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A four-strain probiotic exerts positive immunomodulatory effects by enhancing colonic butyrate production *in vitro*

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ABSTRACT

Poorly formulated probiotic supplements intended for oral administration often fail to protect bacteria from the challenges of human digestion, meaning bacteria do not reach the small intestine in a viable state. As a result, the ability of probiotics to influence the human gut microbiota has not been proven. Here we show how (i) considered formulation of an aqueous probiotic suspension can facilitate delivery of viable probiotic bacteria to the gut and (ii) quantitate the effect of colonisation and proliferation of specific probiotic species on the human gut microbiota, using an *in-vitro* gut model. Our data revealed immediate colonisation and growth of three probiotic species in the luminal and mucosal compartments of the proximal and distal colon, and growth of a fourth species in the luminal proximal colon, leading to higher proximal and distal colonic lactate concentrations. The lactate stimulated growth of lactate-consuming bacteria, altering the bacterial diversity of the microbiota and resulting in increased short-chain fatty acid production, especially butyrate. Additionally, an immunomodulatory effect of the probiotics was seen; production of anti-inflammatory cytokines (IL-6 and IL-10) was increased and production of inflammatory chemokines (MCP-1, CXCL 10 and IL-8.) was reduced. The results indicate that the probiotic species alone do not result in a clinical effect; rather, they facilitate modulation of the gut microbiota composition and metabolic activity thereby influencing the immune response.

1. Introduction

The global market for products containing probiotic species, "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014) is large (predicted to reach over $46 bn by 2020 (O'Toole et al., 2017)). Poorly formulated probiotic supplements intended for oral administration often fail to protect bacteria from the challenges of human digestion, meaning bacteria do not reach the small intestine in a viable state and that any feeling of improved health often reduces to a placebo effect. Further, the ability of probiotic species to influence established gut microbiota has not been proven. As a result, healthcare professionals and the general public often view the use of probiotics to alleviate clinical symptoms with scepticism.

The main process occurring in the human colon is the fermentation of non-digestible carbohydrates by the resident colonic microbiota yielding various metabolic end-products (primarily the short-chain fatty acids (SCFA) acetate, propionate and butyrate, (Tremaroli and Bäckhed, 2012)). Lactate, which is not an SCFA, is also produced as a result of fermentation but does not accumulate in the colon because it is a substrate for several SCFA-producing bacteria, such as *Eubacterium hallii* and *Anaerostipes caccae* or *Megasphaera* spp., resulting in the production of butyrate or propionate, respectively (Louis et al., 2014; Flint et al., 2015). SCFAs are important for general gut health (Ríos-Covían et al., 2016) as an energy source for peripheral tissues (acetate and propionate) and colonocytes (butyrate) (Hamer et al., 2008), but they also influence inflammation, vasodilation, gut motility and wound healing (Bergman, 1990). For instance, dysbiosis in ulcerative colitis (UC) patients has been linked to a reduction in butyrate-producing species (Machiels et al., 2014), reduced levels of propionate-producing species have been linked with asthma in children (Arrieta et al., 2015) while broad changes in the gut microbiota have been linked with

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irritable bowel syndrome (IBS) (Rajilic-Stojanovic et al., 2011; Simren et al., 2013; Jalanka-Tuovinen et al., 2014; Distrutti et al., 2016) and irritable bowel disease (IBD) (Matsuoka and Kanai 2015). It has also been proposed that butyrate produced from a high-fiber diet can improve brain health (Bourassa et al., 2016) and function (Stiling et al., 2016). Ensuring the gut microbiota is optimally balanced and so producing high SCFA, especially butyrate, levels is therefore an important focus in maintaining and improving general health and wellbeing.

The introduction and integration of probiotic species to the gut microbiota has the potential to reduce dysbiosis and/or improve general health by influencing a range of factors (for instance, extent of bacterial diversity, production of SCFAs and/or colonisation of the mucosal layer/reduction in inflammation response) but achieving clinical efficacy requires formulation of bacteria in a nutritional supplement in a way that offers protection from the challenges of oral delivery (Coghetto et al., 2016); primarily, these challenges result from exposure of bacteria to gastrointestinal fluids (the low pH of stomach acid and the bile salt/pancreatic enzyme concentrations in the small intestine). Probiotics are often added to dairy products or formulated in freeze-dried capsules or tablets, but *in-vitro* testing has shown many probiotic supplements cannot protect bacteria against stomach acid pH (Fredua-Agyeman and Gaisford, 2015). Alternative formulation strategies, such as microencapsulation of bacteria (Mirtic et al, 2018), enteric coating (Dodoo et al., 2017) or gelation of emulsions (Picone et al., 2017) show efficacy *in-vitro* but are technologies that are not ready for commercial use. It is also necessary that probiotic bacteria, once they reach the gut, are able to infiltrate, colonise and proliferate in the luminal and mucosal compartments. A recent *in-vivo* study on an 11-strain probiotic suggested that mucosal colonisation by the bacteria was a challenge and that any effect on the gut microbiota was at best transient (Zmora et al., 2018).

Recently, a 4-strain aqueous probiotic supplement (Symprove™, containing *Lactobacillus acidophilus* NCIMB 30175, *Lactobacillus plantarum* NCIMB 30173, *Lactobacillus rhamnosus* NCIMB 30174 and *Enterococcus faecium* NCIMB 30176) has been shown to reduce clinical symptom severity scores in IBS (Sisson et al., 2014) and to reduce abdominal pain scores and significantly reduce constipation, diarrhoea and mucorrhoea in diverticular disease (Kvasnovsky et al., 2017). Symprove also showed a high degree of gastric acid tolerance during *in-vitro* testing (Fredua-Agyeman and Gaisford, 2015) and anti-pathogenic activity against *Clostridium difficile* (Fredua-Agyeman et al., 2017). Understanding, and more importantly quantifying, the effect of probiotics in modulating the human gut microbiota is very challenging *in-vivo*, because of the complexity in sampling and analysing the microbiota, its metabolic products and its influence on immune response in real-time. However, recently developed *in-vitro* gut models, which contain viable microbiota from healthy human donors and that have both luminal and mucosal compartments, allow the complex interactions between probiotics, commensal gut bacteria and the gut wall to be monitored and quantified. Hence, the principal aim of this work was to assess the ability of the probiotic bacteria in Symprove to influence three healthy human gut microbiotas using an *in-vitro* gut model (Simulator of the Human Intestinal Microbial Ecosystem, equipped with mucosal compartments (M-SHIME®)); the effects on bacterial diversity, SCFA production and inflammatory markers, following dosing with Symprove over a three-week period, were quantified.

2. Materials and methods

2.1. M-SHIME® Testing

Symprove™ was obtained from Symprove Ltd and used as received. Experiments were performed using the M-SHIME® system. Briefly, the system comprised four reactors vessels (V). The first two reactors are of the fill-and-draw principle and simulate the initial stages in food uptake and digestion. Peristaltic pumps add feed (140 mL, 3x per day) and pancreatic/bile juice (60 mL, 3× per day) to the stomach (V1) and small intestine (V2) respectively and empty each reactor after defined time intervals. The remaining 2 reactors simulate the conditions of the proximal (V3) and distal (V4) colon. Colon vessels are constantly stirred, retain a fixed volume (PC = 500 mL; DC = 800 mL) and their pH is constant (PC = 5.6–5.9; DC = 6.6–6.9). The retention time of media in each vessel is selected to mimic human *in-vivo* conditions (Possemiers et al 2004). The colon reactors were inoculated with fecal microbiota from healthy human donors (consuming a Western-style diet) and the microbial community was allowed to stabilise over a 2-week period. The microbiota was then maintained for a further 2-week control period. During this period, the baseline microbial community composition and activity were recorded. Three donors were used in this study to address inter-individual variability. A three-week treatment phase then commenced; Symprove was added to V1 and was progressed through V2 prior to being fed to the colonic microbiota. One set of V1-V2 vessels was used to minimise variability of the feed material, meaning the feed arriving in the proximal colon vessels was the same for all donors. Mucin-covered microcosms were added to all colonic vessels, enabling maintenance of not only a luminal microbiota but also a specific mucosal microbiota in the colonic regions (Van den Abbeele et al., 2012, 2013).

2.2. Quantification of viable and non-viable bacteria by flow cytometry

Samples were collected from different stages at various time intervals in V1 and V2 to investigate upper gastro-intestinal survival of the probiotic species. A ten-fold dilution series was initially prepared in phosphate buffered saline. Assessment of the viable and non-viable populations of the bacteria was done by staining the appropriate dilutions with SYTO 24 and propidium iodide. Samples were analyzed on a BDFacs verse, using a high flow rate. Bacterial cells were separated from medium debris and signal noise by applying a threshold level of 200 on the SYTO channel. Proper parent and daughter gates were set to determine all populations. Results are reported as average log (counts) ± sd of the three independent biological replicates.

2.3. Measurement of SCFA/BCFA, lactate and ammonium

SCFA levels, including acetate, propionate, butyrate and branched SCFA (isobutyrate, isovalerate and isocaproate) were monitored as described by De Weirdt et al. (2010). Lactate quantification was performed using a commercially available enzymatic assay kit (R-Biopharm, Darmstadt, Germany) according to the manufacturer's instructions. Ammonium analysis was performed as previously described by Van de Wiele et al (2004). Briefly, the ammonium in the liquid samples was quantified by initially performing a steam distillation. Subsequently, the ammonium in the distillate was determined titrimetrically with HCl.

2.4. Microbial community analysis

During the reference and treatment periods, samples for microbial community analysis were collected once per week from each colon vessel. DNA was isolated using the protocol as described by Vilchez-Vargas et al. (2013), starting from pelleted cells originating from 1 mL luminal or 0.1 g mucus samples. Numbers of the probiotic species were determined with a qPCR protocol, using the species-specific primers and probes listed in Table S1. Although the primers are *species* but not *strain* specific, the microbiota were established over a 4-week period prior to dosing with Symprove, so any increase in the numbers of bacterial species during the treatment period is a result of probiotic treatment, meaning strain specific primers were not required. The qPCR was performed on a QuantStudio 5 Real-Time PCR system (Applied Biosystems, Foster City, CA USA). Each sample was analysed in triplicate. Results are reported as average log(copies/mL) for the luminal

samples and as average log(copies/g) for the mucosal samples ± sd of the three technical replicates.

The microbiota profiling of each colon compartment was established by 16S-targeted sequencing analysis. The 16S rRNA gene V3-V4 hypervariable regions were amplified by PCR using primers 341F (5′-CCT ACG GGN GGC WGC AG -3′) and 785Rmod (5′-GAC TAC HVG GGT ATC TAA KCC-3′), with the reverse primer being adapted from Klindworth et al. (2013) to increase coverage. Quality control PCR was conducted using Taq DNA Polymerase with the Fermentas PCR Kit according to the manufacturers' instructions (Thermo Fisher Scientific, Waltham, MA, USA). The obtained PCR product was run along the DNA extract on a 2% agarose gel for 30 min at 100 V. 10 µl of the original genomic DNA extract was sent out to LGC genomics GmbH (Germany) for library preparation and sequencing on an Illumina Miseq platform with v3 chemistry with the primers mentioned above.

2.5. Caco-2/THP1-blue™ co-culture model

The co-culture experiment was performed as previously described (Daguet et al., 2013). Briefly, Caco-2 cells (HTB-37; American Type Culture Collection) were seeded in 24-well semi-permeable inserts (0.4 µm pore size) at a density of 1 x 10^5 cells/insert. Caco-2 monolayers were cultured for 14 days, with three changes of medium per week, until a functional monolayer with a transepithelial electrical resistance (TEER) of more than 300 Ω cm^2 was obtained (measured with a Millicell ERS-2 epithelial volt-ohm meter, Millipore). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing glucose (25 mM) and glutamine (4 mM), supplemented with HEPES (10 mM) and heat-inactivated fetal bovine serum (HI-FBS, 20% v/v).

THP1-Blue™ cells (InvivoGen) were seeded in 24-well plates at a density of 5 × 10^5 cells/well and treated with phorbol 12-myristate 13-acetate (PMA) for 48 h and maintained in Roswell Park Memorial Institute (RPMI) 1640 medium containing glucose (11 mM) and glutamine (2 mM), supplemented with HEPES (10 mM), sodium pyruvate (1 mM) and HI-FBS (10% v/v).

Before setting up the co-culture, the TEER of the Caco-2 monolayer was measured (the TEER of an empty insert was subtracted from all readings). Caco-2 bearing inserts were then placed on top of the PMA-differentiated THP1-blue™ cells. The apical compartment (containing Caco-2 cells) was filled with sterile-filtered (0.22 µm) colonic SHIME media (diluted 1:5 v/v in Caco-2 complete medium). Cells were treated apically with sodium butyrate (Sigma-Aldrich) as a positive control. The basolateral compartment (containing THP1-blue™ cells) was filled with Caco-2 complete medium. Cells were treated for 24 h, after which the TEER was measured. The basolateral supernatant was then discarded and cells were stimulated on the basolateral side with Caco-2 complete medium containing ultrapure lipopolysaccharide (LPS, *E. coli* K12, InvivoGen). Cells were also stimulated at the basolateral side with LPS in combination with hydrocortisone (HC, Sigma-Aldrich) and medium without LPS as controls. After LPS stimulation (6 h) the basolateral supernatants were collected for cytokine measurement (human IL-1β, IL-6, IL-8, IL-10, CXCL10 and MCP-1) by Luminex® multiplex (Affymetrix-eBioscience) and for NF-κB activity. All measurements were performed in triplicate and cells were incubated at 37 °C in a humidified atmosphere of air/CO2 (95:5 v/v).

3. Results

Fecal samples were obtained from three healthy adult donors and used to establish three discrete gut models, each with a representative human microbiota. Following a two-week stabilization period and a further two-week control period, during which the donor microbiotas were established and vibrant, Symprove was dosed daily over a three-week period into the M-SHIME® gut simulator. This exposed the bacteria to stomach acid conditions for 45 min (*in-vivo* MRI imaging has shown that the half-emptying time for pure water (240 mL) in humans is 13 ± 1 min, (Mudie et al., 2014)) after which they transferred to small intestinal fluid for 180 min. The data in Fig. 1 show the total and viable cell counts following exposure to these phases; 99.3% of bacteria remained viable during this challenge. This indicates that the aqueous formulation of Symprove protected the bacteria against the low gastric pH and the high concentrations of bile salts present in the small intestine, consistent with the results of earlier *in-vitro* acid-tolerance testing (Fredua-Agyeman and Gaisford, 2015). Following this period of exposure to gastric and small intestinal fluids, bacteria were transferred to the established microbiotas from three healthy adult donors.

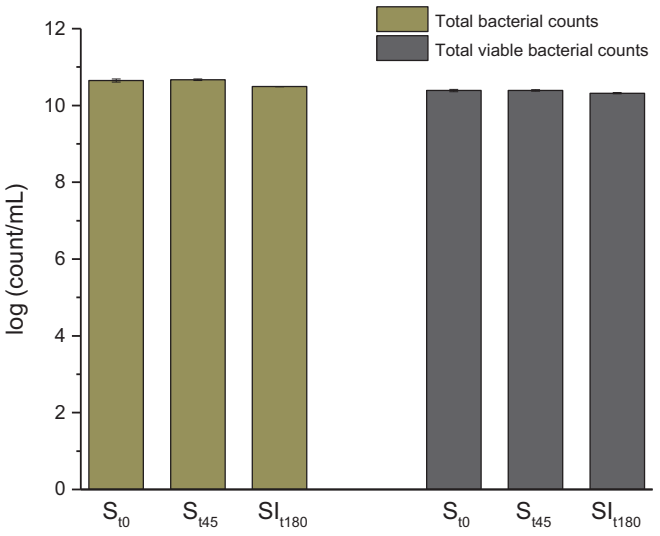

Fig. 1. Total bacterial counts (left) and total viable cell counts (right), determined with flow cytometry, for Symprove bacteria upon addition to stomach juice (S_{t0}), after 45 min in stomach juice (S_{t45}) and after 180 min in small intestinal fluid (SI_{t180}).

Fig. 2 shows how the probiotic species colonised the luminal and mucosal compartments of the proximal and distal colons. *L. acidophilus* was not detected in the control samples, indicating it is not natively present in the human microbiota, and only appeared at a detectable level in the proximal colon after two weeks. It did not colonise the lumen of the distal colon, nor the mucosal compartments of the proximal and distal colons, during the dosing period. This probably reflects the fact that during production of Symprove, *L. acidophilus* is added as a facilitator to aid the growth of *L. rhamnosus* and in the final product it is not present at greater than 10^4 copies/mL. *L. rhamnosus* was also not detected in the control samples, but immediately colonised the luminal compartments upon dosing with Symprove, reaching ca. 10^6 copies/mL in the proximal colon and ca. 10^7 copies/mL in the distal colon after 1 week. It remained detectable at these concentrations throughout the rest of the dosing period. It also immediately colonised the mucosal compartment of the proximal colon, reaching 10^4 copies/g but was never detected in the mucosal compartment of the distal colon. *L. plantarum* was sporadically detected in the lumen of the proximal colon during the control period but immediately colonised the luminal and mucosal compartments of the proximal (10^8 copies/mL luminal and 10^5 copies/g mucosal) and distal (10^7 copies/mL luminal and 10^5 copies/g mucosal) colons. *E. faecium* was abundantly present in all compartments during the control period, but its numbers increased upon dosing with Symprove, reaching ca. 10^8 copies/mL in the luminal and ca. 10^6 copies/g in the mucosal compartments.

Fig. 3 reports the lactate and SCFA concentrations in the proximal and distal colon before and during dosing with Symprove. Concentrations of lactate rose after dosing with Symprove, and increased with continued dosing. Lactate is a major by-product of carbohydrate fermentation by lactobacilli (Moens et al., 2017) and bifidobacteria (De Vuyst et al., 2014) but is also consumed by propionate-producing

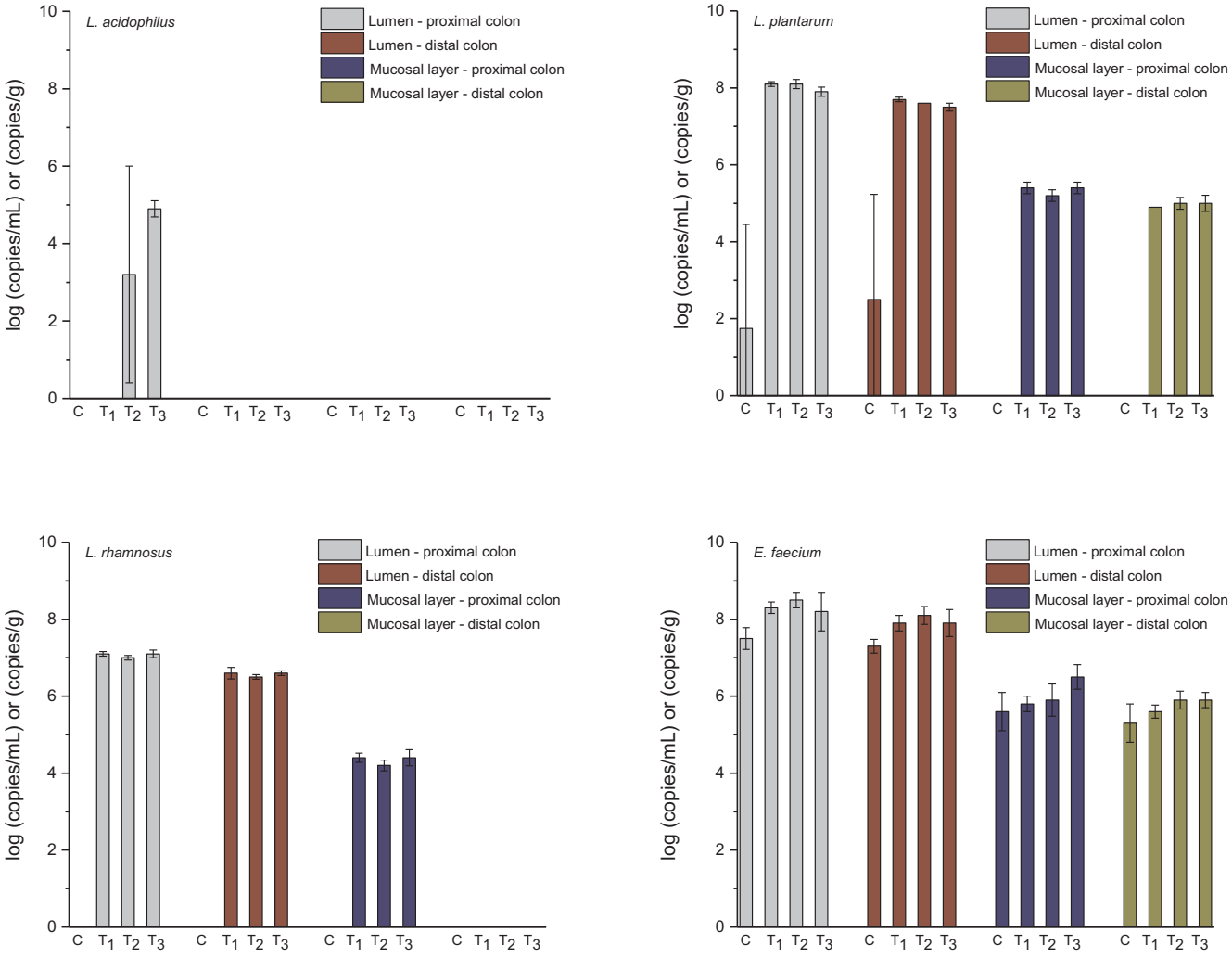

Fig. 2. Average log(copies/mL) ± sd (lumen; n = 3) or average log(copies/g) ± sd (mucus; n = 3) for the Symprove bacteria in the luminal and mucosal compartments of the proximal and distal colon of the three donors for the donor control samples (C) and following 1 week (T_1), 2 weeks (T_2) and 3 weeks (T_3) daily dosing with Symprove. *L. acidophilus* (top left), *L. plantarum* (top right), *L. rhamnosus* (bottom left) and *E. faecium* (bottom right).

species, such as *Veillonella* and *Megasphaera* (Reichardt et al., 2014), and butyrate-producing species, such as *A. caccae* and *E. hallii* (Duncan et al., 2004a). Thus, the measured lactate concentrations will always be the net difference between production and consumption.

The SCFA data show that acetate is the most abundant (50.9% across the proximal and distal colon), followed by butyrate and propionate. This correlates with *in-vivo* data showing acetate comprises more than half the total SCFA detected in human faeces (Louis et al., 2007) and arises because numerous bacterial groups (including bifidobacteria (De Vuyst et al., 2014), bacteroidetes (Macy et al., 1978) and acetogenic bacteria (Ragsdale and Pierce 2008) produce it as a by-product of saccharolytic fermentation. Acetate is itself a substrate for many butyrate-producing species (such as *Faecalibacterium prausnitzii* and *Roseburia* spp. (Duncan et al., 2004b) and is an essential co-substrate that needs to be consumed to complete butyrate synthesis from lactate or carbohydrate (Duncan et al., 2002). Thus, as in the case of lactate, the concentration of acetate is always the net difference between production and consumption. Propionate levels were variable in the donor microbiotas and were not significantly altered during dosing with Symprove. Butyrate concentrations were significantly increased, relative to the control, in both the proximal and distal colons. Unlike acetate and lactate, butyrate is the end product of fermentation and so it is not consumed in the *in-vitro* gut model, but *in-vivo* butyrate is a major energy source for colonocytes (which utilise up to 90% of butyrate (Hamer et al., 2008) and high butyrate concentrations are generally linked with improved health (Tan et al., 2014; Ríos-Covían et al., 2016).

Once carbohydrate is depleted the colonic microbiota will switch from saccharolytic fermentation to proteolytic fermentation of protein, resulting in production of ammonium, branched-chain fatty acids (BCFA, typically isobutyrate, 2-methylbutyrate and isovalerate) and various amines, phenols/indoles and sulphides; these compounds generally impair colon health and so their presence is undesirable (Scott et al., 2013). Fig. 4 shows that dosing with Symprove actually reduced both BCFA and ammonium levels compared with the control.

Fig. 5 shows the diversity of the gut microbiota, in terms of the six major phyla, for the three donors during the control and dosing periods (familial detail of operational taxonomic units (OTU) within phyla are given in Tables S2 and S3). In general, dosing with Symprove enriched the proximal and distal luminal levels, and the distal mucosal level, of Actinobacteria of donors 1 and 2 at the expense of Bacteroidetes. In particular, *Bifidobacterium pseudocatenulatum* was increased in donors 1 and 2 and *Bifidobacterium adolescentis* was increased in donor 3, mainly at the expense of *Bifidobacterium longum*. For donors 2 and 3 there was also a marked increase in the mucosal numbers of *Bifidobacterium bifidum*. Bifidobacteria belong to the Actinobacteria, so the increase in this phylum could explain the higher acetate concentrations but also the higher butyrate concentrations, mediated to acetate-driven cross-

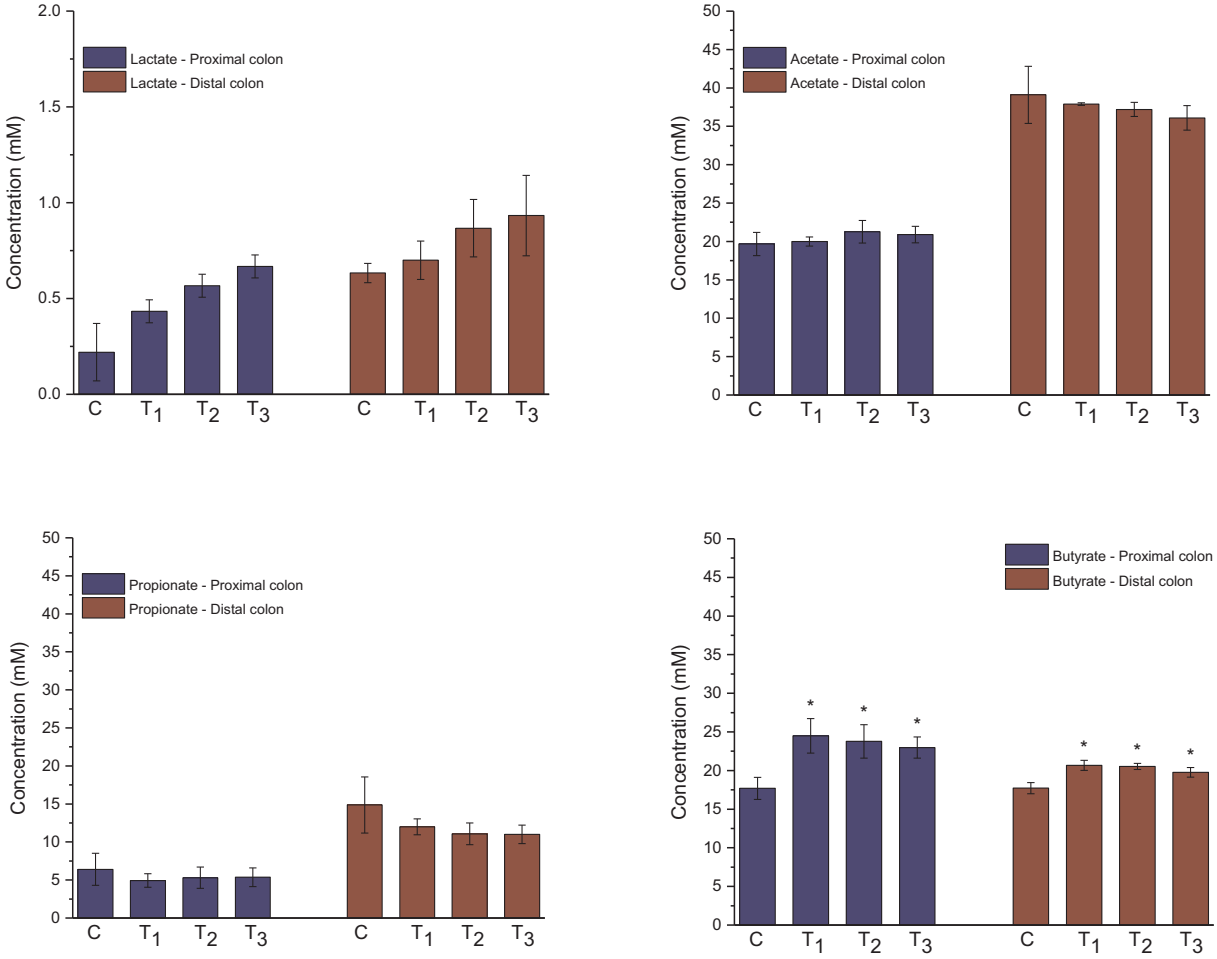

Fig. 3. Average SCFA and lactate concentrations ± sd (n = 3) in the luminal compartments of the proximal and distal colon of the three donors for the donor control samples (C) and following 1 week (T_1), 2 weeks (T_2) and 3 weeks (T_3) daily dosing with Symprove. Lactate (top left), acetate (top right), propionate (bottom left) and butyrate (bottom right). Results that are of statistical significance compared with the control are indicated with * ($P < 0.05$).

feeding interactions with butyrate-producing bacteria discussed above, while the reduction in *Bacteroidaceae* could explain the low propionate concentrations. Luminal levels of Firmicutes increased in the proximal colon of donors 1 and 2 and in the distal colon of all three donors. At OTU level, the main changes were attributed to OTU 33 (*L. plantarum*) and OTU 125 (*L. rhamnosus*), reflecting successful colonisation by the probiotic bacteria in Symprove. *Ruminococcaceae* numbers increased in all three donors, with OTU 64 (*F. prausnitzii*) numbers raised in donor 2 (and the mucosal compartments of all donors) and OTU 29 (*Subdoligranulum* spp.) in donors 1 and 3. Interestingly, *Lachnospiraceae,* a strongly butyrate-producing family, were suppressed in the mucosal compartments of all donors. *Veillonellaceae* numbers were increased for all donors but particularly for donor 2 (OTU 1, *Megasphaera* spp.). Many butyrate-producing bacteria belong to the Firmicutes, so the increase in proportion of this phylum also correlates with the raised butyrate levels discussed above. Other lactate-producing families that were enriched following dosing with Symprove included *Enterococcaceae* in the luminal and mucosal proximal colon and luminal distal colon of donors 1 and 3, reflective of the wide colonisation by *E. faecium*, and *Streptococcaceae* in all compartments of all donors; these increases are manifest in the general increase in the Actinobacteria and Firmicutes phyla. Synergistetes colonised the distal colon of donors 2 and 3 and their numbers increased after dosing with Symprove.

A Caco-2-THP1-Blue^{TM} co-culture *in-vitro* model was used to assess the inflammatory response of SHIME samples (control and after dosing with Symprove). Following dosing with Symprove, no reduction in transepithelial electrical resistance (TEER) was seen in the cell culture model (Table S4), indicating integrity of the cell wall was maintained during experimentation. Fig. 6 shows the levels of the anti-inflammatory cytokines (NK-κB, IL-6, IL-10 and IL-1β) and inflammatory chemokines (MCP-1, CXCL 10 and IL-8). Dosing with Symprove did not alter the levels of NF-κB or IL-1β, but increased the levels of IL-6 and IL-10 and decreased the levels of MCP-1, CXCL 10 and IL-8.

4. Discussion

The data reported here show that the probiotic species in Symprove are capable of surviving the challenges of oral delivery under simulated human conditions. Exposure to stomach acid for 45 min and small-intestinal juice for 3 h did not significantly reduce viable bacterial numbers (99.3% viability), a result that is in agreement with a previous *in-vitro* stomach acid-tolerance and growth test (Fredua-Agyeman and Gaisford, 2015). The primary factor in this stability is probably the fact that the bacteria are suspended in an aqueous wort, rather than in a freeze-dried compact/sachet or an oil-in-water emulsion (such as a yoghurt); *in-vivo*, consumption of water does not trigger production of stomach acid (which is primarily secreted to facilitate digestion of proteins by denaturing them and activating pepsinogen by converting it to pepsin (Smith and Morton, 2010; Wang et al., 2015)). Indeed, ingestion of appreciable volumes of water will dilute gastric juice, raising local pH. Without fat, the stomach will empty water into the small intestine rapidly (the half-emptying time in humans is 13 ± 1 min (Mudie et al., 2014)), where local pH rises again (the small intestine pH gradually increases along its length from ca. 5.6 to 7.4 (Ibekwe et al.,

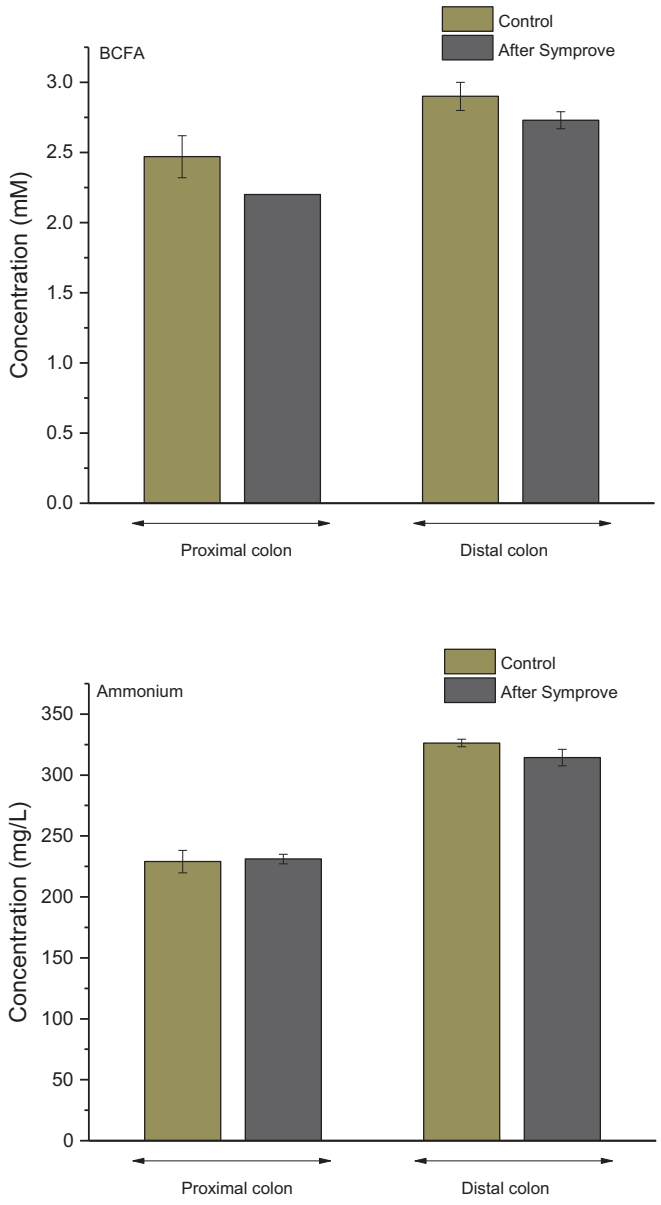

Fig. 4. Average total BCFA (top) and ammonium (bottom) concentrations ± sd (n = 3) in the proximal and distal colon of the three donors for the donor control samples and following 3 weeks daily dosing with Symprove.

2008)). Lactobacilli have been shown to have appreciable acid-tolerance; for instance, *L. acidophilus* strains remain viable at pH 3.5 (Chou and Weimer, 1999) while *L. rhamnosus* strains can remain viable for several hours at pH 3 (Succi et al., 2005). When fat is a component of the ingested foodstuff, water empties at the same rate but the fat is retained for a longer period (Chang et al., 1968). When glucose is present above 6% w/v, gastric emptying is further delayed (Shi et al., 2017).

Following transit through the upper GIT, three of the probiotic bacteria (*L. plantarum*, *L. rhamnosus* and *E. faecium*) were able to establish, colonise and proliferate in the luminal and mucosal compartments of the proximal and distal colon while *L. acidophilus* was able to proliferate in the proximal lumen. Importantly, the data show that three of the probiotic species were able to colonise the mucosal layer; this suggests that *in-vivo* consumption of Symprove would lead to colonisation of the gut by the probiotic species, rather than the luminal numbers rising transiently, which helps to explain the positive, long-term effects seen during clinical studies (Sisson et al., 2014; Kvasnovsky et al., 2017). Proliferation occurred despite the existence of an established, and vibrant, microbiota, suggesting that the probiotic species were not out-competed by the commensal bacteria for nutrients. Once established, the probiotics had a positive influence; the principal effect was caused through an increase in lactate concentration. Cross-feeding interactions from this substrate encouraged growth of commensal gut bacteria, particularly those of the Firmicutes phyla, leading to increased SCFA levels.

Changes in composition of the microbiota was seen for all donors, although the specific changes varied, reflecting both the complexity and diversity of human gut flora. Broad changes in the gut microbiota have been linked with gut disease; for instance, reduced levels of Firmicutes and Actinobacteria are typically seen in IBS (Rajilic-Stojanovic et al., 2011; Simren et al., 2013; Jalanka-Tuovinen et al., 2014; Distrutti et al., 2016) while reduced levels of Firmicutes and increased levels of Proteobacteria are typically seen in IBD (Matsuoka and Kanai, 2015).

As well as being produced by the *Lactobacillus* spp. in Symprove, numbers of bifidobacteria were also seen to increase and these are known to be lactate-producing (De Vuyst et al., 2014). This raises the question of why bifidobacteria were stimulated to grow. One possibility is that the wort used to produce and suspend the probiotic bacteria in Symprove is itself a nutrient source for bifidobacteria, since it contains germinated barley extracts. Untreated barley has been shown to increase *Bifidobacterium* spp. and *Lactobacillus* spp., as well as increase butyrate concentrations, in growing pigs (Moen et al., 2016) and in rats fed low-fat diets (Zhong et al., 2015), while xylooligosaccharides from barley have been shown to increase *Lactobacillus* spp. in simulated GIT conditions (Gullón et al., 2014). The increase in bifidobacteria numbers would in itself have a beneficial impact on general health; for instance, consumption of *B. bifidum* for 4 weeks modulated the microbiota in healthy adults, reducing the numbers of *Prevotellaceae* and *Prevotella*, increasing the numbers of *Ruminococcaceae* and *Rikenellaceae* and raising butyrate concentrations (Gargari et al., 2016).

While acetate levels remained relatively constant throughout the control and dosing periods, raised concentrations of acetate were suggested by the increased proportion of acetate-producing bacteria (bifidobacteria, bacteroidetes and acetogenic bacteria), but since measured acetate concentrations always reflect the net difference between production and consumption, overall levels were not significantly increased.

Conversely, concentrations of butyrate were significantly higher, because this is the end-point of fermentation; *in-vivo*, the majority of butyrate is utilized by colonocytes (Hamer et al., 2008) but since these are not present in the *in-vitro* gut model, butyrate accumulates in the luminal medium. Since dysbiosis in UC patients has been linked to a reduction in butyrate-producing species (Machiels et al., 2014), the increase in butyrate seen here might confer a positive clinical effect. Given the numerous positive effects of butyrate on human health, many attempts have been made to formulate butyrate supplements; unfortunately, butyrate has a strongly unpleasant odour (Bedford and Gong, 2018) and is largely absorbed in the upper GIT (Pituch et al., 2013) and formulation of sodium butyrate in coated pellets proved unsuccessful in modulating gut function in rats (Tuleu et al., 2001). The data presented here suggest that a properly formulated probiotic supplement may be a better approach, stimulating the existing microbiota to produce butyrate rather than supplying it as a dietary supplement.

Since many gut conditions are diseases of inflammation, the effect of probiotics on modulating inflammatory responses is a critical factor in their clinical effectiveness. Here, the *in-vitro* cell culture model showed no degradation in the integrity of the epithelial barrier, as well as reduced markers of inflammation, when exposed to SHIME media following dosing with Symprove. Levels of the anti-inflammatory cytokines NK-κB and IL-1β were unchanged, while IL-6 was increased and IL-10 was significantly increased. Concomitantly, levels of the inflammatory chemokines MCP-1, CXCL 10 and IL-8 were reduced. These results correlate with previous clinical studies, in which fecal

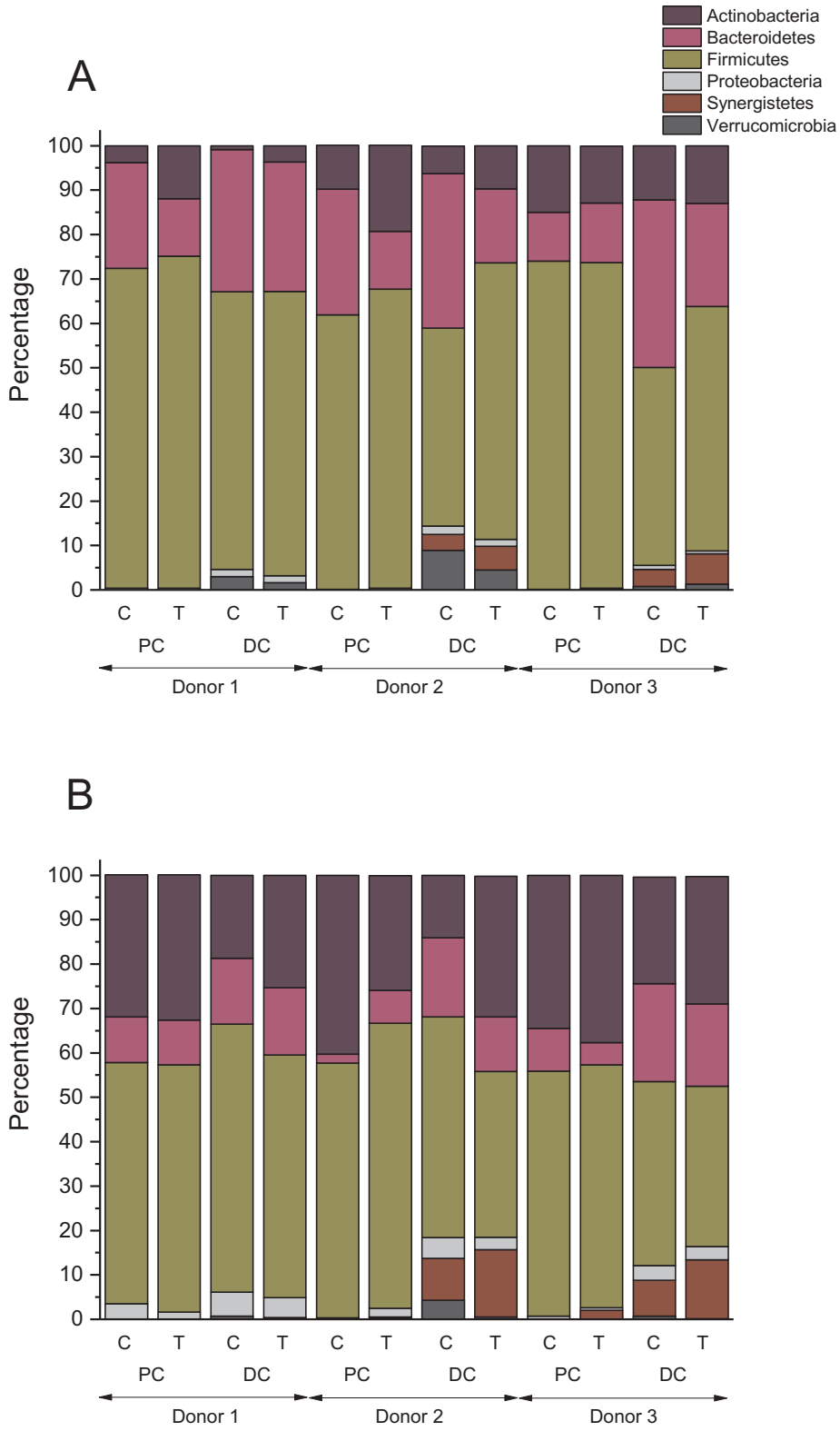

Fig. 5. Abundance (%) of the dominant phyla in the luminal (A) and mucosal (B) compartments of the proximal (PC) and distal (DC) colons at the end of the control (C) and treatment (T) periods for three human donors (n = 1).

calprotectin levels were reduced in diverticular disease (Kvasnovsky et al., 2017) and IBS (Sisson et al., 2014).

5. Summary

The data show that when a probiotic suspension is formulated in such a way as to address the challenges of oral delivery in humans, then viable probiotic species can be delivered to the gut. Once there, the bacteria can infiltrate, colonise and proliferate in the luminal and mucosal compartments. It is important to remember that the cell culture model is exposed to M-SHIME media, not the individual probiotic species, meaning it is the *change in the microbiota as a whole* that is

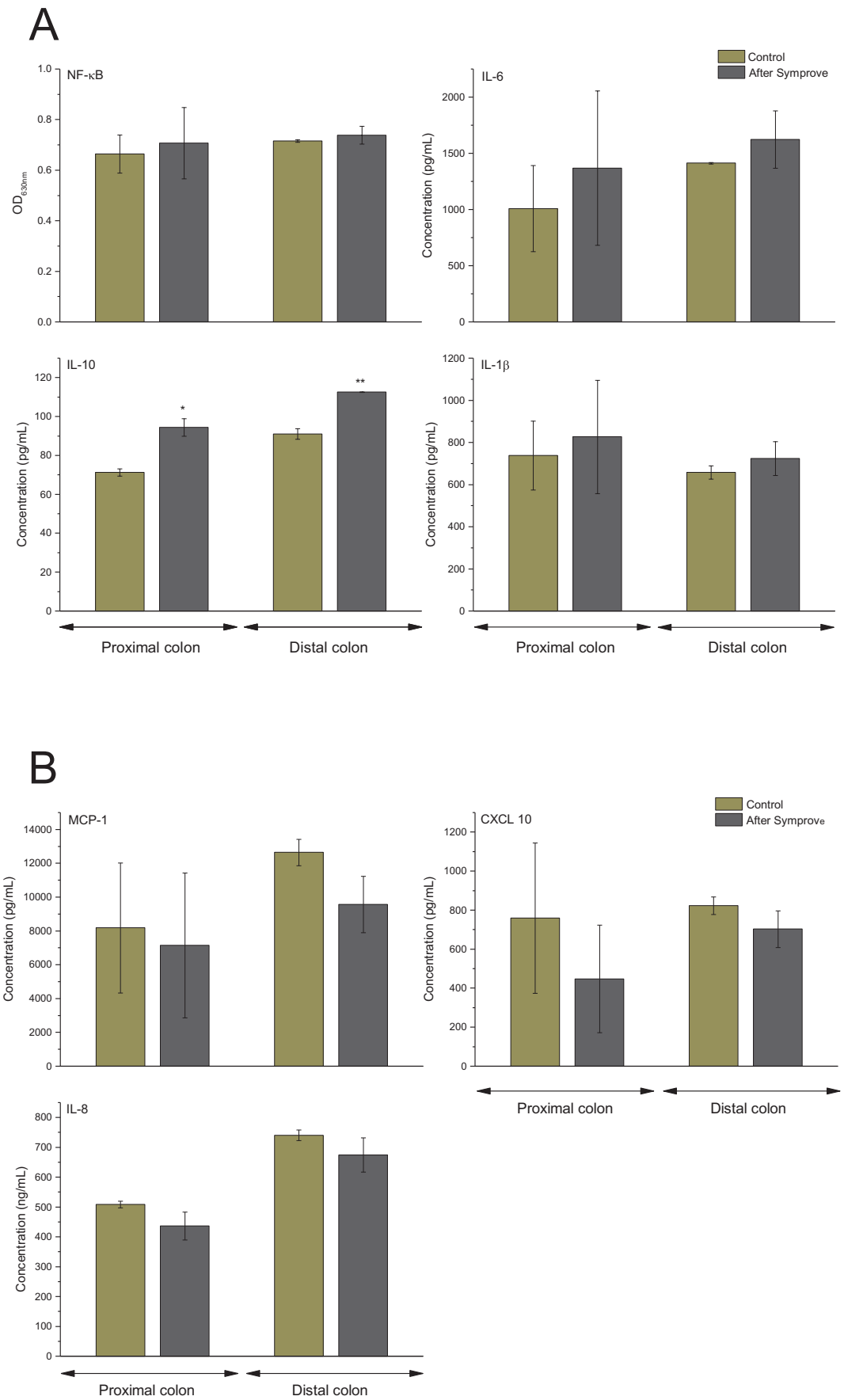

Fig. 6. Effect of SHIME samples (control and after dosing with Symprove) on the secretion of (A) anti-inflammatory cytokines (NF-κB, IL-6, IL-10 and IL-1β) and (B) inflammatory chemokines (MCP-1, CXCL 10 and IL-8). Results that are of statistical significance compared with the control are indicated with * ($P < 0.05$) and ** ($P < 0.01$).

modulating the immune response. This is a critical distinction; the World Health Organisation definition, which requires probiotics to "confer a health benefit on the host" (Hill et al., 2014), is often interpreted as meaning it must be demonstrated that the probiotic species itself must by some metabolic mechanism cause a positive effect *in-vivo*. The data presented here clearly suggest that in fact integration and colonisation of the probiotic species within the existing microbiota, and the generation of a utilizable nutrient (lactate), stimulates growth of the largely beneficial phyla meaning that it is the *rebalancing* of bacterial families that confers health benefits to the host. This rebalancing effect is seen here even though the microbiota were obtained from three healthy donors; since many gut diseases, as noted above, are linked to dysbiosis it seems likely that the mechanism of rebalancing is the major cause of improvement in clinical symptoms, rather than any effect from an individual probiotic species. It is notable also the data show no negative influences on gut health.

Previous work (Fredua-Agyeman et al., 2017) has shown that *Lactobacillus* spp. and *Bifidobacterium* spp. can exert anti-pathogenic action against *Clostridium difficile*. Thus, delivery of these probiotic species may offer an alternative treatment option for patients with recurrent gut infections, before more radical measures such as fecal microbiota transplant (Petrof and Khoruts, 2014).

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Declaration Of Interest

None

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2018.11.020.

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