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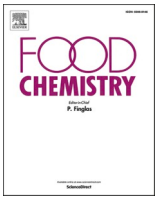

Exploring *in vitro* production of colonic microbial metabolites from diverse protein sources using human ileal digesta

Nikkie van der Wielen [a,d,1], Hanlu Zhang [a,1], Pien J.C. Schouten [b], Erik Meulenbroeks [b], Natascha Stroebinger [c], Suzanne M. Hodgkinson [c], Marco Mensink [d], Wouter Hendriks [a], Edoardo Capuano [b,*]

[a] *Animal Nutrition Group, Department of Animal Sciences, Wageningen University & Research, Wageningen, the Netherlands*
[b] *Food Quality and Design Group, Department of Agrotechnology and Food Science, Wageningen University & Research, Wageningen, the Netherlands*
[c] *Riddet Institute, Massey University, Palmerston North, New Zealand*
[d] *Division of Human Nutrition and Health, Department of Agrotechnology and Food Sciences, Wageningen University & Research, Wageningen, the Netherlands*

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ABSTRACT

We explored the relationship between protein fermentation metabolites and ileal digesta composition, using ileal digesta from ileostomates, who ingested nine different protein sources, incubated in the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®). NH_3, short-chain fatty acids, branched-chain fatty acids (BCFA), H_2S, tryptophan derivatives, and biogenic amines were measured in proximal and distal colon vessels. The relative changes in most metabolites were positively correlated with their amino acid precursors in ileal digesta. In both colon vessels, the relative change of NH_3 was a good predictor for the production of other metabolites. Indole was strongly associated with oxindole, 5-HT, and tryptamine and the sum of Trp metabolites in the distal colon. Per gram ingested protein, zein and whey may produce the highest levels of NH_3 and BCFA in the proximal colon and BCFA in the distal colon, whereas whey and pigeon peas may result in the highest levels of H_2S.

1. Introduction

There is wide consensus nowadays that the intestinal microbiome, *i.e.* the collective genome of all the microorganisms that resides in human gastrointestinal tract, greatly impacts human health (McCarville et al., 2020; Turnbaugh et al., 2009); and that diet represents one of the most important factors that modulate microbiota composition and activity (David et al., 2014). To decipher the complex interaction between diet, gut microbiota and human health, much attention has been devoted to fermentation of undigested polysaccharides, *i.e.* dietary fibre (Makki et al., 2018; Patnode et al., 2019). Comparatively, the effect of protein catabolism in the large intestine on host health is vastly understudied (Bartlett & Kleiner, 2022). Dietary, but also endogenous, proteins that escape digestion and absorption in the small intestine can become substrate for the microbial community thriving in the large intestine. These proteins can be used as building blocks for bacterial cells or can enter different catabolic pathways and become a source of energy for the residing microbiota (Lammers-Jannink et al., 2022). Historically, protein fermentation in the large intestine has been considered detrimental due to the production of potentially toxic metabolites such as ammonia (NH_3), phenols, and hydrogen sulphide (H_2S), which can disrupt cellular functions and increase the risk of inflammation and cancer (Windey, De Preter, & Verbeke, 2012; Yao et al., 2016; Zhang et al., 2020). However, polyamine-deficient diets result in a significant hypoplasia of the small intestinal and colonic mucosa (Löser et al., 1999) while research has shown that some tryptophan catabolites, like tryptamine and indole-3-propionic acid may actually exert protective functions for the human host *via e.g.*, the activation of the Aryl Hydrocarbon Receptor (Agus et al., 2018). Short-chain-fatty acids (SCFAs) are the primary metabolic by-products of carbohydrate fermentation but they can also be produced from protein fermentation. SCFA play an important role in intestinal homeostasis and affect the tissues and organs beyond the gut (Van der Hee & Wells, 2021). Instead, the physiological relevance of branched chain fatty acids (BCFA), exclusive products of protein fermentation, is still debated.

The extent of protein fermentation in the large intestine has been

* Corresponding author.
E-mail address: edoardo.capuano@wur.nl (E. Capuano).
[1] Shared first authors.

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positively associated with the amount of protein in the diet as well as negatively associated with protein digestibility (Corpet et al., 1995; Evenepoel et al., 1999). Low dietary protein concentrations and highly digestible proteins are, therefore, considered as strategies to improve intestinal health (Heo et al., 2010; Zhang et al., 2020). Other aspects, such as the role of the type of proteins, of the structural properties of the food at mesoscopic and microscopic level, as well as of the composition of the ileum effluent on the extent of protein fermentation have also been studied. The availability of fermentable carbohydrates in the ileum effluent as energy source for the gut microbiota is also reported as a potent modulator of the extent of protein fermentation (Lammers-Jannink et al., 2023). It is generally assumed that the presence of sufficient amounts of carbohydrates (*e.g.* dietary fibre) will reduce microbial degradation of proteins and increase the utilization of proteins to form bacterial proteins and eventually bacterial growth in the intestine (Macfarlane & Macfarlane, 2012). Different types of fibre can produce different effects on protein fermentation depending on their fermentability (Jha et al., 2011; Jha & Leterme, 2012). The presence of other dietary components *e.g.* polyphenols may also alter the microbial population (Rodríguez-Daza et al., 2021) and consequently the biochemical pathways of protein utilization.

Because of all the factors discussed above, intervention studies in humans focusing on protein fermentation in the large intestine have often resulted in contradictory outcomes regarding the effect on modulation of gut microbiota and level and profile of produced metabolites (Beaumont et al., 2017; SEPPUR_135499; Cai et al., 2022; Diether & Willing, 2019; Windey, De Preter, Louat, et al., 2012). This may be due to high interindividual variability among participants and the metabolite absorption by gut epithelium. For this reason, the use of *in vitro* models of colon fermentation may allow a more mechanistic understanding of the fermentation processes, through the careful measurement of the ileum effluent composition, standardization of the microbial activity and the capacity to accurately assess what is produced (and could potentially be absorbed) in the colon.

In the present study we have applied a hybrid approach to study protein fermentation in the human large intestine, combining *in vivo* and *in vitro* experiments, to compare the profile of several protein fermentation metabolites from a variety of food sources of proteins including plant and animal sources. To the best of our knowledge, this is the first time that ileal effluent collected from ileostomy patients have been directly used as inputs in *in vitro* colonic fermentation experiments. The advantage of this approach is that the substrate provides a better representation of *in vivo* inputs into the colon compared to *in vitro* digested meals or artificially simulating ileal digesta as it does not only contain undigested food but also endogenous materials. This approach has been previously applied with porcine ileal digesta in subsequent batch fermentations (Lammers-Jannink et al., 2023; Montoya et al., 2021; Zhang et al., 2024). Here, the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®) was used because this semi-continuous fermentation vessel is designed to study the effect of repeated feeding and multiple segments of the colon on the production on the microbial metabolites.

Important questions that were addressed in this study were: 1) the relationship between the content in precursors AA in the fermentable substrate and the concentration of protein fermentation metabolites produced therefrom; 2) the correlation among protein fermentation metabolites in the same colonic segment (proximal or distal colon); 3) the correlation between protein fermentation metabolites produced in the different colonic segments (proximal and distal colon); 4) the comparison of the test meals in terms of potential formation of protein fermentation metabolites.

2. Materials and methods

2.1. Ethics

The ileal digesta samples used in the present study originated from two ileostomy studies previously conducted for which approval of the medical ethical committee of Wageningen University (NL63446.081.17; NCT04207372) or the Massey University Human Ethics Committee Southern A (ref. 16/59) and the New Zealand Ministry of Health Central Health and Disability Ethics Committee (ref. 18/CEN/215) was obtained.

2.2. Ileal digesta samples

Ileal digesta was obtained as described by (Hodgkinson et al., 2022; van der Wielen et al., 2023). Briefly, ileostomy patients fasted overnight and ingested a meal containing 25–30 g of test protein as the sole protein source. After consumption, ileal digesta was collected over a 9-h period. Each protein source was tested on two study days and ileal digesta of one participant consuming one protein source was pooled. For the *in vitro* fermentation described in the present study, for each of the diet tested, the ileal digesta that was fermented *in vitro* was the results of the pooling of individual ileal digesta of n individuals consuming that test diet. These samples include the freeze-dried ileal digesta collected from different Dutch participants after ingestion of either whey protein isolate (hereafter whey, $n = 7$), zein (n = 7) or protein-free meal (n = 7); the freeze-dried ileal digesta from different participants from New Zealand collected following ingestion of pigeon peas ($n = 5$), black beans (n = 5), wheat bran (n = 5), bovine collagen ($n = 6$), toasted wheat bread (n = 5) and chickpeas ($n = 4$).

2.3. In vitro fermentation

SHIME® system (*ProDigest*, Ghent, Belgium) was used to simulate the proximal and distal colon of the human intestinal tract. The setup consisted of three double-jacketed vessels (TRIPLE-SHIME® set-up) each simulating a different section of the human intestinal tract: combined stomach and small intestine, the proximal colon (PC, pH 5.6–5.9), and the descending colon (DC, pH 6.6–6.9).

A freshly donated fecal sample from one healthy donor was collected in 2019 and stabilized for two weeks in the SHIME vessels with SHIME feed. After this stabilization period, the microbiota was collected and stored with 50 % *w*/w cryoprotectant at −80 °C till its use in the present study to inoculate the SHIME® system as previously described by (Koper et al., 2019). During the 3-days stabilization, 70 mL Adult L-SHIME growth medium basal feed (PD-NM001B, *ProDigest*, pH 2, composition: 1.2 g/L arabinogalactan, 2.0 g/L pectin, 0.5 g/L xylan, 0.4 g/L glucose, 3.0 g/L yeast extract, 1.0 g/L special peptone, 3.0 g/L mucin, 0.5 g/L l-cysteine-HCl, and 4.0 g/L starch) and 30 mL pancreatic juice (BD Biosciences, The Netherlands, 12.5 g/L $NaHCO_3$, 6 g/L oxgall, 0.9 g/L pancreatin; pH 7) were added in feeding cycles of 8 h.

After 2 days into the stabilization phase, the combined stomach and small intestine vessel was fed in feeding cycles of 8 h with either 70 mL pooled ileal digesta samples (1.05 g in water, pH 2) + 30 mL water (treatments) or with 70 mL basal feed (1.05 g, in water) + 30 mL pancreatic juice (control), resulting in a final concentration of 10.5 g/L. After 3 h, the total volume of the combined stomach and small intestinal vessel (100 mL) was transferred to the PC vessel and simultaneously a same volume was transferred to the DC vessel. The vessel volumes (250 mL in the PC and 400 mL in the DC), pH, temperature (37 °C), and retention times were always kept constant. The experimental design included 9 separate experiments. Each separate experiment in the TRIPLE-SHIME had 3 treatments with a control treatment with the standard basal feed and two ileal digesta samples. Every ileal digesta sample was tested in two independent experiments.

Each experiment had a total duration of 56 h. Samples were taken

from the PC and DC after different time points: 25 mL was taken from PC and 40 mL from DC at 0, 24, 32, 48, and 56 h; 23 mL was taken from PC and 38 mL from DC at 8 h; 1 mL was taken from both PC and DC at 2, 4, 50, and 52 h. H_2S analysis was carried out immediately. For quantification of Trp and indole derivatives, aliquots were stored at −80 °C. For all other analyses samples were stored at −20 °C. The *in vitro* colon simulation part of this study does not fall within the remit of the 'Medical Research Involving Human Subjects Act'. All this part of the work has been done in agreement with the WMA Declaration of Helsinki about Ethical Principles for Medical Research Involving Human Subjects. The healthy volunteer gave consent to take part in the study and use their information and all the privacy rights of the human subject have been observed.

2.4. Chemical analyses

2.4.1. Composition basal feed and ileal digesta samples

Information about the pooled ileal digesta samples and basal feed is show in Table S1. The C and N content were analysed using the Dumas combustion method ISO 16634 (ISO, 2008). Procedures according to ISO13904 and ISO13903 were followed to determine AA composition of basal feed and individual ileal digesta samples and the composition of pooled samples subsequently was calculated (Fig. S1).

2.4.2. Metabolites in SHIME samples

For the determination of NH_3, samples were deproteinized with trichloroacetic acid, followed by reaction of NH_3 with C_6H_6O and NaClO in an alkaline solution (pH 11.3–11.7) to form indophenol and absorbance measurement photometrically at 623 nm (Rhine et al., 1998).

For the determination of short chain and branch chain fatty acids (SCFA and BCFA), samples were diluted in H_3PO_4 solution, containing $C_6H_{12}O_2$ (3.69 g/L) as an internal standard. The metabolites were separated using gas chromatography and detected by Flame Ionization Detector (Mcwilliam & Dewar, 1958).

Biogenic amines were measured using two different methods due to a change in the internal standard in the laboratory. Some of the samples were measured using cation-exchange chromatography. Briefly, biogenic amines were extracted with dilute HCl, and the clear supernatant obtained after centrifugation was injected into a liquid chromatograph (Sykam S433, Germany) equipped with a fluorescence detector (Shimadzu RF-20a, Kyoto, Japan) using a Phenomenex Kinetex 1.7 μm EVO C18 100 Å LC column (100 ×) and a cation-exchange column (Sykam LCA K17/K). The biogenic amines were separated using potassium buffers (Sykam KA1, KB1) and detected fluorometrically following post-column derivatization with OPA. Biogenic amines from the remaining samples were measured using reversed-phase chromatography after dansylation, according to (Saarinen, 2002).

For the determination of H_2S, the samples were mixed 1:1 (*w/v*) with 200 μL 5 % ZnAc, followed by the simultaneous addition of 500 μL reagent A (0.2 % $C_{10}H_{10}O_4$ in 20 % H_2SO_4) and 50 μL reagent B (9 % $FeCl_3 \bullet 6\ H_2O$ in 2 % H_2SO_4) and incubated at room temperature. After exactly 30 min, the absorbance was measured at 660 nm (Cary 50 UV–Vis Spectrophotometer, Varian, United States) and corrected for the absorbance of the digesta samples without the addition of the reagents. For quantification of H_2S, a calibration curve ranging from 0 to 35 μM was made with a dilution stock containing Na_2EDTA, ZnAc, and Na_2S. Analysis was carried out in triplicate.

For the quantification of Trp derivatives, the PC and DC samples were defrosted and centrifuged at 9000 ×*g* for 5 min at 4 °C where after 0.5 mL supernatant was collected and filtered through a 0.20 μm regenerated cellulose filter. Derivatives of Trp in the supernatant were quantified as previously described (Huang, Boekhorst, et al., 2023). Briefly, the centrifuged and filtered PC supernatants were diluted 10-fold with Milli-Q water, and DC supernatants were used undiluted. The samples were then subjected to targeted analysis for Trp derivatives, including indole-3-propionic acid (IPA), indole-3-acetic acid (IAA), indole-3-lactic acid (ILA), indole (Ind), oxindole (Oxi), indole-3-aldehyde (I3A), tryptamine (TA), kynurenine (Kyn), and serotonin (5-HT). Derivatives were measured *via* a Shimadzu Nexera XR LC-20ADxr UPLC system coupled with a Shimadzu LCMS-8050 mass spectrometer (Kyoto, Japan). Chromatographic separation was accomplished on a Phenomenex Kinetex 1.7 μm EVO C18 100 Å LC column (100 × 2.1 mm) using 0.1 % CH_2O_2 in water (*v*/v) as mobile phase A and 0.1 % CH_2O_2 in MeOH (v/v) as mobile phase B. Tryptophan derivatives were identified by comparing the transitions (*m/z*) and retention time with reference standards (Huang, de Vries, et al., 2023). Data analysis was performed on LabSolutions LCMS 5.6 (Shimadzu Corporation, Japan).

2.5. Data analysis

In general, the concentration of any metabolite in any vessel of the SHIME system is the net result of its microbial production, transfer from the previous vessel and its elimination from the vessel through the liquid transferred to the next unit of the system every 8 h. Since the volume of liquid transferred from any two vessels is the same in all the experiments and treatments, the relative change of each metabolite compared to the baseline was selected as a measure of the net amount produced by the gut microbiota.

The relative change of metabolites in the system compared to baseline values (metabolite concentration at $t = 0$ h prior to the first infusion of test feed) was calculated by the equation:

$$\Delta X_t = \frac{CX_t - CX_0}{CX_0}$$

where ΔX_t = relative change of metabolite X at time t (%); CX_t = concentration of X at time t (h); CX_t = concentration of X at 0 h.

The relative change of metabolites at $t = 56$ h (ΔX_{56}) was used in the further described data analyses. Additionally, an overall relative changes of Trp derivatives were calculated from the sum of Trp derivatives at 56 h and 0 h. The relative change in metabolites produced was further recalculated and expressed as relative change per gram of ingested protein, by considering the apparent nitrogen content in ileal digesta and the apparent ileal digestibility reported in Table S1. Pearson correlation was applied to explore the relationships between different metabolites as well as metabolites and their precursors. Outliers of relative change of metabolites and precursor in ileal digesta were detected using the interquartile range method. $P < 0.05$ is considered significant.

3. Results

3.1. Change in metabolite levels over time

During the 56-h stabilization phase with the basal feed, the concentration of most of the protein-derived microbial metabolites, as exemplified by the NH_3 concentration, remained relatively constant over time (Fig. 1). When the feeding was switched to human ileal digesta, the concentrations of NH_3 decreased over time and resulted in a negative relative change. After 56 h the relative decrease in NH_3 concentration was ~50–80 % in PC for all the diets except zein, while the relative decrease was between 12 and 45 % in DC for all the diets (Fig. 1).

3.2. Relationships between metabolites and precursors

The correlation between relative change in the concentration of a metabolite against the concentration of the corresponding precursor AA (or AAs) in the ileal digesta or basal feed is shown in Fig. 2. For NH_3, the total concentration of N in the ileal digesta was used as precursor since NH_3 can be produced from the deamination of any AA (Lewis & Emery, 1962). A significant positive correlation for most of the metabolites

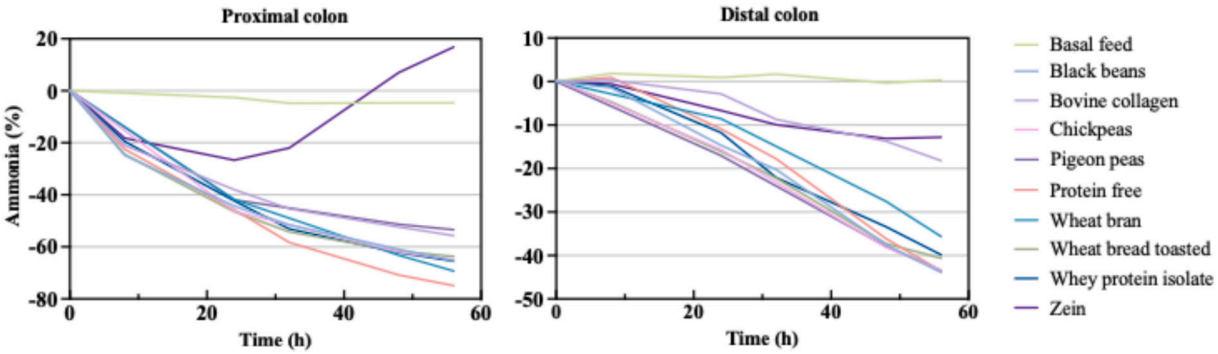

Fig. 1. Example of metabolite change over time. Relative change in ammonia concentration over 56 h compared to baseline in proximal and distal colon SHIME vessels after infusion with basal feed and human ileal digesta obtained after ingestion of different test protein meals.

(NH_3, isobutyrate, isovalerate, cadaverine, spermidine and H_2S) with the corresponding precursor(s) was observed. For spermine, only in the PC vessel a significant correlation with their corresponding precursors Arg and Met was observed. No significant correlation was found between the concentrations of putrescine and Arg, Met or Glu (data not shown). Spermine in PC and spermidine in both vessels were significantly correlated with Glu as well ($P < 0.01$, data not shown).

No general trend was observed in terms of correlations between specific Trp metabolites and the concentration of Trp in the ileal digesta with some tryptophan metabolites showing a positive correlation, while others showing a negative or no correlation (Fig. S2). A significant negative correlation was found between the concentration of the sum of the monitored Trp metabolites and the concentration of Trp in the ileal digesta in the DC vessel of the SHIME (Fig. S2). No consistent trend was observed in the Trp related correlations/concentrations between PC and DC either. C:N ratio was negatively correlated with all the protein fermentation metabolites except for some Trp metabolites.

3.3. Relationships between metabolites

Pearson correlation analysis was conducted to explore the underlying relationships within the metabolite data obtained from different substrates (Figs. 3 & 4). Ammonia was highly correlated to BCFA in PC and DC, and highly correlated to SCFA and H_2S in DC. Correlation between NH_3 and all biogenic amines except putrescine was also high and positive in PC, while less in DC. In PC, spermine and spermidine (both from Arg, Met or Glu) were highly correlated.

Correlation among individual SCFA was stronger in DC than in PC, whereas the correlation among individual BCFA was strong in both PC and DC. In both PC and DC, the sum of Trp metabolites is strongly correlated to indole since its concentration was much higher (up to 100 times in PC and to 2000 times in DC) than other Trp metabolites. In PC, the sum of Trp metabolites was negatively correlated to all the other markers of protein fermentation except for putrescine, although the correlations were not statistically significant. In contrast, in the DC the sum of Trp metabolites were all positively correlated to protein markers with most of the correlations statistically significant. No clear trend could be observed for individual Trp metabolites. In general, indole, oxindole, 5-HT and tryptamine showed strong positive associations in DC and this group was negatively associated with ILA, IPA and IAA.

3.4. Relationships between metabolites in PC and DC

A very strong positive correlation ($r > 0.8$) between the relative change in the concentration in the two colon vessels was shown for NH_3, butyric acid, valeric acid and BCFA. Strong positive ($0.6 < r < 0.8$) correlations were shown for acetic acid, propionic acid, cadaverine, tyramine and spermidine. Putrescine, spermidine and Trp derivatives did not show any correlation between the two colon vessels (Table 1).

3.5. Metabolites relative to ingested food proteins

The data provided so far refer to the level of metabolites produced upon fermentation of an equal aliquot of ileal digesta. However, that aliquot corresponds to different amounts of test meals because of their different digestibility. To explore the potential impact of the same amount of each protein to produce microbial metabolites, the relative changes of each metabolite have been recalculated and expressed per gram of protein intake in the meal (Fig. 5). In the PC, zein showed the highest relative change, indicating the highest NH_3 production among all diets. Whey and pigeon peas showed the second and the third highest value. In the DC, whey showed the highest value, followed by pigeon peas and collagen. The relative change in BCFA from the different meals is highly consistent in the two segments of the colon, and largely overlapping with the relative changes in NH_3, with zein the largest potential producer followed by whey and pigeon peas. A similar trend, in terms of ranking of the different meals, can also be observed for H_2S (only detected in the DC segment) with the notable exception of zein which produced the lowest levels. On the contrary, whey proteins, pigeon peas and toasted wheat bread produced the highest level of SCFA in both the colon segments whereas bovine collagen and zein produced the lowest levels of SCFA.

4. Discussion

Our study aimed to further explore the relation between formation of protein fermentation metabolites throughout the colon and the composition of the ileal digesta as well as the correlation among protein fermentation metabolites in each segment of the colon. For this purpose, we used a unique combination of human ileal digesta and the SHIME system to test a number of protein sources of animal and plant origin.

In the SHIME system, the concentration of any metabolite in each vessel is determined by its production by the microbial population, its transfer from the previous vessel, and its elimination *via* liquid transferred to the next unit. Since the volume of liquid transferred is consistent across experiments and treatments, the relative change in metabolite concentration from the baseline was used here to measure production by the gut microbiota. However, since the protein level of the SHIME basal feed was richer in protein than any of the ileal digesta, a decrease in the concentration was observed during the 56-h exposure. The extent of the drop in concentration at 56 h compared to the baseline therefore indicates the extent of production of the metabolite, with larger drops indicating a lower production of the metabolite. This justifies the use of relative change as indicator for comparing protein sources; and still allows to explore the correlation between precursor amino acids and fermentation metabolites.

All together, we showed that the concentration of several metabolites was clearly correlated to the composition of ileal digesta that would flow into the colon, although there were also exceptions. Ammonia

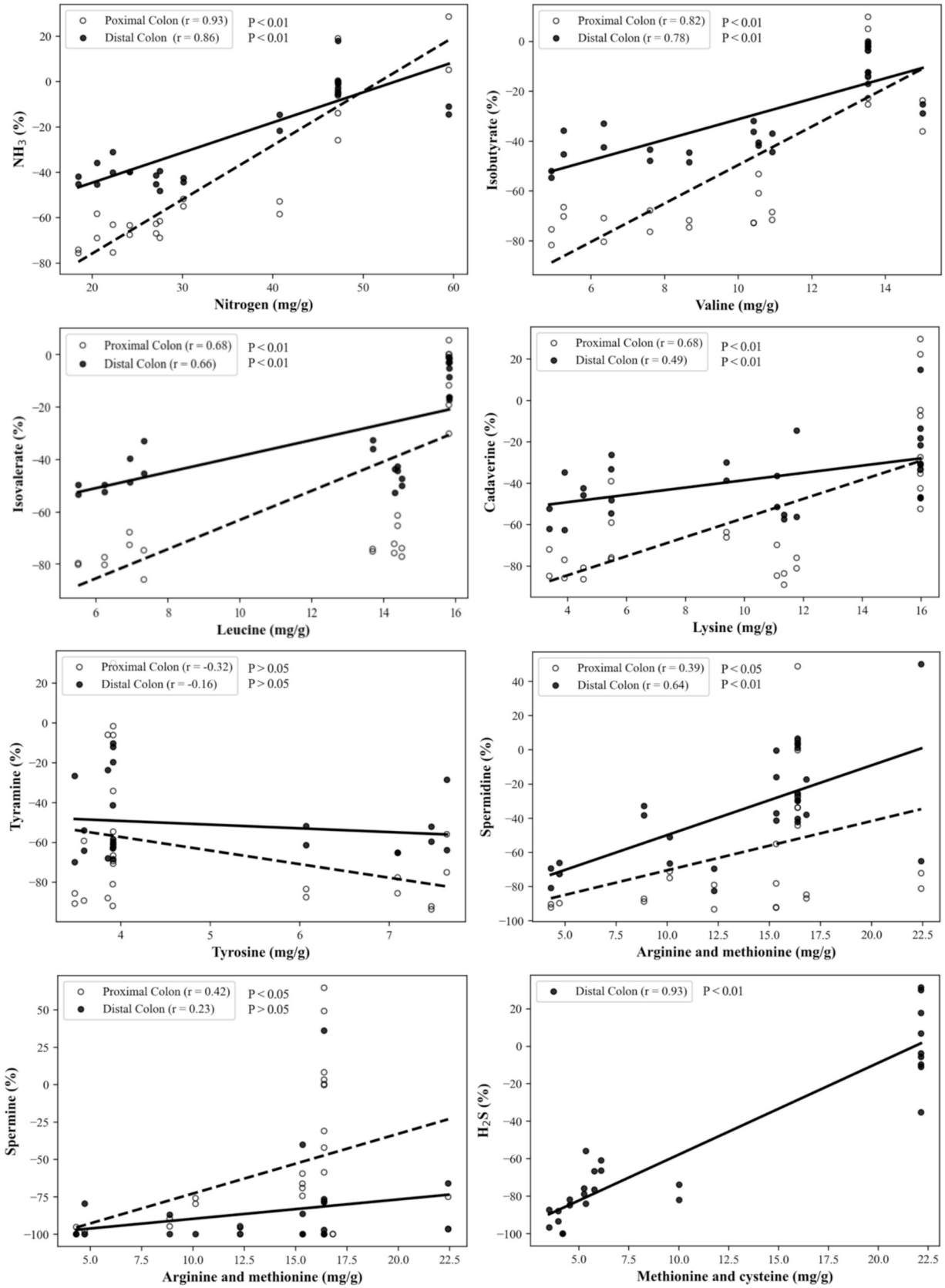

Fig. 2. Correlation between metabolites and their precursor(s). Pearson correlation between the relative change in the concentration of different metabolites compared to baseline in proximal and distal colon SHIME vessels after 56 h infusion with basal feed and the concentration of amino acid(s) in the human ileal digesta obtained after ingesting different protein meals.

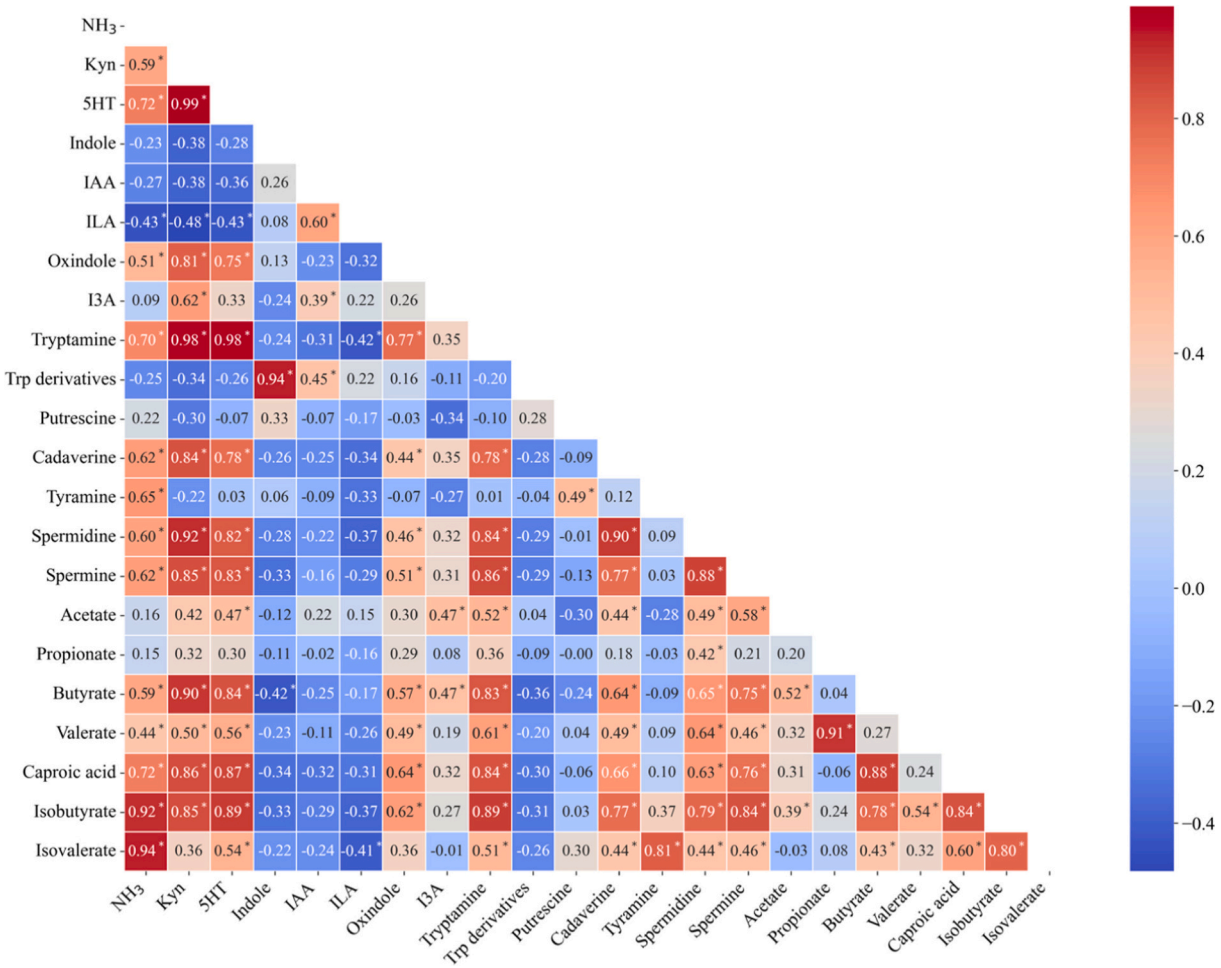

Fig. 3. Correlation between metabolites in proximal colon. Pearson correlation coefficient between the relative changes of metabolite concentrations compared to baseline in the proximal colon vessel of SHIME after 56 h infusion with basal feed and human ileal digesta obtained after ingesting different protein meals. Kyn: Kynurenine, 5HT: 5-Hydroxytryptamine (serotonin), IAA: Indole-3-acetic acid, ILA: Indole-3-lactic acid, I3A: Indole-3-acetaldehyde. *$P < 0.05$.

production was highly correlated to BCFA production in both the PC and DC. Ammonia also correlated well with most biogenic amines in the PC and H_2S in the DC. Many Trp derivatives in the indole pathway on the other hand showed an inverse relation with other protein fermentation metabolites in the PC. The different test meals showed a different potential in producing protein metabolites when the same amount of protein is consumed, with whey, zein and pigeon peas producing relatively more than other test meals. These findings will be discussed in the following sections.

4.1. Relationships between metabolites and precursors

Nowadays the origin of several protein fermentation metabolites (*i.e.* the precursor AA) is well established and several studies have reported changes in the level of protein fermentation metabolites, mostly in response to varying level of protein intake. In the present study we aimed to determine correlations between protein fermentation metabolites and their precursor AAs. We found significant correlations for many of the metabolites with the corresponding precursor (or precursors). For NH_3 the high correlation with N input was similar to that reported by many other *in vitro* studies and rumen fermentation (Castro et al., 2021; Figueiras et al., 2010; Sampaio et al., 2010). In contrast, one study found decreased NH_3 concentrations with increased N in batch cultures with ruminal microorganisms, which the authors explained by the incorporation of AAs and peptides into microbial protein without being first deaminated into NH_3 (Vanegas et al., 2017). Similarly, *in vitro* as well as *in vivo* studies have shown that an increased BCFA concentration was measured when increasing dietary protein intake (Aguirre et al., 2016; David et al., 2014). Our results are in contrast to Lammers-Jannink et al. (Lammers-Jannink et al., 2023), who reported no correlation between BCFA and its precursors in a batch fermentation assay with ileal digesta of pigs that ingested the same batch of protein sources compared to our study. The positive relationship between H_2S production and sulphur-containing AA levels in the substrate was also previously shown both in *in vitro* and in a controlled feeding study in humans (Chiku et al., 2009; Magee et al., 2000). In general, H_2S is regarded as a harmful metabolite due to its potential toxicity and negative effects on gut health (Beauchamp et al., 1984). Besides sulphur-containing AAs, mucin, that contains relatively high levels of sulphate, can also act as precursor for H_2S (Willis et al., 1996). Even though our ileal digesta will likely contain considerable quantities of mucin, it contributed comparatively little considering the high correlation with sulphur-containing AA found here ($r = 0.93$). Altogether, the correlation of NH_3, BCFA and H_2S and their corresponding precursors were high ($r > 0.75$), and these correlations were higher in the PC compared to DC vessel. It should be noted that we do not have data on the precursor concentrations for the digesta flowing into the DC, as the ileal digesta precursor concentration is likely already affected by the metabolism in the PC.

Not all amines correlated well to their precursor and the observed significant correlations were weaker (0.39–0.68) compared to the previously mentioned metabolites. Limited data are available in the literature on the correlations between amines and their precursors. For

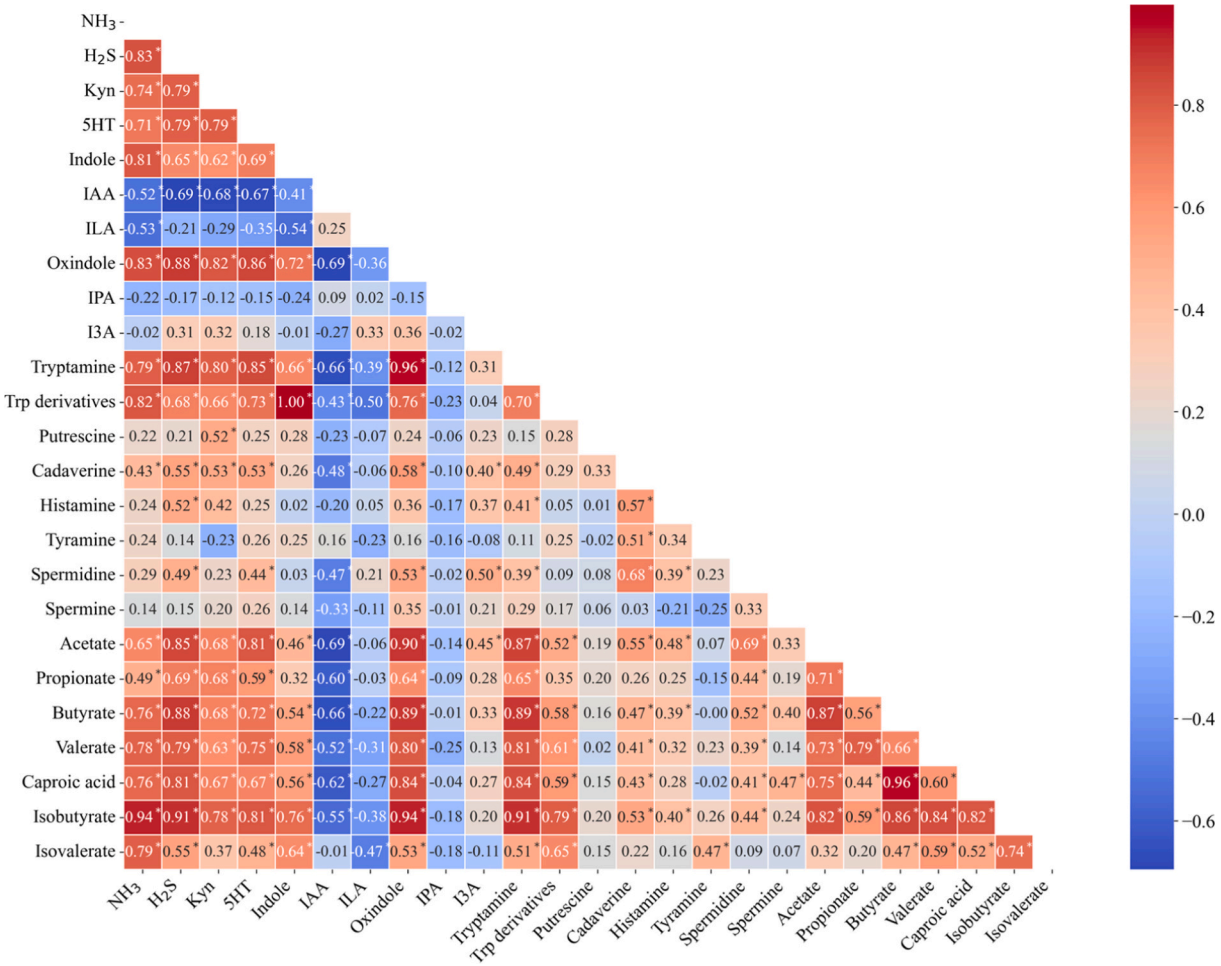

Fig. 4. Correlation between metabolites in distal colon. Pearson correlation coefficient between the relative changes of metabolite concentrations compared to baseline in the distal colon vessel of SHIME after 56 h infusion with basal feed and human ileal digesta obtained after ingesting different protein meals. Kyn: Kynurenine, 5HT: 5-Hydroxytryptamine (serotonin), IAA: Indole-3-acetic acid, ILA: Indole-3-lactic acid, IPA: Indole-3-propionic acid, I3A: Indole-3-acetaldehyde. *$P < 0.05$.

Table 1
Correlation between proximal and distal colon metabolites. Pearson correlation coefficient (r) between the relative changes of metabolite concentrations compared to baseline in the proximal and distal colon vessel of SHIME after 56 h infusion with basal feed and human ileal digesta obtained after ingesting different protein meals.

Metabolite	r	*P*-value
Ammonia	0.87	< 0.01
Tryptophan derivatives	0.004	0.99
Putrescine	−0.2	0.33
Cadaverine	0.75	< 0.01
Tyramine	0.59	< 0.01
Spermidine	0.49	< 0.01
Spermine	0.26	0.22
Acetic acid	0.78	< 0.01
Propionic acid	0.75	< 0.01
Butyric acid	0.85	< 0.01
Valeric acid	0.84	< 0.01
Isobutyrate	0.95	< 0.01
Isovalerate	0.97	< 0.01

cadaverine, it was earlier reported that only trace levels were observed after incubating Lys in an *in vitro* batch fermentation system (Smith & Macfarlane, 1997). Additionally, cadaverine concentrations were previously reported to decrease in batch fermentations (Lammers-Jannink et al., 2023), suggesting a potential negative feedback mechanism or further conversion by the microbiome. For example, His induces the expression of the His decarboxylase gene, but histamine itself can reduce this expression in lactic acid bacteria, indicating a feedback mechanism (Landete et al., 2006). Moreover, amine production can be affected by the bacterial species present and their respective metabolic capabilities. These metabolic activities are widely spread across different taxa present within the human gut microbiota (Pugin et al., 2017). The metabolic fate of histamine itself can also contribute to the observed weak correlation. Histamine can be further metabolized by histamine-degrading bacteria, leading to fluctuations in its concentration during fermentation (Pugin et al., 2017). Overall, these complex interactions between synthesis, regulation, and degradation pathways result in a less straightforward relationship between precursor levels and amine productions, explaining the weak correlations observed in experimental studies. Furthermore, some of the metabolites (putrescine and spermidine) are intermediate products in a metabolic pathway which may explain the lack of a correlation with its precursor.

The complex trends found for Trp metabolites is not in line with previous reports that have shown that supplementation of Trp increases the microbial production of Trp metabolites in an *in vitro* gut model of colonic fermentation with human gut microbiota (Koper et al., 2022) as well as in mice and pigs using a Trp-rich diet (Huang, de Vries, et al., 2023; Lamas et al., 2016; Liang et al., 2018; Yin et al., 2021). It must be noted though that the level of Trp supplementation in the study of Koper et al., 2022 was much larger than the window of Trp content across ileal

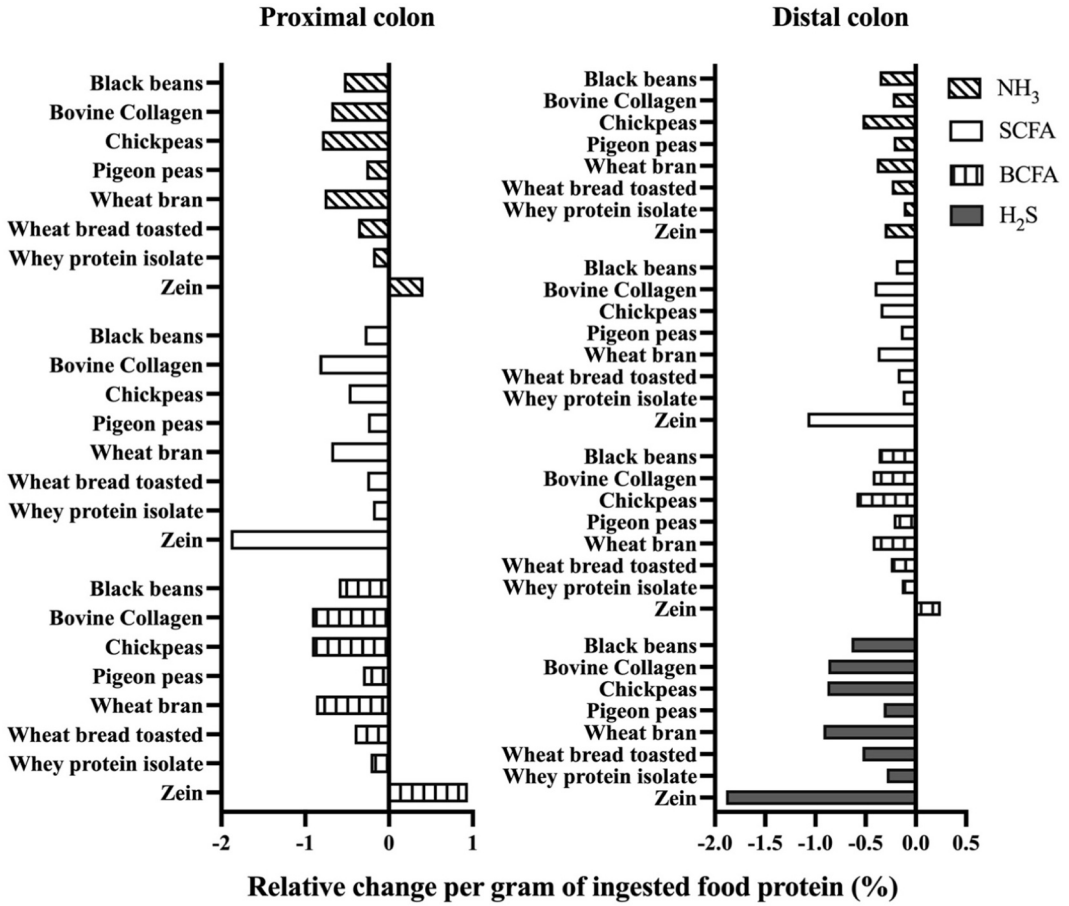

Fig. 5. Relative change in ammonia (NH_3), short-chain fatty acids (SCFA), branched-chain fatty acids (BCFA) and H_2S over 56 h compared to the baseline in proximal and distal colon of the SHIME system per gram of ingested protein. Values were calculated from concentration of each (group) of metabolites in the SHIME vessels corrected by the digestibility of the different sources. The less negative the value, the higher the potential of the unit mass of a protein to produce a certain metabolite.

digesta of the present study and that the increase in Trp metabolites in the other studies was not always proportional to the level of the supplementation, *i.e.* the content dropped at the highest level of supplementation (Liang et al., 2018; Yin et al., 2021).

Similar to previous studies (Smith & Macfarlane, 1996a, 1996b; Lammers-Jannink et al., 2023), C:N ratio of the ileum digesta was found to be negatively correlated to all the protein fermentation metabolites except for a few Trp derivatives. It has been reported that carbohydrates can reduce the production of protein metabolites by providing an alternative substrate for microbial fermentation (Jha & Berrocoso, 2016). However, since the N level across test proteins was not standardized, this correlation is mostly driven by the different N level in the substrate, *i.e.* high level of N produced higher concentrations of metabolites, independently of the C level which did not vary much in our samples. Another study where the C:N ratio was standardized during the incubation, the production of isobutyric, isovaleric acid and tyramine was found largely dependent on the fermentable carbohydrate level (Lammers-Jannink et al., 2023).

4.2. Correlation among metabolites

Here for the first time, we report a high positive correlation between the levels of NH_3 and H_2S as well as with several Trp metabolites in a human fermentation system. This reveals the potential of using NH_3 as a biomarker for H_2S in the future. Similarly, a high correlation between NH_3 and BCFA was found in our study, which has been already reported in previous studies (Lammers-Jannink et al., 2023; Trefflich et al., 2021; Yang & Rose, 2016). However, this may be biased by a relation between nitrogen content and the precursor AA content in the samples.

The correlation of NH_3 with many of the amines observed in the PC, disappeared in the DC. This is in contrast to results from batch fermentations of ileal digesta of pigs, where NH_3 remained correlated to tryptamine and tyramine over time (Lammers-Jannink et al., 2023). This suggests that our SHIME set up, that differentiates between segments of the colon, provides relevant additional insight in functional differences in microbiota in proximal or distal colon compared to batch fermentations. This site-specific difference is also shown in a study where NH_3 and amines were found only increased in PC of the piglets fed with high-protein diet but not in DC (Pieper et al., 2014).

Although in the PC only butyrate was positively correlated with NH_3, all three SCFA were correlated with NH_3 in the DC. At first sight, this positive correlation between SCFA and NH_3 seems contradictory as SCFA are the main products of saccharolytic fermentation and NH_3 is a product of proteolytic fermentation, nevertheless SCFA are known to also be produced during proteolytic fermentation (Smith & Macfarlane, 1997). The negative correlation between propionic acid and NH_3 in the PC is similar to that previously reported in batch fermentation with ileal digesta of pigs ingesting similar protein sources (Lammers-Jannink et al., 2023), whereas the correlation became positive in the DC. This once again underlines the additional value of differentiating between different segments in the colon.

In PC, spermine and spermidine (both from Arg, Met or Glu) were highly correlated, as expected from spermine being a precursor of spermidine (Portune et al., 2016), however both were poorly correlated

to putrescine, which is a precursor of both. This could be because putrescine has a shorter microbial half-life compared to spermidine and spermine, which are more stable and have longer microbial half-lives (McCormick, 1978).

Correlations of the sum of the Trp metabolites or individual Trp metabolites and other markers of protein fermentation are more erratic and difficult to explain. In PC, the sum of Trp metabolites is negatively correlated to all the other markers of protein fermentation with the exception of putrescine. This negative correlation is probably mostly driven by the negative correlation between indole and the other protein fermentation markers, indole being the most abundant Trp metabolite. Different trends found for individual Trp metabolites may be due to the different metabolic pathways involved in their production (Liu et al., 2022). Tryptophan catabolism occurs *via* four pathways. The first two pathways, the kynurenine pathway and the serotonin pathway produce kynurenine and serotonin, respectively (and catabolites related to each of them). In the other two pathways either indole (and oxi-indole) (through the initial activity of tryptophanase A, TnaA) or several other metabolites, including TA, ILA, I3A, IAA and IPA, (trough the activity of tryptophan 2-monooxygenase, TMO; tryptophan decarboxylase, TrD; aromatic amino acid aminotransferase, ArAT), are produced; these metabolites were all detected in our samples. Similar inconsistent trends between individual Trp metabolites were also observed during a previous SHIME study (Huang, Boekhorst, et al., 2023). After incubating with the basal diet for 6 days, the overall concentrations of Trp metabolites, IAA and indole were higher in DC than PC, despite the concentrations of Kyn, 5-HT, TA, ILA and I3A were lower in DC than PC (Huang, Boekhorst, et al., 2023). The variability in the correlation patterns of these metabolites with other markers of protein fermentation highlights the complexity and specificity of the metabolic pathways involved.

Overall, the correlations among metabolites indicate a potential link between protein fermentation, AA metabolism, and the production of these metabolites. This underscores the complex interactions within the gut microbiome and highlights the importance of investigating these relationships further to understand their implications for gut health and disease.

4.3. Relationships between metabolites in PC and DC

It is often stated that protein fermentation takes place in more distal segments of the colon, and only limitedly in more proximal segments. In the current study, most of the protein fermentation metabolites showed higher concentrations in the DC except for putrescine (data not shown). Nonetheless, after incubation for 56 h, we observed a very high correlation between the relative changes of each metabolite in PC and the relative change of the same metabolite in DC. This correlation can be partly explained by the dynamics of digesta flow in our SHIME set-up, where the vessels simulating PC and DC are connected in series and no system was implemented to remove the products of microbial fermentation, so a high production of one metabolite in PC is somehow also reflected in a high transfer of the same to DC. However, this high correlation may also be due to the microbial population adapted to PC and DC having a similar capacity of generating (and further metabolizing) those metabolites from the corresponding substrates, the only differences in concentrations being related to the availability of the precursor AAs and microbiota density. The poor correlation in concentrations of certain biogenic amines, such as putrescine and spermine, and Trp catabolites between PC and DC may result from the complexity of their metabolic pathways, as previously discussed with precursor AAs. Consequently, the concentrations of biogenic amines and Trp metabolites are more sensitive to variations in the capacity of microbial populations in each colon segment to produce and metabolize these compounds.

Furthermore, the protein source seems to have a more direct impact on metabolite concentrations in the PC compared to the DC as the correlation coefficients were higher in PC compared to DC for all significant correlations except spermidine. This is similar with another study using SHIME that found stronger substrate impact on the microbial metabolic activity in the ascending colon compared to transverse and descending colon (Van den Abbeele et al., 2018). The initial composition of the gut microbiota and its distribution along the colon can influence the site of maximal activity. The microbial community in the DC is typically more diverse and has a higher density of bacteria capable of fermenting complex substrates (Wang et al., 2005). In contrast, the microbial community in the PC might be more active or better suited to metabolize certain dietary components present in the meals studied in our experiment.

4.4. Insights for dietary proteins

When we considered the potential of the same amount of proteins from different test meals to produce microbial metabolites in the large intestine, a relatively consistent pattern could be observed from the data in Fig. 5. The highest levels of NH_3 and BCFA would be produced by ingestion of whey and zein. Whereas the outcome for zein can be explained by the very poor digestibility, and thereby the largest N output in the ileal digesta (Table S1), the outcome for whey was not expected due to its high digestibility. Whey would also produce the highest level of H_2S in both the colon segments, which is consistent with an intense fermentation of its indigested fraction. Contrarily, zein does not produce relatively high levels of H_2S in DC which is surprising given also the highest level of sulphur AAs in this test protein (Fig. S1). Similar to whey and zein, feeding pigeon peas would produce among the highest levels of any of the metabolites investigated. This is rather surprising and different from other legumes included in this study and cannot be explained based on the compositional data of the ileal digesta of Fig. S1 and Table S1. Considering the harmful effect of NH_3, and H_2S on gut health (McCarville et al., 2020; Windey, De Preter, & Verbeke, 2012), our data indicate that the consumption of zein, whey and pigeon peas may contribute to a relatively higher effect.

The highest potential of SCFA production by black beans, pigeon peas and chickpeas may relate to the higher level of fibre expected in these test meals. The same can be hypothesized for wheat bran, given its putatively high content in fibre, but not for whey, which is devoid of fibre. Since SCFA can be produced both from certain AAs and carbohydrates, the results from whey suggest that those are intensely converted also in SCFA. In general, the interpretation of these results is complicated by the simultaneous presence of varying levels of carbohydrates in the ileal digesta, *i.e.*, different C:N ratios. It is usually assumed that increasing the fibre quantity in the diet would reduce protein fermentation as saccharolytic fermentation is preferred by the microbiome over proteolytic fermentation (Smith & Macfarlane, 1996b; Smith & Macfarlane, 1997). However, recently there have been reports that in some cases the addition of fibre might even increase the concentration of protein fermentation metabolites such as ammonia (Lammers-Jannink, 2024, p. 148; Noorman, 2025).

4.5. Strengths and weaknesses

In the present study we have used a hybrid approach to study protein fermentation in the human large intestine, combining *in vivo* and *in vitro* methods. Typically, in *in vitro* simulated colonic fermentation experiments, the composition of the ileum effluent is simulated by either predigesting food materials or creating an artificial feed. To the best of our knowledge, this is the first time that ileal effluents from ileostomy patients have been directly used in *in vitro* colonic fermentation experiments. The advantage of this approach lies in its physiological relevance, as it closely mimics actual gut conditions, leading to more accurate and applicable results regarding protein fermentation. Using real ileal effluents ensures the presence of naturally occurring compounds and microbial communities, enhancing microbial activity and providing a comprehensive understanding of microbial interactions and

fermentation kinetics. This method allows for accurate profiling of fermentation metabolites under conditions that resemble the *in vivo* environment. Combining *in vivo* and *in vitro* methods also enables a comprehensive analysis, validating *in vitro* findings with *in vivo* data, thus enhancing the reliability and applicability of the research outcomes. This integrative approach offers significant advantages in studying protein fermentation, leading to more precise and relevant findings.

The SHIME system has been chosen because this semi-continuous fermentation vessel is designed to study the effects of repeated feeding on the production of microbial metabolites of interest. Additionally, the system can simulate the conditions of different segments of the colon, enabling spatial characterization of the metabolic process under study. This capability is not achievable with simpler batch fermentation experiments. Furthermore, by using relative changes compared to baseline rather than absolute concentrations, we accounted for differences in the metabolic potential of various microbiota populations. This approach mitigates the large variations in control and baseline values observed between different studies. We recommend this method for future research aimed at investigating microbial metabolism and the impacts of diet on gut health.

The SHIME system was initiated with SHIME basal feed prior to the addition of each ileal digesta substrate. This basal feed contains larger concentrations of protein compared to our ileal digesta samples, therefore large quantities of metabolites were present in the vessels at $t = 0$ and thereby with a lower production of metabolites results in a drop of the concentration at 56 h compared to the baseline. This indicates that SHIME basal feed is not very representative for human ileal digesta in terms of N and AA content. This could be explained by the fact that the initial focus of the SHIME system was not to study protein fermentation. The system was validated in three studies that focussed on either carbohydrate, bioconversion of isoxanthohumol or the luminal and mucosal microbiome (Van de Wiele et al., 2015).

In the present study we have used the same inoculums across all the experiments, but the experiments were conducted in different weeks and the inoculums stored at freezing temperature for varying time which may have produced differences in the microbial population, which can be reflected by the variations found between weeks for SHIME basal feed (data not shown).

A direct comparison between absolute concentrations in this study compared to batch fermentations or *in vivo* findings is of course greatly influenced by the conditions used. For example, the absolute concentration of most of the protein fermentation end-products (NH_3, BCFA and amines) in the current study is lower than the level reported in the literature (Macfarlane et al., 1986; Cummings et al., 1987; Smith and Macfarlane, 1996). For instance, mean NH_3 concentrations of 6.5 mmol/L in PC and 18.8 mmol/L in DC in our system were lower compared to batch incubation with human fecal suspension that resulted in ~50 mmol/L NH_3 after 56 h ((Macfarlane et al., 1986). A comparable range (26.5 to 49.8) was also observed with human intestinal contents (Macfarlane et al., 1986), but the unit (mmol/kg digesta) makes direct comparison with our concentration in fermentation liquid (mmol/L) difficult. Moreover, our system lacks any absorption which is likely to take place *in vivo*.

5. Concluding remarks

In current study, protein fermentation metabolite profile from a variety of food sources of proteins were evaluated using ileum effluents from ileostomy patients combined with an *in vitro* model of colon fermentation. We showed 1) a positive correlations between the precursors amino acid content and the concentration of metabolites, as well as among protein fermentation metabolites such as NH_3, BCFA, and H_2S; 2) the concentration of metabolites in the proximal colon also correlates well to its concentration in the distal colon; 3) Whey protein isolate, zein and pigeon peas produced the highest levels of protein fermentation metabolites per gram of ingested protein in the test meal. Altogether, these data provide new insights into protein fermentation and suggest that the production of protein fermentation metabolites can be predicted from ileal digesta composition.

CRediT authorship contribution statement

Nikkie van der Wielen: Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Hanlu Zhang:** Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Pien J.C. Schouten:** Writing – review & editing, Methodology, Investigation, Data curation. **Erik Meulenbroeks:** Writing – review & editing, Methodology, Investigation, Data curation. **Natascha Stroebinger:** Writing – review & editing, Methodology, Conceptualization. **Suzanne M. Hodgkinson:** Writing – review & editing, Supervision, Funding acquisition, Data curation, Conceptualization. **Marco Mensink:** Writing – review & editing, Methodology, Data curation, Conceptualization. **Wouter Hendriks:** Writing – review & editing, Funding acquisition, Conceptualization. **Edoardo Capuano:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Edoardo Capuano reports financial support was provided by Wageningen University & Research. Nikkie van der Wielen reports financial support was provided by Wageningen University & Research. 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.foodchem.2025.146632.

Data availability

Data will be made available on request.

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