

# Gut microbial markers are associated with diabetes onset, regulatory imbalance, and IFN- $\gamma$ level in NOD Mice

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**Abbreviations:** CD, cluster of differentiation; DC, dendritic cell; FoxP3, forkhead box; IFN, interferon; MLN, mesenteric lymph node; NKT, natural killer T cell; PCA, principal component analysis; PCoA, principal coordinate analysis; PLN, pancreatic lymph node; siLP, small intestinal lamina propria; Treg, regulatory T cell.

Gut microbiota regulated imbalances in the host's immune profile seem to be an important factor in the etiology of type 1 diabetes (T1D), and identifying bacterial markers for T1D may therefore be useful in diagnosis and prevention of T1D. The aim of the present study was to investigate the link between the early gut microbiota and immune parameters of non-obese diabetic (NOD) mice in order to select alleged bacterial markers of T1D. Gut microbial composition in feces was analyzed with 454/FLX Titanium (Roche) pyro-sequencing and correlated with diabetes onset age and immune cell populations measured in diabetic and non-diabetic mice at 30 weeks of age. The early gut microbiota composition was found to be different between NOD mice that later in life were classified as diabetic or non-diabetic. Those differences were further associated with changes in FoxP3<sup>+</sup> regulatory T cells, CD11b<sup>+</sup> dendritic cells, and IFN- $\gamma$  production. The model proposed in this work suggests that operational taxonomic units classified to S24-7, *Prevotella*, and an unknown Bacteroidales (all Bacteroidetes) act in favor of diabetes protection whereas members of Lachnospiraceae, *Ruminococcus*, and *Oscillospira* (all Firmicutes) promote pathogenesis.

## Introduction

The etiology of type 1 diabetes (T1D) is complex. The susceptibility to develop insulinitis is polygenetic, particularly alleles encoding antigen-presenting cell functions (NOD MHC class II), immunoregulatory molecules (IL-2, IL21, and CTLA-4), and B-, T-, and Natural killer T (NKT) cell activation and homeostasis are found in strongly contributing loci linked to traits of clinical T1D.<sup>1,2</sup> However, genetics cannot fully account for the development of diabetes. Differences in gut microbiota composition have received a great deal of attention, especially since it was published that disease incidence vary between non-obese diabetic (NOD) mice maintained in different facilities.<sup>3</sup> This is supported by studies in germ-free NOD mice demonstrating the importance of gut microbiota as a disease regulator. The studies by Wen et al. and Suzuki et al. are frequently quoted as they reported a near 100% diabetes incidence in germ-free NOD mice.<sup>4,5</sup>

Experimental evidence from other studies in diabetic mouse models has confirmed that differences in gut microbiota partly explain the incidence of diabetes.<sup>3,6</sup> For example, a recent study highlighted the diabetes protective association with presence of

segmented filamentous bacteria (SFB) in NOD mice from different facilities.<sup>7</sup> In a recent study, SFB was however only found to protect NOD males in contrast to females when mono-colonized, and the mice therefore regained the diabetes gender bias which is lost in germ-free conditions.<sup>8</sup> Moreover, *Lactobacillus* and *Bifidobacterium* were found in higher abundance in feces from bio-breeding diabetes-resistant (BB-DR) rats before clinical onset of T1D compared with bio-breeding diabetes-prone (BB-DP) rats, which contained more *Bacteroides*, *Eubacterium*, and *Ruminococcus* ssp.<sup>9</sup> Furthermore, diabetes incidence in BB-DP rats and NOD mice decrease after selective depletion of gut microbes with antibiotics in the postnatal period.<sup>10,11</sup> This indicates that the early life gut microbiota is involved in the development of T1D. Few recent studies have therefore addressed the gut microbiota composition in children developing T1D, and found less abundance of *Clostridium*, *Prevotella*, *Bifidobacterium*, *Roseburia*, and butyrate producers in cases than controls while *Bacteroides* and the phylum Proteobacteria were relatively more abundant.<sup>12-14</sup>

The exact trigger of T1D and the mechanisms behind the protective or predisposing role of gut microbiota are still unknown. Studies in germ-free and gnotobiotic mice have provided

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evidence that stimulation afforded by the immune recognition of microorganisms is required for both pro- and anti-inflammatory mechanisms to evolve.<sup>15</sup> The presence of *Bacteroides fragiles*,<sup>16</sup> probiotic *Bifidobacterium infantis*,<sup>17</sup> *Lactobacillus reuteri*,<sup>18,19</sup> and several *Clostridium* spp. in the gut<sup>20</sup> have for example been shown to increase the number and suppressive activity of regulatory T (Treg) cells; often associated with a decrease in pro-inflammatory cell numbers, pointing toward a profound effect of microbiota on immune homeostasis. Furthermore, Treg cell deficiency seems important in the pathogenesis of T1D.<sup>21–24</sup> Consequently, altered immune regulation in T1D might very well be caused by intestinal dysbiosis, which is most intriguing as stimulation of the mucosal immune system by the gut microbiota may influence the way the immune system discriminates between self- and non-self antigens.

Current evidence on an autoimmune microbiome in human and animal models is still limited. Hence, it is important to identify bacterial markers as possible candidates for early diagnosis and as useful targets in prevention of T1D development. Due to an immense level of complexity that the mammalian gut microbiota vs. immune system cross talk is characterized by, the eventual bacterial markers will most likely not be consistent between various hosts, environments, diets etc. However, an effort should be taken in order to identify those markers even if their predictive value would be solely limited to defined conditions. Emerging evidence indicates that colonization pattern of the gut and early gut microbiota composition have a profound effect on the host's health status later in life. In this study we sought to investigate how the early gut microbiota is associated with changes in immune regulation that could directly link dysbiosis to pathogenesis of autoimmune diabetes.

## Results

### Intestinal and systemic Treg, CD11b<sup>+</sup> dendritic cells (DCs), and IFN $\gamma$ producing T cells are more abundant in non-diabetic NOD mice

The cumulative diabetes incidence in the 46 female NOD mice was 52%. Data collected from some of these mice were used for correlation analyses and results of the correlations between diabetes onset age and host immune markers are presented in Table 1. The significant correlations are furthermore shown in Figure 1. Forkhead box (FoxP3<sup>+</sup>) T cells correlated positively with delayed diabetes onset age in mesenteric lymph node (MLN) and spleen but not in the intestinal lamina propria. Cluster of differentiation (CD) 11b<sup>+</sup> DCs and activated CD40<sup>+</sup> DCs in MLN and spleen were also significantly associated with a late onset age but tolerogenic CD103<sup>+</sup> DCs known to induce Treg were not. Interestingly, small intestinal lamina propria (siLP) interferon (IFN)  $\gamma$  and splenic IFN $\gamma$  but not IL-10 producing T cells correlated positively to a delayed diabetes onset age. All the significant immune parameters were also significant when diabetic mice were statistically compared with non-diabetic mice except for CD40<sup>+</sup> DCs (Table 1).

### Early microbial profiles clustered according to diabetes development

Fecal gut microbiota collected at weaning was used from 13 mice to compare the diabetic with the non-diabetic group. PCo analysis showed significant differences in the early bacterial community composition between NOD mice later classified as diabetic or non-diabetic at 30 weeks of age. The differences were significant for both weighted and unweighted UniFrac distance metrics (Fig. 2; ANOSIM: \* $p < 0.05$ ,  $R = 0.341$  and \* $p < 0.05$ ,  $R = 0.296$  for unweighted and weighted analysis respectively). The abundance of 3 bacterial taxa: unclassified genus from S24–7 family, unidentified taxon from order Bacteroidales, and genus *Prevotella* were significantly increased in the non-diabetic group. The abundance of an unclassified genus from the Lachnospiraceae family were in contrast significantly increased in the diabetic group together with a tendency for more *Oscillospira* and *Ruminococcus* ( $p = 0.05$  and  $p = 0.07$  respectively) compared to the non-diabetic mice (Fig. 2 and Table S1). According to the qualitative analysis (g-test), no genera were categorically different between the 2 categories meaning that no genera were actually lacking in between the groups but it was only the abundance of genera that differed.

The  $\alpha$  diversity measure expressed by the observed species index was significantly increased in the non-diabetic group compared to the diabetic group ( $146 \pm 23$  vs.  $109 \pm 32$  [OTU]; \* $p < 0.05$ ; data not shown), what is in concordance with studies on humans.<sup>13,14</sup>

### Early life gut microbiota correlates with diabetes onset age and immune parameters later in life

Significant results for correlations between all bacterial taxa (genus level) and diabetes onset age are presented in Table 2 and Figure 3. Significant correlations between immune parameters and taxa are also included in Table 2, but those that were not significantly associated with diabetes onset age or did not differ between diabetic and non-diabetic mice are not included in the table. Interestingly, the unclassified genus from Lachnospiraceae family, *Oscillospira*, and *Ruminococcus*, that were all more abundant in diabetic mice, correlated negatively with delayed diabetes onset age which suggests a role of these bacteria in diabetes development. Furthermore, these taxa correlated negatively with the proportion of splenic FoxP3<sup>+</sup>CD4<sup>+</sup> Treg cells and CD11b<sup>+</sup>CD11c<sup>+</sup> DCs, and while Lachnospiraceae correlated further negatively to CD40<sup>+</sup>CD11c<sup>+</sup> DCs in the spleen, *Oscillospira* and *Ruminococcus* correlated negatively to CD40<sup>+</sup>CD11c<sup>+</sup> DCs in MLN. This strongly indicates a diabetes protecting function of these immune cell subsets. In support of this, the unclassified genus belonging to the S24–7 family, the unidentified taxon from order Bacteroidales, and genus *Prevotella* that were all found more abundant in non-diabetic mice also correlated positively with delayed diabetes onset age, splenic FoxP3<sup>+</sup>CD4<sup>+</sup> Treg cells, CD11b<sup>+</sup>CD11c<sup>+</sup> DCs, and CD40<sup>+</sup>CD11c<sup>+</sup> DCs. There was furthermore a tendency for S24–7 and Bacteroidales taxon to positively correlate with splenic IFN- $\gamma$ <sup>+</sup>CD4<sup>+</sup> Th1 cells, while a tendency was evident for Lachnospiraceae and *Oscillospira* to correlate negatively with splenic IFN- $\gamma$ <sup>+</sup>CD4<sup>+</sup> Th1 cells.

**Table 1.** Correlation of immune parameters and diabetes onset age in NOD mice

Parameter	Organ	Group size	Correlation with diabetes onset age <sup>1</sup>		
			P value	R	T-test between diabetic vs. non-diabetic mice <sup>2</sup>
Diabetes onset age	—	46	—	—	—
FoxP3/CD4 Treg	pancreatic lymph nodes	13	0.090	0.489	* < 0.05
FoxP3/CD4 Treg	small intestine lamina propria	18	NS	-0.197	NS
FoxP3/CD4 Treg	colon lamina propria	12	NS	-0.130	NS
FoxP3/CD4 Treg	mesenteric lymph nodes	27	** < 0.01	0.663	*** < 0.001
FoxP3/CD4 Treg	spleen	28	*** < 0.001	0.732	*** < 0.001
CD11b/CD11c DC	spleen	21	*** < 0.001	0.742	*** < 0.001
CD11b/CD11c DC	mesenteric lymph nodes	32	** < 0.01	0.463	** < 0.01
CD40/CD11c DC	spleen	21	* < 0.05	0.480	NS
CD40/CD11c DC	mesenteric lymph nodes	32	* < 0.05	0.374	NS
CD103/11c DC	pancreatic lymph nodes	13	NS	-0.061	NS
CD103/11c DC	mesenteric lymph nodes	28	NS	0.197	NS
INF- $\gamma$ /CD4 Th1	small intestine lamina propria	25	** < 0.01	0.432	*** < 0.001
INF- $\gamma$ /CD4 Th1	spleen	23	*** < 0.001	0.780	** < 0.01
IL-10/CD4	small intestine lamina propria	9	NS	0.244	NS
IL-10/CD4	spleen	12	NS	-0.209	NS

<sup>1</sup>Results of Pearson correlation of the given parameter with diabetes onset age [days]. <sup>2</sup>Results of T test between diabetic and non-diabetic mice for the given parameter.

#### Diabetic and non-diabetic NOD mice cluster separately according to early gut microbiota and correlating immune parameters in a PC analysis

In order to illustrate a combined effect of fecal microbes and the immune system on the development of diabetes a combined Principal component analysis (PCA) analysis was used solely with bacteria suspected to be alleged diabetes markers in early life and significantly correlating immune cell subsets. This illustrated a significant separation between diabetic and non-diabetic mice (Fig. 4, \*\* $p < 0.01$  for PC1). The total variance explained with the first 3 components was 86.1%.

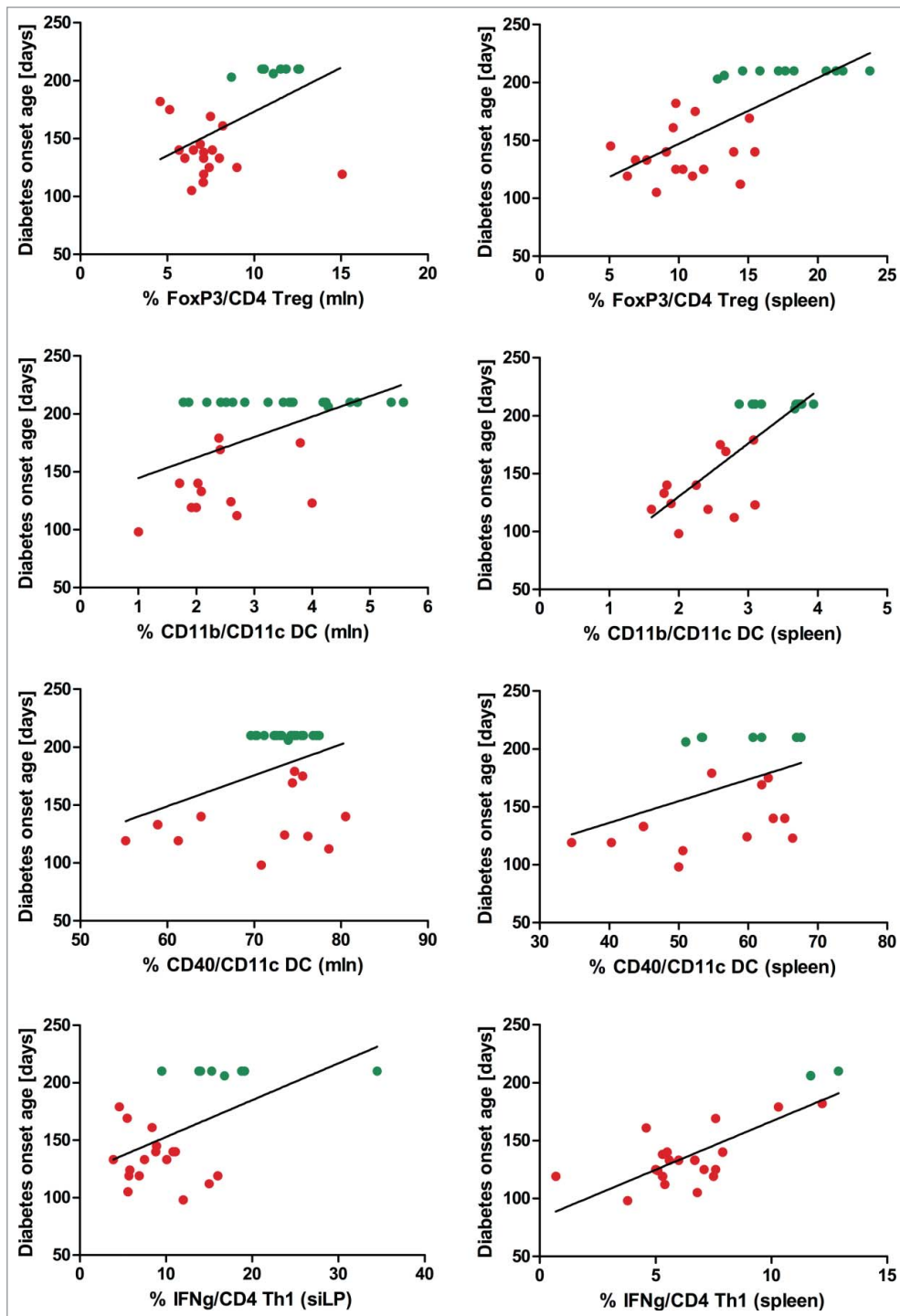
## Discussion

Bacterial markers for T1D may likely be useful in diagnosis and prevention of T1D. The most striking result presented here was the high abundance of S24-7 in feces from non-diabetic NOD mice, whereas the Lachnospiraceae in the Clostriales XIVa cluster was found primarily in the early diabetic mice at weaning. The Lachnospiraceae family was also in pre-diabetic BB-DP rats one of the most prominent differences in the gut microbiota compared with BB-DR rats,<sup>9</sup> but in humans, this taxa decreased over time in cases and was therefore more abundant in controls at the time of diabetes onset.<sup>14</sup> It is however also consistent with a previous study in which non-diabetic NOD mice had significantly fewer microaerophilic bacteria than diabetic NOD mice, primarily due to a difference in the Gram-positive microbiota.

The relevance of these 2 bacterial markers together with the minor but significant differences in *Ruminococcus*, *Oscillospira*, Bacteroidales, and *Prevotella* is further augmented by their simultaneous correlation to systemic FoxP3<sup>+</sup> Treg cells. This is important because a high level of splenic Treg cells was clearly associated with diabetes protection in the NOD mice; an

association that was also found in other rodent studies.<sup>25,26</sup> In addition, jejunal biopsies from T1D patients showed reduced frequency of FoxP3<sup>+</sup> Treg cells and CD103<sup>+</sup> tolerogenic DCs defective of inducing FoxP3<sup>+</sup> Treg cell differentiation.<sup>27</sup> We therefore propose that a microbiota signature characterized by a decrease in S24-7, Bacteroidales, and *Prevotella* and an increase in Lachnospiraceae, *Ruminococcus*, and *Oscillospira* may contribute to the pathogenesis of T1D by decreasing FoxP3<sup>+</sup> Treg cells that protects against diabetes. OTUs classified to S24-7 seem to be largely unexplored in mice models for the time being. De-novo clustering disclosed 243 species level OTUs falling into this taxon, what constituted to nearly 10% of all registered OTUs. Although, correlation performed using species level OTUs have confirmed several OTUs from this family correlating in a similar way, due to relatively shallow sequencing the results could not be cross-validated with a proper subsampling (data not shown). It is however not unlikely that within this broad family only a fraction of OTUs is influencing the result. However, in order to ensure more accurate identification reaching genus or species level within S24-7 family, not only deeper sequencing, but also updates in the databases along with the use of alternative molecular techniques are required. Certain Bacteroidetes species are already known to promote accumulation of Treg cells and has beside its anti-inflammatory properties also been found relatively more abundant in controls than human T1D children.<sup>14,16</sup>

Significantly reduced abundance of *Prevotella* in diabetic patients was previously observed in prediabetic Finnish children<sup>29</sup> and recently confirmed in a cohort of Mexican children suffering from T1D<sup>30</sup> which supports its possible anti-inflammatory properties. In contrast, *Oscillospira* was negatively associated with diabetes development and low level of Treg cells. This is in accordance with a recent study by Everard et al. showing that treatment with probiotic yeast resulted in (inter alia) reduction of *Oscillospira* abundance, what was further linked with reduced



**Figure 1.** Diabetes onset age correlates with Treg, tolerogenic and activated DCs, and IFN $\gamma$  producing (T) cells. Correlation analyses plots between diabetes onset age and immune cell subsets analyzed by flow cytometry in non-diabetic (green) and diabetic (red) NOD mice illustrate significant results presented in **Table 1**. DC = dendritic cells; MLN = mesenteric lymph node; siLP= small intestinal lamina propria; Th = T helper; Treg = regulatory T cells.

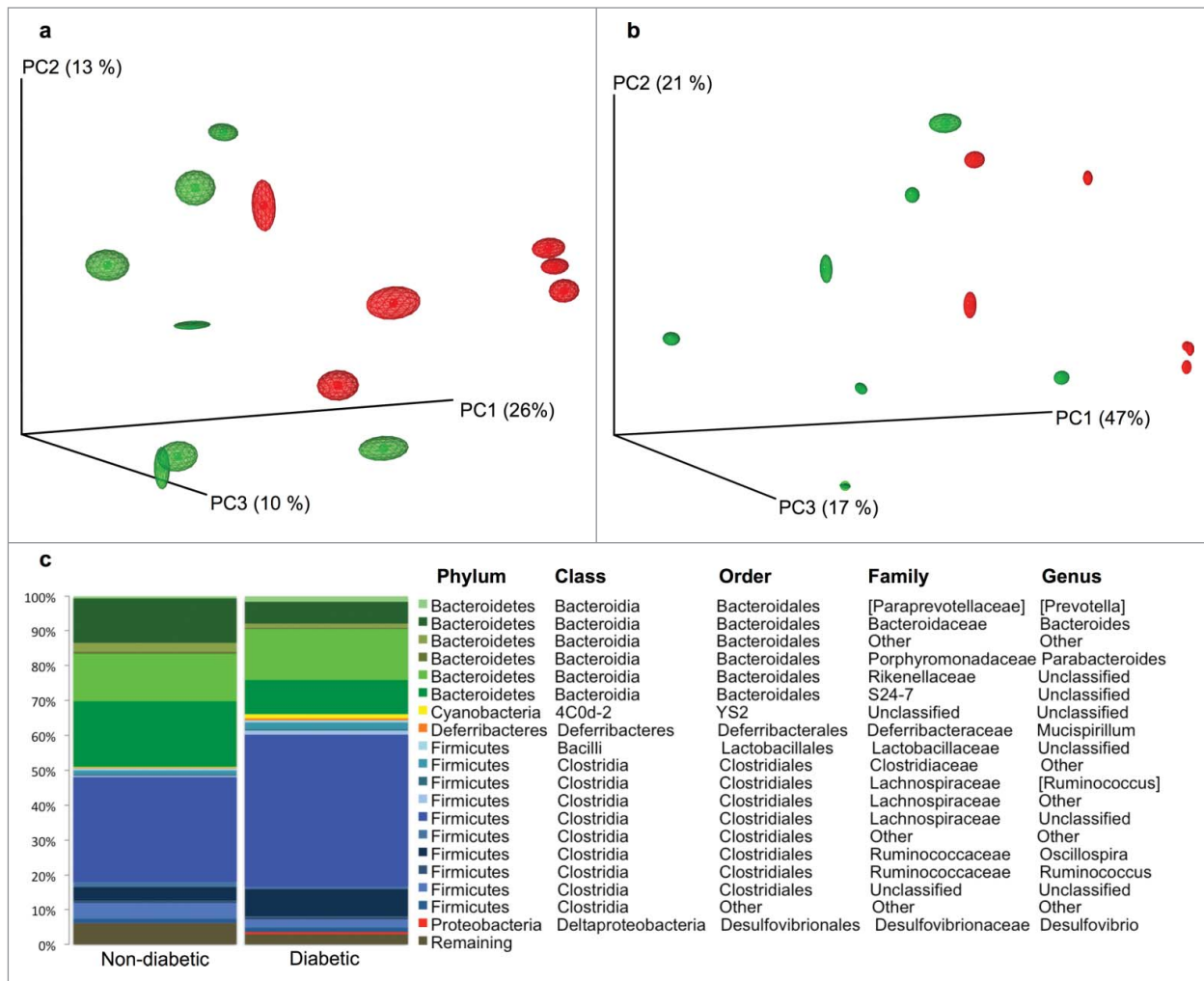
hepatic steatosis, low-grade inflammation, and fat mass in obese and type 2 diabetic mice. Moreover, this clade was positively correlated with the adiposity index and epididymal adipose tissue

complete Adjuvant (CFA) in IFN- $\gamma$  signaling-deficient NOD mice.<sup>32</sup> That study indicated that IFN- $\gamma$  signaling control the susceptibility of pathogenic T cells to the inhibitory activity of

which consolidates its rather negative role in the development of an inflammatory disease state.<sup>31</sup>

All taxa in the early fecal gut microbiota that correlated to diabetes onset age and FoxP3<sup>+</sup> Treg also correlated significantly to CD11b<sup>+</sup> DCs in the spleen and there was a tendency ( $p = 0.07-0.08$ ) for the major bacterial markers S24-7, Lachnospiraceae, and Bacteroidales to also correlate to splenic IFN- $\gamma$  producing T cells. This suggests a combined protective role of these immune cell subsets. This seems in contrast to the fact that IFN- $\gamma$  producing Th1 cells are involved in T1D pathogenesis. However, it is not known whether these IFN- $\gamma$  producing T cells are pathogenic. In parallel, the sexual dimorphism in incidence is lost in IFN- $\gamma$  knock out NOD mice and strikingly, the gene expression pattern in IFN- $\gamma$  signaling pathway was shown to be unique in male specific pathogen free (SPF) NOD mice compared with SPF females and germ-free males and females in which the gender bias in incidence no longer exist.<sup>8</sup> Male PLN and MLN also contained more IFN- $\gamma$  producing T cells, and the authors suggest that IFN- $\gamma$  negatively regulates Th cells required to activate B cells involved in the pathogenesis of T1D. Based on these studies together with our own data, it is likely that the bacterial taxa that strongly correlated with IFN- $\gamma$  producing T cells mediate protection against autoimmune diabetes through induction of excessive IFN- $\gamma$  production as this was characteristic for the non-diabetic NOD mice compared to the diabetic mice; this was significant in both siLP lymphocytes and splenocytes. This hypothesis is further supported by the failure to induce protection with Freund's Com-





**Figure 2.** Early gut microbiota in diabetic and non-diabetic NOD mice differ. PCoA plot based on unweighted (a) and weighted (b) distance matrices calculated from 10 rarefied (1400 reads per sample) OTU tables showing the separation between the fecal gut microbiota at weaning of NOD mice that later in life were diagnosed as diabetic (red,  $n = 6$ ) or non-diabetic (green,  $n = 7$ ). The degree of variation between 10 jackknifed replicates of PCoA is displayed with confidence ellipsoids around each sample. (c) The relative distribution of the major genera (20) determined for diabetic and non-diabetic NOD mice. Taxa denoted as "Unclassified" means that the reference database does not have an official taxonomy for this cluster. Taxa denoted as "Other" (unidentified), indicates ambiguity in the assignment meaning that more than one bacterial taxa could be assigned to this cluster.

Treg cells, which in parallel to our results seem probable as Treg and IFN- $\gamma$  producing T cells simultaneously increased with delayed diabetes onset age. This might also explain why attenuated diabetes incidence in vancomycin-treated NOD mice is accompanied by excessive IFN- $\gamma$  production in siLP lymphocytes.<sup>11</sup>

A positive correlation between delayed diabetes onset age and the proportion of CD11b<sup>+</sup> DCs in MLN and spleen at diabetes onset time indicates a more tolerogenic phenotype of this subset compared with their pathogenic CD11b<sup>-</sup> counterparts that are responsible for breaking peripheral tolerance.<sup>33</sup> An immunosuppressive milieu created by these tolerogenic DCs was previously documented to inhibit autoreactive T cells and suppress autoimmune diabetes in an *in vivo* transfer model.<sup>34</sup> It is, thus, possible that the microbes characteristic for the signature profile of non-

diabetic NOD mice mediate a protective effect through induction of immunosuppressive CD11b<sup>+</sup> DCs in the gut, which migrate to islets to exert its inhibitory effect. However, Kriegel et al. found that the tolerogenic DCs had reduced levels of activation markers CD80 and CD86 which is in contrast to the high level of CD40<sup>+</sup> DCs that in this study correlated positively to delayed diabetes onset age. These cells were however not significantly different between diabetic and non-diabetic NOD mice. Also, another study found an increased proportion of the CD11b<sup>+</sup>CD11c<sup>+</sup> DC subset within spleen in NOD, but not in congenic non-obese diabetes-resistant (NOR) mice, compared with BALB/c and C57BL/6 mice.<sup>35</sup> This was further reinforced by data showing increased percentage of CD11b<sup>+</sup> DCs during insulinitis. Thus, the phenotype of the CD11b<sup>+</sup> DCs associated with late diabetes onset needs further investigation.

**Table 2.** Correlations between given host parameters and fecal gut microbiota taxa at weaning that were significantly associated with diabetes onset

Order	Taxa		Parameter	p value	R	Group size
	Family	Genus				
Clostridiales	Lachnospiraceae	Unclassified	Diabetes onset age	* <0,05	-0,672	13
			FoxP3/CD4 Treg	* <0,05	-0,619	12
			CD11b/CD11c DC	* <0,05	-0,665	13
			CD40/CD11c DC	** <0,01	-0,731	13
Clostridiales	Ruminococcaceae	<i>Oscillospira</i>	Diabetes onset age	* <0,05	-0,583	13
			FoxP3/CD4 Treg	** <0,01	-0,744	12
			CD11b/CD11c DC	* <0,05	-0,615	13
			CD40/CD11c DC (MLN)	* <0,05	-0,661	7
			INF- $\gamma$ /CD4 Th1	0,066	-0,724	7
Clostridiales	Ruminococcaceae	<i>Ruminococcus</i>	Diabetes onset age	* <0,05	-0,586	13
			FoxP3/CD4 Treg	* <0,05	-0,720	12
			CD11b/CD11c DC	* <0,05	-0,685	13
			CD40/CD11c DC (MLN)	* <0,05	-0,614	7
Bacteroidales	S24-7	Unclassified	Diabetes onset age	** <0,01	0,769	13
			FoxP3/CD4 Treg	** <0,01	0,781	12
			INF- $\gamma$ /CD4 Th1	0,081	0,670	7
			CD11b/CD11c DC	* <0,05	0,684	13
			CD40/CD11c DC	* <0,05	0,604	13
Bacteroidales	Prevotellaceae	<i>Prevotella</i>	Diabetes onset age	** <0,01	0,718	13
			FoxP3/CD4 Treg	* <0,05	0,665	12
			CD11b/CD11c DC	* <0,05	0,704	13
			CD40/CD11c DC	0,097	0,502	13
Bacteroidales	Other	Other	Diabetes onset age	** <0,01	0,709	13
			FoxP3/CD4 Treg	** <0,01	0,732	12
			CD11b/CD11c DC	** <0,01	0,735	13
			CD40/CD11c DC	* <0,05	0,651	13

Correlations between splenic or mesenteric lymph nodes (MLN) host parameters at diabetes onset age and taxa relative abundance at genus level (94% sequence similarity OTUs) in samples collected at weaning verified with the Pearson's product-moment correlation coefficient. Results with  $p < 0.1$  are included in the table.

Our proposed model is that bacterial members belonging to S24-7, Bacteroidales, and *Prevotella* act in favor of diabetes protection while Lachnospiraceae, *Ruminococcus*, and *Oscillospira* promote pathogenesis. Their regulatory effect may be mediated through or together with changes in FoxP3<sup>+</sup> Treg cells, CD11b<sup>+</sup> DCs, and IFN- $\gamma$  production. This model is illustrated in the PCA plots that demonstrate how these parameters direct diabetes onset what further indicates the importance of the early life events in the intestinal environment in the development of diabetes.

## Methods

The experiments were carried out in accordance with the Council of Europe Convention European Treaty Series (ETS) 123 on the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, and the Danish Animal Experimentation Act (LBK 1306 from 11/23/2007). The study was approved by the Animal Experiments Inspectorate, Ministry of Justice, Denmark.

### Animals and diet

NOD/BomTac mice (Taconic, NY, USA) were purchased and mated in a 2 y time interval, and an overall number of 46

female offspring were group-housed at our barrier animal facility (Faculty of Health and Medical Sciences, University of Copenhagen, Frederiksberg, Denmark) under standard conditions and used for the analyses. Mice had free access to Altomin 1324 diet (Brogarden, Lyngø, Denmark) and water. From ten weeks of age, measurements of tail blood glucose commenced twice a week. A mouse was considered diabetic and killed when blood glucose levels exceeded 12 mmol/l on 2 consecutive days. At the age of 30 weeks (210 days), all remaining mice were killed by cervical dislocation. Two mice were diabetic at 30 weeks of age (203 and 206 days) but were included in the non-diabetic group throughout the analyses as these 2 mice were very late diabetic and thus resembled the non-diabetic group in both microbiota and immune parameters. The diabetes incidence for male mice was very low and data on diabetic males were therefore too insufficient to include.

### Cell Isolation and Flow Cytometry

Immediately after the mice were killed when diagnosed as diabetic or at 30 weeks of age, organs were placed in cold PBS. Single cell suspensions from spleen, MLN, PLN, and siLP were prepared, PMA/ionomycin stimulated, and analyzed by flow cytometry as described previously.<sup>11</sup> The following immune cell subsets were analyzed as follows: Tregs: % FoxP3<sup>+</sup> cells in CD4<sup>+</sup> cell gate, tolerogenic DCs: % CD11b<sup>+</sup> cells or %

CD103<sup>+</sup> cells in CD11c<sup>+</sup> cell gate, activated DCs: % CD40<sup>+</sup> cells in CD11c<sup>+</sup> cell gate, Th1 cells: % IFN- $\gamma$ <sup>+</sup> producing cells in CD4<sup>+</sup> cell gate, and suppressive T cells: % IL-10<sup>+</sup> producing cells in CD4<sup>+</sup> cell gate. All dead cells were first gated out using 7-AAD viability staining kit (eBioscience, San Diego, CA) except on intracellular stained cells. All antibodies were purchased from eBiosciences. The immunological analyses were performed using an Accuri C6 flow cytometer and software (Accuri Cytometers Inc., Ann Arbor, MI). Same template with fixed gates was used in each analysis to ensure reproducibility between test days. The number of samples collected and used in the correlation analyses for each immune cell subset is shown in **Table 1**. The analyses were performed with Pearson correlation coefficient.

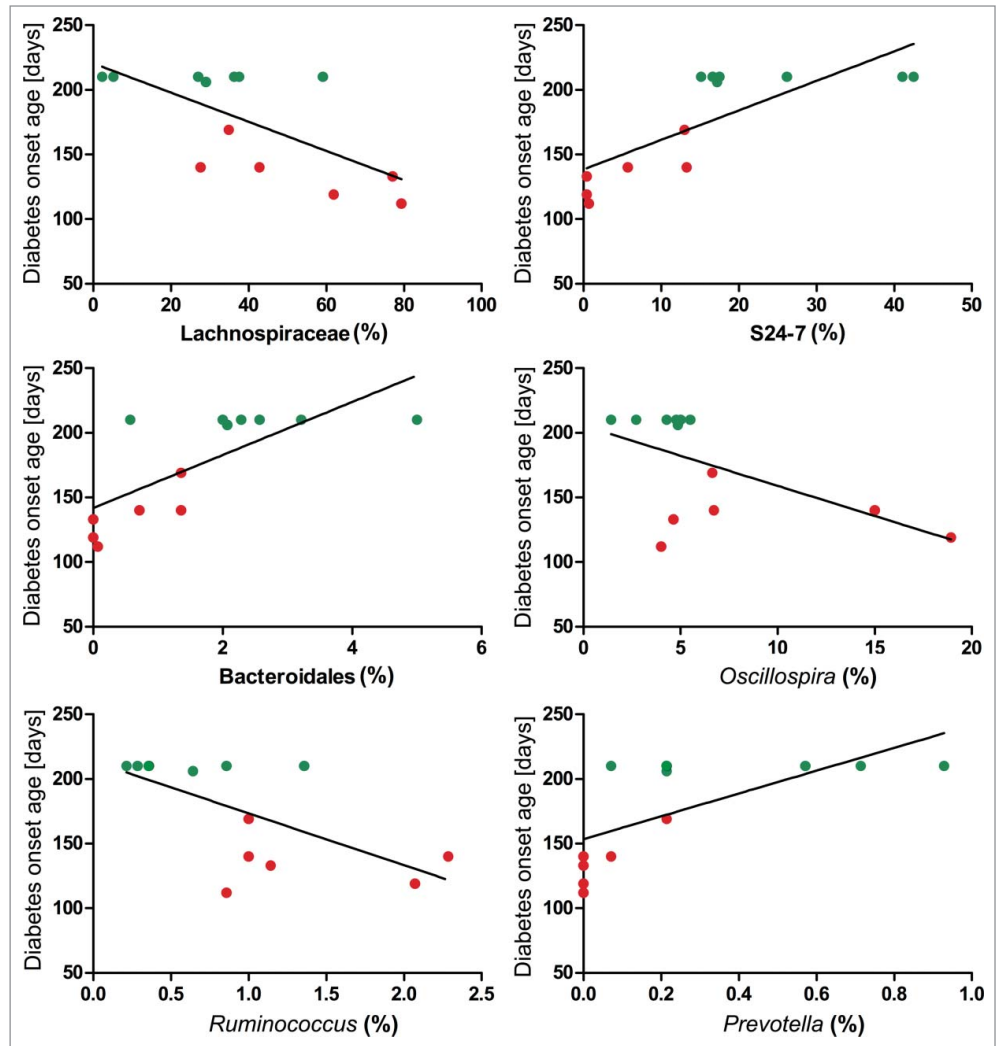
#### Tag-encoded pyrosequencing

Feces samples from 16 NOD mice were collected at 4 weeks of age at weaning. The composition of the bacterial community of feces samples was determined using tag-encoded 454/FLX Titanium (Roche) pyro-sequencing of the V3 and V4 region of the 16S rRNA (rRNA) gene by the National High Throughput DNA Sequencing Center, University of Copenhagen, Denmark as described in Hansen et al.<sup>11</sup>

#### Pyrosequencing data analysis

Quantitative Insight Into Microbial Ecology (QIIME version 1.7.0) was used to analyze the pyrosequencing data (NCBI accession numbers: no. SRS471978, no. SRS270214, no. SRS2704 (25)-SRS2704(35), and no. SRS2704(38)-SRS2704(40)).

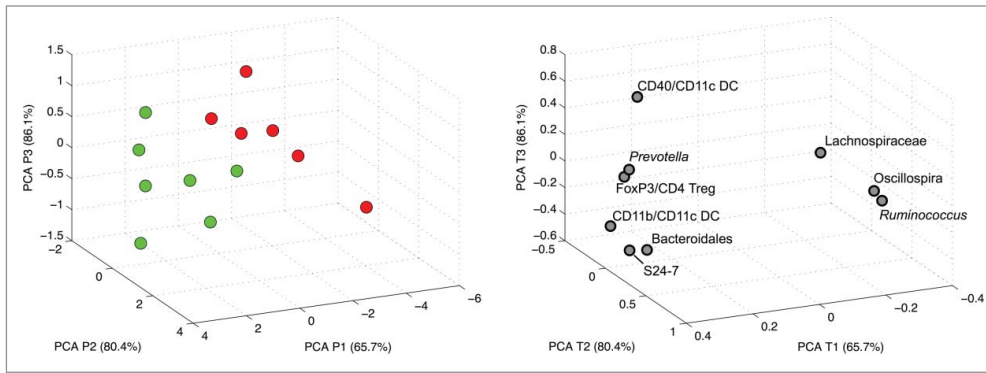
Initial analysis steps such as quality control, de-noising, chimera filtering and operational taxonomic unit (OTU) picking were conducted as previously described.<sup>36</sup> High quality sequences purged from chimeric reads were clustered at 97% relatedness using UCLAST.<sup>37</sup> The representative sequences from each cluster were aligned with pyNAST<sup>38</sup> and subjected to the Greengenes reference database (version 12.10). For each category of data sets, a set of 1000 subsampled OTU tables was generated in order to unify the number of reads per sample.



**Figure 3.** Gut microbial taxa correlates significantly to diabetes onset age. Correlation analyses plots between diabetes onset age and gut microbial taxa analyzed by 454 pyro-sequencing in non-diabetic (green,  $n = 7$ ) and diabetic (red,  $n = 6$ ) NOD mice illustrate significant results presented in **Table 2**. Testing was performed based on 1000 rarefied OTU tables unified to an equal number of reads per sample (1400).

#### Taxa differences

Determining differences in taxa composition between gut microbiota of diabetic and non-diabetic mice was performed using `otu_category_significance.py` script. The G test of independence (`g_test`) and ANOVA determining respectively: qualitative (presence/absence) and quantitative (relative abundance) association of OTUs in the given category, were calculated based on 1000 subsampled OTU tables rarefied to an equal number of reads (1400 per sample). Principal coordinate analysis (PCoA) plot was generated with Jackknifed Beta Diversity workflow based on 10 unweighted UniFrac distance metrics calculated using 10 subsampled OTU tables. The minimum number of sequences taken for each jackknifed subset was set to 1400. Two samples were withdrawn from the plot due to low reads number. Analysis of similarities



**Figure 4.** PCA using selected alleged bacterial and host immune markers. PCA scores (a) of non-diabetic (green,  $n = 7$ ) and diabetic (red,  $n = 6$ ) NOD mice and the corresponding PCA loadings (b) based on taxa and immune parameters that significantly correlated to diabetes onset age.

(ANOSIM) was used to evaluate group differences using unweighted UniFrac distance metrics that were generated based on rarefied (1400 reads per sample) OTU tables. Alpha diversity measure expressed with an observed species (sequence similarity 97% OTUs) value was computed for rarefied OTU tables (1400 reads per sample) using  $\alpha$  rarefaction workflow. Testing on the differences in  $\alpha$  diversity was conducted using t-test employing the non-parametric (Monte Carlo) method (999 permutations) that is implemented in the compare  $\alpha$  diversity workflow.

### Microbiota correlation analysis

Correlations between host parameters at diabetes onset age and taxa relative abundance summarized to the genus level in samples collected at weaning were verified with the Pearson's product-moment correlation coefficient implemented in the `otu_category_significance.py` script (QIIME 1.7.0). Testing was performed based on 1000 rarefied OTU tables unified to an equal number of reads per sample that were constituting 85% of the most read-indigent sample within the subset. An in-house Matlab (Mathworks) script was used to examine all vectors from subsampled OTU tables with a Jarque-Bera test for normal distribution. One sample was excluded from all microbiota analyses as an

outlier because its PCoA values were below mean  $\pm 2 \times$  SD of all samples and changed significant results to/from non-significant results.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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### Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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