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In Vitro Fermentation of Animal and Plant Protein Isolates by the Human Gut Microbiota Under High and Low Carbohydrate Conditions

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Scope: There is a lack of research comparing how different protein isolates influence the microbiome, especially when carbohydrate (CHO) availability is varied. The objective is to determine changes in gut microbiota composition and function during fermentation of digested protein isolates under high and low CHO conditions.

Methods and results: Protein isolates from beef, egg white, milk, pea, and soy are subjected to in vitro digestion and fermentation with human fecal microbiota. Under low CHO conditions, the microbiota is primarily proteolytic with decreased concentrations of peptides and increased variance among microbial taxa and production of ammonia and branched chain fatty acids by the microbiota. Milk protein not only results in the highest production of butyrate and *p*-hydroxyphenylacetate but also has high concentrations of deleterious fermentation metabolites. Amino acid composition of the protein isolates is significantly correlated with abundances of many microbial taxa and metabolites, but the correlations are stronger in the low CHO medium.

Conclusion: This study shows that low CHO conditions increase proteolytic fermentation and result in increased differences in microbiota response to protein isolates. It also showed that amino acid composition is highly associated with microbiota composition and function especially under low CHO conditions.

1. Introduction

Although over 97% of Americans exceed recommendations for protein intake[1] this nutrient is widely sought out among consumers.[2] This has fueled the development of a robust protein isolate market that continues to grow in market share,[3] and to expand to include protein isolates from new sources.[2] In contrast, the average American diet lacks dietary fiber, with more than 90% of women and 97% of men not meeting the recommended daily intakes for dietary fiber.[1] Public health recommendations to increase dietary fiber intake have not been effective despite the associated health benefits.[4,5] Thus, the current dietary habits of Americans reflect a high-protein, low-fiber diet. These dietary habits can influence the gut microbiota because undigested food components escape digestion in the small intestine and enter the colon for fermentation by the microbiota. Depending on intake, digestibility, and composition,

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20–60 g of dietary fiber and 6–18 g of dietary protein enter the colon daily.[6] The microbiome preferentially ferments dietary fiber for energy, using proteins and peptides for anabolic biosynthesis rather than catabolism.[7,8] However, when there is a lack of available carbohydrates (CHOs), the microbiome can metabolize proteins for energy even though saccharolytic fermentation is energetically favored over protein fermentation.[9] Additionally, an overconsumption of dietary protein increases protein entering the colon, as evidenced by increased protein fermentation metabolites in stool samples collected from subjects consuming increased levels of protein.[10–13]

Indeed, during fermentation of either dietary fibers or proteins, the gut microbiota produces metabolites that can influence host health. Saccharolytic metabolites, including short chain fatty acids (SCFAs), are produced by gut microbes during CHO metabolism and have numerous beneficial effects on host health.[14] Butyrate in particular is important for colonic health by serving as the main energy source for colonocytes, maintenance of colonic mucosal health, and acting as an anti-inflammatory agent.[14] In addition to supporting colonic health, butyrate has been shown to protect brain health by increasing the expression of genes related to neural regeneration and plasticity,[15] and to prevent and treat diet-induced obesity and insulin resistance in mouse models.[16]

Fermentation of peptides and amino acids also results in SCFA, but also leads to production of branched chain fatty acids (BCFAs) and a number of metabolites that can negatively influence host health such as ammonia, amines, phenols, and indoles.[9,17] These proteolytic compounds have been linked to damage to the colonic mucus layer, increase in colonic epithelial permeability, and overall DNA damage.[18] Proteolytic fermentation results in the production of general metabolites resulting from deamination of amino acids (e.g., ammonia); however, there are a number of metabolites that arise from specific amino acids, including *iso*-butyrate (valine [Val]), *iso*-valerate (leucine [Leu]), phenol (tyrosine [Tyr]), indole (tryptophan), 4-aminobutyrate (arginine [Arg]), hydrogen sulfide (methionine [Met] and cysteine [Cys]), and cadaverine (lysine [Lys]).[8,18,19] Thus, when proteins and peptides enter the colon for fermentation, the amino acid composition should influence the proteolytic metabolites produced.

While several studies indicate an increase in protein consumption results in a significant increase of protein specific fermentation metabolites that are detrimental to health,[10–13] proteolytic fermentation can be reduced with adequate dietary fiber, even without altering protein intake.[20,21] These results suggest the ability of dietary fibers to attenuate production of harmful proteolytic fermentation metabolites.

There is evidence that protein and dietary fiber intake interact to influence microbiome composition and function. However, there is a lack of research comparing how different protein isolates influence the microbial response, especially when dietary fiber availability is varied. Thus, the objective of this study was to determine the changes in gut microbiota composition and function during fermentation of digested protein isolates from diverse sources under high- and low-CHO conditions simulating high and low dietary fiber intakes. In this study, we tested three hypotheses: 1) low CHO conditions will increase fermentation of protein isolates as evidenced by an increase in proteolytic fermentation metabolites compared with high CHO conditions; 2) under the low CHO conditions, where the microbiota primarily ferments proteins for energy, the differences among individual protein isolates in terms of metabolite production and microbiota composition will be exaggerated compared to high CHO conditions where the proteins are not primarily used for energy; and 3) the amino acid composition of different protein isolates could be associated with the composition of the gut microbiota and to the production of proteolytic fermentation metabolites, especially under low CHO conditions where metabolism of the protein isolates is increased.

2. Results

2.1. Substrate Characterization

The protein isolates contained 82%–99% protein before digestion (**Figure 1A**, undigested). The proteins did not separate by their origin (animal versus plant source), as the beef and soy2 isolates were the highest in protein and pea, egg white (EW), and milk protein were the lowest. The plant protein isolates showed a wide range of proteins with differing molecular weights, while the animal protein isolates lacked in protein diversity (Figure 1B, undigested). The molecular weight profiles showed that EW protein isolate was very different from the other protein isolates (Figure 1C, undigested). Additionally, beef protein isolate clustered with pea and soy1 protein, while the milk protein isolate (MPI) clustered with the soy2 sample. The plant protein isolates showed similarities in amino acid profiles, while the animal protein isolates differed in the concentration and distribution of amino acids (Figure 1D, undigested). Plant proteins had high concentrations of asparagine + aspartate (Asx), Arg, and Lys. For animal proteins, beef had high concentrations of glycine (Gly), alanine (Ala), and proline (Pro); EW and milk protein had high concentrations of Leu, Val, isoleucine (Ile), threonine (Thr), Met, and Cys.

To prepare proteins for in vitro fermentation, samples were subjected to in vitro digestion followed by exhaustive dialysis to remove digested amino acids and peptides <1 kDa. After digestion, the pea protein concentration was low at 63.4% ± 7.9%, but all other samples ranged from 78% to 100% protein (Figure 1A, digested). The SDS-PAGE gel of the digested and dialyzed samples confirmed extensive digestion of proteins into peptides except for the EW, which still contained bands corresponding to the major intact proteins (Figure 1B, digested). This was reflected in the size-exclusion chromatography, which showed that the peptides with molecular weights greater than 7 kDa was >90% for the EW protein isolate (Figure 1C, digested). The percentage of peptides with molecular weights greater than 7 kDa ranged from 38% to 56% for the other protein isolates. The clustering of the protein isolates after digestion was similar to the before digestion samples, except soy1 moved to the cluster with the MPI and soy2 moved to the cluster with the beef and pea protein isolates. The amino acid profiles of the samples after digestion were also similar to the before digestion samples. Plant proteins had high concentrations of Asx, Arg, and Lys, while beef had high concentrations of Gly, Ala, and Pro. EW and milk proteins had high concentrations of Leu, serine (Ser), Var, Ile, Thr, Tyr, Met, and Cys.

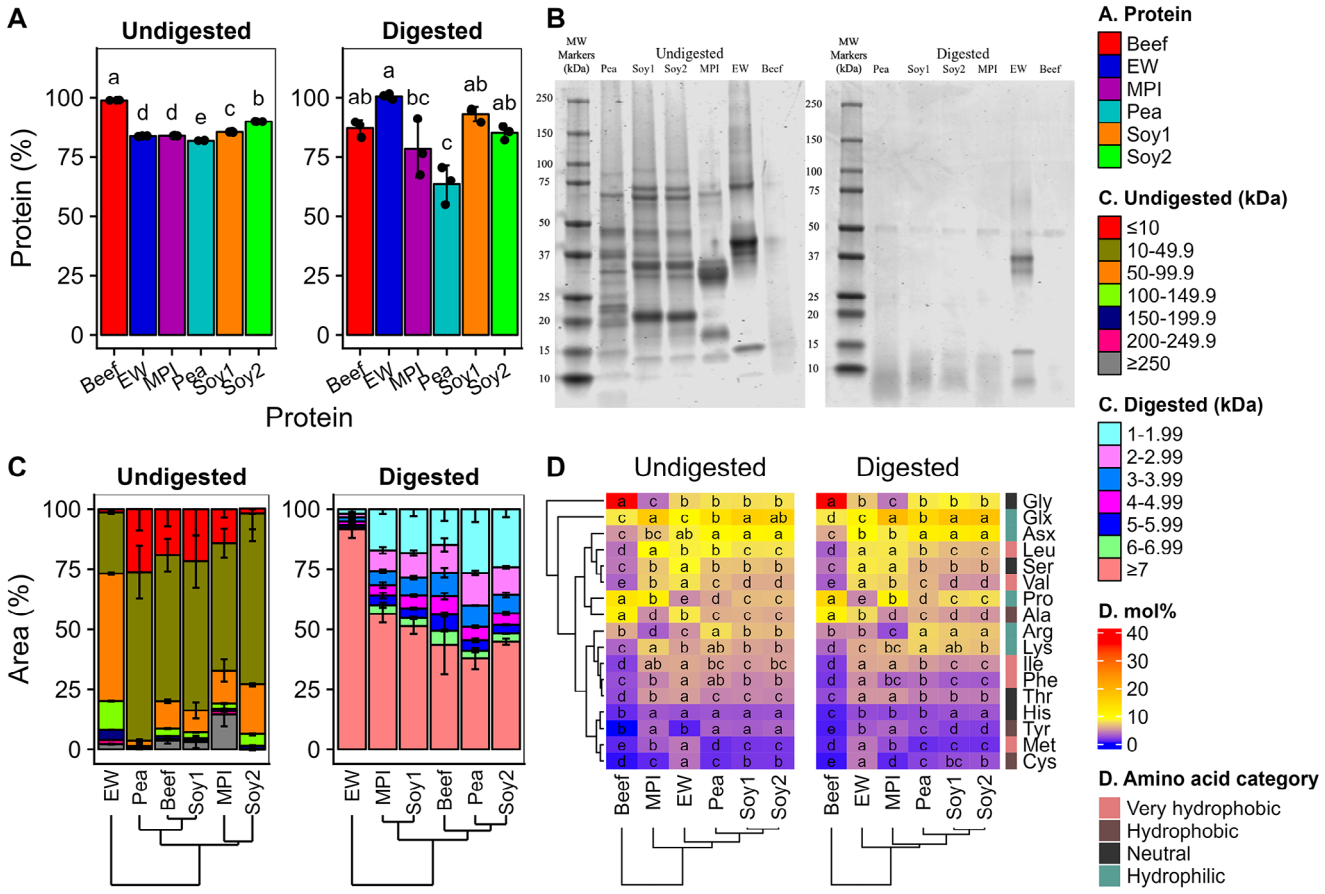

Figure 1. Characterization of protein isolates. A) Protein concentration (%N X 6.25); B) SDS-PAGE; C) molecular weight distribution; D) amino acid composition of protein isolates before (undigested) and after (digested) in vitro digestion; error bars show standard deviation of triplicate measurements; the heatmap shows means of triplicate measurements; bars (a) or rows (d) within subplot marked with different letters are significantly different (Tukey's HSD $p < 0.05$). EW, egg white; HSD, Tukey's honestly significant difference; MPI, milk protein isolate; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

2.2. Peptide Concentration During In Vitro Fermentation

The fermentation of the digested protein isolates was examined when high or low CHO concentrations were available in the medium. Milk protein had significantly higher total peptides, while the EW protein had significantly lower total peptides available at the beginning of fermentation (0 h) than the other protein isolates (**Figure 2**). Under high CHO conditions, the total peptides showed either no change or a slight increase in concentration over the course of the fermentation. In contrast, under low CHO conditions, total peptides showed steep declines in concentration for all proteins except beef and EW over the 24 h fermentation period. This suggested that the microbiomes utilized the peptides during fermentation for energy due to the lack of available CHO.

2.3. Microbiota Composition During In Vitro Fermentation

The β-diversity among samples during fermentation was examined in terms of Bray–Curtis distance. As expected, a principal coordinates biplot of the Bray–Curtis distance matrix showed major clustering by microbiome (PERMANOVA $R^2 = 0.36$, $p < 0.01$), confirming the differences in composition among microbiomes (**Figure 3A**). The shift in microbiome composition as the fermentation time progressed was also evident ($R^2 = 0.18$, $p < 0.01$). Interestingly, it appeared that the composition of the microbiomes became more similar over time, as all microbiomes shifted toward the fourth quadrant on the biplot during fermentation. The ASVs with the highest vectors in this quadrant were from *Providencia*, *Veillonella*, *Enterococcus*, and *Clostridium sensu stricto 1* (data not shown). After correcting for microbiome, minor clustering was evident by medium ($R^2 = 0.04$, $p < 0.01$) and protein type ($R^2 = 0.06$, $p < 0.01$).

Next, we compared the Bray–Curtis distance from the fecal samples (0 h) for all fermented samples (8 and 24 h) under high and low CHO conditions. The responses of the microbiomes under the two media conditions were highly correlated (Figure 3B). Thus, responses to the different digested protein isolates were similar under each condition. However, the slope and intercept of the regression line also revealed that after extended fermentation (i.e., 24 h), the magnitude of the Bray–Curtis distance from the fecal sample was greater in low CHO medium compared with high CHO medium ($p < 0.001$).

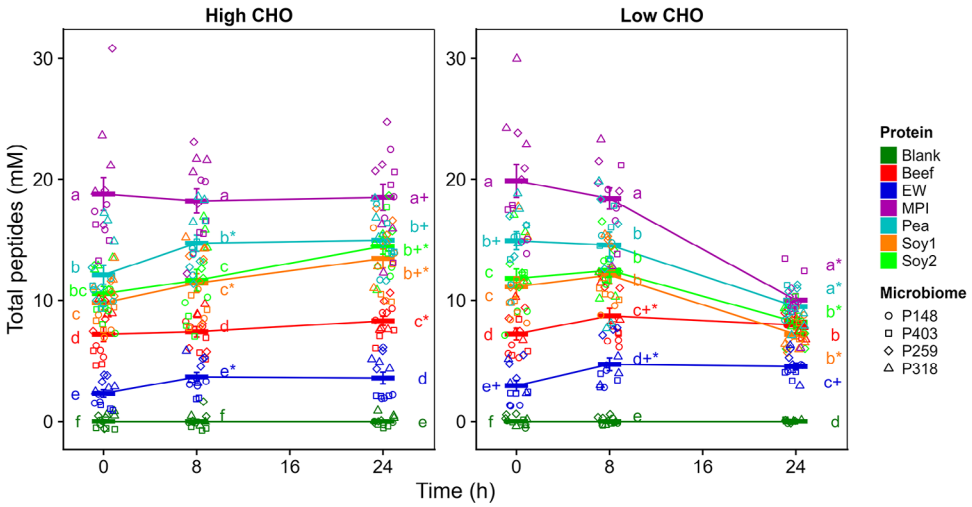

Figure 2. Peptide concentration during fermentation. Crossbars labeled with different letters within medium and time are significantly different among proteins; a "+" sign after the letter annotation indicates the proteins had a significantly higher peptide concentration compared with the same protein in the other medium; a "*" sign indicates a significant difference from the previous time point for the same sample ($n = 4$ microbiotas X 3 replications of each; Tukey's HSD $p < 0.05$). CHO, carbohydrate; EW, egg white; HSD, Tukey's honestly significant difference; MPI, milk protein isolate.

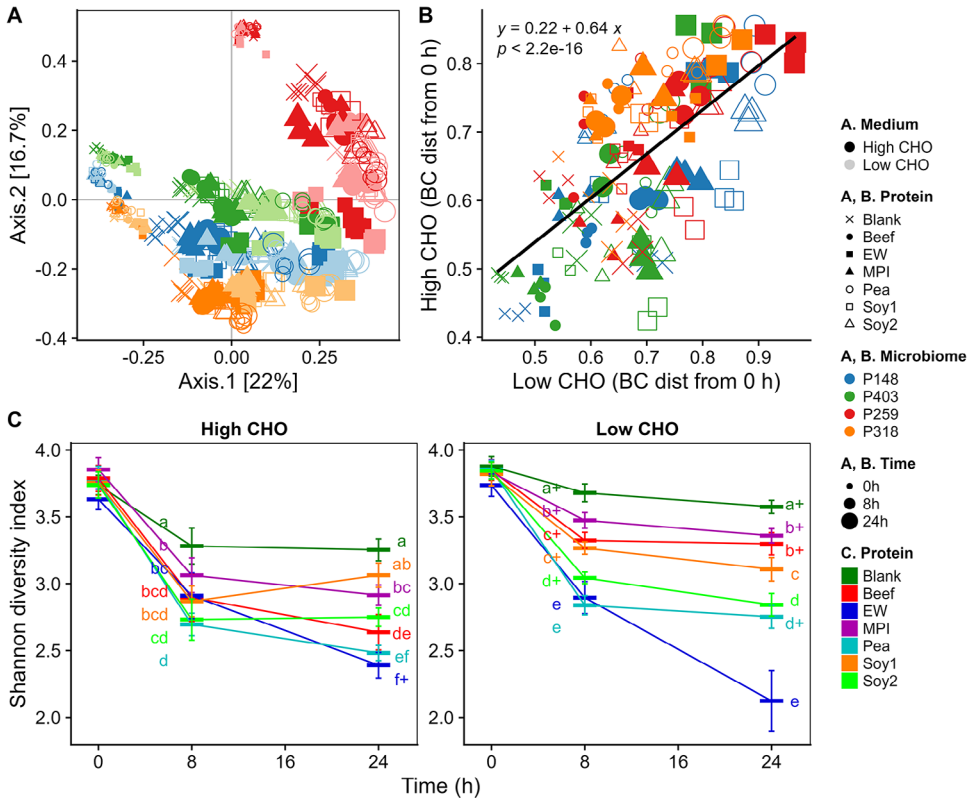

Figure 3. β- and α-diversity among samples during fermentation. A) Principal coordinates biplot of the Bray–Curtis distance (BC dist) matrix among samples; B) correlation between BC dist from fecal sample (0 h) on low and high carbohydrate (CHO) media; C) α-diversity in terms of Shannon's index [crossbar represents the mean across microbiomes; error bar shows standard error; individual data points removed for clarity; proteins marked with different letters within time point are significantly different; a "+" sign after the letter annotation indicates that protein had significantly higher diversity compared with the same protein in the other medium ($n = 4$ microbiotas X 3 replications of each; Tukey's HSD test $p < 0.05$)]. EW, egg white; HSD, Tukey's honestly significant difference; MPI, milk protein isolate.

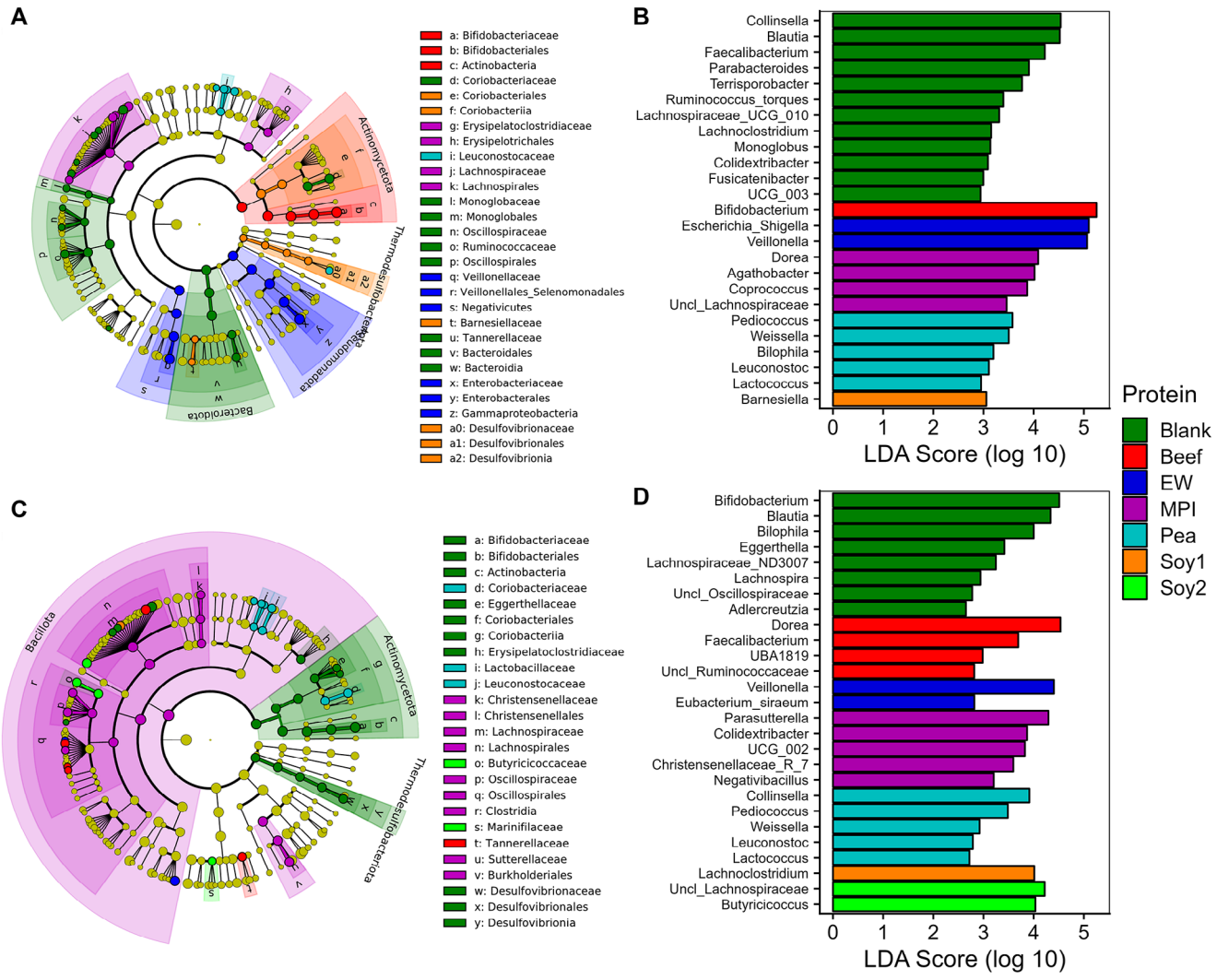

Figure 4. Differentially abundant taxa after 24 h of fermentation depending on protein type. Linear discriminate analysis (LDA) effect size analysis across proteins corrected for microbiome in high carbohydrate (A, B) and low carbohydrate (C, D) media; A, C) cladograms with taxa enriched on each protein type colored by protein; phylum to family levels are labeled; B, C) differentially abundant genera colored by protein ($n = 4$ microbiotas X 3 replications of each; LDA > 2 and $p < 0.05$). EW, egg white; MPI, milk protein isolate.

The α-diversity of fermented samples was examined in terms of Shannon's index. The blank generally supported higher diversity than the protein samples, suggesting that the proteins selectively stimulated or inhibited certain members of the microbiota (Figure 3C). Among proteins, milk together with soy1 in high CHO medium and beef in low CHO medium supported the highest diversity. The EW protein resulted in the lowest diversity on both media. The pea protein also supported low diversity that was not significantly different from EW in the high CHO medium.

Given that the peptide degradation data showed that the microbiota was forced to metabolize the peptides during fermentation under the low CHO conditions, and that the diversity results suggested that the magnitude of the microbiota response to the digested protein isolates was greater in the low CHO medium compared with the high CHO medium, we compared the variance among proteins for each ASV identified in the microbiome for each medium tested. Under low CHO conditions, there were 76 ASVs with significantly greater variance among protein types compared with the high CHO conditions, while only 36 ASVs had significantly higher variance among proteins under the high CHO conditions (Supporting Information Table S1; χ^2 test of equal frequency [36 versus 70]: $p < 0.001$). Thus, there appeared to be more variation in microbiota composition among the protein types under low CHO conditions than high CHO conditions.

Linear discriminant analysis effect size (LEfSe) was then used to determine the differentially abundant taxa among protein types after 24 h of fermentation after correcting for microbiome. In the high CHO medium, there were 107 differentially abundant features across all taxonomic ranks (**Figure** 4A,B). The beef protein resulted in increased *Actinomycetota* (formerly *Actinobacteria*), particularly due to an increase in *Bifidobacterium*, while the EW resulted in an increase in *Pseudomonadota* (formerly

Proteobacteria), especially from *Escherichia/Shigella*. The milk protein did not cause any phylum-level shifts in the microbiome but did result in a significant increase in *Erysipelatoclostridiaceae* as well as several genera from *Lachnospiraceae*. Among the plant proteins, only the soy1 protein caused phylum-level differences. *Thermodesulfobacteriota* were elevated on soy1 proteins due to an increase in *Desulfovibrionaceae*. Soy1 proteins also resulted in elevated *Barnesiella*. No differentially abundant features were identified for the soy2 sample. Pea proteins appeared to target taxa from several *Lactobacillales*, including *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Weissella*.

Under low CHO conditions, LEfSe analysis identified 117 differentially abundant features across all taxonomic ranks (Figure 4C,D). Among protein samples, only the milk protein induced phylum-level changes in the microbiome. Compared with the other protein isolates, the milk protein resulted in increased abundance of *Bacillota* (formerly *Firmicutes*) due to increases in the abundance of taxa across three orders within *Clostridia*: *Oscillospirales*, *Lachnospirales*, and *Christensenellales*. At the family level, several other protein isolates had significant associations. Pea protein was associated with Coriobacteriaceae, Lactobacillaceae, and Leuconosctocaceae due to increases in *Collinsella*, *Pediococcus*, *Leuconostoc*, and *Weissella*. Soy2 had significant associations to Butyricicoccaceae and *Butyricicoccus*, in addition to Marianifilaceae and an unclassified genus in Lachnospiraceae. Beef protein was associated with Tannerellaceae at the family level and *Dorea* and three genera within *Ruminococcaceae* at the genus level. EW and soy1 were only associated with taxa at the genus level: *Veillonella*, *Eubacterium siraeum* for EW, and *Lachnoclostridium* for soy1.

2.4. Microbial Metabolite Production

We analyzed five metabolites arising primarily from protein fermentation by the microbiota after 24 h of fermentation. Like the shifts in microbiota composition during fermentation (β-diversity, Figure 3B), the concentrations of these protein fermentation metabolites were highly correlated between the high and low CHO media (Supporting Information Table S2), and the low CHO medium resulted in higher concentrations of ammonia, *iso*-valerate, and *iso*-butyrate, across most protein types compared with high CHO medium (**Figure 5**). Additionally, these metabolites showed significantly more variation across protein types in low CHO medium compared with high CHO medium (Supporting Information Table S3). This is consistent with the increased degradation of peptides as well as the increase variance across genera in the low CHO medium compared with the high CHO medium. In contrast, cadaverine and *p*-hydroxyphenylacetate production were comparable between the two media.

Among protein types, the milk protein resulted in the highest or among the highest protein metabolite production regardless of media (Figure 5). Second to milk protein, the pea and soy proteins produced relatively high levels of protein metabolites compared with the beef and EW proteins.

We also analyzed SCFA production, which arises from both protein and CHO metabolism by the gut microbiome. Similar to ammonia and the BCFAs, the low CHO medium showed increases in SCFA across most protein types and showed significantly more variation across protein types compared with high CHO medium (**Figure 6**; Supporting Information Table S3). However, only butyrate concentrations were correlated between the high and low CHO media (Supporting Information Table S2). The most striking finding for SCFA production was the high butyrate production with milk protein. This was evident in both media but exaggerated in the low CHO medium. The beef protein and the soy proteins also resulted in appreciable butyrate production in the low CHO medium but not in the high CHO medium.

For acetate and propionate production, the milk protein as well as the plant proteins resulted in an increase in the production of these metabolites compared with the beef and EW proteins in the low CHO medium (Figure 6). In high CHO medium, elevated acetate production was evident from the plant proteins, while relatively high propionate production resulted from the metabolism of EW proteins.

2.5. Correlations of Amino Acids with Microbiota Composition and Metabolite Production

We correlated the concentrations of individual amino acids in the digested proteins with the microbiota composition and metabolite production after 24 h of fermentation of the digested proteins. When analyzing the correlations between amino acid concentrations and microbiota composition, it was immediately obvious that there were more associations in low CHO medium compared with high CHO medium (229 versus 149, respectively, χ^2 $p < 0.001$; **Figure 7A**). The high CHO medium lacked associations between the amino acids and several genera across the taxonomic spectrum that were present in the low CHO medium. Those genera with the most correlations in the low CHO medium and no correlation in the high CHO medium were *Ruminococcaceae UBA1819*, *Oscillospiraceae NK4A214*, *Faecalitalea*, and *E. siraeum*. Additionally, Asx and histidine (His) had several significant correlations in the low CHO medium but none in the high CHO medium. The highest correlations to Asx and His in the low CHO medium were *Phascolarctobacterium*, *Butyricicoccus*, *Sutterella*, and *Muribaculaceae*.

Nevertheless, there were some similarities between the high and low CHO media. For example, the amino acids clustered by hydrophobicity in each medium. Mostly hydrophilic amino acids clustered on the left side of the heatmaps, while mostly hydrophobic amino acids clustered on the right side of the heatmaps. Pro displayed the highest positive correlation to genus abundances, with strong positive correlations to *Bifodobacterium*, *Coprococcus*, *Butyricicoccus*, and *Faecalibacterium* in both media. The basic amino acids, Lys and Arg, showed similar correlations, with the strongest positive associations to several genera from *Lactobacillales*, including *Lactococcus*, *Leuconostoc*, and *Pediococcus*. The (very) hydrophobic amino acids had strong negative correlations to the *Coprococcus*, *Bifidobacterium*, and *Collinsella*.

When analyzing the correlations between amino acid and metabolite concentrations, several positive correlations were observed between different amino acids and propionate, ammonia, *p*-hydroxyphenylacetate, butyrate, and *iso*-valerate in the high CHO medium. Few or no significant correlations were observed for cadaverine, *iso*-butyrate, and acetate. Ala and to a lesser extent, Cys, were unique by showing negative correlations with several

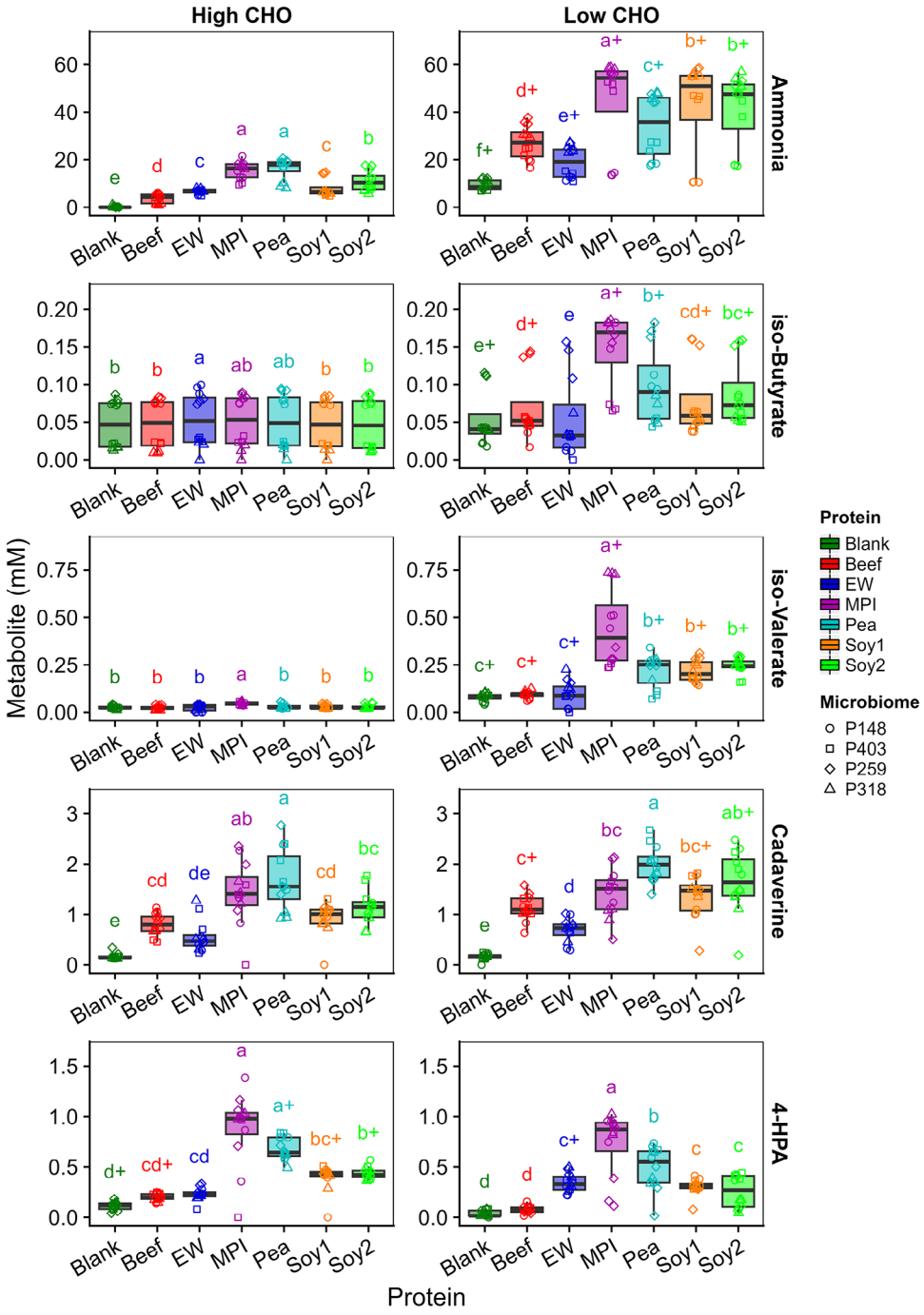

Figure 5. Protein-specific microbial metabolite production after 24 h of fermentation of digested protein isolates. Boxplots labeled with different letters within panel are significantly different among proteins; a "+" sign after the letter annotation indicates that protein had significantly higher SCFA production compared with the same protein in the other medium ($n = 4$ microbiotas X 3 replications of each; Tukey's HSD $p < 0.05$). CHO, carbohydrate; EW, egg white; 4-HPA, 4-hydroxyphenylacetate; HSD, Tukey's honestly significant difference; MPI, milk protein isolate; SCFA, short chain fatty acid.

metabolites. The strong negative correlations between Ala and Cys and metabolite production were also evident in the low CHO medium. Glutamine + glutamate (Glx) was positively correlated with all metabolites analyzed. Many amino acids were correlated, either positively or negatively, with *p*-hydroxyphenylacetate and *iso*-valerate.

3. Discussion

The present research aimed to understand how different protein isolates affect gut microbiota composition and function in high CHO or low CHO conditions. An in vitro fermentation with enzymatically digested and dialyzed protein isolates from animal and

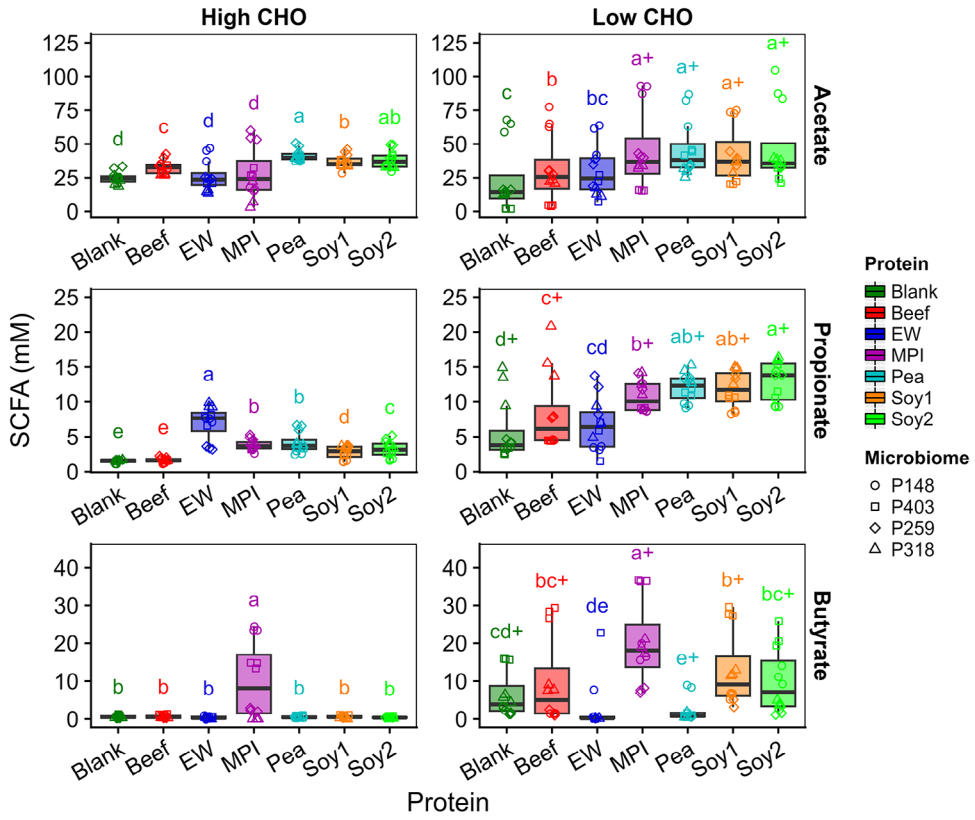

Figure 6. Short chain fatty acid (SCFA) production after 24 h of fermentation of digested protein isolates. Boxplots labeled with different letters within panel are significantly different among proteins; a "+" sign after the letter annotation indicates that protein had significantly higher SCFA production compared with the same protein in the other medium ($n = 4$ microbiotas X 3 replications of each; Tukey's HSD $p < 0.05$). CHO, carbohydrate; EW, egg white; HSD, Tukey's honestly significant difference; MPI, milk protein isolate.

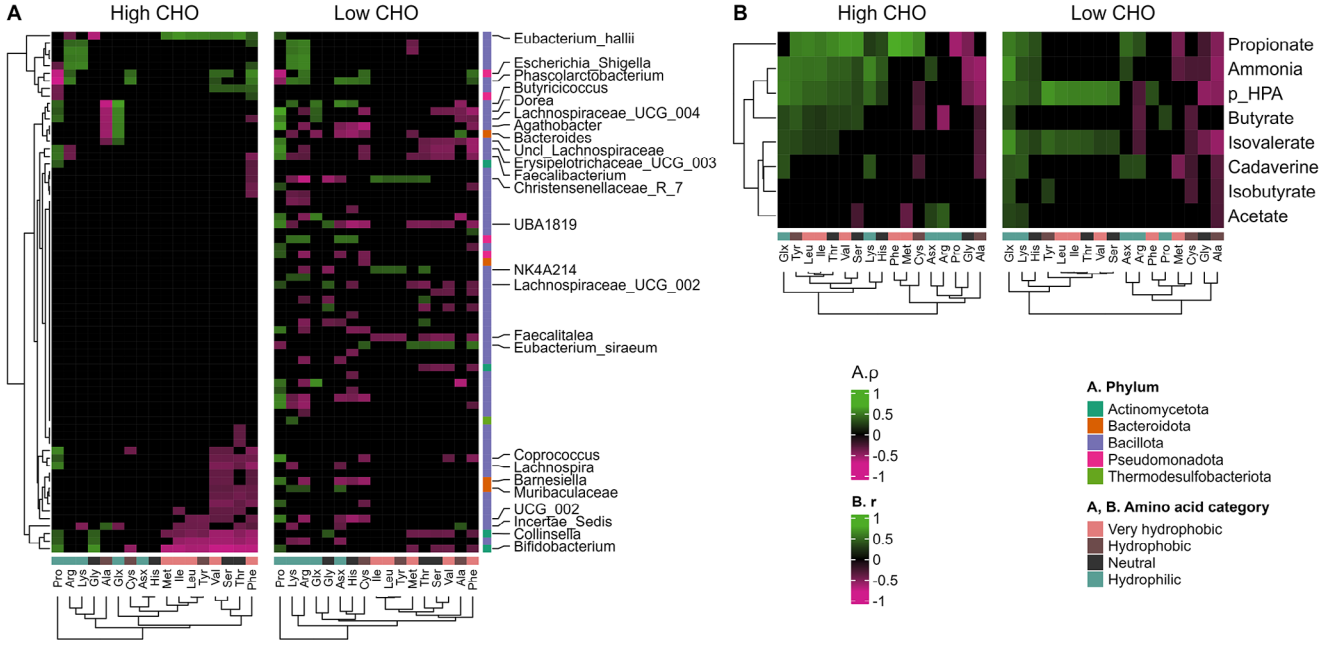

Figure 7. Partial correlations of amino acids with microbiota composition (A) and metabolite production (B). A) Spearman correlations and B) Pearson correlations after 24 h of in vitro fermentation under low and high carbohydrate (CHO) conditions. In Panel (A), for clarity, only genera with at least seven significant correlations with amino acids across both media are labeled. All correlations are significant at $p < 0.05$ with Benjamini–Hochberg adjustment ($n = 72$). Nonsignificant correlations were set to zero and appear black in the heat maps. Partial variable was microbiome.

plant origins was completed with differing CHO concentrations in the medium to mimic high and low dietary fiber intake conditions. Our first hypothesis – that low CHO conditions would force the catabolism of the protein isolates for energy by the microbiota – was confirmed through decreased concentrations of peptides during fermentation accompanied by increased production of protein-specific metabolites by the microbiota. Thus, our results confirmed previously reported observations from both in vitro[22,23] and human trials[10–13] that proteolytic fermentation increases in the absence of available CHO.

Our second hypothesis – that the microbiota response to different protein isolates would be exaggerated in low CHO medium compared to high CHO conditions – was also confirmed. In the low CHO medium, protein isolates induced greater shifts in microbiota composition (quantified through Bray–Curtis distance) and greater variance among microbial taxa and metabolite concentrations than in the high CHO medium. As stated, the microbiome preferentially ferments CHO over proteins for energy.[7,8] Thus, under high CHO conditions, the microbiota was saccharolytic and its composition and function were primarily influenced by the CHO in the medium, which was the same across all samples. In contrast, under low CHO conditions, the microbiota was forced into proteolytic fermentation. Presumably, the varying amino acid compositions, molecular weights, and other unique properties of the protein isolates induced an exaggerated differential effect on microbiome composition and function. This has potential implications for human health, as it suggests that under a high-protein, low-fiber diet adopted by many Americans, the microbiota composition and function may be more influenced by dietary proteins than by dietary fiber. This can result in a metabolite profile generally associated with deleterious effects on human health.[12]

The protein isolates induced unique shifts in microbiota composition and metabolite production that were dependent on media condition. The most striking finding was the high butyrate production during fermentation of the milk protein. The high butyrate production from milk proteins was evident in the high CHO medium, but it was dramatically enhanced in the low CHO medium. The fermentation of milk protein was associated with increased abundances of several genera that have been associated with butyrate production, including *Christensenellaceae* R7 group,[24] *Colidextribacter*,[25] *Nagativibacillus*,[26] and *Oscillospiraceae* UGC-002[27] in the low CHO medium and *Agathobacter*,[26] *Coprococcus*,[28] and an unclassified *Lachnospiraceae* genus[29] in the high CHO medium. Most genera capable of butyrate production are associated with the breakdown of CHOs, although many also have enzymes for the conversion of acetate and acetyl-CoA to butyrate.[30] The conversion of acetate to butyrate may have been the primary pathway of butyrate production from milk protein under low CHO conditions. Alternatively, the glutarate, 4-aminobutyrate, and Lys pathways are also associated with butyrate production from amino acid metabolism and could have been used by the microbiota to produce butyrate from milk proteins.[30,31]

The inclusion of milk proteins in both media also resulted in high concentrations of *p*-hydroxyphenylacetate. *p*-Hydroxyphenylacetate can be a product of the microbial metabolism of Tyr,[32] which was the highest in digested milk proteins compared with the other digested proteins used in this study. While certain phenolic compounds have negative health effects (e.g., phenol and *p*-cresol),[9,33] the biological effects of substituted phenolic acids, including *p*-hydroxyphenylacetate, have been minimally explored. However, because of the antioxidant nature of these compounds, it is believed that they may protect against oxidative stress.[34] Furthermore, hydroxyphenyl derivatives are also produced during the fermentation of plant secondary metabolites, which are associated with positive health outcomes.[35,36] Thus, in addition to butyrate production, the generation of high concentrations of *p*-hydroxyphenylacetate may be a benefit of milk protein fermentation.

The beef and soy proteins also resulted in elevated butyrate production during in vitro fermentation, but only when added to the low CHO medium. These proteins were associated with different butyrate-producing genera than the milk protein fermentation. This included several genera from *Ruminococcaceae* for the beef proteins,[29] and *Lachnoclostridium*,[37] an unclassified *Lachnospriaceae*,[29] and *Butyricicoccus*,[38] for the soy proteins.

While there was significant butyrate and *p*-hydroxyphenylacetate production associated with the fermentation of some protein isolates, these same proteins were also associated with relatively high levels of ammonia and cadaverine. High levels and sustained exposure to ammonia can harm colonocytes by decreasing cell proliferation and causing DNA damage.[39,40] Cadaverine is toxic at high levels and has been associated with increased cardiac output, hypotension, and bradycardia.[8,41–43] Therefore, while butyrate and *p*-hydroxyphenylacetate may be beneficial metabolites that contribute to host health during protein fermentation, the formation of additional negative fermentation metabolites can be a drawback.

The presence of each individual protein isolate in the medium induced its own unique effect on the microbiome, rather than inducing shifts based on the broader origin of the protein isolates, i.e., animal proteins versus plant proteins. In general, milk protein fermentation was more similar to the plant proteins than the other animal-derived proteins in terms of microbiota composition and metabolite production. This is consistent with the amino acid profiles and molecular weight distributions of the digested protein isolates, where milk proteins clustered with the plant proteins instead of other animal proteins. This suggests the important role of amino acid composition in directing how digested proteins will influence the microbiome.

Accordingly, our third hypothesis – that the amino acid composition of different protein isolates could be associated with the composition of the gut microbiota and to the production of proteolytic fermentation metabolites, especially under low CHO conditions where metabolism of the protein isolates is increased – was also confirmed. Indeed, while the abundances of many microbial taxa after fermentation were correlated with amino acids at the beginning of fermentation in both high and low CHO media, far more associations were significant in the low CHO medium. This reiterates the strong influence that protein isolates had on microbiota composition and function in the low CHO medium.

Much is known about the fermentation of individual amino acids and the types of metabolites that are produced. For example, cadaverine is a metabolite of Lys metabolism; Tyr utilization results in the production of *p*-hydroxyphenylacetate; and

branched chain amino acids are converted to BCFAs through microbial metabolism.[8,18,19] These associations were confirmed through the significant correlations observed in both high and low CHO medium. However, there were several other significant correlations that were notable. Glx carried the most significant correlations in both media and was in fact positively correlated with all microbial metabolites in the low CHO medium. This suggests an important role of Glx in microbiota function. During anaerobic fermentation, glutamine (Gln) is converted to glutamate (Glu) through deamidation (producing ammonia), and Glu is broken down into acetate, butyrate, ammonia, and carbon dioxide.[44,45] Glx supplementation is known to affect the gut microbiota composition, and active research exploring how these amino acids might improve human health are underway.[45]

In contrast to the correlations between amino acids and protein-specific metabolites, the correlations of amino acids with the SCFA were not as expected. For example, Gly, Thr, and Glu are known to be metabolized to acetate; propionate is primarily produced from Ala and Thr metabolism; and Lys, Glu, and tryptophan breakdown can produce butyrate.[46,47] Except for a significant correlation between Glx and butyrate, none of the other amino acids were correlated with the metabolites they are known to generate. This is likely because the simultaneous metabolism of CHO, even at low levels in the low CHO medium, masked these relationships. Also, the sequence of amino acids in the digested proteins may also influence their metabolism by the gut microbiota. Furthermore, unlike the protein-specific metabolites, acetate and propionate production were not correlated with each other in the high versus the low CHO media.

Among the correlations between amino acid concentrations and microbial taxa, the apparent strong influence of Pro on microbiota composition is notable. The availability of Pro is fiercely competitive among gut microbes, and reduction in the availability of Pro in the gut contributes to colonization resistance against *Escherichia* coli O157:H7 and *Clostridioides difficile* infections.[48,49] Dysregulated Pro metabolism in the guts of rats with dextran sodium sulfate-induced colitis has also been shown to contribute to intestinal barrier dysfunction.[50] The high correlations between Pro concentration and many gut microbial taxa suggest its central role in determining microbiota composition.

Other properties of digested proteins besides amino acid composition may also affect their utilization by the gut microbiota. Research on dietary fibers has shown that CHO chain length can influence microbial metabolism because some species are unable to grow on long chain polysaccharides.[51,52] Likewise, the chain length of peptides in the digested protein isolates may affect microbial metabolism. For example, *E. coli* can only take up small peptides of less than 650 Da, while *Lactococcus lactis* and *Bacillus megaterium* can take up peptides larger than 2140 and 10 000 Da, respectively.[53] However, there is a lack of research deciphering the impact of peptide length on the microbiota composition. While such a topic is worthy of investigation, the differences in molecular weight profiles among protein isolates in the present study were confounded by differences in amino acid composition making it impractical to examine the impact of peptide length on the microbiota composition.

One limitation of our study is that the varying proportion of protein digestibility among the protein isolates was not taken into account when weighing samples for the in vitro fermentation experiment. Rather, all digested and dialyzed protein isolates were standardized to equivalent nitrogen concentrations as described above and in Section 4.4. This was to ensure that all proteins were equally comparable. We considered that this was an important comparison since previous studies have not shown differences in in vitro fermentation properties among different protein isolates after being subjected to digestion and dialysis. Additionally, this is a typical approach when performing in vitro fermentation experiments.[54] Should we have taken into account the percent protein digestibility (i.e., added more of a poorly digestible protein and less of highly digestible protein), any differences we observed among the protein isolates would be confounded by differences in sample weights.

However, because we standardized the nitrogen content for each fermentation reaction, varying levels of residual digestive enzymes and enzyme fragments in each sample occurred because of differences in the total digestibility of the protein isolates. However, because >79% of the nitrogen (84% of the sample weight) came from the protein isolates, and because the proportion of the sample contributed by the digestive enzymes was similar to a previous study using whole foods,[55] we considered that the effects of the residual digestive enzymes was small and that the differences among samples were primarily due to the protein isolates. Now that we have shown the differential effects of digested and dialyzed protein isolates on the gut microbiota, future studies could examine the effect of varying the total nitrogen concentration based on the protein digestibility.

Another limitation is that the digested protein isolates were not 100% protein and may have contained trace quantities of compounds from the source material from which they were extracted. Commercial protein isolates, as employed in this study, are known to contain trace quantities of nonprotein material, such as CHOs, lipids, phenolic compounds, and minerals.[56–59] These trace components may have had an influence on the fermentation properties of the protein isolates. However, we expect that this influence was minor due to the high production of protein-fermentation metabolites, especially under low CHO conditions, and because of the significant correlations between many amino acids and their fermentation metabolites.

In conclusion, the in vitro fermentation of protein isolates under low CHO conditions showed greater protein metabolism than under high CHO conditions. The protein isolates were surprisingly butyrogenic in low CHO medium, particularly milk proteins and to a lesser extent soy and beef proteins. However, fermentation of these protein isolates was also accompanied by elevated concentrations of harmful metabolites such as ammonia and cadaverine. Amino acid composition had strong associations with microbiota composition and the production of protein-specific metabolites, especially under low CHO conditions when the microbiota was primarily proteolytic. Overall, the information presented herein can be used to better understand how the microbiome utilizes unabsorbed dietary proteins that likely have a strong influence on the microbiome under the typical low dietary fiber intakes of many consumers.

4. Experimental Section

Protein Isolates: Six commercially available protein isolates were sourced: beef (Prinova, Hanover Park, IL, USA), bovine milk (Idaho Milk Products, Jerome, ID, USA), EW (Henningsen Foods, Omaha, NE, USA), pea (Naked Nutrition, Rochester, NY, USA), and two soy protein isolates (BulkSupplements.com, Henderson, NV, USA and Bob's Red Mill, Milwaukie, OR, USA). Two soy protein isolates were included because other plant protein products obtained contained protein concentrations that were much lower than the animal protein isolates. For example, it sourced faba beans (Artesa Ingredients, Henrico, VA, USA), chickpeas (Artesa Ingredients, Henrico, VA, USA), and lentils (Artesa Ingredients, Henrico, VA, USA), but the protein concentrations of these products were 58.2% ± 0.2%, 56.9% ± 0.2%, and 53.1% ± 0.1%, respectively (measured by combustion as described later). This was substantially reduced compared to the animal protein isolates and the pea and soy protein isolates, which had protein concentrations >80%. In figures and tables, the selected protein isolates are referred to as beef, MPI (milk), EW, pea, soy1, and soy2, respectively.

In vitro Digestion and Dialysis of Protein Isolates: In vitro digestion was performed as described[60] with slight modifications. Specifically, protein isolates containing 8.5 g of protein (1.36 g N) were dispersed in 85 mL water. To the protein slurry, 6.8 mL of simulated salivary fluid was added and mixed for 5 min. Then, 13.6 mL of simulated gastric fluid and 50 µL of 0.3 M calcium chloride were added. The pH was adjusted to 3 with 1 M HCl, followed by the addition of 0.85 mL of pepsin (P7000, Sigma, St Louis, MO, USA) solution (containing 2000 U pepsin activity per mL, i.e., 1700 U pepsin activity per sample) prepared in simulated gastric fluid. The mixture was incubated with shaking at 200 rpm at 37 °C for 2 h. Next, 18.7 mL of simulated intestinal fluid and 68 µL of calcium chloride were added. The pH was adjusted to 7 with 1 M NaOH before the addition of 8.5 mL of pancreatin (P7545, Sigma, St Louis, MO, USA) solution (containing 100 U trypsin activity per mL or 8500 U trypsin activity per sample) prepared in simulated intestinal fluid. This mixture was incubated with shaking at 200 rpm at 37 °C for 2 h. After digestion, the mixture was transferred to dialysis tubing (1000 Da molecular weight cut-off, Fischer Scientific, Hampton, NH, USA) and dialyzed against distilled water at 4 °C for 3 days with a water change at least every 12 h. While di-and tri-peptides primarily undergo intestinal absorption facilitated by transporters, certain studies propose that peptides extending up to eight amino acids in length (approximately 880 Da) may be absorbed through transcytosis or passive diffusion via tight junctions;[61–65] thus, a 100 Da molecualr weight cut off was considered to be physiologically relevant. Finally, the dialysis retentate was freeze-dried (FreeZone Tray Dryer, Labconco, Kansas City, MO, USA) and stored at −80 °C until analysis.

As explained below, the digested and dialyzed protein samples were used for in vitro fermentation experiments where the total nitrogen was standardized across all protein isolates. This is standard practice in in vitro fermentation experiments.[54] This was also done to demonstrate the effects of protein composition on microbiome composition and function without the confounding effects of differences in digestibility (i.e., the quantity of protein added to the fermentation reaction). However, some residual digestive enzymes may remain after digestion and dialysis, and therefore the nitrogen content in each digested and dialyzed sample contributed by residual digestive enzymes may have differed among protein isolates because of differences in total digestibility. Therefore, the weight and nitrogen yield after digestion and dialysis were analyzed for MPI, EW, and soy1 samples and compared them with a blank containing only the buffers and enzymes added during digestion. The digestive enzymes contributed (mean ± standard deviation) 2.62% ± 0.26%, 15.2 %± 0.5%, and 5.13% ± 0.05% of the digested, dialyzed, and freeze-dried sample weight for EW, MPI, and soy1, respectively. On a nitrogen basis, the digestive enzymes contributed 2.58% ± 0.26%, 20.3% ± 0.6%, and 6.09% ± 0.09% of the total nitrogen in the digested, dialyzed, and freeze-dried samples, respectively. This was similar to the residual digestive enzyme concentrations in a previous study using whole foods (i.e., 7.14%–8.39% of the digested weight or 7.99%–27.63% of the nitrogen).[55] Thus, it was considered that, although there was some variability in the quantity of digestive enzymes among samples, the vast majority (i.e., >79.73%) of the nitrogen in the sample came from the digested protein isolates and could be used for in vitro fermentation experiments.

Compositional Analysis of Protein Isolates Before and After In Vitro Digestion: Total nitrogen concentration was measured with a nitrogen analyzer (FP 528, LECO, St Joseph, MI, USA) and converted to protein concentration (%) with the universal conversion factor of 6.25.[66] The nitrogen measurement was determined both before and after in vitro enzymatic digestion and dialysis. Nitrogen was also determined in peptone (Fisher Scientific, Waltham, MA), which was used for calculation of samples weights in the in vitro fermentation experiment (see Section 4.4).

Amino acid composition of the undigested and digested protein isolates was performed by the Proteomics & Metabolomics Facility at the University of Nebraska–Lincoln. Samples were hydrolyzed for 24 h using 0.5% phenol/6 N HCl and derivatized as described.[67] Briefly, the hydrolyzed samples were dried and resuspended with 1 mL of 20 mM HCl. The reconstituted amino acids and hydrolysate standard amino acid mixtures were derivatized with AccQ-Tag derivatization kit (186003836, Waters, Milford, MA, USA). Next, amino acids were separated, detected, and quantified using UPLC (1290 Agilent Infinity II) with a C18 column (ACCQ-TAG Ultra C18 1.7 µM, 2.1 × 100 mm). An external standard curve was run with known concentrations of Ala, Arg, aspartate (Asp), cysteate (Cya), the derivative of Cys, Glu, Gly, His, Ile, Leu, Lys, methionine sulfone ($MetSO_2$), the derivative of Met, phenylalanine (Phe), Pro, Ser, Thr, Tyr, and Val. During the hydrolysis/derivatization process, the asparagine (Asn) and Gln were converted to Asp and Glu and were therefore quantified together and abbreviated Asx and Glx, respectively. Tryptophan was destroyed during this process and was not reported. Metabolite concentrations were reported as mol%. The amino acids were classified as very hydrophobic, hydrophobic, neutral, and hydrophilic as described.[68]

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was completed with undigested and digested protein isolates using a 4%–20% gradient gel (4561096, Bio-Rad, Hercules, CA, USA). Protein isolate stock solutions of 1 mg mL^{-1} were prepared in double distilled water. The stock solution was further diluted to 30 µg with 2X Laemmli loading buffer (1610737, Bio-Rad, Hercules, CA, USA). The solutions were loaded into the gradient gel for electrophoresis and stained with Coomassie R250. After destaining, the gel was scanned with an infrared imaging system, Odyssey CLX Imaging System (Li-Cor Biosciences, Lincoln, NE, USA) and molecular weights were determined using Precision Plus Protein Standards (1610374, Bio-Rad, Hercules, CA, USA).

The molecular weight profile of undigested and digested protein isolates was measured using size exclusion chromatography. Protein isolate stock solutions of 1 mg mL^{-1} were prepared in phosphate buffer (50 mM sodium phosphate, 150 mM sodium chloride, pH 7.2) and further diluted to 0.5 mg of protein per mL with buffer. Five hundred microliters of solution were injected into a protein purification liquid chromatography (ÄKTA pure, Cytiva, Marlborough, MA, USA). For nondigested samples, a Superdex peptide 200 10/300GL column (10 mm × 300–310 mm, GE Healthcare Sweden) was used with a detection wavelength of 280 nm for measuring the absorbance of proteins. For digested samples, a Superdex peptide 30 10/300GL column (10 mm × 300–310 mm, GE Healthcare Sweden) with a 214 nm wavelength was used for measuring the absorbance of peptides. The column was equilibrated with phosphate buffer at 0.8 mL min^{-1} for two column volumes and samples were eluted at 0.5 mL min^{-1} for two column volumes. The molecular weight distribution of samples was determined by a standard curve with compounds of varying molecular weights for proteins (13 700–670 000 Da) and digested peptides (132–6511 Da). For both standard curves, the *x*-axis values (molecular weight) were log-transformed, and the *y*-axis values (elution volume) were not transformed.

In Vitro Fermentation of Digested Protein Isolates: In vitro fermentation was performed as described[69] with slight modifications. First, stool samples were collected from four healthy stool donors with no history of gastrointestinal diseases, no prebiotic or probiotic use, and no antibiotic use within the last 6 months. All procedures involving human subjects were approved by the Institutional Review Board of the University of Nebraska–Lincoln before initiating the study (approval number 20210621206EP). All

subjects provided written informed consent before participating in any study protocols.

Ten grams of fresh fecal sample was stomach mixed (Bag Mixer 400 CC, Interscience, Saint Nom la Breteche, France) with a phosphate-buffered saline with glycerol as a cryoprotectant (8 g L^{-1} sodium chloride, 0.2 g L^{-1} potassium chloride, 1.44 g L^{-1} disodium phosphate, 0.24 g L^{-1} monopotassium phosphate, 100 mL L^{-1} glycerol, pH 7.3) in ratio 1:9 w/v for 4 min. The slurry was filtered with a filtra bag (Filtra-Bag, Thomas Scientific, NJ, USA), divided into 15 mL aliquots in an anaerobic chamber (Bactron X, Sheldon manufacturing, Cornelius, OR, USA, containing 5% H_2, 5% CO_2, and 90% N_2), and stored at −80 °C until further use.

Next, fermentation media was prepared with the peptone removed (to be replaced with digested protein isolates; see Section 4.5) and varying concentrations of CHO to simulate high CHO and low CHO conditions. The medium contained (per L): yeast extract (2 g, Fisher Scientific, Waltham, MA), bile salts (0.5 g, Oxoid, Cheshire, England), sodium bicarbonate (2 g), sodium chloride (0.1 g), dipotassium phosphate (0.08 g), magnesium sulfate heptahydrate (0.01 g), calcium chloride dihydrate (0.01 g), L-Cys hydrochloride (0.5 g, Fisher Scientific, Waltham, MA), hemin (1 mL, 5 mg mL^{-1} dissolved in DMSO), Tween 80 (2 mL, Fisher Scientific, Waltham, MA), vitamin K solution (10 µL dissolved in ethanol, Alfa Aesar, Haverhill, MA), and 0.025% w/v resazurin solution (4 mL dissolved in water, Alfa Aesar, Haverhill, MA). A mixture of soluble CHOs were added to the media to simulate the range of starch and dietary fibers that might enter the colon and be present in a typical diet: soluble starch (S9765, Sigma, St Louis, MO, USA), inulin (Orafti HP, BENEO, Mannheim, Germany), arabinogalactan (A1328, TCI, Tokyo, Japan), xylan (X0064, TCI, Tokyo, Japan), and pectin (J6102, Alfa Aesar, Haverhill, MA).[70,71] The CHO were added at equal concentrations of 0.2 and 2 g L^{-1} for total CHO concentrations of 0.1% and 1% in the low CHO and high CHO media, respectively. The volume was adjusted to 1 L and the pH to 6.8 before autoclaving for sterilization.

The in vitro fermentation was completed in 20 mL glass vials (SU860030, Supleco, Bellefonte, PA, USA) with 18 mm screw caps (SU860101, Supleco, Bellefonte, PA, USA) inside an anaerobic chamber (Bactron X, Sheldon) equipped with an incubator shaker. Protein isolates, freeze-dried after in vitro digestion and dialysis, were added at varying weights equivalent to 16 mg total nitrogen (sample weights ranged from 99.5 to 158 mg as described in Section 4.5). These samples were suspended in 9 mL fermentation media. A blank sample was prepared with no digested protein isolate. Vials were inoculated with 1 mL of fecal slurry and incubated at 37 °C in the anaerobic chamber. Three mL samples were collected at 0, 8, and 24 h, with the 0 h being taken immediately after inoculation. All samples were stored at −80 °C until further analysis.

Dosage Information: About 12–18 g of undigested protein (1.9–2.9 g N) is estimated to enter the large intestine for fermentation by gut bacteria per day.[72] Given that the volume of the large intestine ranges from 0.5 to 3.0 L,[73] this translates into 4–36 g protein L^{-1} (0.63–5.8 g N/L).

In in vitro fermentation experiments, protein (nitrogen) is typically derived from peptone. In vitro fermentation media typically call for 2–15 g peptone per liter.[54] Given that peptone contains 0.16 g N/g (measured by combustion as described in Section 4.1), this translates into 0.32–2.4 g N/L in fermentation media. This is comparable to the physiological concentrations of nitrogen in the large intestine noted previously.

In the present study, the peptone was removed from the fermentation medium formulation and replaced it with the digested protein isolates. The nitrogen concentration was standardized across all digested protein isolates at 1.6 g N/L as a moderate, realistic level based on the concentration ranges in the large intestine (0.63–5.8 g N/L)[72,73] and the ranges typically used in in vitro fermentation studies (0.32–2.4 g N/L).[54] This was equivalent to 16 mg N, derived from the digested protein isolates, in each 10 mL fermentation vessel, and translated into samples weights of 115 mg (beef), 99.5 mg (EW), 128 mg (MPI), 158 mg (pea), 108 mg (soy1), and 117 mg (soy2).

Microbiota Composition: DNA extraction was completed with the BioSprint 96 One-For-All Vet Kit (SP947057, Qiagen, Germantown, MD, USA). One mL aliquots of the fermentation slurries were centrifuged, and the pellets were resuspended in 400 µL of warmed ASL stool lysis buffer (19082, Qiagen, Germantown, MD, USA) following the manufacturer's protocol. Extracted DNA samples were subjected to amplicon sequencing of the V4 region of the 16S rRNA gene on the Illumina MiSeq platform using the MiSeq reagent kit v2 (2 × 250 bp).[74] Prior to sequence analysis using the QIIME 2 platform, sequences were demultiplexed and barcodes were removed.[75] Next, DADA2 was used to perform sequence quality control, trimming, chimera removal, and denoising.[76] To maintain sequence qualities above a Phred score of 30, forward and reverse reads were truncated to 245 and 160 bp, respectively. With DADA2, sequences were dereplicated into 100% amplicon sequence variants (ASVs) for exact sequence matching and taxonomy was assigned using the SILVA database.[77] Data were rarefied to 5022 reads per sample, providing >99.5% coverage, using the phyloseq package[78] in R[79] using R Studio (Build 353). α-Diversity (Shannon's index) and β-diversity (Bray–Curtis distance) were calculated on the rarefied data also using the phyloseq package.[78]

Chemical Composition of Fermented Samples: The concentration of peptides in samples during fermentation was measured by Pierce Quantitative Fluorometric Peptide Assay kit (23290, Fisher Scientific, Waltham, MA, USA). The supernatant from the fermentation slurry after the centrifugation during DNA extraction was diluted 50-fold with distilled water. Next, 10 µL of each diluted sample was transferred into a 96-well fluorometric compatible plate (88378, Fisher Scientific, Waltham, MA, USA). The manufacturer's instructions were followed, and peptide concentration of each fermented sample was determined based on a calibration (7.8–1000 µg mL^{-1}) with the addition of a blank.

SCFA and BCFA were extracted from the supernatant of the fermentation slurry and measured by gas chromatography as described.[80] First, 0.4 mL of fermentation supernatant, 0.1 mL of 7 mM 2-ethylbutyric acid in 2 M potassium hydroxide, 0.2 mL of 9 M sulfuric acid, and 0.16 g of sodium chloride were mixed together. Next, 0.5 mL of diethyl ether was added and vortexed for 30 s. The diethyl ether layer was injected into the gas chromatography (Clarus 580, PerkinElmer, MA USA) equipped with a capillary column (Elite-FFAP, 15 m × 0.25 mm inner diameter × 0.25 µm film thickness, PerkinElmer, Waltham, MA, USA) and the SCFAs were detected by a flame ionization detector at 240 °C. SCFAs were quantified by calculating response factors for each SCFA relative to the 2-ethylbutyrate internal standard.

Ammonia in fermentation samples was determined according to the phenol-hypochlorite method.[81] One mL of fermentation supernatant was diluted 100-fold with distilled water and the ammonia concentrations were determined relative to a standard curve with ammonium chloride standards (1–10 mg L^{-1}).

Targeted one-dimensional (1D) ^{1}H NMR metabolomics was performed for the quantification of other important metabolites arising from protein fermentation.[8,18,19] Standard curves were generated for cadaverine, indole-3-acetate, 2-methylbutyrate, *p*-cresol, and *p*-hydroxyphenylacetate; however, only cadaverine and *p*-hydroxyphenylacetate were detected in fermentation samples. Therefore, only data from these metabolites is presented. Preparation of fecal slurry samples was performed as described[82] with slight modifications. Four hundred and fifty microliters of fermentation slurry supernatant was evaporated to dryness using a centrifugal concentrator (SpeedVac, Fisher Scientific, Waltham, MA, USA), followed by consecutive pellet washes of 500 µL of 100% methanol, 50% methanol, and water. The three washes were combined for a total volume of 1.5 mL and evaporated to dryness overnight. Samples were reconstituted in 550 µL of 50 mM phosphate buffer (pH 7.2) prepared in "100%" D_2O spiked with 50 µM 3-(trimethylsilyl) propionic-2,2,3,3-d_4 acid sodium salt (TMSP-d_4) as an internal chemical shift reference and concentration standard. The 1D ^{1}H NMR spectra were acquired using Topspin version 3.5 on a Bruker AVANCE III-HD 700 MHz spectrometer equipped with a 5 mm quadruple resonance QCIP cryoprobe (^{1}H, ^{13}C, ^{15}N, and ^{31}P) with *z*-axis gradients. A SampleJet automated sample changer system, an automatic tune and match system (ATM), and ICON-NMR software was used to automate the NMR data collection. The 1D ^{1}H NMR spectra was collected with an excitation sculpting pulse sequence for solvent suppres-

sion. The 1D ^{1}H NMR spectra was collected at 298 K with a spectral width of 9803 Hz, 64K data points, 128 scans, 4 dummy scans, and relaxation delay of 1 s. Spectra were Fourier transformed, automatically phased, and baseline corrected. Chemical shifts and metabolite quantification were referenced to the TMSP-d_4 peak at $\delta = 0.00$ ppm. Data were serially processed in TopSpin (v 4.2.0) to extract integral values for peaks of interest. Metabolite concentrations were determined by a standard curve with the corresponding metabolite standard prepared in fermentation media (125–1000 μM).

Data Analysis: All data were analyzed using R (version 4.1.3) and RStudio (Build 353),[79] except for linear discriminate analysis (LDA) effect size (LEfSe) was performed using the online Galaxy module.[83] For protein concentration and amino acid composition of protein isolates before and after digestion, a one-way ANOVA followed by Tukey's honestly significant difference (HSD) test was performed to determine significant differences among proteins. For peptide concentrations and α-diversity during fermentation, three factor ANOVAs were performed by medium where the factors in the model were subject, protein, and time, followed by Tukey's HSD test to determine significant differences among proteins by time, across times by protein within each media type, and across media types by protein and time. The same approach was used to determine significant differences among proteins for the microbial metabolites, except time was not a factor in the model since only 24 h concentrations were analyzed. ANOVA and Tukey's HSD test were performed using base functions in R, and superscript letters corresponding to significant differences from Tukey's HSD test were generated using the multcompView package.[84]

For β-diversity, the Bray–Curtis distance matrix was analyzed using principal coordinates analysis using the phyloseq package.[78] PERMANOVA was used to determine significant differences among microbiomes, media, proteins, and time on the Bray–Curtis distance matrix, calculated using the vegan package.[85] The melt function in reshape2 was used on the Bray–Curtis distance matrix to determine the Bray–Curtis distance from the fecal samples for all fermented samples.[86] Variance calculations by ASV or metabolite across proteins by medium and time were performed using base functions in R and Levene's test for homogeneity of variance was performed using the rstatix package.[87]

LEfSe analysis was used to identify differentially abundant features of the microbiome after 24 h of in vitro fermentation under low CHO and high CHO conditions. Protein isolate was the class variable, and microbiome was the subject variable. Statistical significance was defined as an LDA effect size >2 and $p < 0.05$. All taxonomic ranks (phylum to ASV) were analyzed.

To determine correlations with amino acid concentrations in digested protein isolates with microbiota composition and metabolite production after 24 h of fermentation, partial correlation coefficients were calculated where the partial variable was microbiome. Spearman partial correlation coefficients were calculated for the microbiota data and Pearson partial correlation coefficients were calculated for the metabolite concentrations. All p-values for the correlations were adjusted using the Benjamini–Hochberg procedure in the rstatix package. Nonsignificant correlations (adjusted $p > 0.05$) were adjusted to zero before visualizing in a clustered heatmap. All plots were generated using the ggplot2, ggpubr, lemon, cowplot, magick, and ComplexHeatmap packages in R.[88–93]

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

Conceptualization: M.B., D.C., R.P., and D.R.; methodology: M.B., D.C., Q.Y., W.D., M.M., K.M., R.P., and D.R.; formal analysis: M.B., H.M., and D.R.; investigation: M.B., D.C., Q.Y., W.D., and M.M.; resources: M.M., K.M., R.P., and D.R.; writing—original draft preparation: M.B. and D.R.; writing—review and editing: M.B., D.C., Q.Y., W.D., M.M., K.M., R.P., and D.R.; visualization: M.B., H.M., and D.R.; supervision: R.P. and D.R.; project administration: D.R.; funding acquisition: R.P. and D.R. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement

Raw data are included within the supplementary files of this manuscript (Supplementary Data.xlsx). Raw sequence reads from 16S rRNA gene sequencing are available in the Sequence Read Archive under PRJNA1011328 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1011328). Additional inquiries or clarification should be directed to the corresponding author.

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[1] USDA, Agricultural Research Service, **2021**. Usual Nutrient Intake from Food and Beverages, by Gender and Age, What We Eat in America, NHANES 2015-2018 Available https://www.ars.usda.gov/nea/bhnrc/fsrg.
[2] B. P. Ismail, L. Senaratne-Lenagala, A. Stube, A. Brackenridge, *Anim. Front.* **2020**, *10*, 53.
[3] Grand View Research, Share & Trends Report, 2030, **2021**, https://www.grandviewresearch.com/industry-analysis/protein-supplements-market (accessed: May 2024).
[4] R. Clemens, S. Kranz, A. R. Mobley, T. A. Nicklas, M. P. Raimondi, J. C. Rodriguez, J. L. Slavin, H. Warshaw, *J. Nutr.* **2012**, *142*, 1390S.
[5] A. R. Mobley, J. M. Jones, J. Rodriguez, J. Slavin, K. M. Zelman, *Nutrients* **2014**, *6*, 2540.
[6] J. H. Cummings, G. T. Macfarlane, *J. Appl. Bacteriol.* **1991**, *70*, 443.
[7] K. Korpela, *Annu. Rev. Food Sci. Technol.* **2018**, *9*, 65.
[8] K. Oliphant, E. Allen-Vercoe, *Microbiome* **2019**, *7*, 91.
[9] C. K. Yao, J. G. Muir, P. R. Gibson, *Aliment. Pharmacol. Ther.* **2016**, *43*, 181.
[10] J. H. Cummings, M. J. Hill, E. S. Bone, W. J. Branch, D. J. Jenkins, *Am. J. Clin. Nutr.* **1979**, *32*, 2094.
[11] B. Geypens, D. Claus, P. Evenepoel, M. Hiele, B. Maes, M. Peeters, P. Rutgeerts, Y. Ghoos, *Gut* **1997**, *41*, 70.
[12] W. R. Russell, S. W. Gratz, S. H. Duncan, G. Holtrop, J. Ince, L. Scobbie, G. Duncan, A. M. Johnstone, G. E. Lobley, R. J. Wallace, G. G. Duthie, H. J. Flint, *Am. J. Clin. Nutr.* **2011**, *93*, 1062.
[13] L. A. David, C. F. Maurice, R. N. Carmody, D. B. Gootenberg, J. E. Button, B. E. Wolfe, A. V. Ling, A. S. Devlin, Y. Varma, M. A. Fischbach, S. B. Biddinger, R. J. Dutton, P. J. Turnbaugh, *Nature* **2013**, *505*, 559.

[14] W. R. Russell, L. Hoyles, H. J. Flint, M. E. Dumas, *Curr. Opin. Microbiol.* **2013**, *16*, 246.
[15] M. W. Bourassa, I. Alim, S. J. Bultman, R. R. Ratan, *Neurosci. Lett.* **2016**, *625*, 56.
[16] Z. Gao, J. Yin, J. Zhang, R. E. Ward, R. J. Martin, M. Lefevre, W. T. Cefalu, J. Ye, *Diabetes* **2009**, *58*, 1509.
[17] G. T. Macfarlane, S. Macfarlane, *J. AOAC Int.* **2012**, *95*, 50.
[18] N. E. Diether, B. P. Willing, *Microorganisms* **2019**, *7*, 19.
[19] K. Windey, V. de Preter, K. Verbeke, *Mol. Nutr. Food Res.* **2012**, *56*, 184.
[20] A. Birkett, J. Muir, J. Phillips, G. Jones, K. O'Dea, *Am. J. Clin. Nutr.* **1996**, *63*, 766.
[21] J. G. Muir, E. G. W. Yeow, J. Keogh, C. Pizzey, A. R. Bird, K. Sharpe, K. O'Dea, F. A. Macrae, *Am. J. Clin. Nutr.* **2004**, *79*, 1020.
[22] E. A. Smith, G. T. Macfarlane, *FEMS Microbiol. Ecol.* **1998**, *25*, 355.
[23] M. Aguirre, A. Eck, M. E. Koenen, P. H. M. Savelkoul, A. E. Budding, K. Venema, *Res. Microbiol.* **2016**, *167*, 114.
[24] Z. Chen, D. Radjabzadeh, L. Chen, A. Kurilshikov, M. Kavousi, F. Ahmadizar, M. A. Ikram, A. G. Uitterlinden, A. Zhernakova, J. Fu, R. Kraaij, T. Voortman, *JAMA Netw. Open* **2021**, *4*, e2118811.
[25] W. Zhao, Y. Huang, N. Cui, R. Wang, Z. Xiao, X. Su, *Front. Microbiol.* **2023**, *14*, 458.
[26] Y. S. Liu, S. Li, X. F. Wang, T. Xing, J. L. Li, X. D. Zhu, L. Zhang, F. Gao, *Poult. Sci.* **2021**, *100*, 273.
[27] A. Atzeni, T. F. S. Bastiaanssen, J. F. Cryan, F. J. Tinahones, J. Vioque, D. Corella, M. Fitó, J. Vidal, I. Moreno-Indias, A. M. Gómez-Pérez, L. Torres-Collado, O. Coltell, O. Castañer, M. Bulló, J. Salas-Salvadó, *Front. Endocrinol. (Lausanne)* **2022**, *13*, 662.
[28] K.-H. Schleifer, in *Bergey's Manual of Systematic Bacteriology* (Eds: P. De Vos, G. M. Garrity, D. Jones, N. R. Krieg, W. Ludwig, F. A. Rainey, K.-H. Schleifer, W.B. Whitman), Springer, New York **2009**, pp. 19–1317.
[29] A. Biddle, L. Stewart, J. Blanchard, S. Leschine, *Diversity* **2013**, *5*, 627.
[30] M. Vital, A. C. Howe, J. M. Tiedje, *mBio* **2014**, *5*, e00889.
[31] P. Louis, H. J. Flint, *Environ. Microbiol.* **2016**, *19*, 29.
[32] W. R. Russell, S. H. Duncan, L. Scobbie, G. Duncan, L. Cantlay, A. G. Calder, S. E. Anderson, H. J. Flint, *Mol. Nutr. Food Res.* **2013**, *57*, 523.
[33] K. Windey, V. de Preter, K. Verbeke, *Mol. Nutr. Food Res.* **2012**, *56*, 184.
[34] A. Scalbert, C. Morand, C. Manach, C. Rémésy, *Biomed. Pharmacother.* **2002**, *56*, 276.
[35] W. Lin, W. Wang, H. Yang, D. Wang, W. Ling, *J. Food Sci.* **2016**, *81*, H3026.
[36] Y. T. Loo, K. Howell, M. Chan, P. Zhang, K. Ng, *Compr. Rev. Food Sci. Food Saf.* **2020**, *19*, 1268.
[37] I. Dandachi, H. Anani, L. Hadjadj, S. Brahimi, J. C. Lagier, Z. Daoud, J. M. Rolain, *New Microbes New Infect.* **2021**, *41*, 100863.
[38] T. Takada, K. Watanabe, H. Makino, A. Kushiro, *Int. J. Syst. Evol. Microbiol.* **2016**, *66*, 4125.
[39] I. R. Rowland, C. J. Rumney, J. T. Coutts, L. C. Lievense, *Carcinogenesis* **1998**, *19*, 281.
[40] N. E. Diether, B. P. Willing, *Microorganisms* **2019**, *7*, 19.
[41] H. P. Til, H. E. Falke, M. K. Prinsen, M. I. Willems, *Food Chem. Toxicol.* **1997**, *35*, 337.
[42] A. R. Shalaby, *Food Res. Int.* **1996**, *29*, 675.
[43] B. del Rio, B. Redruello, D. M. Linares, V. Ladero, P. Ruas-Madiedo, M. Fernandez, M. C. Martin, M. A. Alvarez, *Sci. Rep.* **2019**, *9*, 120.
[44] W. Buckel, H. A. Barker, *J. Bacteriol.* **1974**, *117*, 1248.
[45] S. Perna, T. A. Alalwan, Z. Alaali, T. Alnashaba, C. Gasparri, V. Infantino, A. Riva, G. Petrangolini, P. Allegrini, M. Rondanelli, *Int. J. Mol. Sci.* **2019**, *20*, 5232.
[46] A. M. Davila, F. Blachier, M. Gotteland, M. Andriamihaja, P. H. Benetti, Y. Sanz, D. Tomé, *Pharmacol. Res.* **2013**, *68*, 95.
[47] E. P. J. G. Neis, C. H. C. Dejong, S. S. Rensen, *Nutrients* **2015**, *7*, 2930.
[48] C. A. Lopez, T. P. McNeely, K. Nurmakova, W. N. Beavers, E. P. Skaar, *Anaerobe* **2020**, *63*, 102210.
[49] Y. Momose, K. Hirayama, K. Itoh, *Int. J. Gen. Mol. Microbiol.* **2008**, *94*, 165.
[50] G. Song, Q. Gan, W. Qi, Y. Wang, M. Xu, Y. Li, *Nutrients* **2023**, *15*, 782.
[51] V. Van Craeyveld, K. Swennen, E. Dornez, T. Van De Wiele, M. Marzorati, W. Verstraete, Y. Delaedt, O. Onagbesan, E. Decuypere, J. Buyse, B. De Ketelaere, W. F. Broekaert, J. A. Delcour, C. M. Courtin, *J. Nutr.* **2008**, *138*, 2348.
[52] G. Falony, K. Lazidou, A. Verschaeren, S. Weckx, D. Maes, L. De Vuyst, *Appl. Environ. Microbiol.* **2009**, *75*, 454.
[53] J. R. Walker, E. Altman, *Appl. Environ. Microbiol.* **2005**, *71*, 1850.
[54] S. Pérez-Burillo, S. Molino, B. Navajas-Porras, Á. J. Valverde-Moya, D. Hinojosa-Nogueira, A. López-Maldonado, S. Pastoriza, J. Á. Rufián-Henares, *Nat. Protoc.* **2021**, *16*, 3186.
[55] W. Ding, S. Liu, Q. Yang, Y. Bai, C. E. Perez-Donado, D. J. Rose, *Food Res. Int.* **2024**, *178*, 113953.
[56] C. Chen, Y. J. Chi, W. Xu, *Food Bioprocess Technol.* **2012**, *5*, 2342.
[57] A. Cosson, E. Meudec, C. Ginies, A. Danel, P. Lieben, N. Descamps, V. Cheynier, A. Saint-Eve, I. Souchon, *Food Chem.* **2022**, *385*, 132615.
[58] C. Wang, Q. Ma, S. Pagadala, M. S. Sherrard, P. G. Krishnan, *J. Am. Oil Chem. Soc.* **1998**, *75*, 337.
[59] P. Salunke, A. Syamala, L. E. Metzger, *Dairy* **2023**, *4*, 462.
[60] A. Brodkorb, L. Egger, M. Alminger, P. Alvito, R. Assunção, S. Ballance, T. Bohn, C. Bourlieu-Lacanal, R. Boutrou, F. Carrière, A. Clemente, M. Corredig, D. Dupont, C. Dufour, C. Edwards, M. Golding, S. Karakaya, B. Kirkhus, S. Le Feunteun, U. Lesmes, A. Macierzanka, A. R. Mackie, C. Martins, S. Marze, D. J. McClements, O. Ménard, M. Minekus, R. Portmann, C. N. Santos, I. Souchon, *Nat. Protoc.* **2019**, *14*, 991.
[61] L. Amigo, B. Hernández-Ledesma, *Molecules* **2020**, *25*, 4479.
[62] P. Tyagi, S. Pechenov, J. Anand Subramony, *J. Control. Release* **2018**, *287*, 167.
[63] T. Suzuki, *Anim. Sci. J.* **2020**, *91*, e13357.
[64] H. J. Freeman, *World J. Gastrointest. Pharmacol. Ther.* **2015**, *6*, 22.
[65] B. Spanier, F. Rohm, *Compr. Physiol.* **2018**, *8*, 843.
[66] D. B. Jones, in *Circ. Ser. 183*, US Department of Agriculture **1931**, pp. 1–21.
[67] M. K. Dhillon, S. Kumar, G. T. Gujar, *Indian J. Exp. Biol.* **2014**, *52*, 73.
[68] O. D. Monera, T. J. Sereda, N. E. Zhou, C. M. Kay, R. S. Hodges, *J. Pept. Sci.* **1995**, *1*, 319.
[69] J. Yang, I. Martínez, J. Walter, A. Keshavarzian, D. J. Rose, *Anaerobe* **2013**, *23*, 74.
[70] M. Aguirre, A. Eck, M. E. Koenen, P. H. M. Savelkoul, *Res. Microbiol.* **2016**, *167*, 114.
[71] P. Van Den Abbeele, K. Venema, T. Van De Wiele, W. Verstraete, S. Possemiers, *J. Agric. Food Chem.* **2013**, *61*, 9819.
[72] J. H. Cummings, G. T. MacFarlane, *Clin. Nutr.* **1997**, *16*, 3.
[73] O. Olsson, *Acta Radiol.* **1952**, *37*, 348.
[74] J. J. Kozich, S. L. Westcott, N. T. Baxter, S. K. Highlander, P. D. Schloss, *Appl. Environ. Microbiol.* **2013**, *79*, 5112.
[75] E. Bolyen, J. R. Rideout, M. R. Dillon, N. A. Bokulich, C. C. Abnet, G. A. Al-Ghalith, H. Alexander, E. J. Alm, M. Arumugam, F. Asnicar, Y. Bai, J. E. Bisanz, K. Bittinger, A. Brejnrod, C. J. Brislawn, C. T. Brown, B. J. Callahan, A. M. Caraballo-Rodríguez, J. Chase, E. K. Cope, R. Da Silva, C. Diener, P. C. Dorrestein, G. M. Douglas, D. M. Durall, C. Duvallet, C. F. Edwardson, M. Ernst, M. Estaki, J. Fouquier, et al., *Nat. Biotechnol.* **2019**, *37*, 852.
[76] B. J. Callahan, P. J. McMurdie, M. J. Rosen, A. W. Han, A. J. A. Johnson, S. P. Holmes, *Nat. Methods* **2016**, *13*, 581.
[77] C. Quast, E. Pruesse, P. Yilmaz, J. Gerken, T. Schweer, P. Yarza, J. Peplies, F. O. Glöckner, *Nucleic Acids Res.* **2013**, *41*, D590.
[78] P. J. McMurdie, S. Holmes, *PLoS ONE* **2013**, *8*, e61217.
[79] R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, **2023**. https://www.R-project.org/.

[80] A. L. Hartzell, M. X. Maldonado-Gomez, R. W. Hutkins, D. J. Rose, *Bioact. Carbohydr. Diet. Fibre* **2013**, *1*, 81.
[81] L. Solorzano, *Limnol. Oceanogr.* **1969**, *14*, 799.
[82] A. Boulaka, P. Christodoulou, M. Vlassopoulou, G. Koutrotsios, G. Bekiaris, G. I. Zervakis, E. K. Mitsou, G. Saxami, A. Kyriacou, M. Zervou, P. Georgiadis, V. Pletsa, *Molecules* **2020**, *25*, 3554.
[83] N. Segata, J. Izard, L. Waldron, D. Gevers, L. Miropolsky, W. S. Garrett, C. Huttenhower, *Genome Biol.* **2011**, *12*, R60.
[84] S. Graves, H.-P. Piepho, L. Selzer, S. Dorai-Raj, MultcompView: Visualizations of Paired Comparisons, **2023**, https://cran.r-project.org/package=multcompView.
[85] J. Oksanen, F. G. Blanchet, M. Friendly, R. Kindt, P. Legendre, D. McGlinn, P. R. Minchin, R. B. O'Hara, G. L. Simpson, P. Solymos, M. H. H. Stevens, E. Szoecs, H. Wagner, Vegan: Community EcologyPackage, **2022**, https://cran.r-project.org/package=vegan.
[86] H. Wickham, *ggplot2: Elegant Graphics for Data Analysis*, Springer-Verlag, New York **2016**.
[87] A. Kassambara, **2020**.
[88] J. Ooms, **2023**.
[89] S. Edwards, **2022**.
[90] A. Kassambara, **2023**.
[91] H. Wickham, *Ggplot2: Elegant Graphics for Data Analysis*, Springer-Verlag, New York **2016**.
[92] Z. Gu, R. Eils, M. Schlesner, *Bioinformatics* **2016**, *32*, 2847.
[93] C. O. Wilke, **2020**.