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Research Article

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Using *in vitro* models to ascertain whether multi-probiotic supplementation influences neurotransmitter and SCFA production in the absence of human cells

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Abstract

Aims: The present study aimed to explore microbial production of neurotransmitters related to cognitive function in the faecal microbiota of healthy older adults, and assess whether a multi-strain probiotic formula may influence production of these neuroactive metabolites, short-chain fatty acids, and the bacterial community.

Method and results: The current study employed a three-stage continuous culture system with faecal microbiota from three healthy older adult donors. Neuroactive compounds were quantified using liquid chromatography mass spectroscopy, SCFAs using gas chromatography, and the bacterial community was assessed using fluorescence *in situ* hybridization with flow cytometry and 16S rRNA sequencing. Addition of the probiotic supplement (*Bifidobacterium lactis* W51, *Bifidobacterium lactis* W52, *Lactobacillus acidophilus* W37, *Lactobacillus salivarius* W24, *Lactobacillus casei* W56, *Bifidobacterium bifidum* W23, *Lactobacillus brevis* W63, *Lactococcus lactis* W19, *Lactococcus lactis* W58) significantly increased the relative abundance of *Lactococcus lactis* in the transverse region, alongside a trend for increased *Roseburia* across the three colon regions modelled, valerate in the distal region, and GABA in the proximal region.

Conclusions: While administration of the probiotic only had a small effect of trending increases in the synthesis of GABA and valerate, this highlights important mechanisms by which probiotics could be involved in the gut-brain axis. The model also enabled the observation of limited microbial production of other neurotransmitters. Further exploration in human studies is therefore warranted. Probiotics were confirmed to lead to microbial changes, both directly (*Lactococcus*) and indirectly (*Roseburia*). This research helps to support mechanistic understanding of probiotics and the gut-brain axis.

Impact Statement

The present work provides support for microbial production of neurotransmitters under physiologically relevant conditions, and highlights the potential for probiotic supplementation to influence the gut-brain axis via alterations in microbially derived GABA.

Keywords: probiotics; human gut microbiome; in vitro models; applied microbiology; fermentation

Introduction

Cognitive decline is a common characteristic of ageing, even in the absence of age-related disease such as Mild Cognitive Impairment (MCI) and Alzheimer's disease (AD). In particular, the domains of learning, memory, and executive function tend to be affected (Burke and Barnes 2006, Mattson and Arumugam 2018), posing daily challenges to the individual as well as reducing quality of life and often causing poorer mental health (Montejo et al. 2014). Given the increasing population, there is a growing need to identify lifestyle interventions which may mitigate against age-related cognitive decline and support healthy neural function.

One such intervention that has gained support in recent years is the use of probiotics, which are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit to the host (Hill et al. 2014). Probiotic microorganisms have gained interest as potential modulators of neural function due to the discovery of the gut-brain axis (GBA), which describes multiple pathways via which the gut microbiota (GM) may interact with and affect the brain, including the vagus nerve, the neuroendocrine system, immune pathways, and microbially derived metabolites (Margolis et al. 2021, Mayer et al. 2022). To date, several randomized control trials (RCTs) have explored the effects of probiotic interventions on cognitive function in older adults, with consistent evidence for a positive effect of probiotics on cognition compared to placebo controls, particularly in those with MCI and AD (Eastwood et al. 2021, Xiang et al. 2022, Handajani et al. 2023). Additionally, in healthy older adults, a recent study found reduced cognitive reactivity to sad mood (lowering the risk of depression) and improvements to executive function following chronic supplementation with a multi-strain probiotic (Ecologic® Barrier) in addition to quicker reaction times during cognitive testing following just a single dose of the probiotic supplement (Eastwood et al. 2025).

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Despite promising results from RCTs exploring the effect of probiotics on cognitive function, the potential mechanism(s) of action underlying these behavioural effects remain unclear. A number of studies, namely in animals, have reported increased concentrations of neurotransmitters (NTs) in the lumen, serum, and brain following chronic probiotic intervention (Pokusaeva et al. 2017, Leblhuber et al. 2018, Cao et al. 2019), indicating that probiotics may increase the synthesis of these neuroactive metabolites. GABA is known to be produced by several microbes via the glutamic acid decarboxylase mechanism, which in turn provides a protective mechanism for the bacteria against acidic environments (Das and Goyal 2015, Otaru et al. 2021), but there is increasing evidence that specific strains of bacteria, including those found enterically, can produce other NTs. While much of this evidence stems from pure microbial culture work (Girvin and Stevenson. 1954, Tsavkelova et al. 2000, Landete et al. 2007, Özoğul et al. 2012, Das and Goyal 2015, Danilovich et al. 2021, Rich et al. 2022), the presence of several NTs has been reported in faecal batch culture (Eastwood et al. 2023) and continuous culture models (Liu et al. 2021), which mimic the human colonic environment without the presence of epithelial cells, suggesting these metabolites to be, in part, microbially derived. Increasing the availability of these metabolites may be of particular significance for cognitive function, as NTs such as serotonin, acetylcholine, norepinephrine, GABA, and dopamine are essential for cognitive processes (Handra et al. 2019, Murari et al. 2020, Holland et al. 2021). Additionally, reductions in acetylcholine and altered tryptophan metabolism through the kynurenine and serotonin pathways have been linked with cognitive decline and AD (Chen et al. 2022, Savonije and Weaver 2023).

Gut microorganisms also produce short-chain fatty acids (SCFAs) as a result of polysaccharide fermentation (Fusco et al. 2023). SCFAs such as butyrate, acetate, and propionate regulate the expression of precursors tryptophan 5-hydroxylase and tyrosine hydroxylase, which in turn influence the synthesis of serotonin (5-HT) and biosynthesis of catecholamines dopamine, epinephrine, and norepinephrine, respectively (Reigstad et al. 2015). Further to their role in neurotransmitter synthesis, SCFAs play an integral role in the production of brain-derived neurotropic factor (BDNF), blood-brain-barrier integrity, gut permeability, and regulating neuroinflammation, all of which have a significant effect on cognitive function (Dalile et al. 2019).

In vitro models are useful tools for determining how microbial communities behave in the presence of different nutrients while under physiologically relevant conditions, without the need for human participants. Three-stage continuous culture models such as that developed by Macfarlane et al. (1998) allow for modelling of the human colon from the proximal through to the distal and transverse colon, mimicking nutrient availability, temperature, and pH at each region. Such models can be run for several weeks, allowing bacteria to reach a steady state before initiating treatment and reassessing after a second steady state is reached. To that end, these models also allow for repeat dosing of nutrients of interest over consecutive days, mimicking a daily dietary intervention *in vivo*.

As such, the current study employed a three-stage continuous culture system with faecal microbiota of healthy older adults with the aim to explore microbial production of neurotransmitters related to cognitive function in the faecal microbiota of healthy older adults, and assess whether a multi-strain probiotic formula may influence production of these neuroactive metabolites, short-chain fatty acids, and the bacterial community. Based on previous data (Eastwood et al. 2023), it was hypothesized that supplementation would increase the production of GABA in the proximal (pH 5.5) region of the model, and could increase general production of SCFAs. No prediction was made as to the effect on other neurotransmitters of interest, given the exploratory nature of the work.

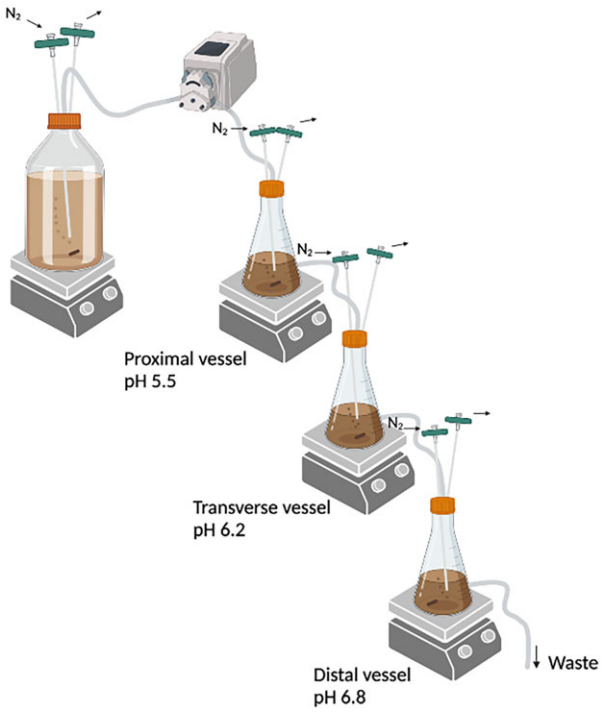

Figure 1. Schematic illustration of the three-stage continuous gut models utilized in the present work. Vessels containing culture medium, faecal microbiota, and a magnetic stirring flea are placed on magnetic stirrers. Temperature is controlled and maintained by connecting vessels to a circulating water bath, which floods the outer cavity of the vessels. Anaerobic conditions are maintained using a steady flow (15 mL/min) of N2, and pH is maintained using pH controllers connected to solutions of HCl and NaOH. A continuous flow of media is pumped into the proximal vessel via a peristaltic pump. Each region is then modelled as a separate vessel and connected to allow for gravitational feed from the proximal through to the transverse, then distal and finally a waste vessel, mimicking the typical transit through the human colon. This therefore provides a simulation model when the proximal, transverse, and distal vessels are well-controlled analogues of the colon regions, and not exact equivalents (illustration made with BioRender.com).

Materials and methods

Ethics statement

The fermentation experiment received favourable ethical opinion from the University of Reading Research Ethics Committee (UREC 15_20), and was performed in accordance with the principles of the Declaration of Helsinki.

Continuous 3-stage model

A modified version of the three-stage continuous culture system (Fig. 1) described by Macfarlane et al. (1998) was employed to simulate the proximal (vessel 1, 80 mL, pH = 5.5),

transverse (vessel 2, 100 mL, pH = 6.2), and distal colon (vessel 3, 120 mL, pH = 6.8). Each region, modelled using glass fermenter vessels, was connected to the next in series to allow for a continuous flow of gut model media to mimic the nutritional input to each region of the colon. Components (g/L) included in the gut model media were as follows: potato starch (5 g), peptone water (5 g), tryptone (5 g), yeast extract (4.5 g), casein (4 g), guar gum (1 g), inulin (1 g), pectin (2 g), arabinogalactan (2 g), xylan (2 g), potassium chloride (KCl, 4.5 g), sodium chloride (NaCl, 4.5 g), sodium bicarbonate ($NaHCO_3$, 1.5 g), magnesium sulphate heptahydrate ($MgSO4{\cdot}7H_2O$, 1.25 g), potassium dihydrogen phosphate (KH_2PO_4, 0.5 g), dipotassium phosphate, (K_2HPO_4, 0.5 g), calcium chloride hexahydrate ($CaCl2{\cdot}6H_2O$, 0.15 g), haemin (0.5 g), ferrous sulphate heptahydrate (FeSO4.7H2O, 0.005 g), and vitamin K, 10 μL. Additionally, each litre of media contained 0.8 g L-cysteine HCI, 1 mL Tween 80, 4 g mucin (porcine gastric type III), and 0.4 g bile salts, representing human secretions, and 4 mL resazurin solution (0.025 g/100 mL, pH 7) as an anaerobic indicator. Physiologically relevant conditions were maintained throughout via continuous supply of N_2 to ensure an anaerobic environment, a circulating water bath to maintain vessels at a temperature of 37°C, and maintenance of pH per vessel via pH controllers attached to 0.5 N NaOH and 0.5 N HCl solutions (Electrolab, Gloucestershire, UK). Vessels were placed on magnetic stirrers to ensure continual homogenization of the contents and therefore more accurate pH detection.

To maintain an anaerobic environment faecal samples were collected in anaerobic jars (AnaeroJarTM 2.5 L, Basingstoke, UK, Oxoid Ltd.) with anaerobic sachets (AnaeroGen, Oxoid). Samples were used for inoculation within 2 h of production. To prepare the faecal sample, a 20% (wt: v) faecal slurry with PBS (anaerobic phosphate buffered saline; 0.1 mol/L; pH 7.4) was homogenized in a stomacher (Stomacher 400, Seward, West Sussex, UK) for 2 min (240 paddle beats/min). Vessels were inoculated to give a final concentration of 6% faecal slurry. Models were conducted in triplicate with a different faecal sample used for each run. Faecal donors were 3 healthy adults aged 65–72 (2 female, 1 male, all white British) with no antibiotic, pre-, or probiotic consumption within 3 months of sample collection.

Following inoculation, the vessels were left for 24 h to allow the faecal bacteria to grow within the new environment. After 24 h, the flow of gut model media was initiated by connecting a 3.5 L vessel of media to vessel 1 via a media pump. The flow was maintained at a retention rate to mimic the GI transit time of a healthy adult (48 h, 6.25 mL/h). After 8 turnovers (384 h, 2400 mL), the first equilibrium (SS1) was reached [this was established by stabilizing of SCFA over 3 consecutive days (± 10%)], and samples taken. After which, the probiotic (as detailed below) was added to vessel 1 as a supplement every morning until a second steady state (SS2) was achieved after a further 384 h. As such, the effect of additional probiotic bacteria on bacterial enumeration and metabolite production could be assessed by comparing SS2 with SS1.

Multi-strain probiotic supplement

The probiotic intervention used in the present study was a multi-strain probiotic supplement, commercially known as Ecologic® Barrier, containing the following 9 probiotic strains: *Bifidobacterium lactis* W51, *Bifidobacterium lactis* W52, *Lactobacillus acidophilus* W37, *Lactobacillus salivarius* W24, *Lactobacillus casei* W56, *Bifidobacterium bifidum* W23, *Lactobacillus brevis* W63, *Lactococcus lactis* W19, and *Lactococcus lactis* W58. To provide the equivalent of consuming 4 g of the supplement/day (1×10^{10} CFU per/day), 1.3 g of supplement was administered daily to the proximal vessel of the model.

Preparation of samples

One millilitre, 1.5 mL, and 0.75 mL of sample were aliquoted to Eppendorfs for liquid chromatography—mass spectroscopy (neurotransmitters), gas chromatography (short-chain fatty acids), and fluorescence *in situ* hybridization (FISH) coupled to flow cytometry (enumeration of bacteria), respectively. One millilitre samples were immediately stored at −20°C. For GC and sequencing, samples were centrifuged at 11, 600 *g* for 10 min, before transferring the supernatant and storing the pellet at −20°C. For FISH, samples were centrifuged at 11,600 *g* for 5 min. After removing the supernatant, the pellet was resuspended in 375 μL of PBS before adding 1125 μL of 4% paraformaldehyde. These samples were then stored at 4°C for 4–8 h before being washed twice with 1 mL of PBS and resuspending the pellet in 150 μL of PBS. Finally, 150 μL of ethanol was added, the samples were vortexed to homogenize, and stored at −20°C.

FISH with flow cytometry (flow-FISH)

Preparation of samples followed the protocol of Grimaldi et al. (2017). Briefly, samples were removed from storage at −20°C and vortexed to redisperse. Seventy microlitre of sample was suspended in 500 μL of PBS before vortexing and centrifuging for 3 min at 11, 600 *g* (consistent for all centrifuging during this process). For permeabilization of the bacterial cell wall, supernatant was discarded, and the pellet resuspended in TE-FISH containing lysozyme (1 mg/mL) and incubated in the dark for 10 min at room temperature. Samples were then re-centrifuged and washed using 500 μL PBS. For *in situ* hybridization, pellets were resuspended in 150 μL of hybridization buffer (0.9 M NaCl, 0.2 M Tris-HCl (pH 8.0), 0.01% sodium dodecyl sulphate, 30% formamide), centrifuged, and resuspended again in 1 mL. Fifty microlitre of this solution was added to each Eppendorf containing 4 μL of the oligonucleotide probe solutions, which were vortexed and incubated overnight at 36°C using heating blocks. Following incubation, 125 μL of hybridization buffer was added, and Eppendorfs were vortexed and centrifuged as standard. After discarding the supernatant, pellets were resuspended in 175 μL of washing buffer (0.064 M NaCl, 0.02 M Tris/HCl (pH 8.0), 0.5 M EDTA (pH 8.0), 0.01% sodium dodecyl sulphate), vortexed to homogenize, and incubated at 35°C for 30 min in the heating block. The washed pellets were then centrifuged once again, resuspended in 300 μL of PBS, vortexed, and stored in the dark at 4°C ready for flow cytometry. Enumeration of bacteria was conducted using the Accuri C6 flow cytometer and analysed using the Accuri CFlow Sampler software (BD, Erembodegem, Brussels).

Ten oligonucleotide probes (Table 1) were selected for inclusion, targeting a range of functionally relevant bacterial populations. Additionally, a mixed 338EUB probe was used to enumerate total bacteria.

Table 1. Oligonucleotide probe sequences and corresponding target species.

Probe	Sequence	Target species	Reference
Non-Eub	ACTCCTAGGGAGGCAGA	Control probe for EUB338	Wallner et al. (1993)
Eub338I+	GCTGCCTCCCGTAGGAGT	Most bacteria	Daims et al. (1999)
Eub338II+	GCAGCCACCCGTAGGTGT	*Planctomycetales*	Daims et al. (1999)
Eub338III+	GCTGCCACCCGTAGGTGT	*Verrucomicrobiales*	Daims et al. (1999)
Bif164	CATCCGGCATTACCACCC	*Bifidobacterium* spp.	Langendijk et al. (1995)
Lab158	GGTATTAGCAYCTGTTTGGA	*Lactobacillus* and *Enterococcus*	Harmsen et al. (2000)
Bac303	CCAATGTGGGGGACCTT	*Bacteroidaceae*, *Prevotellaceae*	Manz et al. (1996)
Erec482	GCTTCTTAGTCARGTACCG	Most of the *Clostridium coccoides-Eubacterium rectale* group	Franks et al. (1998)
Rrec584	TCAGACTTGCCGYACCGC	*Roseburia*	Walker et al. (2005)
Ato291	GGTCGGTCTCTCAACCC	*Atopobium* cluster	Harmsen et al. (2000)
Prop853	ATTGCGTTAACTCCGGCAC	*Clostridium* cluster IX	Walker et al. (2005)
Fprau655	CGCCTACCTCTGCACTAC	*Feacalibacterium prausnitzii* and relatives	Hold et al. (2003)
DSV687	TACGGATTTCACTCCT	*Desulfovibrio* genus	Devereux et al. (1992)
Chis150	TTATGCGGTATTAATCTYCCTTT	Most of the *Clostridium histolyticum* group	Franks et al. (1998)

16S rRNA sequencing

In addition to flow-FISH, bacterial community was assessed using 16S rRNA sequencing, allowing for a more detailed understanding of how the probiotic supplement may affect different genera of interest, including those present in the supplement (*Lactobacillus, Bifidobacterium,* and *Lactococcus*), and those previously implicated in age-related change and health outcomes in older adults, such as *Alistipes*, *Akkermansia*, *Blautia*, *Clostridium*, *Desulfovibrio*, *Faecalibacterium*, *Gemmiger*, *Prevotella*, *Roseburia*, and *Ruminococcus* (Duncan et al. 2013, Ragonnaud and Biragyn 2021, Wilmanski et al. 2021, Zeng et al. 2023, Borrego-Ruiz and Borrego 2024, Ramos et al. 2025).

Pellets were resuspended in 300 μL of sterile H_2O before DNA extraction was performed using QIAamp PowerFecal Pro DNA kits (QIAGEN, Germany) according to manufacturer's instructions. The concentration of extracted DNA as well as purity (260/280 ratio) was measured using a Nanodrop (NanoDrop^{TM} ND-1000 Spectrometer). As per instructions, concentration was deemed acceptable if between 20 and 100 ng/μL. If greater than 100 ng/μL, additional C6 solution (10 mM Tris buffer) was added in 25 μL quantities until satisfactory.

16S rRNA gene sequencing and bioinformatics were outsourced to Microsynth AG (Schützenstrasse 15, 9436 Balgach, Switzerland). 25 uL of extracted bacterial DNA per sample was shipped on dry ice in sealed 96-well plates. To sequence the V3 and V4 regions of the bacterial 16S rDNA gene, two-step, Nextera barcoded PCR libraries using the locus-specific primer pair 341F (5′- CCT ACG GGN GGC WGC AG -3′) and 805R (5′- GAC TAC HVG GGT ATC TAA TCC -3′) with 20 PCR cycles for the first step and 20 PCR cycles for the second step were created.

Subsequent sequencing of PCR libraries was performed on an Illumina MiSeq platform using a v2 500 cycle kit (2 × 300 pb, V3-V4). The produced paired-end reads that passed Illumina's chastity filter were subject to de-multiplexing and trimming of Illumina adaptor residuals using Illumina's bcl2fastq software version v2.20.0.422. The quality of the reads was checked with the software FastQC version 0.11.8 and sequencing reads that fell below an average Q-score of 20 or had any uncalled bases (N) were removed from further analysis. The locus-specific primers were trimmed from the sequencing reads with the software cutadapt v3.2 and discarded if the primer could not be trimmed. Trimmed forward and reverse reads of each paired-end read were merged to in-silico reform the sequenced molecule considering a minimum overlap of 15 bases using the software USEARCH version 11.0.667. Merged reads that contained ambiguous bases or were outliers regarding the expected amplicon size distribution were also discarded. Samples that resulted in less than 5000 merged reads were discarded, to not distort the statistical analysis. The remaining reads were denoised using the UNOISE algorithm implemented in USEARCH to form operational taxonomic units (OTUs) discarding singletons and chimaeras in the process. The resulting OTU abundance table was then filtered for possible barcode bleed-in contaminations using the UNCROSS algorithm. OTU sequences were compared to the reference sequences of the RDP 16S database (https://www.drive5.com/usearch/manual/sintax_downloads.html) and taxonomies were predicted considering a minimum confidence threshold of 0.5 using the SINTAX algorithm implemented in USEARCH.

Gas chromatography

Preparation of samples for GC was carried out in line with the method previously described by Richardson et al. (1989). Samples were defrosted, vortexed, and 1 mL transferred to 100 mm × 16 mm glass vials, in addition to 50 µL internal standard (0.1 M 2-ethylbutyric acid), 0.5 mL concentrated HCl, and 2 mL diethyl ether. Vials were vortexed for 1 min and centrifuged for 10 min at 2000 *g* (Eppendorf 5804 R). The upper diethyl ether layer was extracted and transferred to new vials, from which 400 µL was taken and added to a screwcap HPLC vials with 50 µL of MTBSTFA. The vials were protected from light and stored at room temperature for 72 h prior to analysis to allow for all SCFAs, including lactate, to derivatize.

Samples were analysed using a 5690 series Gas Chromatograph (Hewlett Packard, UK) with HP-5 ms column (L × I.D. 30 m × 0.25 mm, 0.25 μm film thickness) coating of crosslinked (5%-phenyl)-methylpolysiloxane (Hewlett Packard, UK). One microlitre of each sample was injected with a run time of 17.7 min. Injector and detector temperatures were 275°C and the column temperature programmed from 63 to 190°C by 5°C and held at 190°C for 30 min. Helium was used as the carrier gas at a flow rate of 1.7 mL/min (head pressure, 133 KPa). The external standard solution included: acetic acid (30 mM); propionic acid (20 mM); n-butyric acid

Table 2. LC-MS/MS conditions used for quantification in faecal supernatant.

Compound	Retention time (min)	Retention time window (min)	Precursor ion (m/z)	Product ion (m/z)	Fragment or (V)	Collision energy (V)	Classification
GABA	1.90	3	104	87	50	4	Organic acid
		3	104	45	50	20	
Norepinephrine	2.50	3	152	107	116	16	Catecholamine
			152	77	116	30	
Epinephrine	4.60	3	184	166	70	8	Catecholamine
			184	107	70	24	
Dopamine	7.00	3	154	137	75	8	Catecholamine
			154	91	75	28	
Serotonin	9.70	3	177	160	45	4	Amino acid derivative
			177	115	45	30	
Kynurenic acid	9.77	3	190	144	100	16	Organic acid
			190	172	100	4	
Tryptophan	10.20	3	205	188	78	4	Amino acid
			205	146	78	20	

(20 mM); n-valeric acid (5 mM); iso-butyric acid (5 mM); iso-valeric acid (5 mM) (all Sigma-Aldrich). Quality control (QC) samples of external standard solution were included between donors to maintain accurate calibration. Peak integration was performed using Agilent Chemstation software (Agilent Technologies, Basingstoke, UK), and quantification of each SCFA (mM) was calculated using internal response factors as described previously (Liu et al. 2016).

LC-MS/MS

Samples were first removed from storage at −20°C and centrifuged for 5 min at 2000 *g*. Hundred microlitre of supernatant were added to 9.9 mL of HPLC water to form a 1:100 dilution, which was then filtered using 0.22 μm nylon syringe filters. One microlitre was added to a screw-cap HPLC vial for analysis. In addition, 1 mL of batch culture medium was prepared in the same manner to be analysed as a control. Individual stock solutions were prepared using analytical standards powders of dopamine hydrochloride (99%, Alfa Aesa), serotonin (Sigma–Aldrich), tryptophan (98%, Sigma–Aldrich), GABA (99%, Sigma–Aldrich), L(-)-epinephrine (99%, Acros Organics), L-noradrenaline (98%, Alfa Aesar), and kynurenic acid (98%, Sigma–Aldrich), each at 1000 ng/mL. A mixed standard solution was then prepared from the individual stock solutions and used to create a 7-level calibration series with the following dilutions: 10, 5, 1, 0.5, 0.25, 0.125, and 0.0625 ng/mL. Additionally, a 1 ng/mL standard was run every 20 samples as a quality control.

Samples were analysed by liquid chromatography−mass spectrometry/mass spectrometry (LC–MS/MS) using an Agilent 1200 HPLC system with a 6410 triple-quadrupole mass spectrometer with electrospray ion source in positive ion mode (Agilent Technologies, Basingstoke, UK). A gradient separation was carried out using a 150 × 2.1 mm Discovery HS F5–3 column, with a 2 × 2.1 mm Discovery C18 Supelguard precolumn (both 3 μm particle size; Supelco, Dorset, UK). The column was maintained at 40°C. Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. The column flow rate was maintained at 0.4 mL/min. The timetable was as follows: 0–2 min, 100% A; 5 min, 75% A; 11 min, 65% A; 15–20 min 5% A; 20.1–30 min, 100% A. The injection volume was 25 μL. The eluent from the column was run to waste from 0 to 1 min, and data were collected from 1 to 18 min. Data were acquired in dynamic MRM mode. The transitions studied and voltages used are shown in Table 2. Two transitions were acquired for each compound.

Data analysis

All statistical analyses were performed using R statistical software (R Core Team 2022). The effect of time (SS1 vs SS2) and vessel (proximal, transverse, and distal) on specific bacterial groups, relative abundance of a priori specified genera, SCFAs, and neurotransmitters was assessed using repeated-measures two-way ANOVAs with post-hoc pairwise comparisons (Bonferroni corrected) using the R Stats package (R Core Team 2022). Statistical significance was set to $P < 0.05$ and data presented as mean ± standard error unless otherwise stated.

Results

Donors

The experiment was performed in triplicate using faecal samples from 3 healthy donors between 65 and 72 (2 female and 1 male, all white British). No antibiotics, pre-, or probiotics were consumed within 3 months of sample collection.

FISH

Table 3 outlines the log $_{10}$ bacterial numbers per mL within each functional group. No significant changes in bacterial enumeration from SS1 to SS2 were observed, although the effect of time on *Roseburia* subcluster (RREC) was trending [$F(1,12) = 3.83$, $P = 0.074$], where bacterial numbers were higher at SS2 following probiotic feeding than SS1 [$P = 0.074$].

16S rRNA sequencing

The effect of time on *Lactococcus* was significant [$F(1,12) = 9.32$, $P = 0.010$], where relative abundance increased significantly from SS1 to SS2 in the transverse vessel ($P = 0.008$). This appears to be driven by an increase in one particular subspecies of *Lactococcus—Lactococcus lactis* ssp *hordinae*. As was suggested in the flow FISH data, relative abundance of *Roseburia* increased between SS1 and SS2 in all vessels, but these shifts were non-significant. Similarly, relative abundance of *Bifidobacterium* increased between SS1 and SS2, but again did not reach statistical significance.

Table 3. Enumeration of bacteria by Flow-FISH at steady state 1 (SS1) and steady state 2 (SS2) within the analogous proximal, transverse, and distal vessels represented as $\log_{10}$ cells/mL culture.

Vessel	Time	Total bacteria	Bacterial groups detected by flow-FISH								
			BIF	LAB	BAC	EREC	RREC	ATO	PROP	FPRAU	DSV	CHIS
Proximal	SS1	8.55 ± 0.48	5.41 ± 1.19	6.52 ± 1.35	7.54 ± 1.64	7.09 ± 2.01	6.12 ± 0.99	5.69 ± 0.59	6.38 ± 1.72	6.29 ± 1.69	6.29 ± 1.69	6.81 ± 1.37
	SS2	8.90 ± 0.25	6.38 ± 0.91	6.99 ± 1.32	7.90 ± 1.08	7.24 ± 2.37	6.72 ± 0.75	6.22 ± 0.88	6.98 ± 0.72	6.85 ± 1.23	6.85 ± 1.23	6.77 ± 0.51
Transverse	SS1	8.60 ± 0.25	5.54 ± 0.79	7.28 ± 0.49	7.87 ± 0.06	8.06 ± 0.29	5.80 ± 0.82	5.84 ± 0.72	7.40 ± 0.17	7.06 ± 0.88	7.06 ± 0.88	6.07 ± 1.71
	SS2	8.59 ± 0.30	6.03 ± 1.28	6.52 ± 0.77	7.69 ± 0.56	8.24 ± 0.44	6.38 ± 0.42	5.75 ± 0.49	7.21 ± 0.34	6.92 ± 1.04	6.92 ± 1.04	5.61 ± 1.01
Distal	SS1	8.26 ± 0.29	5.21 ± 0.75	6.44 ± 1.70	7.23 ± 0.17	6.59 ± 1.86	5.47 ± 0.67	5.40 ± 0.86	7.00 ± 0.40	6.69 ± 0.56	6.69 ± 0.56	5.54 ± 1.81
	SS2	8.32 ± 0.78	5.73 ± 1.75	6.25 ± 1.53	6.75 ± 0.76	7.99 ± 0.73	6.20 ± 0.10	5.99 ± 0.32	7.12 ± 0.44	6.43 ± 0.81	6.43 ± 0.81	5.69 ± 1.03

Target bacteria: Bifidobacterium spp. (BIF), *Lactobacillus* spp. (LAB), most Bacteroidaceae and Prevotellaceae (BAC), Clostridium coccoides–Eubacterium rectale group (EREC), Roseburia subcluster (RREC), *Faecalibacterium prausnitzii* (FPRAU), *Clostridium* cluster IX (PROP), Atopobium-Coriobacterium spp. (ATO), Desulfovibrio (DSV), and Clostridium histolyticum group (CHIS). The effect of time on Roseburia subcluster (RREC) was trending [$F(1,12) = 3.83$, $P = 0.074$], where bacterial numbers were higher at SS2 following probiotic feeding than SS1 [$P = 0.074$]. Values are presented as mean ± standard deviation. SS1 is following fermentation of standard gut model media, while SS2 is following daily addition of the probiotic consortia until the next equilibrium was reached. Vessel 1, 2, and 3 are analogous to proximal, transverse, and distal colon regions, respectively.

Lactobacillus was not detected in 16S rRNA gene data and therefore a significant change could not be assessed.

Gas chromatography

Figure 2 illustrates change in concentration for each SCFA/BCFA of interest between SS1 and SS2. Generally, SCFA levels increased between SS1 and SS2 following daily probiotic feeding, particularly in the distal region. While the main effect of Time was trending for valerate [$F(1,12) = 3.67$, $P = 0.08$], no significant main effect of time or vessel was seen for any of the SCFAs measured. Concentrations of lactate were below that of minimum detection and are therefore not presented.

LC-MS/MS

Figure 3 illustrates change in concentration for each neuroactive compound of interest between SS1 and SS2. GABA, dopamine, norepinephrine, tryptophan, and kynurenic acid were detected at sufficient quantities, but concentrations of epinephrine and serotonin were below that of minimum detection and are therefore not presented. Despite increases in GABA, dopamine, and tryptophan following probiotic feeding, no statistically significant changes in concentration were detected between SS1 and SS2 for any of the neuroactive metabolites measured. Substantially higher values for tryptophan in the proximal vessel driven by one donor make it difficult to visualize changes in concentration between SS1 and SS2 in the other vessels. As such, a second graph for tryptophan is presented below (Fig. 3f), where these extreme values have been replaced with the mean concentration of the other two donors, for the purpose of improving visual clarity.

Discussion

This work aimed to assess how the addition of a multi-strain probiotic supplement may affect both bacterial composition and the production of neuroactive metabolites within faecal bacterial communities from healthy older adults, using comprehensive three-stage continuous culture systems modelling the large intestine.

FISH indicated little change in bacterial composition between steady states, although an increase in numbers of *Roseburia* spp. following probiotic feeding was trending towards significance, and this was mirrored in the sequencing data, particularly in the distal region. *Roseburia* is a genus of Gram-positive bacteria belonging to the phylum Firmicutes under the family Lachnospiraceae (Rainey 2009), which have gained attention for being prolific butyrate producers (Duncan et al. 2002, Nie et al. 2021). Maintenance of *Roseburia* in older adults has been associated with healthier ageing (Claesson et al. 2012). In particular, the incidence of frailty and cognitive decline was found to be reduced in older adults adhering to a Mediterranean dietary intervention that promoted SCFA-producing bacteria such as *Roseburia* and *Faecalibacterium prausnitzii* (Ghosh et al. 2020), likely due to the beneficial effects of butyrate on energy metabolism, immune function, and histone deacetylase inhibition, which in turn positively impact cognitive function (Stilling et al. 2016, Alpino et al. 2024). Trends in the present work therefore suggest that the probiotic formulation utilized here may have the potential to support levels of *Roseburia* in older age, which could have a beneficial effect on cognitive function. While *Roseburia* is not within the probiotic consortia, these changes are likely to be

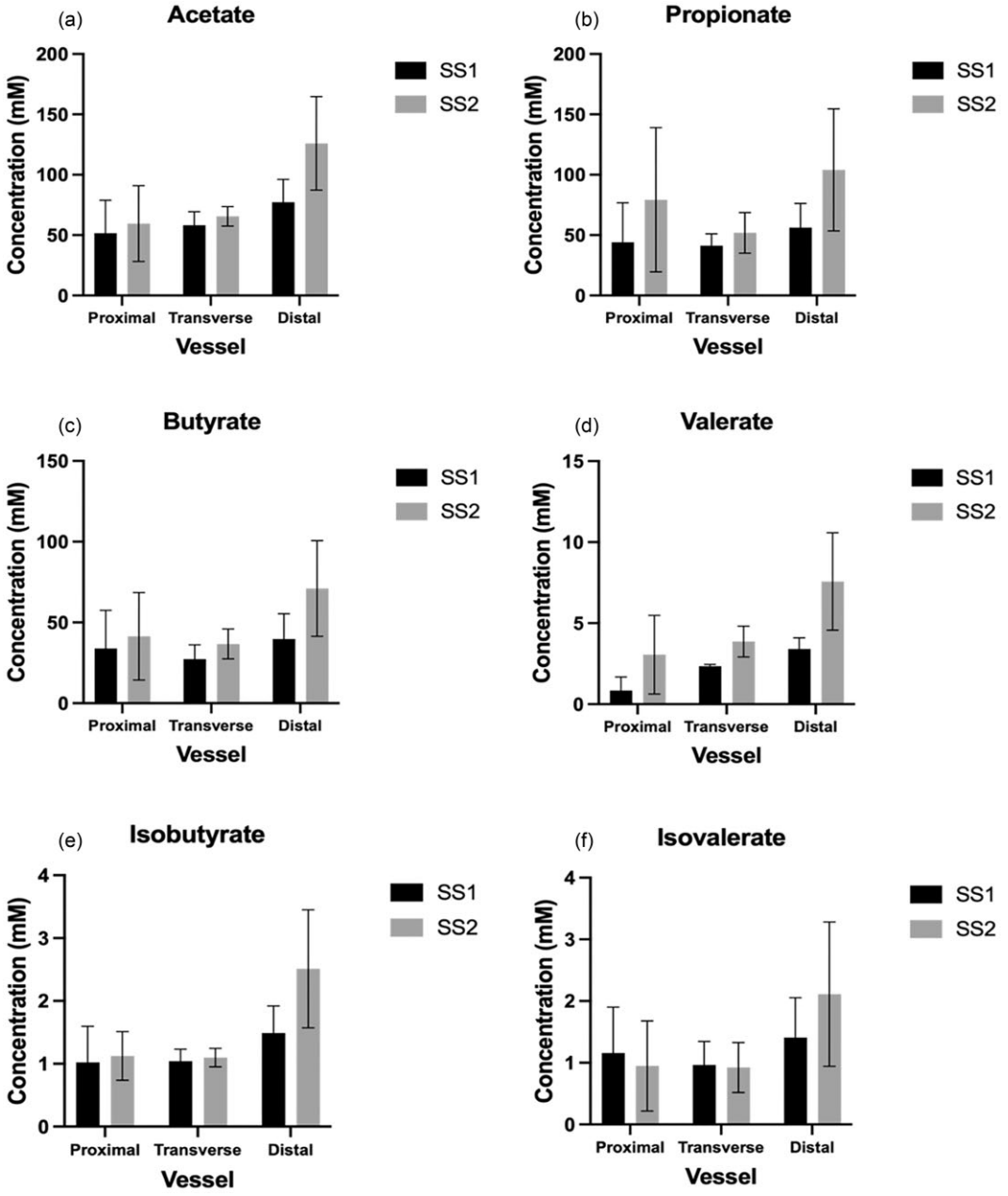

Figure 2. SCFA concentrations of acetate (a), propionate (b), butyrate (c), valerate (d), isobutyrate (e), and isovalerate (f) (mM) per vessel at steady state 1 (SS1) and steady state 2 (SS2). Two-way ANOVA indicated a trending main effect of time on valerate [$F(1,12) = 3.67$, $P = 0.08$]. Values are presented as mean ± standard error. SS1 is following fermentation of standard gut model media, while SS2 is following daily addition of the probiotic consortia until the next equilibrium was reached. Vessels 1, 2, and 3 are analogous to proximal, transverse, and distal colon regions, respectively.

an indirect consequence of cross-feeding due to the probiotics within the model system.

16S sequencing data illustrated a significant increase in the relative abundance of *Lactococcus lactis* following probiotic feeding. *Lactococcus lactis* is a Gram-positive lactic acid-producing bacterium thought to be of particular importance for immune function, with *in vitro* and animal models reporting enhanced immune response against pathogenic bacteria (Santibañez et al. 2021), inhibition of cancer cells and proinflammatory cytokines (Han et al. 2015), and stimulation of ileal mucosal immunity (Yu et al. 2021). In the latter study, authors reported alterations in serum tryptophan and ileal GABAA$\alpha 5$ receptor gene expression, suggesting *L. lactis* may influence immune function by regulating amino acid profiles and the GABAergic system. As such, *L. lactis* species may influence the GBA via immune pathways.

Several SCFAs were detected at SS1 and SS2, including acetate, propionate, butyrate, valerate, and branch-chain fatty acids isobutyrate and isovalerate. Lactate was not detected at either sampling timepoint across any of the modelled regions, likely due to the fact that lactic acid is utilized for the production of SCFAs via various pathways, including the acrylate pathway (Flint et al. 2015), the succinate pathway (Louis et al. 2017), and the butyryl-CoA: acetate-CoA transferase route (Duncan et al. 2004). SCFAs were produced in the expected relative quantities, such that acetate was greater than

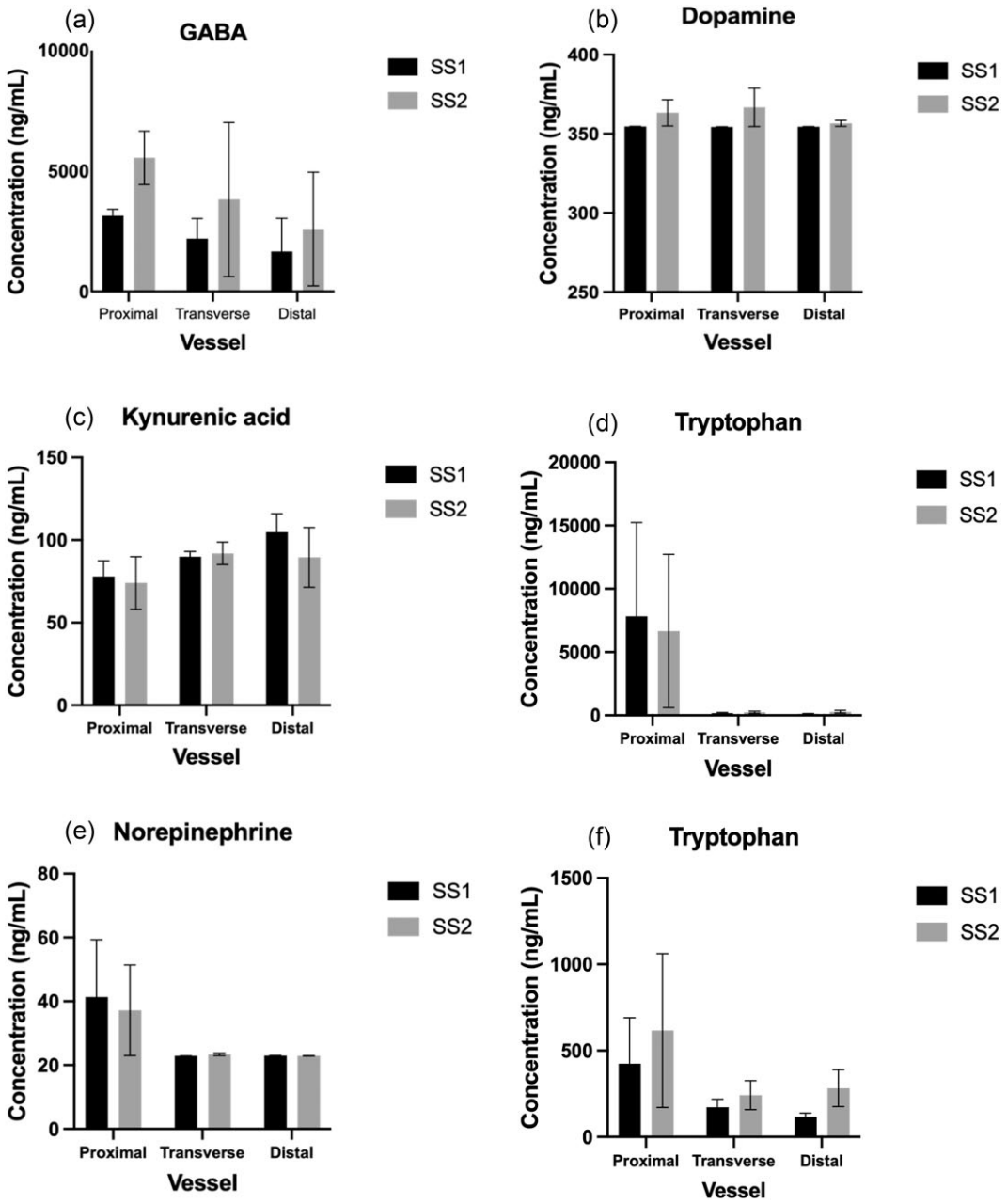

Figure 3. Concentrations of GABA (a), dopamine (b), kynurenic acid (c), tryptophan (d), norepinephrine (e), and tryptophan with extreme values removed (f) (mM) per vessel at steady state 1 (SS1) and steady state 2 (SS2). Two-way ANOVAs identified no statistically significant main effects of time or vessel. Values are presented as mean ± standard error. SS1 is following fermentation of standard gut model media, while SS2 is following daily addition of the probiotic consortia until the next equilibrium was reached. Vessels 1, 2, and 3 are analogous to proximal, transverse, and distal colon regions, respectively.

propionate and butyrate, which were greater than valerate. Although concentrations generally increased following probiotic feeding, particularly in the distal region, inter-donor variability may have occluded statistically significant change. This is perhaps unsurprising given that no additional carbohydrate or protein sources were available for SCFA production, just the additional microorganisms. Having said that, the increase in valerate in the distal region from SS1 to SS2 was trending. Valerate is perhaps less understood than the more abundant SCFAs, but microbial synthesis has been reported *in vitro* via number of pathways by select bacteria such as *Escherichia coli*, *Prevotella copri*, and *Megasphaera* (Oliphant and Allen-Vercoe 2019, Akhtar et al. 2022). Reported benefits of valerate on the host include improved epithelial barrier integrity (Gao et al. 2022) and anxiolytic effects, likely through GABAergic type activity (Vishwakarma et al. 2016), meaning valerian derivatives are often consumed to reduce anxiety, insomnia, and pain. Additionally, valeric acid appears to have a neuroprotective effect against pro-inflammatory cytokines and neurodegeneration in mouse models of AD and Parkinson's disease (PD) (Jayaraj et al. 2020, Dulla and Bindhu 2022), and is therefore actively being investigated as a potential therapeutic for neurodegenerative disorders (Jayaraj et al. 2020). Altered valerate production is often not reported following probiotic

intervention, likely as it is difficult to observe *in vivo* due to being produced in relatively small quantities, prior to absorption within the body. As such, use of these *in vitro* models enables production to be better mapped. The trends in the data suggest the current probiotic formula may enhance microbially derived valerate, which, given that the aforementioned issues of epithelial permeability, increased inflammation, pain, and low mood are often encountered in older age, could be beneficial.

Regarding neurotransmitters, GABA, dopamine, norepinephrine, tryptophan, and kynurenic acid were present at both steady states, while serotonin and epinephrine were not detected. While both serotonin and epinephrine have been reported in gut models previously (Liu et al. 2021), therefore suggesting some level of microbial production, levels below minimum detection in the present work indicate that microbial synthesis is likely a minor pathway, and more abundant neurotransmitter production may require host-led pathways, combining host cells and end products of microbial fermentation (Reigstad et al. 2015, Dicks 2022). Despite concentrations of GABA increasing after probiotic feeding as predicted, particularly under proximal conditions, inter-donor variability once again made it difficult to establish statistical significance. However, it is of interest to note that, as reported in previous work (Eastwood et al. 2023), quantities of GABA were far greater than that of the other metabolites, particularly at the lower pH found in the proximal vessel. This collective evidence suggests that, of the neuroactive metabolites analysed, gut microbes provide a major production pathway for GABA and therefore highlights GABA as a putative target for gut-brain axis interventions.

Although GM composition varies greatly within older adult populations making it difficult to compare the present data with a population norm (Claesson et al. 2011), the microbiota profiles of the present donors may represent healthy ageing microbiomes and therefore limit the scope for a potential benefit of the probiotic. Use of standard gut model media could also have contributed to a healthy microbial profile by SS1, which may not accurately reflect the colonic environment of ageing volunteers. As such, there may be a need to alter model media in future to more accurately mimic the diet of a healthy older adult population (Smith et al. 2022, Norton et al. 2024). Additionally, as mentioned, the three donors elicited high inter-donor variability (see Supplementary Materials) in the production of both SCFAs and neuroactive metabolites, making it challenging to observe whether changes in metabolite production as a result of probiotic feeding were statistically significant. This highlights that exploring how the starting consortia of bacteria interact with the effect of probiotics is a necessary avenue of future research. With that said, the continuous culture models utilized provide a highly controlled environment with which to meet the present study aim of exploring the effect of probiotics on faecal metabolite production. It should also be noted that the metabolites targeted here are by no means an exhaustive list of metabolites with the potential to influence gut-brain activity. Future work utilizing continuous culture systems would likely benefit from looking at a wider range of metabolites, including other derivatives of the tryptophan pathway and bile acids (Connell et al. 2022), to continue expanding our understanding of GBA pathways and the role the probiotic bacteria may play. Finally, these modelling systems do not incorporate human cells, which, as is implied by the low levels of production in the current data, are likely necessary for sufficient production of neurotransmitters in the gut microbiota. Exposing the supernatant from these models to colon cells to see the impact on subsequent metabolite production, such as in enterochromaffin cells and enteroendocrine cells, would provide further insight into these potential pathways.

In conclusion, the present study did not find compelling evidence for a beneficial effect of probiotics on microbially derived metabolites in the faecal microbiota of healthy older adults. Trends in the current data suggest administration of this multi-strain probiotic supplement may support the prevalence of *Lactococcus* and *Roseburia*, and synthesis of valerate, but the effect on other SCFAs and neuroactive metabolites remains unclear. However, the data does provide further support for the microbial production of neurotransmitters, and highlights that microbial production of GABA under low pH may be a particularly relevant target for the gut-brain axis.

Author contributions

Jessica Eastwood (Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft), Saskia van Hemert (Conceptualization, Writing – review & editing), Carlos Poveda (Methodology, Writing – review & editing), Stephen Elmore (Methodology, Writing – review & editing), Claire Williams (Conceptualization, Supervision, Writing – review & editing), Daniel Joseph Lamport (Conceptualization, Funding acquisition, Supervision, Writing – review & editing), and Gemma E Walton (Conceptualization, Methodology, Supervision, Writing – review & editing)

Supplementary data

Supplementary data is available at *JAMBIO Journal* online.

Conflict of interest: S.v.H. was at the time of this research being conducted employed by Winclove Probiotics, who partially funded the Ph.D. studentship and provided the probiotic bacteria for this research.

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Data availability

Data will be available upon request.

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