Streptozotocin-induced type-1-diabetes disease onset in Sprague–Dawley rats is associated with an altered intestinal microbiota composition and decreased diversity

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INTRODUCTION

Type-1-diabetes (T1D) is an organ-specific autoimmune disease involving the selective destruction of insulin-producing pancreatic β-cells. While the pathophysiology of T1D is not yet entirely clear, it is the genetic predisposition of an individual combined with environmental factors which initiate an autoimmune response (Boerner & Sarvetnick, 2011). Genetically, the human leukocyte antigen (HLA) complex is the strongest predictor of T1D risk, whereby after HLA, the strongest susceptibility locus is in the insulin gene itself (Noble & Erlich, 2012). Over time, genetic susceptibility activates both the innate and adaptive immune systems but remains subclinical, until approximately 80% of the β-cell mass is destroyed (Notkins & Lernmark, 2001). Over time, β-cell destruction results in insulin deficiency, worsening hyperglycemia and an eventual call for insulin therapy. As the incidence of T1D is increasing at a rate far beyond the rate of population growth, additional emphasis has been placed on the contribution of environmental factors towards disease onset (Ehehalt et al., 2010; Patterson et al., 2001; Vaarala et al., 2008). Indeed, the intestinal microbiota is an environmental factor that cannot be overlooked as a contributor towards T1D development or a factor that is significantly altered as a result of T1D development.

Abbreviations: HLA, human leukocyte antigen; OTU, operational taxonomical unit; SCFA, short-chain fatty acid; STZ, streptozotocin; T1D, type-1-diabetes.

Five supplementary figures and three supplementary tables are available with the online Supplementary Material.
Many studies have linked alterations in the intestinal microbiota composition with conditions such as obesity (Ley et al., 2006, 2005; Murphy et al., 2010; Turnbaugh et al., 2006), inflammatory bowel disease (Greenblum et al., 2012), colitis (Bellavia et al., 2013; Klimesova et al., 2013) and T1D (Giongo et al., 2011; Murri et al., 2013; Roesch et al., 2009). Moreover, risk of T1D onset in childhood is higher in children delivered by Caesarean section (Cardwell et al., 2008), where there is also altered microbiota composition (Dominguez-Bello et al., 2010). Previous studies have examined the role of the intestinal microbiota in the development of T1D but these are often limited due to the models used i.e. genetically pre-disposed animals (Roesch et al., 2009) and humans (Brown et al., 2011; de Goffau et al., 2013; Giongo et al., 2011; Murri et al., 2013) who develop T1D, with conflicting results. Oral administration of the probiotic compound VSL#3 to non-obese diabetic mice prevented spontaneous T1D development (Calcinaro et al., 2005), while antibiotics have also been shown to prevent autoimmune diabetes in bio-breeding diabetes-prone rats (Brugman et al., 2006). Thus, the intestinal microbiota have demonstrated a capacity to influence diabetes and become altered due to disease development (Atkinson & Chervonsky, 2012; King & Sarvetnick, 2011).

Originally derived from cultures of *Streptomyces achromogenes*, streptozotocin (STZ) has been clinically used as a chemotherapeutic agent in the treatment of pancreatic β-cell carcinoma (Lenzen, 2008). STZ produces rapid pancreatic β-cell destruction, resulting in hypoinsulinemia and hyperglycemia. The selectivity for β-cells is associated with preferential accumulation of the chemical in these cells after entry through glucose transporter 2, whereby chemical structural similarity with glucose allows STZ to bind to this receptor. It is currently unclear what the direct effect of STZ induced T1D development and progression is on the intestinal microbiota composition of the host. To this end, in this study, diabetes was induced in male Sprague–Dawley rats through a single intraperitoneal injection of STZ. The impact of T1D disease onset and progression on intestinal microbiota composition and microbial fermentation products was investigated. DNA was isolated from faecal pellets at weeks 0, (pre-STZ injection in one of the groups), 1, 2 and 4, and from caecal content at week 5 from T1D and healthy control rats and prepared for 16S rRNA pyrosequencing.

**METHODS**

**Assessment of antimicrobial activity of STZ.** Given the microbial origin of STZ, it was important to first assess its own antimicrobial activity. Bioactivity of STZ (Sigma) was assessed by well-diffusion assays. For this purpose, molten agar was cooled to 48 °C and seeded with fresh overnight cultures of various Gram-positive (*Bifidobacterium, Lactobacillus and Listeria*) and Gram-negative (*Escherichia coli and Cronobacter*) bacterial strains. Twenty millilitres of inoculated medium was dispensed into sterile Petri dishes, allowed to solidify and dried. Wells (4.6 mm in diameter) were then bored in the seeded agar plates and 50 μl volumes of various concentrations (7.5, 4, 2 and 1 mg STZ ml⁻¹) of freshly prepared STZ in filter-sterilized sodium citrate buffer (50 mM, pH 4.5) to be assayed were dispensed into the wells. Petri dishes were incubated accordingly for each of the strains used.

**Animals and treatment.** Male Sprague–Dawley rats were purchased (Harlan Laboratories) at 5 weeks of age and housed under barrier-maintained conditions within the Biological Services unit, University College Cork (UCC). Rats were allowed to acclimatise for 1 week before the start of the study and were fed *ad libitum* with Teklad Global Standard Rodent Diet (#2018S; Harlan Laboratories) with free access to water at all times. Rats were housed in groups of five per cage and kept in a controlled environment at 25 °C under a 12 h light/12 h dark cycle. The rats were divided into two groups: a non-diabetic healthy control group and an STZ-induced T1D group. After 1 week of acclimatization, T1D was induced in the latter group by injection of a single dose (60 mg STZ kg⁻¹) of freshly prepared STZ (Sigma) administered via the intraperitoneal cavity, according to a previously described method (Wu & Huan, 2008). Glucose levels were measured in triplicate using a Contour Next glucometer (Bayer) in blood samples collected from a tail vein each week. Rats with blood glucose higher than 200 mg glucose dl⁻¹ were considered diabetic and STZ induced rats with lower glucose levels were excluded from the rest of the study (n=10 rats per group). Body weight was assessed and fresh faecal pellets were collected weekly. After 5 weeks, the rats were killed by decapitation. Caecal content was removed from individual rats and flash-frozen immediately on dry ice. All samples were stored at −80 °C until processed. All experiments were conducted in accordance with the European Directive 86/609/EEC, the Recommendation 2007/526/EC, and approved by the Animal experimentation Ethics Committee of UCC. All efforts were made to minimize animal suffering and to reduce the number of animals used.

**DNA isolation from faecal pellets and caecal content and high-throughput amplicon sequencing.** Total DNA was isolated from fresh individual rat faecal pellets collected at weeks 0 (pre-STZ-induced diabetes in the T1D group), 1, 2 and 4 post-STZ injection and from the individual caecal content of all rats, 5 weeks following STZ injection using the QIAamp DNA Stool Mini kit (Qiagen) (n=10 per group). Furthermore, total DNA was extracted from faecal pellets at weeks 0, 1, 2 and 4, and from caecal content at week 5 from rats which were injected with STZ but that did not develop T1D to assess the antimicrobial activity of STZ (n=2). DNA extraction was coupled with an initial bead-beating step (three times, 30 s). Isolated DNA was then stored at −20 °C until further processing. The microbiota composition of the samples was established by amplicon sequencing; universal 16S rRNA primers estimated to bind to 94.6 % of all 16S genes, flanking the V4 region [i.e. the forward primer F1 (5'-AYTGGYDTAAAGNG-3') and a combination of four reverse primers: R1 (5'TACCCGCGGTGTCTAACTCC-3'), R2 (5'TACCATGATATTTCTAATC-3'), R3 (5'TACACDSRGGMTTCTAATC-3') and R4 (5'TACANNGGTTACTAATC) (Ribosomal Database Project’s Pyrosequencing Pipeline: http://pyro.cme.msu.edu/pyro/help.jsp)] were employed for PCR amplification. Molecular identifier tags were attached between the 454 adaptor sequence and the target-specific primer sequence, allowing for identification of individual sequences from the pooled amplicons. Ampure purification system (Beckman Coulter) was used to clean the amplicons prior to being sequenced on a 454 Genome Sequencer FLX platform (Roche Diagnostics), in line with 454 protocols at the Teagasc high-throughput sequencing centre.

**Bioinformatics sequence analysis.** Quality trimming of sequence reads was performed using the Qiime suite of tools (version 1.7) (Caporaso et al., 2010); reads shorter than 200 bp, reads that failed to reach a quality score of 25 and those that were not exact matches to barcode tags and primer sequence were discarded. A locally installed version of SILVA 16S rRNA database (version 106) (Pruesse et al., 2007) was used to BLAST (Altschul et al., 1997) the trimmed sequence reads against the SILVA 16S rRNA database (version 106).
using default parameters. Resulting BLAST output files were parsed through MEGAN (Huson et al., 2007); similar to previous studies a bit-score cut-off of 86 was selected (Rea et al., 2011; Urich et al., 2008). Clustering of sequence reads into operational taxonomical units (OTUs) at 97% identity and subsequent alignments were achieved using QIIME suite software tools (Caporaso et al., 2010). ChimeraSlayer was used to remove chimeric OTUs from aligned OTUs and the FastTreeMP tool reconstructed a phylogenetic tree (Haas et al., 2011; Price et al., 2010). Alpha and beta diversities were calculated on the sequence reads. Principal co-ordinate analyses were performed on the samples and viewed within KING viewer (Chen et al., 2009).

**Short-chain fatty acid (SCFA), lactic and formic acid analysis of caecal content.** SCFA analysis was performed as previously described (Wall et al., 2012). Briefly, caecal content was vortex-mixed with MilliQ water, incubated at room temperature for 10 min and centrifuged to pellet bacteria and other solids. The supernatant was filtered, transferred to a clear GC vial and 2-ethylbutyric acid was added as an internal standard. The concentrations of SCFA were measured using a Varian 3800 GC flame-ionization system, fitted with a ZB-FAP column (30 m x 0.32 mm x 0.25 μm; Phenomenex). The initial oven temperature was set at 100 °C for 0.5 min, raised to 180 °C at 8 °C min⁻¹ and held for 1 min, then increased to 200 °C at 20 °C min⁻¹, and finally held at 200 °C for 5 min. The temperature of the injector and the detector were set at 240 °C and 250 °C, respectively. Helium was used as the carrier gas at a flow rate of 1.3 ml min⁻¹. A standard curve was built with different concentrations of a standard mix containing acetic acid, propionic acid, n-butyric acid and iso-butyric acid (Sigma). Peaks were integrated by using the Varian Star Chromatography Workstation, version 6.0 software. Formic acid and lactic acid were measured using formic acid and DL-lactate commercial kits (Megazyme).

**Statistical analysis.** All results are presented as means ± SEM (per group). To assess whether differences between treatment groups were significant, the unpaired Student’s t-test was used with the use of GraphPad Prism version 6.0 for Windows (GraphPad Software). Statistical significance was accepted at P<0.05. Compositional data were statistically analysed using the non-parametric Mann–Whitney and Kruskal–Wallis tests (Kruskal & Wallis, 1952) to estimate the relationships between different groups using GraphPad Prism version 6.0. Statistical significance was accepted at P<0.05.

**RESULTS**

**Antimicrobial activity of STZ**

Initially, the antimicrobial activity of different concentrations of STZ was tested against Gram-positive (*Bifidobacterium*, *Lactobacillus* and *Listeria*) and Gram-negative (*E. coli* and *Cronobacter*) bacterial strains. At a concentration of 7.5 mg STZ ml⁻¹, STZ produced a clear zone of inhibition of strains *Lactobacillus bulgaricus*, *Lactobacillus plantarum*, *Lactobacillus gasseri* and *Bifidobacterium infantis* which decreased at concentrations of 4, 2 and 1 mg STZ ml⁻¹ *(data not shown)*. A concentration of 7.5 mg STZ ml⁻¹ had a bacteriostatic effect on *Bifidobacterium breve*, *Lactobacillus acidophilus*, *E. coli* and *Cronobacter sakazakii*, which decreased at concentrations of 4, 2 and 1 mg STZ ml⁻¹ *(data not shown)*. No zone of inhibition was observed for any concentration of STZ on *Bifidobacterium bifidus* and *Listeria monocytogenes* *(data not shown)*.

**Induction of T1D by STZ injection in rats**

One week after STZ injection, rats were severely diabetic as indicated by elevated blood glucose levels (572.6 ± 10.3 mg glucose dl⁻¹), compared with the normoglycemic range observed in healthy controls (104.2 ± 2.1 mg glucose dl⁻¹) *(P<0.05; Fig. 1a)*. The chronic increases in blood glucose levels were maintained throughout the study period *(Fig. 1a)*. Diabetic rats exhibited polyuria, polydipsia, hyperphagia and excessive fecal output *(data not shown)*. Diabetic rats displayed significant decreases in percentage body weight gain from week 1 onwards *(following STZ injection)*, compared with healthy controls *(P<0.05; Fig. 1b)*.

**STZ induced T1D decreases α-diversity of the rat intestinal microbiota over time**

A total of 777 796 reads were sequenced, corresponding to a total of 388 898 reads per group and an average of 7857 reads per rat. At the 97% similarity level, a total of five metrics were used to estimate α-diversity; Shannon index, Simpson index, Chao1 richness estimation (Figs. S1–S3, available in the online Supplementary Material), phylogenetic diversity and observed species *(Fig. 2)*. At week 0, pre-STZ injection and T1D induction in one of the groups, no differences were observed across any of the five metrics calculated between the groups *(Figs 2 and S1–S3)*. This indicated that all rats in the T1D group had similar levels of diversity pre-STZ injection. However, 1, 2, 4 and 5 weeks post-STZ injection, Chao1, phylogenetic diversity and observed species were all significantly reduced in the T1D group compared with healthy controls *(P<0.05; Figs 2 and S1)*. While Shannon diversity data revealed no change to the biodiversity of the microbiota within the healthy controls and the T1D group at week 2, significant reductions to the diversity profile were found between the T1D group and the healthy controls at all other time points taken *(P<0.05; Fig. S2a, b)*. Similarly, the Simpson diversity index-based analysis, which also takes into account the number of species present and the relative abundance of each species, highlighted a significant reduction in diversity in animals induced with T1D at 1, 4 and 5 weeks following STZ injection, compared with healthy controls *(P<0.05; Fig. S3a, b)*. Rarefaction curves were seen to be parallel *(Fig. S4)*, indicating that additional sampling would yield a limited increase in species richness.

**β-Diversity highlights an STZ-induced T1D effect on microbial population variation over time as disease progresses**

Principal co-ordinate analyses, based on unweighted Unifrac distances of the 16S rRNA sequences, illustrated changes to the microbial populations following STZ-induced T1D, compared with healthy controls. Fig. 3 shows data points corresponding to healthy controls *(red)* cluster closely together between weeks 0, 1, 2, 4 and 5 regardless of the time point. Similarly, Fig. 3 also shows
Impact of STZ-induced T1D on microbiota composition

Fig. 1. (a) Blood glucose levels of healthy control and T1D rats (n=10 per group) and (b) Percentage body weight gain over the eight-week trial. At week 3, T1D was induced in one of the groups by injection of a single dose of STZ, administered via the intra-abdominal cavity. Error bars represent SD.

Taxonomical analysis highlights the effect of STZ-induced T1D on intestinal microbial composition over time

Taxonomy based analysis of the assigned sequences highlighted few differences in the intestinal microbiota populations between the groups at week 0, pre-STZ injection. While no significant differences were observed at the phylum level at week 0 (Fig. 4), significantly lower relative proportions of Clostridiaceae (P<0.05; Fig. 5) and Clostridium (P<0.05; Fig. 6) were found at the family and genus levels, respectively, while the proportions of Parabacteroides were significantly increased in the group yet to be induced with T1D, compared with the control group (P<0.05; Fig. 6).

Analysis of the microbial profile of the intestine at week 1 (post-STZ injection in one of the groups) revealed many statistically-significant differences between the groups at all levels. While the relative abundance of the Tenericutes population was increased in the T1D group (P<0.05; Fig. 4), the proportions of Actinobacteria and Deferribacteres were reduced (P<0.05; Fig. 4), compared with healthy controls. Most notably, just 1 week after STZ injection, the proportions of Bacteroidetes were increased (P<0.05; Fig. 4) in the T1D group, while the Firmicutes population was reduced (P<0.05; Fig. 4), compared with healthy controls. At the family level, the proportions of S24-7, Prevotellaceae, Erysipelotrichaceae, Porphyromonadaceae and Anaeroplasmataceae were increased in the T1D group (P<0.05; Fig. 5), while Bifidobacteriaceae and Peptococcaceae were reduced, compared with healthy controls (P<0.05; Fig. 5). At the genus level, both the proportions of Parabacteroides and Mucispirillum were increased (P<0.05; Fig. 6) while the proportions of Ruminococcus were reduced (P<0.05; Fig. 6) in the T1D group, compared with healthy controls.

While the significant changes in the relative proportions of certain intestinal microbiota populations at week 2 remained the same as week 1, many more changes to the intestinal microbiota were observed at the family and genus levels 2 weeks post-STZ-induced T1D, compared with healthy controls. At the phylum level, the proportions of Firmicutes remained lower (P<0.05; Fig. 4), while the proportions of Deferribacteres increased (P<0.05; Fig. 4) in the T1D group, compared with healthy controls. Similar to week 1, the proportions of Prevotellaceae and Erysipelotrichaceae remained higher while Peptococcaceae remained lower in the T1D group, compared with healthy controls (P<0.05; Fig. 5), while the proportions of Porphyromonadaceae shifted from being higher at week 1 to lower in the T1D group at week 2 (P<0.05; Fig. 5). Progression of T1D increased the proportions of Lactobacillaceae, Peptostreptococcaceae and Clostridiaceae (P<0.05; Fig. 5), while reducing the proportions of RF-9 gut group (P<0.05; Fig. 5), after 2 weeks in the T1D group when compared with healthy controls. At the genus level, the proportions of Ruminococcus and Mucispirillum remained lower (P<0.05; Fig. 6) and higher (P<0.05; Fig. 6), respectively, in the T1D group.

data points corresponding to T1D rats (blue) initially cluster close together with healthy controls (red) at week 0, since T1D had not been induced at this time point. However, subsequent data points corresponding to the T1D rats (blue), as the trial progressed from week 1 to 5 following T1D induction, became increasingly distant from the healthy control data points (red). Individual data points within particular time points for the T1D group (blue) became much more dispersed and failed to form distinct clusters with each other (Fig. 3). These results are in line with the α-diversity and taxonomical data presented since data points corresponding to T1D rats cluster away from those corresponding to healthy controls in all time points from week 1 onwards (post-STZ injection). Furthermore, Fig. S5 shows data points corresponding to the STZ-injected group that did not develop T1D superimposed on the principal co-ordinate analyses plot of Fig. 3. Green data points correspond to this group (n=2) over the five time points and highlight that STZ injection did not alter the intestinal microbiota composition after week 0 (data points corresponding to this group cluster well together and do not disperse after STZ injection).
Fig. 2. Phylogenetic diversity (a) and observed species (b) metrics of $z$-diversity between healthy control and T1D rats ($n=10$ per group) at weeks 0, 1, 2, 4 and 5. The non-parametric Mann–Whitney test was used to estimate the relationship between the groups. Statistical significance was accepted at $P<0.05$. **$P<0.01$, ***$P<0.001$. 

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group, similar to week 1, compared with healthy controls. Significant increases in the proportions of *Bacteroides*, *Lactobacillus*, *Turicibacter* and *Clostridium* were also observed 2 weeks after STZ-induced diabetes \( (P < 0.05; \text{Fig. 6}) \), compared with healthy controls. *Parabacteroides* were reduced 2 weeks after STZ-induced T1D, compared with healthy controls \( (P < 0.05; \text{Fig. 6}) \). The proportions of *Ruminococcaceae incertae sedis*, one of the most dominant populations at the genus level, was reduced at week 2 in the T1D group, compared with healthy controls \( (P < 0.05; \text{Fig. 6}) \).

Four weeks after STZ-induced T1D, the relative proportions of *Tenericutes*, *Actinobacteria* and *Proteobacteria* were higher in the T1D group \( (P < 0.05; \text{Fig. 4}) \), compared with healthy controls. The abundance of *Firmicutes* remained lower in the T1D group, compared with healthy controls \( (P < 0.05; \text{Fig. 4}) \). At the family level, *Prevotellaceae* recovered in the healthy controls, while *Lactobacillaceae* and *Clostridiaceae* remained higher \( (P < 0.05; \text{Fig. 5}) \), and *Peptococcaceae* and RF-9 gut group remained lower in the T1D group, compared with healthy controls \( (P < 0.05; \text{Fig. 6}) \). The abundance of *Porphyromonadaceae* increased in the T1D group, compared with healthy controls \( (P < 0.05; \text{Fig. 5}) \). *Bacteroidaceae*, *Bifidobacteriaceae* and *Alcaligenaceae* proportions increased \( (P < 0.05; \text{Fig. 5}) \), while *Ruminococcaceae* and *Lachnospiraceae* proportions decreased \( (P < 0.05; \text{Fig. 5}) \), compared with healthy controls. Correlating with the changes seen at the family level, the abundances of *Parasuterella*, *Bifidobacterium*, *Lactobacillus* and *Clostridium* were all increased \( (P < 0.05; \text{Fig. 6}) \), while the proportions of
Alistipes, Ruminococcus and Ruminococcaceae incertae sedis were reduced \((P<0.05;\text{ Fig. 6})\) in the T1D group when compared with healthy controls.

Data highlighting the intestinal microbiota changed 5 weeks following T1D induction, compared with healthy controls, corresponding to microbiota populations from the caecal content of the rats after they had been killed. Thus, week 5 data were graphed separately from the other time points as variations between the microbiota of faecal and caecal content may skew results. The proportions of Actinobacteria and Proteobacteria remained higher compared with healthy controls \((P<0.05;\text{ Fig. 7a})\). The abundance of Deferribacteres decreased \((P<0.05;\text{ Fig. 7a})\) in the T1D group, compared with healthy controls. Similar to week 4, the proportions of Bacteroididaeae, Bifidobacteriaceae and Alcaligenaceae were increased \((P<0.05;\text{ Fig. 7b})\), while Ruminococcaceae and Peptococcaceae were reduced \((P<0.05;\text{ Fig. 7b})\) in the T1D group, compared with healthy controls. The proportions of Rikenellaceae and Peptostreptococcaceae were also reduced \((P<0.05;\text{ Fig. 7b})\) in the T1D group, compared with healthy controls, while Erysipelotrichaceae was higher \((P<0.05;\text{ Fig. 7b})\), in the T1D group, compared with healthy controls. At the genus level, Parasutterella, Bifidobacterium, Bacteroides and Phascolarctobacterium were higher \((P<0.05;\text{ Fig. 7c})\), while the proportions of Alistipes, Ruminococcus, Ruminococcaceae incertae sedis, Peptostreptococcaceae incertae sedis and Mucispirillum were lower \((P<0.05;\text{ Fig. 7c})\) in the T1D group, compared with healthy controls.

Throughout the study, from week 0 to 5, the relative populations of the intestinal microbiota at phylum, family and genus levels fluctuated only slightly within the healthy control group (Table S1). In contrast, the proportions of the intestinal microbiota at all levels in the T1D-induced group fluctuated significantly at all weeks following T1D induction (Table S2). Most notably, the relative proportions of Actinobacteria and Proteobacteria were higher \((P<0.05;\text{ Table S2})\) at the later stages of T1D development, compared with week 0 (pre-STZ injection). In addition, while the intestinal microbiota of rats injected with STZ but did not develop T1D fluctuated between the time points taken, changes to the relative abundance did not reflect the same changes observed in the STZ-induced T1D group and were most likely natural fluctuations caused by environmental factors (Table S3).

**STZ-induced T1D altered the production of SCFA and lactic acid**

T1D was associated with a significant decrease in caecal propionate \((P<0.05)\) and butyrate \((P<0.05)\) and a significant increase in caecal acetate \((P<0.05)\) and lactate levels \((P<0.05)\), compared with healthy controls (Fig. 8). No differences in formate were exhibited between the groups (Fig. 8).

**DISCUSSION**

This study provides a comprehensive account of the impact STZ-induced T1D on the intestinal microbiota composition, diversity and microbial fermentation metabolite production over time. The antimicrobial effect of STZ has previously been described (Vavra et al., 1973), however, very little is understood of the antimicrobial impact a single intraperitoneal injection of STZ could have on intestinal microbiota composition. In man, STZ has a circulating half-life of only 15 min, following intravenous infusion (Schein et al., 1973) or 30–40 min following intravenous bolus injection (Adolphe et al., 1975). Approximately 70% of \(^{14}\text{C}\)-labelled-STZ administered intravenously to rats in single doses of 70 mg STZ kg\(^{-1}\) was recovered in the urine over the first 6 h, thus indicating rapid excretion (Karunanayake et al., 1976). It is therefore unlikely that the changes to microbiota composition found in this study were caused by the antimicrobial activity of STZ and were most likely a result of T1D onset and progression. Further, the antimicrobial activity of STZ was assessed on a separate group of rats that received the STZ injection but did not develop T1D. Their failure to develop T1D excluded them from the study as they did not meet

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**Fig. 6.** Genus level distributions of the microbial communities either significantly increased or decreased in the faecal content of T1D rats, compared with healthy controls \((n=10\text{ per group})\) between weeks 0, 1, 2 and 4. The non-parametric Mann–Whitney test was used to estimate the relationship between the groups. Statistical significance was accepted at \(P<0.05\). Vertical bars represent significant increases and decreases in the percentage relative abundances in the microbiota composition in the T1D group versus healthy controls.

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the requirements of the T1D group; however, DNA was isolated from faecal pellets collected from these rats ($n=2$) at each time point and caecal content at week 5. The STZ toxin did not appear to have a strong antimicrobial effect on these rats and reflected changes to the microbiota composition did not correlate with the changes seen in the T1D group over time. Despite the low sample size, these rats provided a valuable dataset on the antimicrobial activity of STZ.

The data revealed that STZ-induced T1D increased the Bacteroidetes:Firmicutes ratio, as previously demonstrated in various T1D models (de Goffau et al., 2013; Giongo et al., 2011; Murri et al., 2013). Initially, pre-T1D induction, no differences in the Bacteroidetes:Firmicutes ratio was observed between the groups. In contrast, the intestinal relative proportions of Actinobacteria and Proteobacteria were significantly increased at weeks 4 and 5 following T1D induction, compared with healthy controls. Interestingly, the proportions of Proteobacteria are often negatively associated with inflammatory bowel conditions (Sartor & Mazmanian, 2012). The Bacteroidetes:Firmicutes ratio has frequently been associated with the obese phenotype, with a greater capacity to extract energy from the diet (Murphy et al., 2010). In addition, work carried out recently by Liou et al. (2013) demonstrated conserved shifts in the intestinal microbiota composition resulting from weight loss following Roux-en-Y gastric bypass surgery (Liou et al., 2013). While T1D rats in this study did experience dramatic weight loss following diabetes induction, due to the high loss of calories derived from sugar in the urine and the consequential breakdown of fat

**Fig. 7.** Phylum (a), family (b) and genus (c) level distributions of the microbial communities either significantly increased or decreased in the caecal content of T1D rats, compared with healthy controls ($n=10$ per group) at week 5. The non-parametric Mann–Whitney test was used to estimate the relationship between the groups. Statistical significance was accepted at $P<0.05$.

**Fig. 8.** Caecal content SCFA, lactate and formate concentrations in T1D and healthy control rats ($n=10$ per group). Unpaired Student’s t-test was used to compare the levels of caecal acetate, propionate, butyrate, lactate and formate between the groups. Values are means ± SEM, represented by vertical bars. Statistical significance was accepted at $P<0.05$. *$P<0.05$, **$P<0.01$, ***$P<0.001$. 

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cells for energy, they also experienced hyperphagia in an attempt to maintain body weight. Changes in the microbiota coincide with the loss of body weight in the T1D rats following 1 week of diabetes development, suggesting that the changes to body weight may have affected microbiota composition. However, since weight loss is a symptom of untreated T1D, both factors should be considered in this scenario as insulin therapy was not administered.

Sequencing technologies have highlighted a positive association between a diverse microbiota and health. Decreases in the number and abundance distribution of distinct intestinal microbes has been linked with various intestinal diseases (Manichanh et al., 2006; Sartor, 2008; Turnbaugh et al., 2009) and, in particular, T1D (Brown et al., 2011; de Goffau et al., 2013; Giongo et al., 2011; Knip et al., 2011). Microbial diversity was significantly decreased just 1 week post-STZ-induced T1D and failed to recover during disease progression. Principal co-ordinate analyses revealed a temporal decrease in diversity in T1D rats, compared with healthy controls. As disease progressed over the 5 weeks, the individual microbiota populations of T1D rats became less diverse, compared with healthy controls and failed to cluster together distinctly, suggesting vast alterations in the intestinal environmental conditions created following T1D onset and progression, compared with healthy controls. Interestingly, the phylogenetic diversity and observed species z-diversity metrics demonstrated a reduction in diversity between weeks 0 and 1 compared with week 4, in the healthy group with age as a possible contributing factor towards this. Age-related decreases in intestinal microbial diversity have previously been described (O’Toole & Claesson, 2010). It is likely that the intestinal environmental conditions created following T1D onset decreased microbial diversity, creating an opportunity for members of the phyla Actinobacteria and Proteobacteria to thrive. These data correlated with previous observations (Giongo et al., 2011), that T1D was associated with decreased abundances of Ruminococcaceae and Lachnospiraceae and increased abundances of Bacteroidaceae, compared with healthy controls at the later stages of T1D development. However, in contrast to this study, which found Porphyromonadaceae significantly more abundant in healthy control children, the data generated in this study revealed that the proportions of Porphyromonadaceae were higher in T1D rats, 1 and 4 weeks after T1D induction and lower at week 2, compared with healthy controls. This highlights temporal variations to intestinal microbiota composition in response to T1D progression. The relative proportions of Bifidobacteriaceae, Lactobacillaceae and Clostridiaceae were all increased in T1D rats at the later stages of T1D progression.

The intestinal surface barrier is one of the most important components of the innate immune system (Vaara et al., 2008), whereby T1D compromises intestinal integrity (Carratù et al., 1999; Kuitunen et al., 2002; Sapone et al., 2006). Abnormalities of the intestinal barrier, the so-called ‘leaky gut’, expose the intestinal immune system to antigens in the setting of T1D (Vaara et al., 2008). An impaired intestinal microbiota composition in children pre-diagnosed with autoimmune diabetes has previously been associated with altered microbial fermentation metabolite production (Brown et al., 2011). Butyrate producers such as Eubacterium, Fusobacterium, Anaerostipes and Roseburia were higher in the faeces of a control group of healthy children. Lactic acid producers such as Lactobacillus, Lactococcus, Bifidobacterium and Streptococcus were higher in the faeces of children pre-diagnosed with autoimmune diabetes (Brown et al., 2011). The fate of lactate is crucial in determining intestinal health as conversion to butyrate results in mucin synthesis (Barcelo et al., 2000; Burger-van Paassen et al., 2009; Finnie et al., 1995; Shimotoyodome et al., 2000) and tighter junctions (Peng et al., 2007, 2009), while conversion to other SCFA, such as propionate and acetate does not induce mucin synthesis (Brown et al., 2011). Mucin is a glycoprotein made by the host that maintains the integrity of intestinal epithelium (Brown et al., 2011). In addition, butyrate contributes to colonic health through its anti-inflammatory properties (Hamer et al., 2008; Louis & Flint, 2009; Pryde et al., 2002) and decreases bacterial transport across metabolically stressed epithelia (Louis & Flint, 2009), thus preventing the development of the so-called ‘leaky gut’. The data in this study revealed increases in the abundances of lactic acid producing bacteria such as Bifidobacterium and Lactobacillus in T1D rats, as were the concentrations of lactate and acetate, while butyrate was decreased in the caecum of T1D rats, compared with healthy controls. Furthermore, the proportions of Bacteroides were higher 2 and 5 weeks after T1D onset, compared with healthy controls. Bacteroides have recently been implicated in both diabetic animal and human studies (Brown et al., 2011; Brugman et al., 2006; de Goffau et al., 2013; Giongo et al., 2011; Roesch et al., 2009). Such studies have highlighted an association between Bacteroides and enhanced intestinal bacterial translocation, resulting in β-cell autoimmunity, associated with T1D (de Goffau et al., 2013). Bifidobacteria have previously been associated with an inhibition of bacterial translocation (Duffy, 2000; Romond et al., 2008; Wang et al., 2004) and it has recently been hypothesized (de Goffau et al., 2013) that bifidobacteria may inhibit the translocation and growth of Bacteroides as they compete for space and/or adherence (Stecher & Hardt, 2008) and nutrients (Gibson et al., 1996). Furthermore, bifidobacteria enhance the intestinal epithelial barrier function (Lièvin et al., 2000) by increasing the thickness of the mucus layer (Kleessen & Blaut, 2005; Kleessen et al., 2003). Thus, increased proportions of Bifidobacterium in the T1D group, as seen in this study at the later stages of diabetes progression may enhance intestinal epithelial barrier function. This study highlighted temporal variations in the intestinal microbiota of T1D rats. Although these data did not provide any data on functional changes related to the...
observed bacterial diversity, the results provide an extensive account of the impact STZ-induced T1D onset and progression has on the intestinal microbiota composition, diversity and microbial metabolite production in the host. Moreover, the data clarified that the changes observed in the intestinal microbiota of T1D rats resulted from disease development only and did not have any causative role in the onset of diabetes.

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