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Development of a bio-functional fermented soy beverage supplemented with microbial exopolysaccharides and its effect on the human gut microbiome *in vitro*

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The gut microbiome plays a key role in modulating human health and well-being. Exopolysaccharides (EPS) produced by lactic acid bacteria (LAB) are emerging as novel polymers that could exert a prebiotic effect *via* modification of this microbiome. Thus, incorporation of EPS to enhance food functionality is of interest. This study investigates the impact of a fermented soy beverage, supplemented with EPS produced by *Leuconostoc mesenteroides* DSA_O or DSA_F, on the faecal microbiota as assessed using an *ex vivo* model of the human distal colon. The soy beverage (SM) was prepared by fermentation with *Lactiplantibacillus paraplantarum* GB3 followed by supplementation with EPS_O (SMO) or EPS_F (SMF). Faecal samples from healthy donors were inoculated into a faecal fermentation medium with SM, SMO and SMF and incubated anaerobically at 37 °C for 24 h. After incubation, samples were subjected to shotgun metagenomic and short-chain fatty acid (SCFA) analysis. SMO and SMF were more effective than SM at enhancing the alpha diversity of the faecal microbiota after 24 h incubation. In addition, SMO promoted the growth of the health-associated species *Bifidobacterium longum* and *Faecalibacterium prausnitzii*, the latter of which is considered a next-generation probiotic. Butyrate and propionate levels were higher in faecal samples fermented with SMO and SMF than in SM. Taken together, these preliminary results indicate a potential role of EPS produced by *Leuc. mesenteroides* to be used as a functional food ingredient, modulating the gut microbiome as well as increasing the levels of SCFAs.

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Introduction

The human body is characterised by a complex microbiome, and the gut microbiome has garnered considerable attention from researchers. The gut microbiome is a complex microbial ecosystem composed of approximately 100 trillion cells, including microorganisms from thousands of different species, some being regarded as beneficial, many as having neutral impact, but some with undesirable properties.[1] This complex microbial consortium has a significant effect on human health. Indeed, a suboptimal microbiome has been associated with a wide variety of conditions, such as autoimmune diseases, inflammation of the gut and bowel disorders, cardiometabolic dysfunction, and mental health issues.[2,3] The gut microbiome can also contribute to host health through different pathways, *i.e.*, vitamin and essential amino acid biosynthesis, as well as the generation of metabolites such as short-chain fatty acids (SCFAs) from dietary components.[4] Even when not directly contributing to health or disease, gut microbiome profiles can serve as biomarkers.[5]

Exopolysaccharides (EPS) are microbial extracellular biopolymers composed of repeating units of the same monosaccharide (homopolysaccharides; HoPs) or different monosaccharides (heteropolysaccharides).[6] In recent years, these polymers have garnered attention due to their potential health-promoting properties, such as antimicrobial, antioxidant, and prebiotic activity.[7] EPS can be produced by various microorganisms, including bacteria, yeasts, fungi, and microalgae. Among bacteria, lactic acid bacterium (LAB) species, such as *Leuconostoc mesenteroides*, are well known for their ability to produce EPS. Indeed, *Leuc. mesenteroides* can convert sucrose to glucose and fructose through the action of dextransucrase, resulting in the

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production of α-glucans like dextran, composed mainly of α-(1 → 6) linked glucose units, and β-fructans like levan, made of β-(2 → 6) linked fructose units.[8,9] The indigestible properties of EPS produced by *Leuc. mesenteroides* can make these compounds recalcitrant to gastrointestinal digestion,[10,11] increasing their availability to intestinal microbes and potentially enabling a role for EPS as a prebiotic,[12] *i.e.*, "substrates that are selectively utilised by host microorganisms conferring a health benefit".[13] Indeed, gut microbes such as *Bifidobacterium* spp., *Bacteroidota* spp., and *Faecalibacterium prausnitzii* can produce SCFAs from fermentable polysaccharides, such as EPS, which are resistant to enzymatic hydrolysis in the upper gastric tract and arrive intact in the colon.[14] SCFAs play a pivotal role in modulating host health and have been linked to many health benefits, such as reducing the incidence of cardiovascular diseases, diabetes, and colon cancer.[15]

While EPS produced *in situ* in foods have been studied in depth, the incorporation of EPS into foods as ingredients (*ex situ*) has received less attention but merits consideration. Moreover, to date, just a few studies have investigated the potential of EPS produced by *Leuc. mesenteroides* to modulate the gut microbiome. Notably, Miyamoto *et al.*[16] found that an indigestible dextran was metabolised to SCFAs (mainly acetate and propionate) by gut microbes, leading to a modulation of the gut microbiome. Over the past decade, EPS-producing LAB have been increasingly used for yogurt production due to the capacity of EPS to improve textural properties.[17] Worldwide, yogurt is regarded as a healthy food due to its nutrient-rich profile and the presence of compounds derived from the fermentation process.[18] However, plant-based alternatives have gained popularity as a healthy and sustainable substitute for yoghurt due to a demand for dairy alternatives, including, for example, amongst individuals with lactose intolerance. To date, the use of EPS in plant-based fermented products is limited to the production of EPS *in situ*, where production could improve rheological properties.[19] However, the addition of EPS *ex situ* as an ingredient is also worthy of consideration. For this reason, we aimed to investigate the effect of fermented soy beverages fortified with two different EPS (1% w/v) in a model of the colon microbiome. The EPS tested were previously characterised for their structure and *in vitro* bioactivities.[20,21] Thus, in this study, a fermented soy beverage was formulated using these microbial EPS as ingredients, and the impact of soy beverage fermentates on the human gut microbiome was studied using the micro-Matrix bioreactor platform as an *ex vivo* model of the human distal colon.

Materials and methods

Microorganisms, EPS, media and reagents

The strains of *Lactiplantibacillus plantarum* subsp. *plantarum* GB1, *Lb. plantarum* subsp. *argentoratensis* GB2, *Lb. paraplantarum* GB3 and *Lb. paraplantarum* GB4 were isolated from pickled cabbage, fermented cassava roots (fufu), and Cheddar cheese and were sourced from the Teagasc Food Research Centre collection (Cork, Fermoy, Ireland). Overnight cultures were prepared in de Man Rogosa Sharpe (MRS) broth at 30 °C in an anaerobic environment. Soy beverage (soya drink with zero sugar, Alpro, Gand, Belgium) was purchased from a local supermarket. Bacteriological agar, bacteriological peptone, Maximum Recovery Diluent (MRD), MRS broth, tryptone water, and yeast extract were purchased from Oxoid (Milan, Italy). Bile salts, calcium chloride hexahydrate, D-(+)-glucose, hemin, L-cysteine hydrochloride, magnesium sulphate heptahydrate, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, sodium bicarbonate, sodium chloride, trichloroacetic acid (TCA), Tris HCl, Tween 80, and vitamin K1 were purchased from Sigma Aldrich (Milan, Italy and Vale Road, Arklow, Ireland).

EPS extraction and purification

EPS_O and EPS_F were obtained as previously reported.[20,21] Briefly, 1 L of MRS-S broth (bacteriological peptone 10 g L^{-1}, Lab-Lemco powder 8 g L^{-1}, yeast extract 4 g L^{-1}, D-(+)-sucrose 20 g L^{-1}, dipotassium hydrogen phosphate 2 g L^{-1}, sodium acetate trihydrate 5 g L^{-1}, ammonium citrate tribasic 2 g L^{-1}, magnesium sulfate heptahydrate 0.2 g L^{-1}, manganese sulfate tetrahydrate 0.05 g L^{-1}, Tween 80 1 mL L^{-1}, pH adjusted to 6.2 ± 0.2 before sterilisation at 121 °C for 15 min) was inoculated at 1% (v/v) with the overnight culture of *Leuc. mesenteroides* (DSA_O[20] or DSA_F[21] for EPS_O and EPS_F, respectively) and incubated at 25 °C for 48 h under aerobic conditions. 4% (w/v) TCA was added to the broth and left for 30 min at 4 °C to precipitate proteins. Then, cells and proteins were separated by centrifugation at 13 000*g* for 10 min at 4 °C (Avanti Centrifuge™ J-25; Beckman Coulter, Indianapolis, Indiana, USA) and discarded. Three volumes of cold absolute ethanol were added to the supernatant and kept at 4 °C overnight to allow EPS precipitation. Then, EPS was washed and centrifuged at 13 000*g* for 10 min at 4 °C three times with three volumes of cold absolute ethanol. Then, the pellet was resuspended in Milli-Q water and dialysed using a Slide-A-Lyzer Dialysis Cassette (MW cut-off 10 000 g mol^{-1}) (Thermo Fisher Scientific) using Milli-Q water as the exchange buffer and following manufacturers' instructions. Then, EPS were freeze-dried using an Epsilon 2–4 LSCplus (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany), setting the vacuum pressure at 0.8 mBar and the condenser temperature at −45 °C. Freeze-dried EPS samples were stored under vacuum at room temperature.

Production of fermented soy beverages

For the preparation of the fermented soy beverages *Lb. plantarum* subsp. *plantarum* GB1, *Lb. plantarum* subsp. *argentoratensis* GB2, *Lb. paraplantarum* GB3 and *Lb. paraplantarum* GB4 were tested. Ninety-six mL of soy beverage was supplemented with 2 mL of a 25% w/v glucose solution (final concentration 0.5% w/v). Then, for each strain, soy beverage was inoculated with 2 mL of the overnight culture (prepared as described in the previous section) to reach a cell concentration of about 10^7 CFU per mL and fermented at 30 °C for 24 h under aerobic

conditions. At the beginning and the end of fermentation, viable plate counts on MRS agar and pH were evaluated. At the end of fermentation, EPS (EPS_F or EPS_O; final concentration 10 mg mL^{-1}) were added to the soy beverage (SM) and mixed until completely dissolved, to obtain SMF and SMO, respectively.

Donor recruitment and preparation of faecal fermentation media (FFM)

Faecal samples were collected from up to 10 healthy volunteers as part of the study APC108, which was approved by the Clinical Research Ethics Committee (CREC, Cork, Ireland). Faecal samples were pooled together to generate a frozen standardised inoculum (FSI)[22] that was used for all *ex vivo* human distal colon model experiments described herein. The faecal fermentation broth was composed of the following: tryptone water, 2.0 g L^{-1}; yeast extract, 2.0 g L^{-1}; NaCl, 0.1 g L^{-1}, dipotassium hydrogen phosphate, 0.04 g L^{-1}, potassium dihydrogen phosphate 0.04 g L^{-1}; calcium chloride hexahydrate, 0.01 g L^{-1}; magnesium sulphate heptahydrate, 0.01 g L^{-1}; sodium bicarbonate, 2.0 g L^{-1}; Tween 80, 2 mL L^{-1}; hemin, 0.05 g L^{-1}; vitamin K1, 10 μL L^{-1}; L-cysteine hydrochloride 2 g L^{-1}; and bile salts, 2 g L^{-1}, and was prepared as described by Fooks & Gibson.[23] Before each test, the faecal fermentation media (FFM) were prepared by mixing 12 mL of FSI with 80 mL of double-strength faecal fermentation broth under anaerobic conditions.

Ex vivo human distal colon model experiments

The micro-Matrix bioreactor platform (Applikon Biotechnology, Delft, the Netherlands) was used as an *ex vivo* model of the human distal colon. The experiments were conducted in sealed micro-Matrix cassettes, having 24 wells per cassette (1–10 mL), occupying, for each sample, two wells of the cassette. Samples were prepared by adding 3.5 mL of the FFM and 3.5 mL of fermented soy beverage (SM, SMF and SMO) in each well of the micro-Matrix cassette under anaerobic conditions. The control sample contained 3.5 mL of FFM mix added with 3.5 mL of sterile deionised water. The experiments were conducted as described by O'Donnell *et al.*[22] At 0 and 24 h, 1 mL of sample was taken in an anaerobic chamber from each well for DNA extraction and SCFA determination.

DNA extraction, preparation, and sequencing

Samples were centrifuged in a Micro20 centrifuge (Hettich Italia S.r.l., Milan, Italy) at 16 000*g* for 15 min, and DNA was extracted from faecal cell pellets with the QIAmp PowerFaecal Pro DNA Kit (Qiagen, Hilden, Germany) following the manufacturer's specifications. DNA samples were diluted 1 : 10 in 10 mM Tris-HCl, and the total DNA concentration was estimated using a Qubit 4.0 fluorimeter (Invitrogen, Carlsbad, CA). DNA was then diluted to 1 ng μL^{-1} with 10 mM Tris-HCl and used as a template for paired-end shotgun sequencing library preparations using the Illumina DNA Prep kit (Illumina Inc., San Diego, CA) following the manufacturer's instructions. After library preparation, DNA samples were normalised to 4 nM, and libraries were pooled together. The size of the library was determined with a 2100 Bioanalyzer (Agilent Technologies, Ireland) with a high-sensitivity DNA chip. Then the library was subjected to sequencing using the Illumina NextSeq 2000 (Illumina Inc.) platform using a high-output chemistry (2 × 150 bp) following the manufacturer's instructions.

Bioinformatics sequencing processing and analysis

Mean sequencing output was 4.5 M (±1.6 M) read pairs per sample. Read quality was evaluated using FastQC (v 0.11.8) and MultiQC (v 1.9). Low quality and artefactual sequences were removed using Trimmomatic (v 0.32; CROP:144, HEADCROP:20, ILLUMINACLIP:ref.fa:2:30:10:5, SLIDINGWINDOW:6:15, MINLEN:120), while contaminants were removed *via* alignment to the human ($GRCh_38$) and decoy (build 37d5) genomes using BowTie2[24] (v 2.4.4, default parameters), providing a mean filtered output of 3.8 M (±1.3 M) read pairs.[25,26] Taxonomic identities were assigned using Kraken2 (v 2.1.1; –confidence 0.1, –minimum-hit-groups 5, minimum-base-quality 20) and Bracken (v 2.2; sequence length 124, threshold of 50 counts).[27] Taxonomic diversity indexes were explored in R using the vegan library (version 2.6–4) for alpha diversity indices, NMDS (Bray–Curtis relative abundances), and PERMANOVA.[28] Figures were composed using the ggplot (version 3.4.2) and patchwork (version 1.1.2) libraries for R (version 4.1.2).[29]

Determination of SCFAs

1 mL samples from *ex vivo* distal colon fermentations were centrifuged at 13 000*g* for 15 min at 4 °C and filtered through 0.22 μm polyvinylidene fluoride (PVDF) membrane filters (Millipore, MA). For the analysis, an LC-4000 HPLC system (Jasco Europe, Cremella, Italy) equipped with an Aminex HPX—87H ion exclusion column (330 mm × 7.8 mm × 9 μm; Biorad, Hercules, CA), an AS-4050 autosampler, a 20 μL sample loop, and an oven set at 35 °C was used. Isocratic elution was performed at 0.6 mL min^{-1} with 0.005 M H_2SO_4 as the mobile phase.[30] SCFAs were detected using the MD-4010 PDA module (Jasco Europe, Cremella, Italy) set at 210 nm. Data analysis was performed using the ChromNav software (Jasco Europe), and the amount of each SCFA was determined by external calibration with standard solutions at different concentrations.

Statistical analysis

Statistical analysis was performed using R v.3.0.2 for Windows. All trials were carried out in duplicate, and values were presented as means ± SD. Differences among means ($p < 0.05$) were determined using one-way ANOVA, and Tukey's-HSD *post hoc* test was used to assess significant differences between means. For each *Lb. plantarum* strain, an independent *t*-test ($p < 0.05$) was used to compare the viable counts (Log CFU per mL) at the beginning (0 h) and at the end (24 h) of the soy beverage fermentation.

Results and discussion

Selection of suitable strains for soy beverage fermentation

In a preliminary step, the ability of four strains of *Lb. plantarum* and *Lb. paraplantarum* to grow and ferment the soy beverage was assessed using viable cell counts and pH measurements, respectively (Table 1). Glucose-supplemented soy beverage supported the growth of all strains tested. After 24 h of fermentation, all the tested strains successfully grew ($p < 0.05$), with an observed increase ranging from 1.25 to 1.93 Log CFU per mL.

However, the final cell concentration was not significantly different among the tested strains ($p > 0.05$). The ability of soy beverages to support the growth of other strains of *Lb. plantarum* and *Lb. paraplantarum* strains has been reported previously.[31–33]

The initial pH was 7.09 ± 0.08, and after 24 h of fermentation, pH values decreased by 2.02–2.85 units, owing to the ability of lactobacilli to ferment glucose. Among the tested strains, *Lb. paraplantarum* GB3 exhibited the most significant pH decrease after fermentation ($p < 0.05$). When choosing a LAB strain for plant-based fermentations, the ability to grow and ferment the substrate, leading to a decrease in the pH, is of high importance for maintaining product stability and shelf life, as well as product quality and sensory characteristics.[34] In this study, based on the pH decrease during fermentation, *Lb. paraplantarum* GB3 was selected for the formulation of an EPS-added fermented soy beverage.

Effect of EPS-containing fermented soy beverage on alpha diversity

Alpha diversity is a measure of the microbial diversity within an ecological community/sample.[35] In the colonic environment, a high alpha diversity is reported to lead to a more stable ecosystem, closely related to a healthy status.[36–38] To evaluate the impact of the addition of EPS to the fermented soy beverage on the composition of the model colonic microbiome, DNA was extracted from the samples at the beginning (T0) and the end of fermentation (T24) and subjected to shotgun metagenomic sequencing to determine the relative abundance of taxa in the microbial communities. The Shannon's index highlighted that following 24 h incubation, the alpha diversity of the gut microbiome decreased both in the control (FFM) and soy fermentate beverage samples without EPS (SM) (Fig. 1a). On the other hand, the presence of EPS_F (SMF) in the beverage slightly increased the alpha diversity. A very notable result was obtained with respect to sample SMO, where the addition of EPS_O, a high M_w dextran produced by *Leuc. mesenteroides* DSA_O, led to a much higher Shannon's index (Fig. 1a) at the end of incubation with respect to the other samples. The increased faecal community diversity in the presence of SMO was also highlighted by the Inverse Simpson index (Fig. 1b). This result differed from what has been observed by other authors with EPS produced by *Lacticaseibacillus paracasei* CIDCA 8339, CIDCA 83124 and GL1, which have been shown to reduce the microbiome diversity in *in vitro* models compared with control samples, possibly due to the inhibition of enteric microorganisms.[39,40] In this regard, it should be noted that both EPS added to soy milk fermentates in this study had been shown to possess antimicrobial activity against pathogenic bacteria.[20,21] Despite this, no reduction in diversity was observed in this study. It could be hypothesised that EPS were metabolised by many faecal microbial species present, which resulted in increased diversity compared to samples without added EPS.

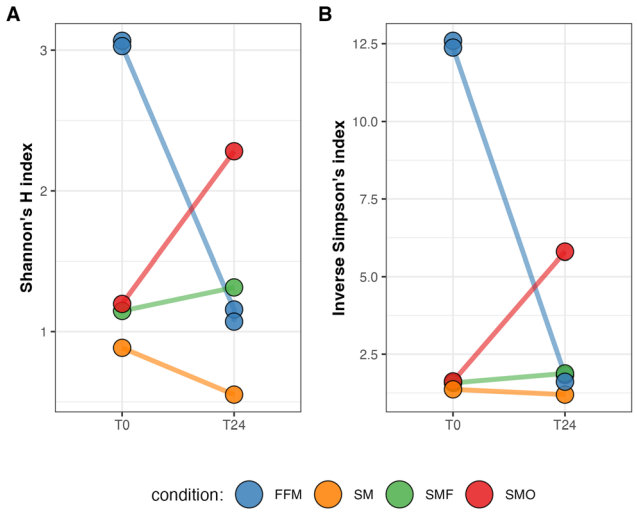

Fig. 1 Shannon's H index (A); inverse Simpson's index (B); within samples during *ex vivo* human distal colon model experiments.

Effect of EPS-containing fermented soy beverage on the gut microbial composition

The changes in the microbiota composition during colonic fermentation are shown in Fig. 2. At T0, the dominant phylum

Table 1 Viability of lactobacilli (Log CFU per mL) at 0 and 24 h and the final pH of fermented soy beverages. In the same column, means indicated by different lowercase letters are significantly different ($p < 0.05$ Tukey's test); in the same row, means indicated by different uppercase letters are significantly different ($p < 0.05$ independent samples *t*-test)

Strain	Log CFU per mL		pH
	0 h	24 h	24 h
Lb. plantarum GB1	$7.37^{aB} \pm 0.32$	$9.14^{aA} \pm 0.13$	$5.06^{a} \pm 0.25$
Lb. plantarum subsp. *argentoratensis* GB2	$7.08^{aB} \pm 0.43$	$8.70^{aA} \pm 0.28$	$4.87^{a} \pm 0.16$
Lb. paraplantarum GB3	$7.49^{aB} \pm 0.20$	$8.74^{aA} \pm 0.35$	$4.23^{b} \pm 0.11$
Lb. paraplantarum GB4	$6.84^{aB} \pm 0.51$	$8.77^{aA} \pm 0.10$	$4.72^{a} \pm 0.02$

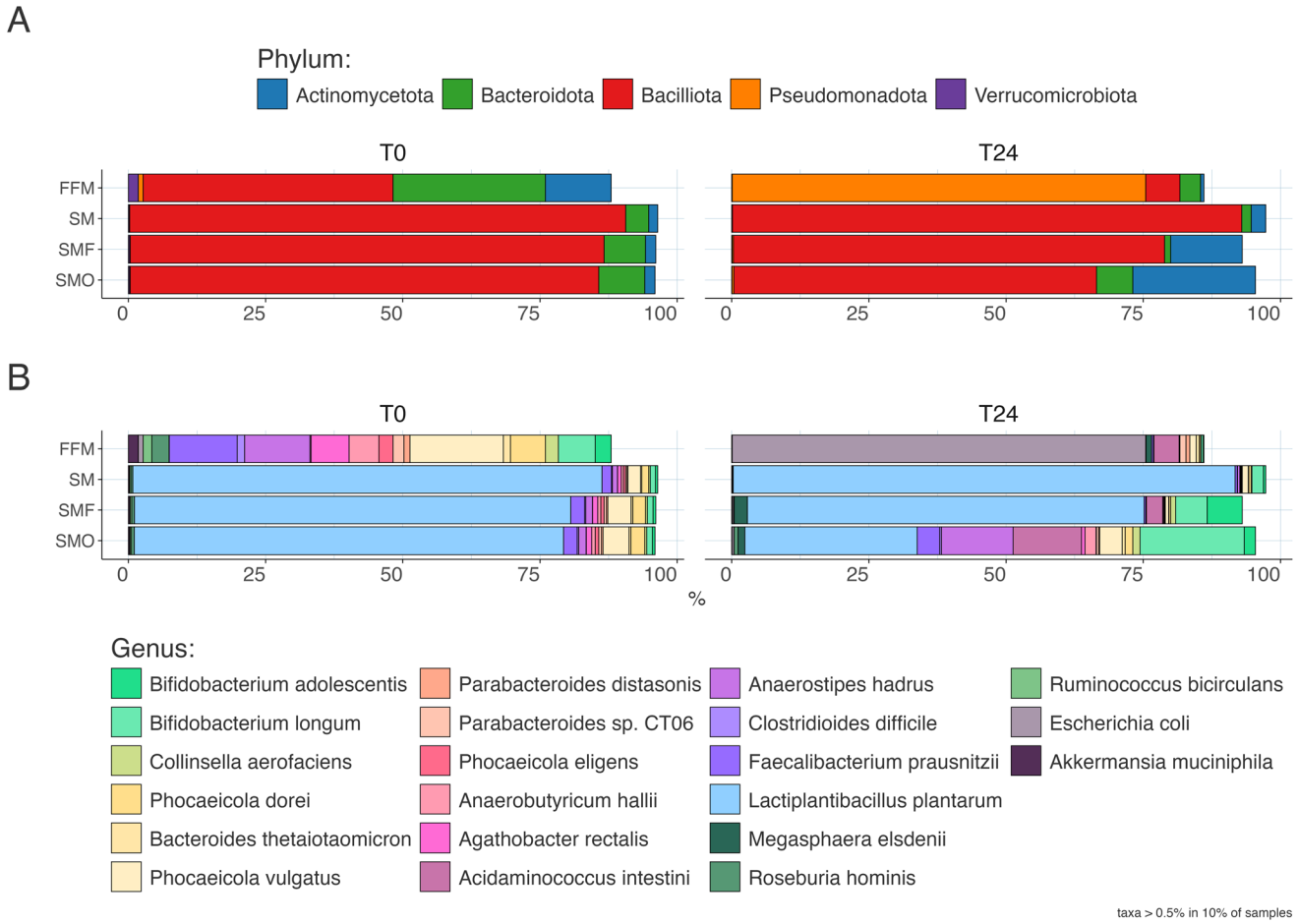

Fig. 2 Relative abundance (%) of bacterial taxonomic profiling at the phylum (A) and species (B) levels (taxa > 0.5% in 10% of samples).

was *Bacillota* in all samples (Fig. 2a). The much higher prevalence of *Bacillota* in SM, SMF and SMO than in FFM can likely be attributed to the presence of *Lb. paraplantarum* GB3 as a starter culture in the soy beverage fermentation. By T24, the dominant phylum of the FFM was identified as *Pseudomonadota*, whereas SM, SMF and SMO were characterised by higher proportions of *Bacillota.* The increase in the prevalence of *Pseudomonadota* in the control FFM could probably be related to the phenomenon termed "*Escherichia coli* bloom", which has been described very recently in *ex vivo* colon model experiments using the micro-Matrix bioreactor.[41] The species *E. coli* was indeed the prevalent one in the FFM control sample at T24 (Fig. 2b).

In the presence of fermented soy beverage, at T24, the prevalent phylum was again *Bacillota*, and in samples supplemented with EPS, the prevalence of *Actinomycetota* significantly increased. This increase can be attributed to the prevalence of bifidobacteria, particularly *Bifidobacterium adolescentis* and *Bifidobacterium longum* (Fig. 2b). Compared to SMF and SMO, the SM sample contained a smaller abundance of *Bifidobacterium* (2.5%); thus, the addition of both EPS_F and EPS_O strongly impacted the microbiome composition and stimulated the growth of bifidobacteria. This result is notable as the presence of bifidobacteria in the intestine is closely related to the maintenance of the structural integrity of the intestinal mucosa as well as the regulation of inflammatory cytokine production.[42]

The increased prevalence of *Bifidobacterium* in SMF indicates the utilization of EPS_F (*i.e.*, a mixture of dextran and levan) by *Bifidobacterium* spp. at the intestinal level and suggests prebiotic potential. Notably, the stimulation of the growth of bifidobacteria after levan-type polysaccharide supplementation has been reported in other studies.[43,44] The effect of stimulating bifidobacteria was also observed in the case of SMO. Moreover, the addition of EPS_O to the fermented soy beverage led to a higher proportion of other bacterial genera, such as *Bacteroides*, *Acidaminococcus*, *Anaerostipes*, *Anaerobutyricum, Faecalibacterium*, and *Phocaeicola*, compared to SM and SMF. These results indicated that both SMF and SMO could potentially modulate the gut microbiome composition. However, the effects on the specific microbes were different.

The microbiome following incubation with SMO was characterised by the presence of beneficial bacterial species such as *Bif. longum*, *Anaerostipes hadrus*, *F. prausnitzii*, *Anaerobutyricum hallii*, and *Bif. adolescentis*, whose

relative abundances were 18.75%, 13.1%, 4.36%, 2.5%, and 1.87%, respectively. These abundances were higher than the corresponding values at T0, indicating that SMO elicited an increase in these species upon fermentation. Some *Bifidobacterium* spp. have health-promoting attributes, and this study indicates that EPS could have a bifidogenic effect. Indeed, HoPS, both glucans and fructans, could be metabolised by bifidobacteria and stimulate their growth in the intestine.[43–45]

F. prausnitzii is an anaerobic bacterium commonly present in the large intestine. Its presence in the gut has been correlated with a decreased risk of inflammatory bowel disease and type-2 diabetes, which makes this species one of the most promising next-generation probiotics.[46] Indeed, the preservation and growth of *Faecalibacterium* spp. is likely to be a beneficial trait, and the presence of sufficient levels of this genus in the colon can potentially be used as a biomarker for colonic health with low levels associated with inflammatory conditions.[47] In addition, species such as *F. prausnitzii* are associated with butyrate production in the colon which can have many beneficial effects.[48] Although knowledge about its carbohydrate metabolism is limited, the presence of glycoside hydrolases was reported in *F. prausnitzii*, which might explain the effect of SMO on this species.[49] *Anaerostipes* spp. are human gut commensals able to produce butyrate from carbohydrate metabolism.[50] Moreover, their presence in the human gut has been linked to the health status of the host, decreasing the incidence of inflammatory bowel diseases and allergies.[51,52] While *A. hallii* is an important gut microbe due to its ability to produce SCFAs such as butyrate from glucose, this species does not utilize complex polysaccharides.[53,54] Thus, the effect of SMO might not be due to the direct metabolism of the EPS but potentially a result of some cross-feeding mechanism involving other bacterial species. For instance, from the degradation of oligosaccharides, bifidobacteria can generate L-lactate, which is converted into acetyl-CoA by *A. hallii*, thus leading to butyrate synthesis.[55] Other interactions between bifidobacteria and *A. hallii* led to the formation of SCFAs with potential benefits for the host.[56]

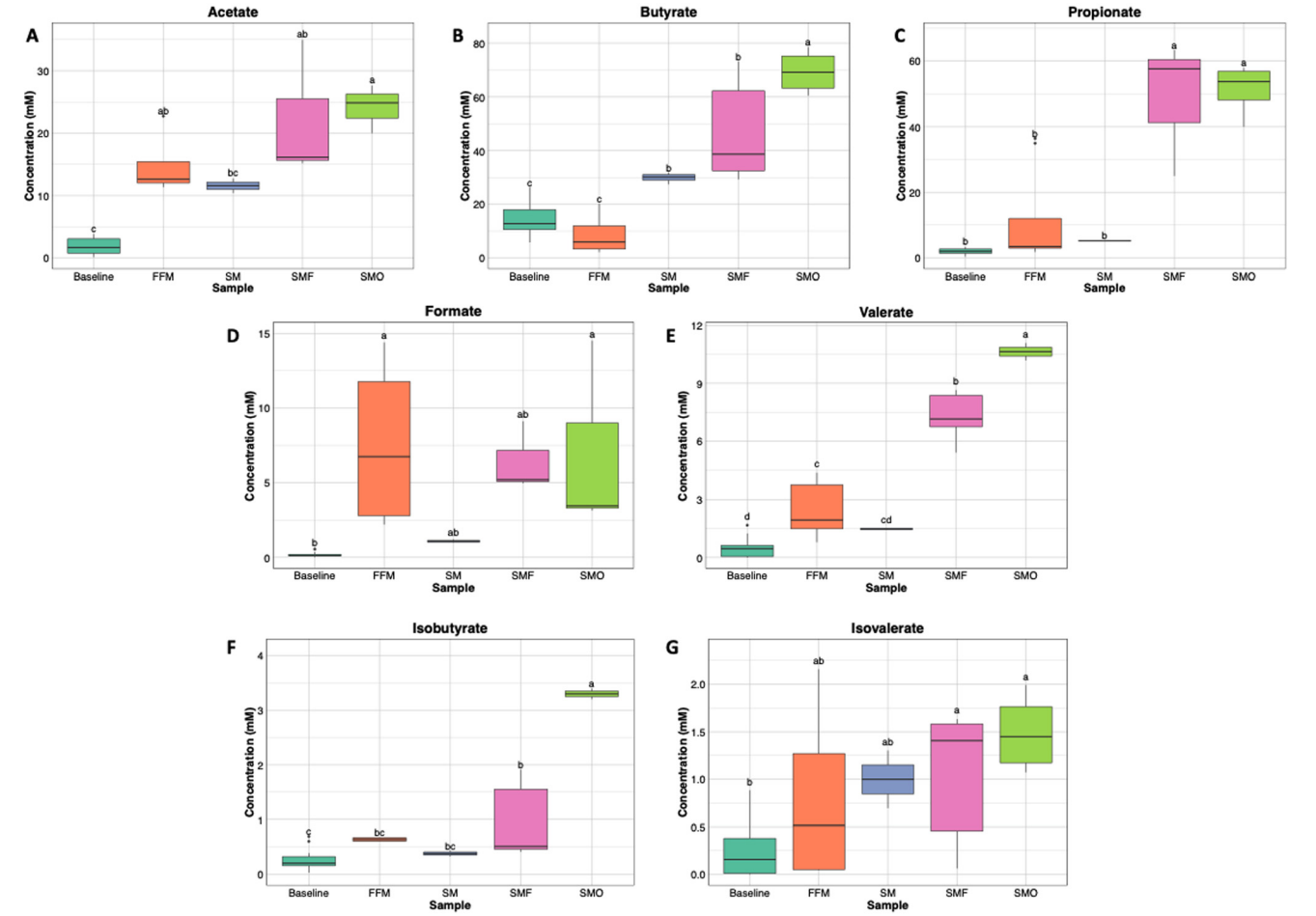

Fig. 3 Box plots of short-chain fatty acid (SCFA) concentrations (mM) at T0 (baseline) and at T24 of blank (FFM), soy milk fermentate (SM), soy milk fermentate supplemented with 10 mg mL^{-1} EPS_F (SMF), and soymilk fermentate supplemented with 10 mg mL^{-1} with EPS_O (SMO). (A) Acetate, (B) butyrate, (C) propionate, (D) formate, (E) valerate, (F) isobutyrate and (G) isovalerate. Different letters indicate significant differences between samples (Tukey's *post hoc* test; $p < 0.05$).

Effect of EPS-containing fermented soy beverage on SCFA production

SCFAs are the primary metabolic products of anaerobic fermentation by the human gut microbiome. These compounds are absorbed by the gut mucosa, leading to relevant impacts on the host physiology.[57,58] The amounts of SCFAs such as acetate, butyrate, propionate, formate, valerate, isovalerate and isobutyrate produced in the *ex vivo* colonic model were determined using HPLC in this study (Fig. 3).

SMO samples showed increased ($p < 0.05$) production of acetate (24.16 ± 3.85 mM), butyrate (69.32 ± 8.41 mM), propionate (51.34 ± 8.13 mM), valerate (10.63 ± 0.64 mM), and isobutyrate (3.30 ± 0.14 mM) with respect to SM at T24, whereas, compared to SM, at T24, SMF led to a higher production of propionate (57.64 ± 30.09 mM) and valerate (7.27 ± 1.31 mM). These results are consistent with those of the microbial changes reported in Fig. 2. SMO may have had a functional role in increasing the production of SCFAs through the enrichment of *F. prausnitzii*, *Bacteroides* spp., and *Bifidobacterium* spp., indicating a strong prebiotic potential. Indeed, compared to the other samples tested, both SMF and SMO stimulated the growth of *Bifidobacterium* spp., particularly that of *Bif. adolescentis* and *Bif. longum* (Fig. 2b). *Bifidobacterium* is known for its positive outcomes in host health and plays a key role in fermenting insoluble polysaccharides, such as dextran[59] and levan[43] and producing SCFAs.[60,61] It should also be highlighted that in SMO, the relative abundance of *F. prausnitzii* increased compared to the other samples studied. This species is considered one of the most important butyrate producers in the gut microbiome.[62] SMO samples were also characterised by a higher abundance of *Phocaeicola vulgatus*, which is recognised as a butyrate and propionate producer and has been suggested as a potential probiotic.[63,64]

In conclusion, both SMF and SMO were characterised by higher SCFA production relative to SM, and this correlated with changes in the microbiome composition observed, suggesting a role of the microbiome in SCFA production. However, although both EPS could stimulate the growth of beneficial bacteria in the faecal microbiota, this effect depended on the EPS tested.

To the best of our knowledge, this is the first study investigating the effect of EPS as a functional ingredient in a food matrix in the modulation of the gut microbiome. Indeed, the effect of EPS from LAB on the gut microbiome has been investigated only testing EPS alone.[10,45,65] Thus, our preliminary results indicate the possible use of EPS as functional ingredients in a food matrix which the human fecal microbiota could use to produce high amounts of SCFAs, important metabolites with proven beneficial effects on human health.[57]

Conclusions

In this study, the effects of EPS-supplemented fermented soy drink on the human faecal microbiome were evaluated using an *ex vivo* distal colon model. The results indicate that both types of EPS samples investigated increased the diversity within the microbial faecal community, with a more marked effect obtained with SMO which was supplemented with EPS_O, a dextran produced by *Leuc. mesenteroides* DSA_O. Moreover, SMO was shown to be bifidogenic and stimulated the growth of bacterial species whose presence is related to host health. The addition of SMF elicited a slight increase in *Bif. longum* and *Bif. adolescentis* abundance when compared to the SM control without added EPS. The different microbiome responses may be attributed to the different structural characteristics of the EPS used, which could have potentially influenced their fermentability and selective utilization by the gut microbiome. Additionally, both SMO and SMF increased the production of specific SCFAs, which was correlated to the changes in the microbiome composition observed, further supporting their role in modulating the gut environment. These findings provide promising preliminary evidence supporting the prebiotic potential of EPS derived from *Leuc. mesenteroides*, which enhanced microbial diversity and stimulated the production of beneficial metabolites, and could serve as a solid foundation for future research on this topic. However, future research is needed to validate these findings through larger, replicated studies and to investigate the effects of EPS on other health-related aspects, such as inflammatory markers, mucosal integrity assessments, or metabolomic profiling.

Author contributions

Giulia Bisson: investigation, formal analysis, writing – original draft preparation, and writing – review & editing; Clara Comuzzi: investigation and writing – review & editing; Jamie A. FitzGerald: formal analysis and writing – review & editing; Arghya Mukherjee: data curation and writing – review & editing; Niccolò Renoldi: investigation and writing – review & editing; Nadia Innocente: resources, supervision, and writing – review & editing; Tom Beresford: supervision and writing – review & editing; Harsh Mathur: supervision, investigation, and writing – review & editing; Paul D. Cotter: supervision, funding acquisition, and writing – review & editing; Marilena Marino: conceptualization, resources, supervision, funding acquisition, and writing – review & editing.

Conflicts of interest

G. B., C. C., J. A. F., A. M., N. R., N. I., T. B., H. M., and M. M. declare no competing interests. Research in P. D. C.'s laboratory has been funded by Friesland Campina, PrecisionBiotics Group, PepsiCo and Danone. P. D. C. has received support from PepsiCo, Yakult and H&H to attend/present at scientific meetings/conferences and is the CTO and a co-founder of SeqBiome Ltd.

Ethical statement

The study was approved by the Clinical Research Ethics Committee (CREC, Cork, Ireland; project number APC 108). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their informed consent to participate in this study.

Data availability

Raw shotgun sequencing data have been deposited at the European Nucleotide Archive (ENA) under accession numbers ERA31158192 and ERA31157780. All other data are available upon request.

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