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Gamma aminobutyric acid production by commercially available probiotic strains

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Abstract

Aims: Certain bacteria can produce gamma aminobutyric acid (GABA) from glutamate in the human intestinal tract, leading to the possibility of altering GABA levels through diet. To this end, we assessed the ability of seven commercially available probiotic supplements to produce GABA.

Method and results: Probiotic strains were compared for GABA production in pure culture. The bacteria were inoculated at a concentration of 10^7 CFU ml^{-1} in 10 ml MRS supplemented with monosodium glutamate (1% w/v), both with and without oligofructose-enriched inulin (OFI) (1% w/v). Two strains with the highest production of GABA were further assessed for 48 h in pH-controlled anaerobic batch cultures inoculated with faecal bacteria. Liquid chromatography-mass spectrometry (LC–MS) was used for quantification of GABA and microbiota composition was determined through 16S rRNA gene sequencing. *Levilactobacillus brevis* LB01 (CGMCC 16921) and *Lactiplantibacillus plantarum* 299v (DSM 9843) were the most efficient producers of GABA. High GABA levels (28.32 mmol l^{-1} ± 0.29) were produced by the probiotic strain *L. brevis* LB01 at pH 5.4–5.6. This was significantly higher than the levels of GABA produced by *L. plantarum* (4.8 mmol l^{-1} ± 6.8) and a negative control (2.9 mM ± 3.1). The addition of OFI did not further stimulate GABA production under the conditions tested. The ability of these strains to produce GABA *in-vitro* was further evaluated in a faecal microbiota environment. Once again, *L.brevis* LB01 produced the highest levels of GABA (40.24 mmol l^{-1} ± 20.98).

Conclusions: *L. brevis* LB01 was found to be the most efficient probiotic strain, of those tested, for GABA production.

Significance and impact of the study

High GABA levels obtained by *L. brevis* LB01 make this probiotic strain a candidate for potential dietary interventions aimed at increasing GABA production in the gut.

Keywords: gamma aminobutyric acid, probiotic, gut microbiota, gut-brain, batch cultures

Introduction

The human large intestine harbours a complex microbial ecosystem comprising trillions of microorganisms, including bacteria, fungi, protozoa, and viruses. Commensal bacteria play an important role in host health, contributing, for instance, to development and functioning of the immune system, food digestion, and host metabolism/homeostasis (Hooper et al. 2012, Tremaroli and Bäckhed 2012). However, metabolic and immunomodulatory effects of bacteria on human health are not only limited to the gut but can also have systemic influences (Budden et al. 2017, Milosevic et al. 2019). In this context, emerging evidence has linked gut microbiota as a contributor to gut–brain axis signalling. Numerous studies, mainly in animal models, suggest that gut microbiota can modulate brain, behavioural, and cognitive functions (Heijtz et al. 2011, Needham et al. 2022). However, potential mechanisms through which gut bacteria could influence brain and behavioural function remains largely unknown. Gut bacteria are known to produce a range of compounds that function as neurotransmitters in the host, such as γ-aminobutyric acid (GABA), serotonin, catecholamines, and acetylcholine among others (Yunes et al. 2016, Villageliú and Lyte 2018, Villageliú et al. 2018).

GABA is the main inhibitory neurotransmitter in the central nervous system, playing a critical role in sensory perception and emotion (Möhler 2012, Porges et al. 2017). Clinical and animal studies have suggested an antihypertensive effect of GABA (Tanaka et al. 2009, Zareian et al. 2020). Some species of gut bacteria synthesize and export GABA by a pyridoxal-5′-phosphate-dependent glutamate decarboxylase (GAD) enzyme through irreversible α-decarboxylation of L-glutamate and consumption of one proton (Barrett et al. 2012). Although more research is needed to elucidate whether GABA produced in the gut can reach the brain by crossing the blood brain barrier, GABA synthesized by the microbiota in the intestine may also modulate brain function by GABAergic signalling in the small and large intestines (Bravo et al. 2011). Therefore, the potential to alter GABA levels through diet could open up possibilities for appropriate interventions.

Genetically, *gad* genes have been detected in many lactic acid bacteria (LAB) from food sources (Siragusa et al. 2007), and their presence has been linked with acid tolerance (Otaru et al. 2021). Some research has been directed towards isolating and characterizing GABA-producing bacteria to be used as starters for the production of GABA-enriched fermented food (Seo et al. 2013, Linares et al. 2016). Another approach to

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Table 1. List of commercial probiotics formulations tested for GABA producing ability.

Commercial probiotic strains	Company provider	Manufacturer	Origin
L. brevis LB01 CGMCC 16921	Epigenetics	Wecare Probiotics	Cheese
L. plantarum 299v DSM 9843	Metagenomics	Probi	Healthy intestinal mucosa
L. reuteri (2-strain mixture) • *L. reuteri* DSM 17938 • *L. reuteri* ATCC 6475	Biogaia	Biogaia	Breast milk
L. brevis D18	Creative Enzymes	Creative Enzymes	Yoghurt
B. adolescentis Q9	Creative Enzymes	Creative Enzymes	Faeces of healthy infants
L. plantarum (3-strain mixture) • *L. plantarum* CECT 7527 • *L. plantarum* CECT 7528 • *L. plantarum* CECT 7529	Optibac	Optibac	Faeces of healthy infants
B. infantis Bi02 (DSM 24687)	Epigenetics	Probiotical	Human faeces

increase levels of GABA in the gut is the use of GABA-producing probiotic supplements (Yunes et al. 2016). Probiotics are defined as 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host' (Hill et al. 2014). In this regard, while the supply of GABA by pharmaceutical products and enriched food supplements would be restricted to dose and times of intake, the administration of appropriate probiotic strains would imply a constant and continuous supply of GABA *in vivo* to the intestine as well as provide additional benefits to potentially improve human health.

Recently, such benefits have been extended to psychological conditions, and the concept of psychobiotics has been introduced to describe probiotics that confer mental health benefits (Sarkar et al. 2016). *Lactobacillaceae* and *Bifidobacterium* species have been extensively used as probiotics due to their health promoting potential and long history of safe use (Sanders et al. 2018). Notably, *Lactobacillaceae* species are considered primary GABA producers, and the presence of *gad* genes has also been reported in *Bifidobacterium* species (Yunes et al. 2016, Duranti et al. 2020). However, GABA production ability is strain-specific, and the mere presence of *gad* genes does not ensure GABA export to the extracellular milieu (Yunes et al. 2016). A major contributor to this could be that many bacteria only use an intracellular GAD system (Feehily and Karatzas 2013), which produces intracellular GABA. However, this GABA is catabolized intracellularly and not exported, as described previously (Karatzas et al. 2012). Furthermore, in other cases, the extracellular GAD system that exports GABA might be inactive or may require specific signals to function. Thus, *in vitro* screening of bacterial species for their ability to produce GABA from monosodium glutamate (MSG) is an essential step in assessing levels of GABA production in the gut.

To this end, the current study sought to determine GABA produced by bacteria from commercially available probiotics. Two supplements containing *Lactiplantibacillus plantarum* strains, two containing *Levilactobacillus brevis* strains, one supplement with *Limosilactobacillus reuteri* strains, one supplement with *Bifidobacterium adolescentis* and one supplement with *B. infantis* were assessed. The choice of these species was based on their potential for GABA production (Su et al. 2011, Barrett et al. 2012, Teixeira et al. 2014, Yunes et al. 2016, Wu and Shah 2018, Duranti et al. 2020) and probiotic health benefits (Fuentes et al. 2013, Steenbergen et al. 2015, Gutierrez-Castrellon et al. 2017, Krumbeck et al. 2018, Rudzki et al. 2019, Lai et al. 2021) as reported in the literature. These products had already undergone genotyping, functional characterization, and safety checks. The aim of this work was to identify a commercially available probiotic with high GABA production ability, and demonstrate that production can also occur in a simulated intestinal environment using pH-controlled anaerobic batch cultures inoculated with faecal samples.

Materials and methods

Maintenance and culture of bacteria strains

Seven commercial probiotic products were selected for investigation of GABA production (Table 1).

Capsules were opened under aseptic conditions and the powder content dissolved in 10 ml phosphate-buffered saline (PBS). For bacterial isolation in the single commercial preparations, 100 µl of seed culture was spread on MRS agar for *Lactobacilli* strains and MRS agar + 0.05% (w/v) cysteine for *Bifidobacterium* strains. Agar plates were incubated for 48 h at 37°C in anaerobic conditions. Glycerol stocks from single colonies were prepared and stored at −80°C. For multistrain probiotic formulations, powder from one capsule was dissolved before each experiment in sterile PBS (1×) at pH 7.

Pure cultures of probiotic strains in the presence of MSG

GABA production by the probiotic strains was tested both with and without oligofructose-enriched inulin (OFI) (1% w/v; Beneo-Orafti, Tienen, Belgium), which contains a 50:50 mixture of short (P95) and long-chain (HP) inulin. Thus, GABA was assessed at two different starting pH values (pH 5.6 and 6.7) to mimic pH in the proximal and distal colon, respectively (Fig. 1a). Prior to the assessment of GABA production, strains were subcultured in MRS broth for 24 h at 37°C in an anaerobic chamber (80% N_2, 10% H_2, and 10% CO_2) from glycerol stocks (single probiotic strains) or directly from the capsule dissolved in sterile PBS for multistrain probiotic products (*L. plantarum* multistrain and *L. reuteri* multistrain; Table 1). Subsequently, strains were inoculated (1% v/v) in Hungate tubes containing 10 ml of MRS supplemented with 1% w/v (59 mmol l^{-1}) MSG, both with and without the prebiotic OFI (1% w/v) at pH 6.7 (pH in distal colon) or 5.6

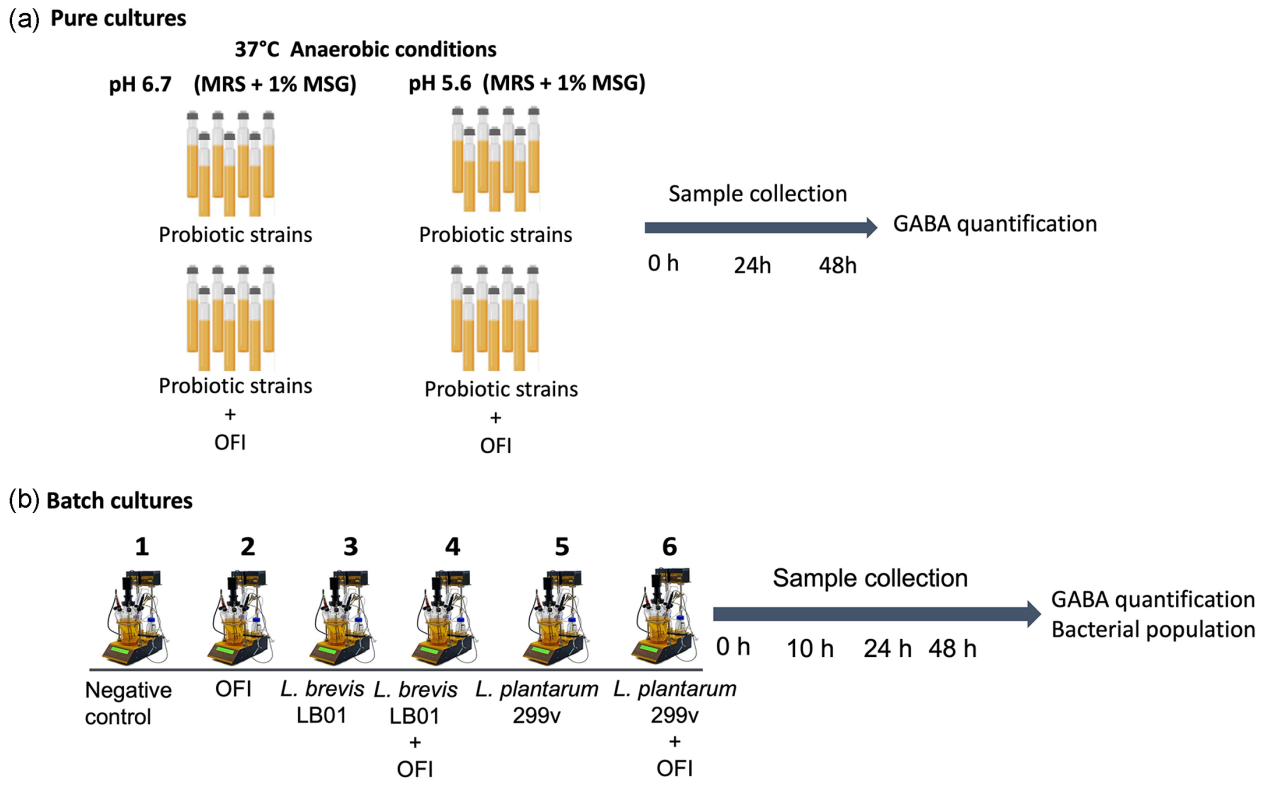

Figure 1. Schematic representation of the experimental conditions conducted in this study. (a) GABA production assessed in pure cultures using MRS supplemented with MSG (1% w/v) at two different starting pH (5.7 and 6.7) at 37°C under anaerobic conditions. GABA production was tested from commercial probiotic strains alone or in combination with the prebiotic OFI (1%). Seven commercial probiotic strains were tested in this study: *L. brevis* LB01, *L. brevis*, *L. adolescentis*, *L. plantarum* 299v, *B. infantis*, *L. plantarum* (multistrain), and *L. reuteri* (multistrain) (Table 1). (b) GABA production assessed in an anaerobic pH-controlled batch culture system inoculated with faecal samples to mimic microbiota conditions in the colon. GABA production was only studied in the two top GABA-producing probiotic strains from the previous experiment—*L. plantarum* 299v and *L. brevis* LB01—alone or in combination with the prebiotic OFI. The prebiotic OFI alone was also tested and a negative control without probiotic and prebiotic supplementation was included in the experiment design. Four biological replicates were carried out for each condition.

(pH in proximal colon). Technical duplicates were performed for each condition. Bacterial cultures were incubated for 48 h at 37°C in the anaerobic chamber. Samples (1 ml) were collected at baseline (0), 24, and 48 h. Samples were centrifuged at 10 000 *g* for 10 min and supernatants stored at −20°C until analysis. Growth curves were obtained using a FLUOstar Omega microplate reader equipped with an atmospheric control unit (BMG Labtech, Aylesbury, UK) under anaerobic conditions (90% N_2 and 10% CO_2).

GABA production by selected probiotic strains in anaerobic pH-controlled faecal batch cultures

Faecal inoculum preparation

Batch culture fermentation vessels were inoculated with non-pooled faecal samples provided by four healthy donors (males; age 25–42 years; omnivores). Ethical approval for collecting faecal samples from healthy volunteers was obtained from University of Reading Research Ethics Committee (UREC 15/20). Donors were healthy, with no known metabolic or gastrointestinal conditions, and had not received any antibiotic, probiotic, or prebiotic intervention for at least 3 months prior to the experiment. Faecal samples were collected in a clean plastic container placed in an anaerobic jar containing AnaeroGen sachets (Oxoid, Basingstoke, UK) and were used within 2 h of collection. Faecal samples were diluted in sterile PBS at 10% (w/v) and homogenized in strainer bags to remove large particles (Seward, Worthing, UK) using a stomacher (Stomacher 400, Seward, Worthing, UK) for 2 min at 200 rpm. Resulting faecal slurries were used to inoculate the batch culture systems.

In vitro anaerobic faecal batch cultures

Sterile batch fermentation vessels of volume 100 ml (50 ml working volume) were aseptically filled with 45 ml sterile gut model medium (GMM) in a reduced state, following autoclaving (Macfarlane et al. 1998) (Supplementary Table S1) supplemented with MSG 1% (w/v)—equivalent to 59 mmol l^{-1}—and sparged with O_2-free N_2 overnight to establish anaerobic conditions. Within the medium, the anaerobic indicator resazurin was used to confirm the absence of oxygen. The next day, vessels were inoculated with 5 ml of fresh faecal slurry and incubated at 37°C using a circulating water bath and pH was controlled between 5.4 and 5.6 using an automated pH controller (Fermac 260, Electrolab, Tewkesbury, UK) to mimic the proximal colon. GABA production was tested with the two commercial probiotic preparations that produced the highest GABA levels in the previous pure-culture assay (*L. brevis* LB01 and *L. plantarum* 299v, see the 'Results' section), both with and without the prebiotic OFI. The following six conditions were therefore assessed in the *in vitro* batch system: (1) *L. brevis* LB01; (2) *L. brevis* LB01 + OFI; (3) *L. plantarum* 299v; (4) *L. plantarum* 299v + OFI; (5) OFI; and (6) negative control (no probiotic or prebiotic added; Fig. 1b).

Probiotic strains were grown in MRS for 24 h at 37°C under anaerobic conditions. Probiotic cells were prepared for addition to the fermenter vessels by centrifuging at 5000 *g* for 10 min. The supernatant was removed, cells washed, resuspended in PBS, and adjusted to an optical density (OD_{600}) corresponding to 5×10^9 CFU ml^{-1}. One millilitre of bacterial suspension was added to each of the 50 ml fermenter vessels to have a final bacterial load of 10^8 CFU ml^{-1}.

The supernatant was removed, pellet resuspended in PBS and inoculated in the vessels to give an initial number of 10^8 CFU ml^{-1}. The prebiotic OFI was added at 1% (w/v). Batch cultures were conducted for 48 h at 37°C and samples were collected at baseline (0), 10, 24, and 48 h for GABA quantification and analysis of bacterial populations. Batch cultures were carried out four times independently for each condition using faecal samples from four different donors ($n = 4$).

Quantification of GABA production by LC–MS

LC–MS was used for quantification of GABA on a Shimadzu triple quadrupole (QQQ) equipped with a Discovery HS F5 HPLC Column (3 mum particle size, L × I.D. 15 cm × 2.1 mm) maintained at 40°C, mobile phase gradient. The mobile phase comprised a gradient mixture of solvent A (0.1% v/v formic acid) and solvent B (acetonitrile containing 0.1% formic acid) at a flow rate of 0.25 ml min^{-1}. The gradient elution programme was as follows: 2–5 min solvent B from 0%–25%, 5–6 min solvent B from 25%–95%, then holding for 2 min, 8–9 min from 95%–0% and then until 15 min.

A LC/MS-8050 QQQ detector was operated in the multiple reaction monitoring (MRM) mode using the polarity-switching electrospray ionization mode. Dry gas temperature was set at 200°C with a flow of 10 l min^{-1} and volume of the sample injected was 4 µl. For the analysis of GABA, LC/MS Method Package for Primary Metabolites (Shimadzu Corporation, Kyoto, Japan) was used. The MRM transition for GABA was 104.10 > 87.05 m/z. A linear calibration curve was generated based on the detected signal proportional to concentration of the analyte. Good linearity with $R^2 > 0.98$ was obtained across the set calibration in the range from 1.56 ng ml^{-1} to 1000 ng ml^{-1}.

Bacterial population analysis in batch culture fermentations

DNA extraction

Batch culture samples of 1 ml were centrifuged in Eppendorf tubes at 10 000 *g* for 10 min. Pellets were stored at −80°C. Cell pellets were defrosted on ice. Then, DNA was extracted from the pelleted cells using the QIAamp PowerFecal Pro DNA Kit (Qiagen, Hilden, Germany). Modifications in the lysis step using the Tissue Lyser LT (Qiagen, Manchester, UK) of the supplied protocol were made to avoid partial degradation of genomic DNA. Briefly, tubes were placed in a Tissue Lyser and homogenized for 20–30 sec at 35 Hz, 3 min at 30 Hz, and 2 min at 30 Hz. Samples were kept on ice for 1 min between cycles. Subsequent steps were performed according to manufacturer's protocol.

16S rRNA gene sequencing and data analysis

DNA samples were sent to Novogene Europe (Cambridge, UK) for 16S rRNA gene sequencing. Aliquots of extracted DNA were amplified with universal primers for the V4 and V5 regions of the 16S rRNA gene. A 393 bp hypervariable region V4–V5 of the 16S rRNA gene was amplified using universal primers 515F (5′-GTGCCAGCMGCCGCGGTAA) and 907R (5′-CCGTCAATTCCTTTGAGTTT) and phusion® High-Fidelity PCR Master Mix (New England Biolabs, UK). PCR products were purified, libraries generated using NEBNext® UltraTM DNA Library Prep Kit (Illumina, UK) and sequenced on a paired-end Illumina NovaSeq 6000 platform to generate 250 bp paired-end raw reads.

Data output were demultiplexed using the in-built RTA software on the instrument.

Demultiplexed sequence data were then processed by applying the following workflow. First, forward and reverse reads were merged using FLASH (V1.2.7) (Magoč and Salzberg 2011) and next fastp software (version 0.20.0) was used to obtain high-quality clean tags. Subsequently, the Divisive Amplicon Denoising Algorithm 2 was run in QIIME2 (version 2020.06) for noise reduction and clustering sequences into amplicon sequence variants (ASVs). ASVs were taxonomically assigned by using the function QIIME feature-classifier classify-sklearn, a naïve Bayes machine learning classifier. Subsequent analyses of alpha diversity and beta diversity were all performed based on output normalized data using QIIME2 and displayed with R software (Version 2.15.3).

Nucleotide sequence accession number

The sequence data obtained by sequencing of the V4–V5 region of the 16S rRNA gene have been submitted to the Sequence Read Archive (SRA) of NCBI (https://www.ncbi.nlm.nih.gov/sra) under accession number PRJNA866336.

Statistical analysis

Differences in GABA concentration among probiotic strains at the same time point were analysed using one-way analysis of variance (ANOVA) followed by post hoc Tukey's multiple comparison tests using SPSS for Mac, version 27 (IBM, Armonk, NY, USA).

For microbiota composition, differentially abundant taxa were identified with two methods. First, LEfSe [linear discriminant analysis (LDA) Effect Size, http://huttenhower.sph.harvard.edu/lefse/] was used to determine significant differences in taxon abundance using an alpha of 0.05 and a threshold LDA log score of ±4. Second, a *t*-test with false discovery rate correction was performed to determine species with significant variation between groups using a standard threshold of $P < 0.05$.

Results

Screening of probiotic strains for the production of GABA

Seven commercially available probiotic formulations were assessed for their ability to produce GABA using MRS growth medium supplemented with MSG (1%) (Table 1). The final concentration of GABA in the culture medium after 48 h ranged between 0.04 mmol l^{-1} to 28.32 mmol l^{-1} (Table 2). *Levilactobacillus brevis* LB01 was the most efficient probiotic strain tested for MSG conversion to GABA at 24 h and 48 h in both pH conditions. A maximum concentration (28.32 mmol l^{-1} ± 0.29; 48% conversion rate) was obtained by *L. brevis* LB01 after 48 h at an initial pH of 5.6 (Table 2). Although *L. brevis* LB01 was the highest producer of GABA under both pH values tested, the final GABA concentration was lower at

Table 2. GABA production (mmol l^{-1}) by selected probiotic bacteria grown in MRS supplemented with MSG (59 mmol l^{-1}) and in combination or not with the prebiotic OFI (1%) at pH 5.6 and 6.7.

Species	MRS pH 5.6		MRS pH 6.7		MRS + OFI pH 5.6		MRS + OFI pH 6.7	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
B. infantis	$1.57^{a} \pm 0.02$	$1.06^{b} \pm 0.09$	$0.39^{ab} \pm 0.05$	$0.13^{a} \pm 0.01$	$0.16^{a} \pm 0.00$	$0.84^{c} \pm 0.06$	$0.35^{a} \pm 0.00$	$1.62^{ab} \pm 0.35$
L. brevis LB01	$15.5^{c} \pm 2.60$	$28.32^{d} \pm 0.29$	$5.14^{c} \pm 0.33$	$13.83^{b} \pm 0.40$	$12.46^{b} \pm 0.26$	$24.18^{e} \pm 0.10$	$7.06^{b} \pm 0.88$	$15.45^{c} \pm 1.24$
L. brevis D18	$3.11^{ab} \pm 0.10$	$1.45^{b} \pm 0.09$	$0.25^{ab} \pm 0.02$	$0.10^{a} \pm 0.03$	$0.09^{a} \pm 0.01$	$0.66^{bc} \pm 0.02$	$0.24^{a} \pm 0.01$	$2.60^{b} \pm 0.35$
B. adolescentis Q9	$2.71^{ab} \pm 0.80$	$1.36^{b} \pm 0.19$	$0.48^{ab} \pm 0.02$	$0.30^{a} \pm 0.02$	$0.18^{a} \pm 0.02$	$0.18^{ab} \pm 0.02$	$0.43^{a} \pm 0.00$	$2.74^{b} \pm 0.16$
L. reuteri (2- strain mixture)	$0.04^{a} \pm 0.00$	$0.04^{a} \pm 0.00$	$0.04^{a} \pm 0.00$	$0.04^{a} \pm 0.00$	$0.04^{a} \pm 0.00$	$0.03^{a} \pm 0.00$	$0.04^{a} \pm 0.00$	$0.03^{a} \pm 0.00$
L. plantarum (3-strain mixture)	$1.33^{a} \pm 0.01$	$0.44^{a} \pm 0.03$	$0.25^{ab} \pm 0.03$	$0.18^{a} \pm 0.02$	$0.26^{a} \pm 0.02$	$0.42^{b} \pm 0.03$	$0.22^{a} \pm 0.02$	$0.92^{ab} \pm 0.01$
L. plantarum 299v	$5.81^{b} \pm 1.18$	$3.03^{c} \pm 0.12$	$0.62^{b} \pm 0.01$	$0.27^{a} \pm 0.05$	$0.17^{a} \pm 0.01$	$4.13^{c} \pm 0.23$	$0.46^{a} \pm 0.01$	$1.52^{ab} \pm 0.13$

Values are the means of two independent experiments ± standard error of the mean. Values that are significantly different between probiotic strains at the same time point and growth medium are labelled with different letters (one-way ANOVA, $P < 0.001$ followed by Tukey's HSD test).

pH 6.7 (15.45 mmol $l^{-1} \pm 1.24$; 26.2% conversion rate). Final pH in the growth medium was higher for *Le. brevis* LB01 compared to the other strains tested (Supplementary Table S2).

The remaining six probiotic strains tested showed a GABA production yield significantly lower than *L. brevis* LB01, with a final GABA concentration ranging between 0.03 and 5.8 mmol l^{-1} (0.05%–9.83% conversion rate; Table 2). In this regard, *L. plantarum* 299v was the strain with the second highest capacity to produce GABA, reaching maximum production (5.81 mM ± 1.18) at pH 5.7 after 24 h. Overall, the prebiotic OFI did not influence GABA production as it did not stimulate growth of any strain tested (Supplementary Figure S1).

GABA production by *L. brevis* LB01 and *L. plantarum* 299v in anaerobic pH-controlled batch cultures

The GABA-producing ability of the top two probiotics from the screening stage, *L. brevis* LB01 and *L. plantarum* 299v, were assessed for 48 h using anaerobic pH-controlled batch cultures at pH 5.4–5.6 (pH in proximal colon) inoculated with faecal samples both with and without the prebiotic OFI (1%; Fig. 2). Another set of batch cultures was conducted at pH 6.7–6.9 (pH in distal colon) but no GABA was detected under these conditions (Supplementary Table S3). Batch cultures supplemented with the probiotic strain *Le. brevis* LB01 or with the synbiotic combination *L. brevis* LB01 + OFI resulted in significant higher production of GABA after 24 h (24.63 mmol $l^{-1} \pm 15.69$; 41.7% conversion rate) and 48 h of fermentation (40.03 mmol $l^{-1} \pm 20.21$; 67.85% conversion rate), compared to negative control ($p < 0.01$) and to the other conditions tested ($p = 0.01$) whose concentration did not exceed 5 mmol l^{-1} (conversion rates < 9%; Fig. 2). No significant differences were detected between the probiotic alone and in combination with the prebiotic OFI. Similar GABA concentration (50.67 mmol $l^{-1} \pm 2.79$) (85.9% conversion rate) was obtained with three out of four donors for *L. brevis* LB01 at the end of the fermentation. However, a lower production (8.95 mmol l^{-1};15.16% conversion rate) was detected in batch cultures inoculated with faecal samples from donor 4.

In contrast, no significant differences ($p > 0.05$) in GABA production were observed between *L. plantarum* 299v alone (4.93 mmol $l^{-1} \pm 5.90$) or with the prebiotic (4.27 mmol $l^{-1} \pm 2.66$), and negative control (4.11 mmol $l^{-1} \pm 4.12$). The prebiotic OFI itself did not stimulate GABA production by faecal microbiota (Fig. 2).

Effect of probiotic and/or prebiotic supplementation in the bacterial population of batch cultures

16S rRNA gene amplicon NGS showed a reduction of observed species in all conditions tested between baseline (502.54 ± 11.02) and at the end of the fermentation at 48 h (379.63 ± 17.96) ($p = 0.06$). However, comparisons between tested conditions at the end of fermentation (48 h) showed that supplementation with the probiotics and/or prebiotic did not impact diversity of the microbiota ($p > 0.05$) (Fig. 3a).

Weighted UniFrac distance plotted on principal coordinate analysis (PCoA) plots showed that samples from baseline clusters separately from samples collected after 48 h but no clusters were observed between conditions at 48 h indicating that probiotic/prebiotic supplementation did not induce

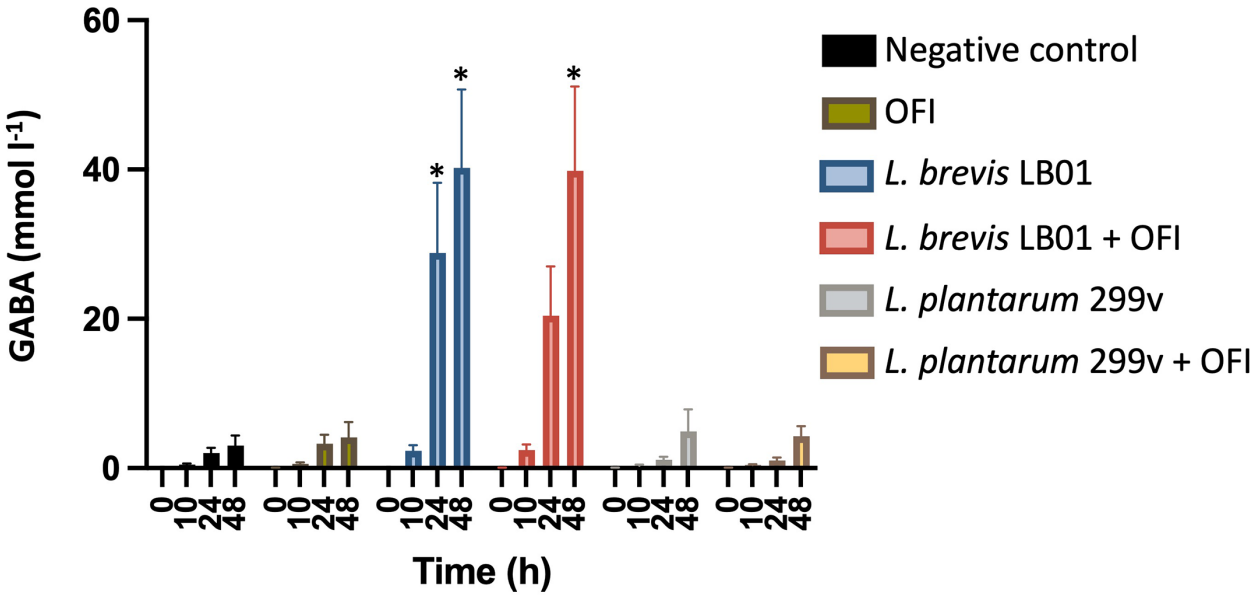

Figure 2. GABA production by selected probiotic strains in anaerobic pH-controlled faecal batch cultures. GABA production by *L. brevis* LB01 and *L. plantarum* 299v in combination or not with the prebiotic OFI in anaerobic pH-controlled faecal batch cultures with GMM supplemented with 1% MSG at pH 5.4–5.6. Fermentations were carried out four times with faecal samples from four different healthy donors. Asterisk denotes significantly different $P < 0.05$ compared to the other conditions at the same time point (one-way ANOVA, $p < 0.001$ followed by Tukey's HSD test).

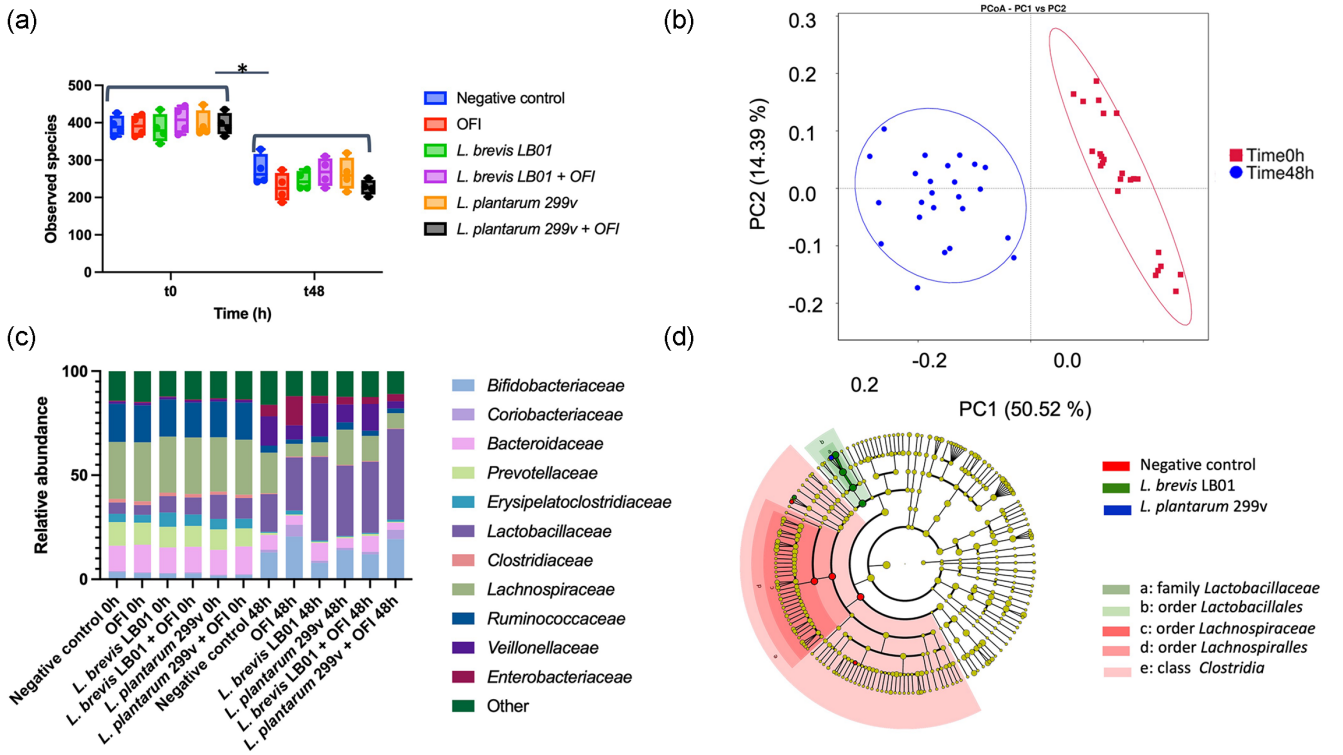

Figure 3. Impact of probiotic and/or prebiotic supplementation on the faecal microbiota: single-stage anaerobic batch culture fermentations inoculated with nonpooled faecal samples from four healthy donors were carried out using GMM containing 1% (59 mmol l^{-1}) MSG supplemented with the probiotic strains *L. brevis* LB01 or *L. plantarum* 299v in combination or not with the prebiotic OFI. (a) Alpha diversity measured as observed species at baseline and after 48 h of fermentation in all tested conditions. (b) Principal coordinates analysis of weighted UniFrac distances of microbiota composition profiles (as determined by 16S rRNA gene amplicon sequencing) obtained at baseline (0 h) and 48 h for all tested conditions. (c) Microbiota composition at family level at baseline and after 48 h of fermentation with different probiotic and/or prebiotic supplements. (d) Taxonomic cladogram generated from LefSe analysis showing significant difference in microbiota profile of three groups at 48 h (negative control, *L. brevis* LB01, and *L. plantarum* 299v). Taxa identified by LEfSe analysis were statistically significant using an alpha of 0.05 and exceeded an LDA log score of ±4 to explain the differences between the different testing groups after 48 h of fermentation.

drastic changes in the composition of microbiota (Fig. 3b). In general, in response to the adaptation of the faecal microbiota to *in vitro* conditions, an increase of the families *Bifidobacteriaceae*, *Lactobacillaceae*, *Enterobacteriaceae*, *Coriobacteriaceae*, and *Veillonellaceae* was observed. In contrast, a reduction of the families *Ruminococcaceae*, *Prevotellaceae*, and *Lachnospiraceae* was detected after 48 h of fermentation (Fig. 3c). Pairwise comparisons between treatments and control showed some specific changes at species level, which were not significant after adjusting for multiple correction. LEfSe analysis confirmed that *Lactobacillaceae* family was the most differentially abundant taxa enriched in microbiota from batch cultures inoculated with *L. plantarum* 299v and *L. brevis* LB01 compared to negative control (Fig. 3d). Control without probiotic supplement was enriched with species from the family *Lachnopiraceae* and *Clostridium*.

Discussion

GABA is the most important and abundant inhibitory neurotransmitter in the human brain. While neurons are known to be the principal source of its production, an increasing number of studies have indicated that several bacterial species in the human colon can produce GABA. Identifying bacteria that could alter the production of GABA in the gut opens up the possibility of designing experimental dietary interventions to elicit effects. The current study evaluated the ability of seven commercially available probiotics to produce high levels of GABA. *Levilactobacillus brevis* LB01 was found to produce the highest levels of GABA both in pure culture as well as in a faecal microbial environment.

Some animal studies and human interventions have suggested certain psychological benefits to the consumption of GABA-rich food supplements (Nakamura et al. 2009, Kanehira et al. 2011, Yoto et al. 2012). These reports have led to growing commercial demand of GABA food supplements and GABA-enriched foods as neuro-nutraceuticals. However, there is a dearth of studies that test GABA production in environments that closely mimic the human colon. While previous studies have reported GABA production in some *Lactobacillus* and *Bifidobacterium* strains, these have been tested primarily in pure cultures with an aim to produce GABA-enriched food products (Komatsuzaki et al. 2005, Yunes et al. 2016, Kanklai et al. 2021). These studies have not tested GABA producing bacteria in the presence of commensal intestinal microbiota. Here, we used an *in vitro* pH-controlled anaerobic batch culture approach, to simulate *in-vivo* conditions more closely. This approach, when complemented with the results from the data on the pure cultures and validated by a direct assessment of the relative abundance of species in the bacterial population, provides evidence for the potential of probiotics to produce GABA *in-vivo*.

The first part of the study assessed probiotic formulations in single cultures without pH control, and identified *L. brevis* LB01 and *L. plantarum* 299v as the most efficient GABA-producers. This result was consistent with previous reports of *L. brevis* strains as GABA-producers (Li et al. 2010, Barrett et al. 2012, Wu and Shah 2017, Cataldo et al. 2020).

GABA production bacteria depends on the activity of the GAD operon. GAD is encoded by either *gadA* or *gadB* genes (Smith et al. 1992). While most *Lactobacillaceae* and *Bifidobacterium* strains possess only one *gad* gene (*A* or *B*), *L. brevis* is the only known species that encodes two biochemically identical isoforms of the GAD enzyme (Lyu et al. 2018). Interestingly, the final concentration of GABA obtained with *L. brevis* D18 in our study was 20 times lower than with *L. brevis* LB01, showing that levels of GABA production were strain specific. In this regard, different GABA concentrations from different *L. brevis* strains were also observed by another group (Banerjee et al. 2021).

In our study, *L. brevis* LB01 also showed high GABA production in the presence of commensal mixed bacteria when this probiotic was added to batch cultures. In both experiments, pH of the growth medium seemed to be a critical factor for GABA production. In single cultures, *L. brevis* LB01 synthesized higher amounts of GABA (between 1.5 and 2 times) at a lower initial pH of the growth medium (pH 5.6). In batch cultures, whilst a low concentration of GABA (<1 mmol l^{-1}) was obtained by *L. brevis* LB01 at pH 6.7–6.9 (typically noted in distal colon), around 40 mmol l^{-1} was obtained when the pH was 5.4–5.6 (typically noted in proximal colon). To the best of our knowledge, only one previous study (Barrett et al. 2012) tested GABA production by a *L. brevis* strain in a pH-controlled batch culture system inoculated with faecal samples. In this case, MSG added to the medium (3% w/v) was three times higher than in our study (1% w/v). Although the strain had a 100% conversion rate from MSG to GABA in single cultures, only 70–72 μg ml^{-1} (equivalent to 0.69 mmol l^{-1}) was obtained in batch cultures at pH 6.7. Although GABA production was not tested at a lower pH by Barret et al. (2012), these results together suggest that GABA production would be higher in the proximal part of the colon where bacteria are exposed to a lower pH environment. GABA production by LAB using the GAD operon is one the most important mechanisms for survival in acidic conditions. In this regard, the highest GABA producer, *L. brevis* LB01, was also the strain in single cultures with a higher final pH in the growth medium.

Although *L. plantarum* 299v was the second highest GABA producer in monoculture, no significant difference in the final concentration of GABA was observed between this probiotic and negative control in the faecal microbial environment. This highlights the need to screen candidate GABA-producing probiotics in a simulated intestinal environment as different growth medium and conditions found in the gut could affect transcription and function of the GAD system, subsequently.

This study also investigated the use of synbiotics by testing the addition of probiotics with the prebiotic OFI, which could enhance probiotic growth in order to maintain high levels of the probiotic strain in the colon. However, the prebiotic did not stimulate growth of any tested strain. Nevertheless, we also added the prebiotic OFI in the batch cultures as while it may not stimulate the selected probiotic strains, it may enhance growth of other indigenous *Bifidobacterium* or *Lactobacillaceae* species, which could contribute to overall GABA production. However, OFI itself or in combination with the tested probiotics was not an effective strategy to enhance probiotic growth or increase GABA production in our study, although this option should not be excluded in future work testing other prebiotics and probiotic strains.

Levilactobacillus brevis LB01 identified in this study produced around 40 mM in our *in vitro* models. This concentration was equivalent to 206 mg of GABA in the vessel simulating the colonic environment. Although, there is a limited number of studies that have examined doses of oral GABA and duration of interventions on stress and sleep in humans,

beneficial effects have been reported with lower doses than those obtained in our *in vitro* work. For instance, very small amounts of GABA-fortified tea (2.01 mg GABA/200 mL tea) resulted in an improvement in acute stress levels reflected by alterations in heart rate variability measures in a university student cohort (Hinton et al. 2019). Moreover, Yamatsu et al. (2016) reported that intake of 100 mg GABA capsule (vs. control) improved feelings upon awakening scores, objectively measured reduced sleep latency, and increased total non-REM sleep time after intervention as well as observed trends for sleep satisfaction, and ease of falling asleep scores.

Regarding microbiota composition in batch cultures, no significant compositional changes were observed between the negative control and additional of probiotics after 48 h of fermentation. PCoA revealed that time, not treatment, was the main driver in microbiota composition across the study. This fact could be caused by reduced diversity, measured by number of species, observed in fermented samples at 48 h due to a loss of low-abundance species after inoculation and increase in abundance of some fast-growing facultative anaerobic genera such as *Lactobacilli* and *Enterobacteria* in the *in vitro* system. These results are in line with other *in vitro* work using fermentation systems (Rajilić-Stojanović et al. 2010, Takagi et al. 2016, Miguez et al. 2020). LEfSee analysis confirmed an enrichment with *L. brevis* and *L. plantarum* in the fermenters where the probiotic was administered. However, no significant changes were observed in other microbial taxa between the fermenters with probiotic supplementation and negative control. This result was expected and consistent with other probiotic studies as the bacteria used do not need to alter the gut microbiota composition to exert an impact (Kristensen et al. 2016, Gutiérrez-Castrellón et al. 2022).

In summary, screening of commercially available probiotic strains for GABA production is a valuable strategy to identify microbes that could potentially be used in human interventions as they have already passed quality and safety controls to be used as food supplements. This study highlights the importance of testing the GABA producting ability of probiotic strains not only in monocultures but also in an *in vitro* fermentation system simulating the intestinal environment. A commercially available probiotic strain—*L. brevis* LB01—was identified in our study as a valuable candidate probiotic to be studied in prospective human interventions aimed at increasing GABA levels in the gut, and to test its impact on brain and behaviour.

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Supplementary data

Supplementary data is available at *JAMBIO* Journal online.

Conflict of interest

None declared.

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Author contributions

Conceptualization: B.C., G.G., A.K., and A.M.; methodology: B.C., G.G., A.K., A.W., and A.M.; software: A.M., V.F., and C.R.; formal analysis: A.M.; investigation: A.M.; data curation: A.M., V.F., and C.R.; writing—original draft preparation—: A.M.; writing—review and editing—: B.C., G.G., A.K., and C.R. All authors have read and agreed to the published version of the manuscript.

Data availability

All data generated or analysed during this study are included in this published article and its supplementary information files. The sequence data obtained by sequencing of the V4-V5 region of the 16S rRNA gene are available from Sequence Read Archive (SRA) of NCBI (https://www.ncbi.nlm.nih.gov/sra) under accession number PRJNA866336.

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