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Influence of GABA and GABA-producing Lactobacillus brevis DPC 6108 on the development of diabetes in a streptozotocin rat model

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Received: 16 October 2015 / Accepted: 26 January 2016
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RESEARCH ARTICLE

Abstract
The aim of this study was to investigate if dietary administration of γ-am inobutyric acid (GABA)-producing Lactobacillus brevis DPC 6108 and pure GABA exert protective effects against the development of diabetes in streptozotocin (STZ)-induced diabetic Sprague Dawley rats. In a first experiment, healthy rats were divided in 3 groups (n=10/group) receiving placebo, 2.6 mg/kg body weight (bw) pure GABA or L. brevis DPC 6108 (~109 microorganisms). In a second experiment, rats (n=15/group) were randomised to five groups and four of these received an injection of STZ to induce type 1 diabetes. Diabetic and non-diabetic controls received placebo [4% (w/v) yeast extract in dH2O], while the other three diabetic groups received one of the following dietary supplements: 2.6 mg/kg bw GABA (low GABA), 200 mg/kg bw GABA (high GABA) or ~109 L. brevis DPC 6108. L. brevis DPC 6108 supplementation was associated with increased serum insulin levels (P<0.05), but did not alter other metabolic markers in healthy rats. Diabetes induced by STZ injection decreased body weight (P<0.05), increased intestinal length (P<0.05) and stimulated water and food intake. Insulin was decreased (P<0.05), whereas glucose was increased (P<0.001) in all diabetic groups, compared with non-diabetic controls. A decrease (P<0.01) in glucose levels was observed in diabetic rats receiving L. brevis DPC 6108, compared with diabetic-controls. Both the composition and diversity of the intestinal microbiota were affected by diabetes. Microbial diversity in diabetic rats supplemented with low GABA was not reduced (P>0.05), compared with non-diabetic controls while all other diabetic groups displayed reduced diversity (P<0.05). L. brevis DPC 6108 attenuated hyperglycaemia induced by diabetes but additional studies are needed to understand the mechanisms involved in this reduction.

Keywords: streptozotocin, type-1 diabetes, probiotic, γ-am inobutyric acid

1. Introduction
Type 1 diabetes (T1 D) is a chronic and progressive disorder in which genetically susceptible individuals may develop an autoimmune response leading to pancreatic β-cell damage and insulin insufficiency (Atkinson and Eisenbarth, 2001). Recent research has described an altered intestinal microbiome in individuals with type 1 diabetes (Brown et al., 2011; De Goffau et al., 2013; Giongo et al., 2011; Murri et al., 2013; Patterson et al., 2015) and type 2 diabetes (Cani, 2012; Karlsson et al., 2012; Qin et al., 2012; Zhang et al., 2013). Insulin administration is the main therapeutic strategy for T1 D and although there have been improvements in treatment achieving euglycaemia without risk of severe hypoglycaemia requires strict control of glucose levels (Atkinson and Eisenbarth, 2001). γ-am inobutyric acid (GABA) may constitute a very effective therapy for T1 D. Soltani et al. (2011) have previously demonstrated that injections of GABA not only promoted β-cells proliferation, but also inhibited immune responses, reversing the disease in severely diabetic mice. GABA had also previously demonstrated its effectiveness towards preventing type 2 diabetes progression by improving glucose tolerance and insulin sensitivity in high fat diet induced obese mice (Tianet et al., 2011 b). Furthermore, GABA-producing bacteria have been isolated from several food sources and from the human gut (Barrett et al., 2012; Park and Oh, 2007; Soo et al., 2013). GABA is the major inhibitory neurotransmitter in the central nervous system (CNS). In peripheral tissues, GABA acts not only as a neurotransmitter in the enteric and parasympathetic nervous systems but also as a hormone in non-neuronal tissues (Erdő, 1992). In the endocrine pancreas, GABA is produced by β-cells and once released, activates ionotropic GABAA and metabotropic GABAB receptors in both α- and β-cells (Bonaventura et al., 2008; Dong et al., 2006). Through a paracrine signal, GABAreleased from β-cells inhibits glucagon release from α-cells (Bailey et al., 2007), whereas an autocrine signal increasesor decreases insulin secretion from β-cells, depending on extracellular glucose levels (Dong et al., 2006). GABA is produced through the conversion of L-glutamate by the enzyme glutamate decarboxylase and
different types of bacteria produce GABA as a response to adverse acidicoenvironmental conditions (Cotter et al., 2001; Jung and Kim, 2003; Sanders et al., 1998). Indeed, potential probiotic bacteria isolated from the human gastrointestinal tract have been shown to efficiently convert glutamate to GABA in vitro (Barrett et al., 2012). A disturbed microbiota is a potential environmental factor contributing toward metabolic disease, affecting the immune system and even influencing the perception of pain and behaviour (Brown et al., 2011; Cryan and Dinan, 2012; Lam et al., 2011). Interestingly, the use of dietary strategies, such as probiotics and prebiotics, may be effective for the alleviation of these disorders through the production of bioactive compounds that may act directly or indirectly on host metabolism (Lytte, 2011). Thus, considering the role of GABA on glucose homoeostasis and the evidence showing how probiotics may affect host health, we investigated the impact of oral administration of pure GABA and GABA-producing bacteria Lactobacillus brevis DPC 6108 on the protection against the development of T1D in streptozotocin (STZ)-induced diabetic rats.

2. Materials and methods

**Animals**

All experimental procedures were performed in accordance with the protocols approved by the University College Cork Ethics Committee, under a license issued from the Department of Health and Children. Male Sprague-Dawley rats, 5 weeks of age, were obtained from Harlan UK (Loughborough, UK) and housed under barrier-maintained conditions. All animals were allowed to acclimatise for one week prior to commencement of the experiment. Animals were group-housed, with 5 animals per cage at standard conditions (room temperature of 21 °C, with a 12-h light-dark cycle, lights on at 07:00). All groups were fed ad libitum with Teklad Global rodent standard diet (#2018S; Harlan Laboratories, Loughborough, UK) and allowed free access to food and water. Water containing either L. brevis DPC 6108, GABA (Sigma Aldrich, Arklow, Ireland) and/or placebo freeze-dried powder was the only watersupply provided to the animals throughout the experiment. Bottles were replaced daily. Two experiments were conducted in this study – Experiment 1 using healthy rats and Experiment 2 using STZ-induced diabetic rats.

**Lactobacillus brevis DPC 6108 and placebo preparation**

L. brevis DPC 6108 is an efficient GABA producer, with maximum conversion in vitro when growing on DeMan Rogosa and Sharpe (MRS; Difco Laboratories, BD, Franklin Lakes, NJ, USA) broth supplemented with 30 mg/ml monosodium glutamate (MSG) (Barrett et al., 2012). Rifampicin-resistant variants of L. brevis DPC 6108 were isolated by spread-plating ~109 cfu from an overnight culture (1% inoculum) onto MRS agar (Difco Laboratories) containing 500 μg rifampicin/ml (Sigma Aldrich Ireland) and stored at -80 °C. Before freeze-drying, frozen stock vials were plated in MRS agar and a single colony was isolated for inoculation in 10 ml fresh MRS broth supplemented with 30 mg/ml MSG. The culture was incubated overnight at 37 °C under anaerobic conditions and then inoculated into 1 l MRS broth containing MSG and allowed to grow overnight at 37 °C under anaerobic conditions. The overnight culture aliquots were then inoculated into large volumes of MRS containing MSG and allowed to grow overnight at 37 °C under anaerobic conditions. The overnight culture aliquots were then inoculated into large volumes of MRS containing MSG and allowed to grow overnight at 37 °C under anaerobic conditions. The overnight culture aliquots were then inoculated into large volumes of MRS containing MSG and allowed to grow overnight at 37 °C under anaerobic conditions. The overnight culture aliquots were then inoculated into large volumes of MRS containing MSG and allowed to grow overnight at 37 °C under anaerobic conditions.

**Faecal sample microbial analysis and GABA production assay**

Fresh faecal samples were taken every week for microbial analysis in order to verify if the strain survived gut transit. Rats were receiving enough bacterial cells and there was no cross-contamination to the groups not receiving the strain. Microbial analysis involved enumeration of the L. brevis DPC 6108 strain after plating serial dilutions on MRS agar supplemented with 100 μg rifampicin/ml (Sigma Aldrich Ireland) and incubating anaerobically for 48 h at 37 °C. In addition, isolated colonies were tested for GABA production as described previously (Barrett et al., 2012). Briefly, isolated colonies were grown anaerobically in MRS containing 3% (w/v) MSG at 37 °C for 55 h. Samples were then deproteinised by mixing equal volumes of 24% (w/v) trichloroacetic acid and culture, allowed to stand for 1 h and centrifuged at 1,400 x g for 10 min. Supernatants were removed and diluted with 0.2 mol/l sodium citrate buffer, pH 2.2 to yield 250 nmol of each amino acid residue. Samples were then diluted with the internal standard, norleucine, to give a final concentration of 125 nmol/ml. Amino acids were quantified using a Jeol
Effects of GABA powder and Lactobacillus brevis DPC 6108

Experiment 1 – effects on healthy rats

Animals were divided into 5 groups with 15 rats per group: non-diabetic control group and diabetic control group received only placebo freeze-dried powder [4% (w/v) yeast extract], diabetic low dose GABA group received 2.6 mg/kg bw GABA powder, diabetic high dose GABA group received 200 mg/kg bw GABA powder, and diabetic L. brevis DPC 6108 group received 109 bacterial cells in 4% (w/v) yeast extract. GABA, L. brevis DPC 6108 and placebo freeze-dried powders were diluted in fresh drinking water every day throughout the 9-week trial period. Every group preceived 4% (w/v) yeast extract as this solution was used as cryoprotectant while freeze-drying L. brevis DPC 6108. On experimental week 3, type 1 diabetes was induced in rats in four of the groups by intraperitoneal injection with a single dose of 60 mg/kg STZ (≥75% α-anomer basis; SigmaAldrich) freshly prepared in 50 mM sodium citrate buffer (pH 4.5) and injected within 10 to 15 min after dissolution, according to a previously described procedure (Wu and Huan, 2008). The non-diabetic control group received an injection of citrate buffer only. Seven days post STZ injection, glucose levels were measured in triplicate using a Contour Next blood glucose meter (Bayer) in blood samples collected from a tail vein. Rats with glucose levels higher than 200 mg/dL were considered diabetic and STZ-induced rats that had lower glucose level were excluded. From a total of 60 rats treated with STZ, three died 1-2 days after injection, whereas ten rats did not develop diabetes and were excluded. In the remaining 47 rats, body weight and glucose levels were assessed weekly, while food intake and water consumption were measured daily. After 9 weeks of dietary intervention rats were killed by decapitation and blood samples were collected, allowed to clot at 4 °C, centrifuged for 20 min at 2,000×g and the serum collected into clean microtubes. Liver was removed and flash-frozen on dry ice. All samples were stored at -80 °C prior to analyses.

Serum analyses

Commercial kits were used for measurement of GABA and metabolic markers in serum. GABA was determined using the GABA research ELISA kit (Invitech Ltd., Huntingdon, UK). Glucose was determined using the Quantichrom glucose assay (BioAssay Systems, Hayward, CA, USA). Triglycerides were measured using EnzyChrom Triglyceride Assay kit (BioAssay Systems) and cholesterol using LabAssay Cholesterol kit (Wako, Japan). Insulin, glucagon, c-peptide, peptide YY (PYY), active glucagon-like peptide-1 (GLP-1), gastric inhibitory polypeptide (GIP) and leptin were measured using Rat Metabolic Hormone Magnetic Bead Panel (Merck Millipore, Schwalbach am Taunus, Germany).

Liver analyses

Hepatic lipids were extracted according to the method of Folch et al. (1957). After extraction, samples were dried under a stream of nitrogen and resuspended in 5% (v/v) solution of Triton X-100 in distilled water. Triglyceride concentration was determined using the commercial kit EnzyChrom Triglyceride Assay (BioAssay Systems) and cholesterol using LabAssay Cholesterol kit (Wako, Japan).

Microbial DNA extraction, 16S rRNA amplification and Illumina MiSeq sequencing

Caecal contents were collected from individual rats 6 weeks post-STZ injection. Total metagenomic DNA was extracted from caecal contents with the QIamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) where an additional bead beating step was incorporated into the protocol. Extracted DNA was quantified using the Nanodrop 1000 spectrophotometer (Thermo Scientific, Dublin, Ireland). The V3-V4 variable region of the 16S rRNA gene was amplified from each extracted DNA sample according to the 16S metagenomics sequencing library protocol (Illumina, San Diego, CA, USA). Initially the template DNA was amplified using primers specific to the V3-V4 region of the 16S rRNA gene which also allowed for the Illumina overhang adapter, where, the forward primer (5TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG) and reverse primer
were used. Each PCR reaction contained 2.5 μl DNA template, 5 μl forward primer (1 μM), 5 μl reverse primer (1 μM)/Sigma Aldrich) and 12.5 μl Kapa HiFi Hotstart Readymix(2×) (Kapa Biosystems Inc., Wilmington, MA, USA). The template DNA was amplified under the following PCR conditions for a total of 25 cycles: 95 °C for 3 min and 30 s respectively (initialisation and denaturation), 55 °C for 30 s (annealing), 72 °C for 30 s (elongation) followed by a final elongation period of 5 min. A negative control reaction, whereby the template DNA was replaced with PCR grade water, was employed to confirm lack of contamination and PCR products were visualised using gel electrophoresis (1 x-tris-acetate-EDTA buffer, 1.5% agarose gel, 1 00v) post-PCR reaction. Successful amplicons were then cleaned using the AMPure XP purification system (Beckman and Coulter, Takeley, UK). A second PCR reaction was then completed using the previously amplified and purified DNA as the template. Two indexing primers (Illumina Nextera XT indexing primers, Illumina) were used per sample to allow samples to be pooled, sequenced on one flow cell and subsequently identified bioinformatically. Each reaction contained 25 μl Kapa HiFi HotStart ReadyMix (2×), 5 μl template DNA, 5 μl index 1 primer (N7xx), 5 μl index 2 primer (S5xx) and 10 μl PCR grade water. PCR conditions were the same as previously described with the samples undergoing just 8 cycles instead of 25. PCR products then underwent the same electrophoresis and cleaning protocols as described above. Samples were then quantified using the Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA) in conjunction with the high sensitivity DNA quantification assay kit (Life technologies, Carlsbad, CA, USA). All samples were then pooled to an equimolar concentration and the pool underwent a final cleaning step. Quality of the pool was determined using the Agilent Bioanalyser prior to sequencing. The sample pool was then denatured with 0.2M NaOH, diluted to 4 pM and combined with 10% (v/v)/denatured 4 pM PhiX. Samples were then sequenced inhouse on the MiSeq sequencing platform at the Teagasc Next Generation Sequencing Platform Centre (Teagasc Food Research Centre, Moorepark, Ireland) using a 2x300cycle V3 Kit following protocols as described by Illumina.

Bioinformatic analysis
300 base pair paired-end reads were joined using FLASH (fast length adjustment of short reads to improve genoassemblies) (Magoc and Salzberg, 2011). Reads were further processed with the inclusion of quality filtering based on a quality score of >20 followed by subsequent removal of sequences below length threshold using QIIME (Caporaso et al., 2010b). USEARCH v7 (64-bit) was then used for denoising and chimera detection ad well as clustering into operational taxonomic units (OTUs) at 97% identity (http://www.drive5.com/usearch). PyNAST (Caporaso et al., 2010a) was used to align OTUs and taxonomy was assigned using BLAST (Altschul et al., 1990) against the SILVA SSURef database release 111 (Quast et al., 2013). QIIME was used to generate alpha (Shannon, Simpson, Chao1, observed species and phylogenetic diversity) and beta diversities, (Bray Curtis, Weighted and UniWeighted-Unifrac) distance matrices (http://qiime.org). Principal coordinate analysis (PCoA) plots were generated based on the beta diversity distance matrices and were visualised using EMPorer v0.9.3-dev (Vazquez-Baeza et al., 2013).

Statistical analysis
To assess whether differences between treatment groupswere significant, statistical analysis was performed by using one factor ANOVA followed by Tukey’s or Dunnett’s post hoc multiple comparison tests (GraphPad Prism Software (Version 6.0), San Diego, CA, USA). Non-parametric Kruskall-Wallis analysis using Dunn’s multiple comparison test was carried out to determine statistically significant taxonomic differences between groups (GraphPad Prism).

3. Results
Survival of Lactobacillus brevis DPC 6108 through the gastrointestinal tract
Quantification of the numbers of administered rifampicin resistant L. brevis strain in the faeces of rats confirmed its survival during gastrointestinal transit. Stool recovery of L. brevis DPC 6108 was ~1.1×10^7 cfu/g faeces after 1 week of feeding and remained at similar numbers until the end of experiments. Colonies isolated from the plates were tested for GABA production in order to assess if gastric transit affected ability of L. brevis DPC 6108 to produce GABA. All colonies tested produced similar quantities of GABA invitro when compared to the wild type strain (average of 5.8 mg/ml, similar to the wild type (5.5 mg/ml)). There was no cross-contamination between groups as L. brevis DPC 6108 was not detected in the stool of rats not receiving the strain.

Effects of Lactobacillus brevis DPC 6108 on metabolic markers of healthy rats
After 5 weeks supplementation with L. brevis DPC 6108, no differences in body weight gain, final body weight, liver cholesterol and serum glucose were observed (data not shown). However, serum insulin levels were increased (P<0.05) in rats receiving L. brevis DPC 6108, when compared with rats in the GABA supplemented and control groups (Table 1).
Effects of GABA and Lactobacillus brevis DPC 6108 supplementation in induced-diabetic rats

Effects on body weight, small intestine length, water and food intake

Post-STZ injection, rats that developed diabetes continued to lose body weight until the end of the experiment. Final body weights were significantly reduced in all diabetic rats, compared with non-diabetic control rats (P<0.001). Dietary supplementation with GABA and L. brevis DPC 6108 did not improve weight gain in diabetic rats. The abdomens of diabetic rats were distended, filled with a swollen intestine and small intestinal length was significantly longer, compared with those of non-diabetic control rats (P<0.001). Moreover, mean daily food and water consumption were significantly higher in diabetic rats, compared with the non-diabetic control group (P<0.001), with no significant differences between treatment groups observed (Table 2). Diabetic rats exhibited polyuria and excessive faecal output.

Effects on GABA levels in serum

Serum analysis revealed a significant increase in GABA levels in the diabetic-high GABA group, compared with the diabetic-low GABA, diabetic L. brevis DPC 6108 and both diabetic and non-diabetic control groups (P<0.05; Table 3).

Effects on serum glucose levels and other metabolic markers

Serum glucose levels were significantly increased in all diabetic groups, compared with the non-diabetic control group (P<0.001). However, lower glucose levels were observed in rats receiving L. brevis DPC 6108 supplementation relative to rats in the diabetic control group (P<0.01). Insulin and C-peptide levels were significantly decreased, whereas glucagon was increased in all diabetic groups, compared with the non-diabetic control group (P<0.05; Table 3). GLP-1 and GIP serum levels were not affected in any of the groups. PYY was 4- to 5-fold significantly higher in diabetic rats, compared with the non-diabetic control group (P<0.05), with no differences between treatments. Leptin is a hormone produced by adipose cells. Therefore, probably due to the lower adipose tissue mass in diabetic rats, serum leptin levels were significantly decreased in all diabetic groups, compared with the non-diabetic control group (P<0.001; Table 3).

**Table 1.** Final body weight and metabolic markers concentrations in healthy rats receiving γ-aminobutyric acid (GABA) powder, Lactobacillus brevis DPC 6108 or placebo for 5 weeks (n=10 per group).^a^

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>GABA</th>
<th>L. brevis DPC 6108</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final body weight (g)</td>
<td>385.3±7.1</td>
<td>380.5±1.1</td>
<td>378.3±7.7</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>99.1±4.8</td>
<td>94.3±5.0</td>
<td>104.1±7.2</td>
</tr>
<tr>
<td>Insulin (mg/dL)</td>
<td>1.5±0.194</td>
<td>1.5±0.194</td>
<td>2.5±0.399</td>
</tr>
<tr>
<td>Liver cholesterol (mg/dL)</td>
<td>1.88±0.15</td>
<td>2.16±0.22</td>
<td>1.71±0.13</td>
</tr>
</tbody>
</table>

^a^All values are means ± standard error of the means. Values in the same row with different superscript letters are significantly different, P<0.05 (ANOVA followed by post hoc Tukey’s multiple comparisons tests).

**Table 2.** Final body weight (bw), small intestine length, food and water intake in rats after streptozocin injection (n=10 per group).^a^

<table>
<thead>
<tr>
<th></th>
<th>Non-diabetic control</th>
<th>Diabetic control</th>
<th>Low GABA</th>
<th>High GABA</th>
<th>L. brevis DPC 6108</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final bw (g)</td>
<td>372.6±7.9</td>
<td>297.0±6.8</td>
<td>297.6±6.8</td>
<td>297.2±5.9</td>
<td>238.7±8.8</td>
</tr>
<tr>
<td>Small intestine length (cm)</td>
<td>98.6±2.8</td>
<td>129.6±4.9</td>
<td>133.8±6.9</td>
<td>135.3±7.9</td>
<td>132.2±4.7</td>
</tr>
<tr>
<td>Average food intake (g/100 g bw)</td>
<td>6.0±1.1</td>
<td>14.3±0.4</td>
<td>12.8±0.9</td>
<td>13.6±0.4</td>
<td>12.7±0.9</td>
</tr>
<tr>
<td>Average water intake (ml/100 g bw)</td>
<td>12.1±1.1</td>
<td>72.2±2.5</td>
<td>68.7±1.9</td>
<td>66.3±2.0</td>
<td>62.6±3.9</td>
</tr>
</tbody>
</table>

^a^All values are means ± standard error of the means. Values in the same row with different superscript letters are significantly different, P<0.05 (ANOVA followed by post hoc Tukey’s multiple comparisons tests).

^1^GABA = γ-aminobutyric acid
Effects on serum cholesterol

Serum cholesterol was significantly higher in diabetic rats supplemented with the high dose of GABA, compared with rats in the diabetic and non-diabetic control groups (P<0.05). No differences in liver cholesterol were observed between groups. Serum and liver triglyceride levels were significantly higher in all diabetic groups, compared with the non-diabetic control group (P<0.05). No significant differences were observed in serum and liver triglyceride levels between treatment groups (Table 3).

Effects on microbial diversity and microbial composition

The microbial composition in caecal contents of individual rats was elucidated through high-throughput DNA sequencing (Illumina MiSeq) of 16S rRNA (V3-V4) amplicons 9 weeks post GABA and L. brevis DPC 6108 supplementation and 6 weeks post STZ injection. At the 97% similarity level, alpha diversity values were calculated for species richness (Chao1), biodiversity (Shannon Index), observed species and species number relative to abundance within the sample (Simpson diversity index) (Figure 1). All alpha diversity metrics were significantly lower in the diabetic control, diabetic high GABA and diabetic L. brevis DPC 6108 groups, compared with the non-diabetic control group (P<0.05) (Figure 1). No significant differences across any of the five alpha diversity metrics were found between the diabetic low GABA and non-diabetic control groups (Figure 1). Principal coordinate analysis (built upon the unweighted unifrac algorithm) revealed distinct clustering in the non-diabetic control group only (Figure 2). This demonstrates a similarity in the microbial composition of these rats within the non-diabetic control group, commonly associated with such a controlled environment. All diabetic groups, irrespective of treatment, failed to cluster distinctly but did separate from the non-diabetic control group (Figure 2). The failure of individual rats within a group to cluster distinctly suggests that the microbial compositions of rats within each of the diabetic groups are significantly different from each other. None of the treatments appeared to restore clustering either within a particular group or with the non-diabetic control group. This demonstrates that STZ-induced T1D is a strong factor driving the differences in microbial populations, compared with the non-diabetic control group. Four rats from the diabetic-low GABA group appeared closest to the non-diabetic control cluster, possibly indicating a more similar microbial composition to the non-diabetic control group (Table 4).

Relative abundances of Actinobacteria were higher in the diabetic-control and diabetic L. brevis DPC 6108 groups, compared with the non-diabetic control group (P<0.01) (Table 4). Cyanobacteria were higher in the diabetic-low GABA group, compared with the diabetic control and diabetic L. brevis DPC 6108 groups (P<0.05) (Table 4). Proteobacteria were higher in the diabetic-low GABA group (P<0.05) and the diabetic L. brevis DPC 6108 (P<0.01) groups, compared with the non-diabetic control group (Table 4). A comprehensive list of the most abundant proportions that significantly differed between the groups at family and genus levels are outlined in Table 4.
Figure 1. Effects of γ-aminobutyric acid (GABA) powder and Lactobacillus brevis DPC 6108 on alpha diversity of diabetic rats. Values which do not share a common superscript letter are significantly different, P<0.05 (Kruskal-Wallis algorithm).

Figure 2. Principal coordinate analysis using unweighted UniFrac distances for diabetic rats supplemented with either γ-aminobutyric acid (GABA) powder or Lactobacillus brevis DPC 6108 for 9 weeks.
4. Discussion

In the present study, L. brevis DPC 6108 supplementation for 5 weeks significantly increased (69%) serum insulin levels in healthy rats. From this, we subsequently conducted a second experiment to investigate whether dietary GABA and/or L. brevis DPC 6108 could prevent STZ-induced T1D. Oral administration of GABA and L. brevis DPC 6108 for 9 weeks had no effect on the prevention of STZ-induced T1D as rats developed an overt basal hypoinsulinemia and extreme hyperglycemia, suggesting a drastic decrease in β-cell numbers. Our data support the results of a previous study which reported a significant decrease in cell mass of the pancreas isolated from STZ-induced rats with concomitant insulin depletion, not restored by GABA treatment (Adeghate and Ponery, 2002). However, they contradict studies showing that oral GABA administration attenuated hyperglycemia and oxidative stress in STZ-induced diabetic rats (Nakagawa et al., 2005), whereas GABA intraperitoneal injections prevented and reversed high glucose levels caused by multiple low doses of STZ in mice (Soltani et al., 2011). GABA therapy was also reported to be beneficial in other models of diabetes, such as the non-obese diabetic (NOD) mouse model of T1D (Tian et al., 2011 a) and high-fat diet (HFD)-fed mouse model of T2D (Tian et al., 2011 b). In these studies, the positive effect of GABA was related to the modulation of the immuneresponse present in diabetes.

The results obtained in this study support the hypothesis that the beneficial effect of GABA may be primarily due to modulation of immune cell function. In animal models, such as the NOD mouse, HFD-induced T2D mouse and the multiple low-dose STZ-induced diabetes mouse, pancreatic islets are partially damaged and the inflammatory processes cause the further loss of β-cells. In contrast, the procedure commonly applied in rats and used in this study induces T1D with one single dose of STZ, destroying the β-cells rapidly and completely, with absence of an immune response (Wu and Huan, 2008). In this case, GABA supplementation may have no effect, as there is no immune response to be attenuated.
However, beside its anti-inflammatory effect, evidence suggests that GABA may stimulate β-cell replication and inhibit apoptosis in vitro (Soltani et al., 2011) and in vivo (Tian et al., 2013). If a sufficient residual β-cell mass is present to permit recovery, the high levels of glucose and low levels of insulin in diabetic rats indicated an extensive loss of β-cell mass that could not be reversed by dietary GABA in this study.

Probiotic administration in STZ-induced diabetic animals previously displayed contrasting results on glucose homeostasis. Davari et al. (2013) and Lin et al. (2014) reported a significant decrease in glucose levels in anilamis receiving a probiotic mix containing Lactobacillus acidophilus, Bifidobacterium lactis and Lactobacillus fermentum and Lactobacillus reuteri, respectively, compared with diabetic controls, as was seen in this study. Tabuchi et al. (2003) and Yadav et al. (2008) observed an improvement in glucose tolerance following Lactobacillus rhamnosus GG, L. acidophilus and Lactobacillus casei supplemented, whereas Zarfeshani et al. (2011) found no significant differences in blood glucose levels following L. casei supplementation. Increased serum insulin levels in probiotic-fed groups were demonstrated only in the studies by Davari et al. (2013) and Tabuchi et al. (2003). Although we observed a significant increase in serum insulin levels in healthy rats receiving L. brevis DPC 6108 (Experiment 1: 69%), the same was not seen in diabetic rats in Experiment 2, most likely because the β-cell mass was considerably destroyed. However, although supplementation of L. brevis DPC 6108 did not affect serum glucose levels in healthy rats, it significantly reduced glucose levels in diabetic rats, compared with diabetic controls (23%). The balance between glucose uptake, regulated mainly by the CNS and glucose production from the liver is complex and involves several mechanisms. Leptin has been shown to induce ceduction of food intake in diabetic animals, lowering plasma glucose levels (Sindelar et al., 1999), and acting on the CNS inhibiting hepatic glucose production and increasing glucose utilisation by other tissues (German et al., 2011). Food intake and leptin levels were not altered in the diabetic group receiving the bacteria, compared to the other diabetic groups in the present study. Moreover, other metabolic markers involved in glucose homeostasis (insulin, glucagon, GLP-1 and GIP) were similar among all diabetic groups and did not account for this reduction in glucose levels in the L. brevis DPC 6108 supplemented group. Explanations for the difference in glucose levels may be related to increased glucose excretion in the urine, reduced hepatic glucose production or increased glucose uptake by tissues.

GABA released by β-cells plays a role in regulating glucagon secretion from α-cells (Bailey et al., 2007; Bansal et al., 2011). In this study, the extensive damage to the β-cell mass caused by STZ most likely reduced the amount of endogenous GABA (Adeghate and Ponery, 2002), stimulating glucagon release in all diabetic groups. Despite higher circulating levels of GABA detected in the diabetic high GABA supplemented group, this did not suppress glucagon. The increased glucagon secretion together with the low level of circulating insulin in all diabetic rats, most likely caused insufficient suppression of hormone-sensitive lipase activity in the adipose tissue, causing hyperglycaemia and hypertriglyceridaemia. Increased food intake also contributed to hypertriglyceridaemia. Moreover, high levels of glucose may have stimulated hepatic triglyceride production leading to the fatty liver observed in diabetic rats (Dallman and Bhatnagar, 2011; Tamura and Shimomura, 2005).

The marked hyperphagia in diabetic animals has been associated with insulin and leptin deficiencies (Dallman and Bhatnagar, 2011; Sindelar et al., 1999; Sipols et al., 1995). Hyperphagia might have caused small intestine hypertrophy (Saudek and Young, 1981) in all diabetic rats. With increased intestinal mass, the density of gastrointestinal cells also increases, altering gut motility and secretion/absorption (El-Salhy, 2001). Increased faecal output, diarrhoea and significantly higher PYY levels were found in the serum of all diabetic rats. PYY secretion has also been shown to delay intestinal transit and gastric emptying, thus reducing diarrhoea (El-Salhy, 2001). Small intestinal hypertrophy is also associated with increased intestinal cholesterol synthesis and absorption in diabetes (Feingold, 1989; Gleeson et al., 2000). Interestingly, total serum cholesterol was increased only in diabetic rats receiving the high-dose GABA, compared with the non-diabetic controls. As food intake and intestinal length were similar among all diabetic groups as well as hepatic cholesterol levels, the mechanism by which the high dose of GABA mediated the changes in serum cholesterol observed in the current study remains to be elucidated.

Intestinal microbiota composition, diversity and metabolite production were previously shown to be greatly affected by STZ-induced T1D (Patterson et al., 2015). Supplementation with low dose GABA attenuated the significant reduction to microbial diversity induced by T1D, apparent in all other diabetic groups. Distinct phylogenetic alterations in microbial composition were apparent between all diabetic groups, compared with non-diabetic controls. Most notably and similar to the previous study by Patterson et al. (2015), the ratio of Bacteroidetes: Firmicutes was reduced following T1D induction. This ratio was further reduced following all treatments. Interestingly, the treatment with high-GABA had the most pronounced effect on the relative proportions of Bacteroidetes and Firmicutes. Of course, the literature has implicated this ratio to be of significance in numerous disease states (Frank et al., 2011; Graessler et al., 2013). However, our results are in contrast to previous studies which have described a decrease in the Firmicutes: Bacteroidetes ratio associated with T1D (Giongo et al., 2011; Murri et al., 2013). Actinobacteria, previously reported as
significantly increased following STZ-induced T1 D (Patterson et al., 2015), was only significantly increased in the caecal contents of the diabetic control and diabetic L. brevis DPC 61 08 rats, compared with the non-diabetic control rats. The increased Actinobacteria linked with diabetes previously correlated with the reduced microbial diversity of these rats (Patterson et al., 2015) and so it appears that GABA supplementation normalised Actinobacteria levels to those seen in the non-diabetic control group. In accordance with this increase in Actinobacteria, Bifidobacteriaceae and Bifidobacterium were also increased in the diabetic control, diabetic-low GABA and diabetic L. brevis DPC 61 08 groups, compared with the non-diabetic control group. Furthermore, taxa at lower relative proportions drove the significant differences between the groups, but no clear pattern existed between the non-diabetic control and diabetic control groups, compared to any of the treatment groups. This emphasises that STZ-induced T1 D severely altered microbial compositions of the rats, by a random and uncontrolled method. While compositional changes in the relative abundances of intestinal microbiota were present between diabetic groups and the non-diabetic control groups, the treatments did not appear to have any significant effect on taxonomy and so we cannot conclude that GABA or L. brevis DPC 61 08 supplementation had any significant health-promoting effect on the intestinal microbiota.

In conclusion, our results indicate that the use of GABA and GABA-producing probiotic L. brevis DPC 61 08 is not effective in the prevention of diabetic symptoms, as a minimum number of healthy β-cells may be necessary for the efficacy of treatments against T1 D.

Acknowledgements
We acknowledge the technical assistance of Paula O’Connor, Patrick Fitzgerald, Colette Manley and Ruairi Robertson. The authors and their work were supported by the APC Microbiome Institute. The APC Microbiome Institute is funded by Science Foundation Ireland (SFI). This publication has emanated from research supported by a research grant from Science Foundation Ireland (SFI) under Grant Number SFI/12/RC/2273.

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