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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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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 atten uated hyperglycaemia induced by diabetes but additional studies are needed to understand the mechanisms involved in this reduction.

Keywords: streptozotocin, type-1 diabetes, probiotic, γ-aminobutyric 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 microbialecosystem associated with both type 1 (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-aminobutyric 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 al., 2011 b). Furthermore, GABA-producing bacteriahave been isolated from several food sources and fromthe human gut (Barrett et al., 2012; Park and Oh, 2007;Seo et al., 201 3).GABA is the major inhibitory neurotransmitter in thecentral nervous system (CNS). In peripheral tissues, GABAacts not only as a neurotransmitter in the enteric andparasympathetic nervous systems but also as a hormonein non-neuronal tissues (Erdö, 1992). In the endocrinepancreas, GABA is produced by β-cells and once released,activates ionotropic GABAA and metabotropic GABABreceptors 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 onextracellular glucose levels (Dong et al., 2006). GABA isproduced through the conversion of L-glutamate by theenzyme glutamate decarboxylase and

different types ofbacteria produce GABA as a response to adverse acidicenvironmental conditions (Cotter et al., 2001; Jung andKim, 2003; Sanders et al., 1998). Indeed, potential probioticbacteria isolated from the human gastrointestinal tract havebeen shown to efficiently convert glutamate to GABA invitro (Barrett et al., 2012).A disturbed microbiota is a potential environmentalfactor contributing toward metabolic disease, affectingthe immune system and even influencing the perceptionof pain and behaviour (Brown et al., 2011; Cryan andDinan, 2012; Lam et al., 2011). Interestingly, the use ofdietary strategies, such as probiotics and prebiotics may beeffective for the alleviation of these disorders through theproduction of bioactive compounds that may act directlyor indirectly on host metabolism (Lyte, 2011). Thus,considering the role of GABA on glucose homeostasisand the evidence showing how probiotics may affect hosthealth, we investigated the impact of oral administration ofpure GABA and GABA-producing bacteria Lactobacillusbrevis DPC 6108 on the protection against the developmentof T1 D in streptozotocin (STZ)-induced diabetic rats.

2. Materials and methods

Animals

All experimental procedures were performed in accordancewith the protocols approved by the University CollegeCork Ethics Committee, under a license issued fromthe Department of Health and Children. Male SpragueDawley rats, 5 weeks of age, were obtained from Harlan UK(Loughborough, UK) and housed under barrier-maintainedconditions. All animals were allowed to acclimatise forone week prior to commencement of the experiments.Animals were group-housed, with 5 animals per cage atstandard conditions (room temperature of 21 °C, with a 1 2-hlight-dark cycle, lights on at 07:00). All groups were fed adlibitum with Teklad Global rodent standard diet (#2018S;Harlan Laboratories, Loughborough, UK) and allowedfree 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 experimentsand bottles were replaced daily. Two experiments wereconducted in this study – Experiment 1 using healthy ratsand Experiment 2 using STZ-induced diabetic rats.

Lactobacillus brevis DPC 6108 and placebo preparation

L. brevis DPC 6108 is an efficient GABA producer, withmaximum 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., 201 2).Rifampicin-resistant variants of L. brevis DPC 6108 wereisolated by spread-plating ~109 cfu from an overnightculture (1% inoculum) onto MRS agar (Difco Laboratories)containing 500 μg rifampicin/ml (Sigma Aldrich Ireland)and stocked at -80 °C. Before freeze-drying, frozen stockswere plated in MRS agar and a single colony was isolated forinoculation in 10 ml fresh MRS broth supplemented with 30mg/ml MSG. The culture was incubated overnight at 37 °Cunder anaerobic conditions and then inoculated into 1 lMRS broth containing MSG and allowed to grow overnightat 37 °C under anaerobic conditions. The overnight culturealiquots were then inoculated into large volumes of MRScontaining MSG and allowed to grow overnight at 37 °Cunder anaerobic conditions. The overnight culture waswashed twice in phosphate buffered saline (Sigma AldrichIreland) and the pellet resuspended in 15% (w/v) trehalose(Sigma Aldrich Ireland) in dH2O (Experiment 1) or 4% (w/v)yeast extract (Difco Laboratories) in dH2O (Experiment 2).1 ml aliquots of bacterial culture were freeze-dried by usinga 24-h program (freeze temperature, -40 °C; condenserset point, -60 °C; vacuum set point, 600 mTorr). Vialscontaining 1 ml of placebo solution (15% (w/v) trehaloseor 4% (w/v) yeast extract) were freeze-dried using thesame programme. All the vials containing freeze-driedpowder were stored at -20 °C until use. Each animal thatreceived the bacterial strain consumed ~1×1 09 cfu/day.This was achieved by resuspending the freeze-dried powderin water, which rats consumed ad libitum. Freeze-driedaliquots were prepared every 2 weeks and were stored at-20 °C. The groups that did not receive the bacterial strainreceived placebo freeze-dried powder [15% (w/v) trehaloseor 4% (w/v) yeast extract in dH2O]. Freeze-dried powderunderwent continuous quality control of cell counts for theduration of the experiments by plating serial dilutions on MRS agar supplemented with 100 μg/ml rifampicin andincubating plates anaerobically for 48 h at 37 °C.

Faecal sample microbial analysis and GABA production assay

Fresh faecal samples were taken every week for microbialanalysis in order to verify if the strain survived gut transit,rats were receiving enough bacterial cells and there wasno cross-contamination to the groups not receiving thestrain. Microbial analysis involved enumeration of the L.brevis DPC 6108 strain after plating serial dilutions onMRS agar supplemented with 100 μg rifampicin/ml (SigmaAldrich Ireland) and incubating anaerobically for 48 h at37 °C. In addition, isolated colonies were tested for GABAproduction as described previously (Barrett et al., 201 2).Briefly, isolated colonies were grown anaerobically in MRScontaining 3% (w/v) MSG at 37 °C for 55 h. Samples werethen deproteinised by mixing equal volumes of 24% (w/v)trichloroacetic acid and culture, allowed to stand for 1 0min and centrifuged at 1 4,000×g for 10 min. Supernatantswere removed and diluted with 0.2 mol/l sodium citratebuffer, 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 nm/ml.Amino acids were quantified using a Jeol

JLC-500/V aminoacid analyser (Jeol Ltd., Garden City, UK) fitted with a JeolNa+ high-performance cation exchange column.

Effects of GABA powder and Lactobacillus brevis DPC 6108

Experiment 1 – effects on healthy rats
Animals were divided into 3 groups with 10 rats per group,receiving either GABA (2.6 mg/kg bw + 15% w/v trehalose),L. brevis DPC 6108 (-'109 cells in 15% w/v trehalose) orplacebo (15% trehalose) mixed in the drinking water for5 weeks. All groups received 15% (w/v) trehalose as thissolution was used as cryoprotectant while freeze-dryingthe L. brevis DPC 6108. The standard diet used contained3.4% (w/w) glutamic acid and was a sufficient substrate forthe conversion to GABA by L. brevis DPC 6108 (Barrettet al., 2012). Body weight was assessed weekly. After 5weeks dietary intervention, animals were sacrificed bydecapitation and blood samples were collected, allowedto clot at 4 °C, centrifuged for 20 min at 2,000×g and theserum collected into clean microtubes. Liver was removedand flash-frozen on dry ice. All samples were stored at-80 °C prior to analyses.

Experiment 2 – effects on streptozotocin-induced diabetic rats
Animals were divided into 5 groups with 15 rats per group:non-diabetic control group and diabetic control groupreceived only placebo freeze-dried powder [4% (w/v) yeastextract], diabetic low dose GABA group received 2.6 mg/kg bw GABA powder, diabetic high dose GABA groupreceived 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 placebofreeze-dried powders were diluted in fresh drinking waterevery day throughout the 9-week trial period. Every groupreceived 4% (w/v) yeast extract as this solution was usedas cryoprotectant while freeze-drying L. brevis DPC 6108.On experimental week 3, type 1 diabetes was induced inrats in four of the groups by intraperitoneal injection with asingle 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 dissolving,according to a previously described procedure (Wu andHuan, 2008). The non-diabetic control group receivedan injection of citrate buffer only. Seven days post STZinjection,glucose levels were measured in triplicate using aContour Next blood glucose meter (Bayer) in blood samplescollected from a tail vein. Rats with glucose levels higherthan 200 mg/dl were considered diabetic and STZ-inducedrats that had lower glucose level were excluded. From atotal of 60 rats treated with STZ, three died 1-2 days afterinjection, whereas ten rats did not develop diabetes andwere excluded. In the remaining 47 rats, body weight andglucose levels were assessed weekly, while food intake andwater consumption were measured daily. After 9 weeksof dietary intervention rats were killed by decapitationand blood samples were collected, allowed to clot at 4 °C,centrifuged for 20 min at 2,000×g and the serum collectedinto clean microtubes. Liver and caecal contents wereremoved and flash-frozen on dry ice and small intestinallength was measured from individual rats. All samples werestored at -80 °C prior to analyses.

Serum analyses
Commercial kits were used for measurement of GABA andmetabolic markers in serum. GABA was determined usingthe GABA research ELISA kit (Invitech Ltd., Huntingdon,UK). Glucose was determined using the QuantiChromglucose assay (BioAssay Systems, Hayward, CA, USA),triglycerides were measured by using EnzyChromTriglyceride Assay kit (BioAssay Systems) and cholesterolusing LabAssay Cholesterol kit (Wako, Japan). Insulin,glucagon, c-peptide, peptide YY (PYY), active glucagon-likepeptide1 (GLP-1), gastric inhibitory polypeptide (GIP)and leptin were measured using Rat Metabolic HormoneMagnetic Bead Panel (Merck Millipore, Schwalbach amTaunus, Germany).

Liver analyses
Hepatic lipids were extracted according to the method of Folch et al. (1957). After extraction, samples were driedunder a stream of nitrogen and resuspended in 5% (v/v)solution of Triton X-1 00 in distilled water. Triglyceride concentration was determined using the commercial kitEnzyChrom Triglyceride Assay (BioAssay Systems) andcholesterol using LabAssay Cholesterol kit (Wako, Japan).

Microbial DNA extraction, 1 6s rRNA amplification and Illumina MiSeq sequencing
Caecal contents were collected from individual rats 6 weekspost-STZ injection. Total metagenomic DNA was extractedfrom caecal contents with the QIamp DNA Stool Mini Kit(Qiagen, Hilden, Germany) where an additional bead beatingstep was incorporated into the protocol. Extracted DNA wasquantified using the Nanodrop 1000 spectrophotometer(Thermo Scientific, Dublin, Ireland). The V3-V4 variableregion of the 16s rRNA gene was amplified from eachextracted DNA sample according to the 16S metagenomicsequencing library protocol (Illumina, San Diego, CA, USA).Initially the template DNA was amplified using primersspecific to the V3-V4 region of the 16s rRNA gene whichalso allowed for the Illumina overhang adaptor, where, theforward primer (5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGG NGGCWGCAG) andreverse primer

(5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC) wereused. 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 Readmix(2×) (Kapa Bioscystems Inc., Wilmington, MA, USA). Thetemplate DNA was amplified under the following PCRconditions for a total of 25 cycles: 95 °C for 3 min and 30 srespectively (initialisation and denaturation), 55 °C for 30s (annealing), 72 °C for 30 s (elongation) followed by a finalelongation period of 5 min. A negative control reaction,whereby the template DNA was replaced with PCR gradewater, was employed to confirm lack of contamination andPCR products were visualised using gel electrophoresis (1 ×tris-acetate-EDTA buffer, 1.5% agarose gel, 1 00v) post-PCRreaction. Successful amplicons were then cleaned using theAMpure XP purification system (Beckman and Coulter,Takeley, UK). A second PCR reaction was then completedusing the previously amplified and purified DNA as thetemplate. Two indexing primers (Illumina Nextera XTindexing primers, Illumina) were used per sample to allowall samples to be pooled, sequenced on one flow cell andsubsequently identified bioinformatically. Each reactioncontained 25 μl Kapa HiFi HotStart ReadyMix (2×), 5 μltemplate DNA, 5 μl index 1 primer (N7xx), 5 μl index 2primer (S5xx) and 10 μl PCR grade water. PCR conditionswere the same as previously described with the samplesundergoing just 8 cycles instead of 25. PCR products thenunderwent the same electrophoresis and cleaning protocolsas described above. Samples were then quantified using theQubit 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA) inconjunction with the high sensitivity DNA quantificationassay kit (Life technologies, Carlsbad, CA, USA). Allsamples were then pooled to an equimolar concentrationand the pool underwent a final cleaning step. Quality of thepool was determined using the Agilent Bioanalyser prior tosequencing. 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 TeagascNext Generation Sequencing Platform Centre (TeagascFood Research Centre, Moorepark, Ireland) using a 2×300cycle 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 genomeassemblies) (Magoc and Salzberg, 2011). Reads were furtherprocessed with the inclusion of quality filtering based ona quality score of >20 followed by subsequent removal ofsequences below length threshold using QIIME (Caporasoet al., 2010b). USEARCH v7 (64-bit) was then used fordenoising and chimera detection ad well as clusteringinto operational taxonomic units (OTUs) at 97% identity(http://www.drive5.com/usearch). PyNAST (Caporasoet al., 2010a) was used to align OTUs and taxonomy wasassigned by using BLAST (Altschul et al., 1990) against theSILVA SSURef database release 111 (Quast et al., 201 3).QIIME was used to generate alpha (Shannon, Simpson,Chao1, observed species and phylogenetic diversity) andbeta diversities, (Bray Curtis, Weighted and UnWeightedUnifrac) distance matrices (http://qiime.org). Principalcoordinate analysis (PCoA) plots were generated basedon the beta diversity distance matrices and were visualisedusing EMPeror v0.9.3-dev (Vazquez-Baeza et al., 2013).

Statistical analysis

To assess whether differences between treatment groupswere significant, statistical analysis was performed by usingone factor ANOVA followed by Tukey's or Dunnett's posthoc multiple comparison tests (GraphPad Prism Software(Version 6.0), San Diego, CA, USA). Non-parametricKruskall-Wallis analysis using Dunn's multiple comparisonstest was carried out to determine statistically significanttaxonomic differences between groups (GraphPad Prism).

3. Results

Survival of Lactobacillus brevis DPC 6108 through the gastrointestinal tract

Quantification of the numbers of administered rifampicinresistantL. brevis strain in the faeces of rats confirmed itssurvival during gastrointestinal transit. Stool recovery of L.brevis DPC 6108 was ~1.1×107 cfu/g faeces after 1 week offeeding and remained at similar numbers until the end ofexperiments. Colonies isolated from the plates were testedfor 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 5.8mg/ml, similar to the wild type (5.5 mg/ml)). There was nocross-contamination between groups as L. brevis DPC 61 08was 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, livercholesterol and serum glucose were observed (data notshown). However, serum insulin levels were increased(P<0.05) in rats receiving L. brevis DPC 6108, whencompared with rats in the GABA supplemented and controlgroups (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 continuedto lose body weight until the end of the experiment. Finalbody weights were significantly reduced in all diabetic rats,compared with non-diabetic control rats (P<0.001). Dietarysupplementation with GABA and L. brevis DPC 6108 didnot improve weight gain in diabetic rats. The abdomensof diabetic rats were distended, filled with a swollenintestine 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 weresignificantly higher in diabetic rats, compared with thenon-diabetic control group (P<0.001), with no significantdifferences 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 GABAlevels in the diabetic-high GABA group, compared with thediabetic-low GABA, diabetic L. brevis DPC 6108 and bothdiabetic 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 inall diabetic groups, compared with the non-diabeticcontrol group (P<0.001). However, lower glucose levelswere observed in rats receiving L. brevis DPC 6108supplementation relative to rats in the diabetic controlgroup (P<0.01). Insulin and C-peptide levels were significantly decreased, whereas glucagon was increasedin all diabetic groups, compared with the non-diabeticcontrol group (P<0.05; Table 3). GLP-1 and GIP serum levels were not affected in any of the groups. PYY was4- to 5-fold significantly higher in diabetic rats, comparedwith the non-diabetic control group (P<0.05), with nodifferences between treatments. Leptin is a hormoneproduced by adipose cells. Therefore, probably due to thelower 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).[1]

	Control	GABA	*L. brevis* DPC 6108
Final body weight (g)	385.2±9.7	380.5±10.1	378.3±6.7
Glucose (mg/dl)	99.1±4.8	94.3±5.0	104.1±7.2
Insulin (ng/ml)	1.51±0.19^{a}	1.52±0.19^{a}	2.55±0.35^{b}
Liver cholesterol (mg/g)	1.88±0.15	2.16±0.22	1.71±0.13

[1]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 streptozotocin-injection (n=9-10 per group).[1]

	Non-diabetic control	Diabetic control	Low GABA[2]	High GABA	*L. brevis* DPC 6108
Final bw (g)	372.6±7.5^{a}	247.0±5.6^{b}	246.3±8.4^{b}	247.2±5.8^{b}	238.7±8.0^{b}
Small intestine length (cm)	98.6±2.8^{a}	129.4±4.0^{b}	133.8±4.6^{b}	135.7±3.7^{b}	132.2±4.7^{b}
Average food intake (g/100 g bw)	6.0±0.1^{a}	14.3±0.4^{b}	12.8±0.6^{b}	13.6±0.4^{b}	12.7±0.5^{b}
Average water intake (ml/100 g bw)	12.1±1.1^{a}	72.2±2.5^{b}	68.7±1.9^{b}	66.3±2.0^{b}	62.6±3.9^{b}

[1] 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).

[2] GABA = γ-aminobutyric acid.

Table 3. Metabolic markers concentrations in non-diabetic rats and diabetic rats receiving γ-aminobutyric acid (GABA) powder, *Lactobacillus brevis* DPC 6108 or placebo for 9 weeks (n=9-10 per group).[1]

Marker[2]	Non-diabetic control	Diabetic control	Low GABA	High GABA	*L. brevis* DPC 6108
Serum GABA (ng/ml)	144.1±9.8^{a}	145.0±2.9^{a}	157.7±9.4^{a}	195.1±7.7^{b}	137.4±9.4^{a}
Glucose (mg/dl)	125.8±5.9^{a}	579.5±34.3^{b}	513.9±28.7^{b,c}	508.6±23.8^{b,c}	444.5±18.6^{c}
Insulin (ng/ml)	1.45±0.10^{a}	0.12±0.02^{b}	0.13±0.03^{b}	0.19±0.05^{b}	0.11±0.03^{b}
Glucagon (pg/ml)	9.0±3.2^{a}	24.1±1.8^{b}	24.0±3.7^{b}	26.3±3.8^{b}	31.2±3.8^{b}
C-peptide (ng/ml)	1.23±0.13^{a}	0.20±0.02^{b}	0.21±0.02^{b}	0.23±0.03^{b}	0.19±0.02^{b}
PYY (pg/ml)	38.5±4.3^{a}	167.5±30.3^{b}	150.8±10.0^{b}	186.7±22.9^{b}	185.5±33.7^{b}
GLP-1 (pg/ml)	80.5±17.2	182.3±41.4	113.5±21.0	170.5±20.8	151.9±17.3
GIP (pg/ml)	275.5±37.1	230.5±29.9	196.2±25.1	227.0±42.9	237.7±40.2
Leptin (ng/ml)	1.38±0.20^{a}	0.07±0.01^{b}	0.06±0.01^{b}	0.08±0.01^{b}	0.06±0.01^{b}
Serum cholesterol (mg/dl)	58.2±3.0^{a}	63.7±3.4^{a}	68.1±6.0^{a}	103.4±13.4^{b}	90.0±7.4^{a}
Liver cholesterol (mg/g)	1.81±0.06	1.92±0.06	1.81±0.08	1.79±0.05	1.94±0.08
Serum triglycerides (mg/dl)	155.8±9.1^{a}	712.8±87.1^{b}	634.8±106.3^{b}	1,034.5±127.2^{b}	1,013.5±114.9^{b}
Liver triglycerides (mg/g)	1.83±0.08^{a}	5.38±0.31^{b}	5.10±0.21^{b}	5.30±0.14^{b}	5.46±0.28^{b}

[1] 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).

[2] PYY = peptide YY; GLP-1 = active glucagon-like peptide-1; GIP = gastric inhibitory polypeptide.

Effects on serum cholesterol

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

Effects on microbial diversity and microbial composition

The microbial composition in caecal contents ofindividual rats was elucidated through high-throughputDNA sequencing (Illumina MiSeq) of 16S rRNA (V3-V4)amplicons 9 weeks post GABA and L. brevis DPC 6108supplementation and 6 weeks post STZ injection. At the97% similarity level, alpha diversity values were calculatedfor species richness (Chao1), biodiversity (Shannon Index),observed species and species number relative to abundancewithin the sample (Simpson diversity index) (Figure 1).All alpha diversity metrics were significantly lower inthe diabetic control, diabetic-high GABA and diabeticL. brevis DPC 6108 groups, compared with the nondiabeticcontrol group (P<0.05) (Figure 1). No significantdifferences across any of the five alpha diversity metricswere found between the diabetic-low GABA and nondiabeticcontrol groups (Figure 1). Chao1, phylogeneticdiversity and observed species were also significantly higherin the diabetic-low GABA group, compared with all otherdiabetic groups (P<0.05) (Figure 1). Principal coordinateanalysis (built upon the unweighted unifrac algorithm)revealed distinct clustering in the non-diabetic controlgroup only (Figure 2). This demonstrates a similarity in themicrobial composition of these rats within the non-diabeticcontrol group, commonly associated with such a controlledenvironment. All diabetic groups, irrespective of treatmentfailed to cluster distinctly but did separate from the nondiabeticcontrol group (Figure 2). The failure of individualrats within a group to cluster, distinctly suggests that them icrobial compositions of rats within each of the diabeticgroups are significantly different from each other. None ofthe treatments appeared to restore clustering either withina particular group or with the non-diabetic control group.This demonstrates that STZ-induced T1 D is a strong factordriving the differences in microbial populations, comparedwith the non-diabetic control group. Four rats from the diabetic-low GABA group appeared closest to the nondiabeticcontrol cluster, possibly indicating a more similarmicrobial taxonomy (Figure 2).Phylogenetic analysis (examined at phylum, family andgenus levels) detected major changes in microbial taxonomybetween the diabetic groups and the non-diabetic controlgroup (Table 4). At the phylum level, the rat intestinalmicrobiota is dominated by Firmicutes and Bacteroidetes(together harbouring on average 94.08% of sequences;Table 4). The relative abundances of Bacteroidetes andFirmicutes were higher and lower, respectively in thediabetic-high GABA group, compared with the non-diabeticcontrol group (P<0.05) (Table 4). The ratio of Bacteroides:Firmicutes was subsequently lowest in the diabetic-highGABA group, compared with the non-diabetic controlgroup (Table 4). Relative abundances of Actinobacteriawere higher in the diabetic-control and diabetic L. brevisDPC 6108 groups, compared with the non-diabetic controlgroup (P<0.01) (Table 4). Cyanobacteria were higher in thediabetic-low GABA group, compared with the diabeticcontrol and diabetic L. brevis DPC 6108 groups (P<0.05)(Table 4). Proteobacteria were higher in the diabeticlowGABA (P<0.05) and the diabetic L. brevis DPC 6108(P<0.01) groups, compared with the non-diabetic controlgroup (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.

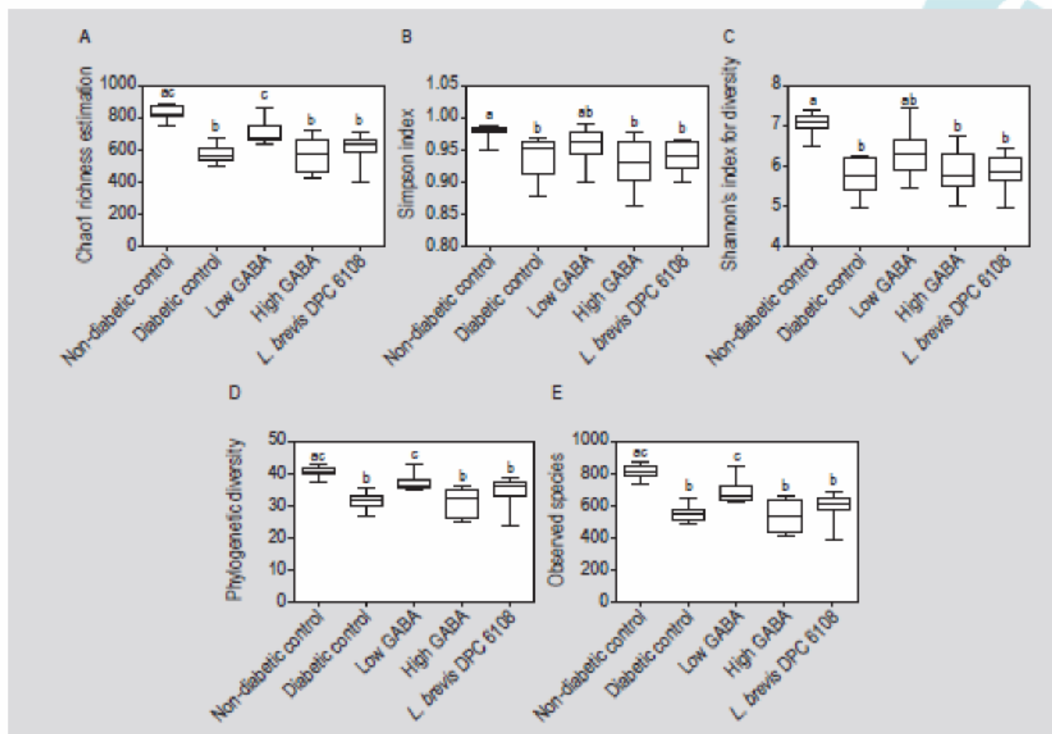

Figure 1. Effects of γ-aminobutyric acid (GABA) powder and *Lactobacillus brevis* DPC 6108 on alpha diversity of diabetic rats Values which do not share a common superscript letter are significantly different; *P*<0.05 (Kruskal-Wallis algorithm).

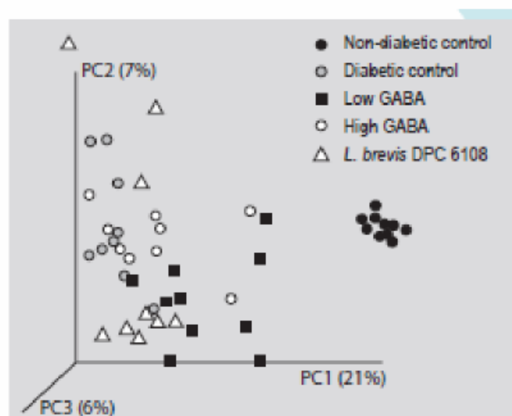

Figure 2. Principal coordinate analysis using unweighted Unifrac distances for diabetic rats supplemented with either γ-aminobutyric acid (GABA) powder or *Lactobacillus brevis* DPC 6108 for 9 weeks.

Table 4. Intestinal microbial composition in caecal contents in non-diabetic rats and diabetic rats receiving γ-aminobutyric acid (GABA) powder, *Lactobacillus brevis* DPC 6108 or placebo for 9 weeks. All values are presented as % reads for each group (n=9-10 per group).[1]

	Non-diabetic control	Diabetic control	Low GABA	High GABA	*L. brevis* DPC 6108
Phylum					
Actinobacteria	0.04^{a}	2.75^{b}	1.11^{ab}	0.57^{ab}	1.31^{b}
Bacteroidetes	30.26^{a}	30.05^{ab}	35.08^{ab}	38.74^{b}	34.28^{ab}
Cyanobacteria	0.49^{abc}	0.11^{ac}	0.99^{b}	1.15^{abc}	0.21^{c}
Firmicutes	67.92^{a}	60.09^{ab}	59.59^{ab}	56.23^{b}	58.17^{ab}
Proteobacteria	0.59^{a}	1.18^{ab}	1.66^{b}	1.10^{ab}	2.06^{b}
Family					
Bifidobacteriaceae	0.00^{a}	2.72^{b}	0.99^{b}	0.55^{ab}	1.27^{b}
Bacteroidaceae	2.30^{a}	6.57^{ab}	5.63^{b}	6.32^{b}	7.59^{b}
Rikenellaceae	2.45^{a}	0.60^{b}	0.99^{ab}	0.84^{b}	0.51^{b}
S24-7	18.86^{a}	18.09^{ab}	22.53^{b}	24.76^{ab}	20.39^{ab}
Peptococcaceae	0.60^{a}	0.11^{b}	0.10^{b}	0.11^{b}	0.10^{b}
Peptostreptococcaceae	0.78^{ac}	0.04^{b}	0.26^{c}	0.09^{abc}	0.02^{b}
Veillonellaceae	1.00^{a}	4.84^{b}	2.23^{ab}	2.60^{b}	2.39^{ab}
Erysipelotrichaceae	0.11^{a}	0.73^{b}	0.40^{ab}	0.58^{b}	0.92^{b}
Rhodospirillaceae	0.08^{a}	0.12^{ab}	0.62^{b}	0.58^{b}	0.67^{b}
Alcaligenaceae	0.04^{a}	1.03^{b}	0.74^{b}	0.41^{ab}	1.27^{b}
Desulfovibrionaceae	0.47^{a}	0.02^{b}	0.14^{b}	0.09^{b}	0.12^{b}
Ruminococcaceae	26.19^{a}	19.87^{ab}	21.71^{ab}	17.31^{b}	21.49^{ab}
Genus					
Bifidobacterium	0.00^{a}	2.72^{b}	0.99^{b}	0.55^{ab}	1.27^{b}
Bacteroides	2.30^{a}	6.57^{ab}	5.63^{b}	6.32^{b}	7.59^{b}
Prevotella	1.26^{a}	0.08^{b}	0.42^{ab}	0.80^{ab}	0.58^{ab}
Alistipes	2.18^{a}	0.56^{b}	0.77^{ab}	0.71^{b}	0.44^{b}
S24-7_uncultured	18.86^{a}	18.09^{ab}	22.53^{ab}	24.76^{b}	20.39^{ab}
4COd-2_uncultured	0.38^{ab}	0.11^{ab}	0.89^{ab}	1.09^{a}	0.19^{b}
Roseburia	0.36^{a}	0.09^{b}	0.14^{ab}	0.52^{b}	0.11^{b}
Peptostreptococcaceae_uncultured	0.78^{a}	0.03^{b}	0.26^{a}	0.09^{abc}	0.02^{c}
Flavonifractor	0.48^{a}	0.00^{b}	0.00^{b}	0.12^{b}	0.00^{b}
Papillibacter	0.38^{ab}	0.17^{ab}	0.17^{ab}	0.06^{b}	0.11^{b}
Ruminococcaceae_uncultured	16.39^{a}	13.84^{ab}	15.79^{ab}	10.83^{b}	16.01^{ab}
Phascolarctobacterium	1.00^{a}	4.84^{b}	2.22^{ab}	2.60^{b}	2.39^{ab}
Allobaculum	0.00^{a}	0.41^{bc}	0.14^{c}	0.08^{abc}	0.70^{b}
Thalassospira	0.08^{a}	0.12^{ab}	0.62^{b}	0.58^{b}	0.67^{b}
Parasuterella	0.04^{a}	1.03^{b}	0.74^{ab}	0.40^{b}	1.27^{b}
RF-9_uncultured	0.30^{b}	5.60^{b}	0.67^{ab}	0.47^{ab}	3.54^{b}

[1] Values in the same row which do not share a common superscript letter are significantly different $P<0.05$ (Kruskal-Wallis algorithm).

4. Discussion

In the present study, L. brevis DPC 6108 supplementationfor 5 weeks significantly increased (69%) serum insulinlevels in healthy rats. From this, we subsequently conducteda second experiment to investigate whether dietary GABAand /or L. brevis DPC 6108 could prevent STZ-inducedT1 D.Oral administration of GABA and L. brevis DPC 6108 for9 weeks had no effect on the prevention of STZ-inducedT1 D as rats developed an overt basal hypoinsulinemia andextreme hyperglycemia, suggesting a drastic decrease inβ-cell numbers. Our data support the results of a previousstudy which reported a significant decrease in cell massof the pancreas isolated from STZ-induced rats withconcomitant insulin depletion, not restored by GABA treatment (Adeghate and Ponery, 2002). However, theycontradict studies showing that oral GABA administrationattenuated hyperglycemia and oxidative stress in STZinduceddiabetic rats (Nakagawa et al., 2005), whereasGABA intraperitoneal injections prevented and reversed high glucose levels caused by multiple low doses of STZ inmice (Soltani et al., 2011). GABA therapy was also reportedto be beneficial in other models of diabetes, such as thenon-obese diabetic (NOD) mouse model of T1 D (Tian etal., 2011 a) and high-fat diet (HFD)-fed mouse model ofT2D (Tian et al., 2011 b). In these studies, the positive effectof GABA was related to the modulation of the immuneresponse present in diabetes.

The results obtained in this study support the hypothesisthat the beneficial effect of GABA may be primarily dueto modulation of immune cell function. In animal models,such as the NOD mouse, HFD-induced T2D mouse and themultiple low-dose STZ-induced diabetes mouse, pancreaticislets are partially damaged and the inflammatory processcauses the further loss of β-cells. In contrast, the procedurecommonly applied in rats and used in this study inducesT1 D with one single dose of STZ, destroying the β-cellsrapidly and completely, with absence of an immune response(Wu and Huan, 2008). In this case, GABA supplementationmay have no effect, as there is no immune response tobe attenuated.

However, beside its anti-inflammatoryeffect, evidence suggests that GABA may stimulate β-cellreplication and inhibit apoptosis in vitro (Soltani et al.,2011) and in vivo (Tian et al., 2013), if a sufficient residualislet mass is present to permit recovery. The high levels ofglucose and low levels of insulin in diabetic rats indicatedan extensive loss of β-cell mass that could not be reversedby dietary GABA in this study.

Probiotic administration in STZ-induced diabeticanimals previously displayed contrasting results onglucose homeostasis. Davari et al. (2013) and Lin et al. (2014) reported a significant decrease in glucose levels inanimals receiving a probiotic mix containing Lactobacillusacidophilus, Bifidobacterium lactis and Lactobacillusfermentum and Lactobacillus reuteri, respectively,compared with diabetic controls, as was seen in this study.Tabuchi et al. (2003) and Yadav et al. (2008) observed animprovement in glucose tolerance following Lactobacillusrhamnosus GG, L. acidophilus and Lactobacillus caseisupplementation, whereas Zarfeshani et al. (2011) foundno significant differences in blood glucose levels followingL. casei supplementation. Increased serum insulin levels inprobiotic-fed groups were demonstrated only in the studiesby Davari et al. (2013) and Tabuchi et al. (2003). Althoughwe observed a significant increase in serum insulin levelsin healthy rats receiving L. brevis DPC 6108 (Experiment 1:69%), the same was not seen in diabetic rats in Experiment2, most likely because the β-cell mass was considerablydestroyed. However, although supplementation of L. brevisDPC 6108 did not affect serum glucose levels in healthyrats, it significantly reduced glucose levels in diabeticrats, compared with diabetic controls (23%). The balancebetween glucose uptake, regulated mainly by the CNS andglucose production from the liver is complex and involvesseveral mechanisms. Leptin has been shown to inducereduction of food intake in diabetic animals, loweringplasma glucose levels (Sindelar et al., 1999), and actingin the CNS inhibiting hepatic glucose production andincreasing glucose utilisation by other tissues (German etal., 2011). Food intake and leptin levels were not alteredin the diabetic group receiving the bacteria, compared tothe other diabetic groups in the present study. Moreover,other metabolic markers involved in glucose homeostasis(insulin, glucagon, GLP-1 and GIP) were similar amongall diabetic groups and did not account for this reductionin glucose levels in the L. brevis DPC 6108 supplementedgroup. Explanations for the difference in glucose levelmay be related to increased glucose excretion in the urine,reduced hepatic glucose production or increased glucoseuptake by tissues.

GABA released by β-cells plays a role in regulating glucagonsecretion from α-cells (Bailey et al., 2007; Bansal et al.,201 1). In this study, the extensive damage to the β-cellmass caused by STZ most likely reduced the amountof endogenous GABA (Adeghate and Ponery, 2002),stimulating glucagon release in all diabetic groups. Despitehigher circulating levels of GABA detected in the diabetichighGABA supplemented group, this did not suppressglucagon. The increased glucagon secretion together withthe low level of circulating insulin in all diabetic rats, mostlikely caused insufficient suppression of hormone-sensitivelipase activity in the adipose tissue, causing hyperglycaemiaand hypertriglyceridemia. Increased food intake alsocontributed to hypertriglyceridemia. Moreover, highlevels of glucose may have stimulated hepatic triglycerideproduction leading to the fatty liver observed in diabetic rats(Dallman and Bhatnagar, 2011; Tamura and Shimomura,2005).

The marked hyperphagia in diabetic animals has beenassociated with insulin and leptin deficiencies (Dallman andBhatnagar, 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 increasedintestinal mass, the density of gastrointestinal cells alsoincreases, altering gut motility and secretion/absorption(El-Salhy, 2001). Increased faecal output, diarrhoea andsignificantly higher PYY levels were found in the serumof all diabetic rats. PYY secretion has also been shown todelay intestinal transit and gastric emptying, thus reducingdiarrhoea (El-Salhy, 2001). Small intestinal hypertrophyis also associated with increased intestinal cholesterolsynthesis and absorption in diabetes (Feingold, 1 989;Gleeson et al., 2000). Interestingly, total serum cholesterol was increased only in diabetic rats receiving the high doseGABA, compared with the non-diabetic controls. As foodintake and intestinal length were similar among all diabeticgroups as well as hepatic cholesterol levels, the mechanismby which the high dose of GABA mediated the changes inserum cholesterol observed in the current study remainsto be elucidated.

Intestinal microbiota composition, diversity and metaboliteproduction were previously shown to be greatly affected bySTZ-induced T1 D (Patterson et al., 2015). Supplementationwith low dose GABA attenuated the significant reductionto microbial diversity induced by T1 D, apparent in allother diabetic groups. Distinct phylogenetic alterationsin microbial composition were apparent between alldiabetic groups, compared with non-diabetic controls.Most notably and similar to the previous study by Pattersonet al. (2015), the ratio of Bacteroidetes: Firmicutes wasreduced following T1 D induction. This ratio was furtherreduced following all treatments. Interestingly, thetreatment with high-GABA had the most pronouncedeffect on the relative proportions of Bacteroidetes andFirmicutes. Of course, the literature has implicated thisratio to be of significance in numerous disease states(Frank et al., 2011; Graessler et al., 2013). However, ourresults are in contrast to previous studies which havedescribed a decrease in the Firmicutes: Bacteroidetes ratioassociated with T1 D (Giongo et al., 2011; Murri et al.,201 3). Actinobacteria, previously reported as

significantlyincreased following STZ-induced T1 D (Patterson et al.,201 5), was only significantly increased in the caecal contentsof the diabetic control and diabetic L. brevis DPC 61 08rats, compared with the non- diabetic control rats. Theincreased Actinobacteria linked with diabetes previouslycorrelated with the reduced microbial diversity of theserats (Patterson et al., 2015) and so it appears that GABAsupplementation normalised Actinobacteria levels to thoseseen in the non-diabetic control group. In accordancewith this increase in Actinobacteria, Bifidobacteriaceaeand Bifidobacterium were also increased in the diabeticcontrol,diabetic-low GABA and diabetic-L. brevis DPC61 08 groups, compared with the non-diabetic controlgroup. Furthermore, taxa at lower relative proportionsdrove the significant differences between the groups, but noclear pattern existed between the non-diabetic control anddiabetic-control groups, compared to any of the treatmentgroups. This emphasises that STZ-induced T1 D severelyaltered microbial compositions of the rats, by a random anduncontrolled method. While compositional changes in therelative abundances of intestinal microbiota were presentbetween diabetic groups and the non-diabetic controls,the treatments did not appear to have any significant effecton taxonomy and so we cannot conclude that GABA orL. brevis DPC 61 08 supplementation had any significanthealth-promoting effect on the intestinal microbiota.

In conclusion, our results indicate that the use of GABAand GABA-producing probiotic L. brevis DPC 61 08 isnot effective in the prevention of diabetic symptoms, as aminimum number of healthy β-cells may be necessary forthe efficacy of treatments against T1 D.

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