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TITLE PAGE

Gut microbiome dysbiosis and increased intestinal permeability in children with islet autoimmunity and type 1 diabetes : a prospective cohort study

Running title: Gut microbiome and islet autoimmunity

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ABSTRACT

Aims/hypothesis: To investigate the longitudinal relationship between the gut microbiome, circulating short chain fatty acids (SCFAs) and intestinal permeability in children with islet autoimmunity or type 1 diabetes and controls.

Methods: We analysed the gut bacterial microbiome, plasma SCFAs, small intestinal permeability and dietary intake in 47 children with islet autoimmunity or recent-onset type 1 diabetes and in 41 unrelated or sibling controls over a median (range) of 13 (2-34) months follow-up.

Results: Children with multiple islet autoantibodies (e2 IA) or type 1 diabetes had gut microbiome dysbiosis. Anti-inflammatory *Prevotella* and *Butyricimonas* genera were less abundant and these changes were not explained by differences in diet. Small intestinal permeability measured by blood lactulose:rhamnose ratio was higher in type 1 diabetes. Children with e2 IA who progressed to type 1 diabetes (progressors), compared to those who did not progress, had higher intestinal permeability (mean [SE] difference +5.14 [2.0], 95% CI 1.21, 9.07, p=0.006), lower within-sample (alpha) microbial diversity (31.3 [11.2], 95% CI 9.3, 53.3, p=0.005), and lower abundance of SCFA-producing bacteria. Alpha diversity (observed richness) correlated with plasma acetate levels in all groups combined (regression coeff [SE] 0.57 [0.21], 95% CI 0.15, 0.99 p=0.008).

Conclusions/interpretation: Children with e2 IA who progress to diabetes, like those with recent-onset diabetes, have gut microbiome dysbiosis associated with increased intestinal permeability. Interventions that expand gut microbial diversity, in particular

SCFA-producing bacteria, may have a role to decrease progression to diabetes in children at-risk.

KEYWORDS Gut microbiome, Intestinal permeability, Islet autoimmunity, Short chain fatty acids, Type 1 diabetes

ABBREVIATIONS

ACAES: Australian child and adolescent eating survey
CSS: cumulative sum scaling
IA: islet autoantibody
IAA: insulin autoantibody
IA2: tyrosine phosphatase-like insulinoma antigen
GAD: glutamic acid decarboxylase 65
PCoA: principal coordinates analysis
SCFA: short chain fatty acid
SNP: single nucleotide polymorphism
TGAb: transglutaminase autoantibody

The gut bacterial microbiome and its products are implicated in the pathophysiology of type 1 diabetes both before the development of islet autoimmunity and later in progression from islet autoimmunity to hyperglycemia and clinical disease.[1–13] An imbalance of the gut microbiome composition, called microbiome dysbiosis, characterised by decreased microbial diversity and decreased relative abundance of short chain fatty acids (SCFAs)-producing anti–inflammatory bacteria, has been detected before and after the development of islet autoimmunity.[1,3,6,12-14] SCFAs such as butyrate, propionate and acetate are produced by fermentation of insoluble dietary fibre and resistant starch to promote gut epithelial integrity and immune homeostasis.[15] Gut microbiome dysbiosis may therefore impair epithelial integrity and increase intestinal permeability to allow dietary antigens and immune stimulants such as endotoxins to enter the circulation to promote systemic inflammation and aberrant immunity.

Studies of the gut microbiome in the development of type 1 diabetes have been confined to Northern Hemisphere children[1,2,7,8,10,12,13,16] and few have compared children with type 1 diabetes with unrelated healthy controls.[4,8,11,17] At the time that this study was initiated only three small longitudinal studies, in Finland and Estonia, had described changes in the gut microbiota associated with progression from islet autoimmunity to type 1 diabetes.[1,2,9] Very recently, however, the TEDDY study has

provided details of the gut microbme and its function from a large cohort of at-risk children followed from infancy. Children who did not develop islet autoimmunity or type 1 diabetes had gut microbiomes that supported SCFA production, with diverse taxa sequenced across research sites in USA (Colorado, Georgia/Florida, Washington) and Europe (Finland, Germany, Sweden).[12,13]

Small intestinal permeability is reported to be increased in children with islet autoimmunity or clinical type 1 diabetes.[18–22] To our knowledge, only one study has investigated longitudinally gut microbiome dysbiosis in relation to intestinal permeability in health,[23] and none in children progressing to type 1 diabetes. We therefore aimed to analyse the gut microbiome, circulating SCFA concentrations and intestinal permeability in Australian children with islet autoimmunity who did or did not progress to diabetes, or with recent-onset type 1 diabetes, in relation to sibling and unrelated controls, prospectively at 6-monthly intervals for up to 3 years.

RESEARCH DESIGN AND METHODS

Study design and setting

Children with recent-onset type 1 diabetes (n=29), or islet autoantibodies (IA) (n=18) including e2IA (n=12) and single IA (n=6) were recruited consecutively from three tertiary centres. Controls (n=41) were either islet autoantibody-negative siblings (n=31: termed sibling controls), or friends (n=10: unrelated controls) of type 1 diabetes and islet autoimmune children. Children with IA were identified as part of a screening program of first-degree relatives with type 1 diabetes at three centres in Australia [Women's and Children's Hospital (WCH), South Australia; Royal Melbourne Hospital, Victoria; Children's Hospital at Westmead, New South Wales]. Single IA all had autoantibodies to GAD. e2IA children included one participant with two IA, seven with three IA and four with four IA, to GAD, IA2, insulin and/or ZnT8. Progression to hyperglycemia was determined by 6-monthly oral glucose tolerance tests and/or post-prandial plasma glucose levels. An abnormal oral glucose tolerance test or a post-prandial laboratory blood glucose concentration > 11.1 mM two hours after food defined participants who were progressors, and this was the endpoint for their follow-up . Children with recent-onset type 1 diabetes were recruited from the type 1 diabetes clinic at the WCH, South Australia, from 4 weeks post-diagnosis and all had detectable islet autoantibodies. Exclusion criteria were known celiac disease or celiac autoimmunity, another chronic disease, or past

involvement in an intervention trial. Gastrointestinal symptoms, infection or fever, or probiotic or antibiotic ingestion did not occur within 4 weeks of sample collection.

Recruitment occurred from January 2014 to January 2017; the last observation was completed in January 2018. A total of 88 children, 76 from SA, 6 from Victoria and 6 from NSW were investigated longitudinally. Children attended the hospital clinic at 6-monthly intervals for measurement of small intestinal permeability and plasma SCFA concentrations, and to complete a dietary questionnaire which was paired with a home stool collection within 24 hours of the visit. The protocol was approved by the Women's and Children's Health Network, Melbourne Health and Childrens Hospital Westmead Human Research Ethics Committees. Parents/Guardians and participants over 18 years gave informed written consent.

Main outcome measures

Microbiome analysis Stool samples were collected at home within 24 hours of the study visit using the OMNIgene GUT (OMR-200; DNA Genotek, Ontario, Canada) tube.[24,25] An EasySampler collection device (GP Medical Devices ApS, Holstebro, Denmark) and gloves were provided. Samples were stored at room temperature until arrival at the clinical laboratory within 24 hours, and immediately aliquoted in a Biosafety Level 2 cabinet into sterile screw-cap vials stored at -80˚C.[25] Frozen samples were shipped to the sequencing laboratory on dry ice and were not thawed at any point until required for DNA extraction. Samples were thawed on ice and DNA

extracted with the MoBio PowerSoil kit (MoBio Laboratories, Carlsbad, CA).[26] The V4 hypervariable region of the bacterial 16S rRNA marker gene (16Sv4) was PCR-amplified with primers containing unique sequences to provide targets for the Illumina sequencing adaptors (Illumina, San Diego, CA) and dual-index barcodes to the amplicon target for paired-end sequencing on the Illumina MiSeq instrument as previously described.[25]

Sequences were clustered into Features using QIIME2 (version 2017.12). Amplicon primers were trimmed with q2-cutadapt. Q2-dada2 was used to filter sequences by quality, joining paired-end reads, chimera removal, denoising, error correction, and Feature inference. Truncation length was set to 180 bp for forward reads and 160 bp for reverse reads and reads with more than three unexpected errors were discarded. Taxonomic classification was performed in QIIME2 using as reference the Silva 16S rRNA reference database. An abundance filter was applied to the Feature table, leaving only those Features for which total relative abundance across all samples was higher than 0.01%. After the abundance filter, the resulting table had a total of 450 different Features and 201 samples (1 sample was excluded due to suspected contamination). Mean (SD) library size by group was 19,093 (6,262) sequences for type 1 diabetes, 19,341 (5,929) for e2IA, 25,623 (8,364) for single IA, 17,697 (5,823) for sibling control and 19,310 (6,345) for unrelated control. Library sizes after abundance filter ranged from 5,793-36,486 per sample with a median of 18,250. Alpha and beta diversity analyses were performed in R using Phyloseq[27] and Vegan.[28] For alpha diversity analysis (Observed Richness or Number of Features, and Inverse

Simpson index or 'Evenness'), the Features table was rarefied without replacement to the number of sequences (5,432) in the smallest sample. For beta diversity analysis, the Feature tables was normalized using cumulative sum scaling (CSS) and log-transformed. PCoA plots of the Bray-Curtis distances between pairs of samples were used for ordination analysis. DNA sequences from differentially abundant taxa were further searched against the non-redundant nucleotide database with the Basic Local Alignment Search Tool.[29]

Intestinal permeability Small intestinal permeability was measured by the lactulose-rhamnose absorption test in which an increase in blood lactulose-rhamnose ratio due to increased permeability of lactulose through the tight junctions of the small intestinal epithelium occurs with increased small intestinal permeability.[30] Lactulose (5 g) (Alphapharm) and rhamnose (1 g) (Sigma-Aldrich) were dissolved in 100 mL of tap water and consumed orally after an overnight fast. Blood was collected 90 minutes later by venepuncture using EDTA evacuated blood collection tubes (Vacuette, Greiner Bio-one, Kremsmünster, Austria). Plasma was collected in accordance with the manufacturer's instructions and stored at -80°C until assayed. Lactulose and rhamnose were measured using the Dionex BioLC High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection system (HPAE-PAD (Dionex Pty LTD, Sunnyvale, CA) and chromatograms recorded and evaluated with Chromeleon Client Version 6.80 software (ThermoFisher Scientific,

Waltham, MA). Data are presented as lactulose/rhamnose (L/R) ratio x 100.[30] Samples were run in two batches: mean (SD) CVs for rhamnose were 9.7% (4.1) and 6.1% (2.7), and for lactulose 8.1% (3.4) and 8.5% (2.7), respectively.

Plasma SCFAs Plasma acetate, propionate and butyrate were measured by gas chromatography after liquid-liquid extraction. 50 µL of cold 200 mM heptanoic acid in 1 M phosphoric acid was added to 200 uL of plasma on ice. 4 mL of ether was added, mixed vigorously, centrifuged and the top layer transferred to a tube containing 50 uL of 200 mM sodium hydroxide. After further mixing and centrifuging the ether layer was aspirated off and the remainder dried under nitrogen at 40°C. The residue was re-dissolved in 30 uL of 1 M phosphoric acid in a glass insert GC vial. Samples were analysed on an Agilent 7890A gas chromatograph (Agilent Technologies, Santa Clara, CA). 0.2 µL of each sample was injected into a Phenomenex Zebron ZB-FFAP column (Phenomenex, Torrance, CA) through a splitless injector at 210°C (gas flow 7.7 mL/min) and held at 90°C for 1 minute then heated at 20°C/min to 190°C and held for 2.5 minutes. Peaks were detected with a flame ionisation detector at 210°C and identified and quantitated against calibration standards over the range 0-400 mM. Intra-assay CVs were 14.2%, 11.8% and 10.3% for acetate, propionate and butyrate, respectively.

Food frequency questionnaire The Australian Child and Adolescent Eating Survey (ACAES version 1.2; University of Newcastle, Australia) was administered on one

occasion by the one registered dietitian (RB). The ACAES is a 120-item, semi-quantitative food frequency questionnaire (FFQ) that is validated in Australian children and adolescents.[31] Nutrient intakes were computed from current food composition databases of Australian foods: the Australian AusNut 1999 database Revision 17 for all foods and AusFoods Revision 5 for all brands (Australian Government Publishing Service, Canberra, Australia) to generate individual daily macro and micro-nutrient intakes.

Other measures

Islet autoantibodies. Autoantibodies to glutamic acid decarboxylase 65 (GADAb), tyrosine phosphatase-like insulinoma antigen (IA2Ab), insulin (IAA) and beta-cell specific zinc transporter 8 (ZnT8) were measured by fluid phase radioimmunoprecipitation assays as described.[32,33] Assays for GADAb, IA2Ab IAA and ZnT8 scored 87%, 88%, 25% and 52% for sensitivity and 99%, 100%, 98% and 97.6% for specificity, respectively, in the IASP 2016 serum exchange. Cut-offs for autoantibody positivity were defined as GADAb >5 U, IA2Ab >3 U, IAA e0.7 mU/mL and ZnT8 e3.1 U. All positive results were confirmed by repeat testing in separate assays.

Transglutaminase antibodies (TGAb) were measured using a Quanta Lite h-tTG IgA kit (INOVA Diagnostics Inc., San Diego, CA). Blood glucose was measured using routine glucose oxidase method.

HLA typing. Saliva was collected into OG-500 Oragene DNA tubes (DNA Genotek, Ontario, Canada) for DNA extraction. Three SNPs (rs3104413, rs2187668 and rs9275495) were PCR-typed using the TaqMan assay (Applied Biosystems, Warrington, U.K.) for imputation of HLA-DR as described.[34]

Statistical analysis

Linear mixed models were fitted for each of alpha diversity (Observed Richness, Inverse Simpson), intestinal permeability and SCFAs (acetate, propionate, and butyrate) to investigate differences between the groups. All models were adjusted for age. Random effects for participant were included in all statistical models, with an unstructured covariance matrix assumed.

The effect of each alpha diversity measure on intestinal permeability or SCFAs was separately investigated using linear mixed models. Interactions between group and alpha diversity were included in each model, to assess if any observed relationship differed between groups. The fit of simpler nested models was examined using likelihood ratio tests. In participants with type 1 diabetes or ≥ 2 IA, intestinal permeability was compared for participants who did or did not have HLA DR3 using a linear mixed model. Random effects for participant were again included in these

statistical models. Stata v14.0 (StataCorp, College Station, TX) was used to fit the linear mixed models.

For analysis of beta diversity in time-invariant metadata, the PERMANOVA permutation procedure was altered such that it permutes the participants rather than levels within participants and the data was analysed thus with adjustment for age.[35]

Differential abundance hypothesis tests were performed using log-counts per million (log-cpm) and with the R limma package[36] with adjustment for age and the correlation between participant's serial measurements accounted for in the model.

A one-way ANOVA compared FFQ across groups.

All available data were used in the analyses. Results are expressed as mean differences between groups, with standard errors (SE) and 95% confidence intervals (95% CI) shown, or mean (SD).

RESULTS

Of 171 children approached, 5 were excluded due to exclusion criteria (3 coeliac disease; 1 had participated in an intervention trial and 1 had nephrotic syndrome) and 78 declined consent. A total of 88 children from 53 families enrolled. During follow-up 8 participants withdrew: 6/8 citing burden of follow up, and 2/8 moved overseas/interstate. 1 participant was lost to follow-up. The 88 participants were studied at a total of 216 time points for a median (range) of 13 (2-34) months.

Baseline characteristics are summarised in Table 1. Age or gender was not different between the groups. Participants were all non- indigenous except for one participant with type 1 diabetes. At baseline, duration of diabetes was 8 (1-22) months in the 29 children with type 1 diabetes. The six children with e2 IA who progressed to type 1 diabetes during follow-up (termed progressors) were studied at 21 time points and followed for 28 (13-30) months up until the time that hyperglycemia was first detected.

Data collected 6 monthly were 94% complete for both microbiome analysis (202/216 time points with complete data in 83/88 participants) and intestinal permeability measurement (204/216 time points with complete data in 87/88 participants). Data collected 6 monthly were 91% complete for SCFA analysis (196/216 time points with complete data in 81/88 participants). 81/88 children completed a food frequency questionnaire on one occasion.

Gut microbiome

Alpha diversity There was no difference between children with type 1 diabetes, e2 IA or single IA, and sibling and unrelated controls (Fig 1 A). Within the e2 IA group, progressors from islet autoimmunity to type 1 diabetes had reduced observed richness of microbiota within samples (lower alpha diversity) compared with non-progressors (mean difference [SE] 31.3 [11.2], 95% CI 9.3, 53.3, p = 0.005) adjusted for age (Figure 1 B). The other measure of within- sample diversity, the InvSimpson index of the evenness of the distribution of the microbiota, was not different between groups.

Inverse Simpson, adjusted for age, was greater in participants with DR4,X [mean (SE) 17.2, (1.4); 95% CI 14.6 – 19.8) than DR3,4 [mean (SE) 11.4 (1.4); 95% CI 8.7 – 14.0] or DR3,X [mean (SE) 11.7 (2.0); 95% CI 7.8 – 15.6] p= 0.01.There was a similar trend for observed richness. Observed Richness, adjusted for age, was greater in participants with DR4,X [mean (SE) 95.3 (3.9); 95%CI 87.7 – 102.9] than DR3,4 [85.3 (4.1); 95% CI 77.3 – 93.4] or DR3,X [81.3 (5.8); 95%CI 70.0 – 92.6] p=0.09.

Beta diversity Pairwise comparisons of Bray-Curtis distances at the Features level did not show differences in beta diversity between type 1 diabetes and unrelated and sibling controls. No significant differences in beta diversity were observed between children with e2 IA and unrelated and sibling controls, (Figure 1C), and children with single IA only, compared with e2 IA, type 1 diabetes and sibling or unrelated controls. Within the e2 IA group, beta diversity was not different between progressors to type 1 diabetes and non-progressors Figure 1 D)..Beta diversity did not differ according to HLA DR type.

Differential abundance of taxa The abundances of *Butyricimonas paravirosa, Prevotella mareseille* and *Prevotella copri* were lower in type 1 diabetes ($p<0.005$) and e2 IA ($p<0.005$) than in unrelated controls (Table 2). These deficits were not detected in children with single IA. Within the e2 IA group, progressors had lower *Oscillibacter valericigenes* ($p = 0.02$) classified to the Ruminococcaceae Family, and lower relative abundance of Verrucomicrobia ($p = 0.02$) and Tenericutes ($p = 0.02$) phyla in comparison with non-progressors (Table 2).

No gender differences were observed in gut microbiome measures.

Small intestinal permeability

Children with type 1 diabetes had higher small intestinal permeability as measured by lactulose/rhamnose (L/R) than unrelated controls [mean difference (SE) +3.67 (1.45), 95% CI 0.83, 6.5, p = 0.01] (Figure 2A) and sibling controls [+2.49 (1.03), 95% CI 0.47, 4.51, p = 0.02]. There was no difference between all children with e2 IA or single IA and controls. However, within e 2 IA, progressors had higher L/R ratios than non-progressors [mean (SE) difference +5.14 (2.0), 95% CI 1.21, 9.07, p = 0.006] (Figure 2B) and unrelated [mean (SE) difference +5.35 (1.82), 95% CI 1.78, 8.91, p = 0.003] and sibling controls [+4.10 (1.57), 95% CI 1.02, 7.19, p = 0.01]. The L/R ratio declined over time in progressors (p = 0.03). There was no difference in L/R in type 1 diabetes and IA participants combined between those who had HLA DR3/4 or HLA DR3/X or HLA DR3/3 (age adjusted L/R 6.55 [SE 1.25]; 95% CI 4.10, 9.00) and those without HLA DR3 (5.86 [SE 1.29]; 95% CI 3.34, 8.38, p = 0.6).

SCFA

Plasma acetate [mean (SE) type 1 diabetes or e2 IA 141.9 (7.3), sibling or unrelated control 129.4 (8.0) μmol/L, p = 0.24], butyrate [2.61 (0.21), 2.18 (0.24) μmol/L, p = 0.16] or propionate [9.04 (0.63), 7.56 (0.70) μmol/L, p = 0.11)] did not differ between children with e2 IA or type 1 diabetes, and sibling or unrelated controls. Plasma acetate levels did not differ according to HLA DR type.

Relationship between SCFA and alpha diversity

Plasma acetate correlated with alpha diversity (observed richness) within samples [age-adjusted regression coeff (SE) 0.57 (0.21), 95% CI 0.15, 0.99, p = 0.008)], with no differences in this relationship observed between groups. Thus, for an increase of one Feature in observed richness, plasma acetate increased by 0.57 (SE 0.21) µmol/L. Plasma butyrate [age-adjusted regression coeff (SE) 0.015 (0.06), 95% CI 0.003, 0.028, p=0.01)] and propionate [age-adjusted regression coeff (SE) 0.047 (0.019), 95% CI 0.011, 0.083, p=0.01)] were also correlated with observed richness, but the associations were very small most likely due to the low plasma concentrations of these SCFAs.

The Inverse Simpson measure of microbial diversity 'eveness' was not associated with plasma acetate, propionate or butyrate.

Dietary Intake

Analysis of FFQ from 81 children showed minimal differences in macro or micronutrient intake between groups, or in fiber-rich foods (fruit, vegetables, whole grains and legumes) between type 1 diabetes, e2 IA and control groups. Estimated daily fiber intake was: type 1 diabetes 32.32 g (11.04) [mean (SD)]; e2 IA 30.14 g (13.57); single IA 26.63 g (9.21); sibling control 33.93 g (15.20); unrelated control 33.62 g (9.38) (p = 0.7). Children with type 1 diabetes consumed a lower proportion of saturated fat as a percentage of total dietary fat [49.0 (3.9)%, mean (SD)] compared to those with single IA [52.5 (3.9)%] or sibling controls [50.3 (3.5)%] (p = 0.008).

The incidence and duration of breast feeding did not differ between type 1 diabetes, e2 IA and control groups (data not shown). Most (78%) particpants had been breast-fed at least once and 50% had been breast-fed > 6 months.

DISCUSSION

Whilst gut microbiome dysbiosis and increased intestinal permeability have been reported separately in children with IA or type 1 diabetes, our study is unique in several ways. It is the first longitudinal study to describe gut microbiome dysbiosis in IA and type 1 diabetes in the Southern Hemisphere, and the first to describe gut microbiome dysbiosis with accompanying increased intestinal permeability, measured by the gold standard method, longitudinally in children progressing from IA to diabetes. Geography is relevant because distinct regional and inter-continental differences exist in the composition of the gut microbiome in children at-risk of type 1 diabetes.[12,13,37] Progressors from islet autoimmunity to type 1 diabetes in comparison with non-progressors had increased small intestinal permeability accompanied by reduced biodiversity of their gut microbiomes and decreased abundance of particular SCFA producing bacteria. Lower observed richness of the gut microbiome was in general associated with lower circulating concentrations of the SCFA, acetate.

Prevotella and *Butyricimonas* genera, both with known SCFA-producing properties, were less abundant in children with e2 IA or type 1 diabetes and distinct differences in microbiota were detected between progressors and non-progressors. Metagenomic sequencing would have provided more detail of the species and strains of these bacteria. While it is postulated that gut dysbiosis with lower SCFA production impairs

epithelial barrier function and increases intestinal permeability and thereby contributes to the pathogenesis of type 1 diabetes[38,39] previous reports analysing the gut microbiome, intestinal permeability and SCFAs have been limited to experimental models. Circulating SCFA concentrations other than acetate are very low due to first-pass hepatic metabolism. We detected an association of some magnitude only between gut microbiome richness and plasma acetate. Plasma acetate concentrations did not differ between children with type 1 diabetes or islet autoimmunity and controls, countering a role for plasma acetate as a potential biomarker of gut dysbiosis. Our results were consistent with those from the large TEDDY cohort[12,13] in which an increase in the pathway of bacterial fermentation to produce SCFAs was detected in children who did not develop type 1 diabetes.[12,13] Preceding, and considerably smaller, studies have also shown a lower proportion of SCFA-producing bacteria as part of a gut dysbiosis in European and US children with multiple islet antibodies[2,3,8] and in children with type 1 diabetes,[4] but not consistently.[7]

Hyperglycemia in experimental models increases intestinal permeability by reprogramming intestinal epithelial cells and reducing the integrity of their tight junctions.[40] Prospective continuous glucose monitoring (CGM) would have been a more sensitive method to detect the first appearance of hyperglycemia in the progressors than 6-monthly oral GTTs and postprandial blood glucose levels, as we used, but CGM was beyond the scope of our study design. Certainly, we

demonstrated a rise in intestinal permeability prior to the onset of persistent hyperglycemia. The fall in intestinal permeability over time in progressors to type 1 diabetes was unexpected and could not be explained by laboratory batch effect.

Species within the *Prevotella* and *Butyricimonas* genera would be expected to be more abundant with high fibre and vegetarian diets[41] although there are variations in the impact of different diets described, for example for different *Prevotella* species.[42] We found no significant differences in nutrient or fibre intake between children with type 1 diabetes, islet autoimmunity or controls, apart from lower saturated fat intake in type 1 diabetes. The latter, previously reported,[43] is most likely due to systematic nutrition advice at the time of clinical presentation of type 1 diabetes. The food frequency questionnaire was administered to the type 1 diabetes children 6 months after their dietary education at clinical presentation to standardise for this. Diet, therefore, did not obviously explain the detected deficiencies in anti-inflammatory bacteria.

A question is how small intestinal permeability aligns with the microbial composition of the colon. We suggest that the effects of butyrate on epithelial barrier function may extend beyond the colon[44] and/or that dysbiosis in the colon may reflect related changes in the small intestine, about which much less is known. Large intestinal permeability can be measured by sucralose absorption, but the assay is less well established than the L/R ratio.

We chose the OMR-200 kit for its reproducibility, reliability, and stability for home stool collections, as we have demonstrated.[25] This may have provided an advantage over earlier studies in at-risk cohorts for which samples were not necessarily collected and processed for the primary purpose of gut microbiome analysis. However, the choice of the OMR-200 kit did preclude the measurement of fecal SCFAs. A further limitation of our study is that the cohort was relatively small and few children progressed from islet autoimmunity to type 1 diabetes over the course of follow-up; nevertheless, the frequency and duration of longitudinal sampling for over 2 years with minimal missing data provided adequate power to detect differences in the microbiome and intestinal permeability. As participants had extant islet autoimmunity, we could not examine changes before and around the time of onset of islet autoimmunity.

In conclusion, we report distinct perturbations in the gut microbiome with deficiencies in anti-inflammatory bacteria in conjunction with increased intestinal permeability, in at-risk children and children with type 1 diabetes. These findings support interventions to restore balance to the gut microbiome in children with islet autoimmunity, potentially with butyrylated starches, other prebiotics or targeted bespoke probiotics, in an attempt to slow or halt the development of type 1 diabetes.

Author contributions:

JEH: researched data, wrote manuscript, oversaw project and is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of data analysis. AJRS: performed bioinformatic analysis of DNA, and generated figures, wrote manuscript. LCG: analysed data, wrote manuscript CDT: reviewed manuscript and contributed to discussion. KMN: performed DNA extraction and sequencing. MASP: developed sample collection methodology, reviewed/edited manuscript and contributed to discussion. JMW: facilitated participant recruitment and study visits, reviewed/edited manuscript, contributed to discussion. PGC: supervised islet autoantibody assays, reviewed/edited manuscript. GM: performed HLA typing, reviewed/edited manuscript. RLT: analysed data, reviewed/edited manuscript/ MEC: facilitated participant recruitment, oversaw NSW study site, reviewed/edited manuscript. ATP: oversaw bioinformatic analysis. SCB: analysed data, reviewed/edited manuscriptLCH: supervised DNA sequencing and analysis, oversaw Victorian study site and wrote manuscript. JJC: oversaw project and wrote manuscript and is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of data analysis.

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Table 1: Baseline characteristics of cohort

	Type 1 diabetes	**e 2 IA**	**1 IA**	**IA-negative sibling controls**	**Unrelated controls**	**Total cohort**
N	29	12	6	31	10	88
F/M	14/15	6/6	2/4	13/18	6/4	41/47
Age in years	10.7 (5.3-16.3) †	10.8 (4.8-23.2)	11.8 (6.8-21.9)	11.2 (4.1-20.2)	11.5 (5.1-26.7)	10.9 (4.8-26.7) *
Caucasian ethnicity %	97%	100%	100%	100%	100%	99%
HLA n (%)						
3/4	9 (32%)	3 (25%)	0 (0%)	4 (13%)	0 (0%)	16
3/X	6 (21%)	2 (17%)	1 (17%)	6 (19%)	4 (40%)	19
4/X	11 (39%)	7 (58%)	5 (83%)	15 (48%)	2 (20%)	40
X/X	2 (7%)	0 (0%)	0 (0%)	6 (19%)	4 (40%)	11
Unknown	1	0	0	0	0	1
Follow-up in months	11 (2-32)†	26.5 (6-34)	3 (2-25)	5(2-30)	16.5(6-24)	13 (2-34)
Stool samples per individual	2 (1-6)†	3 (1-6)	1.5 (1-4)	2 (1-6)	2 (1-5)	2 (1-6)
Plasma samples per individual	2 (1-6)†	3 (1-6)	1.5 (1-4)	2 (1-6)	2 (1-5)	2 (1-6)

†median (range)
* P=0.2

Table 2: Differential abundance analysis comparing type 1 diabetes and multiple islet antibody with unrelated control groups, and progressors to diabetes with non-progressors.

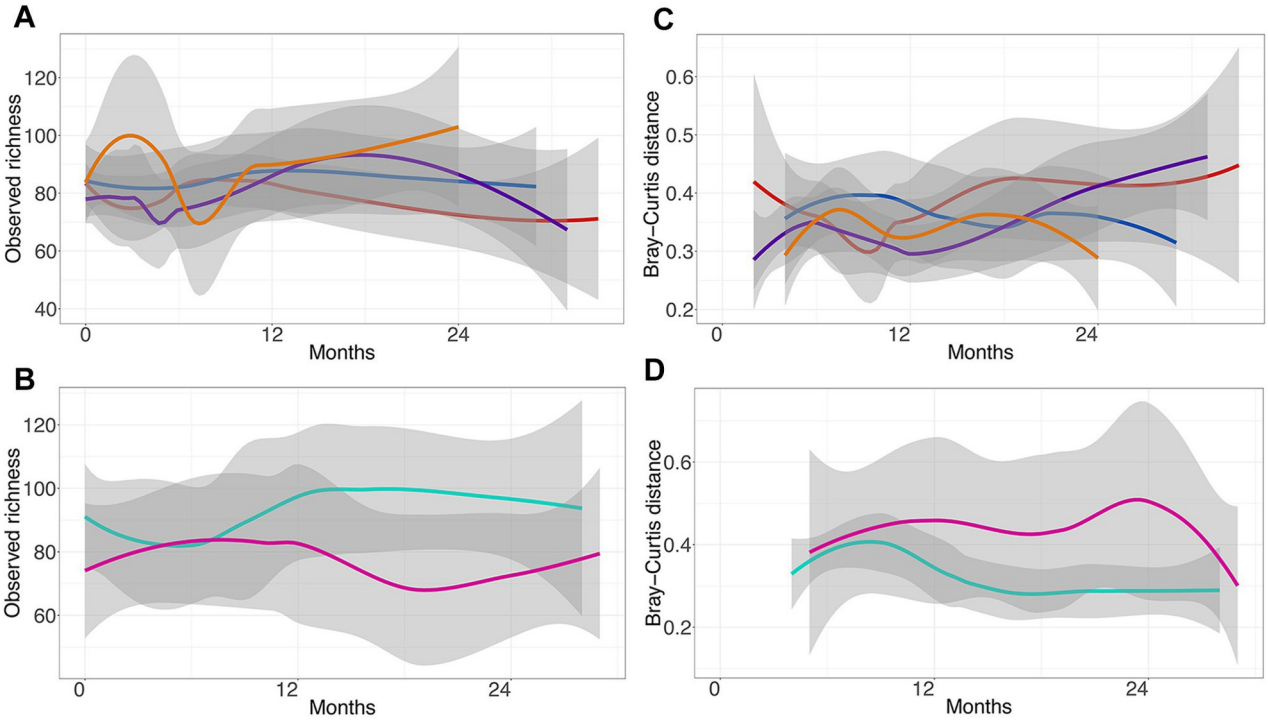
Taxon classification	**Average relative abundance (proportion %)**	**P value (adjusted P-value)**
Feature:6ec8fcc47f7d8dd964c78d47d4bd7419 Genus: Butyricimonas BLASTn: *Butyricimonas paravirosa*†	Type 1 diabetes = 0% (0%); UC = 0.25% (38%)	P = 6.8^{-7} (0.0003)
Feature:2c52774c279d3c51231c7136b83fce52 Genus: Prevotella BLASTn: *Prevotella* sp. *Marseille* and *Prevotella copri*‡	Type 1 diabetes = 0% (0%); UC=0.33% (7.7%)	P = $1.3e^{-5}$ (0.003)
Feature: a222792df90e47dc2b94f66d56a37ea1 Genus: Prevotella BLASTn**:** *Massiliprevotella massiliensis*‡	Type 1 diabetes = 0.35% (8.7.%); UC=5.4% (50%)	P = $1.8e^{-5}$ (0.003)
Feature: a222792df90e47dc2b94f66d56a37ea1 Genus: Prevotella BLASTn: *Massiliprevotella massiliensis*‡	e2IA = 0.02% (3%); UC = 5.3% (50%)	P = $2e^{-5}$ (0.006)
Feature: 6ec8fcc47f7d8dd964c78d47d4bd7419 Genus: Butyricimonas BLASTn: *Butyricimonas paravirosa*†	e2IA = 0 % (0%); UC = 0.25% (38%)	P = $2.7e^{-5}$ (0.006)
Feature:aa98e11290afb2e258ee1f729d1198bb Family: Ruminococcaceae BLASTn: Oscillibacter valericigenes§	P = 0.22% (31%); NP = 0.85% (95%)	P = $5.4e^{-5}$ (0.02)
Verrucomicrobia (Phylum)	P = 0% (0%); NP = 0.41% (62%)	P = 0.003 (0.02)

Tenericutes (Phylum)	P = 0.01% (71%); NP = 1.0% (77%)	P = 0.007 (0.02)

† Coverage 100%, Identity 99%
‡ Coverage 100%, Identity 100%
§ Coverage 100%, Identity 94%
UC = unrelated control
e2IA = multiple islet antibodies
P = Progressor (e2 IA who progressed to hyperglycaemia/type 1 diabetes)
NP = Non-progressor (e2IA children who did not progress to hyperglycaemia/type 1 diabetes)

Figure 1 Gut Microbiome in islet autoimmunity and type 1 diabetes
A Alpha diversity measured by observed number of Features (i.e. observed richness) over the study period in type 1 diabetes (red), e2 IA (blue), sibling control (purple) and unrelated control (orange) groups. The solid line represents the locally weighted smooth regression (Loess) function, the limits of the grey shaded areas represent the 95% confidence intervals. There were no differences between the groups.
B: Alpha diversity measured by observed number of Features (i.e. observed richness) over the study period in progressors from e2 IA to type 1 diabetes (pink) and non-progressors (aqua). The solid line represents the locally weighted smooth regression (Loess) function, the limits of the grey shaded areas represent the 95% confidence intervals. Progressors had lower observed richness than non-progressors [mean difference (SE) 31.3 (11.2), 95% CI 9.3,53.3, p=0.005].
C: Beta diversity measured by pairwise Bray-Curtis distances between consecutive samples over the study period in the type 1 diabetes (red), e2 IA (blue), sibling control (purple) and unrelated control (orange) groups. The solid line represents the locally weighted smooth regression (Loess) function, the limits of the grey shaded areas represent the 95% confidence intervals. There were no significant differences between (i) type 1 diabetes and unrelated controls and sibling controls (ii) e2 IA and unrelated and sibling controls.
D: Beta diversity measured by pairwise Bray-Curtis distances between consecutive samples over the study period in progressors from e2 IA to type 1 diabetes (pink) and non-progressors (aqua) at the Feature level. Beta diversity was not significantly different between the 2 groups.

Figure 2
A: Intestinal permeability measured by blood lactulose/rhamnose (L/R) concentrations over the study period in type 1 diabetes (red), sibling control (purple) and unrelated control (orange) groups. The solid line represents the adjusted regression line, the limits of the grey shaded areas represent the 95% confidence intervals, and individual L/R concentrations are represented by the dots. The L/R was higher over time in type 1 diabetes than in sibling controls [mean difference (SE) +2.49 (1.03), 95% CI 0.47, 4.51, p = 0.02] and unrelated controls [+3.67 (1.45), 95% CI 0.83, 6.5, p=0.01]
B: Intestinal permeability measured by blood L/R concentrations over the study period in progressors from e2 IA to diabetes (pink), and in non-progressors (aqua). The solid line represents the adjusted regression line, the limits of the grey shaded areas represent the 95% confidence intervals and individual L/R concentrations are represented by the dots. Progressors had higher L/R ratios over time than non-progressors (mean [SE] difference +5.14 [2.0], 95% CI 1.21, 9.07, p=0.006)



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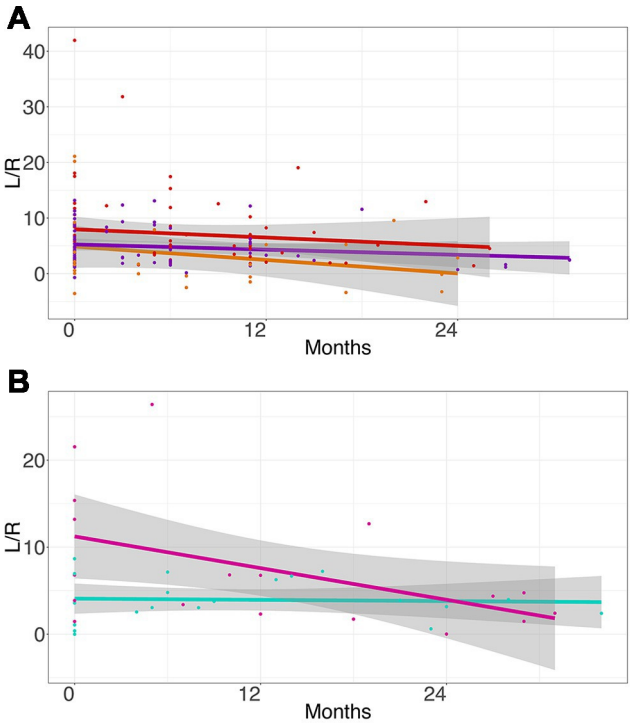

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