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Original Research Article

Protein-fermentation biomarkers in plasma and urine differ between bovine plasma protein and whey protein isolate in a randomized fully controlled dietary intervention

Roseanne Minderhoud [1,2,3,4,*], Edoardo Capuano [2], Sonja de Vries [3], Aniek Even [4,5], Andrea Anesi [6], Guido Hooiveld [1]

[1] *Division of Human Nutrition and Health, Wageningen University & Research, Wageningen, The Netherlands;* [2] *Food Quality and Design Group, Wageningen University & Research, Wageningen, The Netherlands;* [3] *Animal Nutrition Group, Wageningen University & Research, Wageningen, The Netherlands;* [4] *OnePlanet Research Center, Wageningen, The Netherlands;* [5] *imec, Wageningen, The Netherlands;* [6] *Department of Food Quality and Nutrition, Unit of Metabolomics, Research and Innovation Centre, Fondazione Edmund Mach, San Michele all'Adige, Italy*

A B S T R A C T

Background: Microbial fermentation of undigested proteins in the large intestine can produce potentially harmful metabolites. Although protein intake increases large intestinal protein inflow, the effects of dietary protein composition and digestibility are largely unknown.

Objectives: This study aimed to investigate the effects of protein sources differing in amino acid (AA) composition and digestibility on protein-fermentation biomarkers (primary outcomes), digesta transit, and gut microbiome composition (secondary outcomes), by contrasting protein and precursor AA delivery into the large intestine using 2 purified protein sources.

Methods: Fifteen healthy adults participated in a randomized, controlled, crossover dietary intervention consuming bovine plasma protein (BPP, poorly digestible) or whey protein isolate (WPI, highly digestible) at 30 g/d, divided over 3 meals, for 7 d. Intervention periods were separated by a 7-d washout. Plasma, urine, and fecal samples were analyzed for protein-fermentation biomarkers, followed by linear mixed-effects model analysis.

Results: In vitro degree of hydrolysis did not differ between BPP and WPI (32.6 ± 1.38% compared with 26.3 ± 0.05%, $P = 0.10$). Postprandial plasma total AA concentrations were also similar (BPP: 8997 ± 2673 μM, WPI: 9787 ± 3380 μM, $P = 0.57$, $n = 13$). Compared with WPI, BPP consumption induced higher plasma concentrations of p-cresyl sulfate (41.0 ± 27.4 compared with 28.5 ± 19.6, $P < 0.01$), phenyl sulfate (2.1 ± 0.6 compared with 1.8 ± 0.4, $P = 0.03$), phenylacetyl-L-glutamine (PAG) (2.3 ± 1.5 compared with 1.7 ± 1.2, $P < 0.01$), and indoxyl sulfate (4.8 ± 1.8 compared with 3.6 ± 1.4, $P = 0.03$). Urinary p-cresyl sulfate (111.4 ± 63.6 compared with 90.9 ± 56.7, $P = 0.03$), phenyl sulfate (13.6 ± 6.1 compared with 9.7 ± 2.6, $P = 0.03$), and PAG concentrations (55.1 ± 32.2 compared with 41.6 ± 25.6, $P = 0.01$) were also higher during BPP consumption. Protein source did not affect fecal ammonia, branched-chain fatty acid, or microbial diversity. BPP consumption tended to prolong colonic transit time (11:54 hh:mm, $P = 0.07$).

Conclusions: Consumption of BPP resulted in higher plasma and urinary concentrations of aromatic AA-derived microbial metabolites than WPI. These differences were most likely driven by AA composition rather than digestibility, as in vitro digestibility and postprandial AA responses differed less than expected between the protein sources.

This trial was registered at clinicaltrials.gov as NCT06161155.

Keywords: protein fermentation, protein digestibility, amino acid composition, gut microbiota, ammonia, p-cresol sulfate, indoxyl sulfate, phenylacetyl-L-glutamine, dietary protein

Introduction

Protein intake in Western countries typically exceeds recommended levels, with meat and dairy products being the primary contributors [1]. High protein intake has been associated with gastrointestinal (GI) discomfort and problems, including malodorous flatulence and inflammatory bowel disease [2–4]. These problems are hypothesized to be partially caused by microbial fermentation of undigested protein in the GI tract [5].

Abbreviations: AA, amino acid; BCFA, branched-chain fatty acid; BPP, bovine plasma protein; BSC, Bristol stool chart; DM, dry matter; GI, gastrointestinal; PAG, phenylacetyl-L-glutamine; PPAA, postprandialamino acid; SCFA, short-chain fatty acid; TAA, total amino acid; WPI, whey protein isolate.

* Corresponding author.
E-mail address: roseanne.minderhoud@wur.nl (R. Minderhoud).

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Apart from protein intake quantity, protein digestibility also influences protein flow into the large intestine but has been relatively understudied in humans. It depends on intrinsic protein properties, the food matrix, and processing methods, illustrated by standardized ileal digestibility values observed in pig studies that range from 60% to 100% [6]. Differences in apparent digestibility can also be caused by the addition of dietary fiber [7], variation in endogenous losses [8], and antinutritional factors [9].

Undigested proteins that flow into the large intestine can either support microbial growth or be fermented by microbiota. A study with piglets showed that feeding low-digestible protein sources resulted in higher colonic ammonia flows compared with feeding highly digestible protein [10]. Ammonia is a nonspecific metabolite produced during the degradation of nitrogen-containing compounds and can be harmful to the colonic epithelium at high concentrations (~20 mmol/L) [11]. Fermentation of amino acids (AAs) produces specific metabolites; therefore, the AA composition of protein sources will affect metabolite concentrations [12,13]. Aromatic AAs—tyrosine, phenylalanine, and tryptophan—are converted into p-cresyl, phenolic, and indolic compounds [14–16]. p-Cresyl impaired cell membrane integrity and barrier function in Caco-2 cell monolayers [17], whereas phenol may similarly disrupt intestinal barrier function by increasing paracellular permeability [18]. However, the extent to which protein composition and digestibility, when consumed as part of a complete diet, affect colonic protein fermentation remains poorly understood. Most evidence comes from studies in pigs, with limited human data available.

Fermentation kinetics can also be affected by GI transit time. Colonic transit time has been shown to correlate with urinary concentrations of protein-derived fermentation metabolites such as p-cresyl sulfate, p-cresyl glucuronide, and phenylacetyl-L-glutamine (PAG) [19]. Longer descending colonic transit time has likewise been associated with greater fecal microbiome diversity [19,20].

Therefore, the aim of this study was to explore the relation between dietary protein sources varying in protein composition and digestibility and protein-fermentation–derived metabolites as primary outcomes and digesta transit and microbiome as secondary outcomes. To achieve this, 2 purified protein sources: whey protein isolate (WPI) and bovine plasma protein (BPP), which differ in AA composition and presumed digestibility, were compared in a fully controlled 7-d crossover dietary intervention study. This design was expected to generate a clear contrast in proteins and AAs entering the large intestine. We hypothesized that BPP consumption would increase protein fermentation and the production of protein-fermentation–related metabolites compared with WPI.

Methods

Study design

The PROFUN study was a randomized crossover, fully controlled dietary intervention study conducted at Wageningen University & Research, The Netherlands (Figure 1). The study investigated the effects of protein digestibility and composition on protein fermentation by comparing 2 protein sources: WPI, representing a highly digestible protein source, and BPP, representing a low-digestible protein source. Each protein source was consumed during a 7-d fully controlled dietary intervention period. The study protocol was approved by the local medical ethics committee (METC Oost-Nederland, NL84483.09123) and registered at clinicaltrials.gov (identifier NCT06161155). All participants provided written informed consent prior to enrollment. The study was carried out between November 2023 and February 2024.

Subjects

Fifteen healthy adults (n = 8 female and 7 male) aged 18 to 79 y with a BMI (in kg/m^2) of 24.6 ± 3.1 were included in the study. Participants reported regular bowel movements at the time of recruitment (defecation at least once every 48 h) and had no history of swallowing disorders or GI conditions that could affect the study outcomes. Full inclusion and exclusion criteria are provided in Supplementary Table 1. The sample size was not based on a formal power calculation because of the exploratory nature of the study and the lack of prior data required to estimate effect sizes. The crossover design increased statistical efficiency by allowing each participant to serve as their own control, thereby reducing inter-individual variability.

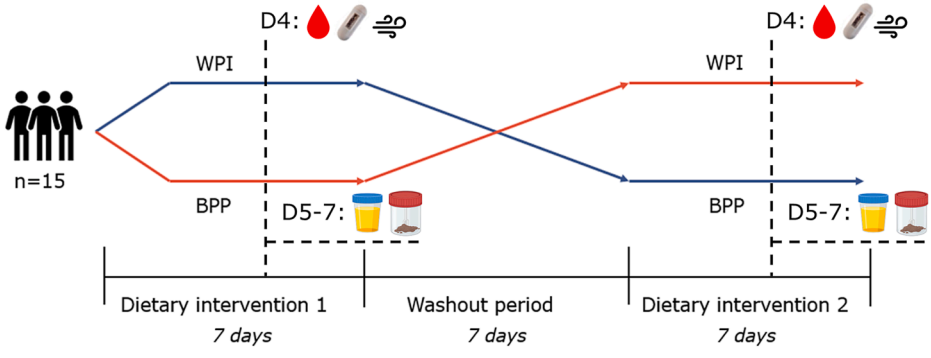

FIGURE 1. Schematic overview of the crossover dietary intervention study that consisted of 2 7-d dietary intervention periods, separated by a 7-d washout period. Each intervention consisted of a fully controlled diet, providing 11.6% energy from protein, 35.4% from fat, 51.0% from carbohydrates, and 2.0% from fiber. The diet included either whey protein isolate (WPI) as high digestible protein source or bovine plasma protein (BPP) as low-digestible protein source (30 g/d, divided over 3 meals). On day 4 (D4), postprandial plasma and breath samples were collected, and participants also swallowed 2 ingestible sensor capsules. Total feces and urine spot samples were collected from days 5–7 (D5–7).

Dietary interventions

Participants were randomly allocated to 1 of 2 diet sequences. Randomization was generated by an independent dietitian. Researchers were aware of dietary allocation, but participants were blinded and did not know which protein source they were consuming during the intervention periods. During each 7-d dietary intervention period, participants consumed 30 g/d of protein (divided over 3 meals) from either BPP (Beef PP70B; Sonac Loenen B.V.) or WPI (MyProtein; THG Nutrition Limited). WPI is a highly digestible protein source (92%–98% true ileal digestibility [21,22]), whereas BPP was selected based on previous findings showing substantially lower postprandial AA (PPAA) concentrations after BPP consumption compared with WPI [23]. Product inclusion levels were adjusted to account for protein content, as BPP contains 70% protein and WPI 90% protein. The AA composition of both protein products was analyzed (ISO 13903, 2005; Table 1). The proteins were dissolved in 50-mL water and added to carrot juice or soup immediately before consumption, without additional heat treatment. Diets were formulated to provide comparable macronutrient content: 11.6% of total daily energy intake (kcal:kcal) from crude protein, based on standard metabolizable energy values [24], 35.4% from fat, 51% from carbohydrates, and 2% from fiber. Participants were allocated to 1 of 4 energy groups (1372–2344 kcal/d; Table 2) according to estimated individual energy requirement, which was calculated using basal metabolic rate and self-reported physical activity level via the Schofield equation [25]. The full menu cycle is provided in Supplementary Table 2. The diet also contained 4.5 g/d Celite (diatomaceous earth; Natural Heroes) as indigestible acid-insoluble ash (AIA) marker, provided in 3 daily doses of 1.5 g incorporated into cookies (21.5 g each, Supplementary Table 3) adapted from Hodgkinson et al. [26]. Participants were instructed not to add any foods or condiments to the provided diet and completed a daily compliance questionnaire. After a 3-d adaptation period, participants consumed their meals at the research facility on day 4 of each dietary intervention. The breakfast on day 4 differed from that on other days and included 2 blue-colored muffins [27], 0.5 g ^{13}C-lactosyl ureide (see "Breath samples"), 10 g BPP or WPI dissolved in carrot juice, and 1 AIA cookie. A 7-d washout period separated the 2 dietary interventions, during which participants returned to their habitual diet (no restrictions). They recorded food intake on 3 d using the TRAQQ application (Wageningen University & Research). During both dietary interventions and the washout period, participants were requested to maintain their regular routine and physical activities.

TABLE 1
Amino acid (AA) composition[1] of the test protein sources (grams of total AA), corrected to anhydrous base

	WPI	BPP
Alanine	48.8	44.9
Aspartate	111.1	104.0
Arginine	20.5	60.3
Cysteine	23.5	31.9
Glutamate	175.9	136.1
Glycine	14.5	32.8
Histidine	17.1	30.9
Isoleucine	67.8	31.6
Leucine	105.5	92.7
Lysine	87.6	85.7
Methionine	22.9	11.0
Phenylalanine	29.8	52.1
Proline	59.0	52.0
Serine	45.4	69.0
Threonine	70.9	68.5
Tryptophan	15.2	16.5
Tyrosine	26.9	11.7
Valine	57.6	68.4
Total	1000.0	1000.0

Abbreviations: BPP, bovine plasma protein; WPI, whey protein. Isolate; ISO.
[1] Amino acids were determined using the ISO 13903 method (2005).

Measurements

Ingestible sensor capsule

GI pH and temperature were measured using an ingestible sensor capsule (GISMO GEN1; OnePlanet Research Center). Measurements were assigned to the stomach, small intestine, or large intestine based on characteristic pH patterns. The full methodology and pH profiles are described in detail in Even et al. [28]. In this article, we focus specifically on the dietary effects. On day 4 of each dietary intervention, participants came to the research facility after an overnight fast and swallowed the ingestible with 200 mL of water after 3 d of diet adaptation. Immediately after ingesting the ingestible, pH and temperature data were monitored in real-time, and once a characteristic rise in pH indicated pyloric passage, participants drank a small volume of room temperature water to confirm gastric exit; if the capsule were still in the stomach, a drop-in temperature would be observed. After confirming entry into

TABLE 2
Formulated nutrient composition of experimental diets[1] in grams per day, unless stated otherwise, presented per energy level

Energy level	WPI				BPP			
	1	2	3	4	1	2	3	4
Energy, kcal/d	1372	1822	2042	2344	1371	1821	2041	2343
Total protein	60.6	70.3	77.5	89.5	58.8	68.5	75.7	87.7
Animal-based protein	31.3	31.6	32.3	32.5	31.7	32.0	32.7	32.9
Of which test protein	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0
Plant-based protein	25.4	32.7	39.2	49.6	23.2	30.5	36.9	47.3
Carbohydrates	243.1	294.8	357.0	423.5	240.3	292.1	354.2	420.7
Mono- and disaccharides	116.3	127.6	154.5	161.7	114.4	125.7	152.5	159.7
Polysaccharides (starch)	126.9	167.3	202.7	262	126	166.5	201.9	261.2
Dietary fiber	17.4	22.9	27.1	31.4	17.4	22.9	27.1	31.4
Fat	76.0	97.2	113.3	131.1	76.2	97.4	113.5	131.3

Abbreviations: BPP, bovine plasma protein; WPI, whey protein isolate; NEVO; RIVM.
[1] All values were determined using NEVO-online version 2021/7.1, RIVM, 2022. Values are averaged across a 7-d menu cycle (Supplemental Table 2).

the small intestine, participants consumed a standardized breakfast meal. Approximately 9 h after the first ingestible was ingested, participants swallowed a second ingestible simultaneously with their third meal of that day.

Participants collected all stool produced from the moment they swallowed the ingestible until its confirmed retrieval. The collected stool was cooled and brought to the research facility within 48 h of defecation, where it was weighed.

Fecal samples

Participants collected a separate stool spot sample from each defecation until the ingestible was excreted, or from 2 defecations after swallowing the ingestible if it was excreted during the first defecation. All collected stool samples were immediately frozen at −20°C. After each defecation, participants recorded stool consistency and shape using the Bristol Stool Chart (BSC) and wrote down whether the stool appeared blue, which served as an additional indicator of transit time.

Before chemical analyses, fecal spot samples were mixed and pooled for each participant per dietary intervention. Samples were homogenized by breaking the frozen fecal samples into smaller pieces with a hammer, followed by grinding the feces into a fine powder using a liquid nitrogen-cooled mill (IKA Tube Mill Control; IKA-Werke GmbH & Co. KG) as described by Kruger et al. [29]. The finely ground homogenate was then divided into aliquots for subsequent analyses. Half of each homogenized fecal mixture was freeze-dried for 48 h at −80°C using a laboratory-scale freeze dryer (ilShinBioBase; Scala Scientific) for additional measurements.

Total fecal output was estimated by weighing all stool produced from the moment the ingestible capsule was swallowed until its confirmed retrieval. Total fecal output per day was calculated by adding 29 g (average of 15 stool samples, SD = 9 g) to each recorded stool weight to account for the average fecal spot sample removed from each defecation for analysis. The corrected weights were summed across all defecations and divided by the number of collection days (from swallowing the ingestible capsule till confirmed excretion).

Dry matter (DM) content was determined by drying the homogenized fecal mixture samples at 103°C for 4 h and weighing samples before and after drying. DM digestibility was calculated as fecal DM output divided by DM intake over the corresponding collection period (from ingestion of the ingestible capsule until its confirmed excretion), expressed as a percentage (%).

Nitrogen content was determined in freeze-dried fecal samples using the Kjeldahl method (ISO 5893, 2005). Apparent total tract nitrogen digestibility was calculated as: apparent nitrogen digestibility (%) = [1 − (fecal nitrogen output/dietary nitrogen intake)] × 100%, using fecal nitrogen and nitrogen intake from the same days.

For NH_3 analyses, 1 g of fresh mixed fecal samples was mixed with 1 mL of 10% trichloroacetic acid (TCA), mixed for 5 min, and centrifuged at 2500 × *g* for 10 min. The supernatant was mixed with 10% TCA 1:1 in duplicate and mixed. Of the mixture, 0.1 mL was taken, and 1 mL phenol solution 4% to 7% and 1 mL hypochlorite buffer solution were added, mixed, and incubated at 37°C for 30 min to form indophenol blue. The absorbance of indophenol blue was measured photometrically at 623 nm using a UV-spectrophotometer (LAMBDA 365+ UV/Vis Spectrometer; PerkinElmer) and linearly correlated to the ammonia concentration in the sample.

Short-chain fatty acids (SCFA), including branched-chain fatty acids (BCFA), were analyzed by dissolving and mixing 200 mg of frozen fecal mixture in distilled water. Samples were suspended in a solution of internal standard 2-ethylbutyric acid, hydrochloric acid, and oxalic acid, and the concentration of each SCFA and BCFA was determined by gas chromatography with flame ionization detector (Agilent 6890N; Agilent Technologies) using identification mixtures of known SCFA [29]. Ten percent of the samples were randomly selected and analyzed in duplicate.

DNA was isolated from fecal samples using the DNeasy PowerSoil Pro Kit (QIAGEN). Samples were bead-beated and homogenized using TissueLyser II (QIAGEN). After multiple centrifugation steps, the supernatant was loaded onto an MB spin column and centrifuged twice to ensure complete binding and purification. DNA concentration was quantified using a NanoDrop One Spectrophotometer (Thermo-Fisher). Extracted DNA was subsequently prepared for amplicon sequencing targeting the bacterial 16S rRNA gene. Library preparation and sequencing were performed by BGI Genomics. Briefly, the V3–V4 region of the 16S rRNA gene was amplified using the primers 338F (5′- ACT CCT ACG GGA GGC AGC AG-3′) and 806R (5′- GGA CTA CHV GGG TWT CTA AT-3′). Sequencing was then conducted on a DNBSEQ G400 platform (PE300, 100K tags). Demultiplexed reads were analyzed with QIIME2 software [30] using DADA2 for ASV calling and the SILVA database, version 138.1 [31] for taxonomic classification. Ten percent of the samples submitted for sequencing were randomly selected and processed in duplicate to assess reproducibility.

Fecal water pH was measured in the supernatant obtained from 1 g of fecal sample. Samples were centrifuged at 21,000 × *g* for 10 min, followed by centrifugation at 30,000 × *g* for 10 min, after which pH was measured using a Sentron CupFET probe (Sentron Europe B.V.). Ten percent of all samples were randomly selected for duplicate measurements to assess analytical consistency.

Urine samples

During baseline and from day 4 onward of each dietary intervention period, participants collected 3 consecutive morning samples, which were frozen immediately after collection. Equal volumes from each sample were pooled and thoroughly mixed prior to analysis. Pooled urine samples were analyzed for urea and creatinine at the Gelderse Vallei Hospital. Urea was quantified using the Atellica CH Urea Nitrogen assay (Siemens) based on the Roch-Ramel enzymatic reaction employing urease and glutamate dehydrogenase. Creatinine was measured using the Atellica CH Creatinine_2 assay (Siemens), which is based on the reaction of picric acid with creatinine in an alkaline medium. Urine pH was measured using a Sentron ConeFET probe (Sentron Europe B.V.).

In addition, urine samples were analyzed for p-cresyl sulfate, p-cresyl glucuronide, phenyl sulfate, PAG, and indoxyl sulfate using the ultra-HPLC (UHPLC) electrospray ionization-tandem mass spectrometry method described by Anesi et al. [32].

Plasma samples

A peripheral venous cannula was placed in the participant's arm on the morning of day 4 of each dietary intervention, following an overnight fast. Blood was collected into EDTA tubes coated with aprotinin (BD Vacutainer K3EDTA Aprotinin Tube; BD) via the cannula 10 min before consumption of the standardized breakfast meal, which included 10 g of the test protein source, and continuing for 5 h postprandially at the following time points: 30 min, 45 min, 60 min, 90 min, 120 min, 150 min, 180 min, 240 min, and 300 min. Blood samples were placed on ice immediately after collection and centrifuged at 1200 × *g* for 10 min at 4°C to obtain plasma.

Individual AAs were quantified in plasma samples at each time point to assess the PPAA response. Plasma aliquots (40 µL) were mixed 1:1 with an internal standard working solution consisting of isotopically labeled AAs. Proteins were precipitated using acetonitrile followed by centrifugation for 15 min at 13,000 × *g*. The resulting supernatant was used for analysis performed using an Acquity H-class Plus UPLC (Waters Chromatography Europe BV) coupled to a Xevo TQ-S micro tandem mass spectrometer (Waters Chromatography Europe BV) based on a hydrophilic interaction liquid chromatography method [33]. An ACQUITY UPLC BEH Amide VanGuard Pre-column 1.7 µm 2.1 × 5 mm coupled to an ACQUITY UPLC BEH Amide Column 1.7 µm 2.1 × 100 mm (Waters Chromatography Europe BV) was used for chromatographic separation. Electrospray ionization was operated in positive mode, and AAs were analyzed using multiple reaction monitoring. Fasted plasma samples were also analyzed for p-cresyl sulfate, p-cresyl glucuronide, phenyl sulfate, PAG, and indoxyl sulfate following UHPLC-electrospray ionization-tandem mass spectrometry [32].

Breath samples

On day 4 of each dietary intervention, breath samples were collected in aluminum bags before breakfast (−10 min) and every 30 min thereafter for 8 h. After 3 d of dietary adaptation, participants ingested 1 g of ^{12}C-lactosyl ureide with the final meal of day 3 as a microbiota pretreatment to improve measurement accuracy (Campro Scientific GmbH, 2011). On day 4, participants ingested 0.5 g of ^{13}C-lactosyl ureide. Fermentation of ^{13}C-labeled lactosyl ureide in the large intestine results in an increased $^{13}C/^{12}C$ ratio in exhaled CO_2 and serves as an established marker for oro-cecal transit time (OCTT) [34]. The $^{13}C/^{12}C$ in exhaled CO_2 was measured using the HeliFANplus (Fischer Analysen Instrumente GmbH) and expressed as $^{13}C/^{12}C$ enrichment above background ($\delta^{13}C$) using the following equation: $\delta^{13}C = 1000 \times (R_{sample} - R_{reference})/R_{reference}$, where $R = {}^{13}C/{}^{12}C$, and the reference is Pee Dee Belemnite, a calcium carbonate standard derived from the fossil Belemnitella of the Cretaceous Pee Dee formation in South Carolina, United States. To validate the method, 10% of all samples (randomly selected) were re-analyzed using isotope ratio mass spectrometry. OCTT was defined as the first of ≥2 consecutive measurements showing an increase in $\delta^{13}C$ relative to baseline. A measurement was considered to indicate fermentation onset if it exceeded the analyzed average of the preceding values by >2.5 times the SD.

Questionnaires

At the end of each day, participants completed a questionnaire assessing their compliance with the provided diet. Compliance was calculated as the proportion of food consumed relative to the total amount of food prescribed (wt/wt). After each period, participants also completed a questionnaire on GI symptoms using the validated GI Symptom Rating Scale [35–37].

In vitro digestibility

In vitro digestion of BPP and WPI was performed following the INFOGEST protocol [38] with minor modifications. Simulated oral, gastric, and intestinal fluids were prepared according to the standard protocol. Protein sources (in duplicate) were diluted 1:1 (wt/wt) with simulated salivary fluid containing 1.5 mM $CaCl_2$ and incubated for 2 min at 37°C. Samples were collected in triplicate (oral phase), and digestion was stopped by heating the samples at 95°C for 5 min (heat-shock treatment) using an Eppendorf Thermomixer Comfort (Eppendorf). The mixture was then mixed with simulation gastric fluid 1:1 (vol/vol) containing 0.15 mM $CaCl_2$ and pepsin (final activity 2000 U/mL), and the pH was adjusted to 3.0. Then, the mixture was incubated for 2 h at 37°C. After the incubation, the gastric chyme was mixed 1:1 (vol/vol) with simulation intestinal fluid containing 10 mM bile salts, 0.6 mM $CaCl_2$, and trypsin (final activity 100 U/mL) and incubated at 37°C for another 2 h. Samples were then taken and heat-shock treated to terminate intestinal digestion (intestinal phase). Simultaneously, duplicate aliquots of each protein source were mixed in 6 M HCl to prepare 5 mg/mL protein solutions for acid hydrolysis. These solutions were incubated at 95°C for 24 h, after which samples were placed on ice, neutralized to pH 7, and sampled (acid hydrolysis). Free amino group (NH_2) concentrations were determined in triplicate using the OPA assay as described by Nielsen et al. [39]. For quality control, 15% of samples were randomly selected for duplicate measurement. Absorbance was measured at 340 nm using the SpectraMax M2 microplate reader (Molecular Devices) with 120 s of shaking prior to each reading. Degree of hydrolysis (DH%) was calculated using the formula: DH (%) = (NH_2 intestinal phase − NH_2 oral phase)/(NH_2 acid hydrolysis − NH_2 oral phase) × 100%.

Data analysis

Analyses were done in singlicate unless indicated otherwise. The coefficient of variation (CV%) between technical duplicates was calculated for each duplicate pair. Duplicate measurements of SCFA and fecal pH were averaged prior to statistical analysis, as the CV% for all duplicates was <3%. For microbiome data, duplicates were averaged because the Bray–Curtis distance between duplicates was ≤0.1.

Data from the 13 participants who completed both interventions were included in the statistical analyses. Missing data were handled by excluding observations with missing values while retaining all available data from each participant in the analysis. All statistical analyses were performed using R 4.4.2 (R Core Team, 2024). AA concentrations in plasma were modeled for each AA and each participant using a modified Wood curve: $y(t) = d + at^{mc}e^{-ct}$, where y(t) represents the concentration of AA at time t, d is the baseline concentration, a relates to peak height, m determines the time to peak, and c describes the return toward baseline. Curve fitting was performed using the "aaresponse" package [40] in R 4.4.2 (R Core Team, 2024). From the fitted curves, incremental AUC, time to peak, and height of peak were calculated [40].

Plasma, urine, and fecal metabolite concentrations; plasma AUC, time to peak and peak height; fecal nitrogen, water, and DM content, pH; urine pH; nitrogen and DM digestibility; and transit times were analyzed using a linear mixed model with diet as a fixed effect and participant as a random effect. The mixed model analyses were implemented using the "lmerTest" package [41], followed by a post hoc Tukey test to determine differences between groups using the "emmeans" package [42]. GI pH was analyzed using a linear mixed model with diet and ingestion timing [moment of taking in the ingestible capsule after fasting overnight (fasted) or after a day of eating (fed)] and the interaction between these as fixed effects, and participant as a random effect. Ordinal data from the BSC questionnaires were analyzed with a cumulative link mixed model with diet as a fixed effect and participant as a random effect using the "ordinal" package [43]. In vitro digestibility values were compared using a t-test with protein as a fixed effect. Assumptions of normality and homogeneity of residuals were assessed visually by inspecting residual-compared with-fitted plots and Q-Q plots. No substantial violations of model assumptions were observed. Data are presented as

estimated means ± SD, unless indicated otherwise. Differences were considered significant at $P < 0.05$.

Microbiome data were analyzed using the "microeco" package [44]. Potential contaminants were removed using negative control samples with the "decontam" package [45]. α-Diversity was assessed using the Shannon diversity index and compared between diets using the Wilcoxon rank-sum test. β-Diversity was calculated using the Bray–Curtis dissimilarity index and evaluated using PERMANOVA. Homogeneity of multivariate dispersion was assessed prior to PERMANOVA. No substantial violations of model assumptions were observed.

A repeated measures correlation analysis was performed across all measured variables, including microbial relative abundance at the genus level, using the "rmcorr" package [46], which corrects for repeated measures within subjects [47]. Except for microbiota, resulting repeated measures correlation coefficient P values were corrected for multiple testing using the Benjamini–Hochberg false discovery rate procedure.

Results

Participants and diet compliance

Of the 15 participants enrolled, 13 completed both dietary intervention periods (Figure 2). One participant dropped out on the first day of the first dietary intervention because of illness, which the study physician deemed unlikely to be related to the diet. Another participant dropped out during the second intervention because of not being able to adhere to the diet, collect fecal samples, and attend the test day; therefore, this participant only completed 1 intervention period.

According to the daily compliance questionnaires, adherence to the supplemental protein intake was 100% (wt/wt) for all participants, and overall compliance with the fully controlled diet was 97% (wt/wt).

In vitro protein digestibility

After in vitro digestion, the DH after the intestinal phase was similar between BPP (32.6 ± 1.38%) and WPI (26.3 ± 0.05%; $P = 0.10$).

Plasma AA concentrations

The AUC of the postprandial total AAs (TAA) in plasma was 8997 ± 2673.0 μM after consumption of BPP and 9787 ± 3380.5 μM after consumption of WPI ($P = 0.57$). Estimated maximum concentrations were 1028 ± 319.0 μM for BPP and 1150 ± 374.4 μM for WPI ($P = 0.34$), and the time to reach maximum concentration was 61 ± 10.7 min for BPP and 52 ± 16.1 min for WPI ($P = 0.12$). The AUC, maximum concentrations, and time to reach maximum concentrations for individual AAs are shown in Supplementary Figure 1.

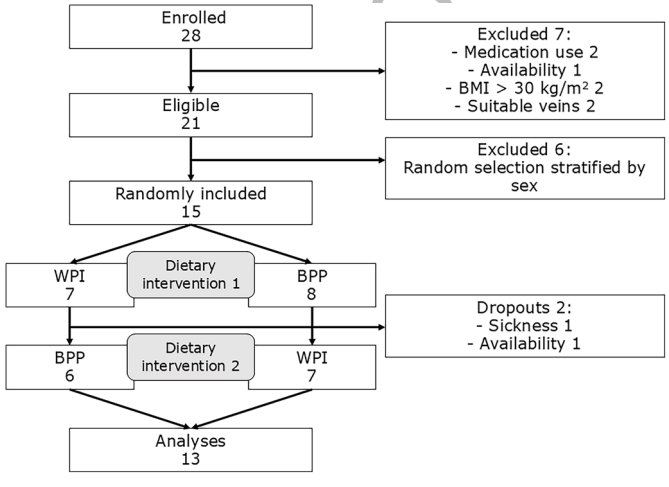

FIGURE 2. Overview of participant inclusion and dropouts. BMI, body mass index; BPP, bovine plasma protein; WPI, whey protein isolate.

Transit time

Colonic transit time, measured by the ingestible, tended to be longer after consumption of BPP compared with WPI (65:19 ± 00:49 hh:mm compared with 53:25 ± 00:41 hh:mm; $P = 0.07$). A similar trend was observed for total tract transit time (73:10 hh:mm ± 00:50 compared with 61:00 ± 00:42 hh:mm; $P = 0.07$). No differences were observed in gastric or small intestinal transit time (Table 3). Mean transit times measured by the blue dye method and OCTT based on breath ^{13}C enrichment were also longer for BPP than for WPI, although these differences were not significant.

GI pH

The pH in the stomach, small intestine, and large intestine did not differ between the 2 dietary protein sources. Stomach pH was significantly lower when the ingestible was taken in a fed state (after a day of eating; 2.0 ± 0.63) compared with the fasted state (after an overnight fast; 3.5 ± 1.24; $P < 0.01$). In contrast, small intestinal pH was significantly higher when the ingestible was ingested in the fed (7.4 ± 0.63) compared with the fasted state (7.2 ± 0.19; $P = 0.03$). No interaction between diet and ingestion timing (fed compared with fasted) was observed in the stomach ($P = 0.91$), small intestine ($P = 0.95$), or large intestine ($P = 0.86$).

Plasma, urine, and fecal measurements

During the consumption of BPP, compared with WPI, plasma concentrations of all measured aromatic AA-derived microbial metabolites were significantly higher. Specifically, concentrations of p-cresyl sulfate (BPP: 41.0 ± 27.41 μmol/L compared with WPI: 28.5 ± 19.62 μmol/L; $P < 0.01$), p-cresyl glucuronide (BPP: 0.2 ± 0.14 μmol/L compared with WPI: 0.1 ± 0.08 μmol/L; $P = 0.01$), phenyl sulfate (BPP: 2.1 ± 0.62 μmol/L compared with WPI: 1.8 ± 0.44 μmol/L; $P = 0.03$), PAG (BPP:

TABLE 3
Mean transit time of ingestible capsule and digesta as measured by the blue dye method and breath enrichment in healthy adults ($n = 13$) after 4 d of a 7-d intervention period consuming a fully controlled diet containing either bovine plasma protein or whey protein isolate, expressed in hours:minutes (hh:mm)

	Protein source		Mean difference (95% CI)	P value[1]
	BPP	WPI		
Transit time measured by an ingestible after fasting				
Stomach	01:22	01:47	−00:15 (−00:59, 00:28)	0.45
Small intestine	06:29	05:47	00:25 (−00:11, 01:01)	0.15
Large intestine	65:19	53:25	11:54 (−01:00, 24:57)	0.07
Total tract	73:10	61:00	12:10 (−00:59, 25:19)	0.07
Transit time measured using blue dye method				
Total tract	40:17	35:54	04:23 (−07:53, 16:39)	0.45
Transit time measured by breath enrichment				
Oro-cecal	06:20	05:36	00:44 (−00:15, 01:02)	0.13

Abbreviations: BPP, bovine plasma protein; CI, confidence interval; WPI, whey protein isolate.

[1] P values are determined using a linear mixed model with diet as a fixed factor and participant as a random factor.

2.3 ± 1.52 μmol/L compared with WPI: 1.7 ± 1.17 μmol/L; $P < 0.01$), and indoxyl sulfate (BPP: 4.8 ± 1.84 μmol/L compared with WPI: 3.6 ± 1.39 μmol/L; $P = 0.03$) were all elevated (Table 4). Urinary concentrations of these metabolites were also higher during the consumption of BPP compared with WPI: p-cresyl sulfate (BPP: 111.4 ± 63.61 μmol/mmol compared with WPI: 90.9 ± 56.69 μmol/mmol creatinine; $P = 0.03$), p-cresyl glucuronide (BPP: 4.5 ± 3.22 μmol/mmol compared with WPI: 3.3 ± 2.83 μmol/mmol creatinine; $P = 0.02$), phenyl sulfate (BPP: 13.6 ± 6.12 μmol/mmol compared with WPI: 9.7 ± 2.60 μmol/mmol creatinine; $P = 0.03$), PAG (BPP: 55.1 ± 32.23 μmol/mmol compared with WPI: 41.6 ± 25.59 μmol/mmol creatinine; $P = 0.01$), and indoxyl sulfate (BPP: 50.1 ± 24.74 μmol/mmol compared with WPI: 40.3 ± 17.13 μmol/mmol creatinine; $P = 0.05$) (Table 4). Protein source did not affect urea (BPP: 19.8 ± 4.53 μmol/mmol compared with WPI: 21.6 ± 3.46 μmol/mmol creatinine; $P = 0.26$) and urine pH (BPP: 6.9 ± 0.66 compared with WPI: 6.7 ± 0.47; $P = 0.11$). Similarly, fecal output, fecal water content, fecal pH, and fecal concentrations of ammonia, SCFA, BCFA, and nitrogen were not affected by the dietary protein source (Table 4). BSC scores did not differ between protein sources ($P = 0.99$) and ranged from 2 to 5 (median = 4) during BPP and 1 to 5 (median = 4) during WPI.

Microbiota

α-Diversity in fecal samples was not affected by the consumption of BPP or WPI ($P = 0.76$; Supplementary Figure 2). Likewise, no differences in overall microbiota composition between the 2 dietary interventions were observed within participants ($P = 0.99$; Figure 3). Inter-individual variability in microbial responses to the protein sources is illustrated in Figure 4, shown as relative abundance at the genus level. No significant correlations were detected between microbial taxa and plasma, urine, or fecal protein-fermentation metabolites, nor with fecal or urinary pH, fecal properties, plasma AA concentrations, or transit time.

Correlations

Correlation analysis of protein-fermentation biomarkers revealed a strong positive correlation between fecal isobutyric acid and isovaleric acid ($r = 0.98$, $P < 0.001$; Table 5). Fecal isobutyric acid also tended to positively correlate with fecal ammonia concentrations ($r = 0.80$, $P = 0.09$). Fecal valeric acid showed a trend toward a negative correlation with total tract transit time measured using the blue dye method ($r = -0.84$, $P = 0.08$; Supplementary Figure 3). In urine, p-cresyl sulfate was strongly correlated with urinary p-cresyl glucuronide ($r = 0.97$, $P < 0.001$) and PAG ($r = 0.93$, $P < 0.001$). Urinary p-cresyl glucuronide was also positively correlated with urinary PAG ($r = 0.93$, $P < 0.001$), and urinary PAG was positively correlated with urinary indoxyl sulfate ($r = 0.87$, $P = 0.02$). In plasma, p-cresyl sulfate correlated positively with p-cresyl glucuronide ($r = 0.86$, $P = 0.02$) and PAG ($r = 0.91$, $P < 0.001$). Plasma p-cresyl glucuronide was also positively correlated with plasma PAG ($r = 0.95$, $P < 0.001$). No significant correlations were observed between fecal, plasma, or urine protein-fermentation biomarkers.

TABLE 4
Concentrations of protein-fermentation biomarkers measured in plasma (day 4 of the 7-d intervention), urine, and feces, as well as fecal properties (collected during days 5–7 of intervention), in healthy adults ($n = 13$) consuming a fully controlled diet containing either bovine plasma protein or whey protein isolate

		Protein source		Mean difference (95% CI)	P value[1]
		BPP	WPI		
Peripheral plasma fasted					
p-Cresyl sulfate	μmol/L	41.0	28.5	12.43 (4.94, 19.93)	<0.01
p-Cresyl glucuronide	μmol/L	0.2	0.1	0.08 (0.02, 0.13)	0.01
Phenyl sulfate	μmol/L	2.1	1.8	0.27 (0.03, 0.52)	0.03
Phenylacetyl-L-glutamine	μmol/L	2.3	1.7	0.64 (0.24, 1.03)	<0.01
Indoxyl sulfate	μmol/L	4.8	3.6	1.18 (0.16, 2.21)	0.03
Urine					
p-Cresyl sulfate	μmol/mmol creatinine	111.4	90.9	20.48 (3.01, 37.95)	0.03
p-Cresyl glucuronide	μmol/mmol creatinine	4.5	3.3	1.26 (0.28, 2.24)	0.02
Phenyl sulfate	μmol/mmol creatinine	13.6	9.7	3.89 (0.56, 7.23)	0.03
Phenylacetyl-L-glutamine	μmol/mmol creatinine	55.1	41.6	13.49 (4.30, 22.68)	0.01
Indoxyl sulfate	μmol/mmol creatinine	50.1	40.3	9.85 (0.16, 19.55)	0.05
Urea	mmol/mmol creatinine	19.8	21.6	−1.83 (−5.17, 1.51)	0.26
pH		6.9	6.7	0.18 (−0.06, 0.42)	0.11
Feces					
Ammonia	mg/g fresh	0.7	0.7	−0.02 (−0.11, 0.07)	0.68
Acetic acid	mg/g fresh	2.3	2.4	−0.06 (−0.28, 0.16)	0.56
Butyric acid	mg/g fresh	0.8	0.8	0.00 (−0.11, 0.10)	0.95
Isobutyric acid	mg/g fresh	0.1	0.1	−0.01 (−0.03, 0.02)	0.66
Isovaleric acid	mg/g fresh	0.3	0.3	0.00 (−0.06, 0.05)	0.84
Propionic acid	mg/g fresh	0.8	0.8	0.04 (−0.10, 0.17)	0.54
Valeric acid	mg/g fresh	0.1	0.1	0.01 (−0.02, 0.03)	0.52
Total SCFA	mg/g fresh	4.6	4.6	−0.03 (−0.48, 0.42)	0.88
Nitrogen	mg/g fresh	14.9	14.9	0.00 (−1.25, 1.26)	1.00
Fecal water content	g/kg feces	714.7	711.1	3.66 (−20.75, 28.08)	0.75
Fecal output	g/d	147.2	163.7	−16.55 (−70.13, 37.02)	0.51
Nitrogen digestibility	%	81.4	84.5	−3.08 (−7.40, 1.24)	0.15
DM digestibility	%	90.6	91.8	−1.25 (−3.30, 0.80)	0.21
pH		6.8	6.9	−0.11 (−0.35, 0.12)	0.32

Abbreviations: BPP, bovine plasma protein; CI, confidence interval; DM, dry matter; SCFA, short-chain fatty acid; WPI, whey protein isolate.
[1] P values are determined using a linear mixed model with diet as a fixed factor and participant as a random factor.

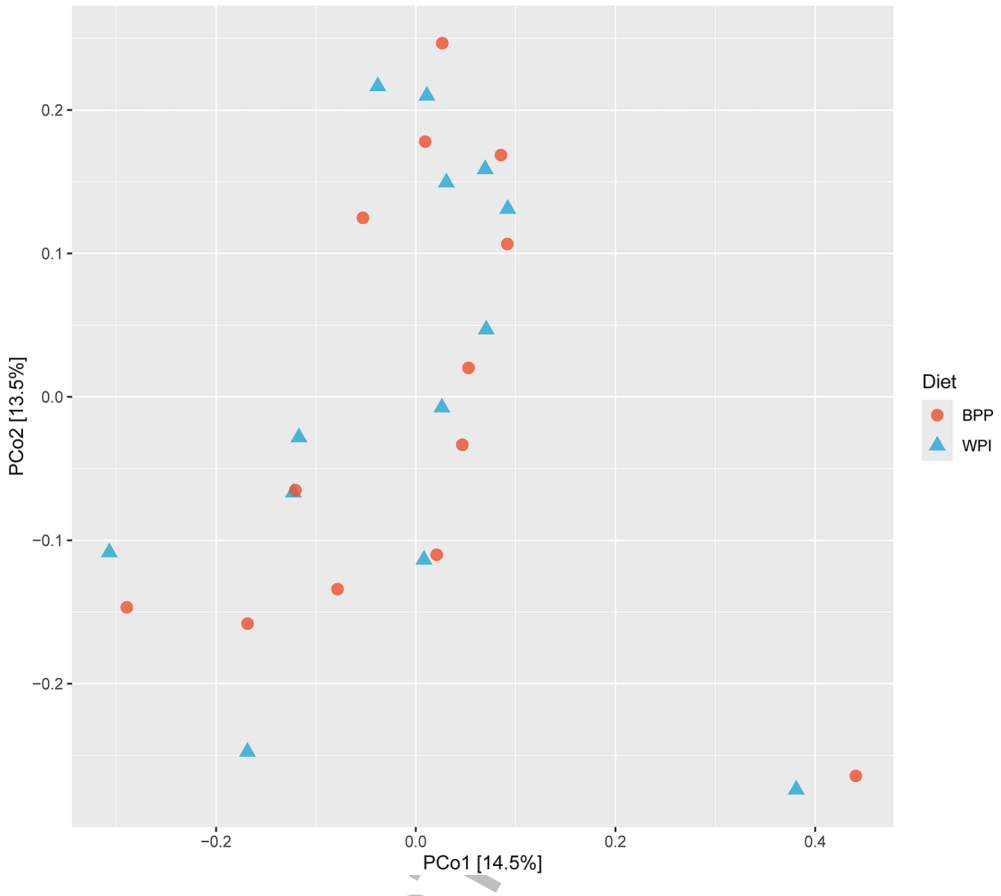

FIGURE 3. Principal Coordinate Analysis (PCoA) plot based on Bray–Curtis dissimilarities of fecal microbiota from all participants (n = 13) during each dietary intervention. Samples collected during the bovine plasma protein (BPP) diet are shown as red dots, and the samples collected during the whey protein isolate (WPI) diet are shown as blue triangles. The first 2 principal coordinates explain 14.5% and 13.5% of the variance in β-diversity, respectively.

Discussion

The aim of the study was to compare gut microbial fermentation of proteins that differ in AA composition and digestibility. We used highly digestible WPI and the presumed less digestible BPP in a fully controlled dietary intervention, assessing the formation of protein-derived fermentation metabolites in plasma, urine, and feces. We hypothesized that higher precursor AA availability, due to a combined effect of higher AA content and lower digestibility, would increase protein fermentation and metabolite production. To our knowledge, this is the first human study to investigate how dietary protein sources varying in composition and digestibility relate to digesta transit, gut microbiome, and protein-derived fermentation metabolites in the GI tract. The fully controlled diet contained relatively low protein concentrations (12% of total daily energy intake) compared with typical Western intakes of 12% to 20% [48,49], reflecting realistic, if not slightly conservative, protein consumption. We observed higher concentrations of protein-fermentation metabolites in plasma and urine but not feces during consumption of BPP compared with WPI.

Differences in total PPAA between the 2 protein sources were unexpectedly small, despite using the same product from the same manufacturer as a previous study [23]. In our study, the protein dose used for PPAA measurements was half the amount administered by Esser et al. [23], potentially reducing differences in AA absorption. WPI's rapid absorption likely affected the PPAA profiles [50,51]. Nevertheless, PPAA concentrations for TAA were similar to or higher than those reported by Esser et al. [23]. Given the smaller-than-anticipated PPAA contrast and the known limitations of AUC as a measure of AA bioavailability, we additionally applied a standardized GI digestion model [38,39] to assess in vitro digestibility and confirm whether the intended difference in digestibility was realized. Surprisingly, in vitro digestibility did not differ between BPP and WPI, consistent with earlier in vitro findings [52]. However, it is important to note that BPP was a relatively insoluble substrate that required forced solubilization during the in vitro procedure, which may have potentially overestimated BPP's in vitro digestibility compared with its actual true ileal digestibility. Although we could not measure ileal digestibility, in vivo nitrogen total tract apparent digestibility was also not different between the protein sources. Taken together, these findings suggest that the observed differences in plasma and urine metabolites are less likely due to large differences in digestibility and more likely due to differences in AA composition, but differences in digestibility cannot be excluded.

Plasma and urine concentrations of p-cresyl sulfate, p-cresyl glucuronide, phenyl sulfate, PAG, and indoxyl sulfate were all higher after BPP consumption compared with WPI. Previous studies reported increased urinary excretion of p-cresyl and phenolic compounds with higher protein intake [53–55] or with increased protein flowing into the large intestine through reduced protein digestion [56]. BPP contains higher amounts of phenylalanine and tryptophan than WPI. As these are essential AAs and cannot be synthesized endogenously, their higher availability may directly explain increased PAG and indoxyl sulfate production. Although tyrosine concentrations are lower in BPP than in WPI, tyrosine can be formed from phenylalanine via

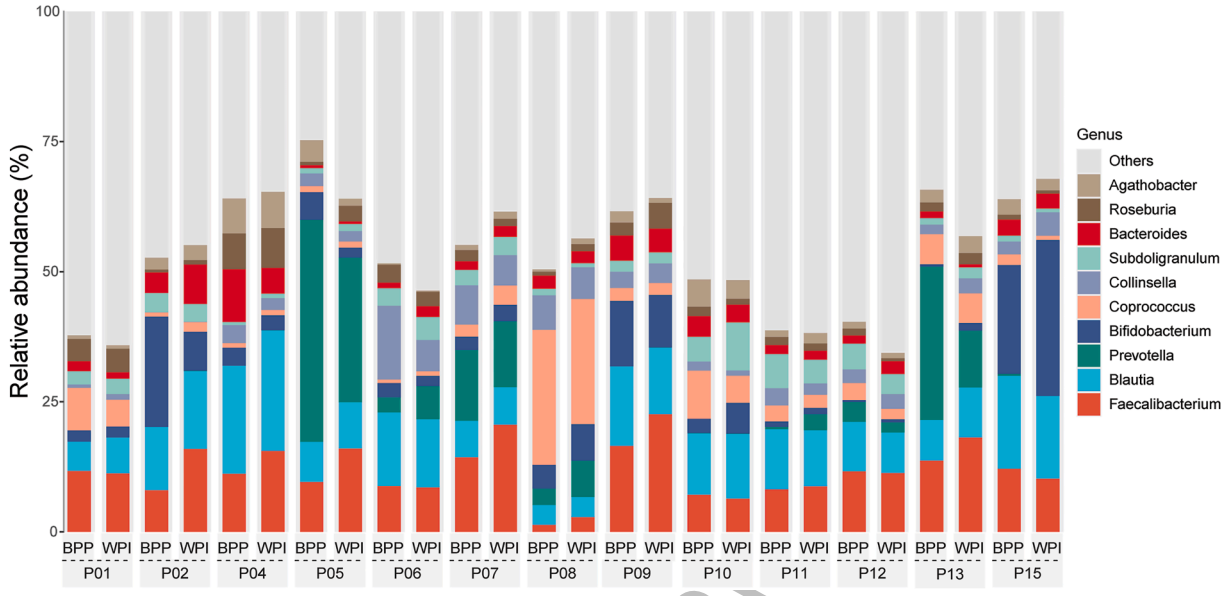

FIGURE 4. Relative abundance of the bacteria genera in the fecal samples of all participants ($n = 13$) per protein source (P) collected during the consumption of bovine plasma protein (BPP) and whey protein isolate (WPI) clustered per participant.

TABLE 5
Correlations among plasma, urine, and fecal metabolites, pH, digestibility measures, and fecal characteristics

	Peripheral plasma fasted					Urine				
	p-Cresol sulfate	p-Cresol glucuronide	Phenyl sulfate	PAG	Indoxyl sulfate	p-Cresol sulfate	p-Cresol glucuronide	Phenyl sulfate	PAG	Indoxyl sulfate
Peripheral plasma fasted										
p-Cresol glucuronide	0.86*									
Phenyl sulfate	0.49	0.37								
PAG	0.91*	0.95*	0.47							
Indoxyl sulfate	0.79*	0.69**	0.46	0.78*						
Urine										
p-Cresol sulfate	0.63	0.67**	0.35	0.73*	0.56					
p-Cresol glucuronide	0.69**	0.74*	0.39	0.76*	0.60	0.97*				
Phenyl sulfate	0.34	0.32	0.30	0.42	0.35	0.56	0.59			
PAG	0.63	0.73*	0.42	0.70**	0.57	0.93*	0.93*	0.55		
Indoxyl sulfate	0.45	0.61	0.22	0.54	0.40	0.76*	0.66	0.61	0.87*	
Urea	−0.35	−0.32	0.10	−0.31	−0.18	0.20	0.13	0.19	0.15	0.24
pH	0.64	0.58	0.17	0.54	0.24	0.29	0.35	0.22	0.27	0.15
Feces										
Ammonia	0.17	0.36	0.11	0.24	0.00	0.26	0.11	−0.02	0.32	0.26
Acetic acid	0.14	0.10	−0.24	0.09	−0.02	0.24	0.24	−0.28	0.09	0.06
Butyric acid	0.14	0.18	−0.05	0.07	−0.11	0.22	0.17	−0.28	0.24	0.18
Isobutyric acid	0.10	0.29	0.18	0.24	−0.06	0.20	0.16	−0.09	0.21	0.00
Isovaleric acid	0.10	0.33	0.23	0.27	−0.05	0.26	0.22	−0.01	0.29	0.08
Propionic acid	0.32	0.22	0.10	0.20	0.13	0.43	0.44	−0.01	0.36	0.32
Valeric acid	0.27	0.37	0.52	0.30	−0.05	0.31	0.27	−0.09	0.37	0.17
Total VFA	0.22	0.23	−0.03	0.18	−0.02	0.36	0.33	−0.23	0.28	0.19
Nitrogen	−0.04	−0.01	0.48	0.04	−0.02	0.05	0.14	0.34	0.04	−0.04
Nitrogen digestibility	−0.16	−0.03	−0.36	−0.17	−0.49	−0.13	−0.24	−0.07	−0.13	0.09
DM digestibility	−0.14	−0.04	−0.20	−0.16	−0.52	−0.21	−0.28	−0.14	−0.21	−0.03
pH	−0.25	−0.15	−0.22	−0.23	−0.03	−0.31	−0.53	−0.64	−0.17	−0.15

Correlations were corrected for repeated measurements, and the Benjamini–Hochberg procedure was applied to correct for multiple testing.
Abbreviations: DM, dry matter; NH_3, ammonia; PAG, phenylacetyl-L-glutamine; VFA.
*= correlation is significant ($P < 0.05$)
**= correlation is a trend ($P < 0.1$).

phenylalanine hydroxylase in the liver [57]. However, evidence that this occurs in the GI tract is lacking, making it unlikely to contribute substantially to colonic tyrosine availability. The higher plasma and urinary concentrations of tyrosine-derived metabolites during BPP consumption therefore remain more difficult to explain, as they suggest differences in protein digestibility. However, we cannot be certain whether these differences reflect differences in protein digestibility, variations in endogenous protein losses, or differential availability of specific AAs within the undigested BPP fraction.

Fecal protein-fermentation biomarkers did not differ between protein sources, consistent with the possibility that similar amounts of protein reached the large intestine, as suggested by the evidence discussed above, PPAA AUCs, in vitro digestibility values, and total tract apparent nitrogen digestibility. Despite the relatively low dietary protein concentrations in our study, fecal ammonia concentrations were comparatively high relative to other studies with similar protein intakes [58–60]. Elevated ammonia concentrations have been associated with increased intestinal permeability and impaired barrier function [18,61]. In contrast, BCFA may reduce pro-inflammatory cytokine expression and enhance intestinal epithelial integrity [62]. In the present study, fecal BCFA concentrations were lower than those previously reported [63]. The absence of differences in ammonia and BCFA may reflect their multiple AA precursors, reducing their sensitivity to dietary protein composition differences, as observed in pigs [13]. Although several plasmas and urinary protein-fermentation biomarkers were correlated, they did not correlate with fecal metabolites. This may be due to differences in fermentation activity throughout colonic segments [64,65], variations in absorption and systemic distribution [66,67], or differential availability of specific AAs. Furthermore, direct comparison across matrices was limited because fecal concentrations of metabolites detected in plasma and urine were not available.

Participants also tended to exhibit longer colonic transit times during BPP consumption. As diets were fully controlled and identical apart from the protein source, prolonged transit time was likely related to consumption of BPP, although the underlying mechanism remains unclear. Colonic transit time may affect fermentation by determining substrate availability for the microbiota [68] and time for metabolite absorption by colonocytes [69,70]. Longer transit times have been associated with lower fecal SCFA concentrations in multiple studies [20,71,72]. In the current study, transit time assessed using the blue dye method was also negatively correlated with valeric acid ($r = -0.84$), isovaleric acid ($r = -0.81$), and isobutyric acid ($r = -0.80$), although none were statistically significant. As BCFA and valeric acid concentrations did not differ between diets despite longer colonic transit during BPP consumption, these negative correlations likely reflect increased absorption rather than reduced metabolite production.

Urine		Feces										
Urea	pH	NH_3	Acetic acid	Butyric acid	Iso-butyric acid	Iso-valeric acid	Propionic acid	Valeric acid	Total VFA	Nitrogen	Nitrogen digestibility	DM digestibility
−0.25												
0.25	0.42											
−0.03	-0.06	−0.23										
−0.15	-0.12	0.01	0.75*									
−0.01	0.19	0.80*	−0.12	0.23								
0.04	0.18	0.77*	−0.08	0.28	0.98*							
0.08	-0.09	−0.21	0.73*	0.78*	−0.13	−0.06						
−0.02	0.13	0.47	0.16	0.61	0.71**	0.76*	0.41					
−0.02	-0.05	−0.02	0.90*	0.93*	0.16	0.22	0.86*	0.54				
0.31	0.17	0.43	−0.27	−0.12	0.50	0.55	−0.28	0.31	−0.13			
0.10	0.21	0.59	0.09	0.29	0.46	0.42	0.03	0.27	0.22	0.26		
0.05	0.29	0.55	0.11	0.30	0.46	0.43	0.01	0.35	0.23	0.38	0.95*	
−0.18	-0.43	0.03	−0.14	−0.04	−0.01	−0.10	−0.26	−0.13	−0.18	−0.52	−0.19	−0.25

Despite the fully controlled diet and high adherence, substantial individual variation was observed in most plasma and urine protein-fermentation biomarkers. Dutch cohort studies indicate that diet and gut microbiome composition contribute more to metabolic variation than genetics [73]. Consistent with this, we observed large inter-individual differences in microbiota composition. These differences, however, were not significantly affected by the dietary interventions, indicating that the microbiota composition remained stable within individuals throughout the study. No correlations were found between microbial taxa and protein-fermentation biomarkers, suggesting that overall fecal microbiota composition was not strongly associated with the observed metabolite differences. However, specific protein-fermentation metabolites may depend on low-abundance microbial species not captured by genus-level relative abundance metrics or overall diversity indices.

Given the exploratory nature of this study and its relatively small sample size, it is difficult to draw hard conclusions from this work. However, despite the small sample size, we were able to find differences in plasma and urine metabolite concentrations. This indicates that protein source can affect protein fermentation. Therefore, we believe that fermentation should not be ignored and that protein composition and digestibility should be considered beyond protein requirements.

Q17 In conclusion, consumption of 30 g/d BPP resulted in higher plasma and urinary concentrations of p-cresyl sulfate, p-cresyl glucuronide, phenyl sulfate, PAG, and indoxyl sulfate than consumption of WPI. These differences were not accompanied by changes in fecal protein-fermentation markers or microbiota composition. AA composition likely contributed to the observed differences, although subtle differences in digestibility or transit time cannot be excluded. Together, these findings indicate that protein source influences microbial protein fermentation in humans.

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Author contributions

The authors' responsibilities were as follows – RM, EC, SdV, AE, GH: designed research; RM, GH, AE, AA: conducted research; RM, GH: analyzed data; RM, EC, SdV, AE, AA, GH: wrote the paper; RM, GH: had primary responsibility for final content; and all authors: read and approved the final manuscript.

Conflict of interest

Q19 The authors report no conflicts of interest.

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Data availability

Data will be made available upon reasonable request.

Declaration of AI and AI-Assisted Technologies in the Writing Process

During the preparation of this work, the authors used language editing tools in order to improve clarity, grammar, and readability. Q21 These tools were not used to generate scientific content, perform data analyses, interpret results, or draw conclusions. After using this tool/service, the authors reviewed and edited the content as needed and takes full responsibility for the content of the publication.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ajcnut.2026.101491.

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