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Specific dietary fibers steer toward distal colonic saccharolytic fermentation using the microbiota of individuals with overweight/obesity

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ABSTRACT

Background: Evidence suggests that increased distal short-chain fatty acid (SCFA) production beneficially impacts metabolic health. However, indigestible carbohydrate availability is limited in the distal colon; consequently, microbes shift toward protein fermentation, often linked to adverse metabolic health effects. We aimed to identify specific fiber(s) that promote saccharolytic fermentation in the distal colon and thereby may (partially) inhibit proteolytic fermentation.

Methods: Potato-fiber, pectin, and inulin were studied individually and in combination against a high (pre-digested) protein background using an *in vitro* model of the colon (TIM-2) inoculated with pooled, standardized fecal microbiota from individuals with overweight/obesity. Microbiota composition and activity were assessed at different timepoints to simulate the travel throughout the colon (proximal: 0-8 h, distal: 8-24 h) and compared to a high protein (HP)_control, receiving only proteins.

Results: Fiber addition increased total SCFA production compared to HP_control (52.11 ± 1.49 vs 27.07 ± 0.26 mmol) whereas total branched-chain fatty acids (BCFA; a marker for protein fermentation) production only slightly decreased (3.31 ± 0.10 vs 4.18 ± 0.40 mmol). Combining potato-fiber and pectin led to the highest total and distal SCFA production and distal SCFA:BCFA. Fiber addition attenuated HP-induced increases in several bacterial taxa including *Mogibacterium* and *Coprococcus,* independent of fiber type. Additionally, time- and fiber-specific microbial signatures were identified: inulin increased *Bifidobacterium* (proximal) relative abundance and pectin and/or potato-fiber increased *Prevotella 9* (distal) relative abundance.

Conclusion: The most marked increase in distal colonic SCFA production was induced by combining potato-fiber and pectin. Further research should elucidate whether this switch toward saccharolytic fermentation translates into beneficial metabolic health effects in humans.

1. Introduction

The gut microbiome, a complex ecosystem of microorganisms inhabiting the human gastrointestinal tract, can break down macronutrients that resist digestion and absorption in the upper gastrointestinal tract. Through this process, metabolites that can affect gut health and various aspects of host physiology, including metabolism and immune function, are produced. A metabolic hierarchy exists for gut microbes, which utilize indigestible carbohydrates including dietary fibers as a preferred energy source, a process referred to as saccharolytic fermentation. However, whereas the bulk of indigestible carbohydrates is large in the proximal colon, saccharolytic energy sources get depleted toward the distal colon. This aligns with an increased use of proteins as an energy source in the distal colon, a process referred to as proteolytic or putrefactive fermentation (Dhingra et al., 2012; Flint et al., 2009). Saccharolytic fermentation produces, among other metabolites, short-chain fatty acids (SCFA), which are considered beneficial for metabolic health. In contrast, proteolytic fermentation results in a diverse set

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of metabolites including branched-chain fatty acids (BCFA), amines, *p*-cresol, and ammonia, which mostly have been implicated with adverse metabolic health effects including insulin resistance and inflammation, two key characteristics in type 2 diabetes mellitus (T2DM) and metabolic syndrome (Blaak et al., 2020; Koh et al., 2016). Keeping in mind the continuing rise in T2DM and obesity prevalence (Guthold et al., 2018; Matthias, 2019), identifying strategies to enhance insulin sensitivity is of significant interest to prevent and/or combat chronic metabolic diseases.

It has been hypothesized that the detrimental effects of proteolytic fermentation can be mitigated by consuming sufficient amounts of dietary fiber, which allows amino acids and peptides to be utilized for the formation of microbial biomass rather than energy production (Dhingra et al., 2012; Flint et al., 2009). Nevertheless, besides the amount of fiber, the location of saccharolytic fermentation and microbial metabolite production may be just as relevant. Previous research has shown that SCFA administration in the distal colon results in pronounced acute metabolic health effects in males with overweight or obesity including an increase in fat oxidation, energy expenditure, and circulating peptide YY (PYY) (Canfora et al., 2017; van der Beek et al., 2016), whereas those effects were not observed with proximal SCFA administration (van der Beek et al., 2016). Additionally, short term administration of fiber mixtures that have been selected to increase distal colonic SCFA production *in vitro* mediate pronounced effects on energy expenditure and postprandial insulin sensitivity in lean individuals *in vivo* (Canfora et al., 2022). SCFA absorption in the distal region can partially bypass the liver through the rectal veins (Canfora et al., 2015) and SCFA absorption is higher in the distal colon compared to the proximal colon (Neis et al., 2019), which may result in higher circulating SCFA levels. SystemicSCFA may exert diverse effects on the pancreas, liver, adipose tissue, and brain and thereby influence substrate metabolism and cognitive function. Through these mechanisms, they have the potential to enhance glucose homeostasis and support weight regulation (Chambers et al., 2018; Korecka & Arulampalam, 2012). Hence, dietary fibers with characteristics that enable fermentation through the entire colon, including the distal colon, may be of major interest in the prevention and treatment of obesity and associated metabolic diseases by promoting a shift from proteolytic to saccharolytic fermentation, (Canfora et al., 2019).

Moreover, individuals with obesity and/or T2DM are reported to have an altered gut microbiota composition compared to lean individuals (Cunningham et al., 2021; Ley et al., 2006; Turnbaugh et al., 2009). To illustrate, the abundance of specific SCFA-producing bacterial taxa, especially those that produce butyrate, appear to be decreased while proteolytic bacteria may be overrepresented (Cunningham et al., 2021; Polidori et al., 2022). Besides differences on a compositional level, animal as well as human studies show functional differences between the microbiota from lean individuals and individuals with obesity pointing toward an increased capacity for protein degradation in obesity (Hu et al., 2024; Ridaura et al., 2013). Consequently, this target population may be more prone to the harmful effects of proteolytic fermentation. High-protein (HP) diets have gained more attention through the years for weight loss interventions because of its proposed association with increased satiety as well as maintenance of muscle mass (Westerterp-Plantenga et al., 2009). Yet, whether HP diets are beneficial in individuals with obesity and/or T2DM remains controversial. Until now, there are no specific dietary guidelines on macronutrient composition for diabetes patients apart from the general nutritional guidelines as meta-analyses do not favor any particular weight-loss diet (Malaeb et al., 2019). From a microbial perspective, these individuals may be more vulnerable for the metabolic consequences of increased protein fermentation. In line, a 12-week dietary intervention comparing a low-protein (LP) to a HP diet in T2DM patients showed that increased protein intake significantly raised the levels of various proteolytic metabolites associated with increased cardiovascular risk (Attaye et al., 2023).

As most proteins (particularly those of animal sources) are efficiently reduced by enzymatic breakdown and absorption in the stomach and small intestine, approximately 10 %–30 % (depending on the type of proteins consumed) escapes digestion and spills over to the colon. Once undigested proteins enter the colon, the absorption capabilities of the colon are limited as it lacks the proteolytic enzymes necessary to digest proteins efficiently. Calculations estimate approximately 4 to 12 g of protein (Blachier, 2023) can reach the colon daily, while others have estimated a slightly higher range from 12 to 18 g (Cummings, 1997a). This aligns with typical protein consumption in Western diets (around 85 g per day), in which a digestion efficiency of about 90 % would result in roughly 8 g of unabsorbed protein (Cummings, 1997a; Portune et al., 2016; Sá et al., 2020). Nonetheless, the amount of protein reaching the colon can vary among individuals and is influenced by several factors such as the type and quality of the proteins (amino acid composition), overall diet composition, and individual differences in protein digestion and absorption (Diether & Willing, 2019). Naturally, increasing the amount of alimentary protein increases the absolute amount of protein directed toward the colon (Cummings, 1997b) and this has been confirmed by *in vitro* (Aguirre, Eck, et al., 2016) as well as *in vivo* (David et al., 2014) studies. Additionally, the source of protein (animal- or plant-based) may influence the protein load directed to the colon (Kaur et al., 2022; Korpela, 2018). A global shift toward plant-based protein consumption is emerging due to its environmental benefits, and several national dietary guidelines now advocate for a transition from animal-based toward plant-based protein sources (Craig et al., 2021; Sabaté & Soret, 2014). Although potential health advantages implicated with components of a plant-based diet have been described (Hemler & Hu, 2019; Satija & Hu, 2018), attention must given to the potential microbial implications of the decreased protein digestibility and subsequently increased protein load and microbial protein degradation in the colon as well as the reduced bioavailability of essential amino acids with consequences for muscle protein synthesis. Microbial implications may be even more troublesome for isolated or purified plant-proteins in which plant-based proteins are removed from their fiber context.

Overall, a nutritional intervention focused on steering toward saccharolytic fermentation in the distal colon through a (mix of) dietary fiber(s) may be of specific interest in individuals with obesity. We hypothesize this increase in saccharolytic fermentation may reduce the microbial switch to increased protein fermentation in the distal colon, resulting in a more optimal gut microbial metabolite profile, potentially conveying beneficial metabolic health effects. Therefore, this exploratory study investigated the ability of different types of fibers and their combinations to steer toward saccharolytic fermentation in a high protein substrate matrix, specifically in the distal colon. Additionally, the effect of protein quantity and protein type on microbial composition and activity were studied. This was achieved by using the validated TNO *in vitro* model of the colon (TIM-2), a dynamic model able to reliably investigate fiber fermentation in the colon (Venema, 2015; Venema et al., 2000). We assessed the production of both classes of microbial metabolites as well as microbial composition in response to three different fibers (either individually or combined): a potato-fiber derived from potato peel (Paselli™), a sugar beet pectin (GENU® BETA), and chicory root inulin (Frutafit® IQ). These fibers were tested against a high (pre-digested) protein background and compared to a HP control without added fibers. The results of this *in vitro* study were used as a screening tool to select fibers, based on their ability to increase distal colonic SCFA (and inhibit BCFA) production, for a clinical study conducted with individuals with obesity and at increased risk of developing T2DM (the DISTAL study, https://www.clinicaltrials.gov./ identifier: NCT05354245).

2. Materials and methods

2.1. Dietary fibers

In this experimental set-up we investigated three different fibers: a

potato-fiber derived from potato peel after the removal of starch (*Solanum Tuberosum spp*) from Avebe (Paselli™, Groningen, the Netherlands), a pectin provided by CP Kelco (GENU® BETA pectin, Großenbrode, Germany) derived from sugar beet (*Beta vulgaris*), and inulin derived from chicory root (*Cichorium intybus*) provided by Sensus (Frutafit® IQ, Roosendaal, the Netherlands). The chemical characterization of Frutafit® IQ inulin and GENU® BETA pectin have been elaborately described elsewhere (Larsen, Bussolo de Souza, et al., 2019; Rösch et al., 2017; Vogt et al., 2013) and several studies have investigated the composition and structural features of fibers extracted from potato peel (Dhingra et al., 2012; Ramaswamy et al., 2013; Rodríguez-Martínez et al., 2021). This selection of fibers was made to include a variety of fibers that differ in solubility and structural complexity and therefore may have different fermentation rates and characteristics.

For each experiment, a total of 7.5 g of fiber(s) was administered to TIM-2, with equal weight contribution for each fiber when tested in combination. We selected a dose of 7.5 g of dietary fiber for our experiments to resemble a typical supplementation dose used by individuals (daily dose of 15 g divided over 2 meals), ensuring physiological relevance and alignment with the DISTAL study.

2.2. Pre-digested proteins

All fibers and fiber combinations were tested against a HP background (12 g, 45 % animal-derived: 55 % plant-derived). We specifically chose this protein quantity and animal:plant-based ratio to promote colonic protein fermentation and align with the global shift toward plant-based protein sources respectively. Additionally, this quantity would be comparable to those achieved by a high (partially plant-based) protein diet in individuals with overweight/obesity. To make the protein mixture, egg protein (Van den Beuken, Meijel, the Netherlands) was used as an animal-based protein source and Tendra fava bean protein isolate (Cosun Protein, Dinteloord, The Netherlands), pea protein isolate (Bulk™, Essex, UK), and SOLANIC 100 F potato protein isolate (Avebe, Groningen, the Netherlands) were used as plant-based protein sources (Supplementary material, Table S1). To also investigate the effect of protein load and type, a LP control with half of the substrate (6 g, 45 % animal derived, 55 % plant-derived) and a high protein control containing solely egg-protein (12 g, 100 % animal-derived) were also tested in TIM-2.

2.2.1. Batch digestion procedure

Since TIM-2 mimics the colon, we performed a batch digestion for each protein. This procedure simulates digestion in the stomach and small intestine to obtain the indigestible protein fraction that would enter the colon. Briefly, 420 g of each protein (separately) was dissolved in a gastric electrolyte solution (GES) (adjusted to, pH 3.0 with 3 mol/L HCl) and the gastric enzymes 1080 mg pepsin from porcine gastric mucosa (Sigma-Aldrich, Gillingham, UK) and 1870 mg lipase from *Rhizopus oryzae* (Sigma-Aldrich) were added and thereafter incubated (30 min, 37 °C). Bottles were shaken manually every 15 min to resemble peristalsis. Thereafter, the pH was adjusted to 7.0 and the small intestine enzymes 315 g of pancreatin (from porcine pancreas (Sigma-Aldrich) dissolved in small intestine electrolyte solution (SIES) and 9 mL of trypsin from bovine pancreas (Sigma-Aldrich), were added and thereafter incubated again (3 h, 37 °C). Bottles were shaken manually every 30 min to resemble peristalsis. Both SIES and GES were prepared as described previously, with minor modifications (no bile was added) (Pentafragka et al., 2022). After enzymatic hydrolysis, samples were centrifuged (20 min, 9000 rpm, Centurion Scientific, K243R, Germany) and supernatants were separated from the pellet. In the supernatant soluble indigestible compounds (free amino acids and oligopeptides) remain. The supernatant was therefore diafiltrated (Sureflux, Nipro Europe NV, Zaventum, Belgium) using a peristaltic pump, removing small digestion products and water. This reduced the volume approximately 20 times. The fraction that was left after dialysis (soluble indigestible fraction) was mixed with the pellet (insoluble indigestible fraction) to get the total indigestible fraction and then freeze-dried (Christ alpha 1–4 LSC plus, Osterode am Harz, Germany) to further reduce volume.

2.2.2. Determination of nitrogen content of each protein before and after batch digestion

For each TIM-2 run, the individual indigestible protein fractions were combined, according to each protein digestibility rate, to form the protein mixture. To determine protein digestibility rate, the total protein content of each protein was determined before and after the batch digestion procedure (Supplementary material, Fig. S1). Nitrogen content was measured by Dumas method (Flash EA 1112 N analyzer, Thermo Scientific), according to manufacturer's protocol. Methionine was used as standard for the nitrogen quantification and 6.25 was used as the average nitrogen conversion factor for protein content. The protein content of the samples was calculated assuming all nitrogen originated from protein. The percentage of undigested protein was calculated for each protein isolate by dividing the protein that was present after digestion procedure (output) by the protein content before the digestion procedure (input).

2.2.3. Amino acid composition of proteins

Each protein sample, both before and after enzymatic hydrolysis, were analyzed for amino acid composition by Qlip B.V., The Netherlands (Supplementary Material, Table S1). Acid hydrolysis and derivatization were performed using the Kairos method. The concentration of each amino acid was determined via liquid chromatography-mass spectrometry (LC/MS), utilizing internal standards for each amino acid and comparison with reference samples. For methionine and cysteine quantification, samples were oxidized using formic acid, followed by hydrolysis with hydrochloric acid at 107.5 ± 2.5 °C. After acid evaporation, norvaline was added as an internal standard. Methionine and cysteine were then separated via liquid chromatography and derivatized post-column with ninhydrin. The final products were quantified at 570 nm and 440 nm using a calibration curve with the Biochrom Bio30+ Lithium Physiological Systems. Tryptophan quantification was performed following alkaline hydrolysis at 100 ± 2.5 °C, followed by neutralization. Separation was achieved using reverse-phase liquid chromatography with a C18 column, and detection was carried out fluorometrically at an excitation wavelength of 281 nm and an emission wavelength of 351 nm. Quantification was based on the internal standard 5-methyl-DL-tryptophan and a single calibration solution.

2.3. Microbiota inoculum: Source, collection, standardization, and processing

Fresh fecal samples were collected from individuals with overweight/obesity and at increased risk of developing T2DM ($n = 14$, male: $n = 4$, female: $n = 10$). These individuals were recruited from a pool of volunteers that were previously enrolled and gave written informed consent in two human studies conducted at Maastricht University Medical Center in the Netherlands: the PERSON study and the AAK study (Trouwborst et al., 2023; Van Deuren et al., 2023), both performed in accordance with the Declaration of Helsinki. Participant's characteristics were as followed: age between 40 and 75 years, body mass index between 25 and 40 kg/m^2, and presence of a disturbed glucose homeostasis (either tissue-specific insulin resistance assessed by a 7-point oral glucose tolerance test or a fasting glucose ≥6.1 mmol/L at the time of enrolment of the beforementioned studies). Exclusion criteria included: BMI < 25 kg/m^2, smoker, regular use of prebiotics, probiotics, or laxatives in the three months prior to donation, gastrointestinal diseases, antibiotic treatment in last three months, >3 kg weight loss is the last three months, and > 3 h/week of intensive physical exercise. To ensure survival of anaerobic bacteria, participants were asked to defaecate in a gastight bag in a fecotainer (DaklaPack

Europe B.V., Lelystad, the Netherlands) and an anaerocult strip (AnaeroGen™, Cambridge, UK) was put on top, whereafter the fecotainer was tightly closed to keep the environment anaerobic. Participants were asked to collect a fresh sample and immediately bring it to Maastricht University, where the samples were put on ice (4 °C) and transported to and processed in the laboratory (Maastricht University Brightlands Campus, Venlo) within 6 h.

Here, samples were immediately homogenized/pooled in an anaerobic cabinet (Sheldon Lab – Bactron IV, Cornelius, OR, USA) as previously described (Aguirre et al., 2014) to create a standardized microbiota stock. Previous studies have revealed standardized pooled microbiota samples closely resemble individual fresh fecal samples, both in composition as well as activity (Aguirre et al., 2014; Logtenberg, Vink, et al., 2020). In brief, pooled fecal samples were mixed with a physiological saline preparation/dialysate fluid and glycerol (10 % final concentration). Subsequently, this mixture was aliquoted, snap-frozen in liquid nitrogen, and stored in −80 °C freezer until inoculation in TIM-2. Before the start of a TIM-2 experiment, the inoculum was thawed in a water bath (1 h, 37 °C).

2.4. TIM-2: A dynamic in vitro model of the colon

TIM-2 is a dynamic, computer-controlled, *in vitro* model developed by TNO (The Netherlands Organization for Applied Scientific Research, the Netherlands) that mimics the colon and has been validated and elaborately described elsewhere (Venema, 2015; Venema et al., 2000). In short, the TIM-2 system has been developed and optimized using microbiota composition and microbial metabolite data from sudden death individuals and the model has demonstrated successful replication of the effects of various dietary components *in vivo* and is considered predictive of *in vivo* conditions (Venema, 2015). The TIM-2 system can simulate peristaltic movement, physiological water content, lumen pH, temperature, metabolites and water removal through dialysis and has a continuous gaseous nitrogen flow to sustain an anaerobic environment. The model contains 4 identical operational units that can run in parallel. Luminal volume is kept constant and controlled by a level sensor, temperature was set at 37 °C and monitored throughout the experiment, and the pH was regulated by a pH sensor and maintained by the addition of NaOH. During the experiment pH was adjusted according to the segment of the colon it should represent (5.8 for 0-8 h mimicking the pH of the proximal colon, 6.4 for 8-9 h, and 7.0 for 9-24 h mimicking the pH of the distal colon).

2.4.1. Growth of standardized microbiota (growth period) and starvation period

After thawing the standardized inoculum, 4 aliquots (each 35 mL) of fecal suspension were transferred to the anaerobic chamber, diluted with dialysate (1:1 ratio) and pooled together. This diluted inoculum was divided over 4 sterile syringes of 60 mL, sealed, and thereafter transferred to administer to each individual TIM-2 unit, which already contained 70 mL of dialysate (so each TIM-2 unit contained approximately 120 mL). The first 16 h after inoculation was a growth period (pH = 5.8), in which each unit was fed with a continuous standard ileal efflux medium (SIEM) drip, which was prepared as described previously (Gibson et al., 1988) with a flow rate of 2.5 mL/h (Fig. 1). This standard growth medium contained a mixture of complex carbohydrates (pectin, xylan, arabinogalactan, amylopectin, and starch), bactopeptone and casein as protein sources, several vitamins and minerals, and bile, which allows for bacterial growth of the inoculum. After this overnight growth period, SIEM feed was discontinued for a 2-h starvation period to ensure any SIEM residue left in the TIM-2 unit was fermented before initiation of the experimental period (Fig. 1). After the starvation period ($t = 0$), samples of both the lumen (inside the system) and dialysate (the liquid that contains the metabolites produced by the microbiota which were dialyzed through the semipermeable hollow fibers) were taken for each unit before adding the fibers and/or proteins, to determine baseline characteristics of each unit.

2.4.2. Administration of protein mixture and fibers (experimental period)

Just before the start of the experimental period, the pre-digested freeze-dried protein mixtures and the fibers and/or fiber mixtures were prepared in the anaerobic chamber by solving them in dialysate solution to allow administration to TIM-2. To simulate ingestion of a meal, all food products were provided though a single shot sample injection. Every unit was first injected with the predigested protein mixture by the port of the lumen. Immediately after, the fiber or fiber mixture was administered through the same port (with exception of the protein controls which did not receive any additional fiber shot). A total of 7.5 g of fiber was administered to TIM-2 and the fibers were tested both separately and in different combinations. Unfortunately, we were

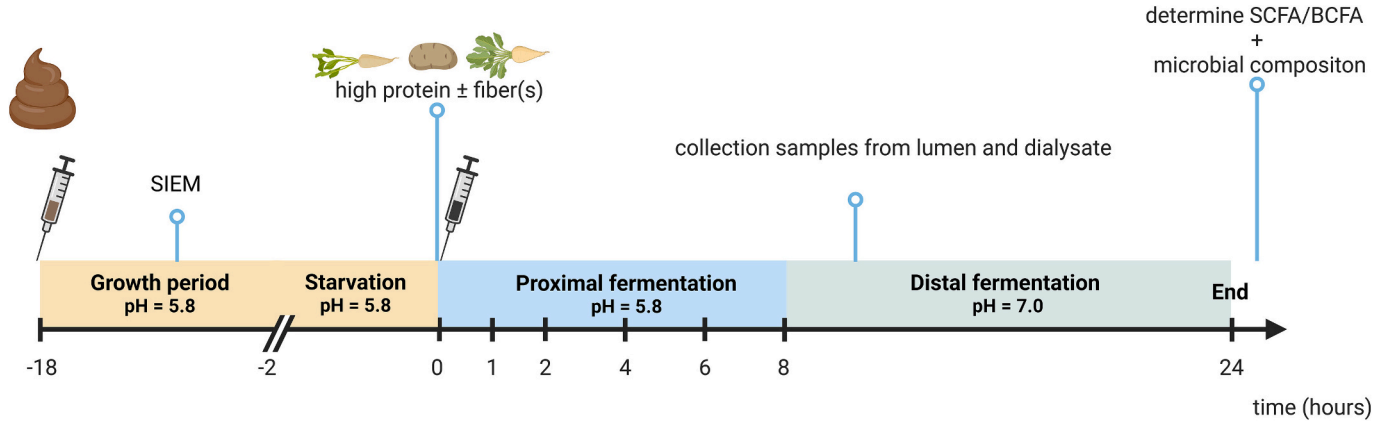

Fig. 1. – Experimental timeline of TIM-2 experiments. Standardized pooled microbiota from individuals with overweight or obesity and at increased risk of developing type 2 diabetes was inoculated in TIM-2 and left to grow overnight (16 h) with standard ileal efflux medium (SIEM). Thereafter, SIEM feed was discontinued, and a starvation period of two hours was initiated to remove SIEM residue and prevent interaction effects. After baseline measurements ($t = 0$), substrates were administered to TIM-2 through the lumen port. To test the effect of protein quantity and protein type, a low protein control, a high protein control (both consisting of a mixture of egg, fava bean, pea, and potato protein), and a high protein control consisting only of egg-protein were administered as a single shot. To investigate the effect of fibers in a high protein context, all the fiber and fiber combinations were provided on top of the high protein control. Samples were taken from the lumen and dialysate 1, 2, 4, 6, 8, and 24 h after administration. The first 8 h were considered proximal fermentation, in which the pH was set at 5.8 whereas the last 16 h (8-24 h) were considered distal fermentation, in which the pH was slowly adjusted to 7.0. Abbreviations: BCFA – branched-chain fatty acids, SCFA – short-chain fatty acids.

unable to test the potato-fiber individually due to the hygroscopic nature of the fiber, which clogged the TIM-2 system thereby making it impossible to draw samples at timepoint 24 h.

The experimental conditions were defined as followed: low-protein control (LP_control), all-egg high protein control (egg_HP_control), high-protein control (HP_control), high-protein + inulin (HP_inu), high-protein + pectin (HP_pec), high-protein + pectin and inulin (HP_pec_inu), high-protein + pectin and potato-fiber (HP_pec_pot), high-protein + potato-fiber and inulin (HP_pot_inu), and high-protein + inulin, potato-fiber and pectin (HP_inu_pot_pec) and were performed in duplicate on different experimental days. 10 mL of sample from the lumen and 10 mL of dialysate was collected after 1, 2, 4, 6, 8, and 24 h and immediately snap-frozen in liquid nitrogen after sampling and stored at −80 °C until analysis. The first 8 h was considered proximal fermentation and between 8 and 24 h was considered distal fermentation (Fig. 1). Luminal samples of $t = 0$, 4,8, and 24 h were transported to the laboratory of The Laboratory of Microbiology of Wageningen University & Research (Wageningen, the Netherlands) for microbiota profiling and fiber degradation experiments.

2.5. Fermentation products: Analysis of short- and branched-chain fatty acids

To investigate the SCFA and BCFA production induced by the protein controls and different fibers and fiber combinations, SCFA and BCFA concentrations were measured in lumen and dialysate samples as previously described (Cuevas-Tena et al., 2019). The weight of the dialysate was recorded at each time point and SCFA concentration was determined to quantify the SCFA in the dialysate, that were removed from the lumen by the filtration system. For determination of total SCFA, acetate, propionate, butyrate, valerate, and caproate and for BCFA, isovalerate and isobutyrate were determined by gas chromatography–mass spectrometry (GC–MS, Agilent 8890 GC Custom system) linked to an Inert Plus MSD Turbo EI/CI and using an automatic sampler (PAL3 RSI 85, Agilent). Luminal samples were first centrifuged (14,000 rpm, 10 min) to separate the liquid phase (containing SCFA and BCFA) from the solid residues whereas this step was not necessary for the dialysate samples. Thereafter, 150 μL of supernatant from the luminal samples was transferred into a glass-GC vial, in which 550 internal standard solution was added consisting of formic acid (20 %), methanol (acidified solvents), and 2-ethyl butyric acid (internal standard), and demineralized water. For the dialysate samples, 300 μL of supernatant was transferred to a glass-GC vial with 400 μL of the same internal standard solution. The GC–MS injected 0.5 μL of each sample onto a GC column (Agilent, Middelburg, the Netherlands). All analyses were conducted at the Maastricht University Brightlands Campus, Venlo, the Netherlands and SCFA concentration in each sample was quantified by using a calibration curve with known standards and then used to derive a cumulative SCFA production over time in which the metabolites at t = 0 were artificially set to zero.

2.6. DNA isolation, purification, and sequencing

DNA was extracted as previously described (Salonen et al., 2010) from approximately 0.25 g of the fecal suspension. Samples were weighted in sterile vials containing 0.5 g of 0.1 mm zirconia beads and 5 glass beads (2.5 mm). Thereafter, 700 μL of STAR (Stool transport and recovery) buffer (Roche Diagnostics, Indianapolis, IN, United States) was added and the samples were homogenized by repeated bead-beating in FastPrep®-24 (MP Biomedicals, Solon OH, USA) in three cycles at room temperature (5.5 ms for 3 × 1 min). Samples then were shaken at 100 rpm for 15 min at 95 °C, and centrifuged at 4 °C (5 min, 15,000 x g). The process was repeated with the remaining pellet with 350 μL of STAR buffer and the supernatant was combined with the previously obtained supernatant. DNA was purified using the Maxwell 16 Tissue LEV Total RNA Purification Kit (cat no. AS 1220) according to manufacturer's instruction on the Maxwell® 16 Instrument (Promega, The Netherlands). DNA concentration was quantified using Qubit™ dsDNA BR assay kit (Invitrogen by Thermo Fisher Scientific and a Denovix DS-11 Fluorometer (Denovix Inc., Washinton, Delaware, USA). Purified DNA was diluted to 20 ng/μL using nuclease-free water.

The DNA library was prepared by PCR using the unique barcode primer pair 515F – 806R to amplify the V4 region of the 16S rRNA gene. Each PCR reaction contained: 1 μL DNA template (20 ng/μL), 20 μL 5× HF buffer green (Thermo Scientific), 1 μL of an uniquely barcoded primer (10 μM), 1 μl dNTPs (Promega), 0.5 μL Phusion Hot start II DNA polymerase (2 U/μL), and 36.5 μL nuclease-free water. PCR products were run in duplicate and negative controls were incorporated in every PCR run. The cycle conditions used for PCR were: initial denaturation: 98 °C for 30 s followed by 25 cycles of denaturation: 98 °C for 10 s, annealing: 42 °C for 10 s, extension: 72 °C for 10 s, final extension: 72 °C for 7 min. PCR products were visualized on a 1 % agarose gel (100 V, 20 min) to verify the presence of the desired amplicon. Duplicate PCR products were pooled and purified using Roche High Pure PCR Cleanup Micro Kit according to manufacturer's instructions. The DNA concentration was measured again by Qubit™. Purified PCR products were pooled in an equimolar mix consisting of 69 unique barcoded primers (with a DNA concentration of 200 ng/μl per sample). Positive controls of two mock communities of known composition and one PCR negative control were added to the libraries to ensure proper sequencing and to check for library biases.

Libraries were sent for sequencing (Novogene, Beijng, China) and were carried out on the Illumina HiSeq 2000 platform (Illumina, CA, USA). The raw sequence data from the 16S rRNA gene amplicons were processed using the default settings of the NG-Tax 2.0 pipeline as previously described (Poncheewin et al., 2020). Each amplicon sequence variant (ASV) was assigned taxonomy according to the SILVA database, version 138.1 (Quast et al., 2012).

2.7. Degradation of dietary fibers during fermentation

The degradation of inulin during a TIM-2 experiment was tracked by High Performance Anion Exchange Chromatography (HPAEC) for one run of HP_inu and one run of HP_pec_inu. Luminal samples from TIM-2 ($t = 0$, 4, 8, and 24) were centrifuged at 19000 *g* for 5 min. The collected supernatant was heated at 100 °C for 10 min to inactivate any enzyme that could be present. 10 μL of undiluted supernatant was injected to a ISC5000 HPLC system (Dionex, Sunnyvale, CA, USA) with a CarboPac PA-1 column (250 mm × 2 mm ID), a CarboPac PA guard column (25 mm × 2 mm ID) and a ISC5000 ED detector (Dionex) in the pulsed amperometic detector (PAD) mode. Mobile phase A (0.1 M sodium hydroxide) and B (1 M sodium acetate in 0.1 m sodium hydroxide) were used as described by Lotenberg et al. (2020) (Logtenberg, Akkerman, et al., 2020): 0–40 min, 0–40 % B; 40–45 min, 40–100 % B; 45–50 min, washing step with 100 % B; 50–51 min, 100–0 % B; 51–66 min, equilibration with 100 % A.

The degradation of sugar beet pectin was monitored by High Performance Size Exclusion Chromatography (HPSEC) as previously described by Broxterman and collagues (2017) (Broxterman et al., 2017) for one run of HP_pec, HP_pec_inu, and HP_pec_pot. 10 μL of previously heated supernatant was injected in an Ultimate 3000 system (Dionex, Sunnyvale, CA, USA) coupled to a Shodex RI-101 detector (Shodex, Tokyo, Japan) and a set of TSK-Gel super 4000, 3000 and 2000 AW columns preceded by a TSK-Gel super AW guard column (6 mm ID × 40 mm) (Tosoh Bioscience, Tokyo, Japan). Mobile phase of 0.2 M NaNO3 was passed through the set of columns at a flow rate of 0.6 mL min-1 at 55 °C. Pectin standards from 10 to 100 kDa were used to estimate the molecular weight of pectin polymers.

2.8. Statistical analysis

R programming and statistical language (4.3.2) was used to perform

all downstream analysis. The tidyverse package collection (Wickham et al., 2019), ggvegan (Simpson & Oksanen, 2023), and ComplexHeatmap (Gu et al., 2016) were used for data handling, filtering, and visualization. The results of NG-Tax 2.0 pipeline were used to construct a phyloseq object (McMurdie & Holmes, 2013). Prior further analysis ASVs that were not assigned to any phylum or assigned to genera Mitochondria and Chloroplast or kingdoms other than *Bacteria* and *Archaea* were removed. In addition, ASVs with total number of reads less than 20 were also removed.

Differences in α-diversity between substrates and over time were examined by calculating different diversity indexes (number of observed species, Shannon, Inverted Simpson, and Phylogenetic diversity) (McMurdie & Holmes, 2013; Paradis & Schliep, 2019). To identify the relative change in α-diversity (within-sample diversity) indexes induced by a substrate in comparison with the reference group (HP_control), the mean value of duplicated runs per substrate at every time point was calculated. Subsequently, differences between the mean value of the reference group and a tested substrate (delta) were calculated per time point and the percentage of change from baseline ($t = 0$) was compared between experimental conditions. Differences in individual taxa were determined using this same methodology. Community differences (β-diversity, between-sample diversity) were elucidated using distance-based Redundancy Analysis (dbRDA) and the significance of the interaction between groups over time was determined by Analysis of variance using distance matrices as implement in the vegan package (ADONIS; function adonis2) (Oksanen et al., 2024). Firstly, dissimilarity

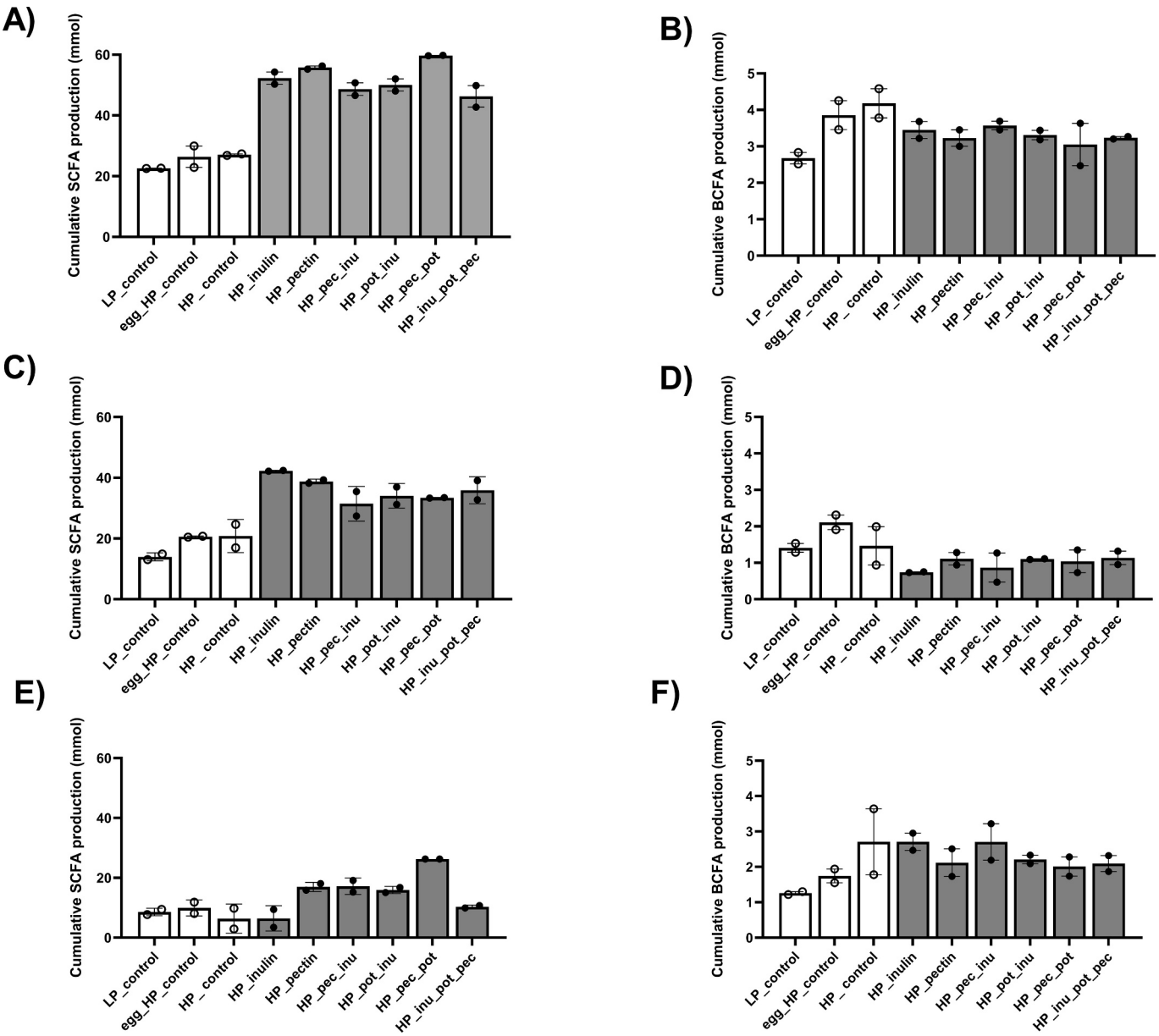

Fig. 2. – Cumulative SCFA and BCFA production in 24 h (total, A, B), the first 8 h (proximal, C, D), or the last 16 h (distal, E, F) after administration of proteins either alone or dietary fibers against a high protein background. Addition of fibers to a high protein background increased total SCFA production and slightly decreased total BCFA production. The combination potato-fiber and pectin stimulated distal SCFA production the most. Providing half the protein load (LP_control) reduced total BCFA but had less effect on SCFA production whereas protein type (egg_HP_control vs HP_control) did no change total SCFA and BCFA production. Protein controls are indicated in white and the high protein control together with different fiber(s) are indicated in black. Dots represent replicates and the bar the mean (±SEM) of both duplicates. Abbreviations: BCFA – branched-chain fatty acids, HP – high protein, LP – low protein, inu – inulin, inu_pot_pec – inulin, pectin, and potato-fiber, pec – pectin, pec_pot – pectin and potato-fiber, pec_inu – pectin and inulin, pot_inu – potato-fiber and inulin, SCFA – short-chain fatty acids. Total SCFA = acetate + propionate + butyrate + valerate + caproate; Total BCFA = iso-butyrate + iso-valerate.

distances (Unweighted UniFrac, Weighted UniFrac, Jaccard, Bray-Curtis) were calculated on ASV taxonomic level with cumulative-sum scaled (CSS) (Paulson et al., 2013) normalized logarithmically transformed count. The Dissimilarity distances were used for dbRDA and andonis2 using Time*Group formula and 999 permutations. The results of dbRDA analysis were plotted as a scatter plot using a custom function. Differences in overall composition were assessed in the complete dataset set - all available samples were used to create a dissimilarity matrix and build ADONIS model. In addition, every substrate was compared with the reference substrate separately - a subset of samples containing the reference and a target substrate was taken to construct a dissimilarity matrix and build ADONIS model.

To see whether there were time specific alterations in microbiota composition, we assessed different time frames to identify changes related to proximal (0–4-8 h) and distal fermentation (8-24 h). All data are presented as mean ± SEM and for β-diversity a p-value of ≤ 0.05 was considered significant and a p-value below 0.10 was considered a trend. We described relevant microbial patterns and signatures based on the presence of consistent duplicates.

3. Results

3.1. Microbial metabolite production profiles in response to proteins and different fiber(s)

3.1.1. SCFA and BCFA production in response to proteins

First, we determined the amount of SCFA and BCFA that can be produced from protein fermentation. SCFA are predominantly produced from indigestible carbohydrates and, to a lesser extent, from proteins, whereas BCFA are derived solely from proteins, specifically the branched chain amino acids (BCAA): leucine, isoleucine, and valine (Diether & Willing, 2019). Cumulative SCFA and BCFA production over 24 h was 27.07 ± 0.26 mmol and 4.18 ± 0.40 mmol respectively when only the protein mixture (HP_control) was provided to TIM-2 (Fig. 2A, B & Supplementary material, Table S2A). If the protein source was solely derived from egg protein (egg_HP_control), the total SCFA and BCFA production remained similar (26.39 ± 3.53 mmol and 3.86 ± 0.40 mmol). Interestingly, decreasing the protein load by 50 % (LP_control) still produced a similar amount of SCFA (22.58 ± 0.04 mmol) whereas BCFA production (2.68 ± 0.16 mmol) was decreased by roughly a third compared to the HP_control (Fig. 2A, B & Supplementary material, Table S2A).

3.1.2. SCFA and BCFA production in response to proteins together with fiber(s)

As expected, total SCFA production was increased when fiber was administered in addition to the HP (Fig. 2A & Supplementary material, Table S2A). On average, the fiber or combination of fibers led to approximately double the amount of total SCFA produced (52.11 ± 1.49 mmol, $N = 12$) whereas BCFA production was only slightly decreased (3.31 ± 0.10 mmol, N = 12) compared to the HP_control. Overall, a higher cumulative SCFA level and a lower BCFA production were observed in the first 8 h (proximal production, Fig. 2C,D & Supplementary material, Table S2B) compared to the last 16 h of fermentation (distal production, Fig. 2E,F & Supplementary material, Table S2C) in all fiber conditions. Slight differences in total, proximal, and distal SCFA production between fiber conditions were observed. To illustrate, the least total SCFA production was observed after administration of the mix of all fibers (HP_inu_pot_pec, 46.26 ± 3.55 mmol) whereas the highest production was achieved by combining pectin and potato-fiber (HP_pec_pot, 59.69 ± 0.09 mmol). Although SCFA production was always highest in the proximal fermentation period, inulin was specifically characterized by a more proximal fermentation pattern with more SCFA production in the first 8 h as compared with the last 16 h (HP_inu, 42.3 ± 0.11 vs 9.97 ± 1.89 mmol) compared to pectin fermentation (HP_pec, 38.78 ± 0.55 vs 16.97 ± 1.08 mmol). Interestingly, combining inulin with either potato-fiber (HP_pot_inu, 34.08 ± 2.87 vs 15.94 ± 0.86 mmol) or pectin (HP_pec_inu, 31.46 ± 4.04 vs 17.20 ± 1.96 mmol) appeared to shift fermentation more distally compared to inulin alone (Fig. 2C-F). Nevertheless, the highest distal SCFA production was achieved with HP_pec_pot (33.41 ± 0.08 vs 26.28 ± 0.01 mmol).

3.1.3. Acetate:butyrate:propionate ratio

To have a closer look at the type of SCFA produced, we calculated the relative contribution of the three most abundant SCFA: acetate, butyrate, and propionate from either total, proximal, or distal cumulative SCFA production. When only protein was supplied (LP_control, HP_control, egg_HP_control, $N = 6$), acetate was the most abundant SCFA, whilst propionate and butyrate nearly equally contributed to the SCFA pool (47:26:27). On average, adding fiber to the HP context, led to an even higher relative abundance of acetate, which was followed by propionate and then butyrate (ratios 57:24:19). Overall, SCFA ratios were relatively similar between fiber conditions. From all fiber conditions, HP_pec_pot resulted in the highest relative (in proportion to total SCFA) and absolute propionate production (29.6 ± 1.3 % & 16.58 ± 0.75 mmol, Fig. 3A & Supplementary material, Table S2A). Interestingly, the SCFA ratios differed between proximal (63:20:17) and distal (41:36:24) SCFA production, with propionate playing a more prominent role in the last 16 h of fermentation at the expense of acetate (Fig. 3B,C & Supplementary material, Table S2,3). Hence, substrate matrix (proteins vs proteins with fibers) may influence the type of SCFA produced. Additionally, SCFA profiles shifted between proximal and distal fermentation, potentially highlighting bacterial cross-feeding and/orchanges in response to substrate availability or pH.

3.1.4. SCFA:BCFA ratio

Given the importance of considering saccharolytic and proteolytic fermentation as interconnected rather than isolated processes, we also investigated the ratio between SCFA:BCFA, which can be used as an indicator for increased saccharolytic fermentation in a protein context (Li et al., 2025; Rios-Covian et al., 2020). Decreasing protein load led to an increase in overall SCFA:BCFA ratio from 6.54 ± 0.69 in the HP_control to 8.49 ± 0.51 in the LP_control after 24 h of fermentation, which was mainly attributed to a lower total BCFA production in LP_control. Protein source did not influence SCFA:BCFA ratio as both HP_control and the egg_HP_control resulted in similar SCFA:BCFA ratios. All fiber combinations more than doubled the SCFA:BCFA ratio (15.99 ± 0.87, $N = 12$) in 24 h compared to the HP_control, yet there were no differences between the tested single fibers and fiber combinations for total SCFA:BCFA ratio (Fig. 4A & Supplementary material, Table S2A). Thus, fibers were able to steer towards saccharolytic fermentation, independent of fiber type in the total fermentation period.

Nevertheless, differences in the SCFA:BCFA ratio were observed in the proximal and distal colon specifically, depending on protein load, protein type, and fiber type (Fig. 4C & Supplementary material, Table S2,3). Overall, SCFA:BCFA ratios were lower in the distal compared to the proximal period, highlighting a change in the balance between saccharolytic and proteolytic processes, shifting more toward protein fermentation (Fig. 4B&C & Supplementary material, Table S2B, C). Decreasing protein load reduced proximal SCFA:BCFA ratio for LP_control (10.04 ± 1.50) compared to HP_control (15.23 ± 2.84), which was mainly attributed to a reduced SCFA production in the LP_control (Fig. 2C). In contrast, the distal SCFA:BCFA ratio showed an opposite trend, with the LP_control resulting in a 3-fold higher distal SCFA:BCFA (6.81 ± 0.51) compared to the HP_control (2.17 ± 0.53), mainly driven by decreased BCFA production in the LP_control (Fig. 2F). Similarly, egg_HP_control showed a lower proximal SCFA:BCFA ratio (9.88 ± 1.03) compared to HP_control, whereas the opposite pattern was observed for distal SCFA:BCFA ratio. These findings suggest that both protein quantity and type appear to influence fermentation processes differently in the proximal and distal colon. Of all fibers and fiber

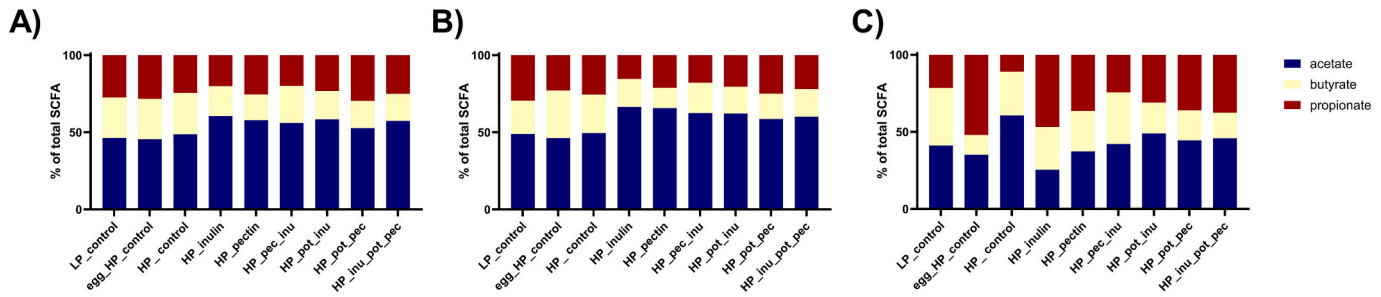

Fig. 3. –The ratio between acetate, butyrate and propionate produced in 24 h (total, A), the first 8 h (proximal, B), the last 16 h (distal, C) after administration of proteins either alone or against a high protein background. Distally, relative SCFA production switched toward propionate production compared to proximally, which was more acetogenic. Overall, fibers, in context of a high protein background, produced relatively more acetate compared to proteins alone. Abbreviations: Abbreviations: BCFA – branched-chain fatty acids, HP – high protein, LP – low protein, inu – inulin, inu_pot_pec – inulin, pectin, and potato-fiber, pec – pectin, pec_inu – pectin and inulin, pec_pot – pectin and potato-fiber, pot_inu – potato-fiber and inulin, SCFA – short-chain fatty acids.

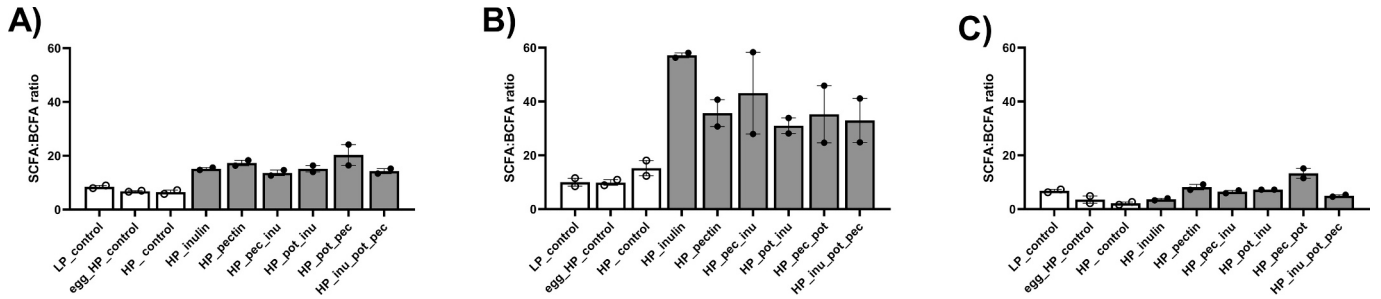

Fig. 4. – The ratio between SCFA and BCFA levels at 24 h (total, A), the first 8 h (proximal, B), the last 16 h (distal, C) after administration of proteins either alone or together with dietary fibers. Increasing protein load had the most effect on distal SCFA:BCFA ratio, decreasing it by approximately 3-fold. Fiber addition increased total, proximal, and distal SCFA:BCFA ratio compared to the high protein control. Inulin induced the most optimal proximal SCFA:BCFA ratio whereas the combination potato-fiber and pectin was most optimal distally. Protein controls are indicated in white and the high protein control together with different fiber(s) are indicated in black. Dots represent replicates and the bar the mean (±SEM) of both duplicates. Abbreviations: BCFA – branched-chain fatty acids, HP – high protein, LP – low protein, inu – inulin, inu_pot_pec – inulin, pectin, and potato-fiber, pec – pectin, pec_inu – pectin and inulin, pec_pot – pectin and potato-fiber, pot_inu – potato-fiber and inulin, SCFA – short-chain fatty acids. Total SCFA = acetate + propionate + butyrate + valerate + caproate; Total BCFA = iso-butyrate + iso-valerate.

combinations, HP_inu had the highest proximal SCFA:BCFA ratio (57.17 ± 0.92) whereas HP_pec_pot resulted in the most optimal distal SCFA:BCFA ratio (13.32 ± 1.79) (Fig. 4B,C & Supplementary material, Table S2B,C). Hence, although overall fermentation was similar between fibers (24 h), fiber type affected metabolite production differently in the proximal and distal fermentation period.

3.1.5. Degradation of dietary fibers

HPAEC showed that inulin started to be fermented actively within the first 4 h (Supplementary material, Fig. S1C) and was completely degraded after 8 h if the fiber was administered individually (Supplementary material, Fig. S1D). In line with the more distally-orientated SCFA production, inulin degradation appeared slowed down when combined with pectin, indicated by the fractions of inulin (DP ~ 25) that were still present in the luminal samples at $t = 8$, despite the lower dose of inulin provided to TIM-2 in HP_pec_inu (3,75 g) compared to HP_inu (7,5 g) (Fig. S2C). Likewise, HPSEC showed that when pectin was added independently, it was fully degraded after 8 h of fermentation time, before reaching the distal period (Fig. S3D). In contrast, when pectin was combined with inulin, the degradation of dietary fibers appeared slightly slower (Supplementary material, Fig. S4). Nevertheless, in both cases, the fibers were depleted at $t = 24$ (Supplementary material, Fig. S2D & S4D). Interestingly, when pectin was combined with potato-fiber, a small peak was still present in the luminal sample at t = 24, which may suggest that it takes longer for the dietary fibers to be fully degrade with this combination of fibers (Supplementary material Fig. S5D).

Thus fiber degradation patterns aligned with the observed shift in SCFA production toward the distal fermentation period when inulin was combined with pectin and pectin was combined with potato-fiber compared to administration of individual fibers.

3.2. Alterations in microbiota composition in response to different fiber(s)

Next to microbial metabolite production, we aimed to assess acute changes in microbial composition in response to these different dietary protein and fiber conditions.

3.2.1. Analysis of microbial α-diversity during fermentation

First, we investigated the richness and evenness of bacterial taxa by evaluating different α-diversity indices over 24 h after one bolus of protein either alone or combined with the different fibers (Fig. 5). On average, the baseline microbiota composition ($t = 0$) consisted of 102 different observed bacterial ASVs after the 2-h starvation period. Observed ASVs (richness) at baseline was not always the same between conditions (range 83–136). However, other α-diversity measures (InvSimpson, Shannon, and Phylodiversity), which also consider the evenness of the bacteria, showed minimal baseline variations in α-diversity.

Protein content and protein type had minimal impact on α-diversity indices as both egg_HP_control and LP_control exhibited similar α-diversity patterns during fermentation compared to HP_control. Nonetheless, in the distal fermentation period, egg_HP_control (−0.1 ± 1.5 %) did not change phylogenetic diversity whereas HP_control increased phylogenetic diversity (+11.5 ± 1.8 %). This may suggest that the addition of plant-based proteins may induce a more phylogenetically diverse set of bacteria in the distal fermentation period. The most pronounced effect on α-diversity was observed 4 h after administration of

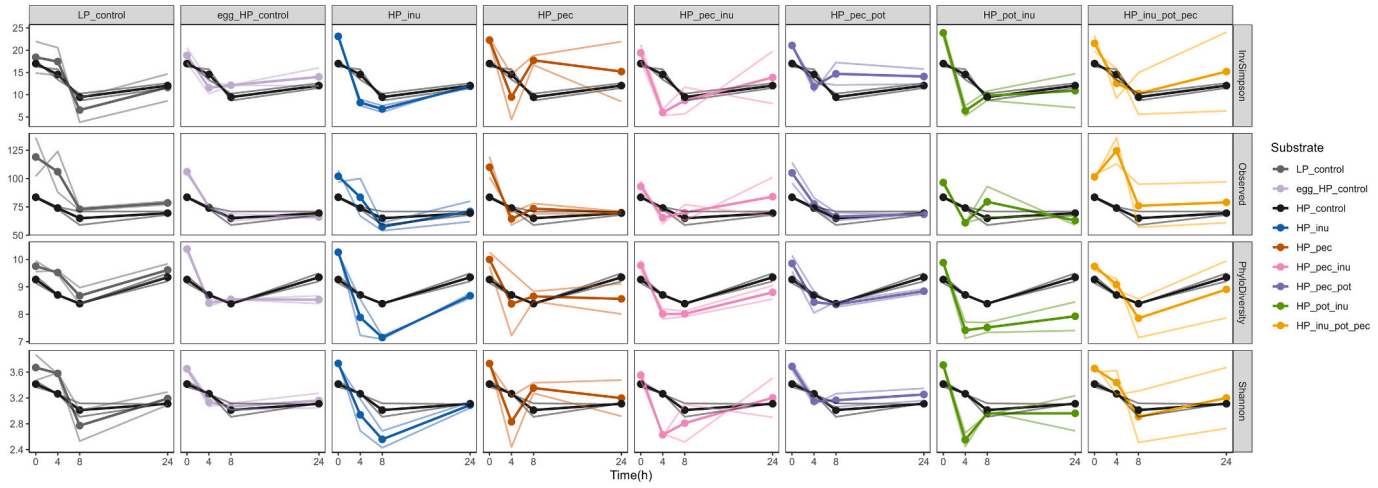

Fig. 5. – The change in bacterial species richness measured by different α-diversity indices: InvSimpson, Observed amount of species, PhyloDiversity, and Shannon index over 24 h after administration of substrates. Overall, α-diversity decreases quickly after administration of substrates. In the first four hours, especially inulin and inulin combinations show a more pronounced reduction in α-diversity compared to HP_control whereas the potato-fiber and pectin combination and the mixture of all fibers did not. In contrast, α-diversity start to slightly increase again in the distal fermentation period (8-24 h) for most substrate conditions. Each thick line represents the mean of duplicate runs (thinner lines) and the black line denotes the reference group (HP_control). Abbreviations: HP – high protein, LP – low protein, inu – inulin, inu_pot_pec – inulin, pectin, and potato-fiber, pec – pectin, pec_inu – pectin and inulin, pec_pot – pectin and potato-fiber, pot_inu – potato-fiber and inulin.

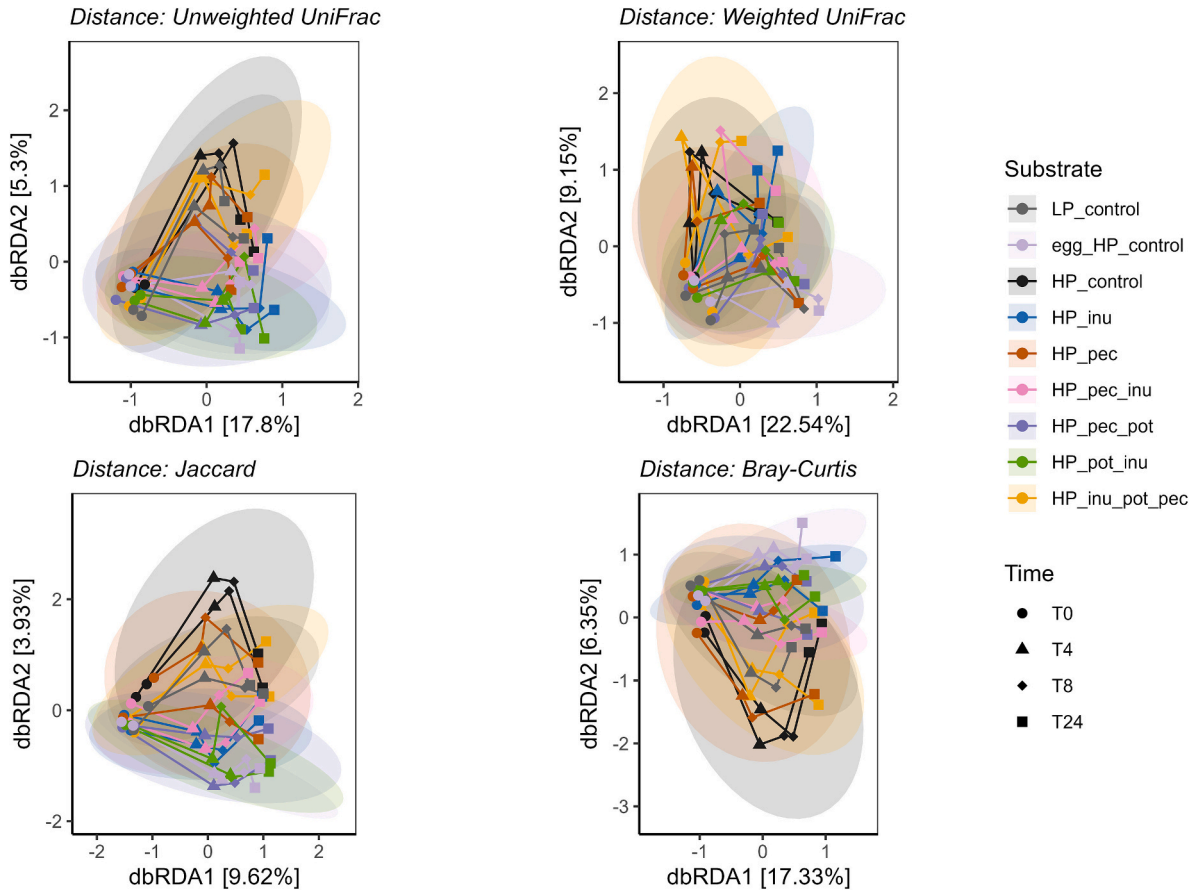

Fig. 6. – The change in bacterial compositions measured by different β-diversity indices: Unweighted Unifrac, Weighted Unifrac, Jaccard similarity, and Bray Curtis dissimilarity. Time and type of substrate significantly impacted β-diversity, although no time interaction effect was observed over 24 h. The biggest changes in microbial composition occurred in the first 4 h, indicated by the longer line between T0 and T4. Overall, the HP_control appeared more similar in microbial composition over time to the mixture of fibers and the LP_control whereas others were more dissimilar. Each condition has two lines representing the duplicates. Abbreviations dbRDA, distance-based Redundancy Analysis, HP – high protein, LP – low protein, inu – inulin, inu_pot_pec – inulin, pectin, and potato-fiber, pec – pectin, pec_inu – pectin and inulin, pec_pot – pectin and potato-fiber, pot_inu – potato-fiber and inulin.

substrates. On average, the level of observed species decreased approximately 20 % in the first 4 h (mean of 81 ± 5 species at $t = 4$, $N = 18$). Only HP_inu_pot_pec increased in level of observed species in the first 4 h although, similar to other conditions, all other α-diversity indices reduced. Thereafter, between 4 and 8 h, α-diversity decreased with an additional 10 % on average yet there was more heterogeneity among different types of substrates. Some conditions including HP_inu and HP_inu_pot_pec continued to decrease in all α-diversity indices whereas most other conditions started to stabilize in this time period. In the proximal period, the most pronounced reduction in α-diversity was induced by HP_inu, followed by HP_pot_inu and HP_pec_inu whereas HP_pec_pot and HP_inu_pot_pec did not differ compared to the HP_control. To illustrate, HP_inu reduced phylogenetic diversity with 30.3 ± 0.9 % after 8 h compared to baseline, which was approximately three times higher than the reduction after HP_control (9.5 ± 1.2 %). Yet, this reduction was attenuated if inulin was combined with pectin (18.1 ± 2.7 %) or potato-fiber (23.9 ± 1.6 %). Thus, administering inulin individually may stimulate a less phylogenetically diverse set of microbes than combining it with either potato-fiber of pectin. HP_pec (12.9 ± 0.5 %) and HP_pec_pot (15.3 ± 3.5 %) showed the smallest reduction in phylogenetic diversity of all fiber conditions at $t = 8$. During the distal fermentation period, overall α-diversity measures started to slightly increase again, which may highlight bacterial cross-feeding and the growth of other bacterial taxa. Overall, the effect on α-diversity appeared to be both substrate- and time-dependent.

3.2.2. Analysis of time- and substrate-dependent microbial alterations (β-diversity)

All baseline ($t = 0$) conditions clustered closely together on the dbRDA plot indicating that the baseline composition of our standardized microbiota was indeed comparable before administration of experimental substrates (Fig. 6). Over the total 24 h, time significantly affected all assessed β-diversity indices (Unweighted UniFrac, Weighted UniFrac, Jaccard, Bray-Curtis, $p = 0.001$). Additionally, the type of substrate also significantly influenced all β-diversity indices ($p = 0.001$), with the exception of Unweighted Unifrac, which showed only a tendency toward significance ($p = 0.062$). The Weighted UniFrac distance matrix best explained separation by substrate type (RDA1: 22.5 % and RDA2: 9.5 %). As Weighted Unifrac takes into account both the richness of bacterial taxa (presence/absence) and the evenness of bacterial taxa (proportion/abundance) whereas Unweighted Unifrac only takes into account richness, this may suggest group separation is predominantly caused by differences in the proportion of bacterial taxa and thus driven by high-abundance taxa rather than low-abundance taxa. Additionally, Unifrac matrices, which take into account phylogenetic distance between bacterial taxa (phylogenetic relatedness), explained more of the compositional variation compared to Bray-Curtis and Jaccard, which do not take into account phylogenetic relatedness. This may suggest compositional variation between conditions may be explained better by bacteria that are phylogenetically more distant from each other.

Nevertheless, no significant substrate x time interaction effects were observed over the total 24 h period. Overall, most pronounced differences in microbiota composition were observed in the first 4 h after substrate administration, as indicated by the longer line on the dbRDA plot between $t = 0$ and $t = 4$ compared to other time points. At $t = 24$, samples seem to cluster together again, suggesting microbial composition may become more similar at this time point (Fig. 6). As HPAEC and HPSEC suggest most fiber substrate is already degraded at $t = 8$, microbial composition may become more similar again as a consequence of dietary fiber depletion between 8 and 24 h.

3.2.3. Bacterial composition of baseline standardized microbiota

On phylum level, the standardized microbiota after the 2-h starvation period (t = 0) was dominated in relative abundance by Bacillota (65.3 ± 0.9 %). Thereafter Bacteroidota (28.5 ± 0.1 %) and Actinomycetota (5.0 ± 0.5 %) were most abundant whereas Pseudonomadota and the archaeal phylum Euryarchaeota (<1 %) appeared to constitute very low amounts at baseline (Fig. S6). On a family level, the standardized microbiota was characterized by Lachnospiraceae, Ruminococcaceae, Bifidobacteriaceae, Prevotellaceae, and Bacteroidaceae (Supplementary material, Fig. S7) whereas *Bacteroides*, *Ruminococcus*, *Faecalibacterium*, *Blautia*, *Dorea*, and *Prevotella 7* were most abundant at genus level. In line with the cluster observed for β-diversity at t = 0, the relative abundance of genera was comparable between experimental conditions (Fig. S8). A slightly higher relative abundance of *Prevotella 9* was observed for HP_control compared to other conditions whereas *Prevotella 7* was lower compared to LP_control and egg_HP_control at baseline.

3.2.4. Changes in relative abundance of bacterial taxa related to proximal or distal fermentation

In general, independent of the substrate used, some uniform patterns could be observed which characterized proximal and distal fermentation. To illustrate, in the first 8 h, the relative abundance of *Coprococcus* and *Dorea* increased and decreased, respectively (Fig. 7). Additionally, the relative abundance of *Faecalibacterium* (t = 0: 13.4 ± 0.8 %, $N = 18$) rapidly decreased to <1 % after 4 h in response to all substrate matrices (Fig. 7). In contrast, distal fermentation was, for instance, characterized by an overall increase in the relative abundance of *Acidaminococcus*, *Peptoclostridium*, *Allisonella*, and *Megasphaera*, which were minimally present after 4 h but slowly started to increase 8 h after administration (Fig. 8). To illustrate, on average, the relative abundance of *Acidaminococcus* increased with 5.4 ± 0.9 % in relative abundance in the distal fermentation period (8-24 h) compared to only 0.5 ± 0.2 % in the proximal fermentation period (0-8 h). Simultaneously, an overall decrease in the relative abundance of *Coprococcus* was observed from 8 to 24 h.

3.2.5. Changes in bacterial abundance in response to protein quantity and protein type

At phylum level, both LP_control and egg_HP_control were characterized by a relative decrease and increase in the relative abundance of Bacillota and Bateroidota respectively compared to baseline. In contrast, an increase in the relative abundance of Bacillota was observed after HP_control administration (Supplementary material, Fig. S6). In the proximal period, LP_control increased the relative abundance of *Parabacteroides* and *Dialister* whereas *Eubacterium hallii group*, *Anaerostipes*, and *Mogibacterium*, were decreased compared to HP_control (Supplementary material, Fig. S9A-E). *Mogibacterium*, for instance, was not detected at baseline but increased to a relative abundance of 12.7 ± 1.2 % at $t = 8$ after HP_control, which was attenuated to a relative abundance of 3.5 ± 1.3 % after LP_control. In the distal fermentation period, the change in relative abundance of *Megasphaera*, *Allisonella*, and *Acidaminococcus* was diminished compared to the HP_control (Fig. 9, Supplementary material, Fig. S10A-C). To illustrate, in this period, the relative abundance of *Acidaminococcus* increased with 5.7 ± 0.8 % in HP_control yet this increase was more than 10-fold lower in LP_control (0.5 ± 0.4 %).

Egg_HP_control resulted in a higher relative abundance of *Ruminococcus* and a lower relative abundance of *Blautia*, *Mogibacterium*, and *Coprococcus* over the total 24 h compared to the HP_control (Fig. 9 & Supplementary material, S11A-D). Similarly to LP_control, the HP-induced increase in *Mogibacterium*, was diminished by egg_HP_control with approximately 4-fold, which unlike LP_control, persisted at $t = 24$ h (Fig. 9 & Supplementary material, Fig. S11C). In the proximal fermentation period, egg_HP_control showed a lower relative abundance in *Anaerostipes*, *Eubacterium hallii group, Streptococcus*, and *Subdoligranulum* (Supplementary material, Fig. S11E-H), compared to the HP_control. In the first 4 h, the egg_HP_control was additionally distinguished by a higher relative abundance of *Paraprevotella* (Supplementary material, Fig. S11H). Hence, both protein quantity or protein type affected changes in microbial composition during fermentation.

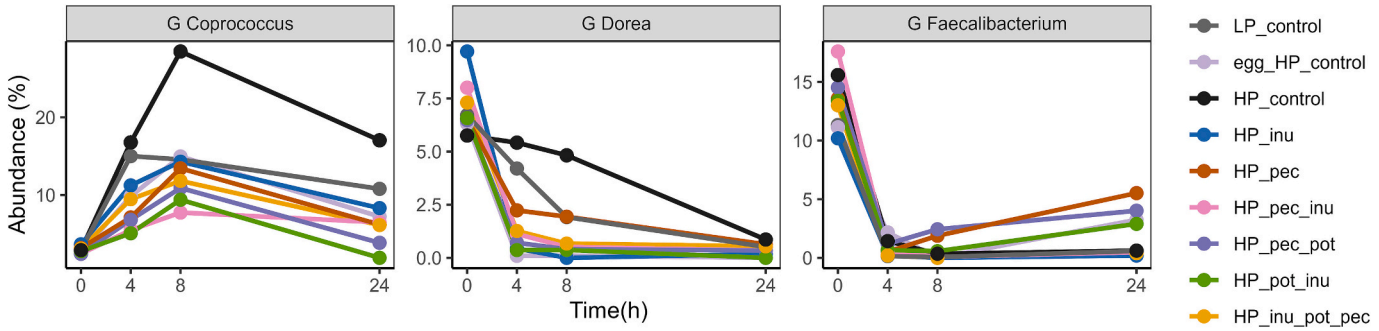

Fig. 7. – Alterations in Coprococcus, Dorea, and Faecalibacterium at genus level for proximal fermentation. The relative abundance of Coprococcus increased whereas the relative abundances of Dorea, and Faecalibacterium decreased in the initial period of fermentation after administration of substrates. Each line represents the mean of duplicate runs. Abbreviations: HP – high protein, LP – low protein, inu – inulin, inu_pot_pec – inulin, pectin, and potato-fiber, pec – pectin, pec_inu – pectin and inulin, pot_inu – potato-fiber and inulin, pec_pot – pectin and potato-fiber.

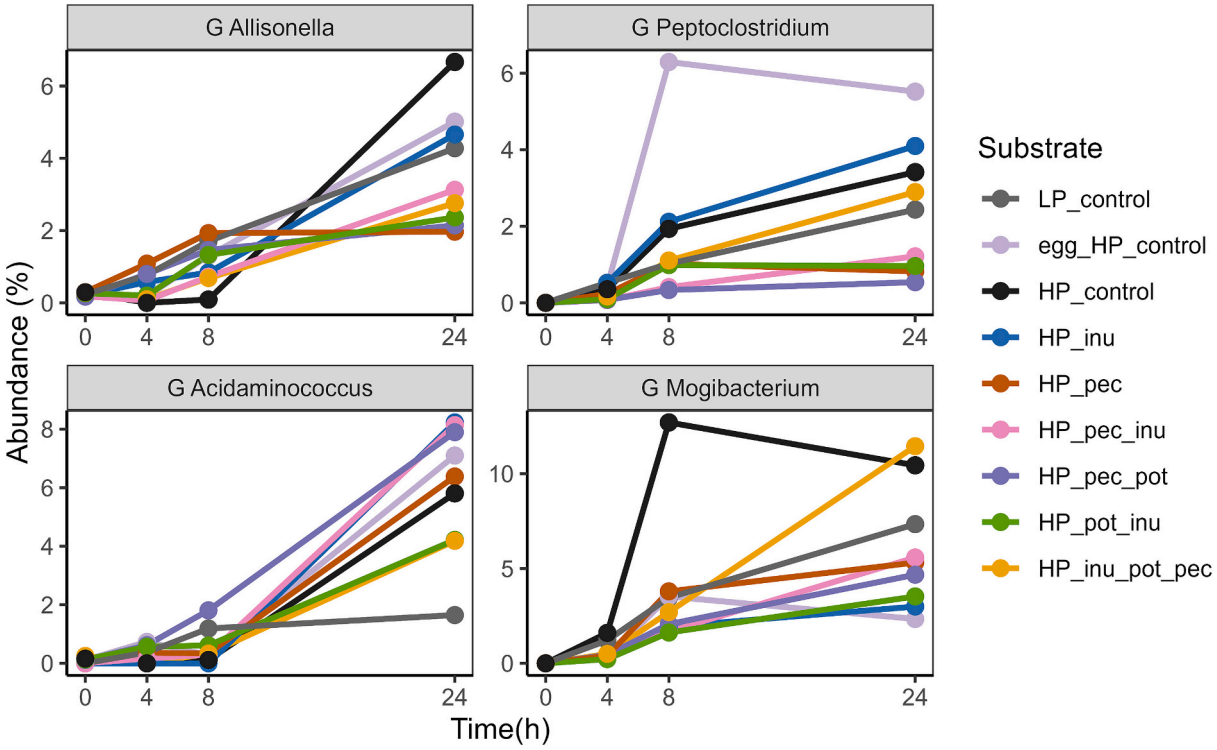

Fig. 8. – Alterations in microbial composition at genus level for distal fermentation. The relative abundance of Allisonella, Peptoclostidium, Acidaminococcus, and Mogibacterium increased appeared to be dominated in the latter part of fermentation. Each line represents the mean of duplicate runs. Abbreviations: HP – high protein, LP – low protein, inu – inulin, inu_pot_pec – inulin, pectin, and potato-fiber, pec – pectin, pec_inu – pectin and inulin, pec_pot – pectin and potato-fiber, pot_inu – potato-fiber and inulin.

3.2.6. The effect of fiber addition to a high protein matrix on microbial composition during fermentation

Next, we aimed to investigate whether adding different fibers or fiber combinations on top of the HP_control would affect microbial alterations compared to HP alone. Unlike the increase in relative abundance of Bacillota observed after HP_control, the addition of fiber, in general, decreased the relative abundance of Bacillota and increased the relative abundance of Actinomycetota and Bacteroidota (Fig. S6).

First, differences in microbial composition over the total fermentation period were identified. The addition of specific fibers or fiber combinations increased the relative abundance of *Ruminococcus* and *Blautia* compared to HP_control over the 24 h period (Fig. 9, S12A-B). To illustrate, *Ruminococcus* ($t = 0$: 15.0 ± 0.8 %), diminished post administration in response to HP_control ($t = 8$: 3.3 ± 2.7 %, t = 24: 1.7 ± 0.5 %), yet HP_inu stimulated *Ruminococcus* levels (t = 8: 29.3 ± 1.7 %, t = 24: 12.7 ± 5.3 %) compared to HP_control. All combinations containing inulin were better at maintaining *Ruminococcus* levels than fiber conditions without inulin. Notably, fibers generally stimulated *Ruminococcus* levels specifically in the proximal fermentation period while a steep reduction was observed during distal fermentation. Similarly, combinations containing pectin or potato-fiber resulted in a higher relative abundance of *Blautia* compared to HP_control over 24 h. Nevertheless, the strongest *Blautia*-inducing effect was observed after pectin was administrated individually in the first 8 h. Interestingly, whereas the HP_control reduced the relative abundance of *Blautia* even further in the distal fermentation period, both HP_inu and HP_pec_pot increased its relative abundance in this period compared to HP_control whereas HP_pec resulted in a more pronounced reduction of *Blautia* in this period. Hence, whereas *Ruminococcus* showed very specific proximal and distal responses predominantly independent of fiber type,

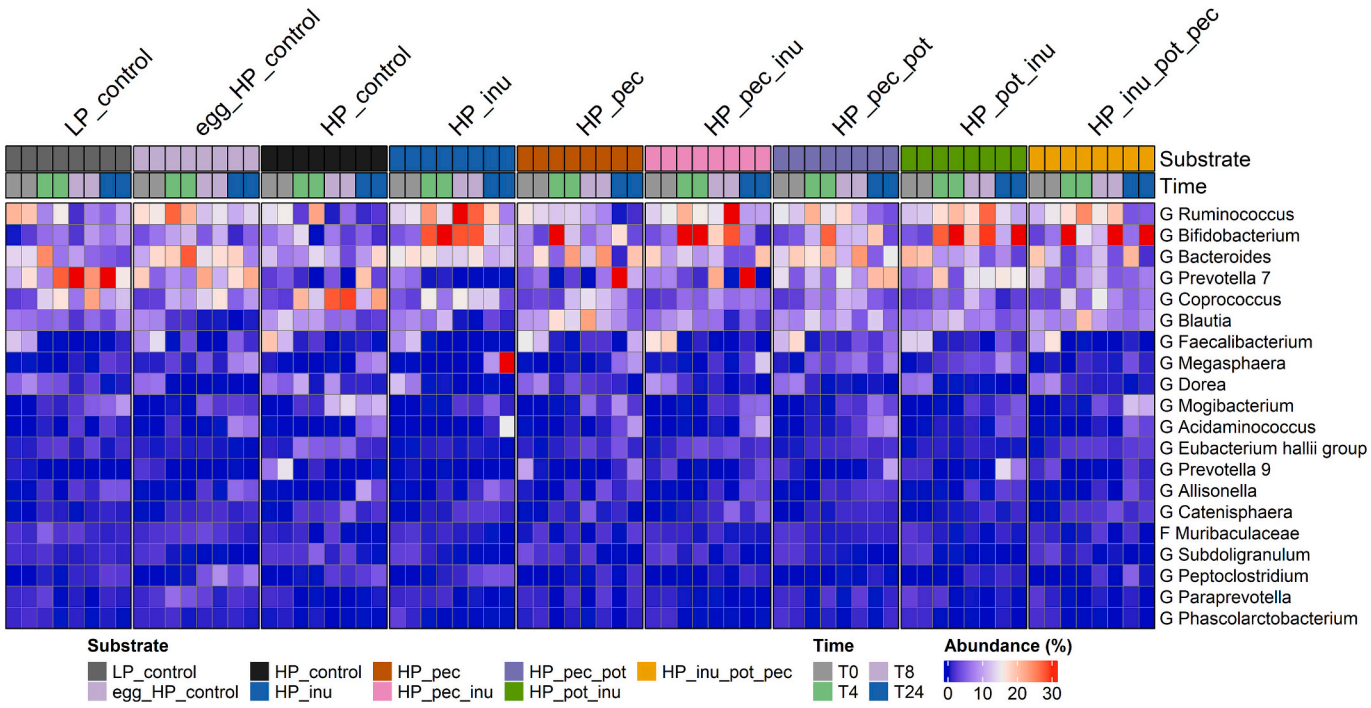

Fig. 9. – Alterations in microbiota composition (as relative abundance), on genus level, in response to either protein alone (LP_control, egg_HP_control, HP_control) or fiber and fiber combinations against a HP background. Both time- and fiber-specific alteration in microbial composition were identified. Abbreviations: HP – high protein, LP – low protein, inu – inulin, inu_pot_pec – inulin, pectin, and potato-fiber, pec – pectin, pec_inu – pectin and inulin, pec_pot – pectin and potato-fiber, pot_inu – potato-fiber and inulin.

Blautia responded differently in each fermentation period depending on the type of fiber(s) supplied. Moreover, pectin specifically stimulated *Blautia* but not *Ruminococcus* whereas the opposite was observed for inulin, indicating fiber-specific responses.

Second, we identified specific fiber induced shifts in bacterial relative abundance patterns compared to HP_control that were more specific to either proximal or distal fermentation. In proximal fermentation, HP_control was specifically characterized by an increase in relative abundance of *Eubacterium hallii group*, *Mogibacterium, Coprococcus*, and *Methanobrevibacter*, which were all minimally present at baseline (Fig. 9, Supplementary material Fig. S13A-D). Fiber addition led to altered responses in all of these microbial genera, predominantly inhibiting the effect induced by HP (Fig. 9, Supplementary material, Fig. S13A-D). The rapid increase in *Eubacterium hallii group* observed 4 h after administration with HP_control (t = 0: 0.8 ± 0.2 %, *t* = 4: 5.8 ± 0.9 %) was almost entirely abolished when inulin (t = 4: 0.8 ± 0.2 %) or potato-fiber and inulin (t = 4: 0.6 ± 0.2 %) were added. Yet, pectin alone and all combinations containing pectin only showed an attenuation in the increase in *Eubacterium hallii group*, but were still able to stimulate it (Supplementary material, Fig. S10A). Similarly, the HP-induced increase in *Mogibacterium* (Fig. 8D, Supplementary material, Fig. S13B) and *Coprococcus* (Fig. 7A, Supplementary material, Fig. S13C) was attenuated by the addition of fiber, although the magnitude of inhibition depended on the type of fiber(s) added. To illustrate, the relative abundance of *Coprococcus* increased with 25.6 ± 1.1 % in the first 8 h after HP_control whereas the addition of fiber minimized this increase (5.3–12.3 %). Similarly, *Mogibacterium* increased with 12.7 ± 1.2 % after 8 h in HP_control but fiber addition diminished this increase (1.6–3.8 %). *Methanobrevibacter* slowly increased more than 15-fold after 8 h (t = 0: 0.1 ± 0.1 %, t = 8: 1.1 ± 0.3 %) when HP_control was administered, yet all fiber mixtures containing potato-fiber were able to attenuate this increase throughout the proximal period (t = 8: 0.2–0.3 %). Thereafter, HP_inu_pot_pec, similar to HP_inu and HP_pec_inu, showed an increase in the distal period, whereas both potato-fiber combinations were still able to maintain its inhibition (Supplementary material, Fig. S13C). In line with the distally-orientated fermentation pattern of potato-fiber combinations, a higher potato-fiber content may suppress the abundance *Methanobrevibacter* for a longer period of time compared to the more proximal-orientated fermentation of inulin.

Specific fibers were also characterized by bacterial taxa that were either absent or less pronounced after HP_control administration in the proximal period. To illustrate, in line with the acute reduction in α-diversity after inulin administration, the relative abundance of *Bifidobacterium* was higher after HP_inu and all combinations containing inulin compared to HP_control. An increase with approximately 30–35 % at t = 4 was observed compared to baseline whereas changes in all protein controls were minimal (−0.3–5.8 %) (Fig. 9, Supplementary material, Fig. S13E). Similarly, *Megasphaera* remained low in the proximal period for most conditions, yet both combinations with potato-fiber displayed a specific increase in *Megasphaera* in this period (Fig. 9, Supplementary material, Fig. S13F).

During the period of distal fermentation, HP_control was characterized by an increase in the relative abundance of *Megasphaera* (Fig. 9, Supplementary material, Fig. S13E), *Allisonella, Peptoclostridium*, and *Dialister* (Fig. 9, Supplementary material, Fig. S14A-C), which were blunted by specific fiber or fiber combinations. To illustrate, both combinations with potato-fiber (HP_pot_inu and HP_pec_pot) reduced *Allisonella* compared to HP_control. Similarly, the relative increase in *Megasphaera* was attenuated by all conditions containing potato-fiber (HP_pot_inu, HP_pec_pot, and HP_ inu_pot_pec) in the distal fermentation period. Interestingly, this inhibiting effect was opposite from the effect of potato-fiber on *Megasphaera* observed in the proximal period. *Peptoclostridium*, which was not present in the baseline microbiota, increased between 4 and 24 h in all protein conditions (3.3 ± 0.8 %). All fiber and fiber combinations, except inulin and the mixture of all fibers, were able to attenuate this increase and the potato-fiber and pectin combination inhibited this increase the most (0.5 ± 0.2 %). For *Dialister*, only inulin individually inhibited the observed HP-induced increase. Interestingly, the observed attenuation of the HP-induced increase in *Mogibacterium* by the addition of fiber in the proximal period, was

opposite to the distal period. Here, HP slowly decreased the relative abundance of *Mogibacterium* whereas the fibers, especially the mixture of all fibers, increased the relative abundance of *Mogibacterium* in this period (Fig. 9, Supplementary material, Fig. S14B). Nevertheless, the relative abundance at $t = 24$ still remained below the levels of HP_control.

Fiber specific signatures were also identified for the distal period. *Prevotella 9*, for instance, only increased when specific fibers were present. Unlike HP_control, in which *Prevotella 9* remained low, adding pectin individually or in fiber combinations containing potato-fiber and/or pectin: HP_pec_pot, HP_pot_inu, and HP_pec_inu resulted in a higher relative abundance of *Prevotella 9* compared to HP_control, with the most pronounced effect for HP_pot_inu (Fig. 9, Supplementary material, Fig. S14D).

Lastly, overall, some very low relative abundance bacterial taxa including *Clostridium_sensu_stricto_1*, *Intestinibacter*, and *Streptococcus* were detected more often in the HP_control compared to when fiber was added to the HP context (Supplementary material, Fig. S15A-C).

Hence, adding fibers to a HP shifted microbial composition and both fiber-dependent (e.g. increase in *Bifidobacterium* and *Prevotella 9*) and fiber-independent (e.g. decrease in *Coprococcus* and *Mogibacterium*) changes were identified.

4. Discussion

The aim of this study was to explore the ability of different fibers and fiber combinations to steer the gut microbiome toward saccharolytic fermentation instead of proteolytic fermentation in context of a HP substrate matrix, specifically in the distal colon. First, we established the effect of protein quantity (LP vs HP) and type of protein (egg protein vs mix of protein from egg, fava bean, pea, and potato) on microbial metabolite profile and composition by using a dynamic *in vitro* model for the colon (TIM-2). Thereafter, three different fibers were evaluated, individually and in combination, against a HP background, for their ability to stimulate (distal) saccharolytic fermentation.

The results of this study confirm a shift toward proteolytic fermentation in the period simulating the distal colon, as evidenced by reduced SCFA production and increased BCFA production compared to the period simulating the proximal colon. In line, microbiota data revealed a substantial uniform increase in the relative abundance of *Coprococcus* in the proximal fermentation period. Members of *Coprococcus* have a diverse set of genes capable of SCFA production and several species also possess genes capable of the production of proteolytic metabolites including isovalerate and *p*-cresol (Fusco et al., 2023; Notting et al., 2023). Other SCFA-producing genera including *Faecalibacterium* and *Dorea* rapidly decreased after administration of fibers and/or proteins, independent of the type of substrate. This rapid decline in relative abundance indicates that the microbial environment was suboptimal for their growth in the acute setting of this study. Distal fermentation was characterized by an increase in the relative abundances of *Acidaminococcus*, *Peptoclostridium*, *Allisonella*, and *Megasphaera*, which are all reported to be involved in microbial protein degradation (Garner et al., 2002; Mei et al., 2020; Peled & Livney, 2021), with a simultaneous decrease in *Coprococcus*. A difference in pH as well as substrate availability between the proximal and distal fermentation period may explain these microbial differences. Proximal fermentation was characterized by a rapid decrease in α-diversity, which gradually began to increase during distal fermentation. The primary decline in α-diversity reflects the response to certain genera flourishing on specific substrates (e.g. *Bifidobacterium* on inulin) whereas, in the distal colon, cross-feeding mechanisms may stimulate the growth of other bacterial species or the initial fiber substrate may slowly become depleted thereby affecting previously stimulated species less. Overall, we only investigated the acute effect of substrate type on microbial composition, which only reflects the use and exchange of substrates whereas chronic effects (>24 h) on α-diversity may be different.

Interestingly, protein quantity did not linearly translate to SCFA and BCFA production. This holds especially true for SCFA production, which increased less than 20 % with double the amount of protein (LP vs HP), suggesting there may be a threshold for SCFA production from proteins. Although we cannot exclude an increased SCFA production due to a higher baseline α-diversity and relative abundance of *Prevotella 7* in the LP_control compared to the HP_control, which may have facilitated increased cross-feeding and the observed expansion of *Prevotella 7* in the LP condition. Nevertheless, increasing protein load may induce more deleterious effects as a result of a lower SCFA:BCFA ratio, especially in the distal fermentation period. The increased BCFA production in response to increased protein quantity coincided with a relative increase in the abundance of several proteolytic bacteria including *Peptoclostridium*, *Megasphaera*, *Acidaminococcus*, and *Allisonella*. Additionally, HP-induced increases in the relative abundance of *Eubacterium Halli group* and *Mogibacterium* were less pronounced in the LP_control. Members of *Mogibacterium* are mainly considered asaccharolytic, which means they retrieves energy from other sources than carbohydrates (Nakazawa et al., 2002). The abundance of *Mogibacterium* may be overrepresented in diabetes (Al Bataineh et al., 2020; Crusell et al., 2018; Jiang et al., 2022) and its abundance has been associated with several metabolites derived from protein degradation in pigs and T2DM patients (Jiang et al., 2022; Yu et al., 2018).

Changing the HP condition to consist of egg-protein alone did not affect total SCFA and BCFA production. The amount of BCAA (valine, isoleucine, valine), from which BCFA can be produced, was comparable between pre-digested egg, pea, fava bean, and potato proteins (Supplementary material, Table S1), which may explain similar BCFA production. Nevertheless, only providing egg protein showed distinct microbial compositional changes compared to the HP_control, which also contained plant-based proteins. Previous studies have indicated plant- and animal-based protein-based diets differentially affect microbiota composition but it is unclear to what extent this can be modulated by the type of protein per se (i.e. plant-based proteins are less digestible) or general differences in nutrient composition of plant and animal-based dietary patterns (e.g. more fiber and polyphenols in plant-based meals) (Korpela, 2018; Tomova et al., 2019). In this study, the decrease in the relative abundance of *Anaerostipes*, *Subdoligranulum*, *Eubacterium hallii group*, and *Blautia* may be partially explained by the residue compounds still present in the plant-based proteins after batch digestion (e.g. resistant starch), as these bacteria all have been predominantly linked to the degradation of plant-based dietary fibers (Bulut et al., 2023; Dobranowski & Stintzi, 2021). Nevertheless, the increased relative abundance of *Ruminococcus* in the egg HP condition is quite striking as this genus has been predominantly associated with complex carbohydrate fermentation (Kim et al., 2024; Salyers et al., 1977) and negative correlations between protein and *Ruminococcus* abundance have been reported *in vitro* (Amaretti et al., 2019). Slight differences in amino acid composition, specifically a higher methionine, cysteine, serine, and tryptophane content in the pre-digested egg protein compared to the pre-digested plant-based proteins (Supplementary material, Table S1), might explain these observed differences. To illustrate, previous studies have reported several *Ruminococcus* species are able to utilize cysteine (Herbeck & Bryant, 1974; Uchiyama et al., 2022).

As expected, all fiber conditions were able to increase total SCFA production compared to HP_control. The observed fiber-induced increase in SCFA production coincided with an increased relative abundance of *Blautia* and *Ruminococcus*, which was more pectin and inulin-dominated, respectively. Both genera have several species that are identified as SCFA producers and are associated with the breakdown of complex carbohydrates (Liu et al., 2021; Ze et al., 2012). In line with the structural complexity of the fibers, inulin resulted in a more proximally-dominated fermentation pattern whereas pectin and combinations containing pectin or potato-fiber were slightly more distally-orientated evidenced by increased distal SCFA production and slower fiber degradation in HPAEC and HPSEC experiments. From all fiber combinations,

combining pectin and potato-fiber resulted in the most pronounced increase in distal SCFA production (and SCFA:BCFA ratio). Frutafit® IQ is a native straight-chain fructan inulin, which is a polydisperse mixture of linear fructose polymers, mainly with a terminal glucose unit and is linked together by β(2–1) bonds, its degree of polymerization (DP) varies between 2 and 60 (Vogt et al., 2013). Due to the linear structure of native inulin, it may be more easily broken down by microbes and rapidly fermented in the proximal colon. GENU® BETA pectin is a soluble high methylester pectin and the degree of esterification typically approximates 55 %. Studies have indicated dietary fiber extracted from potato peel are mainly insoluble (>70 %), with a minor soluble fiber fraction and consist of a mixture of starch and non-starch polysaccharides including potato pectin, cellulose, and hemicelluloses although slight differences in composition may occur depending on manufacturing processes (Dhingra et al., 2012; Rodríguez-Martínez et al., 2021). The complex structures of potato-fiber and pectin may make it more difficult for the bacterial enzymes to access the other dietary fiber present and thereby slow down its fermentation. Interestingly, combining all three fibers together did not result in a synergistic effect, suggesting an optimal dose and/or ratio may be required to facilitate adequate cross-feeding.

The acetate:propionate:butyrate ratio in response to dietary fiber reported in this study (57:24:19) was similar to molar ratios reported in other studies using TIM-2 (Ramasamy et al., 2014) and the molar ratios of 60:25:15 and 60:20:20 that have been measured in the colon of sudden death victims (Cummings et al., 1979; Cummings et al., 1987). Inulin has been reported to have a higher butyrogenic potential compared to pectin (Duncan, Holtrop, et al., 2004; Grootaert et al., 2009; Van de Wiele et al., 2007). Yet, in this study, acetate:propionate:butyrate ratio appeared comparable between all fibers conditions, with an overall acetogenic effect and an equal contribution of propionate and butyrate. This might be due to a different microbial capacity of our baseline microbiota, as heterogeneity in the fermentation capabilities of the human gut microbiota in response to different substrates has been previously proposed (Aguirre et al., 2016; Gurry, Nguyen, Yu, & Alm, 2021)). To illustrate, a specific species of *Bifidobacterium*, *B. adolescentis*, can produce lactate which, in turn, is available for lactate-utilizing bacteria including several species of *Anaerostipes* and *Eubacterium hallii*, to produce butyrate (Dobranowski & Stintzi, 2021; Duncan, Louis, & Flint, 2004; van Deuren et al., 2022). Since the presence of *Anaerostipes* and *Eubacterium hallii group* in our baseline standardized microbiota was minimal (<1 %), insufficient cross-feeding may explain the lack of a pronounced butyrogenic effect in response to inulin. Another explanation might be the high protein background, as our results reflect a combined effect of fibers and proteins. In line, a previous TIM-2 study also observed an equal contribution of propionate and butyrate after administering a high protein diet whereas butyrate was increased after a high carbohydrate diet (Aguirre, Eck, et al., 2016). Additionally, we observed relatively more butyrate and propionate were produced with protein administration alone compared to when fibers were added to the HP context. In agreement, another *in vitro* study comparing different protein and fiber ratios showed increased butyrate and propionate production in response to a relative higher protein content (Jackson et al., 2024). Interestingly, *Coprococcus catus* is known to produce propionate via lactate formation from several amino acids via the acrylate pathway (Louis & Flint, 2017), which may explain the very pronounced increase in *Coprococcus* after HP-administration. Propionate also played a more prominent role in distal fermentation compared to proximal fermentation, which may highlight microbial cross-feeding processes and/or a switch to propionate production from indigestible proteins. If carbon sources are high (high fiber availability), amino acids are not used to form propionate, yet, under energy restricted circumstances (like in distal fermentation as a consequence of fiber depletion), amino acid catabolism can supply propionate biosynthesis (Gonzalez-Garcia et al., 2017).

Total BCFA production was consistently reduced by the addition of fiber, independent of fiber type. Dietary fiber availability can reduce microbial protein degradation in several ways. Carbohydrates are a more efficient source of ATP, therefore an abundance of carbohydrates promotes bacterial growth, increasing the microbial uptake of amino acids and other intermediates for use in biosynthesis pathways instead of fermentation. Additionally, the presence of carbohydrates may inhibit the expression of genes involved in amino acid breakdown, such as those encoding deaminases. SCFA production from fibers can also have a pH-lowering effect and the resulting acidic environment suppresses the activity of bacterial proteases, subsequently limiting amino acid breakdown (Peled & Livney, 2021). Nevertheless the inhibition of BCFA production after fiber addition was minimal, indicating that protein fermentation still continued. The lack of a pronounced inhibition of proteolytic fermentation may be explained by several factors. First, some microbial species in the distally abundant *Allisonella* and *Acidaminococcus* genera, such as *Allisonella histaminiformans* and *Acidaminococcus fermentans* use protein as a sole substrate source (Garner et al., 2002; Rogosa, 1969) and therefore may be less affected by fiber availability. Our findings suggest fibers inhibit the relative abundance of specific proteolytic bacteria (e.g. *Peptoclostridium* and *Mogibacterium*), while other proteolytic bacteria (e.g. *Acidaminococcus*) remain unaffected. Furthermore, HPAEC and HPSEC showed rapid inulin and pectin degradation, with most substrate depleted before 24 h. This depletion may enable proteolytic bacteria to expand at a later stage of fermentation. However, without time points between 8 and 24 h, we cannot determine precisely when this shift occurs. An even more complex dietary fiber or a higher dose of fibers may be required to further limit proteolytic fermentation.

The addition of fiber to a protein matrix resulted in an overall shift in microbial communities and these changes were fiber- and time-dependent. Inulin and inulin combinations resulted in an overall increase in *Ruminococcus* and specifically induced *Bifidobacterium* in the proximal fermentation period, which is in agreement with other human studies which observed an increase in the relative abundance of *Ruminococcus* after inulin exposure (Healey et al., 2018; Holscher et al., 2015) and the well-known bifidogenic effect of inulin (Meyer & Stasse-Wolthuis, 2009; Roberfroid et al., 1998). In contrast, pectin increased *Blautia* and potato-fiber combinations increased *Megasphaera* specifically in the proximal fermentation period, whereas pectin and combinations containing either pectin or potato-fiber were characterized by *Prevotella 9* in the distal fermentation period. A previous TIM-2 study also reported *Prevotella* stimulation by potato-fiber (Larsen, de Souza, et al., 2019) and evidence suggests that some *Prevotella* members have the capability to breakdown pectins (Yüksel et al., 2024). Although specific species of *Megasphaera* have been implicated with amino acid utilization, the genus also has Carbohydrate-Active enZYmes (CAZy) genes which allow for the breakdown of carbohydrates and may have beneficial health effects by converting lactate to butyrate (Shetty et al., 2013).

A key strength of this study is using TIM-2 as an *in vitro* model to mimic physiological conditions, allowing for mechanistic investigation of colonic processes, which was also important to select the fibers for the human intervention study. Based on the most optimal distal SCFA (and distal SCFA:BCFA ratio), the potato-fiber and pectin combination was selected and subsequently tested for the efficacy to improve peripheral insulin sensitivity and other cardiometabolic outcomes in a 12-week clinical study conducted in individuals with obesity and at increased risk of developing T2DM. Furthermore, the dynamic TIM-2 system prevents fermentation product accumulation and simulates physiological uptake of metabolites. In contrast, other models, including batch fermentation, lack sophisticated pH control and metabolite removal, which potentially influences microbiota composition and functionality, making it less physiologically relevant. Moreover, unlike other *in vitro* studies, this experimental design can differentiate between proximal and distal fermentation and pH was adjusted accordingly. Additionally, the protein sources used in these experiments were pre-digested prior to

performing the TIM-2 experiments and a mixture of several protein sources was used to create a more physiologically relevant model.

Naturally, there are also limitations to this study that should be considered. Although baseline β-diversity clustered well, slight baseline microbial composition differences may have influenced SCFA and BCFA production. Fiber purity was not accounted for, with an estimated purity of 70–75 %, 85 %, and 90 % for the PaselliTM potato-fiber, GENU® BETA pectin, and Frutafit® IQ inulin, respectively, SCFA production for inulin might be slightly overestimated. Fibers also slightly varied in protein and sugar content, however given the minor quantities in the fiber dose used for these experiments, these are expected to have minimal effects. Furthermore, since we could not test potato-fiber individually, it remains unclear whether this condition would have produced more SCFA although this is unlikely given its insoluble nature. Although the results presented here were reproducible among duplicates, the sample size of experimental conditions was small and our study therefore remains only explorative in nature. Lastly, we used SCFA and BCFA as important markers for saccharolytic and proteolytic fermentation respectively. Future studies should also consider other metabolites (e.g. succinate and lactate for saccharolytic fermentation, and ammonia, amines, hydrogen sulfide, and indoles for proteolytic fermentation).

5. Conclusions

We demonstrate protein load, protein type, and different types of dietary fiber within a high protein context have differential effects on metabolite production patterns as well as microbial composition, which provides novel insights in substrate fermentation along the gastrointestinal tract. Adding fiber to a HP context increased SCFA production, highlighting a shift toward saccharolytic fermentation, yet inhibition of BCFA production was limited. Time-specific (proximal vs. distal) as well as fiber-specific changes in microbial metabolite profile and microbial composition were identified. A mixture of potato-fiber and sugar beet pectin was best able to increase saccharolytic fermentation in the distal colon compared to the other fiber and fiber combinations tested. Hence, by utilizing various types of complex dietary fibers, the gut microbiota can be strategically modulated, which may ultimately confer health benefits to the host. Based on our results, combining potato-fiber and sugar beet pectin may be an effective approach to improving metabolic health by increasing distal SCFA availability, as previous studies suggest that distal SCFA elicit a more pronounced metabolic health effect (Canfora et al., 2017; van der Beek et al., 2016) possibly due to enhanced absorption in the distal colon, which increases the systemic availability and biological activity of SCFA in circulation.

As the objective of this study was to investigate fibers in a protein context, we used a mixture of different protein sources. However, future studies could use batch fermentation models, which allows for a more extensive set of experimental conditions, to disentangle specific effects of each protein, or different combinations of each protein with fiber(s), thereby providing more insight into specific protein-fiber interactions. Additionally, studies should further elucidate most optimal fiber-protein quantities and ratios in the proximal and distal colon. Nevertheless, *in vitro* models may not fully capture the complexity of fermentation in the context of a living intestinal microbiota and individual differences in microbiota composition may influence the fermentation pattern of different types of dietary fibers. Thus if distal microbial fermentation can be steered toward saccharolytic fermentation in humans remains to be established and, importantly, whether such changes translate into metabolic adaptations in individuals with obesity.

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CRediT authorship contribution statement

Thirza van Deuren: Writing – original draft, Visualization, Project administration, Investigation, Formal analysis, Data curation. **Alexander Umanets:** Writing – review & editing, Visualization, Software, Formal analysis, Data curation. **Koen Venema:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization. **Luis L. Moreno:** Writing – review & editing, Software, Investigation, Data curation. **Erwin G. Zoetendal:** Writing – review & editing. **Emanuel E. Canfora:** Writing – review & editing, Supervision. **Ellen E. Blaak:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Koen Venema reports a relationship with Beneficial Microbes Consultancy that includes: consulting or advisory. If there are 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.2025.116271.

Data availability

Datasets will be made available upon reasonable request. Requests to access the dataset should be directed to e.blaak@maastrichtuniversity.nl

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