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GABA-producing *Bifidobacterium dentium* modulates visceral sensitivity in the intestine

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Abstract

Background—Recurrent abdominal pain is a common and costly health care problem attributed in part to visceral hypersensitivity. Increasing evidence suggests that gut bacteria contribute to abdominal pain perception by modulating the microbiome-gut-brain axis. However, specific microbial signals remain poorly defined. γ-aminobutyric acid (GABA) is a principal inhibitory neurotransmitter and a key regulator of abdominal and central pain perception from peripheral afferent neurons. Although gut bacteria are reported to produce GABA, it is not known whether the microbial-derived neurotransmitter modulates abdominal pain.

Methods—To investigate the potential analgesic effects of microbial GABA, we performed daily oral administration of a specific *Bifidobacterium* strain (*B. dentium* ATCC 27678) in a rat fecal retention model of visceral hypersensitivity, and subsequently evaluated pain responses.

Key Results—Here we demonstrate that commensal *Bifidobacterium dentium* produces GABA via enzymatic decarboxylation of glutamate by *gadB*. Daily oral administration of this specific

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Figures S1, S2, and S3, as well as method employed for Liquid Chromatography coupled with Mass Spectrometry is provided in *Supporting Information File*.

Fig. S1. Growth profile and GABA production by *B. dentium*.

Fig S2. Effects of *B. dentium* administration on GABA concentrations in cecal content of antibiotic treated mice.

Fig S3. *Bifidobacterium* species copy numbers and GABA concentrations in electrophysiological studies in rats.

Contributors KP, CJ, BL, GU, YF, NO, RM, YMA, EH, SD, XZS, DE substantially contributed to the design and acquisition of experiments, as well as analysis and interpretation of the data. They also contributed to drafting the work and revising it critically for important intellectual content. ML and AM substantially contributed to the acquisition of some experiments as well as analysis of the data. TS and JV contributed to study concept and design, analysis and interpretation of the data, and drafting of the manuscript, while JV supervised the conduct of these studies.

Bifidobacterium (but not a *gadB* deficient) strain modulated sensory neuron activity in a rat fecal retention model of visceral hypersensitivity.

Conclusions & Inferences—The functional significance of microbial-derived GABA was demonstrated by *gadB*-dependent desensitization of colonic afferents in a murine model of visceral hypersensitivity. Visceral pain modulation represents another potential health benefit attributed to bifidobacteria and other GABA-producing species of the intestinal microbiome. Targeting GABAergic signals along this microbiome-gut-brain axis represents a new approach for the treatment of abdominal pain.

Keywords

GABA; Microbiome; *Bifidobacterium*; brain gut axis; neuromodulation

INTRODUCTION

Bidirectional communication between the gastrointestinal tract and the central nervous system (CNS) appears to be functionally linked to the intestinal microbiome, collectively termed the microbiome-gut-brain axis (1–6). The microbiome-gut-brain axis may contribute to pathophysiology in human diseases such as autism spectrum disorder (ASD) and Parkinson's disease, whereby GI comorbidities have been associated with disturbances of the intestinal microbiome, and may precede CNS symptoms (7, 8). Whether gut microbes play a role in such disease etiologies is not clearly established, although recent reports in animal models implicated microbiome-gut-brain signaling (9–15).

Functional GI disorders, *e.g.* irritable bowel syndrome (IBS) and constipation, represent a spectrum of diseases with active bidirectional gut-brain communication (9). A subset of patients with IBS (30–40%), have been reported to experience enhanced sensitivity to colonic distension, accompanied by a reduced threshold for pain and increased intensity of sensation (16). Visceral hypersensitivity has therefore been used as a clinical marker in this subset of patients with IBS. The cause of visceral hypersensitivity is unknown; however the recurrent abdominal pain that is attributed in part to visceral hypersensitivity is a common and costly health care problem. This amplification of visceral signals to the brain may occur at various levels, including at the level of the intestinal mucosa. Responses of these visceral afferents are affected by local chemical and mechanical stimuli, but may also be influenced by interactions with the intestinal microbiota. Through these interactions, the intestinal microbiota is proposed to play a regulatory role. Support for regulatory roles of intestinal microbes in human disease is provided by studies of probiotics. Numerous studies have shown that *Lactobacillus* and *Bifidobacterium* species (10, 17–23) ameliorate abdominal symptoms in functional GI disorders such as IBS and constipation. Probiotic induction of μ-opioid and cannabinoid receptor expression on intestinal epithelial cells may promote analgesic effects, as was observed in studies with *L. acidophilus* NCFM supplementation (10). These conceptual findings support a major regulatory role of the intestinal microbiome in enteric neurotransmission and visceral sensitivity. However, interoceptive mechanisms by which gut microbes signal to the enteric and central nervous systems, ultimately influencing pain perception and GI function, are poorly understood.

Metagenomic studies revealed that the human microbiome has the potential to generate numerous bioactive molecules, including but not limited to histamine, epinephrine, and γ-aminobutyric acid (GABA) (3, 4, 24, 25). Although *Lactobacillus* (26) and *Bifidobacterium* (27, 28) species are reported as GABA-producing bacteria, the links between microbial production and neuromodulatory activity *in vivo* have not been established. GABA is a primary inhibitory CNS neurotransmitter in mammals (29), exerting its major functional effects via two GABA receptor subclasses – $GABA_A$ and $GABA_B$ (30). However, GABA bioactivity is evolutionally conserved throughout the animal kingdom. In mammals, GABA has diverse physiologic effects such as modulation of blood pressure (31) and immune function (32), and it is also known to promote stress tolerance and ATP production in microbial communities (33–36). A major source of bioactive GABA is the enzymatic conversion of L-glutamate by glutamate decarboxylase by both microbes and host (37, 38). The potential health benefits of GABA have fueled a growing interest in GABA-enriched dietary supplements and naturally fermented food products (27). We hypothesized that *in vivo* production of GABA by commensal bacterial species would modulate the excitability of colonic sensory afferents. We aimed to demonstrate the neuromodulatory properties of microbial-derived GABA in an animal model of constipation and visceral pain, and to show that GABAergic signaling via the microbiome-gut-brain axis is mechanistically linked to glutamate decarboxylation by the common human commensal bacterium, *Bifidobacterium dentium*.

METHODS

Bacterial strains, cell lines, media, and culture conditions

Bacterial strains and plasmids used in this study are listed in supplemental Table 1. Bifidobacteria were cultured in Reinforced Clostridium Medium (RCM, Oxoid, Mampshire, UK) or modified de Man, Rogosa and Sharpe (mMRS) medium (39), supplemented with 0.05% (w/v) L-cysteine-HCl (Sigma-Aldrich, Steinhein, Germany) and 1% (w/v) of sodium glutamate. Strains were grown under anaerobic conditions in a Modular Atmosphere Controlled System (Davidson & Hardy Ltd.) at 37 °C. *Lactobacillus* sp. strains were grown at 37 °C in mMRS medium, or MRS medium supplemented with 0.05% (w/v) L-cysteine-HCl (Sigma-Aldrich). *Alistipes* sp. strains were cultured in MTGE medium (Anaerobe Systems) or MTGE medium supplemented with 1% L-Glutamate (mMTGE). *Escherichia coli* strains were grown aerobically at 37 °C in Luria Bertani (LB) on a rotary shaker (150 rpm). Where appropriate, media were supplemented with 5 μg mL^{-1} erythromycin (Erm) or 50 μg mL^{-1} to maintain plasmids in *B. breve* or *E. coli*, respectively.

In silico analysis of human microbiome and metagenomic data

To determine the distribution and relative abundances of the glutamate decarboxylase encoding *gad* genes at various sites across the human body, Human Microbiome Project (24) metabolic reconstruction profiles, as generated by the HUMAnN software package (40), were obtained from the Human Microbiome Project Data Analysis and Coordination Center (HMP DACC) (http://www.hmpdacc.org/HMMRC/). From these profiles, the relative abundance of the KEGG ortholog for *gadB* (K01580), across subjects and body sites, was obtained.

To identify potential genomes within the stool microbiome containing glutamate decarboxylases, the Integrated Microbial Genomes/Human Microbiome Project (IMG/HMP) database (http://img.jgi.doe.gov) (41) was used to search the annotations of the stool-associated reference genomes from the Human Microbiome Project (most recent annotation updated 20 April 2016). These genome annotations were queried using the IMG pathway term for glutamate decarboxylation (PathwayID 390: Glutamate decarboxylation to 4-aminobutyrate). This search returned 163 genes from 142 unique genomes. The genomes identified in this analysis were summarized at the genus level to characterize the distribution of putative glutamate decarboxylases among gut-associated bacteria. The data were parsed to the species level for *Bifidobacterium*.

The relative abundance of *B. dentium* in the intestinal tract of healthy children ($n = 22$), young adults ($n = 22$), and older adults ($n = 85$) was evaluated by generating taxonomic profiles of metagenomic sequence libraries from the Human Microbiome Project (24), Metahit cohort (41), and a school-aged pediatric cohort (42) and subjects from the Human Microbiome Project. Raw sequence libraries were obtained from each project's respective SRA repository or project ftp site (HMP: ftp://public-ftp.hmpdacc.org/Illumina/, Metahit:ftp://public.genomics.org.cn/BGI/gutmeta/Raw_Reads/, Pediatric: SRA project accession PRJNA74951). Paired-end libraries were concatenated into a single input file per subject, and each library was profiled using the Metaphlan software package (version 1.7.0) (43) with the Bowtie2 (44) search algorithm and "sensitive-local" settings. The relative abundances of all species detected across the three cohorts, including *B. dentium*, were compared using a Kruskal-Wallis H-test and Tukey-Kramer *post-hoc* test, as employed by the STAMP software package (45).

Bacterial screening

Nine *Lactobacillus* sp., five *Bifidobacterium* sp. and four *Alistipes* sp. strains were screened for their ability to consume L-glutamate and secrete GABA (see Table 1). Lactobacilli and *Bifidobacterium* sp. strains were cultured in MRS or mMRS, while *Alistipes* sp. strains were cultured in MTGE and mMTGE. Growth medium was inoculated with 1% inoculum of overnight cultures (12 h), followed by incubation anaerobically at 37 °C for 48 h. Cells were removed by centrifugation and cell free supernatants were subjected to filtration through 3 kDa MWCO filters (Amicon), following LC-MS analysis (Supporting Information) for the detection of secreted GABA and L-Glutamate.

In Silico Analysis

Sequence data were obtained from the non-redundant sequence database accessible at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov). The genome sequence of *B. dentium* ATCC 27678 (accession number: ABIX02000002.1) in combination with Artemis Informatics Software (Wellcome Trust Sanger Institute) was used for genome resource, display and analysis. BLAST searches were performed using the National Center for Biotechnology Information website. Sequence analysis was performed using the VectorNTI software package. GadB 3D structure modeling was achieved by means of Phyre2 software (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index). Visualization file was generated with Molmol (46).

DNA manipulation

Chromosomal DNA was isolated from *B. dentium* ATCC 27678 using Qiagen All Prep kit (Qiagen) according to the manufacturer's instructions. An initial lysis step involving cell resuspension in lysis buffer with 30 mg mL^{-1} lysozyme (Sigma) and incubation at 37 °C for 30 min was used. Plasmid DNA minipreps were obtained from *E. coli* using the QIAprep Spin Plasmid Miniprep kit (Qiagen) according to the manufacturer's instructions. Microbial genomic DNA was isolated from mouse and rat fecal samples using ZR Fecal DNA Miniprep Kit (Zymo Research) according to the manufacturer's instructions. Single-stranded oligonucleotide primers used in this study were synthesized by Lone Star Labs. Standard PCR reactions were performed using Takara polymerase (Clontech). PCR fragments were purified using Qiagen PCR purification kit (Qiagen). Electroporation of plasmid into *B. breve* NCIMB8807 was performed as described previously (47).

Construction of plasmids pQEgadB and overexpression of recombinant GadB (rGadB) proteins

The entire coding region of *gadB* (gene locus BIFDEN01403) was amplified by PCR with genomic DNA from *B. dentium* ATCC 27678 serving as a template and primer pair GadBFw and GadBRv (see supporting Table 2). These primers allowed the incorporation of a His_{15}-encoding sequence into the 3' end of *gadB* gene (designated here as r*gadB*). The r*gadB* PCR product was amplified with Takara *Ex Taq* DNA polymerase (Clontech). Overlap extension PCR was used to create substitutions in three conserved amino acid residues in *gadB* from *B. dentium* to Alanine (A). Therefore, six individual forward and reverse DNA fragments were designed with amino acid changes in the Threonine225, Aspartate256, and Lysine289 codon positions of the *gadB* gene with mutagenic primers (see supporting Table 2).The amplified DNA fragments were annealed in order to change amino acid residues to GCC, GCT, and GCG Alanine variants and generate a full length *gadB* gene product to express in *E.coli* M15pREP4 cells. Wild type and mutated r*gadB* amplicons were cloned in the pSMARTGC HK cloning vector (Lucigen), transformed into *E. coli* 10G ELITE cells (Lucigen), digested with *NcoI* and *HindIII* endonucleases (New England Biolabs), and ligated (T4 DNA ligase; New England Biolabs) with the expression vector pQE-60 (Qiagen) which had been treated with the same enzymes and transformed into *E.coli* M15pREP4 electrocompetent cells. The selection of *gadB* transformants was accomplished by restriction digestion with *NcoI* and *HindIII* endonucleases and by sequencing of positive clones. The resulting plasmid with the wild type *gadB* was designated as pQEgadB. rGadB proteins were overexpressed and analyzed by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis as previously described (48).

Enzyme assay and determination of glutamate decarboxylase activity by rGadB

In vitro enzymatic reactions with rGadB and its derivatives were performed as previously described by Hiraga et. al. (49). Cell lysates were prepared by sonicating bacterial pellets six cycles for 15 seconds and collected by centrifugation at 6000 rpm for 20 minutes at 4 °C. After centrifugation, soluble and insoluble fractions were collected and separated on a 10% SDS Polyacrylamide gel at 200 V for 30 minutes. 100 μl of soluble and insoluble fractions were suspended in equal volumes of 4 M ammonium sulfate (Sigma) and the resulting

mixture was then incubated for 3 minutes. Subsequently, 1.3 mL of substrate solution, consisting of 20 mM sodium glutamate (Sigma), PLP cofactor (Sigma), and Pyridine-HCl (Sigma), was added to the mixture, followed by an incubation at 37 °C for 20 minutes. The mixture was then heat inactivated by boiling for 5 minutes to stop the decarboxylation reaction. Reaction mixtures were subsequently analyzed for the presence of GABA and glutamate using LC-MS analysis (Details of LC-MS analysis in Supporting Information).

Construction of recombinant *B. breve* NCIMB8807

Construction of *B. dentium* ATCC 27678 *gadB* plasmid was performed as follows. *B. dentium* ATCC 27678 genomic DNA served as a template to amplify *gadB* by PCR using *gadB*-specific oligonucleotide primers (Table 2). The *gadB* PCR product was amplified with Takara *Ex Taq* DNA polymerase (Clontech), cloned in the TOPO 2.1-TA cloning vector (Invitrogen), transformed into *E. coli* Top10 cells (Invitrogen), digested with *XhoI* and *BamHI* endonucleases (New England Biolabs), and ligated (T4 DNA ligase; New England Biolabs) with the expression vector pESH46,(47) which had been treated with the same enzymes. *B. breve* NCIMB8807 cells were transformed, as previously described (50) with the ligated mixture, and several transformants were isolated. The selection of *gadB* transformants was accomplished by restriction digestion with *NdeI* and *BamHI* endonucleases and by sequencing of positive clones. Sequencing of all constructs was performed by Lonestar sequencing labs Inc. The resulting plasmid was designated as pESHgadB and the strain was designated as *B. breve* NCIMB8807 pESHgadB.

The recombinant *B. breve* NCIMB8807 harboring pESHgadB plasmid was screened for its ability to secrete GABA as follows. The recombinant strain, wild type *B. breve* NCIMB8807 (negative control) and *B. dentium* ATCC 27678 (positive control) were grown in MRS medium supplemented with 0.05% (w/v) L-cysteine-HCl for 48 hours and the cell free supernatants were analyzed using LC-MS analysis (Supporting Information).

In vivo GABA measurement

Animals—Six-week old, male, Swiss Webster mice (n=6–8 per group, 30 total) were purchased from Taconic Biosciences and were maintained under Specific-Pathogen-Free (SPF) conditions. Mice were kept under filter-top cages (3–4 mice per cage) and had free access to distilled water and LabDiet 5V5R rodent chow, containing 5.09% glutamate. All mouse experiments were performed in the SPF animal facility according to an Institutional Animal Care and Use Committee (IACUC)-approved mouse protocol at Baylor College of Medicine, Houston, TX.

Bifidobacterial strain administration—*Bifidobacterium* strains (*B. dentium* ATCC 27678, *B. breve* NCIMB 8807, and *B. breve* NCIMB8807 pESHgadB) were grown in culture conditions described above. Bacteria were harvested in early-stationary phase (12h growth in mMRS medium or mMRS supplemented with 5 μg mL^{-1} erythromycin (Erm) to maintain the plasmid in *B. breve* pESHgadB). OD600 of the culture was used to approximate cell viability based on previous growth curve data (Supplemental Figure 1). The culture volume required for approximately 8×10^8 CFU mL^{-1} was centrifuged at 5000g for 5 minutes to pellet the bacterial cells. The cells were then washed in pre-reduced PBS

(PBS + 0.05% L-cysteine) and re-pelleted. The wash was performed to minimize the amount of GABA administered to the mice in the gavage mixture. The cells were suspended to a final concentration of 8×10^8 CFU mL^{-1} in PBS with 0.05% L-cysteine and 1% sodium glutamate. Cell viability in the gavage mixture was also verified by dilution plating on MRS agar immediately before gavage procedure. Mice were administered 4×10^8 CFU mL^{-1} (in 0.5mL) of *B. dentium, B. breve*, or *B. breve* pESH suspended in PBS with 0.05% L-cysteine and 1% glutamate. Control mice received only sterile PBS with 0.05% L-cysteine and 1% glutamate. Oral gavages were administered once per day for 5 days. Weight was monitored over the course of treatment, and mice were euthanized 24 hours after the last gavage. The gastrointestinal tract was carefully removed. Luminal contents in the cecum were collected, flash frozen immediately in liquid nitrogen, and stored at −80°C until use.

Fecal water preparation—Samples of cecal content (50–80mg) were manually homogenized in 1 mL of DEPC-treated water by vigorous vortexing until slurry was produced. The mixture was centrifuged at 5000 × *g* for 5 minutes. The supernatant (fecal water) was carefully decanted into 1.5 mL Eppendorf tubes and stored frozen (–20 °C) until analysis. 100uL of this preparation was used in subsequent GABA ELISA.

GABA Measurement via ELISA—GABA concentrations in cecum content were measured via GABA ELISA (LDN Immunoassays) according to manufacturer instructions. Briefly, after extraction and derivatization, GABA was quantitatively determined by competitive ELISA using an anti-rabbit IgG-peroxidase conjugate with 3,3’,5,5’-Tetramethylbenzidine (TMB) as a substrate. All samples and standards were run in duplicate, and the reaction was monitored at 450 nm. The resulting ng mL^{-1} values were background adjusted using 650 nm as a reference wavelength, and results were normalized to initial weight of sample.

Rat fecal retention model

Animals—Male Sprague-Dawley rats (n=4 per group) of 225 to 250 g were used for this study. The Institutional Animal Care and Use Committee of the University of Texas Medical Branch at Galveston, TX approved the study using Sprague-Dawley rats. Rats were anaesthetized with 2% isoflurane with an E-Z Anesthesia System. A 20-mm-circumference glass rod was inserted into the rat anus. A purse-string suture was placed in the external sphincter to keep the lumen of the anus around the rod, and then the rod was removed. The outlet occlusion was kept for 3 days. This treatment significantly reduced fecal output and led to lumen distention in the distal colon, which significantly increased colon specific sensory neuron excitability. Age-matched control rats were treated similarly, except that the suture was removed immediately after being placed. *B. dentium* ATCC 27678 (4×10^8 CFU in 0.5 mL mMRS) was administered to control and FR rats via oral gavage daily for 5 days (2 days before and 3 days during fecal retention).

Retrograde fluorescence label injections—Labeling of colon specific dorsal root ganglia (DRG) neurons was performed as previously described (51). Under general 2% isoflurane anesthesia, the lipid soluble fluorescence dye, 1,1’-dioleyl-3,3,3’,3’-tetramethylindocarbocyanine methanesulfonate (DiI, Invitrogen, Carlsbad, CA) (50 mg

mL^{-1}) was injected into muscularis externae of the distal colon in 8 to 10 sites (2 μl each site). To prevent leakage, the needle was kept in place for 1 minute following each injection.

Dissociation of DRG neurons—Control and FR rats were euthanized by cervical dislocation, followed by decapitation. Lumbosacral (L6–S2) DRGs were collected in ice-cold and oxygenated dissecting solution, containing (in mM): 130 NaCl, 5 KCl, 2 KH_2PO_4, 1.5 $CaCl_2$, 6 $MgSO_4$, 10 glucose, and 10 HEPES, pH 7.2 (305 mOsm), as described previously (51). After removal of the connective tissue, the ganglia were transferred to a 5 mL dissecting solution containing collagenase D (1.8 mg mL^{-1} ; Roche) and trypsin (1.0 mg mL^{-1} ; Sigma, St Louis, MO), and incubated for 1.5 hours at 34.5 °C. DRGs were then taken from the enzyme solution, washed, and put in 0.5 mL of the dissecting solution containing DNase (0.5 mg mL^{-1} ; Sigma). Cells were subsequently dissociated by gentle trituration for 10–15 times with fire-polished glass pipettes and placed on acid-cleaned glass coverslips. The dissociated DRG neurons were kept in the dissecting solution for at least 1 h in room temperature before recording.

Whole-cell patch clamp recordings from dissociated DRG neurons—Before each experiment, the glass coverslip with DRG neurons was transferred to recording chamber perfused (1.5 mL/min) with external solution containing (in mM): 130 NaCl, 5 KCl, 2 KH_2PO_4, 2.5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 glucose, pH adjusted to 7.4 with NaOH (300 mOsm) at room temperature. Recording pipettes, pulled from borosilicate glass tubing, with resistance of 1–5 MΩ, were filled with solution containing (in mM): 100 $KmeSO_3$, 40 KCl, and 10 HEPES, pH 7.25 adjusted with KOH (290 mOsm). DiI-labeled neurons were identified under fluorescent microscope. Whole-cell currents and voltage were recorded from DiI-labeled neurons using Dagan 3911 patch clamp amplifier. Data were acquired and analyzed by pCLAMP 9.2 (Molecular Devices, Sunnyvale, CA). The currents were filtered at 2–5 kHz and sampled at 50 or 100 μs per point. While still under voltage clamp, the Clampex Membrance Test program (Molecular Devices) was used to determine membrane capacity, C_m and membrane resistance, R_m, during a 10 ms, 5 mV depolarizing pulse form a holding potential of −60 mV. The configuration was then switched to current clamp (0 pA) for determining other electrophysiological properties. After stabilizing for 2–3 min, resting membrane potential (RMP) was measured. The minimum acceptable RMP was −40 mV. Between 20–24 neurons from 4 rats in each group were used for analysis.

(Methods employed for Liquid Chromatography coupled with Mass Spectrometry are provided in *Supporting Information File*.)

RESULTS

Microbial glutamate decarboxylase (*gadB*) is enriched in human intestine

GadB is the primary bacterial enzyme responsible for generating bioactive, microbe-derived GABA. The availability of metagenomic data from the Human Microbiome Project (HMP) allowed us to determine the relative abundances of *gadB* in the microbial genomes found at different body sites in 96 healthy adult individuals. Our analysis demonstrates that although bacterial *gadB* is present at multiple body sites, it is most abundant in adult human stool specimens (Fig. 1*A*). Our results indicate that the *gadB* gene signature is present at nearly 3-

fold greater percentage in the stool microbiome versus the microbiomes at other body sites. This result is not surprising, as the glutamate decarboxylase system is known for its role in acid-resistance, and contributes to bacterial survival during transit through the gastrointestinal tract.

The relative abundance of *gadB* in stool prompted us to investigate which microbes harbor glutamate decarboxylases (Fig. 1*B*). The prevalence of glutamate decarboxylases among the members of the healthy human gut microbiome was estimated from data deposited at the Integrated Microbial Genomes/Human Microbiome Project (IMG/HMP) database (http://img.jgi.doe.gov). From this database, genomes associated with the human gastrointestinal tract were selected for analysis. The genomes of 26 unique genera were found to possess glutamate decarboxylase orthologs, including intestinal bacteria such as *Bacteroides* spp. (relative abundance (RA) is 31.7%), *Escherichia* spp. (RA – 22.5%), and *Fusobacterium* spp. (RA – 9.9%). Interestingly, the commensal bacteria *Bifidobacterium dentium*, which contains a *gadB* gene, was found to be present at a relative abundance of 0.7% (Fig. 1*B*). Thus, microbial GadB is a potentially rich enzymatic source of bioactive GABA in the gut lumen and this species represents a model organism for GABA-producing microbes.

GadB-derived GABA secretion by *Bifidobacterium dentium*

In order to quantify GABA production by specific intestinal bacterial strains, sixteen different commensal microorganisms were screened for GABA production capacity by LC-MS. Based on the metagenomics-based screening, several human-derived isolates of *Alistipes* spp. and *Bifidobacterium* spp. (including *Bifidobacterium dentium*) were selected, along with various commensal and probiotic species (Fig. 2*A*). From this microbial screening library, only four strains actively secreted increased quantities of GABA into MRS medium supplemented with glutamate (Fig. 2*A*) including *L. plantarum* NCIMB8826, *L. plantarum* ATCC14917, *B. angulatum* ATCC27535 and *B. dentium* ATCC 27678. This *B. dentium* strain was the only significant GABA secretor ($p<0.0001$) with the greatest amount of GABA produced among this group, and more than 1 mg mL^{-1} of GABA detected *in vitro*. Our efforts therefore focused on characterizing the mechanisms of GABA production in this human-derived commensal strain.

Taxonomic profiling of metagenomic sequencing data suggests that *B. dentium* may account for up to 0.18% (median value across all subjects evaluated) of the healthy human gut microbiome (Fig. 2*B*). The observed enrichment in pediatric samples ($p = 2.71e^{-6}$) may be partially explained by the increased overall abundance of *Bifidobacterium* species typically detected in children relative to adult samples (42). To characterize the dynamics of GABA production by *B. dentium* ATCC 27678, its growth in MRS medium, glutamate consumption, and GABA secretion were monitored over 72 hrs (Fig. S1). Unlike *B. bifidum* MIMBb13CS, which lacks the *gadB* gene, *B. dentium* secreted GABA by consuming glutamate in a stationary phase environment associated with low pH. These results confirmed and extended prior reports that *B. dentium* is a human-associated GABA-producing microbe (27, 28).

To confirm that GadB is the enzyme responsible for GABA production in *B. dentium*, we generated recombinant His-tagged wild type and mutant GadB proteins to characterize the

catalytic mechanism of glutamate decarboxylation. Homology modeling of GadB in *B. dentium* identified 3 conserved active site amino acids (52)- lysine (K289), threonine (T225), and aspartic acid (D256) (Fig. 3*A*). Each of these amino acids were separately mutated to alanine, and relative enzyme activities were measured in the presence of glutamate and pyridoxal phosphate (PLP) cofactor (Fig. 3*B*). Using this quantitative approach, wild type GadB converted approximately 80% of the substrate, glutamate, into GABA, whereas mutant proteins were deficient in enzymatic activity. Furthermore, to test whether GadB is sufficient for GABA production by bifidobacteria, *gadB* was cloned into the *Bifidobacterium* - *E. coli* shuttle vector pESH46 (47), transformed into *B. breve* NCIMB8807, and analyzed for GABA production. Since it is currently not possible to genetically delete *gadB* in *B. dentium*, we chose this highly transformable *B. breve* strain because it lacks *gadB* and does not produce GABA (53). Constitutively expressed recombinant GadB in *B. breve* harboring pESHgadB, produced GABA in comparable amounts to *B. dentium in vitro* (Fig. 3*C*), demonstrating that GadB functions as a glutamate decarboxylase in *B. dentium* with conserved catalytic amino acid residues involved in PLP cofactor binding and glutamate decarboxylase activity.

Microbial GABA production *in vivo* is mediated by GadB

To confirm that *gadB* mediated GABA production occurs in an *in vivo* setting as well as i*n vitro*, we used a murine model to examine how host GABA concentrations are affected by short-term colonization with these *Bifidobacterium* strains (Figure 3D). Six-week old male Swiss Webster mice (n=6–8 per group) were orally administered1% glutamate plus *B. dentium, B. breve, B. breve* pESHgadB , or saline (PBS) for 5 days. This oral gavage mimicked the natural route of gut microbial colonization. Inter-subject variation was observed in each group, however mice administered *B. breve* pESHgadB had significantly more GABA in their cecal content than mice that received non-modified *B. breve* ($p<0.01$; 18.4±3.4 vs 7.8±1.4 μg g^{-1} cecal content). A trend towards increased GABA production was also observed in the mice that received *B. dentium* relative to those that *B. breve* (10.1±2.5 vs 7.8±1.4 μg g^{-1} cecal content), however GABA concentrations in the PBS-treated mice were also within a similar range (10.3±1.7 μg g^{-1} cecal content).

Increased GABA production in cecal content of *B. dentium* colonized mice has also been demonstrated in previous pilot studies using mice pre-treated with antibiotics (Fig. S2). A trend towards increased GABA production was observed in mice receiving *gadB*-positive *B. dentium* compared with the non-producer, *gadB*-negative *B. breve* ($p>0.05$; 14.2±2.5 vs. 5.4±0.9 μg g^{-1} cecal content; n=6 per group). The increased GABA accumulation was not due to altered survival or transit of the bacteria since similar bacterial counts were measured in extracted cecal content ($p>0.05$; 1.7×10^7 and 6.7×10^7 copies of *B. dentium* and *B. breve*, respectively; n=6 per group) (Fig. S2). Taken altogether, these data demonstrate that the *gadB* gene from *B. dentium* ATCC 27678 is sufficient to facilitate GABA production *in vivo* when cloned into other bacterial species, and that expression of the microbial *gadB* gene can result in significant increases in host intestinal GABA concentrations.

B. dentium ATCC 27678 desensitizes sensory neuron activity in a rat model of visceral hypersensitivity

To determine if GABA producing bifidobacterial strains have beneficial effects on abdominal pain in fecal retention associated constipation, we induced fecal retention (FR) in rats by partial occlusion of the external anal sphincter as previously described (54). This procedure results in abdominal distention, constipation, and visceral pain, as confirmed by behavioral and electrophysiological studies (54, 55). After daily oral gavage of 4×10^8 *B. dentium* or *B. breve* for 5 days, electrophysiologic patch clamp recordings of retro-labeled colon-specific afferent neurons in the dorsal root ganglion (DRG) were used as a measure of afferent neuron sensitivity (56) (Fig. 4*A*–*L*). This technique is well established as a surrogate indicator of visceral sensitization and abdominal pain in animal models (54, 57). Colon-specific DRG neurons in FR rats treated with *B. breve* (n=4 rats, n=24 neurons) yielded significant depolarization of resting membrane potential ($p<0.001$; −48.33±0.48 vs −56.19±0.46), decreased rheobase ($p<0.001$; 0.16±0.01 vs 0.25±0.01), and an increased number of action potentials evoked at 2× rheobase ($p<0.01$; 1.38±0.12 vs 1.00±0.0) and 3× rheobase ($p<0.001$; 2.25±0.20 vs 1.33±0.11), compared to neurons from the sham *B. breve*-treated rats (n=4 rats, n=21 neurons). Importantly, these differences were largely absent in neurons from rats with fecal retention receiving GABA-producing *B. dentium*. There was no significant difference in rheobase (0.25±0.006 vs. 0.23±0.007), or the number of action potentials at 2× rheobase (1.05±0.05 vs. 1.17±0.08) or 3× rheobase (1.25±0.1 vs. 1.5±0.12) in DRG neurons from sham *B. dentium*-treated rats (n=4 rats, 20 neurons) when compared with FR *B. dentium*-treated rats (n=4 rats, n=24 neurons). Fecal retention and treatment with *Bifidobacterium* species by itself did not alter other electrophysiologic characteristics of DRG neurons, including cell diameter, capacitance, input resistance, action potential amplitude, duration, threshold, or overshoot ($p>0.05$ between sham and fecal retention rats treated with both *B. dentium* and *B. breve*; n=4 rats and 20–24 neurons recorded per group; Fig. 4E–L). Furthermore, similar numbers of both *Bifidobacterium* strains were detected in stool specimens from treatment and control animals (Fig. S3) and demonstrated a corresponding trend towards increased GABA in *B. dentium*-treated animals only. These data indicate that colonic-specific sensory neurons are hyper-sensitized following luminal distention associated with fecal retention, and this hyper-excitability phenotype is inhibited by GABA-producing, *gadB*-positive *B. dentium* and not by a genetically similar non-GABA producer.

DISCUSSION

We have shown that visceral hypersensitivity may be modulated by select GABA-producing members of the gut microbiota, implicating neurotransmitter production by the microbiota as a means of microbiome-gut-brain communication. Many amino acids can be metabolized by microbes to form biologically active amine compounds via decarboxylation. These reactions consume intracellular protons, resulting in increased pH within bacterial cells. In this way, the decarboxylation of amino acids (including glutamate) plays a central role in acid stress resistance among enteric (58) and lactic acid bacteria (33–35). Protection against the acidity of the mammalian stomach and acidic microenvironments within the intestinal lumen helps these bacteria survive and even colonize their host.

Amino acid decarboxylation can also generate strong proton motive forces in bacterial cells, which can increase ATP production, and metabolic activity by these cells. Glutamate decarboxylase (GAD or *gadB*, EC 4.1.1.15) catalyzes the irreversible α-decarboxylation of glutamate (glutamic acid) to produce GABA. The *gadB* gene is present in numerous bacterial species residing within the gastrointestinal tract, and we have demonstrated that microbial GadB is a potentially rich enzymatic source of bioactive GABA from the gut lumen. A small subset of bacteria that are especially adept at this particular enzymatic reaction may indeed be sufficient to elicit large changes in the host metabolome.

Our metagenomic analysis confirmed the presence of *B. dentium* in the gut microbiome of healthy children and adults. Bacterial screening revealed *B. dentium* ATCC 27678 as the major GABA producer among those strains encoding the *gadB* gene. Bifidobacteria have been recently hypothesized and demonstrated to produce GABA (27, 28), however this is the first in depth study that investigates GABA secretion and its possible effect on the host. *B. dentium* is a representative taxon of the healthy human gut microbiome (24), and strain ATCC 27678 was isolated from healthy human feces. Analysis of metagenomic datasets demonstrated that *B. dentium* is common in healthy pediatric and adult stool specimens, with a relative enrichment in children suggesting that microbe-derived GABA signaling may be developmentally important during childhood.

With our finding that microbial GadB is highly enriched in human stool, relative to other body sites, we explored the possibility that local GABAergic signals originating from the GI tract regulate gut-brain function. We have shown that the addition of the microbial *gadB* gene *in vivo* can significantly increase luminal GABA concentrations in the intestine. Since GABA is a well characterized inhibitory neurotransmitter involved in central and visceral pain perception, we chose to examine the physiologic effects of *B. dentium*-associated GABA in a rat fecal retention model of visceral sensitivity (54, 55). Here we provide the first experimental demonstration of a direct link between microbial-derived GABA and neuromodulation of colonic sensory afferents in an animal model of constipation and visceral pain. We have shown that sensory afferents are less sensitive to the colonic distension in the presence of *B. dentium*, as is demonstrated by the increased rheobase, and fewer number of action potential spikes. This would indicate that these neurons are less inclined to reach the depolarization threshold, suggesting that nociception is diminished. With the findings in the present study, we suggest that GABA-producing bacterial strains may be potential probiotics, benefitting visceral pain in these conditions.

Neuromodulatory signals that emanate from luminal microorganisms and influence gut-brain function have been studied extensively during the past decade. Proposed mechanisms include activation of Toll-like (59, 60) and histamine receptors (61), as well as short chain fatty acids (62), but these signal transduction pathways likely do not target neuronal synapses exclusively. *In vitro* studies support the notion that intestinal commensals have the capacity to secrete bioactive compounds and neurotransmitters such as histamine (61), nitric oxide (63), hydrogen sulfide (64), and GABA (27) at concentrations that can directly regulate neuronal activity. Changes in the gut microbiome also result in fluctuations of amino acid precursors to serotonin, an important neurotransmitter in the enteric and central nervous systems (65, 66). Serotonin is released by the population of enterochromaffin cells

with consequent effects on enteric neurotransmission and gut motility (67). By responding to host-derived catecholamines, pathogenic *E. coli* elegantly provided an additional example of communication between the enteric nervous system and the gut microbiome (68). However, production of these neuroactive compounds *in vivo* is currently under-studied, and the functional significance of their effects on the host system is not well understood.

We provide *in vivo* evidence that visceral hypersensitivity in response to fecal retention is preferentially inhibited by microbes that produce GABA, possibly by direct modulation of GABAergic signaling via colonic afferent neurons. Previous reports localized $GABA_A$ and $GABA_B$ receptors on murine and human enteric neurons, and these cells are optimally positioned to receive microbial-derived GABA signals (69, 70). As reported by other groups (30), this receptor distribution may coordinate GABAergic regulation of intestinal motility (71), gastric emptying (72), gastric acid secretion (69), and transient lower esophageal sphincter relaxation (73). Interestingly, a recent *in vitro* study showed that the GABA agonist baclofen decreased DRG neuron activity by acting on $GABA_B$ receptors and inhibiting both low-voltage activated and high-voltage activated Ca^{2+} currents (74). GABA has also recently been shown to act via the $GABA_{B1}$ receptor on peripheral nociceptive terminals to modulate nociceptor sensitization and counteract inflammatory pain via interaction with the pain receptor, TRPV1 (75). In our current study, we demonstrate that a GABA-producing species, but not a closely related non-GABA-producing species of commensal gut bacteria can modulate the hyperexcitability of peripheral somatosensory neurons in response to fecal retention. Taken altogether, these findings provide additional support for the role of GABA in visceral nociception, and reinforce the notion that GABA receptors on peripheral somatosensory neurons may be targeted for regulation of pain signaling. In future studies we will examine GABA receptor localization on sensory colonic neurons and the mechanistic role of this signaling pathway in modulation of visceral hypersensitivity by *B. dentium*. Future directions also include using isotope-labeled glutamate to track microbial conversion of glutamate to GABA, and assessing host utilization of GABA. Studies are needed to carefully delineate whether other microbial GABA targets colonic afferents directly, or whether indirect neuromodulatory signals activate receptors on other cell types, such as enteroendocrine cells.

In conclusion, metagenomic data analysis of diverse human microbial communities identified genes involved in GABA synthesis as being particularly enriched in the intestine. Qualitative metabolomic studies supported this observation by reporting GABA in human and mouse luminal contents (76, 77), but a direct demonstration of microbial production in the gut was missing. Here we identify the common commensal microbe, *B. dentium*, as an active GABA producer within the human gut microbiome. The presence of the glutamate decarboxylase encoding gene, *gadB*, in this species provides the genetic basis for glutamate to GABA conversion. By modulating the hyperexcitability of peripheral somatosensory neurons, candidate probiotic *B. dentium* and other GABA-producing gut microbes may represent future therapeutics for recurrent abdominal pain and functional bowel disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Key Messages

- Recurrent abdominal pain is a common and costly health care problem attributed in part to visceral hypersensitivity
- Intestinal bacteria capable of producing the primary inhibitory CNS neurotransmitter, γ-aminobutyric acid (GABA), modulate primary sensory neurons of the enteric nervous system. The production of GABA by the intestinal microbiome can be increased by supplementing bacteria encoding the glutamate decarboxylase gene *gadB*.
- GABA produced by the intestinal microbiome may form the basis of future nutritional interventions or microbiome-based therapeutics that ameliorate abdominal pain.

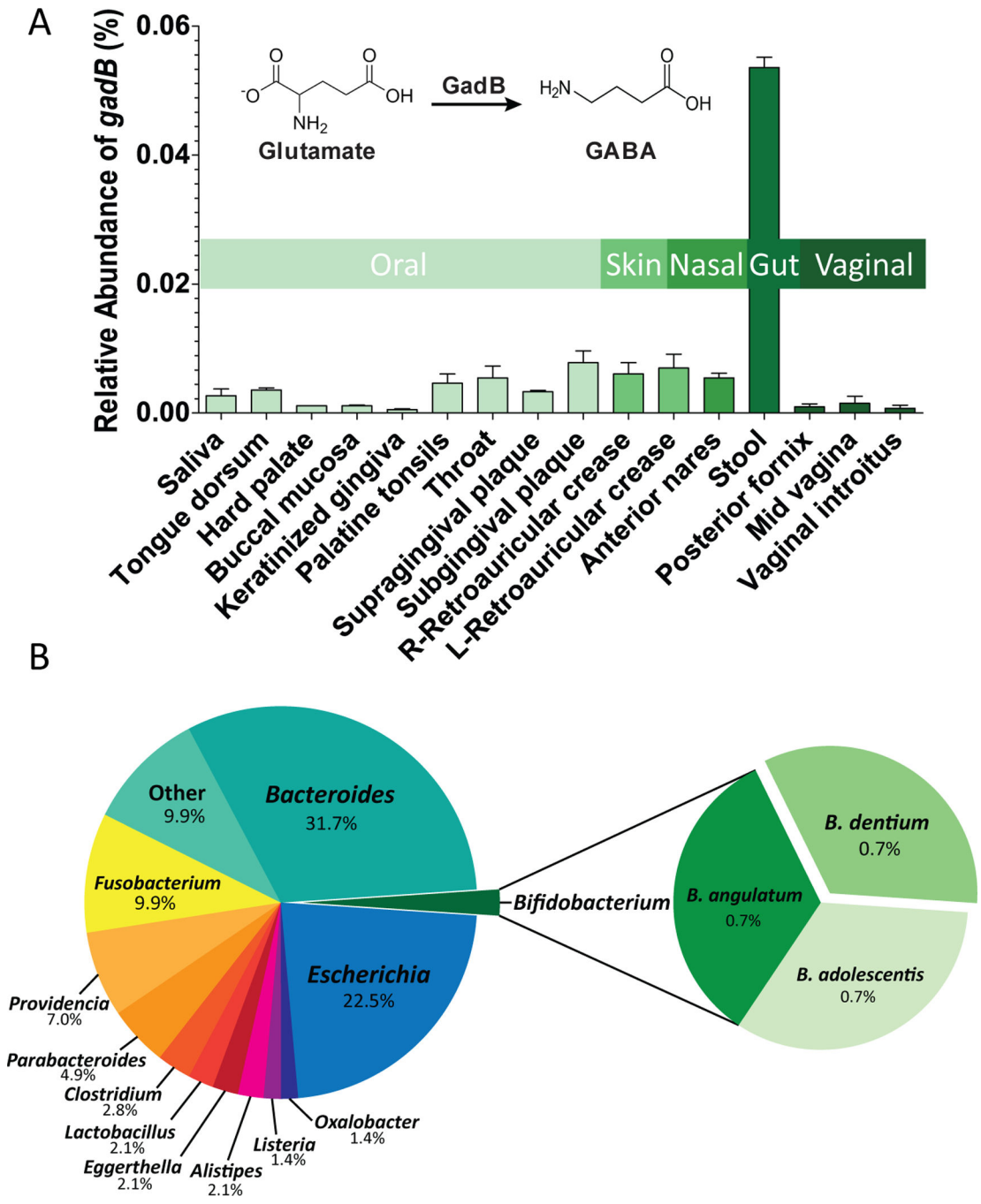

Figure 1. Microbial glutamate decarboxylase gene (*gadB*) in the human microbiome

(A) The relative abundance of the *gadB* gene among different body sites in 96 healthy adult individuals is depicted as a bar graph. The vertical bars indicate the mean relative abundances (and body site) of *gadB* (±sem). The chemical structure of glutamate and GABA and conversion of glutamate to GABA by GadB are shown within the graph. The colored horizontal bars indicate body sites. (B) Bacterial genera/species of the human gut microbiome harboring putative glutamate decarboxylases, are depicted as a pie chart. The prevalence of glutamate decarboxylases among the members of the healthy human gut

microbiome was estimated from data deposited at the Integrated Microbial Genomes/Human Microbiome Project (IMG/HMP) database (http://img.jgi.doe.gov). Percentages displayed represent genus-level distribution among these genomes, with species-level distribution shown for the *Bifidobacterium* species.

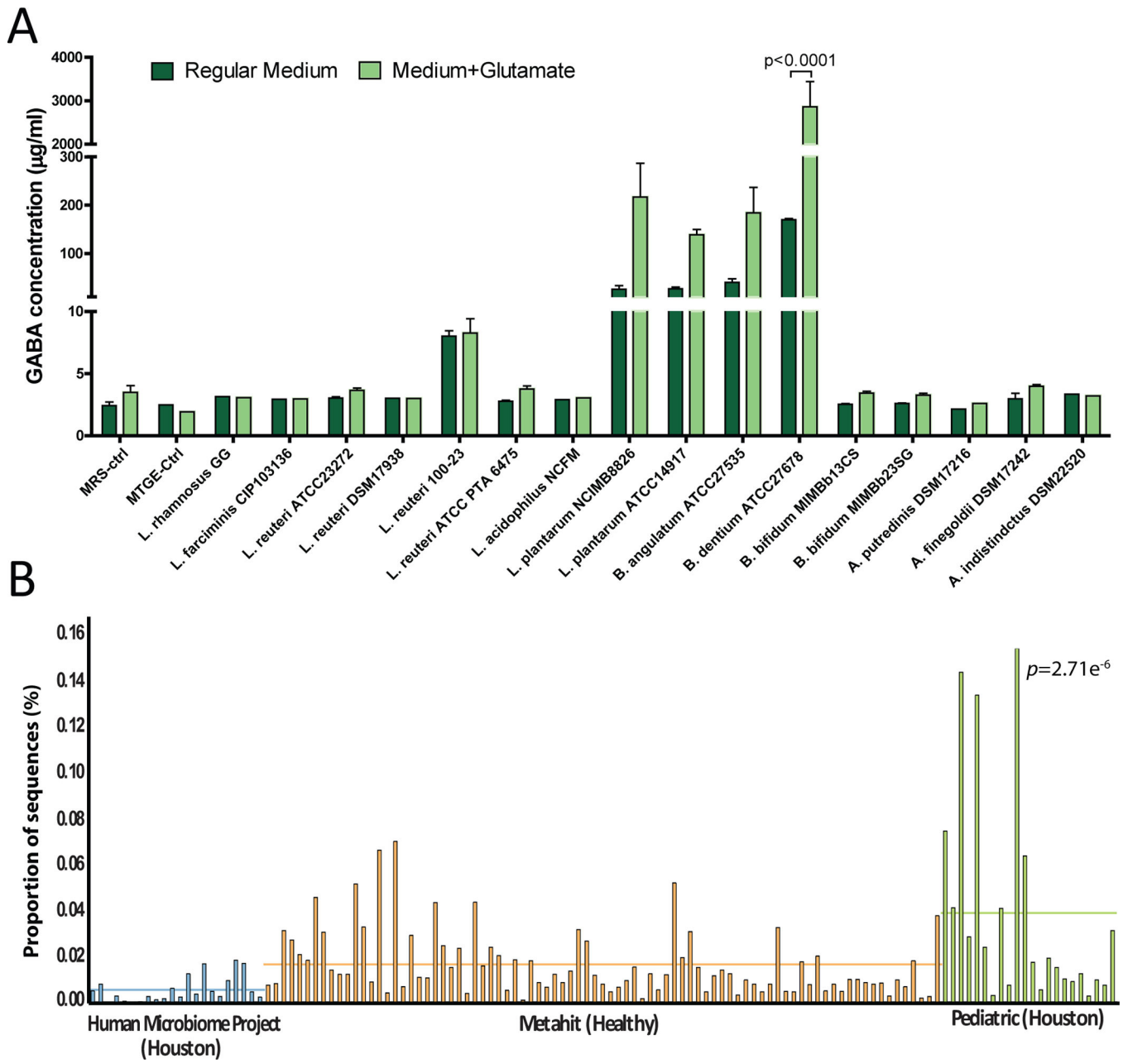

Figure 2. GABA production by commensal intestinal strains and *B. dentium* in the human gut microbiome

(**A**) Screening of 16 different intestinal commensal and/or probiotic isolates identified *B. dentium* ATCC 27678 as a major GABA producer. GABA concentrations were measured using LC-MS after 48 hours of anaerobic growth at 37 °C in either regular MRS (dark green) or MRS medium supplemented with 1% w/v glutamate (light green). Error bars represent standard error of 3 independently performed experiments. B. dentium was the only species that produced significantly more GABA (****p<0.0001; Two-way ANOVA with Bonferroni correction for multiple comparisons) (**B**) *B. dentium* in the healthy human gut microbiome, as detected by Metaphlan profiling of shotgun metagenomic sequence libraries.

Different colors represent subject cohorts, while bars show proportion of sequences with hits to *B. dentium* found in each individual. The horizontal bars represent the average relative abundance (as determined by the proportion of sequences with hits to *B. dentium*) in each cohort.

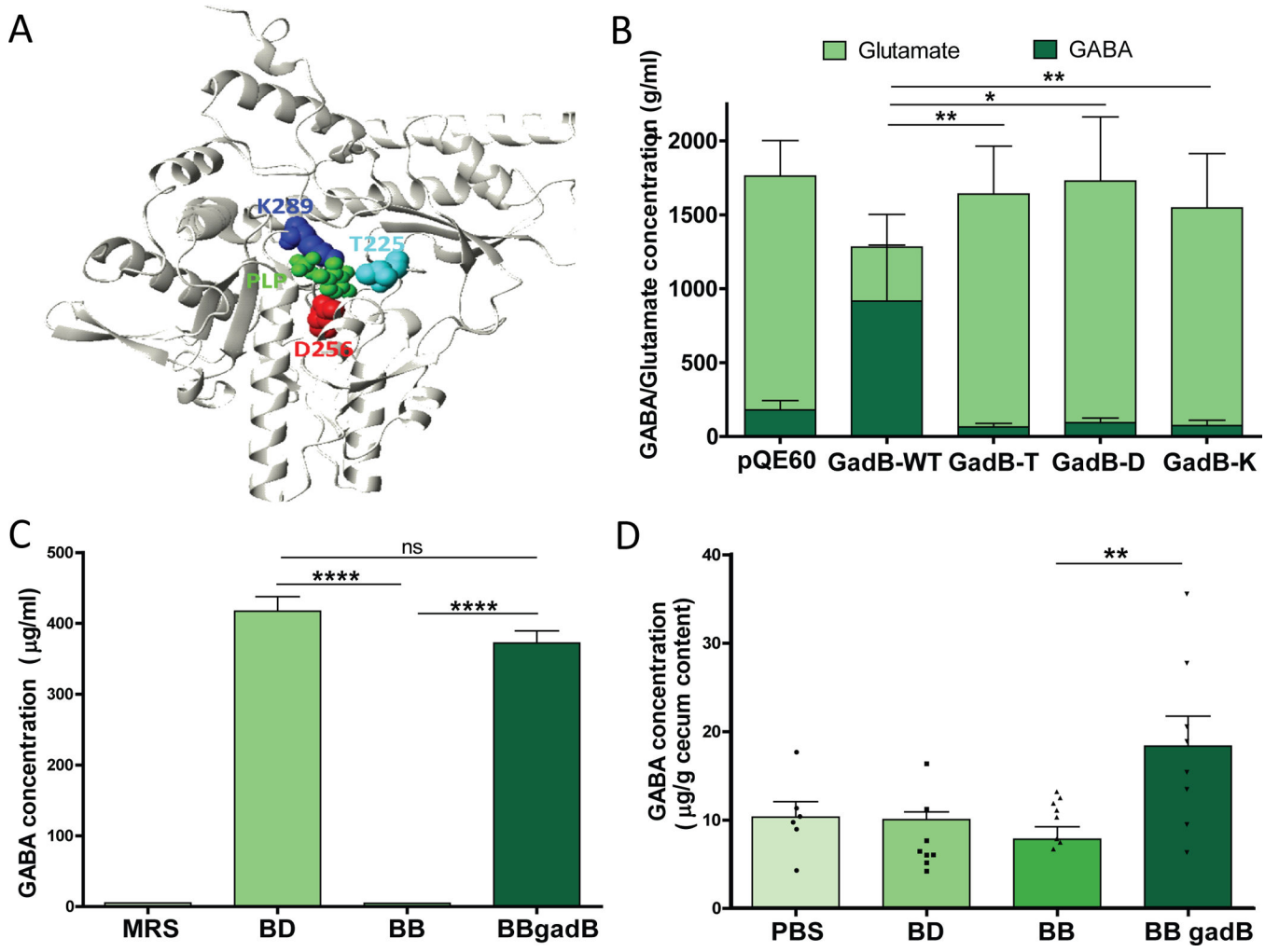

Figure 3. ***In vitro*** **and** ***in vivo*** **activity of glutamate decarboxylase (GadB) from** ***B. dentium***

(**A**) GadB 3D structure with its proposed active site highlighted. Dark blue color depicts catalytic lysine at position 289 (K289), while threonine (T225), and aspartate (D256) are colored in light blue and red, respectively. Position of co-factor pyridoxal-phosphate, PLP, is shown in green. (**B**) Site directed mutagenesis effect on recombinant GadB activity. pQE60 – negative control of crude extract from *E. coli* strain harboring empty pQE60 vector. GadB-WT – crude extract with recombinant wild type GadB overexpressed in *E. coli*. GadB-T, GadB-D and GadB-K are crude extracts of recombinant GadB with mutated amino acids from T225, D256 and K289 to alanine, respectively. Bars demonstrate GABA (dark green) or L-glutamate (light green) concentration. Error bars represent standard error of 3 independently performed experiments (*p<0.01, **p<0.05; One-Way ANOVA of log transformed data, Bonferroni correction for multiple comparisons). (**C**) Expression of GadB from *B. dentium* in *B. breve* by complementation. *gadB* from *B. dentium* ATCC 27678 (BD) was cloned into the pESH46 (pESHgadB) expression vector and transformed into *B. breve* NCIMB8807 (BB) allowing for constitutive expression (BBgadB). GABA was measured via LC-MS method (Supplemental Information). Error bars represent standard error of 3 independently performed experiments (****p<0.0001, ns=not significant; One-Way

ANOVA of log transformed data, Bonferroni correction for multiple comparisons). (**D**) Six-week old male Swiss Webster mice (n=6–8 per group) were orally administered 1% glutamate plus *B. dentium* ATCC 27678 (BD), *B. breve* NCIMB8807 (BB), *B. breve* NCIMB8807 pESHgadB (BBgadB), or saline (PBS) for 5 days. Mice administered *B. breve* pESHgadB had significantly more GABA in their cecal content as measured by ELISA (**p<0.01; One-way ANOVA with Bonferroni correction)

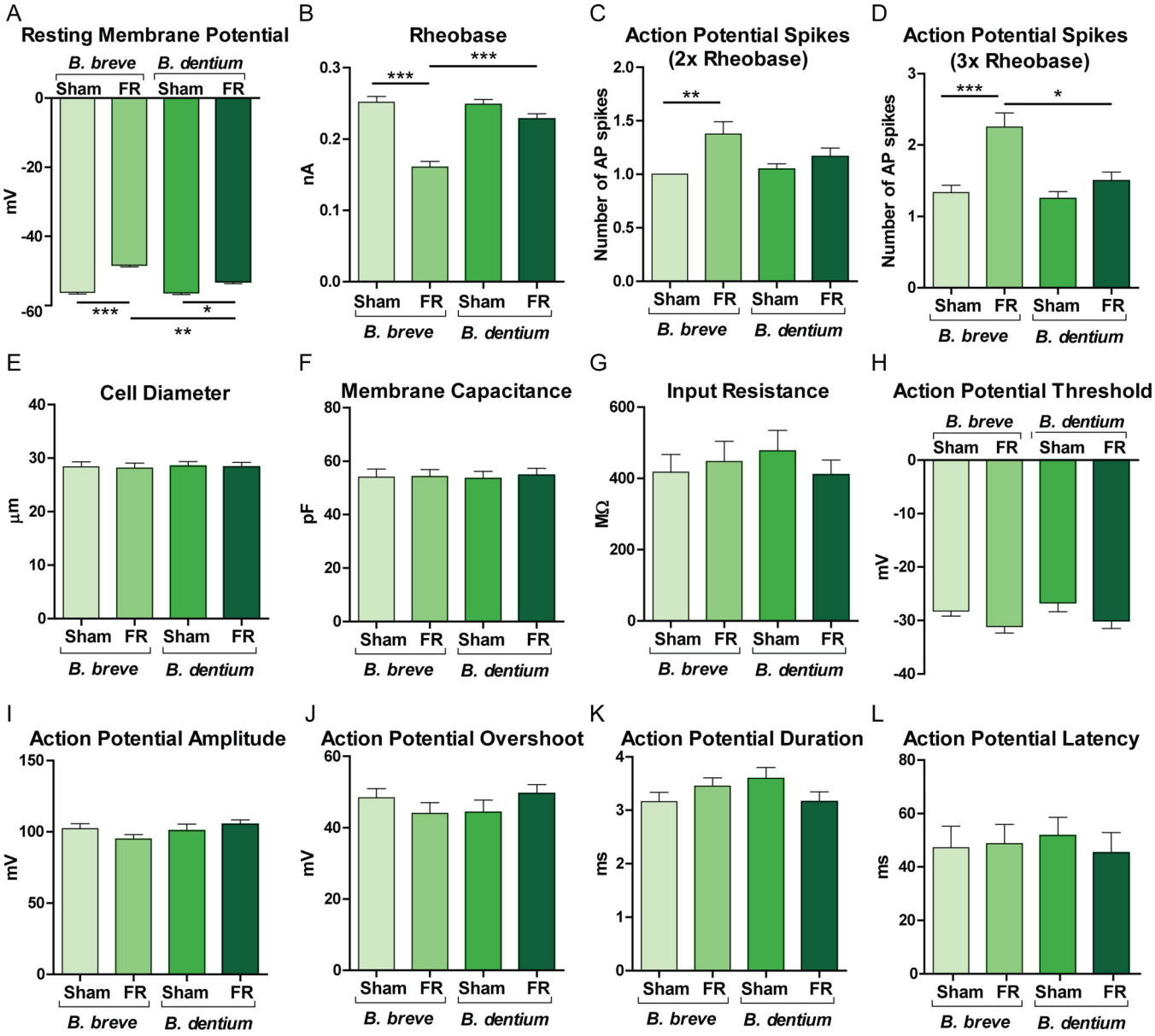

Figure 4. Neuromodulatory effects of GABA-producing *B. dentium* ATCC 27678 administration on colonic sensory neuron activity

Control (Sham) and fecal retention (FR) rats were gavaged daily with GABA-producing, *gadB*-positive *B. dentium* or *gadB*-negative *B. breve* strains (n=4 rats and 20–24 neurons per treatment group). Colon specific DRG neurons were isolated and used for the measurements of cell excitability by patch clamp recordings. The following parameters are displayed: (A) Resting membrane potential, (B) Rheobase, (C) Action potential spikes at 2× rheobase, (D) Action potential spikes at 3× rheobase, (E) Cell diameter, (F) Membrane capacitance, (G) Input resistance, (H) Action potential threshold, (I) Action potential amplitude, (J) Action potential overshoot, (K) Action potential duration, (L) Action potential latency. Bars represent mean values with standard error. (*p<0.05, **p<0.01, *** p<0.001; Kruskal-Wallis with Dunn correction for multiple comparison)

Table 1

Bacterial strains and plasmids used in this study

Strains and plasmids	Relevant features	Reference or source
Strains		
E. coli strains		
E. coli XL1-Blue	(*supE44 hsdR17 recA1 gyrA96 thi relA1 lac* F′ [*proAB*$^{+}$ *lacI*^{q}*lacZΔM15* Tn*10*(Tet^{r})])	Stratagene
E. coli XL1-Blue-pQE-GadB	XL1-Blue harboring pQE60 derivative containing *gadB*	This study
E. coli XL1-Blue-pQE-GadB-T	XL1-Blue harboring pQE60 derivative containing *gadB*, which has mutation of Threonine225 to Alanine	This study
E. coli XL1-Blue-pQE-GadB-D	XL1-Blue harboring pQE60 derivative containing *gadB*, which has mutation of Aspartate256 to Alanine	This study
E. coli XL1-Blue-pQE-GadB-K	XL1-Blue harboring pQE60 derivative containing *gadB*, which has mutation of Lysine289 to Alanine	This study
Bifidobacterium sp. strains		
B. dentium ATCC27678		ATCC
B. angulatum ATCC27535		
B. bifidum MIMBb13CS		
B. bifidum MIMBb23SG		
B. breve NCIMB8807(also known as UCC2003)	Isolate from nursling stool	NCIMB
B. breve NCIMB8807+pESH-GadB	Recombinant *B. breve* NCIMB8807 harboring pESH40 derivative containing *gadB*	This study
Lactobacillus sp. strains		
L. acidophilus		
L. farciminis DSM		DSMZ
L. gasserii ATCC33323		ATCC
L. plantarum NCIMB8826		NCIMB
L. plantarum ATCC14917		ATCC
L. reuteri ATCC23272		ATCC
L. reuteri DSM17932		DSMZ
L. reuteri ATCC PTA 6475		ATCC
L. reuteri 100-23		Su et. al.(34)
L. rhamnosus ATCC53103 (GG)		ATCC
Alistipes sp. strains		

Strains and plasmids	Relevant features	Reference or source
A. indistinctus DSM22520		DSMZ
A. finegoldii DSM17242		DSMZ
A. putredinis DSM17216		DSMZ
Plasmids		
pQE60	Amp^r, IPTG inducible expression vector	De Ruyter et. al. (78) Qiagen
pQE-gadB	Amp^r, pQE60 derivative containing translational fusion of gadB-encoding DNA fragment to IPTG-inducible promoter	This study
pQE-gadB-A	Amp^r, pQE60 derivative containing translational fusion of gadB-encoding DNA fragment to IPTG-inducible promoter	This study
pQE-gadB-B	Amp^r, pQE60 derivative containing translational fusion of gadB-encoding DNA fragment to IPTG-inducible promoter	This study
pQE-gadB-C	Amp^r, pQE60 derivative containing translational fusion of gadB-encoding DNA fragment to IPTG-inducible promote	This study
pESH46	Em^r, Amp^r, $repA^-$, ori^+, cloning vector	Shkoporov et. al. (47)
pESHGadB	Em^r, Amp^r, pESH46 derivative containing translational fusion of gadB-encoding DNA fragment	This study

ATCC, American Type Culture Collection; DSMZ, German Collection of Microorganisms and Cell Cultures; JCM, Japan Collection of Microorganisms; NCIMB, National Collection of Industrial and Marine Bacteria; MIM, Collection from University of Milan.

Table 2

Oligonucleotide primers used in this study

Primer name	Sequence code (5'→3')
GadBFw	AGCTCTCCATGGCGATAATGCATTCCAACATCA[a]
GadBRv	AGCTCTAAGCTTTCA*GTGATGGTGATGGTGATGGTGATGGTGATGGTGATGGTGATG*GAAGCCGGAGACCC[b]
GadB-T-Fw	CATGGGCGTGGCCTACACG[c]
GadB-D-Fw	CCACGTGGCTGCCGCTTCG[d]
GadB-K-Fw	CGGACACGCGTACGGTCTG[e]
GadB-T-Rv	CGTGTAGGCCACGCCCATG[f]
GadB-D-Rv	CGAAGCGGCAGCCACGTGG[g]
GadB-K-Rv	CAGACCGTACGCGTGTCCG[h]
GadB-XhoI-Fw	CAACTCGAGGGAAACAGAAGGTTTGACAG[i]
GadB-BamHI-Fw	CTGGGATCCTCAGTGATGGAAGCCG[j]
Bd_gadB_pL_4_L	GCCAAGGCCTTCTCTAAGTTC
Bd_gadB_pL_4_R	CCACCTGATAGGCGGTCTC
bglC_pL_6_L	TGGAAGGACGAGGTGACTG[k]
bglC_pL_6_R	CTACGCGCTTCGGATCAT[k]
h-gabra1_pL_2_L	GGACAAACAGTAGACTCTGGAATTG
h-gabra1_pL_2_R	TCAAGTGGAAATGAGTGGTCA
h-gabrb2_pL_27_L	ATGGCACCGTCCTTTATGG
h-gabrb2_pL_27_R	TGGGTACCTCCTTAGGTCCA
h-gabbr1_pL_87_L	CATGGACGCTTATCGAGCA
h-gabbr1_pL_87_R	GATCATCCTTGGTGCTGTCA
h-gabbr2_pL_1_L	GACACCATCAGGTTCCAAGG
h-gabbr2_pL_1_R	GATGCTGTAGAGAGGTAGGGAGA
h-gabdh_pL_45_L	GAGTCCACTGGCGTCTTCAC
h-gabdh_pL_45_R	TTCACACCCATGACGAACAT

[a]The underlined sequence corresponds to an *NcoI* site

[b]The underlined sequence corresponds to an *HindIII* site

[c] The underlined sequence corresponds to the introduced mutation site for Threonine.

[d] The underlined sequence corresponds to the introduced mutation site for Aspartate.

[e] The underlined sequence corresponds to the introduced mutation site for Lysine.

[f] The underlined sequence corresponds to the introduced mutation site for Threonine.

[g] The underlined sequence corresponds to the introduced mutation site for Aspartate.

[h] The underlined sequence corresponds to the introduced mutation site for Lysine

[i] The underlined sequence corresponds to an *XhoI* site

[j] The underlined sequence corresponds to an *BamHI* site

[k] *B. breve* NCIMB8807 strain specific primers have been described previously by Pokusaeva et al. (79)