In silico analysis of bacterial metabolism of glutamate and GABA in the gut in a rat model of obesity and type 2 diabetes

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Dysbiosis of gut microbiota has adverse effects on host health. This study aimed to determine the effects of changes of faecal microbiota in obese and diabetic rats on the imputed production of enzymes involved in the metabolism of glutamate, gamma-aminobutyric acid (GABA), and succinate. The levels of glutamate decarboxylase, GABA transaminase, succinate-semialdehyde dehydrogenase, and methylisocitrate lyase were reduced or absent in diabetic rats compared with controls and obese rats. Glutamate decarboxylase (GAD) was significantly reduced in obese rats compared with control rats, while the other enzymes were unaltered; different bacterial taxa are suggested to be involved. Levels of bacterial enzymes were inversely correlated with the blood glucose level. These findings suggest that the absence of GABA and reduced succinate metabolism from gut microbiota contribute to the diabetic state in rats.

Key words: type 2 diabetes, obesity, microbiota, glutamate, succinate

Gut microbiota play a significant role in the maintenance of animal health [1], and dysbiosis contributes to the development of obesity and several metabolic diseases, particularly type 2 diabetes [2–4]. Changes in gut bacterial communities result in changes in bacterial metabolic profile, mainly recognised through in silico analysis, and thus indicate the potential for altered interaction with the host. Examples include changes in the production of short-chain fatty acids along with increases in inflammatory products [3–5]. One consequence of these changes is postulated to be reduced production of mucin, and this is significantly associated with the onset of obesity and diabetes [2, 6, 7]. Neurochemicals such as serotonin, glutamate, and gamma-aminobutyric acid (GABA) [8] are other products of some interest and may have direct effects on host physiology. A previous study indicated that a reduction in the specific activity of the enzyme glutamate decarboxylase (GAD), which is responsible for the conversion of glutamate to GABA, is associated with type 1 diabetes [9], while treatment of nonobese diabetic (NOD) mice with a GAD-derived peptide helped to delay and reduce the incidence of cyclophosphamide-accelerated diabetes [10]. GABA and glutamate are directly linked by interconversion with succinate, a key component of intermediary metabolism (Fig. 1a). Development of software for the prediction of metabolic potential, such as Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt), and Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs (KOs) provide a theoretical picture of metagenome function from 16S rRNA metagenomics [11, 12]. Thus, this study aimed to make predictions about the production of enzymes that form GABA and succinate by the gut bacteria of obese and diabetic rats.

We previously described a rat model of obesity and diabetes [13] produced by feeding adult male Wistar rats a high-fat diet (HFD; 22% fat; #821424, SDS, UK) for 12 weeks in combination with an intraperitoneal (I/P) injection of citrate buffer vehicle (pH 4.4; Obese group) or the HFD for 12 weeks in combination with a single dose of streptozotocin (STZ; 30 mg kg-1 injected IP at week 4; Diabetic group). Two control groups were included; both were fed a normal diet (RM1, Rat and Mouse No. 1 Maintenance Diet, SDS, UK) for 12 weeks and injected IP at week 4 with either citrate buffer vehicle (pH 4.4; Control) or STZ (STZ alone). Animal weights and blood glucose were measured weekly. Glucose levels were measured using a glucometer (Accu-Chek Aviva System, Roche Diagnostics, Indianapolis, IN, USA). After 12 weeks, animals were housed in clean cages individually overnight, and faecal pellets were collected. Bacterial genomic DNA was isolated within one day of collection and immediately stored at −80°C as described previously [13].

Taxonomic profiles were generated for each group by 16S rRNA gene sequencing, and molecular functions were predicted from them by PICRUSt [11]; the detailed methodology for the
bioinformatic analysis has been described previously [13]. The SRA accession number for the sequencing data is “SRP152214”. Here, we extended the metabolic analysis to consider the predictions for glutamate metabolic function and thus suggest mechanisms whereby changes of these metabolites could contribute to health or adverse effects. In addition, we analysed the predicted contribution of bacterial taxa to each individual activity with the metagenomic contributions script (https://picrust.github.io/picrust/scripts/metagenome_contributions.html). Statistical analysis of relative abundances, presented as mean differences within and between groups, was performed using GraphPad Prism 8.4.2 by controlling the False Discovery Rate using the method of Benjamini and Hochberg for corrected multiple comparisons after One-way ANOVA. Data distribution was tested by the Anderson–Darling, D’Agostino–Pearson, Shapiro–Wilk, and Kolmogorov–Smirnov (alpha=0.05) tests. In addition, Spearman’s method was used for nonparametric correlation analysis. Significance was considered to be established when p≤0.05. Principal Coordinates Analysis (PCoA) analysis was conducted with RStudio Version 1.4.1717 (ggplot2 version 3.3.5) and R for Windows Version 3.4.1 (https://cran.r-project.org/bin/windows/base/old/3.4.1/) to compare bacterial communities of the four groups.

**Fig. 1.** a) Schematic pathway for GABA and succinate metabolism with changes for imputed levels of orthologous enzymes along with contributing bacteria. Bacteria in each group: Control group, green box; Obese group, orange box; Diabetic group, blue box. Increase in bacteria: upward arrowhead; decrease in bacteria: downward arrowhead; absence of bacteria: black circle. b) Comparison of imputed levels of orthologous enzyme activity and the predictions for the relative contributions of bacteria in the formation of GABA and succinate. Differences in the relationship between the microbiomes of the rat groups (n=6 per group) were calculated with the False Discovery Rate using the method of Benjamini and Hochberg for corrected multiple comparisons after One-way ANOVA. Values indicated with different symbols (#, +, *, ##, ++, **) are significantly different (p<0.05). Groups (n=6 per group): CL: Control; STZ: STZ alone; Ob: Obese; Diab: Diabetic. c) Comparison of bacterial communities involved in succinate synthesis based on PCoA. PCoA comparisons are pairwise for Log_{10} of bacteria in individual rats in each group (n=6 per group). CL: Control; STZ: STZ alone; Ob: Obese; Diab: Diabetic. d) Correlations between predicted metabolic activities of orthologous enzymes and blood glucose (mmol/L). e) Correlations between the predicted metabolic activity of glutamate decarboxylase and animal weight (g).
Here we extended our earlier work [13] to focus on the predictions for major bacterial functional activities for glutamate and GABA metabolism. As described previously [13], rats fed a HFD and administered a low dose of STZ displayed hyperglycaemia and insulin insensitivity [13], a model of type 2 diabetes, while rats fed a HFD remained normoglycaemic but exhibited significant body weight gain relative to controls.

An analysis of 4 individual orthologous enzymes of this pathway in faecal samples collected from each rat model (Fig. 1a) was carried out using KEGG pathway maps as a reference. Statistical analysis of the enzymes showed them to be significantly higher in the Control, STZ-alone and Obese groups compared with the Diabetic group (Fig. 1b). These enzymes included GAD (EC: 4.1.1.15; gadB, gadA, GAD; K01580), 4-aminobutyrate aminotransferase (EC: 2.6.1.19; GABA transaminase, gabT; K07250), and methylisocitrate lyase (EC: 4.1.3.30; prpB; K03417), and with exception succinate-semialdehyde dehydrogenase (EC: 1.2.1.16, 1.2.1.79; gabD; K00135), which was only significantly higher in the Control group among the compared with the Diabetic group. Only one of these enzymes, glutamate decarboxylase (K01580), was significantly higher in the Control and STZ-alone groups compared with the Obese group and was absent in the Diabetic group, and this enzyme was used for biosynthesis of GABA [8].

Regarding other predictions, bacterial metabolism for the three enzymes involved in succinate synthesis is shown in Fig. 1b. These enzymes catalyse the conversion of GABA to succinic semialdehyde (SSA), bypassing the tricarboxylic acid (TCA) cycle and avoiding α-ketoglutarate dehydrogenase, and all three of them were significantly lower in the Diabetic group compared with the Control and STZ-alone groups.

Many genera are predicted to contribute to these enzyme activities (Fig. 1b). The predicted bacterial taxa involved in the production of GAD activity suggest that the S24-7 family made the predominant contribution in the Control and STZ-alone groups and was greatly reduced in the Obese group and absent in the Diabetic group. In the Obese group, both Ruminococcus flavefaciens and unclassified species from the RF16 family contributed to the activity, and they were not seen in the Control or STZ-alone group. A previous study reported that both Gram-positive and Gram-negative bacteria have GAD genes [8]. Recently, Medvecky et al. [14] reported that glutamate decarboxylase is commonly encoded in representative members of Bacteroidetes. An animal study found that within the phylum Bacteroidetes, the S24-7 family, now renamed Muribaculaceae, was associated with glutamate metabolism and was predominant in control mice, and its abundance was reduced after treatment with vancomycin [15], which is similar to our data in the Control group and STZ-alone groups, though it was significantly reduced in the Obese group and absent in the Diabetic group. In the Obese group, both Ruminococcus flavefaciens and unclassified classed from the RF16 family contributed to the activity, and they were not seen in the Control or STZ-alone group.

In agreement with previous studies, K07250 and K00135 were identified in different bacterial taxa [16, 17], and prpB was found in bacteria such as Escherichia coli [18] and used for the catabolism of propionate [19]. In the present study, the predicted levels of these bacterial enzymes in the synthesis of succinate were reduced in the Diabetic group compared with the other 3 groups. While the levels of the rats in the Obese group were the same level as those in the Controls group, changes in the taxa due to the HFD meant that different bacteria were involved in the production of succinate. Furthermore, the data from the Obese and Diabetic groups highlighted the significant reduction in S24-7 and associated increase of several genera in the phylum Firmicutes involved in succinate formation. In addition, when the Obese group was compared with both the Control and STZ-alone groups, significant enrichment was observed for the genus Bacteroides, while Turicibacter was significantly higher in the Diabetic group compared with the other three groups. It is generally accepted that the succinate produced by primary fermenters is the main precursor for propionate biosynthesis [20] and thus decreased in type 2 diabetes (T2D) rats may be expected. In fact, a study employing genomes assembled from metagenomes of a large panel of human, mouse, koala, and guinea pig hosts [21] found a positive link between the presence of S24-7 and the production of succinate, propionate, and acetate [21]. Cultures of Bacteroides spp. are able to digest a variety of polysaccharides and are described as succinate-producing bacteria [22] under specific growth conditions, such as high CO2 [20, 23]. In addition, Turicibacter spp. were found to be significantly higher in type-2 diabetic patients with chronic kidney disease [24].

In our experiment a high abundance of P. copri was predicted to be involved in the expression of enzymes that synthesise succinate in the Obese rats. There is evidence indicating that colonizing C57BL/6 mice with succinate-producing bacteria, P. copri, provided metabolic benefits that help to improve glucose homeostasis through increased intestinal gluconeogenesis [25] as well as enhanced bile acid metabolism and farnesoid X receptor (FXR) signalling [26]. Indeed, this improvement may be mediated by action at succinate receptor 1 (Sucnr1, or GPR91) [25, 27], which is present in the intestine and liver [28]. A recent study demonstrated that Sucnr1 promotes an anti-inflammatory phenotype in macrophages, and its expression was decreased in obese subjects [29].

A correlation analysis conducted by Spearman’s method to analyse the correlation of GABA and succinate biosynthesis with blood glucose (mmol/L) based on the relative abundances of the three enzymes involved in succinate synthesis indicated a significant inverse correlation (Fig. 1d). Furthermore, a significant inverse correlation was also found between the relative abundance of GAD and animal weight (Fig. 1e). These findings suggest that these enzymes enhance the level of insulin and thus reduce the plasma glucose levels. It has been confirmed that oral administration of GABA to obese and type 2 diabetic mice...
results in decreased fasting blood glucose and improved glucose tolerance and insulin sensitivity [30]. Furthermore, a study of wild-type mice reported that dietary succinate also contributes to improved glucose and insulin tolerance [25]. This suggests that reduction or absence of these bacterial enzymes may have directly impaired the Obese and Diabetic rats in our experiment.

Bacterial expression of these enzymes has beneficial effects on health. A possible mechanism by which bacterial GAD contributes to health is by GABA-stimulated expression of MUC1 in colonic mucosal epithelia, which produce mucus that acts as a protective intestinal barrier [31, 32], preventing the adhesion of harmful microorganisms to mucosal surfaces [33]. In addition, GABA has a role in quorum-sensing signalling that initiates cell-cell communication between bacterial species [34]. This system of cooperation maintains the commensal bacterial population, provides resistance to invasive infectious diseases [35], and stimulates the transport of GABA across the membrane of the intestinal epithelium via Caco-2 [36]. Other benefits of GABA include participation in controlling the cholesterol level and blood pressure and reducing inflammation, and thus it is considered to have anti-diabetic properties [37].

This study indicates that analysing imputed functional contributions provides suggestions for bacterial taxa and orthologous enzymes associated with GABA and succinate production based on the metabolic potential of gut microbiota (Fig. 1a). The findings suggest that deficiencies of GABA and succinate production contribute to the diabetic state.

ETHICAL APPROVAL
All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Rats were treated with full approval of the Institute’s Animal Ethics and Welfare Committee; the procedures complied with the UK Animal Scientific Procedures Act (1986) and were approved by the Home Office.

CONFLICT OF INTEREST
There are no conflicts of interest to disclose.

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