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RESEARCH ARTICLE

The impact of three distinct probiotic supplements on the gut microbiota and its metabolites in healthy adults

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Abstract

The effects of probiotics on the gut microbiota and microbial metabolites in healthy individuals are not well understood. Faecal and serum samples were collected at the start and end of a 3-month, double-blind, placebo-controlled, randomised study with three different probiotic formulations in free-living, healthy adults. The composition of the faecal microbiota and levels of faecal and/or serum short-chain fatty acids (SCFA) and bile acids (BA) were measured and the probiotic formulations were found to impart differing effects including shifts in the composition and structure of the faecal microbiota, enhanced levels of circulating short chain fatty acids such as butyrate and propionate, and elevated levels of sulphated bile acids in faeces. This was in contrast to the outcomes for the placebo population where very little change occurred over the study. These findings demonstrate that probiotic supplementation elicits formulation specific effects and that there are potential benefits for healthy individuals.

Keywords

probiotic – healthy adults – microbiota – metabolites

1 Introduction

A complex relationship exists between the trillions of bacteria residing in the gut (the gut microbiota) and the host (Fan and Pedersen, 2021) and the microbiota is involved in a multitude of functions within and beyond the gut that are fundamental to host health. Two key examples of these functions include the generation of

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short chain fatty acids (SCFAs) and the biotransformation of primary bile acids (BAs) into a wide variety of derivatives (Fan and Pedersen, 2021).

SCFAs such as acetate, butyrate and propionate are generated in the colon during the anaerobic fermentation of non-digestible dietary carbohydrates (and, to a lesser extent, amino acids) by Bacteroidota, Bacillota, Clostridiota and Actinomycetota (den Besten *et al.*, 2013; Fusco *et al.*, 2023). They are key regulators of intestinal immune homeostasis (Fusco *et al.*, 2023) and their acidic properties help prevent the growth of pH sensitive pathogens whilst supporting the growth of commensal bacteria (den Besten *et al.*, 2013). Butyrate is of particular importance, serving as the primary energy source of colonocytes and supporting the integrity of the intestinal epithelium (Recharla *et al.*, 2023). Gut derived SCFAs can reach the peripheral tissues via the circulatory system where they impart beneficial adipogenic, inflammatory and neurogenic effects (van der Hee and Wells, 2021).

Bile acids (BAs) are released into the intestinal lumen during digestion to emulsify dietary fats and fat-soluble vitamins, facilitating their absorption. They are also involved in cellular signalling, immune modulation, and have antimicrobial activities (Larabi *et al.*, 2023). The functional diversity of BAs, and the rate of their synthesis and excretion, is significantly influenced by the biotransformation processes mediated by the gut microbiota (Guzior and Quinn, 2021). Microbial bile salt hydrolases (BSHs) are gateway enzymes and catalyse the deconjugation of BAs to reduce their solubility and promote their excretion. BA deconjugation also enhances their antimicrobial properties, contributing to overall defence against pathogens (Bourgin *et al.*, 2021; Larabi *et al.*, 2023).

Probiotic bacteria are defined as 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host' (Hill *et al.*, 2014) and there is evidence supporting their ability to alleviate gastrointestinal disorders such as irritable bowel syndrome (IBS) (Wu *et al.*, 2024) and constipation (van der Schoot *et al.*, 2022), but there are few studies of the impact of probiotic supplementation on healthy individuals. Little is known about their impact upon the gut microbiota and SCFA and BA metabolism in healthy subjects even though they represent the biggest consumers of probiotic supplements globally (Yilmaz-Ersan *et al.*, 2020).

The tolerability of three distinct combinations of lactic acid bacteria and bifidobacteria was assessed in a double-blind, placebo-controlled, multi-arm, randomised study in healthy adults (Mullish *et al.*, 2023). Probiotic-1 comprised two strains of *Lactobacillus acidophilus*, *Bifidobacterium bifidum* and *Bifidobacterium animalis* subsp. *lactis*, Probiotic-2 comprised the Probiotic-1 organisms with the addition of 13 others, and Probiotic-3 contained two strains of *Lactobacillus rhamnosus* and *Bifidobacterium animalis* subsp. *lactis*. The three formulations were found to exert markedly different effects on participants' gastrointestinal health (described in Mullish *et al.* (2023)) and here we provide details of their impact upon the faecal microbiota composition, the faecal and circulating levels of SCFA, and the faecal bile acid concentration.

2 Methods

Study design, recruitment and randomisation

This follow-up analysis was based on a single-centre, multi-arm, double-blind, randomised and placebo-controlled trial in healthy adults which assessed three different probiotic formulations. Eligible participants were assigned to one of four study arms, Placebo (PL), Probiotic-1 (P1), Probiotic-2 (P2) or Probiotic-3 (P3), at a ratio of 1:1:1:1 and they took the intervention daily for 84 days (3 months).

The intervention

The compositions and doses of the study interventions are shown in Table 1. Compliance to the intervention was monitored via the collection of unused capsules at the end of the study.

The outcomes

The outcomes presented here are changes in the faecal microbiota composition, the faecal and circulating levels of SCFA, and levels of faecal bile acids for those participants who provided faecal and/or serum samples at the start of the study and at the end of the study.

Schedule of sample collection

The schedule of sample collection is shown in Figure 1. Participants visited the study centre on four occasions in total, and faecal and/or serum samples were collected on visit 2 (day 0, Baseline (BL)) and visit 4 (day 84, Endpoint (EP)).

Collection, storage and analysis of faecal samples

Collection and storage

Faecal samples were collected by the participants, frozen at –20 °C immediately after collection and trans-

TABLE 1 Study interventions

Group	Bacterial content	Daily dose
Probiotic-1 (P1)*	*Lactobacillus acidophilus* CUL60 *L. acidophilus* CUL21, *Bifidobacterium bifidum* CUL20 and *B. animalis* subsp. *lactis* CUL34	2 capsules delivering a total of 2.7×10^{11} CFU
Probiotic-2 (P2)*	*L. acidophilus* CUL60, *L. acidophilus* CUL21, *B. bifidum* CUL20, *B. animalis* subsp. l*actis* CUL34, *L. salivarius* CUL61, *L. paracasei* CUL08, *L. plantarum* CUL66, *L. casei* CUL06, *L. fermentum* CUL67, *L. gasseri* CUL09, *Pediococcus pentosaceus* CUL15, *B. breve* CUL74, *S. thermophilus* CUL68, *L. rhamnosus* CUL63, *L. reuteri* JBD301, *B. bifidum* CUL73 and *L. helveticus* CUL76.	2 capsules delivering a total of 2.7×10^{11} CFU
Probiotic-3 (P3)*	*L. rhamnosus* GG, *L. rhamnosus* HN001 and *B. animalis* ssp. *lactis* HN019.	2 capsules delivering a total of 2.7×10^{11} CFU
Placebo (PL)*	None	2 capsules

*Excipients present in all capsules are silicon dioxide, magnesium stearate and potato maltodextrin. Abbreviations: CFU, colony forming units.

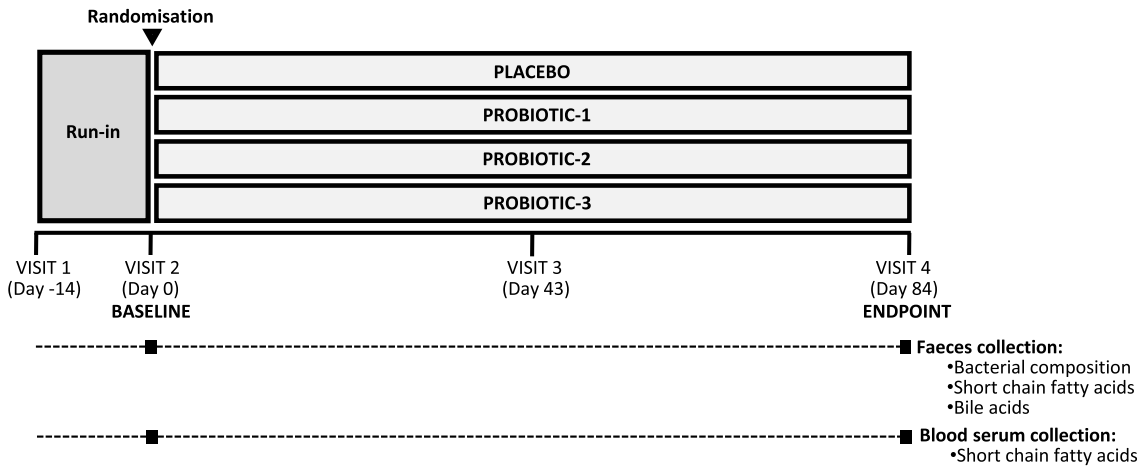

FIGURE 1 Schedule of sample collection.

ported frozen to the trial centre within two days, where they were stored at –80 °C pending analysis.

Genomic DNA extraction

Thawed faecal samples were mechanically lysed by bead beating for 3 × 30 s cycles (5 m/s) with 5 min intervals in Matrix Lysing D tubes and a FastPrep-24® bead beater (MPBIO, United States) and gDNA was extracted using the QIAamp® Fast DNA Stool Mini Kit (Qiagen, Germany) according to the manufacturer's instructions.

Microbial profiling by next generation 16S rRNA gene sequencing

Sample libraries were prepared following Illumina's 16S Metagenomic Sequencing Library Preparation Protocol with the following alterations: amplification of the V1-V2 hypervariable region of the 16S rRNA gene was performed using custom-designed primers (Mullish *et al.*, 2018) and the index PCR reactions were cleaned and normalised using the SequalPrep Normalisation Plate Kit (Life Technologies, UK). Sample libraries were quantified using Qubit fluorometry and NEBNext Library Quant Kit for Illumina (New England Biolabs, UK). Sequencing was performed on an Illumina MiSeq platform (Illumina Inc., UK) with the MiSeq Reagent Kit v3 (Illumina) using paired-end 2 × 300 bp chemistry. Raw sequencing data was processed following the DADA2 pipeline in R using the SILVA taxonomic database (version 138.1) to classify sequence variants, as described in Mullish *et al.* (2024b), and amplicon sequence variants (ASVs) were adjusted to the bacterial biomass within the sample, estimated using 16S rRNA gene qPCR as

described previously (Mullish *et al.*, 2019). To account for multiple sequencing batches, the ASV table was corrected through conditional quantile regression using the ConQuR package with default parameters (Ling *et al.*, 2022).

SCFA analysis

Faecal SCFA were measured according to the method of Valdivia-Garcia *et al.* (2022). using 3-nitrophenylhydrazine derivatisation and liquid chromatography with tandem mass spectrometry (LC-MS/MS). Results are expressed as μmol per gram sample wet weight.

Bile acid profiling

Faecal samples were lyophilised and bile acids extracted according to the method of Mullish *et al.* (2018). Bile acid profiling was performed by the National Phenome Centre (Imperial College London, UK) using Ultra-Performance Liquid Chromatography Tandem Mass Spectrometry (UPLC-MS), as adapted from the method of Sarafian *et al.* (2015). Results are expressed as relative intensity (RI, from UPLC-MS) per gram sample dry weight.

Collection, storage and analysis of blood serum

Collection and storage

At BL and EP, fasted bloods were collected into serum separator tube vacutainers and the serum was separated by centrifugation (2,000 × *g*, 10 min), aliquoted and stored at –80 °C until required.

SCFA analysis

SCFAs were measured using direct-injection gas chromatography with acidified water-extraction (Wang *et al.*, 2020; Zhao *et al.*, 2006). 20 μl of serum was acidified with 3.5 μl of concentrated orthophosphoric acid 2-ethylbutyric acid (Sigma-Aldrich) and was used as an internal standard. A Clarus 500 gas chromatograph with a flame ionisation detector (PerkinElmer 8500, Norwalk, CT, USA) and a TR-FFAP 30 m × 0.32 mm i.d. × 0.25 μm capillary column (Thermo Scientific) was used for separation of SCFAs. The temperature operation conditions for the GC separation/analysis were 90 °C for 1 min, 130 °C at 10 °C/min, hold for 3 min, then 200 °C at 10 °C/min, hold for 8 min. Nitrogen was used as a carrier; the injector temperature was 220 °C; the detector temperature was 240 °C with 5 μl injection volume. SCFAs were identified by comparing retention times of peaks with those of standards: acetic acid, propionic, valeric and butyric acid. PerkinElmer Total Chrom Navigator software was used for data acquisition. Results are expressed as ng/ml.

Data analysis & statistics

Next generation 16S rRNA gene sequencing

Data importation and diversity analyses were performed in R package Phyloseq (McMurdie and Holmes, 2013) with results plotted using ggplot2 (Wickham, 2016) and changes in spatial organisation within the study groups were observed with a non-metric multidimensional scaling (NMDS) plot based on the Bray-Curtis dissimilarity matrix. Pairwise PERMANOVA with 999 permutations were performed using the pairwise Adonis package (Martinez Arbizu, 2020). Homogeneity of dispersion was analysed using betadisper and permutest functions in the R vegan package (Oksanen *et al.*, 2022). Amplicon sequence variants (ASVs) were centred log ratio (CLR) transformed before assessment of changes in relative abundances using the Wilcoxon rank sum test with Bonferroni correction for multiple comparisons. Values of $P < 0.05$ were considered significant. Paired dot plots were created using ggplot2 in R (version 4.4.3).

To identify keystone genera, centrality scores of (*i*) degree (number of direct connections to other genera), (*ii*) betweenness (frequency of appearing on the shortest path between genera), (*iii*) closeness (proximity to other genera) and (*iv*) Eigenvector (influence on highly connected genera) were calculated from networks of genera present in at least 10% of samples after a modified CLR transformation. Networks were generated using the NetCoMi R package (Peschel *et al.*, 2020) and genus abundance correlations were calculated with the Semi-parametric Rank-Based Correlation and Partial Correlation Estimation for Quantitative Microbiome Data (SPRING) method (Yoon *et al.*, 2019). A 'hub score' (representing overall influence of a genus on the microbiota) was determined by combining normalised scores for degree, betweenness, closeness and eigenvector and the 10 genera with highest scores were assigned as 'keystones'.

SCFA and bile acids

Multivariate analysis was performed using principal component analysis (PCA) on log transformed data with *P* values generated by PERMANOVA with Bonferroni correction. Univariate analysis was performed using the Wilcoxon signed rank test (GraphPad Prism, Version 10.20.2) with Bonferroni correction. Where appropriate, data was $\log_{10}(x + 1)$ transformed for presentation in

TABLE 2 Analysis subpopulations

Analysis	Number of participants (n) included in analysis			
	PL (n/23)	P1 (n/24)	P2 (n/24)	P3 (n/25)
Faecal 16S rRNA gene sequencing	18	22	15	20
Faecal SCFA	17	18	21	21
Serum SCFA	20	23	24	21
Faecal bile acids	16	19	21	22

dumbbell plots. Values of $P < 0.05$ were considered significant.

3 Results

Recruitment, compliance and demographics

In total 96 participants were recruited to the study, with 23 allocated to the placebo group, 24 to P1, 24 to P2 and 25 to P3. The baseline characteristics were well matched between groups (Supplementary Table S1A). Participants were aged between 19 and 64 years, had a BMI between 18.99 to 29.90 kg/m^2 and were in good general health. 69.9% of the study population were female. Compliance to the study intervention exceeded 90%. The number of participants providing samples of faeces and/or serum at both BL and EP are shown in Table 2. The baseline characteristics of participants included in each analysis are provided in Supplementary Table S1B and are representative of the complete study groups and each other.

The impact of probiotic supplementation on the faecal microbiota

Shannon's α-diversity (Figure 2A) and Simpson α-diversity index (Supplementary Figure S1) were assessed and there were no differences between the study groups at baseline or endpoint, nor did any changes occur within each study group over the duration of the study (Figure 2B). With regard to β-diversity, there were no differences between the study groups at the baseline, however, at endpoint there were significant differences for the P1 group compared to PL ($P = 0.0120$), P2 ($P = 0.0059$) and P3 ($P = 0.0059$, Figure 2C); β-diversity within the P1 group changed between BL and EP ($P < 0.0001$, Figure 2D). No changes in β-diversity were observed for the PL, P2 or P3 groups between BL and EP. The homogeneity of dispersion changed within in the P3 group between BL and EP ($P = 0.0179$).

Changes in the relative abundance between BL and EP of the 10 most prevalent bacterial genera within each study group are shown in Figure 3 (top 30 shown in Supplementary Figure S2). In P1, there was a significant increase in the abundance of *Agathobacter* ($P = 0.0100$), an increase in *Faecalibacterium* ($P = 0.0780$), and a significant decrease in *Bifidobacterium* ($P = 0.0030$). In P2, *Faecalibacterium* ($P = 0.0463$) significantly increased but there were no changes in PL or P3.

Keystone organisms have a disproportionate influence on the composition, function and stability of a microbiome, irrespective of their abundance (Banerjee *et al.*, 2018; Mills *et al.*, 1993) and Table 3 shows the top 10 keystone genera identified at baseline and endpoint for each study group (rankings of degree, centrality, closeness and eigenvector are provided in Supplementary Table S2). Within the placebo, there was little change in keystone genera between BL and EP with eight taxa detected at both time-points but within the probiotic groups there were considerable changes in keystone profiles. For P1, only two genera appeared at both BL and EP which were *Bacteroides* and *Faecalibacterium*; two groups for P2, *Bacteroides* and *Lachnospiraceae_UCG-004* and two groups for P3, *Bacteroides* and *Paraprevotella*.

The impact of probiotic supplementation on SCFA levels

The overall profiles of faecal SCFA were unchanged both between groups at BL and at EP (Figure 4A) and within each group (Figure 4B). A univariate analysis of changes in faecal SCFA concentrations within each study group is shown in Figure 4C (detailed data in Supplementary Table S3) and no significant changes were observed.

Changes in the overall serum SCFA profiles are shown in Figure 5 (detailed data in Supplementary Table S4) with no between group differences observed at the baseline or at the end point (Figure 5A). Within group serum SCFA profiles changed significantly only for P1 between BL and EP ($P = 0.0196$, Figure 5B); the PL, P2 and P3 groups were unchanged. Univariate analysis of changes in individual serum SCFA levels between BL and EP showed that acetate significantly reduced in the placebo group by EP ($P = 0.0332$, Figure 5C) and propionate

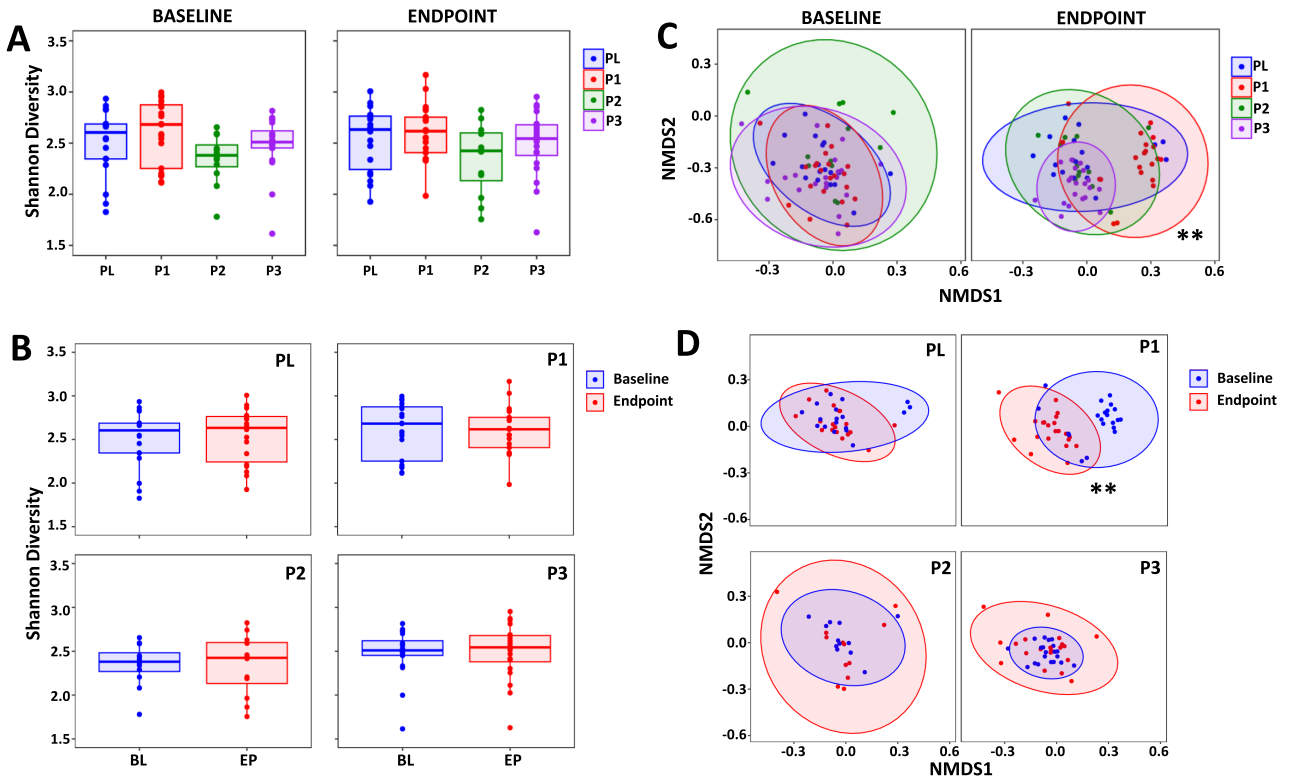

FIGURE 2 Changes in the composition of the gut microbiota. Shannon's α-diversity measure comparing (A) the PL, P1, P2 and P3 groups at the baseline (BL) and at endpoint (EP) and (B) BL with EP within the PL, P1, P2 and P3 groups. (C) non-metric multidimensional scaling (NMDS) plots based on the Bray-Curtis dissimilarity matrix comparing the PL, P1, P2 and P3 groups at BL and at EP and (D) BL vs EP for the PL, P1, P2 and P3 groups. Values of *P*; $^{**}P < 0.01$.

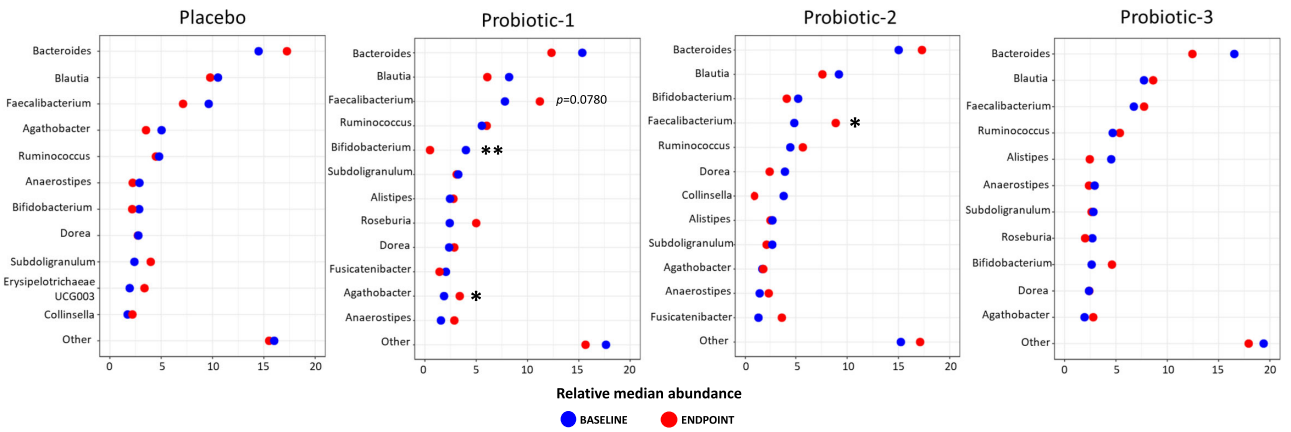

FIGURE 3 Microbiota abundance at genus level. Dumbbell plots showing changes in relative median abundance between baseline (BL) and endpoint (EP) of 10 most prevalent bacterial genera for PL, P1, P2 and P3 groups. Data is presented in descending order of abundance according to the group BL values. Values of *P*; $^{*}P < 0.05$, $^{**}P < 0.01$ or as stated.

significantly increased within P2 ($P = 0.0100$) and P3 ($P = 0.0196$). Butyrate increased in P1 ($P = 0.0216$) and P3 ($P = 0.0048$), while levels of valerate did not change within any of the study groups.

The impact of probiotic supplementation on faecal bile acids levels

There were no significant between (Figure 6A) or within (Figure 6B) group differences in overall faecal bile acid profiles at BL or at EP. Figure 6C summarises the results of a univariate analysis of the changes in bile acids within each study group (detailed data in Supplementary Table S5) and there was a significant increase in

TABLE 3 Top 10 ranked keystone taxa within the study groups

PL		P1		P2		P3	
BL	EP	BL	EP	BL	EP	BL	EP
· *Bacteroides*	· *Parabacteroides*	· *Bacteroides*	· *Bacteroides*	· *Bacteroides*	· *Bacteroides*	· *Bacteroides*	· *Bacteroides*
· *Parabacteroides*	· *Bacteroides*	· *Faecalibacterium*	· *Blautia*	· *Alistipes*	· *Lachnospiraceae_NK4A136_group*	· *Lachnospiraceae_NK4A136_group*	· *Caldicoprobacter*
· *Lachnospiraceae_NK4A136_group*	· *Lachnospiraceae_NK4A136_group*	· *Howardella*	· *Bifidobacterium*	· *Collinsella*	· *Agathobacter*	· *Paraprevotella*	· *Christensenella*
· *Anaerostipes*	· *Anaerostipes*	· *Oscillibacter*	· *Faecalibacterium*	· *Lachnospiraceae_UCG-004*	· *Terrisporobacter*	· *CAG-352*	· *Solobacterium*
· *Ruminococcus*	· *Megamonas*	· *Parabacteroides*	· *Roseburia*	· *Dorea*	· *Romboutsia*	· *Lachnospiraceae_UCG-008*	· *Alloprevotella*
· *Tuzzerella*	· *Tuzzerella*	· *Dialister*	· *Agathobacter*	· *Faecalibacterium*	· *Holdemania*	· *Marvinbryantia*	· *Prevotellaceae_Ga6A1_group*
· *Oscillibacter*	· *Streptococcus*	· *Streptococcus*	· *Alistipes*	· *Haemophilus*	· *Peptococcus*	· *Lachnospiraceae_UCG-004*	· *Acidaminococcus*
· *Coprobacillus*	· *Ruminococcus*	· *Megamonas*	· *Anaerostipes*	· *Lachnospiraceae_UCG-010*	· *Lachnospiraceae_UCG-004*	· *Escherichia/Shigella*	· *Megasphaera*
· *Megamonas*	· *Romboutsia*	· *Tuzzerella*	· *Marvinbryantia*	· *Phascolarctobacterium*	· *Fusobacterium*	· *Holdemanella*	· *Shuttleworthia*
· *Romboutsia*	· *Flavonifractor*	· *Dialister*	· *Collinsella*	· *Coprobacillus*	· *Romboutsia*	· *Defluviitaleaceae_UCG-011*	· *Paraprevotella*

Abbreviations: PL, placebo; BL, baseline; EP, endpoint. Keystone taxa are ordered in descending 'hub score' with those common to BL and EP (within a group) highlighted in grey.

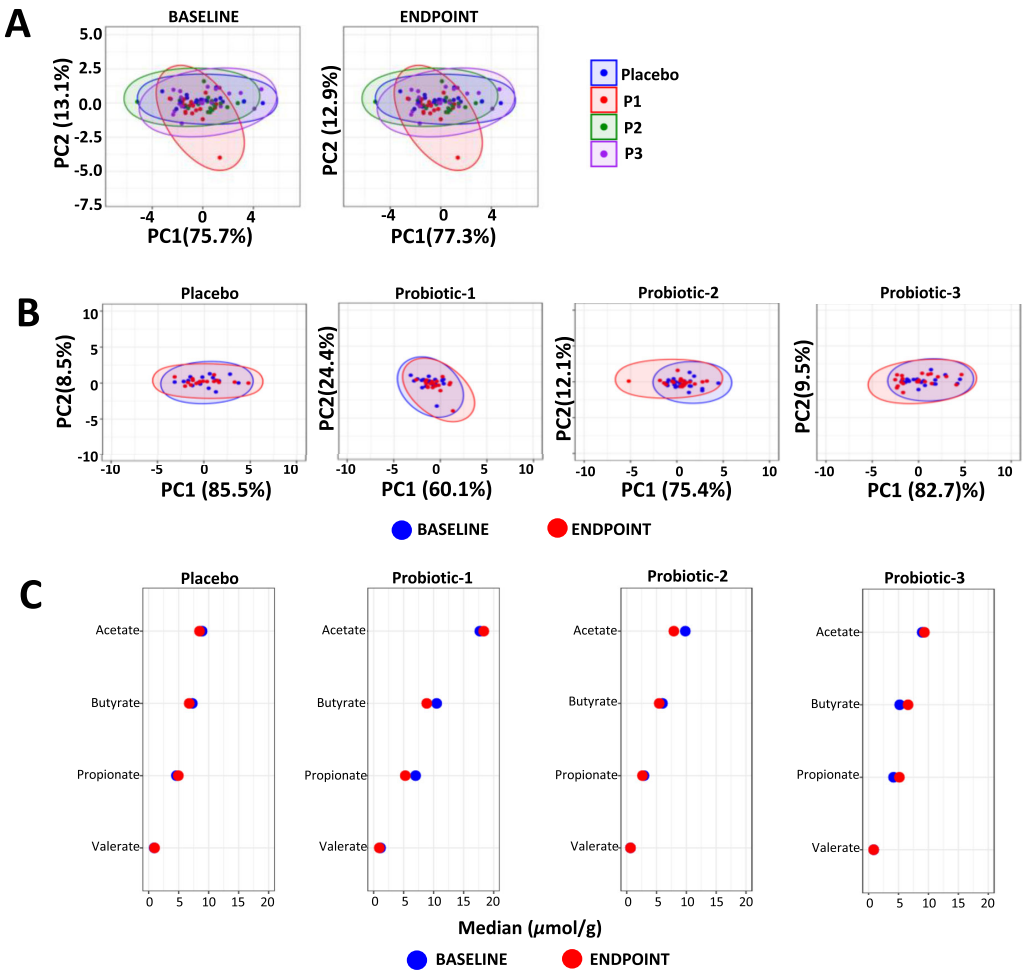

FIGURE 4 Changes in faecal SCFA. Principle component analysis (PCA) comparing faecal SCFA profiles between (A) the PL, P1, P2 and P3 groups at baseline (BL) and at endpoint (EP) and (B) within the PL, P1, P2 and P3 groups at BL and EP. (C) dumbbell plots showing changes in individual SCFA between BL and EP within the PL, P1, P2 and P3 groups. Data is presented as median μmol per g dry weight faeces and is shown in descending order of abundance according to the placebo at BL. Values of P; $^*P < 0.05$.

7KLCA ($P = 0.0040$) in the placebo group and in P1 there were significant increases in LCA-S ($P = 0.0380$) and CDCA-S ($P = 0.0496$). No changes were observed for P2 and P3.

4 Discussion

Faecal and blood serum samples were collected from four groups of healthy adults receiving one of three distinct probiotic supplements or a matching placebo daily for three months. The placebo group was subject to very few changes over the course of the study whereas each of the probiotics exerted varying effects on the composition of the faecal microbiota and on the levels of SCFAs and bile acids.

Probiotic-1 (P1), previously shown to improve gastrointestinal health in both healthy subjects (Mullish *et al.*, 2023) and those with Irritable Bowel Syndrome (Mullish *et al.*, 2024b; Williams *et al.*, 2009), altered the composition and keystone organisms in the faecal microbiota, serum SCFA profile and faecal bile acid composition. Probiotic-2 (P2), comprising the component organisms of Probiotic-1 with 13 others, and Probiotic-3 (P3), comprising organisms with recognised gastrointestinal benefits (Bonfrate *et al.*, 2020; Cheng *et al.*, 2021; Pedersen *et al.*, 2014), altered keystone organisms and serum SCFA levels.

The data presented previously (Mullish *et al.*, 2023) using traditional microbial culture techniques to determine differences in faecal viable numbers compared to the placebo indicated decreases for *Bacteroides* with P1, increased lactobacilli in P2 and for P3, increases in lactobacilli and decreases in *Bacteroides*. Next generation sequencing identified a significant shift in β-diversity within the P1 group (the P1 formulation comprises two

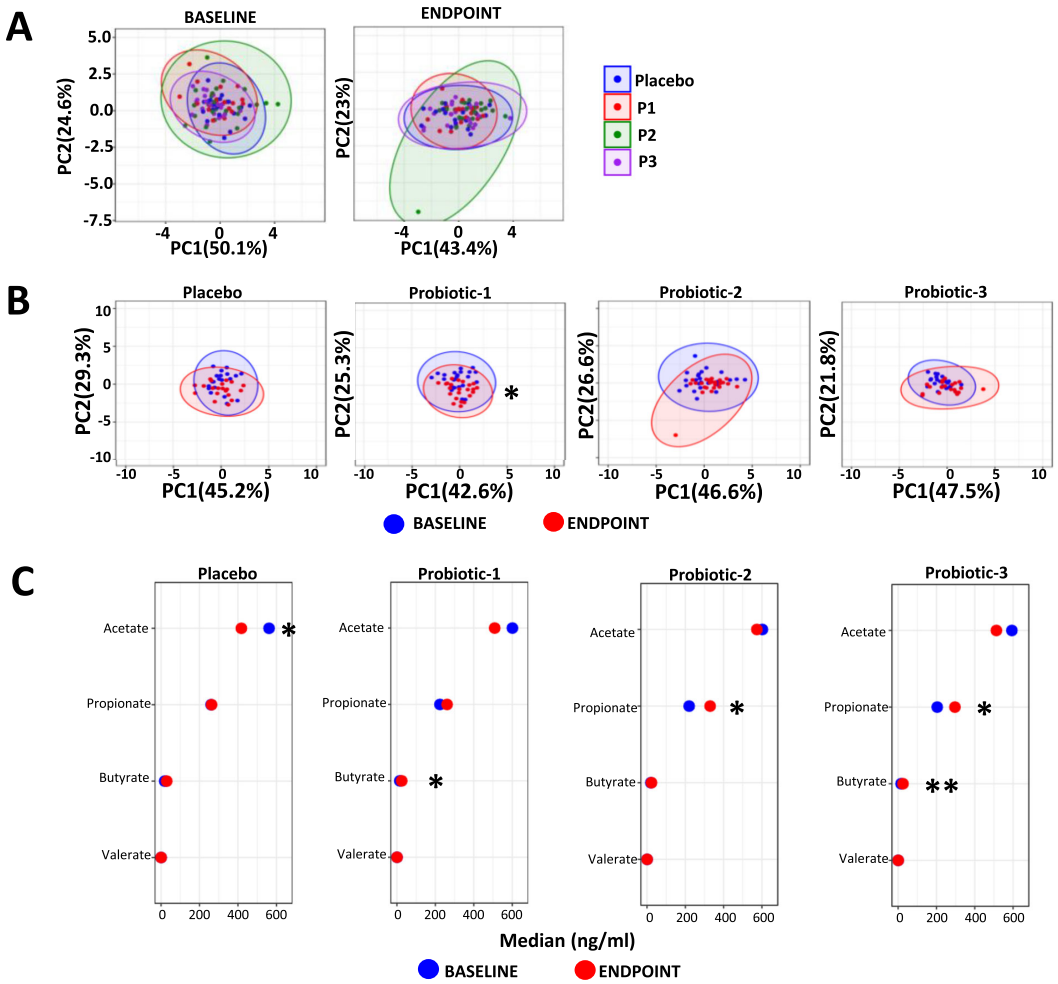

FIGURE 5 Changes in serum SCFA. Principle component analysis (PCA) comparing serum SCFA profiles (A) between the PL, P1, P2 and P3 groups at baseline (BL) and endpoint (EP) and (B) within the PL, P1, P2 and P3 groups between BL and EP. (C) dumbbell plots showing changes in individual SCFA between BL and EP for all groups. Data is presented as median ng/ml and is shown in descending order of abundance according to the placebo at BL. Values of *P*: **P* < 0.05 or ***P* < 0.01.

strains of bifidobacteria and two strains of lactobacilli). Interestingly, no changes in diversity were observed in the P2 probiotic which consists of the organisms present in the P1 formulation together with 13 more organisms. The differing response may be due to 'dilution' by the additional bacteria or, potentially, antagonistic effects among the organisms included in this multistrain population (McFarland *et al.*, 2018; Ouwehand *et al.*, 2018).

At the genus level, within the P1 group there was an increase in the relative abundance of *Agathobacter* and *Faecalibacterium* and a decrease in *Bifidobacterium* and for the P2 group, there was an increase in abundance of *Faecalibacterium*; there were no changes in the PL or P3 groups. The genus *Agathobacter* comprises SCFA producers (Van-Wehle and Vital, 2024) and previously the P1 formulation supported an increased abundance of *Agathobacter* in IBS sufferers alongside a reduced symptom severity (Mullish *et al.*, 2024b). Some members of *Faecalibacterium*, such a *F. prausnitzii*, produce butyrate, and the prevalence of these organisms suggests a 'healthy gut microbiota' (Martín *et al.*, 2023; Van-Wehle and Vital, 2024). The reduced levels of *Bifidobacterium* in the P1 group does not correspond with the viable enumeration results (Mullish *et al.*, 2023)

Keystone organisms are those which have a disproportionately large influence on the composition, stability and/or function of a microbial community, irrespective of their abundance (Banerjee *et al.*, 2018; Mills *et al.*, 1993). The keystone organisms of the PL group changed very little between the start to the end of the study, but for all of the probiotic groups there was near complete re-structuring of keystone organisms, leading us to hypothesise that the probiotics may impact upon the structure/organisation and functionality of the microbiota without imparting gross changes to com-

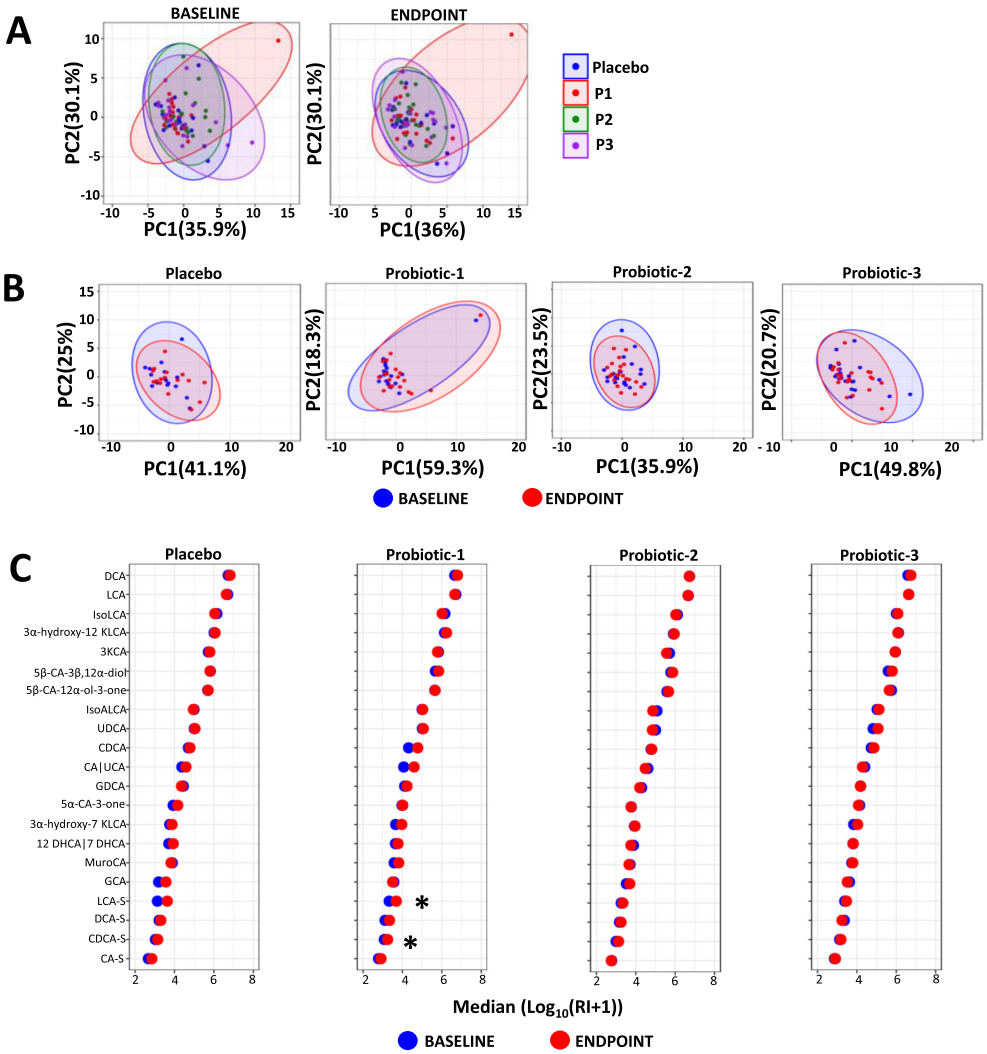

FIGURE 6 Changes in faecal bile acids. Principle component analysis (PCA) comparing faecal bile acid profiles (A) between the PL, P1, P2 and P3 groups at the baseline (BL) and at endpoint (EP) and (B) within PL, P1, P2 and P3 groups. (C) dumbbell plots comparing individual bile acids between BL and EP for the placebo, P1, P2 and P3 groups. Data is presented as the median of $\log_{10}(\text{RI} + 1)$ transformed RI/g of faeces (dry weight) and is shown in descending order of abundance according to the placebo at BL. Values of P; $^*P < 0.05$. Abbreviations: DCA, deoxycholic acid; LCA, lithocholic acid; IsoLCA, isolithocholic acid; 3α-hydroxy-12 KLCA, 3-alpha-hydroxy-12 ketolithocholic acid; 5β-CA-3β,12α-diol, 5-beta-cholanic acid-3-beta, 12-alpha-diol; 3 KCA, 3-ketocholanic acid; 5β-CA-12α-ol-3-one, 5-beta-cholanic acid 12-alpha-ol-3-one; UDCA, ursodeoxycholic acid; IsoALCA, isoallolithocholic acid; CDCA, chenodeoxycholic Acid; GDCA, glycodeoxycholic acid; CA|UCA, cholic acid|ursocholic acid; 5α-CA-3-one, 5-alpha-cholanic acid-3-one; MuroCA, murocholic acid; 3α-hydroxy-7 KLCA, 3-alpha-hydroxy-7 ketolithocholic acid; 12 DHCA|7 DHCA, 12-dehydrocholic acid|7-dehydrocholic acid; DCA-S, deoxycholic acid-3-sulphate; GCA, glycocholic acid; LCA-S, lithocholic acid 3-sulphate; CDCA-S, chenodeoxycholic acid-3-sulphate; CA-S, cholic acid-3-sulphate.

position. In each of the probiotic groups, the endpoint profiles of keystones organisms were rich in carbohydrate metabolizing bacteria (*Roseburia*, *Agathabacter* and *Megasphaera*) and BSH positive bacteria (*Bifidobacterium*, *Romboutsia* and *Christensella*) which could be linked to the changes in SCFAs and bile acids in these healthy subjects. For the P1 group, *Bifidobacterium* was identified as one of the endpoint keystone organisms despite the decreased abundance of this organism. The composition of the post supplementation keystone profile for P1 compares closely with that seen in other studies with the same formulation (Mullish *et al.*, 2024a).

There were no changes in faecal SCFA levels within the placebo nor any of the probiotic groups but faecal SCFA concentrations do not necessarily reflect circulating levels (Nogal *et al.*, 2023). A significant shift in the serum SCFA profile was observed in the P1 group alone. Univariate analyses of P1 revealed a significant increase in circulating levels of butyrate and the benefits linked with butyrate include anti-constipatory, anti-inflammatory, and neuroprotective effects (Xiong *et al.*,

2022). The P2 group was associated with elevated serum propionate levels, while P3 showed increased levels of both butyrate and propionate. Like butyrate, propionate is associated with a range of health-promoting effects (reviewed in Xiong *et al.* (2022)).

Another vital function of the gut microbiota is the biotransformation of bile acids, a process that plays a critical role in maintaining host health (Fogelson *et al.*, 2023). Although there were no changes in overall faecal bile acid profiles the P1 group had increases in the levels of two sulphated bile acids – lithocholic acid sulfate (LCA-S) and chenodeoxycholic acid sulfate (CDCA-S). Increases in sulphated secondary bile acids (glycolithocholic acid-3-sulfate) have been seen in germ-free mice supplemented with *Lactobacillus gasseri* LA39 (Hu *et al.*, 2023).

Bile acid sulphation is a detoxification process, mitigating the cytotoxic effects of bile acids and contributing to gut homeostasis and overall health (Alnouti, 2009; Camilleri, 2022). Sulphation of bile acids is mediated by sulfotransferase enzymes, which are found in gut microbes (Langford and Shah, 2024), but are primarily expressed in the host liver and intestinal epithelium (Chen *et al.*, 2003; Dew *et al.*, 1980; Teubner *et al.*, 2007). In rats, the expression of hepatic and intestinal sulfotransferases is strongly influenced by the gut microbiota (Meinl *et al.*, 2009) and in mice, supplementation with the VSL#3 probiotic has been shown to upregulate hepatic expression of sulfotransferase genes (Jena *et al.*, 2019). BSHs are involved in the deconjugation of bile acids and we have shown that the P1 formulation has BSH activity *in vitro* and *in vivo* (Michael *et al.*, 2017), and may promote deconjugation of bile acids in the intestine, thus enlarging the pool of free bile acids which are more readily available for subsequent host-mediated sulphation. *Lactobacillus rhamnosus GG*, present in P3, has some BSH activity *in vitro* (Hernández-Gómez *et al.*, 2021) and has been shown to promote bile acid deconjugation *in vivo* (Hu *et al.*, 2024).

Future research should include powered studies to confirm the observed microbial and metabolic changes and include faecal shotgun metagenomics and serum bile acid analysis to better understand the probiotic impact upon microbiota functionality. Laboratory-based studies could help to provide a more in-depth evaluation of the ability of the probiotics to influence SCFA absorption and metabolism, and to explore the potential role of probiotics in the process of bile acid sulphation by the intestinal epithelium.

In summary, this study demonstrates that different probiotic formulations impact the gut microbiota composition, SCFA absorption, and bile acid metabolism in healthy adults in a formulation specific manner. It also suggests that there are potential benefits for healthy individuals who choose to take probiotics regularly.

Supplementary materials

Data is available on https://doi.org/10.1163/18762891-bja00096 under Supplementary Materials.

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Authors' contribution

DRM: Conceptualisation, Visualisation, Writing (original draft), Supervision

DAJ: Data curation, Formal analysis, Validation, Visualisation

NC: Data curation, Formal analysis, Validation, Visualisation

IG: Methodology, Investigation

JAKM: Methodology, Investigation

NPD: Methodology, Data curation, Formal analysis, Investigation

MAVG: Methodology, Investigation

GR: Writing (review & editing)

SFP: Conceptualisation, Funding acquisition, Resource, Supervision, Writing (review & editing)

DW: Formal analysis, Writing (review & editing)

JRM: Conceptualisation, Writing (review & editing)

BHM: Conceptualisation, Formal analysis, Methodology, Writing (original draft)

Conflict of interest

BHM was the recipient of an NIHR Academic Clinical Lectureship (CL-2019-21-002) and now is the recipient of a Medical Research Council (MRC) Clinician Scientist award (MR/Z504002/1). The Division of Digestive Diseases at Imperial College London receives financial support from the National Institute of Health Research (NIHR) Imperial Biomedical Research Centre (BRC) based at Imperial College London and Imperial College Healthcare NHS Trust. Metabolomic assays were per-

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Data availability

Data is available from the corresponding author upon reasonable request. Sequence data generated during the current study has been deposited in the European Molecular Biology Laboratory (EMBL) nucleotide sequence database (https://www.ebi.ac.uk/ena) under accession number PRJEB83449.

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