GABA-modulating bacteria of the human gut microbiota

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The gut microbiota affects many important host functions, including the immune response and the nervous system. However, while substantial progress has been made in growing diverse microorganisms of the microbiota, 23–65% of species residing in the human gut remain uncultured1,4, which is an obstacle for understanding their biological roles. A likely reason for this unculturability is the absence in artificial media of key growth factors that are provided by neighbouring bacteria in situ4–6. In the present study, we used co-culture to isolate KLE1738, which required the presence of Bacteroides fragilis to grow. Bioassay-driven purification of B. fragilis supernatant led to the isolation of the growth factor, which, surprisingly, is the major inhibitory neurotransmitter GABA (γ-aminobutyric acid). GABA was the only tested nutrient that supported the growth of KLE1738, and a genome analysis supported a GABA-dependent metabolism mechanism. Using growth of KLE1738 as an indicator, we isolated a variety of GABA-producing bacteria, and found that Bacteroides spp. produced large quantities of GABA. Genome-based metabolic modelling of the human gut microbiota revealed multiple genera with the predicted capability to produce or consume GABA. A transcriptome analysis of human stool samples from healthy individuals showed that GABA-producing pathways are actively expressed by Bacteroides, Parabacteroides and Escherichia species. By coupling 16S ribosomal RNA sequencing with functional magnetic resonance imaging in patients with major depressive disorder, a disease associated with an altered GABA-mediated response, we found that the relative abundance levels of faecal Bacteroides are negatively correlated with brain signatures associated with depression.

We have previously identified quinones as growth factors for uncultured bacteria of the human microbiome4. In this study, we searched for previously undescribed growth factors. On a densely inoculated plate, some uncultured bacteria may be growing because they are in proximity to cultivable organisms producing growth factors. Using this rationale, a faecal sample from a healthy human donor was plated on fastidious anaerobic agar supplemented with yeast extract (FAAy), and newly formed colonies were noted daily for 1 week. Late-forming colonies (appearing after 4–7 days, termed ‘candidate dependent’) were diluted and spread on FAAy, and a heavy inoculum of a neighbouring, early-forming colony (appearing after 1–3 days, termed ‘candidate helper’) was spotted (Fig. 1a). Given that Escherichia coli induced the growth of all quinone-dependent organisms in our previous study4, candidate dependents were counter-screened against E. coli (Fig. 1b).

After screening approximately 200 colonies for the desired dependency phenotype, we identified a single isolate that failed to grow in the presence of E. coli but grew on a plate with B. fragilis. This isolate, KLE1738, required the presence of B. fragilis KLE1758 (100% similar to B. fragilis ATCC 25852 according to 16S rRNA sequencing) for growth (Fig. 1c). KLE1738 is a Gram-positive bacterium of the Ruminococcaceae family and is 93.4% similar to Flavonifractor plautii VPI 0310 [S1] as well as Intestinimonas butyricicaudens SRB-521–5-I(T) based on 16S rRNA gene sequencing. A genome sequence similarity analysis showed that the uncultured Firmicutes bacterium CAG:14 was the closest relative to KLE1738 (Fig. 1d). On the basis of 16S rRNA gene sequencing and recent guidelines for assigning taxonomy9, the dissimilarity of KLE1738 to existing type strains suggests that it is the first representative of a unreported genus of bacteria. KLE1738 is found on the National Institutes of Health’s “Most Wanted” list10, indicating that it has not been cultured. This is despite its relative prevalence in the human gut microbiome, being detectable in 6,001 out of 31,451 (19.08%) of human gut metagenomes available in the Integrated Microbial Next Generation Sequencing database (16S similarity threshold of >99%, with a minimum sequence length of 200 bp)11. However, KLE1738 appears to be a relatively minor constituent of the microbiome, detectable at a relative abundance of >1% in only 60 out of 31,451 human gut metagenomes (0.19%).

The supernatant of a 48-h culture of B. fragilis KLE1758 grown in rich medium induced the growth of KLE1738 (Supplementary Fig. 1a, control shown in Supplementary Fig. 1b), enabling bioassay-driven purification of the growth factor. The supernatant was solvent partitioned with ethyl acetate, and the aqueous fraction
induced the growth of KLE1738. The aqueous fraction was then separated on a HP-20 column, and the most polar fraction induced the growth of KLE1738 (Supplementary Fig. 1c, non-inducing fraction shown in Supplementary Fig. 1d). This active fraction was then further fractionated by preparative high-performance liquid chromatography (HPLC), yielding a single active fraction. A NMR analysis revealed that it contained ten compounds comprising six common amino acids, three carboxylic acids and GABA (Supplementary Table 2). These results indicate that GABA is a required nutrient for KLE1738 in the tested experimental conditions.

To gain insight into the unusual growth requirements of KLE1738, its genome was sequenced and annotated. The annotated draft genome of KLE1738 revealed an unusual profile in that there were no apparent entry points into the central metabolism for common sugars, amino acids or other carbon sources. Bacteria use phosphotransferases to take up a variety of sugars, such as glucose, fructose and mannose. Uptake is coupled to phosphorylation by the membrane transporter Enzyme II, the last component of the following phosphorylation pathway: phosphoenolpyruvate–Enzyme I–Hpr–Enzyme II13. KLE1738 appears to lack Enzyme I. The fact that only one component of the phosphorylation pathway is missing suggests a recent loss of function. Similarly, KLE1738 is predicted to have a limited set of ABC transporters (Supplementary Table 3). This agrees with the inability of KLE1738 to grow on the tested nutrients. The utilization of GABA for carbon and nitrogen by bacteria has been reported before, although not as a required nutrient. The GABA shunt serves as a pathway for its conversion into succinate, which then enters into the tricarboxylic acid cycle14. However, KLE1738 appears to lack succinate semialdehyde dehydrogenase,
an essential enzyme of this pathway. An alternative ATP-generating pathway for GABA consumption was described for the environmental anaerobe *Clostridium aminobutyricum*\(^1\). KLE1738 has homologues of all enzymes of this pathway (Supplementary Fig. 3), with some enzymes present in multiple copies (Supplementary Table 4). Feeding KLE1738 with \(^{13}\text{C}\)- and \(^{15}\text{N}\)-labelled GABA allowed us to detect its metabolites, butyrate and acetate, as well as hexanoic acid, an intermediate of fatty-acid biosynthesis (Supplementary Figs. 4 and 5\(a\)–\(d\)). No incorporation of the \(^{15}\text{N}\) label was found. These results suggest that GABA is a carbon and energy source for KLE1738.

For some species, including *E. coli*, *Lactobacillus* ssp. and *Bifidobacterium* ssp., GABA secretion has been reported to serve as an acid-resistance mechanism. Decarboxylation of glutamate is induced at a low pH and produces GABA, which is then exported from the cell in a protonated form, alkalinizing the cytoplasm\(^14\). While *E. coli* does not induce the growth of KLE1738, a strain overexpressing glutamate decarboxylase (Gad) in *E. coli* K12 does, comparable to *B. fragilis* KLE1758 (Supplementary Fig. 6\(a\)–\(d\)).

*B. fragilis*, the helper of KLE1738, is a common gut bacterium, but we found that similar to *E. coli*, GABA production by *B. fragilis* is only observed at a low pH (≤\(5.5\)) (Supplementary Fig. 7). Apparently, *B. fragilis* growing on a Petri dish acidifies the medium enough to induce GABA production. We therefore sought to find gut microorganisms capable of producing GABA at a physiologically relevant pH for the human large intestine (pH 5.7–7.4)\(^16\). The unique GABA dependence of KLE1738 was exploited to identify additional GABA producers in a co-culture assay. Stool samples were mixed with molten FAAy agar (with or without exogenous buffer to maintain a pH of 7.0), allowed to solidify, and KLE1738 was then spread on top of the medium. By looking for zones of KLE1738 growth, we were able to identify gut bacteria that produce GABA (Fig. 2\(a\),\(b\)). In addition to *Bacteroides* spp., representatives from the *Parabacteroides*, *Eubacterium* and *Bifidobacterium* genera were identified as GABA producers in this assay (Fig. 2\(b\)). Of these, *Bacteroides* spp. (and to a lesser extent *Parabacteroides* spp.) were found to produce GABA within the pH range of the human large intestine (Fig. 2\(b\)).

![Fig. 2 | In vitro and in silico identification of GABA-modulating bacteria. a, To screen for GABA-producing bacteria, a homogenized human stool sample was diluted and mixed with molten FAAy, with or without pH 7.0 MOPS buffer. KLE1738 was then spread on top of the agar, and plates were incubated anaerobically for 1 week. Colonies that KLE1738 grew around were GABA producers (inset). Scale bars, 10 mm. b, Identified GABA producers were grown in liquid medium buffered at pH 5.0 and 7.0, and GABA levels of the spent medium were quantified using LC–MS, and final pH of the medium was tested with pH strips. N = 2 independent samples, and error is based on the s.e.m. c, A total of 1,159 available gut genomes (consisting of 919 species) were analysed for the genetic potential to produce and/or consume GABA (pathways associated with production or consumption highlighted in Supplementary Table 5). d, The biochemical potential of 533 available gut-related metabolic models in KBase were examined for the capability to produce GABA or consume GABA. Shown are genera that represent at least 0.5% of the 533 models and contain either GABA producers or consumers.](https://www.nature.com/naturemicrobiology)
We next sought to profile the GABA-modulating potential of the human gut microbiome in silico. All available bacterial genes encoding for GABA consumption and production were obtained from RAST\(^{11}\) and/or UniProt\(^{4}\). These sequences were then used as input for a bidirectional best hit analysis against genomes of known members of the gut microbiota, as demonstrated by their presence in the faecal 16S rRNA dataset of the American Gut Project, a crowdsourced microbiome sequencing project with over 10,000 participants\(^{19}\). Genomes were scored as ‘producer’ if they had at least one complete metabolic route for GABA production or ‘consumer’ if they had the GABA shunt or GABA transaminase and at least 7 out of 11 enzymes of the KLE1738 pathway (the presence of 7 out of 11 enzymes was chosen due to poor annotation of the remaining enzymes). Of 1,159 genomes analysed (consisting of 919 species), we identified 105, 205 and 211 species harbouring the genetic potential to only produce, to only consume, or to both produce and consume GABA, respectively (Fig. 2C; more detail provided in Supplementary Table 5). Due to its dissimilarity to type strains and its unique growth requirements, we propose the name “Eutepia gabavorous” for KLE1738. It will be described in a future manuscript, using traditional description methods for bacteria\(^{26}\).

To complement this approach, we applied a more rigorous in silico constraint-based modelling method to survey the potential GABA consumption and/or production of the gut microbiota. This was performed using KBase, a database that contains metabolic models computationally derived from genome sequences of many microorganisms\(^{21}\). Of the 919 species used in the above bidirectional analysis, 533 had models represented in KBase, and these were examined for their ability to produce GABA from known precursors—glutamate, arginine, putrescine and ornithine—or consume GABA via the KLE1738 pathway or the GABA shunt. The analysis predicted that 97 organisms had the capability to produce GABA, mostly via Gad (Supplementary Fig. 7). Of these, >25% belong either to the genera Bacteroides or Parabacteroides (Fig. 2D). For consumption, we identified 102 potential consumers of this neurotransmitter, with the majority belonging to the Pseudomonas, Acinetobacter or Mycobacterium genera (Fig. 2D). The number of GABA consumers is probably an underestimate, since the KLE1738 GABA consumption pathway is poorly annotated and thus not captured in KBase.

To validate activity of the genes of interest in humans, we surveyed an existing human transcriptome stool dataset\(^{22}\) for active expression of transcripts associated with bottlenecks of bacterial GABA metabolism (Gad, gamma-aminobutyrate:alpha-ketoglutarate aminotransferase and succinate semialdehyde dehydrogenase). The RNA sequencing data were assembled using the Trinity platform\(^{5}\), and retrieved coding sequences were subjected to BLASTN (90% cutoff at 1×10\(^{-5}\)). No transcripts were identified for enzymes involved in GABA consumption (perhaps attributable to the depth of sequencing), but multiple hits were found for Gad (Supplementary Table 6). These transcripts were successfully mapped to the nearest type strains of some of our GABA-producing panel, suggesting that Bacteroides and Parabacteroides species produce GABA in humans (Supplementary Table 7). Surprisingly, a set of transcripts also mapped to E. coli (Supplementary Table 6). Given the pH restrictions for GABA production by E. coli in vitro\(^{17}\), this result may indicate the presence of acidic microenvironments in the large intestine.

In germ-free mice, GABA levels have been reported to be significantly decreased both in the stool and the blood\(^{36}\) (but an older report showed no difference in GABA blood levels in germ-free and specific pathogen-free rats\(^{37}\)). Similarly, in specific pathogen-free mice, faecal GABA levels can be modifiable by antibiotics\(^{26}\), suggesting that the microbiota may contribute to circulating levels of GABA. This is of interest because multiple diseases are associated with an altered GABAergic profile, such as depression\(^{27}\). Accordingly, we sought to explore whether Bacteroides, perhaps the major bacterial producers of GABA in the human gut, were associated with clinically diagnosed major depressive disorder (MDD). To do this, stool samples from 23 patients with MDD were collected and analysed by 16S rRNA sequencing using the American Gut protocols. Resting-state functional magnetic resonance imaging (fMRI) scans were acquired within 3 days of stool sample collection, and scans were co-registered to the Montreal Neurological Institute (MNI) space. To investigate the relationship between neural circuitry known to be important in depression and faecal Bacteroides abundance we focused, a priori, on the left dorsolateral prefrontal cortex (DLPFC) and the default mode network (DMN). Our choice of the left DLPFC was guided by the highly replicated finding that the left DLPFC is hypoactive in depression\(^{28}\). Similarly, the DMN is involved in self-referential processing\(^{29}\) and negative rumination in depression\(^{30}\). Functional connectivity is elevated both within the DMN and with other networks in depression\(^{31}\) and normalizes with
treatment response. We found an expansive region of the DMN spanning the left anterior medial frontal cortex in which functional connectivity with the left DLPCF was inversely correlated with the relative abundance of faecal Bacteroides (Fig. 3a,b). This region of significance overlapped extensively with both the left medial prefrontal and left frontopolar cortex, regions highly interconnected to the limbic system and thought to be important in emotional reappraisal and reward processing. This cluster was unique, and we found no other clusters in which functional connectivity was positively correlated with the relative abundance of Bacteroides. We found no associations with Klebsiella, possibly due to its low abundance. Interestingly, a recent study of 40 healthy women found that levels of Bacteroides were associated with increased grey matter in the cerebellum, hippocampus and frontal regions of the brain. Moreover, these women exhibited reduced levels of anxiety, distress and irritability after looking at photographs to evoke an emotional response. Furthermore, a high-fat diet has been shown to reduce GABA levels in the rat prefrontal cortex by ~40%, which was associated with reduced levels of Bacteroides and depressive-like behaviour. Nonetheless, our pilot cohort has limitations in that the sample size was small and consequently we did not correct for medications (listed in Supplementary Table 8). Moreover, the 16S rRNA sequencing data reflected relative rather than absolute abundance. As such, this cohort should be expanded to further profile whether Bacteroides, or the GABA they may produce, are involved in affecting behaviours.

Notably, it has been reported that treatment of mice with the probiotic bacterium Lactobacillus rhamnosus JB-1 reduces stress and depression-like behaviour in a vagus nerve-dependent manner. This was accompanied by changes in the expression of GABA receptors in several areas of the brain, including the amygdala and hippocampus. A later study showed that mice exhibited elevated levels of GABA in the brain after 4 weeks of supplementation with JB-1. While JB-1 was not explicitly tested for its ability to produce GABA, other L. rhamnosus strains have been shown to produce GABA around a pH of 3.6–5.2. Some Bifidobacterium have been shown to produce GABA, and introduction of a GABA-producing Bifidobacterium strain resulted in improved visceral sensitivity in a rat model of pain. Importantly, a recent human study found that transplant of a faecal microbiome from lean to obese individuals resulted in increased levels of GABA in the plasma, showing that manipulating the microbiome may alter GABA levels. Our findings, combined with these reports, suggest that microbial-derived GABA may influence the host, and are the first step in understanding the biology of this intriguing connection.

**Methods**

**Human stool collection.** For cultivation experiments, stool samples from an adult healthy human donor were collected using a stool collection vessel (Medline Industries). Within 5 min of collection, 1 g of stool was resuspended in 9 ml of sterile 20% glycerol in PBS and homogenized for 30 s using a vortexer. Aliquots (1 ml) of this mixture were loaded into cryotubes and stored at −80 °C. Stool samples for cultivation experiments were collected with informed consent following an Institutional Review Board–approved protocol at Northeastern University (number 08–11–16).

For the MDD cohort, study participants were provided with sterile plastic 4-oz specimen collection cups at their first visit. They were instructed to collect stool on the day of or the night before their second visit depending on their ability to produce a sample to ensure that no more than 24 h passed between stool sample collection and processing. Study participants were instructed to keep their stool collection and processing. Study participants were instructed to keep their stool samples at room temperature until they brought it to their second visit. Once a sample was received by study personnel, it was processed within 1 h. American Gut samples were shipped at room temperature the day of sample processing (the standard shipping protocol used for the American Gut). Two 1.5-ml screw top plastic tubes per sample were filled with stool and immediately frozen at −80 °C for future studies.

**Cultivation of helper–uncultured pairs from human stool samples.** All cultivation was performed in a Coy Anaerobic Vinyl chamber with an atmosphere of 5% hydrogen, 10% CO2, 85% nitrogen. Stool samples were thawed and serially diluted in PBS under anaerobic conditions and bead-break (7–10 beads per plate) on 1× FAA (Accumedia) plates with 0.5% yeast extract (FAAY). Plates were incubated at 37 °C anaerobically for 1 week, and the appearance of colonies was recorded each day by spotting the samples onto the plates with different colour markers. At the end of the week, serial dilutions of late-coming colonies (appearance after 4–7 days) were prepared in PBS and bead-break on FAAY plates. Nearby (<2 cm), early-forming colonies (appearance after 1–3 days) were then resuspended in PBS at a high density. Five microlitres of this suspension was spotted on plates with their respective plates sold in duplicate and cultured. Plates were inspected for up to 1 week in the chamber, and observed daily. Growth induction of the dependent organism around the spotted helper indicated a positive hit. Strain nomenclature is as follows: KLE represents Kim Lewis, while the numbers represent the strain number.

**Taxonomic assignment.** PCR was performed for candidate dependents, helpers and other isolates using the general bacterial primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGGTACGACTT-3′) to amplify the 16S RNA gene. The following PCR reaction mixture was used: GoTaq Master Mix (12.5 µl; Promega), 27F and 1492R primers (1 µl each of 10 µM; Operon), nuclease-free water (9.5 µl; Promega), and 1 µl of a colony resuspended in 100 µl sterilized distilled water. The amplification conditions were as follows: one cycle of 95 °C for 5 min; 30 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 90 s; and final, one cycle of 72 °C for 7 min. Amplification of PCR products was confirmed using gel electrophoresis on a 0.8% agarose gel containing ethidium bromide.

**16S rRNA sequencing and bioinformatics.** Polymerase chain reactions were conducted using the 27F and 1492R primers using an Applied Biosystems 3730xl DNA Analyzer. Quality control for sequences was performed using DNA Baser (www.DNABaser.com, v.4.36.0), in which ends were trimmed until there were more than 75% good bases (defined by having a quality value score of higher than 25) in an 18-base window. Identification of phylogenetic neighbours and calculation of pairwise sequence similarity were done using the EzTaxon server (http://www.eztaxon.eu). The phylogenetic tree for KLE1738 was built using the Randomized Axelerated Maximum Likelihood (RAxML) method (v.8.3.5.23) via PATRIC45.

**Identification of GABA as a growth factor for KLE1738.** A single colony of B. fragilis KLE1738 was inoculated into brain heart infusion (BHI) broth (Becton Dickinson) with 5.0 mg ml−1 yeast extract, 0.1% cysine–HCL and 15 mg per litre hemin (BHIyH), and incubated in the anaerobic chamber at 37 °C. The 48-h B. fragilis KLE1738 culture was then filtered sterilized using a 0.22-µm syringe filter unit, and 200 µl of the supernatant was loaded into a Millicell Single Well Hanging Insert (pore size of 0.4 µm) and placed on top of a BHIyH agar plate with bead-break (7–10 beads per plate) KLE1738. Induction of KLE1738 growth was observed after 48 h. For all induction experiments, cells of KLE1738 were taken from 48-h cultures on solid BHIyH plates with 1 mg ml−1 GABA, or from plates spotted with B. fragilis.

The supernatant of B. fragilis KLE1738 (1 litre) was solvent partitioned with ethyl acetate (3×500 ml, each 3 h) to yield an ethyl acetate–soluble fraction and water residue. All extractions and fractionations of the supernatant were tested for the ability to induce the growth of KLE1738 by loading 200 µl into a Millicell Single Well Hanging Insert, as described above. The water residue part induced the growth of KLE1738, which allowed the highly polar water fraction to be applied to a HP-20 column for further fractionation, yielding the following six fractions (A–F): A, water–eluted fraction (2 litres); B, 20% methanol–eluted fraction (1 litre); C, 40% methanol–eluted fraction (1 litre); D, 60% methanol–eluted fraction (1 litre); E, 80% methanol–eluted fraction (1 litre); and F, 100% methanol–eluted fraction (2 litres). The most polar fraction (A) turned out to be the active fraction for inducing the growth of KLE1738, and was consequently separated by HPLC using an Agilent 1100 series HPLC system (Agnel Technologies) equipped with a photodiode array detector. The active fraction A (3.8 g) was further fractionated by a preparative HPLC (phenyl–hexyl column; Phenomenex Luna, 250 × 21.2 mm, 5 µm) with a flow rate of 10 ml min−1 using an isocratic solvent system of 1% aqueous acetonitrile for 30 min, then to 100% acetonitrile for the next 6 min, and 100% acetonitrile for the next 7 min to give the following 14 fractions according to HPLC chromatography analysis: A1 (3–5 min); A2 (5–8 min); A3 (8–10 min); A4 (10–13 min); A5 (13–16 min); A6 (16–19 min); A7 (19–22 min); A8 (22–25 min); A9 (25–29 min); A10 (29–35 min); A11 (35–36 min); A12 (36–38 min); A13 (38–40 min); and A14 (40–45 min). Among these fractions, the fraction A2 induced the growth of KLE1738. The active fraction was directly applied to NMR analyses, including 1H, 13C, H–H correlation spectroscopy, total correlation spectroscopy, heteronuclear single quantum coherence and heteronuclear multiple bond correlation experiments, to identify its constituents in the fraction. All NMR experiments were carried out on a Varian INOVA 600 MHz NMR spectrometer equipped with an indirect detection probe.

**Testing other compounds for induction of KLE1738.** Multiple compounds were tested for the ability to induce the growth of KLE1738. Stocks of each compound (purchased from Sigma, excluding the ATCC) were prepared according to their solubility in water (compounds and tested concentrations are provided in Supplementary Table 1). A volume (5 µl) of the stocks were then spotted on FAAY plates spread with KLE1738.
and incubated in the anaerobic chamber at 37 °C for 1 week. Biolog plates were inoculated with a 10-μl sample from a bacterial culture and incubated in the anaerobic chamber at 37 °C for 1 week. Biolog plates were inoculated with a 10-μl sample from a bacterial culture and incubated in the anaerobic chamber at 37 °C for 1 week.

**Whole-genome sequencing and annotation.** DNA from cells of KLE1738 grown anaerobically on FAy agar with 1.0 mg ml⁻¹ GABA and incubated anaerobically as described above.

**Preparation of U-13C, 15N-GABA and 15C feeding experiments.** Recombinant Gad was purified from an E. coli strain harbouring a his-tagged GadB from the ASKA library using the suggested protocol. The His-tagged GadB enzyme was eluted from a Ni-NiTA column, and was further purified by dialysis in PBS at pH 4 for 5 h, with a 2000–10000 molecular weight cutoff filter. GadB was quantitated by ultraviolet spectroscopy (μ = 85000 μm⁻¹ cm⁻¹) and diluted to give a concentration of 1.7 μg μl⁻¹. The following protocol was followed to convert U-13C, 15N-Glu (Cambridge Isotope Laboratories) to U-13C, 15N-GABA. Stocks of pyridoxal-5-phosphate (PLP, 10 μM in water) and dithiothreitol (DTT, 1 mM in water) were prepared fresh each time. In an Eppendorf tube, U-13C, 15N-Glu (9.4 μg, 0.066 mmol 60 mM final) was dissolved in 0.2 M sodium acetate buffer (750 μl, pH 4.6). Then, 10 μM PLP (100 μl, 1 mM final), 1 mM DTT (100 μl, 0.1 mM final) and 1.7 μg μl⁻¹ Gad (30 μl, 85 μg final) was added. This was incubated in a 37 °C water bath for 18 h. The mixture was passed through a Ni-NTA column to remove protein, adjusted to pH 1 with 2 M HCl and lyophilized. This was repeated twice (5–10 min scale) to produce enough U-13C, 15N-GABA for analyses.

13C, 15N-GABA was added to KLE1738 at a final concentration of 100 μg ml⁻¹ on FAy agar plates. After 72 h of incubation in the anaerobic chamber, cells were resuspended in PBS, washed once and pelleted. Agar was also collected. 13C, 15N-GABA was detected by mass spectrometry (MS; notably, no 13C, 15N metabolites were identified). Bacteria were pelleted by centrifugation, the supernatant discarded and 2 M HCl (100 μl) was added. The sample was subsequently vortexed, water (Mill-Q, 500 μl) was added, the sample was vortexed again and then was allowed to stand at room temperature for 10 min. Afterward, the sample was extracted with diethyl ether (3 × 2 ml), and the consolidated organic phase was dried with Na2SO4. The organic phase was then evaporated to dryness and the resulting material was resuspended in diethyl ether (500 μl). For extraction of the agar on which the bacteria was grown, the agar was sliced into small squares and 2 M HCl (10 ml) was added. The sample was vortexed, water (Mill-Q, 50 ml) was then added, and the sample was vortexed again. The mixture was subsequently allowed to stand at room temperature for 10 min, then was extracted with diethyl ether (3 × 50 ml). The consolidated organic phase was dried with Na2SO4, evaporated to dryness, and the resulting material was resuspended in diethyl ether (500 μl). The resuspended material was spun down to pellet insoluble particulate, and the supernatant was passed through a 0.2-μm syringe filter to afford samples suitable for analysis. A chromatography (GC)–MS analysis was performed on an Agilent 6890 GC with a Waters Quattro micro GC–MS–MS triple quadrupole mass spectrometer using electron ionization (EI) with a sample injection volume of 1 μl. A fused-silica capillary column of cross-linked DB-262UI (30 μm × 0.32 mm
organisms from the American Gut Project, 533 models were identified as gut-related (not all 913 microbes identified as gut related in KBase have associated models). These models were forced to produce GABA (cpd00281) intracellularly ([c]) by introducing the constraint “cpd00281[c]=” and maximizing it, with all exchange reactions (except for GABA) allowed to be unbalanced (−1.000 to 1.000). Reversing the GABA constraint revealed that no model can consume free GABA. To identify potential GABA consumers, the biochemical reactions involved in the KLE1738 GABA metabolism pathway were defined, and models were examined for similar reactions. Specificity of cofactor molecules were not considered, only that GABA consumption pathway were defined, and models were examined for similar potential GABA consumers, the biochemical reactions involved in the KLE1738 GABA consumption pathway. The MDD sequences analysed corresponded to the study if they presented with a history of claustrophobia, seizure disorder or previous head injury. Potential subjects were excluded from the study if they presented with a history of claustrophobia, seizure disorder or previous head injury. All subjects participated in the study. Subjects were recruited through referral from the outpatient clinic in the Department of Psychiatry at Weill Cornell Medical College. Subjects were also self-selected by directly contacting our mood disorders research programme or through the American Gut Project. The recruitment procedure and all the other aspects of our experimental protocol were approved by the Institutional Review Board of Weill Cornell Medical College, and all experiments were conducted in accordance with institutional guidelines and regulations. The ROI comprising the DMN was defined a priori based on previously published reports. The left DLPFC seed was an ROI within BA46 (9 mm seed centred on the following MNI coordinates: −44, 40, 29). Functional connectivity analysis. We first quantified functional connectivity between the left DLPFC and DMN areas by testing for correlations between the blood-oxygen-level dependent signal time series derived from the left DLPFC with voxels in the DMN (see below). Next, to identify cortical clusters in the DMN in which left DLPFC–DMN functional connectivity correlated with the relative abundance of Bacteroides, we performed an analysis of covariance (implemented using the 3dAsso covariate). The left DLPFC–DMN functional connectivity were not driven by Bacteroides effects on head motion. This also showed that the effects of relative abundance of Bacteroides on left DLPFC–DMN functional connectivity were indistinguishable in the groups of subjects scanned before and after the scanner upgrade. Next, a Monte Carlo simulation was performed for this map using the 3dClustSim function of the AFNI to determine the statistical thresholds for voxel cluster size needed to achieve a family-wise < 0.01 at voxel-wise P < 0.05. This yielded a threshold voxel cluster size of 213 voxels. In addition, the spatial mean connectivity value (Z score) of each significant cluster was extracted for each subject and a linear regression analysis was performed for each cluster with the relative abundance of Bacteroides. Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The 16S rRNA sequence and genome data for KLE1738 are available from the NCBI (MI636586 and PRJNA482656, respectively). The American Gut sequence data are available from the EBI under accession ERP012803. MRI data are available at the discretion of M.J.D. All other data that support the findings of this study are available from the corresponding author upon request.

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Author contributions
P.S. and K.L. planned the study, analysed the data and wrote the paper. P.S. performed the co-culture screening for helper-dependent pairs and general KLE1738 cultivation. P.S., J.C. and K.H.K. designed and performed the bioassay-driven screening for GABA. P.S. and N.M. performed media exclusion experiments. P.S., E.L.S. and D.D. analysed the KLE1738 genome. P.S., D.D. and T.R.R. performed 13C feeding experiments. P.S. cultivated B. fragilis and D.D. analysed the supernatant for GABA and glutamate. P.S. designed and with A.L. executed the screen for GABA-producing bacteria. P.S. prepared the supernatant of GABA producers identified in the KLE1738 co-culture screen, and D.D. analysed these supernatants for GABA production. P.S., D.M., R.K., J.L., J.K.L. and K.Z. performed the metabolic analysis for GABA producers and consumers, and P.S., A.S., and J.A.G. analysed the human transcriptome data. P.S., K.L., D.T. and M.J.D. designed the MDD study, and P.S., K.L., A.S., J.A.G., D.T., C.L. and M.J.D. analysed the MDD data. All authors helped edit the manuscript.

Competing interests
P.S. and K.L. declare competing financial interests as they are founders of Holobiome, Inc. All other authors have no competing interests.

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- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
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- Clearly defined error bars
- State explicitly what error bars represent (e.g. SD, SE, CI)

Software and code

Policy information about availability of computer code

Data collection 16S sequence data was collected and analyzed using QIIME (v 1.8.0). fMRI data was collected using AFNI (v17.0.03) and FSL (v5.0).

Data analysis Several programs were used throughout this study to analyze the data. Genome assembly and annotation of KLE1738 -- Edena (v3.131028) and RAST (v2.0); GABA quantification in spent mediums of potential GABA producers – ChemStation (Agilent); Metabolic modeling of candidate GABA modulating bacteria – Kbase v 3.0; Transcriptomic Analysis – Prodigal (v2.6.1); 16S sequencing from the American Gut – Deblur v1.0.2; fMRI imaging analysis – AFNI (v17.0.03) and FSL (v5.0).

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The 16S rRNA sequence and genome for KLE1738 are available from NCBI (MH636586 and PRJNA482656, respectively). The American Gut sequence data is in EBI under accession ERP012803. fMRI data is available at the discretion of author M.D. All other data that support the findings of this study are available from the corresponding author upon request. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to K.L. (k.lewis@neu.edu).

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

For the Major Depression Disorder cohort 23 subjects were recruited as a pilot effort. Given no such cohort (combining microbiome and brain imaging in depressed patients) has existed prior to this one, we had no prior cohorts to compare to for sample size. Nonetheless, a sample size of n=23 was large enough to reach significance between an association of GABA producing Bacteroides and fMRI brain signatures of depression (Figure 3A,B; p=0.0005), suggesting we reached appropriate power.

Data exclusions

No data was excluded.

Replication

GABA dependency phenotypes of KLE1738 was repeated in triplicate, and GABA production capabilities of all reported strains were validated in at least two biological replicates, with the exception of media exclusion experiments (Supplemental Data Table 2), C13 feeding experiments, which were both performed as a single experiment. All details on sample sizes can be found in the figure legends. No variation was observed to change the conclusions of the study.

Randomization

The human cohort was observational, so we did not have experimental groups.

Blinding

Blinding was not performed for the MDD cohort as all patients had MDD and no intervention was being performed.

Reporting for specific materials, systems and methods

Materials & experimental systems

| n/a | □ Involved in the study |
|-----|------------------------|
| ☑ | Unique biological materials |
| ☑ | Antibodies |
| ☑ | Eukaryotic cell lines |
| ☑ | Palaeontology |
| ☑ | Animals and other organisms |
| ☑ | Human research participants |

Methods

| n/a | Involved in the study |
|-----|------------------------|
| ☑ | ChiP-seq |
| ☑ | Flow cytometry |
| ☑ | MRI-based neuroimaging |

Human research participants

Policy information about studies involving human research participants

Population characteristics

For cultivation experiments, a single adult male donor provided fecal samples. For the MDD cohort, 23 currently depressed subjects between the ages of 19 and 65 (15 female) participated in fMRI imaging component of the study. Subjects were eligible for inclusion if they met DSM-5 criteria for Major Depressive Disorder and a current major depressive episode. Subjects were
Subjects were recruited through referral from the outpatient clinic in the Department of Psychiatry at Weill Cornell Medical College. Subjects were also self-referred by directly contacting our mood disorders research program or from the local community via flyers, outreach at local events, or direct contact. The recruitment procedure and all other aspects of our experimental protocol were approved by the Institutional Review Board of Weill Cornell Medical College, and all experiments were conducted in accordance with institutional guidelines and regulations. A potential bias to this cohort is that the patients we recruited from the local area -- expansion of this cohort into other regions will help build confidence of the associations.

Recruitment