Beneficial Microbes, 2021; 12(6): 601-612

Wageningen Academic Publishers

A blend of 3 mushrooms dose-dependently increases butyrate production by the gut microbiota

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Received: 6 February 2021 / Accepted: 7 September 2021

RESEARCH ARTICLE

Abstract

The gut microbiota has been indicated to play a crucial role in health and disease. Apart from changes in composition between healthy individuals and those with a disease or disorder, it has become clear that also microbial activity is important for health. For instance, butyrate has been proven to be beneficial for health, because, amongst others, it is a substrate for the colonocytes, and modulates the host's immune system and metabolism. Here, we studied the effect of a blend of three mushrooms (*Ganoderma lucidum* GL AM P-38, *Grifola frondosa* GF AM P36 and *Pleurotus ostreatus* PO AM-GP37)) on gut microbiota composition and activity in a validated, dynamic, computer-controlled *in vitro* model of the colon (TIM-2). Predigested mushroom blend at three doses (0.5, 1.0 and 1.5 g/day of ingested mushroom blend) was fed to a pooled microbiota of healthy adults for 72 h, and samples were taken every day for microbiota composition (sequencing of amplicons of the V3-V4 region of the 16S rRNA gene) and activity (short-chain fatty acid (SCFA) production). The butyrate producing genera *Lachnospiraceae* UCG-004, *Lachnoclostridium*, *Ruminococcaceae* UCG-002 and *Ruminococcaceae* NK4A214-group are all dose-dependently increased when the mushroom blend was fed. Entirely in line with the increase of these butyrate-producers, the cumulative amount of butyrate also dose-dependently increased, to roughly twice the amount compared to the control (medium without mushroom blend) on the high-dose mushroom blend. Butyrate proportionally made up 53.1% of the total SCFA upon feeding the high-dose mushroom blend, compared to 27% on the control medium. In conclusion, the (polysaccharides in the) mushroom blend led to substantial increase in butyrate by the gut microbiota. These results warrant future mechanistic research on the mushroom blend, as butyrate is considered to be one of the microbial metabolites that contributes to health, by increasing barrier function and modulating inflammation.

Keywords: *Ganoderma lucidum*, *Grifola frondosa*, *Pleurotus ostreatus*, *in vitro* colon model, SCFA, butyrate

1. Introduction

The gut microbiota, containing trillions of bacteria, archaea, fungi and yeasts, and bacteriophages and viruses, has been shown over the past decades to play a crucial role in health and disease (Matijasic *et al.*, 2020; Vemuri *et al.*, 2020). Diseases and disorders affected by the gut microbiota range from those occurring in the gastrointestinal (GI) tract, where these micro-organisms reside, to diseases elsewhere in the body. The diseases of the GI tract include inflammatory bowel disease (IBD) (Lavelle and Sokol, 2020), irritable bowel syndrome (IBS) (Collins, 2014) and colorectal cancer (CRC) (Fan *et al.*, 2021). Systemic diseases and disorders include, amongst others, allergy of skin (Petersen *et al.*, 2019) and lungs (Barcik *et al.*, 2020), obesity and associated cardiovascular diseases (Marzullo *et al.*, 2020), type I diabetes (Verduci *et al.*, 2020) and other autoimmune diseases (Reyes-Castillo *et al.*, 2021), and even phenomena related to the brain, such as brain development and cognition (Almeida *et al.*, 2020), Parkinson's and Alzheimer's disease (Ceppa *et al.*, 2020), and autism spectrum disorders (MacFabe, 2015). Although the full spectrum of mechanisms by which the gut microbiota interacts with the host are still being discovered,

ISSN 1876-2883 print, ISSN 1876-2891 online, DOI 10.3920/BM2021.0015

it is clear that the microorganisms influence our immune system and metabolism (Backhed *et al.*, 2004; Geurts *et al.*, 2014), through e.g. interacting directly with immune cells, modulating gene expression in a variety of tissue, and/or production of metabolites which are absorbed through the gut epithelium and transported to distant organs through blood and lymph systems.

One of the metabolites produced by the gut microbiota that has received a lot of attention is butyrate. Butyrate is one of the short-chain fatty acids (SCFA), the others being acetate and propionate, which are organic acids produced within the lumen of the colon by the gut microbiota mainly through fermentation of undigested/indigestible dietary carbohydrates (Blaak *et al.*, 2020). Undigested/indigestible dietary and endogenous protein, such as the protein-part of mucus, and sloughed epithelial cells, can be fermented into SCFA and branched-chain fatty acids (BCFA) (Topping and Clifton, 2001). Butyrate is considered to be health-promoting, as it has been shown to have anti-carcinogenic and anti-inflammatory effects, and it strengthens the gut barrier function (Hamer *et al.*, 2008). Its production from carbohydrate fermentation is thought to be more important for health than from protein fermentation, as protein fermentation also leads to various putrefactive metabolites, which are considered to be toxic to the host (Macfarlane and Macfarlane, 2012).

A prebiotic is defined as 'a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health' (Gibson *et al.*, 2017). Where in the past 'prebiotic' was synonymous to 'bifidogenic' (increase in *Bifidobacterium*) effects (Gibson and Roberfroid, 1995), the latest definition also allows room for health benefits related to microbial activity (amongst other production of SCFA).

Mushrooms have been the subject of intense research due to their bioactive components. Medicinal mushrooms have been used for centuries in Asia, originating in China, and are currently still applied as traditional Chinese medicine (TCM) (Martel *et al.*, 2017; Zhou *et al.*, 2019). Mushrooms produce a large number of pharmaceutically active proteins, which have become popular sources of natural antitumor, antimicrobial or immunoenhancing agents (Zhou *et al.*, 2019). Moreover, polysaccharides and some small-molecule components, such as flavones, other polyphenols and terpenes, are present in mushrooms, and these may interact with the (immune system of the) host or its gut microbiota (Jayachandran *et al.*, 2017). Carbohydrates are the major components in mushrooms, accounting from 35 to 70% of the dry weight, with variations in different species. Among these are polysaccharides, such as β-glucans, consisting primarily of β-(1→3) linkages with some β-(1→6) branches, as well as hetero-polysaccharides containing mannose, galactose, fucose, xylose and rhamnose. Mushroom polysaccharides have been shown to possess a variety of therapeutic and gut microbiota modulation benefits (Cheung, 2013; Ruthes *et al.*, 2016; Sameer Kumar *et al.*, 2016), e.g. the β-glucans are also known to promote butyrogenic bacteria in the colon, thus producing butyrate (Friedman, 2016; Han *et al.*, 2020; Jayachandran *et al.*, 2018).

In vivo, it is quite difficult, if not impossible, to disentangle the direct effects that the mushroom bioactives have on the immune system and those occurring through the gut microbiota (such as butyrate production). Effects on the gut microbiota are therefore usually tested *in vitro*, using models that mimic the colon. One of these models is the TNO *in vitro* model of the proximal colon (TIM-2) (Minekus *et al.*, 1999), which is a validated, dynamic, computer-controlled system that accurately mimics host physiology in the colon with respect to temperature, pH, removal of microbial metabolites (mimicking absorption through the gut epithelium) and presence of a dense, active gut microbiota (Venema, 2015). It has been used extensively to test the effect of prebiotics, potential novel prebiotics and dietary carbohydrates on gut microbiota composition and activity (Larsen *et al.*, 2019; Martina *et al.*, 2019; Miguez *et al.*, 2020a,b; Sayago-Ayerdi *et al.*, 2019; Venema *et al.*, 2020).

The aim of the study described here was to investigate the potential prebiotic effect of a blend of three mushrooms by investigating the effect on composition and activity of the gut microbiota. This was studied using the validated TNO dynamic *in vitro* model of the colon (TIM-2). Changes in composition (using sequencing of the V3-V4 region of the 16S rRNA gene) and activity (SCFA production) of the gut microbiota of healthy adults upon feeding three different doses of the mushroom blend were tested.

2. Materials and methods

Characteristics of the mushroom blend

A proprietary blend of three mushrooms (*Ganoderma lucidum* GL AM P-38, *Grifola frondosa* GF AM P36 and *Pleurotus ostreatus* PO AM-GP37) was provided by Aloha Medicinals (Carson City, NV, USA) as a fine coarse powder.

Predigestion of the mushroom blend

To remove digestible components and components that would normally be absorbed in the small intestine, the mushroom blend was predigested in bulk. This protocol was adapted from Brodkorp *et al.* (2019) and Minekus *et al.* (2014). However, it included dialysing the digestion products using a dialysis unit that is incorporated in TNO *in vitro* model of the stomach and small intestine (TIM-1) (Minekus *et al.*, 1995). This unique dialysis membrane (Sureflux 07 L, Nipro Europe, Zaventem, Belgium) removes

digestion products (and water) and prevents them from reaching the colon (where they would normally also not arrive). After predigestion, this slurry was freeze dried and ground to a fine particle size, to get a homogeneous powder, which was used in subsequent fermentation experiments in TIM-2.

TNO's *in vitro* model of the colon (TIM-2)

TIM-2 (Supplementary Figure S1) simulates the large intestine or colon, consisting of four interconnected compartments containing flexible membranes with a volume of 125 ml. By applying pressure on the flexible membrane, peristaltic movements are accomplished (Venema, 2015). Average conditions of healthy human individuals were simulated including: body temperature (37 °C), pH in the lumen (pH 5.9), composition and rate of secretion fluids, delivery of a predigested substrate, mixing and transport of intestinal contents by peristalsis, absorption of water and microbial metabolites through the means of a dialysis system, and presence of a complex, high density, metabolically active and anaerobic microbiota of human healthy adults as described in detail before (Venema, 2015; Venema *et al.*, 2020). The dialysis system is a crucial component of the model as it prevents accumulation of microbial metabolites, which would otherwise inhibit or even kill the members of the gut microbiota. The model was inoculated with a standardised microbiota of healthy adult human volunteers (Venema *et al.*, 2000). For this, faeces were collected from healthy adult volunteers from the department (n=6; 3 male, 3 female; average age 25.8±3.2 years) and pooled in an anaerobic cabinet to allow for a standardised microbiota (Venema *et al.*, 2000) that could be used throughout the experiments, and allowed for comparison between runs. We have shown before, when studying carbohydrate fermentation, that pooling the microbiota from different individuals leads to a pool with the same metabolic capacity as observed in the individual samples (Aguirre *et al.*, 2014), likely due to high functional redundancy in metabolic capacity of members of the gut microbiota (Moya and Ferrer, 2016; Thursby and Juge, 2017), especially with respect to carbohydrate fermentation (Aguirre *et al.*, 2014). The pooled microbiota was aliquoted, snap frozen in liquid nitrogen and stored at -80 °C until inoculation in the model. Four units were run in parallel. After an adaptation period of 16 h, in which the inoculum adapted to the conditions in the system (Venema *et al.*, 2000), the different doses of the substrates were fed to the microbiota over a period of 3 days through the feeding syringe.

Experimental set-up

Three doses of predigested mushroom blend were tested, corresponding to 0.5 g/day, 1 g/day and 1.5 g/day of ingestion. The predigested blend was added to the standard medium used in TIM-2 (standard ileal efflux medium – SIEM; Supplementary Table S1). The predigested mushroom blend was added to the standard carbohydrates used in the control medium (SIEM). This medium was originally developed by Gibson *et al.* (1988) and mimics the complex undigestible carbohydrates (pectin, xylan, arabinogalactan, amylopectin and resistant starch) on an average Western diet.

Sampling

Samples were taken every day for a period of three days from both the lumen and the dialysate of the system (at 0, 24, 48 and 72 h). Samples from both the lumen and dialysate were measured for SCFA concentrations. The production of metabolites at the moment of addition of the substrates was artificially set to zero, and cumulative production of the different SCFAs was calculated from that moment onwards. Samples from the lumen of the model were analysed on composition of the microbiota by sequencing the V3 – V4 region of the 16S rRNA gene using Illumina sequencing as indicated below.

Short chain fatty acid analyses

Both lumen (1.5 ml) and dialysate (2 ml) samples were centrifuged at 14,000×*g* for 10 min. Fifty µl (lumen) or 150 µl (dialysate) samples were mixed with 650 µl (lumen) or 550 µl (dialysate) internal standard solution, containing methanol, internal standard (2 mg/ml 2-ethyl butyric acid), and formic acid (20%). The analysis was carried out on a GC-MS (8890 GC System; Agilent Technolgies, Amstelveen, the Netherlands) equipped with a PAL3 RSI 85 autosampler (Agilent) by injecting 1 µl sample on a DB-FATWAX Ultra Inert column (30 m, 0.25 mm, 0.25 µm Agilent). The temperature settings of the injector port, oven, flame-ionization detector and mass spectrometer detector were 250, 200, 275 and 225 °C, respectively. The flow rate over the column was 1.2 ml|/min.

Illumina MiSeq sequencing of V3-V4 region of the 16S rRNA gene

Samples for microbiota composition were analysed by sequencing PCR amplicons of the V3-V4 region of the 16S rRNA gene. Briefly, the isolation of genomic DNA from the faecal samples (1 ml lumen sample) was performed using standard molecular biology kits from ZYMO Research as provided by the manufacturer (BaseClear, Leiden, the Netherlands). The PCR amplification of the V3 and V4 regions, the barcoding and the library preparation were carried out according to established protocols provided by Illumina (Nextera XT DNA Library Preparation Kit and TG Nextera® XT Index Kit v2 Set A, Illumina, Eindhoven, the Netherlands) using the following primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and the 785R

(5′-GACTACHVGGGTATCTAATCC-3′) appended with Illumina adaptor sequences. The sequencing was carried out using the Illumina MiSeq system (MiSeq Reagent Kit v3, Illumina) and later the sequences were converted into FASTQ files using the BCL2FASTQ pipeline version 1.8.3. The quality cut was applied based on the Phred quality score. Quantitative Insights Into Microbial Ecology 2 (QIIME 2) software package (2019.4) was used for microbial analyses (Caporaso *et al.*, 2010; Estaki *et al.*, 2020). The sequences were classified using Greengenes (version 13.8) as a reference 16S rRNA gene database.

Statistical analyses

Correlations between Operational Taxonomic Units (OTUs) and test-products were investigated using the non-parametric Kruskal-Wallis test, by using the software package R (3.5.3) (R Core Team, 2013) in RStudio. Multiple comparison was corrected using the Benjamini-Hochberg false discovery rate (FDR), and *q*-values (FDR-adjusted *P*-values) were considered significantly different at a cut off of <0.1.

3. Results and discussion

Predigested material

After predigestion, most of the protein from the mushroom blend was removed. Protein that was left was mostly coming from the digestive enzymes added to predigest the blend (data not shown). The material, from which digestible material was removed through dialysis using a membrane used in hospital for kidney-failure patients, primarily contained carbohydrates (β-glucans and heteropolysaccharides; see below) and material attached to cell-wall structures, such as polyphenols, tannins, etc. For the purpose of the current study the latter were not quantified. Recovery of predigested material was 65% of the material that was initially added to the predigestion, and the material contained 90% carbohydrates (w/w), and 5% protein (w/w) (data not shown).

TIM-2 set-up

To study the potential prebiotic effects of the blend of three mushrooms, the effect of the blend on gut microbiota composition and activity was evaluated in TIM-2. As most carbohydrates are fermented in the proximal part of the colon, the model mimicked the conditions in the ascending colon. After an overnight adaptation period, in which the SIEM (Cuevas-Tena *et al.*, 2019) was fed, the predigested mushroom blend was fed to the standardised microbiota in TIM-2 at 0 (SIEM only), 0.5 g/day, 1.0 g/day and 1.5 g/day for a period of 3 days. The blend was added to SIEM. Each day samples were taken from the lumen and dialysate of TIM-2 and changes in microbiota composition and activity were measured.

Changes in microbiota composition

Changes in composition of the microbiota were evaluated by sequencing amplicons of the V3-V4 region of the 16S rRNA gene. Since a standardised microbiota was used, all experiments started with the same composition at time point zero, as can be seen by the close clustering in the weighted UniFrac principal coordinate analysis (Supplementary Figure S2A), a measure for beta-diversity. Over time, the units fed with the mushroom blend moved away from those fed with SIEM only in the weighted UniFrac (Supplementary Figure 2B) indicating changes in composition at the genus level (see below). There were no differences at phylum level (Supplementary Figure S3).

Using the non-parametric Kruskal-Wallis test, we studied which operational taxonomic units (OTUs), at the level of bacterial genera, were different between treatments. This was done at the level of the individual 'interventions' (each dose of mushroom blend separately), as well as grouping the three doses together and looking at the level of 'substrate' (control vs mushroom blend). After correction for multiple comparison, we apply a strict cut-off for significance. For *q*-values <0.1 (the corrected *P*-value after correction for multiple comparisons using false-discovery rate) we consider the difference to be significant, and for a *q*-value between 0.2 and 0.1 as a trend. Figure 1A-E shows the OTUs that are significantly different when looking at the individual doses. The plots with OTU names in green show a *q*-value <0.1, while those with names in orange have a *q*-value between 0.1 and 0.2. The latter are shown as well, because some of these become significant when all three doses of the mushroom blend are grouped together (Figure 1F-I). The data show a significant difference for *Pediococcus* when tested at the individual doses, which can be attributed to its increase at the highest dose of mushroom blend tested. If all doses are grouped together, the significance is lost (data not shown). The other OTUs, *Lachnospiraceae* UCG-004, *Lachnoclostridium* and the two *Ruminococcaceae* (UCG-002 and NK4A214-group) are all dose-dependently increased when the mushroom blend is fed (significant for *Lachnospiraceae* UCG-004 and *Ruminococcaceae* UCG-002; trend for the others). This is interesting, because these OTUs are well-known butyrate producers. When examining the difference at the level of all three doses of mushroom blend combined (Figure 1F-I), the butyrate producing OTUs *Lachnoclostridium* and *Ruminococcaceae* NK4A214 also become significant. The boxplots show all time-points (T24, T48 and T72) together. Supplementary Figure S4 shows the development of the relative abundance at the individual time-points for *Lachnospiraceae* UCG-004, *Lachnoclostridium* and the two *Ruminococcaceae* (UCG-002 and NK4A214-group). This data shows that these taxa

(dose-dependently) increase over time compared to the condition with SIEM, where these taxa stay more or less at the same abundance or decrease over time.

Production of microbial metabolites

Apart from composition of the microbiota, we also studied its activity. The major microbial metabolites that have been implicated in health are the SCFA (acetate, propionate and butyrate). Especially butyrate has attracted attention over the past decades as it has been shown to be the primary substrates for the colonocytes, epithelial cells of the colon, and has been shown to be beneficial in inflammation in the gut, due to its effects on gene-expression in immune and other host cells (Hamer *et al.*, 2008).

Figure 2 shows the cumulative SCFA production when the various interventions are fed to the gut microbiota. The profile on the control medium (SIEM) shows that acetate is the major SCFA produced. *In vivo* the ratio of acetate:propionate:butyrate is in the order of 60%:20%:20% (Cummings *et al.*, 1987). For SIEM this is also observed in TIM-2 (Table 1). The cumulative amount of total SCFA produced after the 3-day experiment is 143.8 mmol (Table 2). No differences in production of BCFA was observed (data not shown).

Strikingly, but entirely in line with the increase in relative abundance of the butyrate producing OTUs in Figure 1, upon feeding the three different doses of the mushroom blend, a dose-dependent increase in butyrate proportion

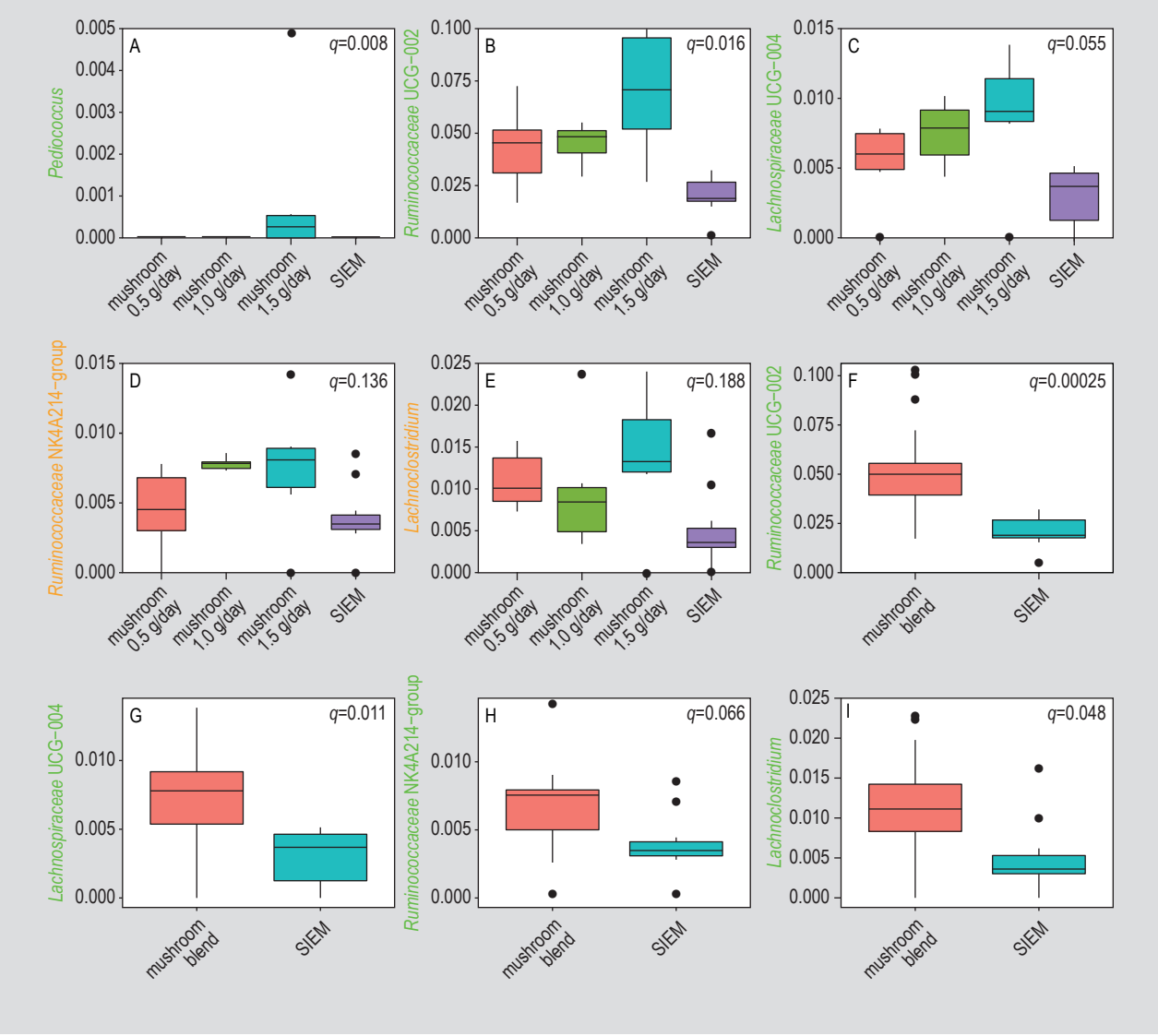

Figure 1. Boxplots of the operational taxonomic units (OTUs) that are different between the different doses and standard ileal efflux medium (SIEM) (A-E) or between the blend (all doses combined) and SIEM (F-I). The *q*-values after false discovery rate-correction of Krukal-Wallis analysis are provided. A *q*-value <0.1 is considered significant (taxa font in green); one between 0.1 and 0.2 is considered a trend (taxa font in orange).

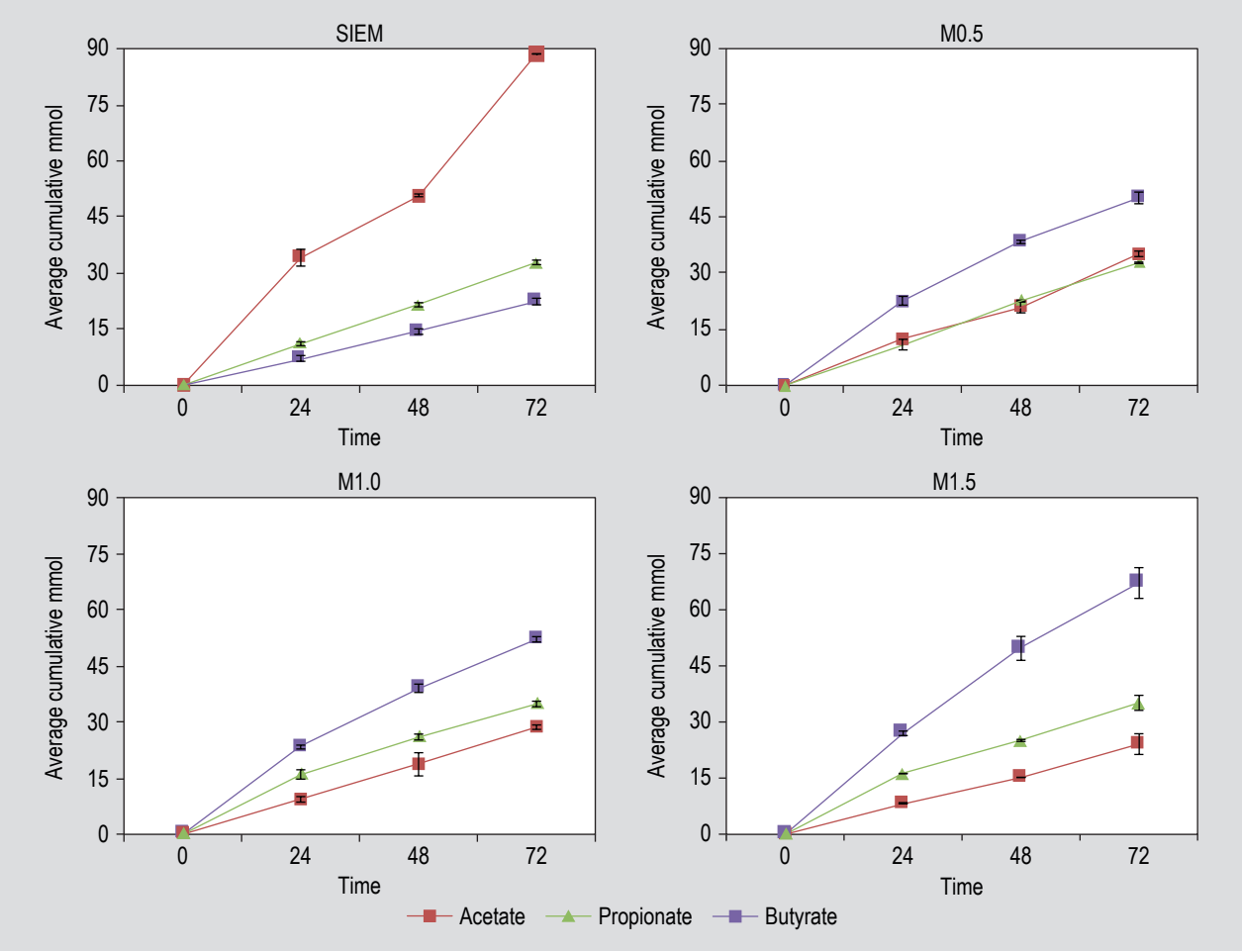

Figure 2. Cumulative production of the short chain fatty acid (SCFA) acetate, propionate and butyrate on the different interventions. M0.5, M1.0 and M1.5: mushroom blend at 0.5 g/day, 1.0 g/day and 1.5 g/day, respectively. SIEM: simulated ileal efflux medium. Error bars indicated range.

Table 1. Ratio of the different short chain fatty acids (SCFA) at time point 72.[1]

Intervention/ metabolite	Acetate	Propionate	Butyrate
SIEM	53.3±0.5%	19.8±0.2%	27.0±0.3%
M0.5	29.8±11.5%	27.8±4.1%	42.4±7.4%
M1.0	24.8±14.4%	30.1±5.3%	45.1±9.1%
M1.5	19.1±8.7%	27.8±4.5%	53.1±14.3%

[1] M0.5, M1.0 and M1.5: mushroom blend at 0.5 g/day, 1.0 g/day and 1.5 g/day, respectively; SIEM: simulated ileal efflux medium, control.

Table 2. Amount of total short chain fatty acids (SCFA) (mmol) and amount of carbon (C) in the microbial metabolites at time point 72.[1]

Intervention/sum of metabolites	Total SCFA (mmol)	Amount of C
SIEM	143.8±1.4	365.3±3.4
M0.5	118.0±1.7	369.0±5.4
M1.0	115.6±2.0	370.3±6.3
M1.5	126.4±3.3	422.3±16.9

[1] M0.5, M1.0 and M1.5: mushroom blend at 0.5 g/day, 1.0 g/day and 1.5 g/day, respectively; SIEM: simulated ileal efflux medium, control.

is observed (Table 1). The ratio of butyrate at the lowest dose of the mushroom blend is 42.4% (compared to 27.0% of the control medium), and this increases to 45.1% for the medium dose, and 53.1% for the high dose. The latter is almost 2-fold that produced on the control medium. Also, the proportion of propionate increases, but not dose-dependently, and is 27-30%. Of course, if the proportions of butyrate and propionate increase, the proportion of acetate has to drop (Table 1). With the changes in proportion of the individual SCFA towards more propionate and butyrate,

the sum of SCFA produced is reduced (Table 2), from 143.8 mmol at T72 for the control down to 118 mmol for the low dose mushroom blend, 115.6 for the middle dose, and 126.4 for the high dose. Although it seems that on the mushroom blend the production of SCFA is thus lower, this is skewed by the fact that acetate only contains 2 carbon-atoms, while butyrate contains 4 (and propionate 3). So, for every molecule of butyrate twice the number of carbon-atoms are needed than for acetate. If we take that into consideration, then rather than a reduction in amount when expressed as mmol, a small increase is observed when expressed in amount of carbon (C) (Table 2). Of course not all C-atoms end up in SCFA, and the results should not be considered an attempt to make a mass-balance, but we have shown before from ^{13}C-labeled substrates that 95% of the labelled C got incorporated in SCFA (Binsl *et al.*, 2010; de Graaf *et al.*, 2010).

It is unlikely that the almost two-fold increase in butyrate proportion can only be attributed to the increases observed in relative abundance of the four butyrate producing taxa. Collectively, in the high dose mushroom blend, these make up approximately 11% of the observed OTUs, while this is 7 to 8% in the other doses. Although it is possible that these taxa are modulated by the mushroom blend to such an extent that their metabolism is geared primarily towards butyrate production, it is likely that other taxa, although not significantly modulated in their relative abundance, contribute to the observed changes in butyrate production. There are some (non-significant) dose-dependent increases in relative abundance of *Ruminococcus gauvreauii*-group, *Ruminococcaceae* UCG-008, *Dorea*, an uncharacterised genus in *Lachnospiraceae*, and an uncharacterised genus in *Erysipelotrichaceae*, that together also account for 2% (low dose) to 5.6% (high dose) of the total relative abundance. It is also possible that the metabolism of other butyrate producers is shifted, without affecting their relative abundance. The conclusions drawn here are based on relative abundance. Although in the current experiments we did not quantitatively measure total bacteria, in previous experiments we have shown that the total number of bacteria remain relatively constant in the model, because we removed an appropriate amount of volume from the system daily to simulate passage to the distal colon (or 'going to the bathroom'). The results obtained here are therefore indeed likely the result of a reduction in acetate/propionate producers as well as an increase in butyrate producers. However, as mentioned above we cannot exclude that the metabolism of certain taxa changed (from acetate/propionate to butyrate), without major shifts in composition. Future studies with ^{13}C-labelled substrates, similar to the ones done by us and others before, might shed a light on that (Binsl *et al.*, 2010; de Graaf *et al.*, 2010; Lamichhane *et al.*, 2018).

In general, ruminococci (including *Ruminococcaceae* UCG-002 and NK4A214-group) are stimulated by dietary fibre (e.g. (De Angelis *et al.*, 2015; Reider *et al.*, 2020)). The *Ruminococcus gauvreauii*-group has been shown before to be stimulated by fibres (e.g. Long *et al.*, 2020) and is not inhibited by polyphenols (Firrman *et al.*, 2016). In addition, recently it was shown that members of the family *Lachnospiraceae* are stimulated by dietary fibre, but depending on the source of fibre (Shang *et al.*, 2021). Similarly, *Lachnoclostridium* has been shown to be stimulated by some dietary fibres (wheat bran and levan; Adamberg *et al.*, 2018; Shang *et al.*, 2020), but not by others (type 2 resistant starch; Zhang *et al.*, 2020). Also *Dorea* is stimulated by dietary fibre (e.g. Chen *et al.*, 2020a) and polyphenols (e.g. Kilua *et al.*, 2020). Moreover, also *Erysipelotrichaceae* has been shown to be stimulated by fibre intake (e.g. Lamichhane *et al.*, 2018), and in this study with ^{13}C-labelled polydextrose was correlated with increased SCFA production.

Fungi are remarkable for the variety of high-molecular-weight polysaccharide structures that they produce, which are found in all parts of the mushroom (e.g. Wachtel-Galor *et al.*, 2011; Yang *et al.*, 2019). Most studies that investigated the effects of medicinal mushrooms on gut microbiota composition and/or activity have used the purified polysaccharides and animal models. Moreover, very few studies have looked at the (potential synergistic) effects of mushroom blends. For instance, the polysaccharide of one of the mushrooms in the blend *G. lucidum*, has been tested in various rodent models (mice, rats and hamsters) for the effect on gut microbiota modulation, usually with the primary aim to look at immuno-modulation, using polysaccharide purified either from spores or from mycelium (Chen *et al.*, 2020b; Jin *et al.*, 2019; Khan *et al.*, 2019; Su *et al.*, 2018; Tong *et al.*, 2020; Xie *et al.*, 2019). *G. lucidum* has been shown to contain linear and branched β-glucans (Liu *et al.*, 2017), including a highly branched β-glucan found in *G. lucidum* spores (Wang *et al.*, 2017). Although the main polysaccharide present in *G. lucidum* is composed of glucose, other monosaccharides were found in varying proportions, such as arabinose, galactose, xylose and mannose, suggesting the presence of polysaccharides with different chemical structures. Heterogalactans composed of rhamnose, galactose and glucose, and side chains composed of glucose have been described (Pan *et al.*, 2012; Ye *et al.*, 2008). Moreover, other isolated fractions, like oil (Wu *et al.*, 2020) and the triterpenoid ganoderic acid A (Guo *et al.*, 2020b) have been shown to modulate the rodent gut microbiota. Similarly, the purified polysaccharide of *G. frondosa* has been shown to modulate the gut microbiota in rodents (mice and rats) in conjunction with beneficial changes in glucose metabolism and lipid metabolism disorders (Chen *et al.*, 2019; Guo *et al.*, 2020a; Li *et al.*, 2019a,b; Pan *et al.*, 2018, 2020). β-glucans, but also α-glucans are known to be the main component of

G. frondosa, but also a heteropolysaccharide composed of (1→6)-α-D-Galp with O-2 linked residues of α-Manp α-Fucp have been found (Jayachandran *et al.*, 2018; Ruthes *et al.*, 2016). Regarding the chemical structure of *P. ostreatus* polysaccharides, β-glucans are the most prevalent, as well as best studied component of *P. ostreatus*. This mushroom was also found to have glucogalactan →1)-α-D-Galp-(→6 with residues of β-L-Glcp at non-reducing ends (Ruthes *et al.*, 2016). The effect of *P. ostreatus* polysaccharides on the gut microbiota composition has been tested in piglets and chickens (Adams *et al.*, 2019; Robinson *et al.*, 2018) and on gut microbiota metabolic activity in *in vitro* batch fermentations using human inocula (Boulaka *et al.*, 2020; Mitsou *et al.*, 2020). Despite the limitations of such batch fermentations, such as accumulations of microbial metabolites, leading to inhibition and/or death of the microbiota, one of these studies found increased proportions of butyrate with feeding of *P. ostreatus* (Mitsou *et al.*, 2020), although the increase was not as evident as observed in our fermentations in a dynamic *in vitro* model with the blend of mushrooms.

To our knowledge whole mushrooms, let alone blends of different mushrooms, have not been tested frequently. Rather purified fractions, mostly the polysaccharide fraction, have been investigated. In our experiments, in a validated *in vitro* model of the proximal colon, that closely mimics physiological conditions in humans, and which has been used for three decades in research on gut microbiology, we show that the blend of *G. lucidum*, *G. frondosa* and *P. ostreatus* has a beneficial effect on gut microbiota composition and activity. The amount of extract used would equal 5-15 gram of fresh mushrooms (taking on average a water-content of 90%). Given that the model is roughly scaled to the *in vivo* situation, one would expect 5-15 g of mushroom ingestion to have the same effect *in vivo*. A number of butyrate producing taxa are increased in relative abundance, which is accompanied by an increase in butyrate proportion. As butyrate is considered to be one of the microbial metabolites that contributes to health, by increasing barrier function and modulating inflammation, it would be good to reproduce these results in a clinical trial.

Supplementary material

Supplementary material can be found online at https://doi.org/10.3920/BM2021.0015

Table S1. Composition of the simulated ileal efflux medium as originally composed by Gibson *et al.* (1988).

Figure S1. Schematic diagram of the dynamic, multi-compartmental TNO in vitro mode of the colon (TIM-2).

Figure S2. Weighted UniFrac of samples coloured by time point and intervention.

Figure S3. Relative abundance of phyla in the different TIM-2 samples treated with 0.5 g/day, 1.0 g/day, 1.5 g/day mushroom blend, or SIEM.

Figure S4. Changes over time of *Lachnoclostridium*, *Lachnospiraceae* UCG-004, *Ruminococcaceae* NK4A214-group and *Ruminococcaceae* UCG-002 upon feeding of 0.5 g/day, 1.0 g/day, 1.5 g/day of the mushroom blend or SIEM.

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