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The impact of Symprove™ multi-strain probiotic on enterotoxigenic *Escherichia coli*- or antibiotic-induced gut microbiome dysbiosis using high-throughput *in vitro* screening

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ABSTRACT

The gut microbiome plays a significant role in host physiology, both in health and disease. Assessment of changes in microbial metabolites beyond short-chain fatty acids (SCFAs) following probiotic supplementation may identify additional metabolic pathways that are activated or suppressed in response to probiotics. This study assessed changes in microbial metabolites in healthy and dysbiosed microbiomes following supplementation with Symprove™, a multistrain probiotic, using the Colon-on-a-plate® miniaturized short-term batch fermentation system with a fractional factorial design. The fecal microbiome from 10 healthy human donors was evaluated under healthy and dysbiosed (enterotoxigenic *Escherichia coli* infection and/or low-, medium-, or high-dose antibiotics) conditions. Samples were supplemented with Symprove™ or water (control) and evaluated for microbial metabolites at 24 h and 48 h using untargeted metabolic fingerprinting, capillary gas chromatography, and targeted metabolic profiling. Favorable impacts were observed with Symprove™ supplementation across the different antibiotic doses. SCFA levels (acetate, propionate, butyrate) were significantly increased and levels of branched SCFAs were significantly decreased with Symprove™ supplementation *versus* control in both the healthy and dysbiosed populations. Significant increases and decreases in several other microbial metabolites were also observed with Symprove™, many of which could be considered to have beneficial effects on intestinal inflammation, intestinal barrier health, and the gut-brain axis. Symprove™ supplementation significantly affected microbial metabolism, with many of the observed changes being considered positive for human health. Importantly, these benefits were shown not only in healthy fecal microbiomes, but also in fecal microbiomes with *in vitro* antibiotic-induced dysbiosis, showing therapeutic potential.

1. Introduction

Probiotics, defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014), have been gaining in popularity. Research demonstrating the health benefits of probiotic supplementation has been expanding, further solidifying the importance of supporting gut microbiome health as a tool to improve human health (Gul & Durante-Mangoni, 2024). Studies of the gut microbiome have clearly demonstrated that microbial metabolites, specifically short-chain fatty acids (SCFAs), are involved in many of the observed beneficial effects of probiotic supplementation. SCFAs, mainly acetate, propionate, and butyrate, have been shown to support intestinal epithelial cell health and survival, to have immunomodulatory effects, and to protect the intestinal barrier (Bidell et al., 2022; Ma et al., 2022). In contrast, gut microbiome dysbiosis has been associated with many diseases, including gastrointestinal conditions such as inflammatory bowel disease (IBD) and neurological conditions such as Parkinson's disease (PD) (Bi et al., 2022; Huang, Chau, et al.,

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2023; Quaglio et al., 2022), strengthening the importance of gut health for overall human wellbeing.

Symprove™ (Symprove Ltd., Farnham, Surrey UK) is a liquid, non-dairy, water-based multistrain probiotic that contains *Lacticaseibacillus acidophilus* (former *Lactobacillus acidophilus*) NCIMB 30175, *Lactiplantibacillus plantarum* (former *Lactobacillus plantarum*) NCIMB 30173, *Lacticaseibacillus rhamnosus* (former *Lactobacillus rhamnosus*) NCIMB 30174, and *Enterococcus faecium* NCIMB 30176. The live bacteria in Symprove™ were shown to survive in a simulated gastrointestinal environment, indicating that they can survive the harsh conditions of gastrointestinal transit and reach the colon alive (Fredua-Agyeman & Gaisford, 2015). Studies using *in vitro* models with the gut microbiome of patients with IBD, liver cirrhosis, and PD have demonstrated that Symprove™ supplementation resulted in changes in the bacterial composition of the gut microbiome, increased SCFA production, immunomodulatory effects (*i.e.*, an increase in anti-inflammatory cytokines and a decrease in inflammatory cytokines), improvement of the epithelial tight junction integrity, and faster wound healing compared with control (Ghyselinck et al., 2020; Ghyselinck et al., 2021; Moens et al., 2022). Studies in humans suggest that Symprove™ supplementation may improve gut-related symptoms in IBS and PD, and reduce intestinal inflammation in patients with ulcerative colitis (Bjarnason et al., 2019).

Short-term batch fermentations facilitate an understanding of the detailed mechanistic interplay between the gut microbiome and test products, such as probiotics (Goya-Jorge et al., 2024; Moens, Duysburgh, et al., 2019). The Colon-on-a-plate® system is a validated system that utilizes deep well plates to offer a miniaturized version of short-term batch fermentation (Perreau et al., 2023). This allows for the simultaneous assessment of multiple test conditions (*e.g.*, testing multiple treatments and/or fecal samples from multiple individuals). Applying a fractional factorial design to this system allows for the testing of a high number of experimental conditions with a minimum number of experimental runs. In this study, these methodologies were employed to evaluate the robustness and effectiveness of Symprove™ supplementation in multiple fecal donors and over a wide variety of conditions, including a healthy fecal microbiome, fecal microbiomes with *in vitro* antibiotic-induced dysbiosis using low, medium, and high doses of antibiotics, and in the presence of enterotoxigenic *Escherichia coli* (ETEC) infection.

Studies of microbial metabolism are often limited to evaluating levels of SCFAs, lactate, branched SCFAs, and ammonium. While evaluating the effects of probiotics on these metabolites is very useful, expansion of research to identify other microbial metabolites that are affected by probiotic supplementation may provide valuable mechanistic insights into how probiotics improve human health and affect general metabolism. Utilizing metabolic fingerprinting may reveal information not reflected in SCFA changes. Thus, the complementary techniques of Laser-Assisted Rapid Evaporative Ionization Mass Spectrometry (LA-REIMS) and liquid chromatography mass spectrometry (LC-MS) were employed in this study, as assessed through the Colon-on-a-plate® *in vitro* simulation. This study evaluated changes in microbial metabolism following supplementation with Symprove™, a multistrain probiotic, under multiple conditions which have not been assessed in previous research though could have a strong impact on human health, including ETEC- and antibiotic-induced microbiome dysbiosis.

2. Materials and methods

2.1. Experimental design

The overall experimental design is illustrated in Fig. 1. Fecal samples from ten healthy donors were evaluated under multiple conditions to assess the fecal microbiome response to Symprove™ supplementation or under control conditions (*i.e.*, no Symprove™). Fecal samples were evaluated with or without ETEC infection and with or without antibiotic pre-incubation to induce dysbiosis. The antibiotic pre-incubation included a total of 15 conditions, with each condition involving pre-incubation with one of five antibiotics (vancomycin, clindamycin, tetracycline, chloramphenicol, gentamicin) at a low, medium, or high dose, prior to the initiation of the Colon-on-a-plate® experiments. The used antibiotics and their applied doses are detailed in Table 1. The

Table 1
Pre-incubation antibiotic conditions used to create dysbiosis in the microbial communities of fresh fecal samples.

Antibiotic	Dose (ppm)			Reference
	Low	Medium	High	
Clindamycin	0.68	3.39	33.9	Murphy et al., 2024
Vancomycin	2.5	12.5	125	Patel et al., 2024
Tetracycline	16.7	83.3	833	Shutter & Akhondi, 2023
Chloramphenicol	4.8	24.0	240	Oong & Tadi, 2023
Gentamicin	11.6	58.0	580	Chaves & Tadi, 2023

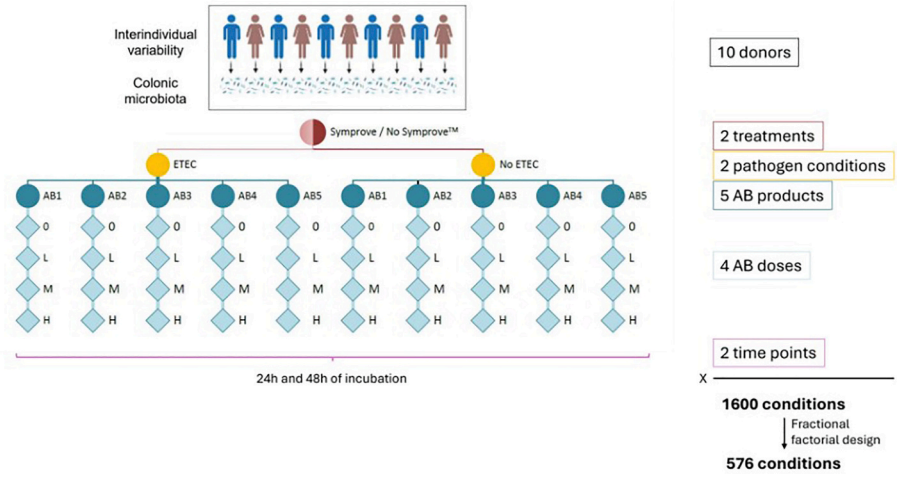

Fig. 1. Study design. Individual fecal samples were collected from 10 healthy donors and used in Colon-on-a-plate® experiments with or without antibiotic pre-incubation (one of five antibiotics at a low, medium, or high concentration). Samples were either infected with ETEC or control media (no ETEC). Under each described condition, samples were supplemented with Symprove™ or control media (no Symprove™). Finally, each condition was set up twice, to allow for evaluation at two timepoints (24 h or 48 h). 0: no antibiotic addition; AB1–5: antibiotic 1–5; ETEC: enterotoxigenic *Escherichia coli*; H: high antibiotic dose; L: low antibiotic dose; M: medium antibiotic dose.

highest antibiotic concentrations were selected based on previously reported fecal levels and standard prescribing guidelines. Medium and low concentrations were defined as tenfold and fiftyfold dilutions of the highest concentrations, respectively. Vancomycin and clindamycin were administered at maximum concentrations of 125 mg/L and 33.9 mg/L, respectively (Table 1). For tetracycline, chloramphenicol, and gentamicin, the highest concentrations were calculated based on recommended oral doses, assuming no intestinal absorption (*e.g.*, capsule formulation), a standard adult body weight of 70 kg, and a colonic volume of 600 mL. This approach yielded estimated *in vitro* concentrations of 833 mg/L for tetracycline, 240 mg/L for chloramphenicol, and 580 mg/L for gentamicin (Table 1). Samples were evaluated at both 24 h and 48 h. In total, 576 conditions were included. These conditions were determined by an optimized fractional factorial experimental design. Specifically, the first step included construction of a fractional factorial design to allow for the estimation of all important effects, including all two-way interaction effects and a few three-way interaction effects. These calculations were performed with JMP® version 15.2.0. Subsequently, this design was further optimized for distributing the donors over the 24-well plates such that the plate-effects did not confound any other effect. The process also aimed to minimize the average covariances between the statistical model parameter estimators; this corresponds to maximizing the overall power of the statistical tests. This optimization step was performed with R statistical software version 4.0.4 (The R Foundation for Statistical Computing, Vienna, Austria). For each experimental condition, microbial metabolism was evaluated using both untargeted LA-REIMS fingerprinting to provide a holistic mapping of metabolic alterations and targeted SCFA analysis to provide insights into these key fermentation metabolites. Eventually, a strategy of targeted metabolic profiling was conducted on a subset of the 576 conditions, to provide detailed insights about changes in specific, identified, metabolites. All 576 conditions were tested in single technical replicate, as the design included already ten biological replicates per experimental test condition (*i.e.*, ten donors per test condition).

2.2. Test product

Symprove™ was supplied by Symprove Ltd. (Farnham, UK). Symprove™ production involves a patented, controlled fermentation process, using a gluten-free barley extract as a nutrient base for four specified bacterial strains (*Lacticaseibacillus acidophilus* (former *Lactobacillus acidophilus*) NCIMB 30175, *Lactiplantibacillus plantarum* (former *Lactobacillus plantarum*) NCIMB 30173, *Lacticaseibacillus rhamnosus* (former *Lactobacillus rhamnosus*) NCIMB 30174, and *Enterococcus faecium* NCIMB 30176) that multiply over 21 h, developing acid resistance throughout fermentation, and resulting in a water-based, live probiotic with a stable shelf-life for four months. Each mL of Symprove™ contains approximately $\sim 2 \times 10^8$ CFU of live bacteria (Dodoo et al., 2019).

2.3. Fecal samples and antibiotic pre-incubation

Fecal samples were collected from 10 healthy donors. All fecal donors were aged between 20 and 50 years (donor A: M, 26y; donor B: F, 40y; donor C: M, 28y; donor D: M, 30y; donor E: F, 30y; donor F: F, 21y; donor G: M, 31y; donor H: F, 24y; donor I: M, 35y; donor J: M, 34y), had a healthy BMI between 18.5 and 24.9, had no history of chronic disease, consumed a Western diet, and had no history of antibiotic use during the 6 months prior to their stool sample donation. Health status of the donors was obtained through a questionnaire. Fecal materials were collected and used as approved by the Ethics Committee of the University Hospital Ghent (reference number ONZ-2022-0267; approved on 29 July 2022). All participants provided informed consent for the use of the fecal materials.

Fecal samples, either fresh or following an antibiotic pre-incubation step, were cryopreserved prior to conducting the experiment. Under anaerobic conditions (Jacomex G-T4, Jacomex, Dagneux, France; gas mixture $N_2/H_2/CO_2$ at a ratio of 90/5/5) the fecal samples were suspended in anaerobic phosphate-buffered saline (Marsaux et al., 2020) and mixed with an in-house cryoprotectant, modified from the cryoprotectant developed by Hoefman et al. (Hoefman et al., 2013) including trehalose, tryptone soy broth, NaCl, cysteine-HCl, and DMSO as main components. The suspensions were sparged with nitrogen gas until anaerobiosis was reached then flash frozen and stored at −80 °C in an anaerobic atmosphere. Aliquots were defrosted and immediately added to a reactor at the start of an experiment.

Fecal samples that underwent a pre-incubation step prior to cryopreservation were treated as follows. The fresh fecal samples were added to reactors containing a background of nutritional medium (fiber-enriched PD01; ProDigest, Belgium) and one of five antibiotic agents (clindamycin, vancomycin, tetracycline, chloramphenicol, gentamicin) at a low, medium, or high concentration as described in Table 1. After 24 h incubation under strictly anaerobic atmosphere, continuous mild shaking (90 rpm) and 37 °C, the obtained antibiotic-induced dysbiotic fecal microbial communities were harvested using mild centrifugation (1250 rcf for 2 min) and then cryopreserved as described above. Highly water-soluble antibiotics were selected for use in this study to ensure that the antibiotics were efficiently removed with the supernatant after centrifugation, together with the nutritional medium.

2.4. In vitro Colon-on-a-plate® simulation

At the start of the experiment, individual wells of the Colon-on-a-plate® (24-well plates; Thomson, Oceanside, Canada) were filled with a nutritional medium (nutritional blend PD01, fiber-depleted; ProDigest, Ghent, Belgium). A single dose of 0.7 mL of the test product (Symprove™), corresponding with $\sim 1.4 \times 10^9$ CFU, or water (control) was added to the respective wells. Designated wells were infected with ETEC, by spiking 2.9 % (*v*/v) of a 16 h ETEC H10407 culture grown in nutrient broth (13 g/L nutrient broth CM0001B; 5.2 g/L K_2HPO_4; 16.3 g/L KH_2PO_4), after a washing step, or an equal volume of saline. Finally, 10 % (v/v) of fecal inoculum, with or without antibiotic pre-incubation as described above, was added. The total volume in each well was 7 mL. Samples were incubated at 37 °C under an anaerobic atmosphere for 24 h or 48 h.

2.5. Metabolite analysis

2.5.1. Untargeted metabolic fingerprinting

Untargeted metabolic fingerprints were obtained using the validated LA-REIMS platform (Plekhova et al., 2021; Van Meulebroek et al., 2020). This platform consisted of an MID infrared laser system (Opolette™ HE2940, OPOTEK, LLC, Carlsbad, California, USA) and a Xevo™ G2-XS Quadrupole Time-of-Flight mass spectrometer (Waters Corporation, Wilmslow, UK). The laser system was used for laser ablation of samples and associated metabolite release, and the mass spectrometer was used for mass analysis. The 50–1200 Da *m/z*-scan range was targeted in negative ionization mode and pre-treatment of the *in vitro* fluid samples was not required. Each measurement was done in a single repetition, for which 100 µL per sample was transferred into the well of a 96-well plate. The entire set of 576 samples was subjected to LA-REIMS analysis. Appropriate quality control measures at the level of sample randomization and (external and internal) quality control samples were taken.

2.5.2. SCFAs

Using capillary gas chromatography coupled with a flame ionization detector as described by De Weirdt et al. (De Weirdt et al., 2010), levels of SCFA (acetate, propionate, and butyrate) and branched SCFA production were measured. Each measurement was done in a single repetition. The entire set of 576 samples was subjected to SCFA and branched SCFA analysis.

2.5.3. Targeted metabolic profiling

Ultra-high performance liquid chromatography-high-resolution mass spectrometry (UHPLC-HRMS) was used to measure a targeted metabolite profile containing more than 400 polar to medium-polar metabolites (De Paepe et al., 2018; Vanden Bussche et al., 2015). Chromatographic separation was performed using a Vanquish quaternary pumping system (ThermoFisher Scientific, California, USA), equipped with an Acquity HSS T3 C18 column (1.8 µm, 150 × 2.1 mm; Waters Corporation, Manchester, UK). A binary solvent system consisting of acidified (0.1 % formic acid) ultrapure water and acetonitrile was used at a constant flow rate and by applying a gradient profile. A Q-Exactive™ standalone bench top quadrupole-Orbitrap high-resolution mass spectrometer (ThermoFisher Scientific, California, USA), preceded by heated electrospray ionization (HESI-II source) in polarity switching mode, was used for detection. A set of 72 samples were subjected to UHPLC-HRMS analysis. This set of samples was selected based on the data of the LA-REIMS metabolic fingerprinting and the SCFAs and branched SCFAs, and were considered to cover the most interesting experimental condition to gain information related to the effects of Symprove™ supplementation. More specifically, samples obtained after 24 h incubation, without ETEC infection, without antibiotic pre-treatment or with antibiotic pre-treatment with clindamycin, vancomycin, or tetracycline at the low and high doses, and with/without Symprove™ supplementation were selected. In general, these selections are representative of sample populations without dysbiosis, with a low degree of dysbiosis, and with a high degree of dysbiosis.

2.6. Statistical analysis

Statistical comparisons between the different experimental test conditions were conducted including ten biological replicate measurements per condition, corresponding to one measurement per donor.

Considering the complex structure of the experimental setup (*i.e.*, the fractional factorial design), an R-based pipeline was utilized for automatic data processing (R version 4.1.2). In this context, absolute concentration values were used when evaluating the impact of specific experimental conditions on SCFA dynamics, and principal components (*i.e.*, linear combinations of the measured metabolome constituents) were used when LA-REIMS metabolic perturbations were assessed. To reduce the dimensionality of the multivariate nature of the LA-REIMS metabolomics data, the data were replaced by their first four principal components. The number of principal components was selected by means of the scree plot of the eigenvalues of the Principal Component Analysis (PCA-X); this number was set at four for both the 24 h and 48 h data. To facilitate interpretation, standard PCA score plots and associated Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) models were constructed as well (for the main experimental parameter, with *vs* without Symprove™ supplementation). It should be noted that additional information about the fractional factorial design was not taken into consideration with this standard multivariate analysis. Data were pre-processed by unit variance scaling and log-transformation to standardize the range of normalized abundances and to induce normality, respectively.

For the LC-MS metabolic profiles, univariate statistics were employed to evaluate the alterations at the metabolite-specific level in relation to the main experimental parameter (with *vs* without Symprove™ supplementation) and in the included subpopulations (without dysbiosis, low-degree dysbiosis, and high-degree dysbiosis). Relative abundances of metabolites that were considered below the limit of quantification (LOQ) were replaced by half of the lowest detected relative abundance value that was considered above the LOQ. Statistical analysis was performed using R (version 4.5.0) with the nlme (version 3.1–168) and emmeans (version 1.11.1) packages. For each subpopulation, linear mixed linear mixed-effects models were fitted to metabolite abundance with donors included as a random intercept. Pairwise treatment comparisons used estimated marginal means for unbalanced repeated measures. Raw *p*-values were adjusted using the Benjamini-Hochberg method to control the False Discovery Rate, with a significance threshold set at 5 %.

3. Results

3.1. Impact of Symprove™ supplementation on general bacterial metabolism

In donors under healthy conditions (*i.e.*, no ETEC infection and no antibiotic use), LA-REIMS data demonstrated relevant changes in microbial metabolism which was most pronounced at 24 h (Fig. 2A) but was also present at 48 h (Fig. S1A). OPLS-DA modelling yielded valid models at both the 24 h timepoint ($Q^2Y = 0.923$, valid permutation testing, and CV-ANOVA *p*-value <0.01, total of 5 principal components) (the analysis based on the first four principal components also resulted in a significant result [Holm-Bonferroni-corrected *p*-value <0.0001 for the first principal component]) and 48 h timepoint ($Q^2Y = 0.730$; valid permutation testing, and CV-ANOVA *p*-value <0.001, total of 8 principal components) (the analysis based on the first four principal components also resulted in a significant result [Holm-Bonferroni-corrected *p*-value <0.01 for the fourth principal component]), thus demonstrating significant metabolic shifts following Symprove™ supplementation.

SCFAs and branched SCFAs showed significant changes upon Symprove™ supplementation in donors under healthy conditions (*i.e.,* no ETEC infection and no antibiotic use). More specifically, Symprove™ supplementation resulted in a significant increase in acetate production at 24 h and 48 h ($p < 0.001$ for both), a significant increase in propionate production at 24 h ($p < 0.001$), and a significant increase in butyrate

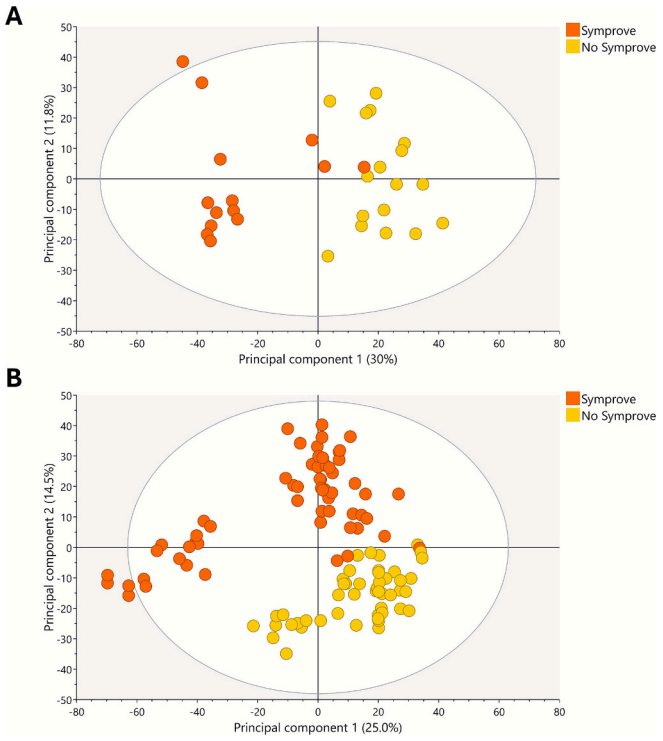

Fig. 2. PCA-X plots based on the LA-REIMS data for the healthy population (no ETEC infection and no antibiotic use) (A) and for the dysbiosed population (no ETEC infection and following antibiotic use) (B). LA-REIMS was used to measure the metabolic activity of colonic fermentations collected after 24 h incubation with Symprove™ or untreated control (water) in the Colon-on-a-plate® model using fecal samples from 10 healthy human donors. ETEC: enterotoxigenic *Escherichia coli*; LA-REIMS: Laser-Assisted Rapid Evaporative Ionization Mass Spectrometry; OPLS-DA: orthogonal partial least squares discriminant analysis; PCA-X: unsupervised principal component analysis.

production at 24 h ($p = 0.007$) and 48 h ($p < 0.001$) (Table 2). Levels of branched SCFAs were significantly decreased with Symprove™ supplementation compared with control at 24 h ($p < 0.001$) and 48 h ($p = 0.013$). Similar changes in SCFA/branched SCFA production were observed under healthy conditions with ETEC infection (Table S1).

Under dysbiotic conditions (following antibiotic use, no ETEC infection), LA-REIMS data demonstrated relevant changes in microbial metabolism which was most pronounced at 24 h (Fig. 2B) but was also present at 48 h (Fig. S1B). OPLS-DA modelling yielded valid models at the 24 h timepoint ($Q^2Y = 0.941$, valid permutation testing, and CV-ANOVA p-value of 0, total of 5 principal components) (the analysis based on the first four principal components also resulted in a significant result [Holm-Bonferroni-corrected p-value <0.01 for the first principal component]) and 48 h timepoint ($Q^2Y = 0.895$, valid permutation testing, and CV-ANOVA p-value of 0, total of 6 principal components) (the analysis based on the first four principal components also resulted in a significant result [Holm-Bonferroni-corrected p-value <0.01 for the first principal component]), suggesting significant metabolic shifts upon Symprove™ treatment.

Under the dysbiosis condition, supplementation with Symprove™ resulted in a significant increase in the production of all three SCFAs at 24 h and 48 h (acetate, $p < 0.001$ for both timepoints; propionate, $p < 0.001$ for both timepoints; butyrate, $p = 0.021$ and $p < 0.001$ for 24 h and 48 h, respectively) (Table 2). Levels of branched SCFAs were significantly decreased upon Symprove™ supplementation at 24 h ($p = 0.002$) and 48 h ($p = 0.001$). Similar changes in SCFA/branched SCFA production were observed under dysbiosed conditions with ETEC infection (Table S1).

3.2. Biological impact of Symprove™ supplementation

Upon UHPLC-HRMS metabolic profiling, 155 metabolites were detected above their set LOQ levels. After applying univariate statistics and pathway mapping, several metabolites relevant to Symprove™ supplementation were selected for further description (additional rationale is provided in the Discussion). Table S2 provides a comprehensive overview of metabolite changes. Levels of tryptophan and indole-3-lactic acid, a metabolite produced by gut bacteria and related to tryptophan catabolism, were strongly increased with Symprove™ supplementation *versus* control in the healthy, low dysbiosis, and high dysbiosis populations (Figs. 3 and S2). Levels of several metabolites of the kynurenine pathway, also related to tryptophan catabolism, were significantly different between Symprove™ supplemented samples and control samples. In healthy conditions, levels of kynurenine as well as the downstream products of kynurenine, nicotinic acid, and picolinic acid, were increased with Symprove™, while levels of kynurenic acid were decreased (Figs. 3 and S3). The increase in kynurenine was also observed for the dysbiosed conditions (low and high antibiotic doses), while the decrease in kynurenic acid was only observed in the low antibiotic dose dysbiosed condition (Figs. 3 and S3).

Levels of 3-hydroxybutyric acid were increased with Symprove™ supplementation in all three microbial populations (Fig. 3). Levels of 2-hydroxyhexanoic acid and 2-hydroxyisocaproic acid were strongly increased in the healthy population, and to a lesser extent in the low and high dysbiosis populations (Fig. S2). Among these three hydroxy fatty acids, the strongest effect was observed for 3-hydroxybutyric acid.

Several metabolites of purine metabolism were increased with Symprove™ supplementation *versus* control. These included adenosine, adenine, adenosine-5-monophosphage (AMP), guanine, and inosine (Figs. 3 and S4). The increases were most pronounced in healthy conditions, and a trend towards increases was observed for most of these metabolites in the low and high antibiotic dose dysbiosed conditions.

Levels of the neuroactive metabolite gamma-aminobutyric acid (GABA) were notably increased with Symprove™ supplementation *versus* control under healthy conditions and in the low and high antibiotic dosed dysbiosed conditions (Fig. 3). This may be linked with increased levels of the precursor of GABA, glutamic acid (Fig. S2), at least in the healthy condition.

Multiple metabolites related to energy metabolism were increased with Symprove™ supplementation *versus* control in the healthy condition (Figs. 3 and S5). These increases were also observed in the low and high antibiotic dose dysbiosed conditions, with the exception of citric acid, which was decreased in these two conditions.

Symprove™ supplementation increased levels of 2,4-dihydroxyphenylalanine (L-DOPA), a precursor of dopamine, in the high antibiotic dose dysbiosed condition (Fig. 4). There was also a trend for decreased levels of tyrosine, the precursor metabolite for L-DOPA. L-DOPA levels tended to be higher with Symprove™ supplementation *versus* control in the low antibiotic dose dysbiosed condition, but this was not confirmed statistically (data not shown).

Some effects of Symprove™ were only observed under healthy conditions. There was an increase in acetylation of several amino acids with Symprove™ supplementation compared with control (Fig. S6). Tryptophan levels increased, which may relate to the observed decrease in indole levels with Symprove™ supplementation *versus* control (Fig. 5). This may have also affected downstream metabolites, as indole-3-acetic acid levels were increased and indole-3-propionic acid levels were decreased. Several metabolites involved in pyrimidine metabolism were increased with Symprove™ supplementation compared with control, including cytidine, uracil, uridine, thymine, and cytosine (Fig. S7). Metabolites of histidine metabolism were also altered with Symprove™ supplementation in the healthy population, including increased levels of histidine, histamine, and urocanic acid (Fig. 5). While there were some slight alterations in the low dysbiosis and high dysbiosis populations, the profile in these populations was less clear (Table S2). Metabolites related to arginine biosynthesis, including ornithine, citrulline, arginine,

Table 2
Impact of Symprove™ treatment on SCFA and branched SCFA production under healthy conditions (no ETEC infection and no antibiotic use) and under conditions with dysbiosis (no ETEC infection but following antibiotic use).

	With Symprove™ – Without Symprove™							
	Acetate		Propionate		Butyrate		Branched SCFA	
	Δ (mM)	p-value	Δ (mM)	p-value	Δ (mM)	p-value	Δ (mM)	p-value
Healthy								
24 h	15.70	<0.001	5.18	<0.001	3.32	0.007	−0.59	<0.001
48 h	23.80	<0.001	4.71	0.06	11.87	<0.001	−3.53	0.013
Dysbiosis								
24 h	17.40	<0.001	4.70	<0.001	1.62	0.021	−0.12	0.002
48 h	18.91	<0.001	5.75	<0.001	4.27	<0.001	−0.48	0.001

Differences (Δ) in concentration of acetate, propionate, butyrate, and branched SCFA between conditions with Symprove™ supplementation and without, including p-values, are shown. Positive values indicate that the concentration was higher in conditions with Symprove™ supplementation.
ETEC: enterotoxigenic *Escherichia coli*; SCFA: short-chain fatty acid.

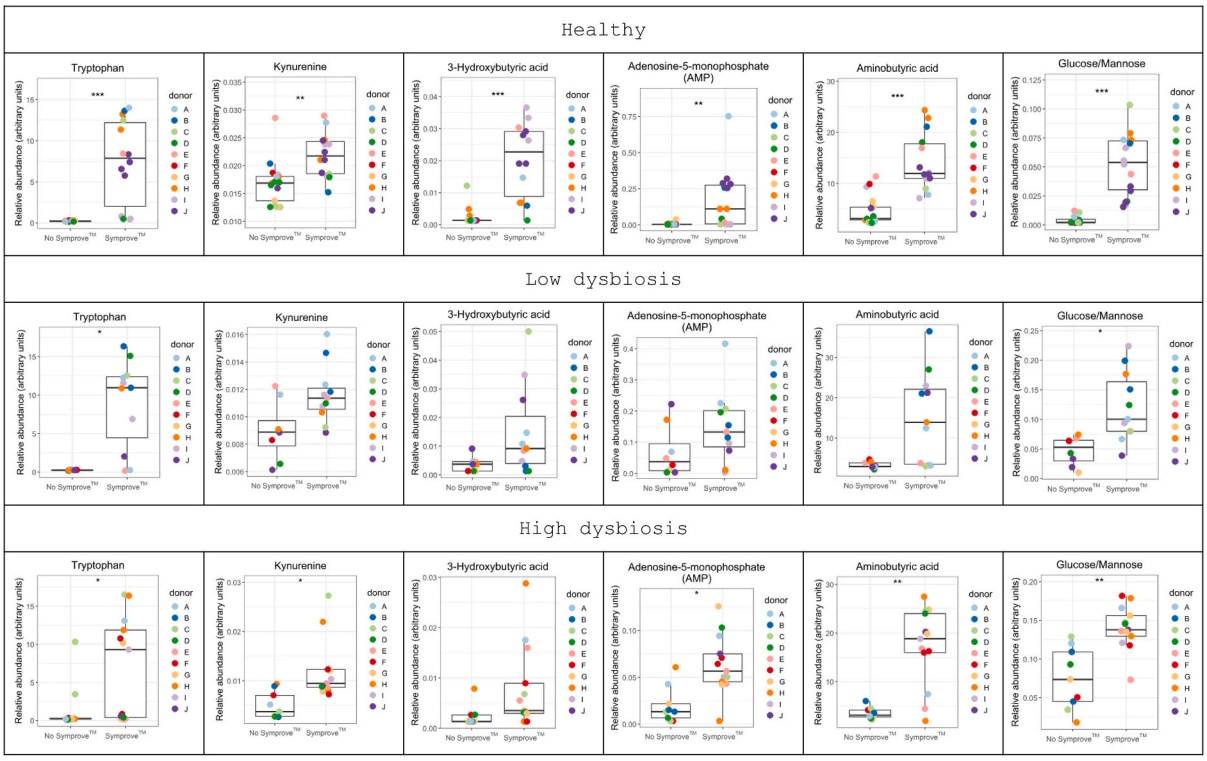

Fig. 3. Impacts of Symprove™ on selected metabolites and metabolic pathways observed in the healthy, low dysbiosis, and high dysbiosis populations. Tryptophan, kynurenine pathway, hydroxy fatty acids, purine metabolism, gamma-aminobutyric acid, and energy metabolism. UHPLC-HRMS profiling was used to quantify relative levels of metabolites from colonic fermentations collected after 24 h incubation with Symprove™ or untreated control (water) in the Colon-on-a-plate® model using fecal samples from 10 healthy human donors. The healthy population included the samples that were not infected with ETEC and were not exposed to antibiotics. The low dysbiosis and high dysbiosis populations included the samples that were not infected with ETEC but were exposed to either low-dose or high-dose antibiotics, respectively, to induce dysbiosis. ETEC: enterotoxigenic *Escherichia coli*; UHPLC-HRMS: ultra-high performance liquid chromatography-high-resolution mass spectrometry.

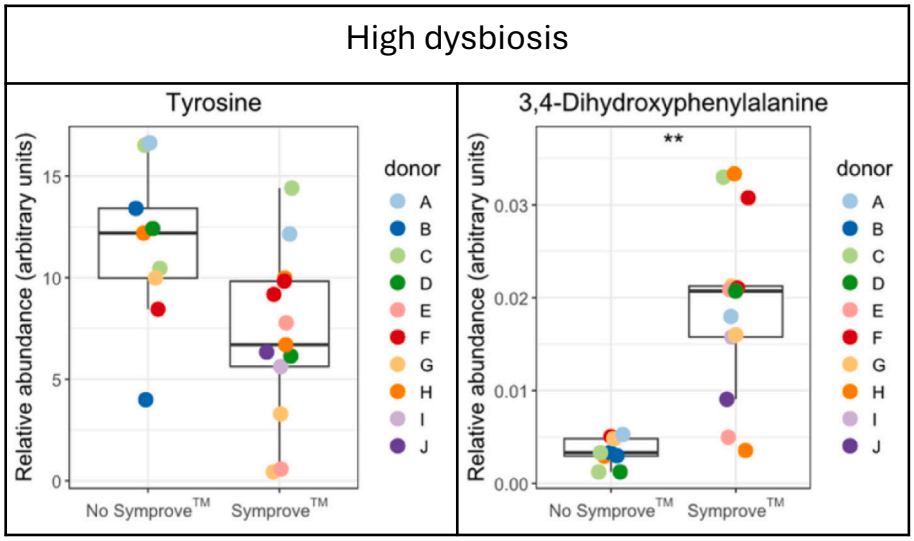

Fig. 4. Impacts of Symprove™ on L-DOPA observed in the dysbiosis populations. UHPLC-HRMS profiling was used to quantify relative levels of metabolites from colonic fermentations collected after 24 h incubation with Symprove™ or untreated control (water) in the Colon-on-a-plate® model using fecal samples from 10 healthy human donors. The low dysbiosis and high dysbiosis populations included the samples that were not infected with ETEC but were exposed to either low-dose or high-dose antibiotics, respectively, to induce dysbiosis. ETEC: enterotoxigenic *Escherichia coli*; L-DOPA: 2,4-dihydroxyphenylalanine; UHPLC-HRMS: ultra-high performance liquid chromatography-high-resolution mass spectrometry.

glutamic acid, and *n*-acetylglutamic acid, were increased with Symprove™ supplementation compared with control (Fig. S8).

4. Discussion

In this study, a high-throughput colonic *in vitro* simulation strategy,

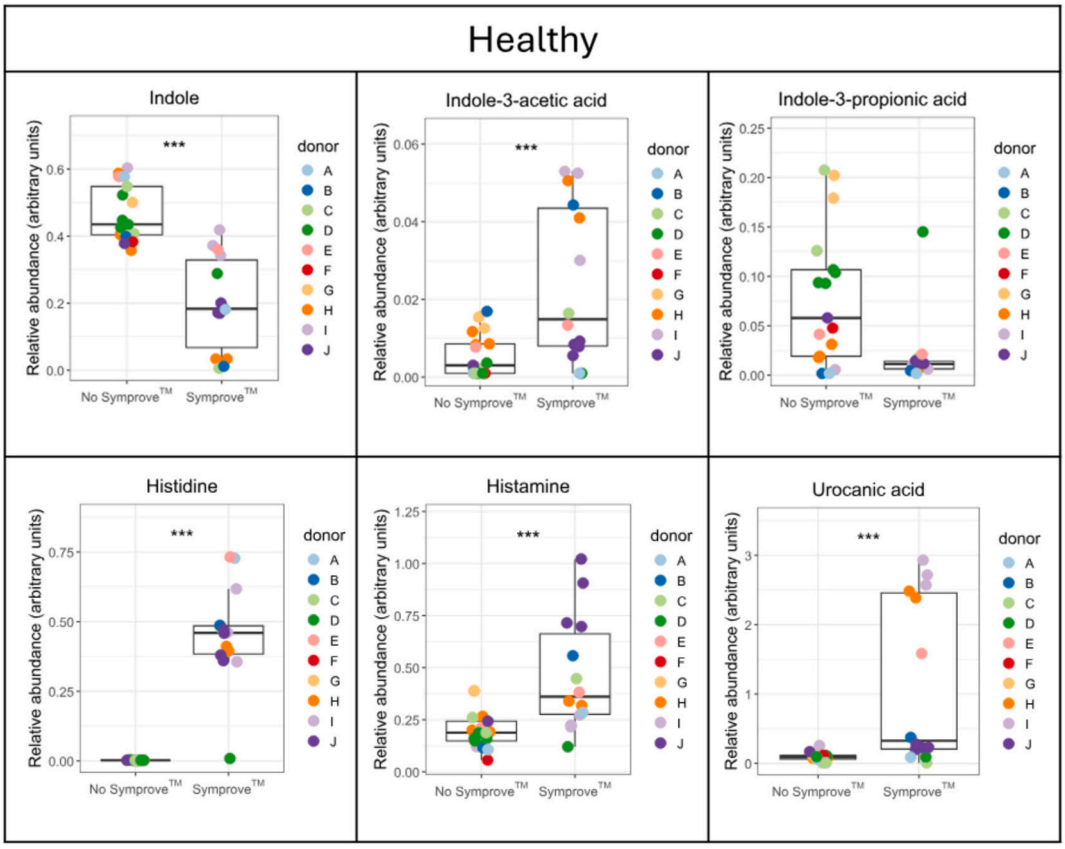

Fig. 5. Impacts of Symprove™ on selected metabolites and metabolic pathways observed in the healthy population. Tryptophan catabolism using the indole route (top), and histidine metabolism (bottom). UHPLC-HRMS profiling was used to quantify levels of metabolites from colonic fermentations collected after 24 h incubation with Symprove™ or untreated control (water) in the Colon-on-a-plate® model using fecal samples from 10 healthy human donors. The healthy population included the samples that were not infected with ETEC and were not exposed to antibiotics. ETEC: enterotoxigenic *Escherichia coli*; UHPLC-HRMS: ultra-high performance liquid chromatography-high-resolution mass spectrometry.

known as Colon-on-a-plate®, was employed to investigate hundreds of experimental conditions in a controlled and reproducible manner. As a powerful screening technology, Colon-on-a-plate® enables the systematic evaluation of numerous variables that may influence the biological effects of the test product, Symprove™. Key experimental factors included inter-donor variability, pathogen challenge, and the baseline state of the fecal microbial community, being either healthy or dysbiotic. These variables are critical for understanding the nuanced interactions between the microbiome and the supplemented product. In particular, accounting for the microbial community state can uncover unique insights, such as the product's potential to support the recovery of microbial activity or restore metabolic balance under dysbiotic conditions.

To fully leverage the scale and throughput of the Colon-on-a-plate® platform, integration with advanced metabolomics techniques such as LA-REIMS is especially valuable. This approach allows for the simultaneous detection of hundreds of metabolites, providing a comprehensive view of microbial metabolic activity and enabling the mapping of dozens of metabolic pathways. Such integration enhances the resolution and interpretability of the data, offering a deeper understanding of the functional impact of Symprove™ across diverse fecal environments. Insights gained from LA-REIMS can guide the design of targeted follow-up assays, enabling more in-depth exploration of specific biological mechanisms and responses.

There were favorable impacts observed across the healthy and dysbiosed condition, including the different antibiotic doses. Symprove™ supplementation significantly affected microbial metabolism, with targeted metabolic profiling revealing increases and decreases in a variety of microbial metabolites, many of which may be considered to have beneficial effects on the gut-brain axis, intestinal inflammation, and intestinal barrier health. Recently, Naso et al. have shown that a multi-strain probiotic supplement, containing *Lactocaseibacillus rhamnosus* LR32, *Bifidobacterium longum* BB536, and *Bifidobacterium lactis* BL04, were able to protect the intestinal barrier against *Salmonella*-induced damage, supporting the hypothesis that multi-species probiotics could have a potential therapeutic role under dysbiosed conditions (Naso et al., 2025).

Changes in SCFA and branched SCFA levels indicated that Symprove™ supplementation increased saccharolytic fermentation and decreased proteolytic fermentation in both healthy and dysbiosed conditions. Considering that all four probiotic species in Symprove™ are capable of producing SCFAs (Wang et al., 2019; L. Wang et al., 2014; Yue et al., 2022; Zhu et al., 2024), it is feasible that these strains contributed to the observed increase in saccharolytic fermentation. Indeed, while the current study design could not investigate persistence of the microbial strains in the colonic environment, previous research has shown that the provided probiotic strains can colonize and proliferate in the human large intestine (Moens, Van den Abbeele, et al., 2019). Interestingly, at least three of the strains (*L. plantarum*, *L. rhamnosus* and *E. faecium*) were even able to colonize the intestinal mucosal layer, which is of key importance for prolonged probiotic activity (Moens, Van den Abbeele, et al., 2019). Symprove™ supplementation significantly stimulated the production of acetate, propionate, and butyrate at 24 h and 48 h in both healthy (except propionate at 48 h) and dysbiosed conditions, demonstrating a strong positive effect on saccharolytic fermentation. The health benefits of these key microbial metabolites are well documented and include anti-inflammatory, anti-obesity, anticancer, hepatoprotective, immunoregulatory, neuroprotective, anti-diabetes, and

cardiovascular protective activities (Tan et al., 2014; Xiong et al., 2022). Reduced levels of SCFAs are considered an indicator of dysbiosis (Bidell et al., 2022). Symprove™ supplementation had an inhibitory effect on the production of branched SCFAs. While some metabolites of proteolytic fermentation are beneficial, many are not and may induce inflammation and increase gut permeability (Diether & Willing, 2019; Windey et al., 2012). The data also revealed that infection with ETEC had no effect on SCFA production in healthy or dysbiosed conditions whether or not Symprove™ was present. Hereby, Symprove™ supplementation was demonstrated to affect SCFA and branched SCFA production even in the presence of ETEC infection. The increased production of SCFAs and decreased production of branched SCFAs with Symprove™ supplementation generally suggests that it may effectively help to restore a dysbiosed fecal microbiome towards a healthy one. This has been shown for other multi-strain probiotics as well. For instance, supplementation of a multi-species probiotic formula to obese postmenopausal women positively impacted cardiometabolic health through the stimulation of SCFA production in the intestinal environment (Loniewski et al., 2023). Furthermore, a probiotic cocktail containing multiple *Lactobacillus* and *Enterococcus* species has been shown to exert propiogenic and butyrogenic effects in both *in vitro* human and *in vivo* mice studies, thereby ameliorating gut microbial dysbiosis by inhibiting the growth of uropathogenic microbial species (Nagpal et al., 2018). Again, these results thus support the therapeutic potential of multi-strain probiotics for human health under multiple conditions.

Several metabolites involved in the gut-brain axis were affected by Symprove™ supplementation. The inhibitory neurotransmitter GABA increased with supplementation in both the healthy and dysbiosed conditions. The GABA pathway is dysregulated in patients with PD, and GABAergic dysfunction may be involved in the development and progression of the disease (Alharbi et al., 2024). Decreased concentrations of GABA in the occipital cortex are associated with visual hallucinations, suggesting that reduced levels may have a negative impact. Interestingly, some strains of *E. faecium,* L. *plantarum* and *L. rhamnosus* are capable of either producing GABA or increasing GABA levels with supplementation (Bs et al., 2021; Tette et al., 2022; Vo & Park, 2019; Yunes et al., 2020). A multi-strain probiotic formulation containing GABA-producing L. *plantarum*, for instance, has been shown to reduce depressive symptoms in a mice model following repeated administration, reaching similar effects as antidepressant medication (Yunes et al., 2020). The upregulation of the GABA pathway thus suggest a potential new therapeutic area for Symprove™; however, much work is needed to confirm these findings and understand populations that may benefit from supplementation.

We reported a significant increase in levels of the dopamine precursor L-DOPA (levodopa) in the high dose antibiotic dysbiosed condition, with a trend towards increasing levels in the low dose dysbiosed condition. In the gastrointestinal tract, L-DOPA can be converted to dopamine; however, dopamine generated in the periphery cannot cross the blood-brain barrier (Maini Rekdal et al., 2019). Thus, the efficacy of L-DOPA is tied to the proportion of the unconverted drug that reaches the brain. The increase in L-DOPA observed in the present study may be the result of increased L-DOPA production and/or decreased decarboxylation of L-DOPA to dopamine by the fecal microbiome. Further studies investigating the potential of Symprove™ to increase L-DOPA levels in the periphery and brain, and its effect on dopamine levels and mood are warranted.

LA-REIMS data showed that Symprove™ supplementation had a strong effect on general microbial metabolism under both healthy and dysbiosed conditions, largely substantiating the SCFA findings. As with SCFA production, the presence of ETEC infection had no effect on general microbial metabolism. The LA-REIMS data were utilized as a tool for the selection of samples for follow-up targeted metabolic profiling. LA-REIMS data provide information on metabolic pathways beyond SCFAs, making it a more suitable option for guiding sample selection, potentially allowing the identification of alternative and important metabolic pathways in the context of health and disease. Based on the LA-REIMS data, samples from the healthy condition and the dysbiosed conditions (low and high antibiotic doses) were chosen for targeted metabolite profiling. Considering that the presence of ETEC infection did not have demonstrable metabolic effects, these samples were not selected for further analysis.

Previous studies have reported that Symprove™ has anti-inflammatory effects (Bjarnason et al., 2019; Ghyselinck et al., 2020; Ghyselinck et al., 2021), improves the intestinal barrier integrity (Ghyselinck et al., 2020; Ghyselinck et al., 2021), and supports a shift to a healthy microbiome (Ghyselinck et al., 2021). Several metabolites and pathways involved in immune regulation were altered with Symprove™ supplementation compared to the control. With Symprove™, there were increased levels of several hydroxy fatty acids (3-hydroxybutyric acid, 2-hydroxyhexanoic acid, 2-hydroxyisocaproic acid), which have anti-inflammatory effects and are involved in dampening of immune responses, suggesting a potential health benefit (Nieminen et al., 2014; Sebag et al., 2024; Suzuki et al., 2023).

Levels of metabolites involved in tryptophan catabolism also changed. These included an increase in the anti-inflammatory molecule indole-3-lactic acid (Meng et al., 2020), increased levels of nicotinic acid which has been reported to inhibit LPS-induced IL-8 production *in vitro* (Santoru et al., 2020), and increased levels of indole-3-acetic acid. The increases in indole-3-lactic acid and indole-3-acetic acid are likely, at least in part, due to the *Lactobacillus spp.* in Symprove™, as lactic acid bacteria are well known to metabolize tryptophan to indole derivatives (Ma et al., 2025). Specifically, indole-3-lactic acid is reported to be a metabolite of L. *acidophilus* and L. *plantarum* (Pan et al., 2024; Zhang et al., 2023) and indole-3-acetic acid is produced by L. *rhamnosus* (Huang et al., 2023). There was an increase in kynurenine and decrease in both indole and indole-3-propionic acid, both of which have anti-inflammatory effects (Ye et al., 2022). Increases in metabolites involved in purine metabolism, including inosine which has strong anti-inflammatory properties, were observed (Y. Zhang, Jia, et al., 2024). Metabolites involved in pyrimidine metabolism (cytidine, uracil, uridine, thymine, cytosine) were also increased, whereby a critical role in nucleotide synthesis and microbial coordination has been suggested, improving immune functions (Daneshmand et al., 2017).

The level of histamine, which can have both anti-inflammatory and inflammatory effects depending on receptor interactions, was increased (Dvornikova et al., 2023). The level of histidine, which has anti-inflammatory and antioxidant characteristics, was increased in the healthy condition with Symprove™ supplementation. This may be linked to the presence of L. *acidophilus* and *L. plantarum* in Symprove™, both of which are linked to increased histidine synthesis (Godzien et al., 2025; Zhang, Yang et al., 2024).

Metabolites involved in arginine biosynthesis were increased. The alterations in 3-hydroxybutyric acid, 2-hydroxyhexanoic acid, 2-hydroxyisocaproic acid, indole-3-lactic acid, and nicotinic acid were reported in the healthy and dysbiosed conditions and the alterations in kynurenine, nicotinic acid, arginine biosynthesis, indole, indole-3-propionic acid, indole-3-acetic acid, inosine, metabolites of pyrimidine metabolism, and histamine were reported in the healthy condition alone. These data may provide insights into the mechanism by which Symprove™ supplementation has been reported to increase the production of anti-inflammatory molecules (IL-10 and IL-6) and decrease production of inflammatory molecules (MCP-1 and IL-8) (Ghyselinck et al., 2020; Ghyselinck et al., 2021).

Targeted metabolic profiling also revealed changes in several metabolites that may be involved in maintaining intestinal barrier integrity (Camilleri et al., 2019). An increase in amino acid acetylation with Symprove™ supplementation was observed in healthy conditions. This might be linked to post-translational acetylation of host and microbial proteins, which is known to affect protein function, stability, and interactions. In the gut, this could influence proteins involved in the regulation of the intestinal mucosal immune barrier. Improvement in

intestinal barrier function is an important clinical goal.

We note that the effects of Symprove™ were more pronounced at 24 h than 48 h, which may suggest that repeat supplementation may be needed to maintain these metabolic changes. Future studies utilizing a long-term model with repeat administration would help to clarify this.

This study had a few limitations that should be considered. First, fractional factorial designs are limited by the potential for confounding effects and involve making assumptions about higher order interactions. Second, the size of the donor populations for the conditions compared were not necessarily similar and may contain multiple samples from the same donor, especially in the UHPLC-HRMS data analysis. However, appropriate statistical tests (linear mixed models) were used. It must be mentioned that donor variation in the microbial community composition can strongly impact metabolic outputs. Even though a solid population of ten donors was included in the current study design, including data on the microbial composition of each individual donor could have provided more insight in specific metabolic effects. Finally, as with any *in vitro* study, the findings reported herein cannot be directly applied to the *in vivo* situation, certainly in the absence of any investigated effects on host-microbiome interactions. However, they do provide some mechanistic insights that cannot be acquired from in-human studies. Indeed, *in vitro* short-term colonic fermentation models, like the used Colon-on-a-plate® system, are extensively used in literature to study the effects of different test compounds on the intestinal microbiome, as they allow to assess production of microbial metabolites and can help to elucidate involved microbial pathways in a rapid, high-throughput and cost-effective way (Isenring et al., 2023; Verhoeckx et al., 2015). However, the accumulation of fermentation products, nutrient depletion towards the end of the incubation period and the single-dosing strategy can affect the *in vivo* relevance and should therefore be considered.

5. Conclusions

Symprove™ significantly affected microbial metabolism, with many of these changes being considered positive for human health. Importantly, these benefits were shown in both healthy and *in vitro* antibiotic-induced dysbiosed fecal microbiomes, showing therapeutic potential. The changes in SCFAs and branched SCFAs were similar for healthy and dysbiosed conditions and were also observed in the presence of ETEC infection. Targeted metabolic profiling revealed changes in metabolites related to the anti-inflammatory response, intestinal barrier health, and the gut-brain axis. Some changes were observed within both healthy and dysbiosed conditions, while others were unique to one condition. These data provide new insights into the potential effects of Symprove™ supplementation and confirm that many of these effects are consistent over different microbiome conditions (*i.e.*, healthy, dysbiosed, ETEC infected). This knowledge provides a springboard for future studies further examining the therapeutic potential and use of Symprove™ in different populations. Additionally, they provide mechanistic insights into the effects of Symprove™ supplementation reported in previous *in vitro* and *in vivo* studies.

CRediT authorship contribution statement

Lieven Van Meulebroek: Writing – original draft, Visualization, Methodology, Formal analysis, Data curation, Conceptualization. **Jonas Ghyselinck:** Writing – original draft, Visualization, Methodology, Formal analysis, Data curation, Conceptualization. **Dries Van Elst:** Visualization, Formal analysis. **Cindy Duysburgh:** Writing – review & editing. **André Gessner:** Writing – review & editing. **Olivier Thas:** Writing – review & editing, Formal analysis, Data curation. **Massimo Marzorati:** Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: This work was financially supported by ProDigest and Symprove. Lieven Van Meulebroek, Jonas Ghyselinck, Dries Van Elst, Cindy Duysburgh, and Massimo Marzorati are employees of ProDigest. André Gressner has received financial support from Symprove as a member of an advisory board. Other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2025.118172.

Data availability

Data will be made available on request.

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Glossary

ETEC, enterotoxigenic Escherichia coli; IBD, inflammatory bowel disease; LA-REIMS, Laser-Assisted Rapid Evaporative Ionization Mass Spectrometry; LC-MS, liquid chromatography mass spectrometry; LOQ, limit of quantification; OPLS-DA, orthogonal partial least squares discriminant analysis; PCA-X, Unsupervised Principal Component Analysis; PD, Parkinson's disease; SCFA, short-chain fatty acid; SVD, singular value decomposition; UHPLC-HRMS, Ultra-high performance liquid chromatography-high-resolution mass spectrometry.: