severity ($r^2 = 0.00001$, 0.8011 for Protecflor and vehicle-treated mice, respectively). An F Test was performed on the linear regression of each sample group to determine of the slope was non-zero. ($p = 0.9949$, 0.0159 for Protecflor and vehicle-treated mice, respectively).
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**Table 4-1: Oligonucleotide primers specific for Protecflor organisms.** Primer sequences and other information were provided by Lallemand (See Methods).
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**Table 4-2: Number of 16S rDNA sequencing reads per sample.** Standard deviation of the number of sequences read was 30178.27, with a range from 190856 to 38771 (range = 152085).
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Table 4-3: PC1 OTU object loadings on Protecflor treated mouse caecal samples. PCA was performed on Protecflor and vehicle-treated caecal samples using Ginkgo. OTU object loadings on PC1 were extracted from Ginkgo and ordered from least to greatest.
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</tr>
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<tr>
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**Table 4-4: PC2 OTU object loadings on Protecflor treated mouse caecal samples.** PCA was performed on Protecflor and vehicle-treated caecal samples using Ginkgo. OTU object loadings on PC2 were extracted from Ginkgo and ordered from least to greatest.
Chapter 5
Discussion
5 Discussion

5.1 The effect of oral gavage transfer of female NOR caecal microbiota into female NOD recipients on microbiome composition and T1D incidence

5.1.1 Summary

We present this set of experiments as our effort to validate qPCR as viable method for the detection and quantification of members of specific bacterial taxa, and the application of such an assay panel to probe the effect of the microbiome on T1D development in the NOD mouse model. Specifically, we applied the assay to quantify changes in the gut microbiome of female NOD mice from weaning to adulthood. We quantified changes associated with gavage treatment of weanling female NOD mice with caecal material from donor adult NOR mice. We followed up on the changes we detected by looking at the effect of gavage treatment with NOR-derived material in NOD mice on the incidence and progression to T1D.

5.1.2 Use of “indicator” taxa

The microbiome is a complex community of organisms, and the ecology and interaction of these organisms is only beginning to be understood. High-throughput 16S sequencing provides deep taxonomical overview of the target microbiome. However, despite continuing advances in sequencing technology, the cost of HTS can be prohibitive for large sample sets, longitudinal studies, and routine verification of experimental conditions. In experimental comparisons where prior sequencing data has distinguished microbes which are consistently indicators of differences in the microbiome, we suggest that taxa-specific qPCR assays can be used to supplement sequencing technology in a more cost-effective manner.

In our study, prior sequencing data from our lab had shown the microbiomes of NOD and NOR animals consistently differ in five bacterial taxa. We designed and validated the specificity of a set of taxa-specific qPCR primers by in silico and in vitro methods. We determined ranges of linear amplification and amplification efficiency of these assays, and validated the ability of designed taxa-specific qPCR assays to produce consistent and sensitive quantification of bacterial OTUs present in the microbiome of NOD mice. We found that these 5 taxa could act as low resolution indicator taxa of the state of the microbiome; differentially abundant taxa we
previously saw by sequencing the microbiome of NOD and NOR mice were reflected by changes in relative abundance in the indicator taxa as quantified by qPCR. We also detected changes in the microbiome of mice as they aged from weaning to adulthood, suggesting that these 5 taxa could also serve as rough indicators of the community state of the microbiome.

5.1.3 Comparison of abundance of caecal bacterial taxa in NOD mice from weaning to adulthood

When we applied our panel of assays to tracking changes in the composition of the microbiome from weaning to adulthood, we found that changes in the gut microbiome from weaning to adulthood could be detected and quantified by taxa-specific qPCR, and a major shift in the microbiome composition was observed between 25d and 41d female NOD mice.

Our data shows major shifts in the qPCR profiles of the gut microbiome at 25, 33 and 41d mice, with the profile stabilizing between 41d and 55d animals. Previously, our lab showed the ability to perturb the developing mouse microbiome by the introduction of male donor caecal contents to weanling (21 days of age) female mice, suggesting that the gut microbiome at weaning is alterable by the introduction of exogenous bacteria[50]. Ivanov et al. also showed successful transfer of SFB and subsequent effects on the T\textsubscript{H}-17 cell population by gavage at 28d[60]. Our results corroborate the unstable state of the gut microbiome between weaning at 21d and puberty at 35-42d. The stabilization of the gut microbiome after puberty at ~40d is in agreement with previous work in our lab showing that puberty is associated with a divergence of the microbiomes of male and female mice[50]. Together, these data suggests a consistent shift in the microbiome of NOD mice around the 30-40 day time point.

We speculate, in agreement with previous work in our lab, that this may be related to the onset of puberty in mice[50], although further evidence is needed to determine the correlation between puberty and this microbiome shift. Previous work in our lab found that the microbiome of male and female NOD mice diverged in composition at the onset of puberty[50]. Other studies have provided evidence that the puberty and sex hormones may have an effect on microbiome composition. In humans, studies have shown changes in the microbiome of other body sites (gingiva, nares, skin, vaginal canal) associated with the onset of puberty[109][110][111]. A study on salivary bacterial communities also found the abundance of selected bacterial taxa (Veillonellaceae and Actinomycetaceae) were correlated with age from adolescence to early
adulthood[112]. The gut microbiome of human adolescents has also been found to differ from that of adults, with significantly higher abundance of genera *Bifidobacterium* and *Clostridium* among adolescent samples[113]. Other studies have also shown that the gut microbiome develops in conjunction with human host development from infancy to adulthood, modified by geography[56]; most notably in infancy at weaning and the introduction of solid food[114].

5.1.4 Comparison of abundance of caecal bacterial taxa in NOD, NOR and NOR-gavaged NOD Mice

We applied the qPCR panel to quantifying bacterial taxa in unmanipulated NOD and NOR mice. We observed differences in the microbiome composition of NOD and NOR mice. *Bacteroidetes* and *Helicobacter* were enriched in NOR animals and *Clostridia IV*, and *Ruminococcus* were enriched in NOD animals. These differences were in agreement in directionality, but not magnitude with previous sequencing data in our lab comparing the microbiomes of NOD and NOR animals. We also found that variance of abundance in Clostridia IV, Helicobacter, and Ruminococcus was higher between NOR animals compared to NOD. Both the differential abundance and differential variance suggest NOR animals may have differing microbiome communities than NOD.

When we compared bacterial taxa in unmanipulated NOD, NOD recipients of NOR caecal material by gavage (NOR-gavaged NOD), and NOR mice, we found significant differences between untreated NOD and NOR-gavaged NOD mice in the *Clostridia IV* and *Clostridia XIVa*. These differences suggest that the gavage treatment has some effect on the composition of microbiome of treated NOD mice. Data also showed statistically significant differences between NOR and NOR-gavaged NOD mice in the *Clostridia IV*, *Clostridia XIVa*, and *Helicobacter* groups. These data suggested that the resulting microbiome of recipient mice is not identical to that of the donor, nor is it clearly partially composed of NOD and NOR contributions. This is consistent with data from previous experiment in our lab comparing female, male-gavaged female and male NOD mice by 16S sequencing. Male-gavaged female NOD mice were found to have microbiomes which were significantly different from either unmanipulated female or male NOD mice[50].

Except in the *Clostridia IV* and *Ruminococcus*, samples from the same strain (NOD/NOR) were clustered. These results are consistent with previous findings in our lab; previous sequencing
results comparing the gut microbiome of NOD and NOR mice found inter-strain differences between NOD and NOR, and similarity between individuals from the same strain. These data support the idea of a stable microbiome “archetype” which is mostly dictated by the genotype of the host mouse. Other studies suggest that microbiomes do develop similarly within mouse strains; Hildebrand et al. found genetic distance was associated with microbiota distance between four strains of inbred mice (B6, BALB/c, FVB and NOD)[115]. Other studies also support the idea that genotypic differences are a significant determinant in microbiome composition. Benson et al. described quantitative trait loci in mice associated with relative abundances of specific microbial taxa in an intercross line between C57BL/6J and an ICR-derived outbred line [116]. Alexander et al. showed intra-strain differences in Altered Schaedler Flora (ASF) colonization between multiple barrier-raised inbred strains were maintained despite co-housing[117].

We suggest that the effects of genetics on the composition or functional capacity of the gut microbiome may be an important phenotypic outcome of genotypic differences. Other studies have also suggested genetic differences amongst inbred mouse strains are an important factor in maintaining distinct microbiome profiles under similar environmental exposures[117]. The NOD mouse, when contrasted with NOR and NOD.NOR congenic animals, provides a good experimental system in which to study the effects of genetics on microbiome composition. Previous work in our lab and others has generated an extensive number of congenic strains in the course of dissecting the genetic contribution of IDD loci to autoimmune defects in the NOD mouse. Analysis of the microbiome, both by composition and functional differences, of these closely related congenic strains may shed more light on the microbiome phenotypic outcomes of T1D risk loci.

5.1.5 Interstrain microbiome variation

Despite most members of a strain showing similar microbiome composition, significant variation was also observed, most notably in the Clostridia IV group for NOR mice. Microbiome divergence between NOR mice was greater than that in NOD. This data is consistent with data presented by Alexander et al., showing that the degree of microbiome divergence between non-littermates depended on the genotype of the mouse[117]. However, other studies have suggested that maternal transmission and housing conditions are more important in determining the composition of the microbiome. Specifically, Benson et al. found in an advanced intercross line
derived from C57BL/6J and an ICR-derived HR line that variation in microbiome composition could be segregated by a mixed-model analysis into parental, sex and cohousing effects[116].

Ubeda et al. also found that genetic defects in innate immune sensing (MyD88<sup>−/−</sup>, TLR2<sup>−/−</sup>, TLR4<sup>−/−</sup>, TLR5<sup>−/−</sup>, and TLR9<sup>−/−</sup>) in C57BL/6 mice did not have a significant impact on the composition, diversity, or antibiotic recovery of the microbiome when compared to wildtype littermates. In contrast, clear maternal and litter effects were observed[118].

Further experiments are needed to narrow down the cause of the intra-strain microbiome variation; but we hypothesize the variation could be due to differences in the composition of the initial colonizing organisms. This hypothesis would include differences in this composition caused by variations in environment, such as those caused by differences in cage environment. In this case, we would expect variations in the microbiome to segregate along these environmental variations. Assorting the data by cage/littermates, we found no clustering of cage/littermates into groups of similar relative abundance. This suggests that housing cages were not related to the intra-strain variation we observed. Of course, our observations were limited by the sample size, in this case. Further experiments or meta-analysis of collected T1D natural history data may shed more on the issue of cage environment.

Other studies have shown that housing environment and cohousing can have a significant effect on the composition of the microbiome, and moreover, these alterations of the microbiome can be linked to inflammatory phenotypes. Elinav et al. found that the colitogenic microbiome of NLRP6<sup>−/−</sup> mice was transferable by cohousing with wildtype mice, and furthermore, in cohoused wildtype mice, the colitogenicity of the dysbiosis was recapitulated[119].

### 5.2 The effect of Protecflor probiotic treatment on NOD microbiome composition and insulitis severity

#### 5.2.1 Summary

We present this set of experiments as our effort to probe effect of Protecflor probiotic treatment on the gut microbiome, and on its effect on T1D development in the NOD mouse model. Specifically, we validated qPCR as viable method for the detection and quantification of members of specific bacterial taxa, and the applied this to determining the gastrointestinal transit time of the Protecflor organisms in female NOD mice to validate the gavage treatment of mice
with Protecflor. We then looked at the effect of gavage and water treatment with Protecflor in NOD mice on insulitis progression and severity. We followed up with 16S high-throughput sequencing of caecal and fecal samples from these mice to quantify changes in the gut microbiome associated with Protecflor treatment.

By in silico and in vitro methods, we validated the specificity of a set of probiotic strain-specific qPCR primers conveyed to us by our collaborators at Lallemand. We determined ranges of linear amplification and amplification efficiency of these assays, and validated the ability of designed taxa-specific qPCR assays to produce consistent and sensitive quantification of probiotic strain present in the guts of Protecflor-treated mice.

5.2.2 Gastrointestinal Transit times of Protecflor Probiotic organisms in the NOD model

We applied our assays to tracking the gastrointestinal transit of the Protecflor probiotic strains in NOD mice gavaged with Protecflor. We found that Protecflor strains could be detected in the stomach, small intestine and, caecum and large intestine of treated animals after gavage. Protecflor organisms were rapidly cleared from the GI tract, and could not be detected by qPCR 4 hours after gavage treatment. Our data shows that Protecflor strains could be detected in the stomach, small intestine and, caecum and large intestine of treated animals by qPCR up to 4 hours after gavage.

In other studies, a wide range of gastrointestinal transit times in mice have been observed, depending on the methodology and mouse strain used. Schwarz et al. found GI transit times of 154 ± 24 minutes in BL/6 mice using a contrast MRI-based measurement method[101]. Padmanabhan et al. found a GI transit time of 6h in 129SvEv mice using a radiolabelled charcoal pellet[102]. One study found, in NOD mice, a bolus consisting of 10% charcoal in 5% Arabic gum aqueous suspension had transited 60% of the small intestine 20 minutes after feeding[120]. Our results corroborate this range of transit times, and add to the evidence that a liquid bolus has faster transit times compared to a solid bolus.
5.2.3 Evaluating the Effects of Protecflor Probiotic on Insulitis Severity in NOD model

To test the effect of Protecflor probiotic on autoimmune insulitis, we assessed insulitis severity in NOD mice treated with Protecflor and vehicle. We found that Protecflor treatment was protective against insulitis and that Protecflor-treated mice showed less immune infiltration of the islets compared to vehicle-treated mice.

Other studies have shown that probiotic formulations containing the organisms in Protecflor are beneficial in irritable bowel syndrome and ulcerative colitis. *Lactobacillus helveticus* R0052 and *Lactobacillus rhamnosus* R0011 have been shown to reduce clinical symptoms of irritable bowel syndrome in open label experiments on human adults with irritable bowel syndrome [121]. *Bifidobacterium longum* R0175, given in combination with inulin in a small pediatric study of ulcerative colitis (UC) was found to improve self-reported quality of life, UC symptoms of blood and mucous in stool, and to reduce relapse rate[71]. Oral administration of probiotic bacterial strains related to the Protecflor organisms has also been found to protect NOD mice from T1D. Specifically, the probiotic mixture VSL#3 (*Bifidobacterium, Lactobacillus* species and *Streptococcus salivarius subsp. Thermophiles*) was given by oral gavage three times a week from 4 to 32 weeks of age, reduced T1D incidence in NOD mice [49].

Our results demonstrate the protective effect of the Protecflor organisms against insulitis, a pre-T1D phenotype, in a single-gavage experimental setting. This is novel compared to three times a week gavage treatment tested using VSL#3. Of particular importance is that multiple gavages increase the risk of systemic exposure of microbes to the immune system, by abrasion of the esophageal wall, or aspiration of the gavage material. It is well documented by multiple studies that bacterial infection or systemic exposure is protective against T1D in NOD mice. Studies showing deliberate infection with multiple organisms, including *Schistosoma mansoni*[42], *Mycobacterium avium*[43], *Trichinella spiralis*[44], *Heligmosomoides polygyrus*[45] and *Salmonella typhimurium*[46], can induce protection in NOD mice. Interperitonal injection of zymosan, a fungal cell wall component, has also been found to be protective in the NOD model[47]. In contrast, our protocol limits the frequency of the gavage procedure, and probiotic dosing is maintained by addition to drinking water.
5.2.4 The Effects of Protecflor Probiotic treatment on the gut microbiome in NOD mice

We used Illumina high-throughput sequencing of the bacterial 16S rDNA gene to quantify changes in the composition of the gut microbiome associated with Protecflor treatment. Sequencing results showed enrichment of the genera *Allobaculum*, *Candidatus Arthromitus*, *Roseburia*, and the order *Clostridiales* in caecal flush and fecal samples from Protecflor-treated animals compared to controls, and depletion of the genera *Helicobacter*, *Parabacteroides* and the order *Bacteroidales*, in Protecflor treated vs. controls.

5.2.4.1 Taxa enriched in Protecflor treated mice

Overall, the OTUs enriched in samples from Protecflor-treated mice were depleted in T\textsubscript{H}1-associated inflammatory disorders or linked to protection from T1D in mice or humans. Our results corroborate the association of some bacterial taxa, in particular SFB and Roseburia, with protection in the NOD T1D setting.

5.2.4.1.1 *Allobaculum*

*Allobaculum* is a newly recognized genus with a single species first isolated from canine feces[122]. The type strain of *Allobaculum stercoricanis* was found to produce butyrate in glucose and other hexose metabolism. This data suggests that *Allobaculum* may be able to modulate host mucosal immunity from SCFA metabolites (see below). There is also evidence *Allobaculum* is associated with T\textsubscript{H}2 immune skewing. *Allobaculum* was found to be the most abundant OTU in arthritis-susceptible HLA transgenic DRB1*0401 mice, compared to arthritis-resistant HLA transgenic DRB1*0402 mice[123]. *Allobaculum* was also enriched in control diet fed mice and depleted in diet-induced obese mice[124].

5.2.4.1.2 Segmented Filamentous Bacteria (SFB)

The association of SFB with Protecflor treatment and protection is consistent with evidence from previous studies. Kriegel et al. found the presence of SFB in feces was correlated with diabetes protection in female NOD mice, but insulitis did not depend on SFB colonization[62]. Yurkovetskiy et al. found SFB monoclonization was protective in male by not female NOD mice, compared to germ-free mice [125]. SFB-positive fecal transfer suppressed the insulitis and T1D incidence in NOD mice that were on acidified (pH 3.2) water but not in those on neutral
water (pH 7.0)[126]. Interestingly, bacterial taxa which were enriched in NOD mice acidified (pH 3.2) water in another study[127] were also loaded positively on PC1 and were associated with protection from insulitis in Protecflor-treated mice. However, it should be noted that these two studies suggest opposite effects of acidified water in NOD T1D incidence[127][126].

5.2.4.1.3 Roseburia

*Roseburia* is a butyrate-producing genus in the *Lachnospiraceae* family. *Roseburia* has been associated in previous studies with T1D-protected populations in both the NOD mouse model and in human T1D case-control studies. Previous work in our lab by Markle et al. found *Roseburia* was enriched in male vs. female NOD mice, and also in female recipients of gavage material derived from male caecal contents vs. female caecal contents[50]. *Roseburia* was found to be enriched in control vs. T1D case subjects, along with other butyrate-producing genera[128]. Goffau et al. found that *Roseburia* was enriched in healthy controls vs. children which developed anti-islet autoantibodies[129].

5.2.4.2 Taxa depleted in Protecflor treated mice

Overall, the OTUs enriched in samples from vehicle-treated mice were linked in small studies to T1D in mice or humans. Our results corroborate the association of some bacterial taxa, in particular SFB and Roseburia, with protection in the NOD T1D setting.

5.2.4.2.1 Helicobacter

*Helicobacter* species have been associated with both protection and disease progression in previous studies. One Japanese case report reported the eradication of a *Helicobacter pylori* infection in an adult (42 year old) patient with a high risk *HLA* haplotype was followed by the diagnosis with T1D[130].

Other studies have suggested that *Helicobacter* is a bystander to T1D, and changes in host physiology affect *Helicobacter* survival. Gasbarrini et al. found *Helicobacter pylori* infection in T1D patients was more resistant to eradication by triple antibiotic therapy. Other studies have suggested Helicobacter seropositive status has a time-dependent relationship with T1D. de Luis et al. found that seroprevalence of *Helicobacter pylori* was higher among younger (<24 years old) T1D patients vs. healthy controls, but lower in older (<24 years old) T1D patients[131].
One study found seroprevalence of *Helicobacter pylori* was significantly higher in patients with T1D than in healthy controls[132]. It has been suggested that *Helicobacter* is a possible trigger for gastric autoimmunity by molecular mimicry, as it expresses lipopolysaccharides which mimic Lewis y, Lewis x, and H type I blood group structures similar to those commonly occurring in gastric mucosa. One study found FUT2 non-secretor status, resulting in a lack of Lewis antigen expression, was linked to T1D susceptibility, as well as resistance to pathogens which mimic the Lewis antigens, such as *H. pylori*[133].

### 5.2.4.2.2 Parabacteroides

Previous work in our lab by Markle et al. found *Parabacteroides* was enriched in 14 week old female NOD mice vs. male NOD mice and 14 week old vs. 34 week old NOD female recipients of male caecal contents[50].

### 5.2.4.2.3 Bacteroidales

Multiple studies have found *Bacteroides*, a genus within *Bacteroidales*, is enriched in patients with T1D vs. healthy controls. Brown et al. found *Bacteroides* were enriched in T1D patients vs. healthy controls in a small cohort of age/HLA matched children[128]. Similarly, Mejía-León et al. found *Bacteroides* were enriched in newly-diagnosed Mexican T1D patients vs. healthy controls[134], and Murri et al. found *Bacteroides* were enriched in T1D cases vs. healthy controls by PCR-DGGE[135]. Giongo et al., in a Finnish T1D case-control study, found *Bacteroides* were enriched in T1D patients vs. healthy controls. *Bacteroides* enrichment was also correlated with a pre-T1D phenotype, anti-islet autoantibodies, in a study by Goffau et al[129].

### 5.2.5 Principal components analysis of Proteclor and vehicle-treated caecal samples

We applied principal components analysis to our sequencing data in order to reduce the dimensionality of the data, reveal hidden structure and correlate changes in the microbiome with insulitis. We found that 53.47% of the total variance could be explained by 2 principal components, PC1 and PC2. Positive loading on PC1 were populated with Order *Clostridiales*, including the *Ruminococcaceae* and *Lachnospiraceae* families. The negative loadings were populated with Order *Bacteroidales*, and Phylum *Proteobacteria*. Samples were distributed along PC1 in two clusters, regardless of treatment. Positive loadings for PC2 were populated
with family *Erysipelotrichaceae*, Order *Clostridales*, specifically from the *Lachnospiraceae* family, and Order *Bacteroidales*, including the *Rikenellaceae* and *Porphyromonadaceae* families. The negative loadings were populated with phylum *Cyanobacteria*, and Order *Clostridales*, including the *Veillonellaceae* and *Lachnospiraceae* families, and *Candidatus-Arthromitus* (SFB). Samples were more evenly distributed along PC2, also independent of treatment. Positive loading on PC1 was correlated with decreased insulitis in Protecflor-treated mice, but not vehicle-treated mice. In contrast, positive loading on PC2 was correlated with decreased insulitis in vehicle-treated mice, but not Protecflor-treated mice.

5.2.5.1 Bacterial taxa associated with T1D in NOD mice and humans

Bacterial taxa found to significantly load in our principal components analysis have been linked in other studies to T1D in other murine experimental settings. *Roseburia*, *Coprococcus*, *Peptococcus*, all members of Order *Clostridales*, found to load positively on PC1 and be associated with protection from insulitis in Protecflor-treated mice in our study, were found to be enriched in NOD female recipients of male-derived microbiome transplant compared to untreated females at 14 weeks of age[50]. Members of Family *Ruminococcaceae*, *Rikenellaceae* and *Erysipelotrichaceae* were found to load positively on PC1 and be associated with protection from insulitis in Protecflor-treated mice in our study, were all also found to be enriched in T1D-protected NOD mice treated with acidified (pH 3.2) water vs. controls treated with neutral (pH 7) water[127]. *Lactobacillaceae*, *Erysipelotrichaceae*, and *Rikenellaceae*, found to load positively on PC2 and be associated with protection from insulitis in untreated mice in our study, were all also found to be enriched in T1D-protected NOD.MyD88<−> vs. SPF NOD controls[48].

Bacterial taxa found to significantly load in our principal components analysis have been linked in other studies to T1D in humans. Families *Ruminococcaceae*, Family *XIII Incertae Sedis*, and *Lachnospiraceae*, all members of Order *Clostridales*, found to load positively on PC1 and be associated with protection from insulitis in Protecflor-treated mice in our study were found to be enriched in healthy controls vs. T1D patients in a small cohort of age/HLA matched children[128].
5.2.5.2 Proteolytic Bacteria

Dietary triggers have been implicated in the etiology of T1D in humans, particularly in association with cow’s milk proteins[136] and gluten, although conflicting evidence has been presented. The epidemiological link between T1D and celiac disease is also suggestive of gluten as a dietary trigger. Previous studies have suggested that microbial peptidases present in the gut may degrade immunogenic peptides and reduce autoimmunity. In our study, *Peptostreptococcaceae, Clostridium, Peptococcus*, taxa with known proteolytic/amino acid fermenting ability[137][138], were found to load positively on both PC1 and PC2 and be associated with protection from insulitis in all experimental groups in our study.

5.2.5.3 Short Chain Fatty Acid Producers

Short chain fatty acids (SCFAs) such as butyrate and propionate are products of the anaerobic bacterial fermentation of dietary fiber in the host gut[139]. Previous studies have highlighted the phylogenetic relationships between the major bacterial taxa involved in SFCA production in the gut[140][141][142]. SCFAs bind and signal through previously orphaned G Protein-coupled Receptors (GPRs) GPR41, GPR43[143][144] and GPR109A[145]. SFCA signaling through GPR43 has been implicated in regulating the gut mucosa-associated immune system. SFCA added to drinking water was found to increase colonic Treg frequency and number in germ free mice, and stimulate *in vitro* production of IL-10, and this effect is thought to be mediated by GPR43 signaling[146]. Bacterially-derived SCFAs were also found to stimulate TGF-β production by the gut epithelium[147].

Propionate and butyrate have also been found to have Histone De-ACetylase (HDAC) inhibitory activity[148]. This HDAC inhibitory activity may also have a role in regulating mucosal immunity in the gut. Treatment of macrophages with butyrate led to the down-regulation of pro-inflammatory mediators, including nitric oxide, IL-6, and IL-12 in a GPR and TLR independent mechanism[149]. Lin− bone marrow cells treated with SCFAs were blocked from DC, but not granulocyte development[150].

Although butyrate and propionate have similar signaling activity in many studies, some have suggested differential activity in these SCFAs. Butyrate and propionate act in an opposed fashion in IL-2 and IFN-γ production in cultured mesenteric lymph node lymphocytes, with butyrate
promoting IFN-γ production, and propionate partially rescuing IL-2 inhibition by butyrate[151]. There is evidence suggesting that GPR109A interacts with butyrate by not propionate, providing another source of differential signaling[145].

In this study, order Clostridiales members *Clostridium, Roseburia, Coprococcus, Peptococcus*, and *Anaerotruncus*, known to specifically produce butyrate (butanoic acid)[140][141], were found to load positively on PC1 and be associated with protection from insulitis in Protecflor-treated mice in our study. In contrast, Bacteroides, Escherichia, Veillonella, Prevotella, bacterial genera known to specifically produce propionate (propanoic acid)[152], were found to load negatively on PC1 and PC2 and be associated with insulitis in both vehicle and Protecflor-treated mice in our study. Together, this evidence suggests there may be a role of the differential regulation of mucosal immunity by propionate and butyrate in the pathogenesis of T1D.

### 5.2.5.4 Enterotypes in NOD and T1D

Hildebrand et al. observed in their study the segregation of other inbred mouse strains into archetypes or “enterotypes,” which were mostly independent of genotype, but not in NOD[115]. They also found that fecal calprotectin levels, an indication of intestinal inflammation in IBD[153] and IBS[154], were elevated in mice harbouring the low-richness, Bacteroides-rich enterotype. In their study, calprotectin levels were significantly negatively correlated to Lachnospiraceae, Rikenellaceae, Ruminococcaceae as well as Prevotellaceae, while the positive correlation to Bacteroidaceae, Verrucomicrobiaceae, Enterobacteriaceae and Burkholderiales[115].

The clustering of our NOD-derived samples into two clusters, primarily differentiated by PC1, provides initial evidence that these enterotypes, with microbial compositions corresponding those found in Hildebrand et al., also exist in NOD mice. Cluster 1 and Cluster 2 correspond by composition to the low-richness, Bacteroides-rich enterotype, and high-richness, Ruminococcus-rich enterotypes in Hildebrand et al.

Based on the presence of similar “enterotypes” in untreated adult mice, both NOD and other genotypes, we suggest that these archetypes represent two basal microbiota communities which can stabilize in the un-manipulated mouse gut. Furthermore, having found protection from insulitis is well-correlated with cluster membership in Protecflor-treated animals, we speculate
that the Cluster 2-type “enterotype” may be a “receptive” microbiome to Protecflor treatment, or may be indicative of a similarly “receptive” host.

The correlation between PC loading organisms and insulitis in untreated NOD mice also raises the intriguing possibility that the abundance of some group of bacterial taxa are directly proportional to insulitis severity, either causally or in response to host factors.

5.2.6 Species richness and diversity in Protecflor and vehicle-treated animals

We applied ecological richness and diversity metrics to the sequencing samples in order to correlate changes in the community structure with changes in the microbiome OTU composition and insulitis. We found that community richness, as measured by the Chao1 statistic, was correlated with changes in microbiome composition associated with positive loadings on PC1 in all mice, and with decreased insulitis severity in Protecflor, but not vehicle-treated mice. We found that community diversity, as measured by Shannon’s H statistic, was correlated with changes in microbiome composition associated with positive loadings on PC2 and with decreased insulitis in vehicle, but not Protecflor-treated mice.

Other studies have related both changes in community richness and diversity with dysbiosis associated with T1D in both NOD mice and humans. A study on NOD mice treated with gluten-free chow found T1D incidence was lower in mice given gluten-free chow, and these mice exhibited increased gut microbial community richness, as measured by the Chao1 statistic. However, community diversity, as measured by Shannon’s H statistic was not altered by gluten-free chow[155]. Wolf et al. also found the consumption of acidic (pH 3.2) water by NOD mice altered both community richness and diversity, although not statistically significantly[127]. In humans, a number of small studies have found correlations between community richness and diversity and T1D. Giongo et al. found microbial community diversity increased over time in both healthy children and T1D patients, but much more rapidly in controls, while diversity in T1D patients plateaued as they approached clinical autoimmunity and T1D diagnosis[156]. Together, these results suggest that diet-based treatment in the host can alter the overall gut microbiome community structure, and these alterations in community richness and diversity may be correlated with T1D pathogenesis.
References


