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Gut microbiome modulation by cricket, pea, and whey protein using the SHIME in vitro simulator

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Entomophagy is increasingly popular, and *Acheta domesticus* offers an ecologically sustainable protein alternative, but the effects on the human gut microbiome need further investigation. In this study, we investigated the impact of the intake of three isolated proteins: pea (plant), whey (animal), and cricket (insect) on gut microbiome of a single-donor using the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®). Cricket protein intake was associated with potential beneficial taxa such as *Bifidobacterium* and *Lactobacillus*, genes related to vitamin biosynthesis and bacteriocin transport, and short and medium-chain fatty acids. Pea protein intake was associated with *Faecalibacterium* and *Slackia*, while whey protein with *Butyricimonas* and *Lactobacillus*. Metagenomic analysis revealed that pea intake led to increased lysine degradation genes, promoting SCFAs production. Each protein has its own unique characteristics that may contribute positively to gut health. Specifically, cricket protein intake appears to have beneficial effects, promoting the growth of potentially beneficial taxa and enhancing short-chain fatty acid production. The results of this study indicate that cricket protein does not exhibit any detrimental effects compared to pea and whey proteins.

Background

Shift in dietary protein is required to meet the demographic growth as well as the new dietary guidelines that encourage populations to increase their protein intake[1]. Sustainability is also an important factor that must be taken into the account when a novel protein is chosen[2].

It is well known that microbiome acts as an intermediary between diet, host physiology and health[3,4] and the different aminoacidic composition of proteins can have a huge impact on the gastrointestinal microbiome[5]. Among various nutrients, proteins in general have garnered significant attention due to their function as primary substrates for short-chain fatty acids (SCFAs), branched-chain amino acids (BCAAs) and nitrogen that can impact host health by reducing disease risks[5,6]. Butyrate, acetate, and propionate are SCFAs crucial for human health as they modulate the immune system, regulate metabolic pathways, and restore the gut barrier. Numerous studies have shown that dietary interventions can enhance the population of SCFA-producing bacteria[7–9]. In vivo, the breakdown of proteins begins in upper part of the gastro-intestinal tract and peptides that are not digested or absorbed in the small intestine were metabolized by the colonic microbiome with the production of SCFAs, BCAAs, ammonia, amines, sulphur compounds, phenols, and indoles among others[5,10]. Animal and plant-based proteins have a substantial environmental impact due to high water consumption and greenhouse gas emissions during farming and post-processing[11,12]. Insect-based proteins are a sustainable alternative[4,11,12], offering several health benefits[11]. Entomophagy is a practice widely used in Asia, Africa, and Latin America were more than 2000 insect species are daily consumed. Edible insects are considered an excellent source of nutrients, such as fibers, vitamins and minerals, mono- and polyunsaturated fats, essential amino acids and biologically active peptides with anti-hyperglycaemia, anti-hypertension, anti-tumour, anti-inflammation, and anti-obesity effects[11,13,14].

At the same time, it is crucial to consider the potential risks associated with the consumption of edible insects, particularly due to the presence of

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Table 1 | Concentration of free amino acids (mg/kg) in whey, pea, and cricket protein powders before (crude proteins) and after in vitro gastrointestinal digestion

	Crude proteins			Digested proteins		
Amino acid	**Cricket**	**Pea**	**Whey**	**Cricket**	**Pea**	**Whey**
Alanine	1941.2 ± 105.6^{b}	9.7 ± 1.0^{a}	1.3 ± 0.1^{a}	1748.7 ± 30.8^{c}	374.4 ± 26.5^{b}	181.5 ± 8.4^{a}
Ammonia	34.0 ± 2.6^{c}	7.6 ± 0.3^{b}	2.2 ± 0.0^{a}	158.5 ± 8.2^{b}	339.8 ± 25.4^{c}	1.0 ± 0.1^{a}
Arginine	21.7 ± 0.8^{c}	2.5 ± 0.2^{b}	0.0 ± 0.0^{a}	386.4 ± 11.5^{b}	1215.2 ± 22.3^{c}	101.8 ± 6.1^{a}
Asparagine	240.0 ± 9.9^{b}	1.1 ± 0.0^{a}	5.4 ± 0.4^{a}	306.6 ± 21.6^{c}	118.4 ± 8.8^{b}	25.5 ± 2.5^{a}
Aspartic acid	283.5 ± 16.4^{b}	7.3 ± 0.1^{a}	1.6 ± 0.2^{a}	327.2 ± 9.8^{c}	73.0 ± 7.4^{b}	1.4 ± 0.1^{a}
Cysteine*	1398.2 ± 39.7^{b}	34.4 ± 4.2^{a}	0.0 ± 0.0^{a}	106.8 ± 5.3^{b}	296.5 ± 33.5^{c}	53.4 ± 6.9^{a}
γ-Aminobutyric acid	99.1 ± 7.0^{c}	18.4 ± 1.6^{b}	2.4 ± 0.1^{a}	1407.6 ± 40.4^{b}	2534.3 ± 19.3^{c}	204.8 ± 24.4^{a}
Glutamic acid	1511.6 ± 49.1^{b}	8.7 ± 0.9^{a}	1.7 ± 0.2^{a}	1226.7 ± 59.9^{b}	96.4 ± 11.6^{a}	11.1 ± 1.2^{a}
Glycine	1531.2 ± 150.5^{b}	8.8 ± 0.6^{a}	1.1 ± 0.1^{a}	2038.0 ± 28.8^{c}	317.8 ± 21.5^{b}	150.6 ± 15.3^{a}
Histidine*	624.8 ± 54.7^{b}	13.6 ± 0.9^{a}	1.2 ± 0.1^{a}	2936.7 ± 102.7^{b}	5394.3 ± 52.4^{c}	482.4 ± 14.7^{a}
Isoleucine	91.5 ± 6.1^{b}	2.6 ± 0.1^{a}	0.0 ± 0.0^{a}	882.7 ± 77.0^{b}	904.5 ± 43.5^{b}	50.3 ± 3.5^{a}
Leucine*	266.7 ± 10.7^{b}	7.8 ± 0.3^{a}	0.0 ± 0.0^{a}	960.7 ± 96.1^{b}	3440.1 ± 207.7^{c}	18.3 ± 1.8^{a}
Lysine*	59.3 ± 3.0^{c}	13.4 ± 0.2^{b}	0.0 ± 0.0^{a}	129.1 ± 4.6^{b}	438.4 ± 27.8^{c}	13.7 ± 1.5^{a}
Methionine*	13.5 ± 0.6^{b}	2.5 ± 0.2^{a}	13.9 ± 3.7^{b}	929.4 ± 20.7^{c}	511.5 ± 27.6^{b}	0.9 ± 0.1^{a}
Ornithine	1878.2 ± 153.9^{b}	501.7 ± 14.0^{a}	286.6 ± 20.4^{a}	2276.8 ± 66.2^{b}	2792.6 ± 22.9^{c}	215.4 ± 14.0^{a}
Phenylalanine*	581.8 ± 71.9^{b}	16.3 ± 1.7^{a}	0.0 ± 0.0^{a}	2630.0 ± 65.6^{b}	2858.9 ± 30.2^{c}	215.1 ± 9.6^{a}
Proline	1939.5 ± 123.7^{b}	4.6 ± 0.2^{a}	2.1 ± 0.1^{a}	5155.4 ± 537.5^{b}	8748.8 ± 640.6^{c}	233.3 ± 15.8^{a}
Serine	492.8 ± 9.1^{b}	2.6 ± 0.2^{a}	0.0 ± 0.0^{a}	469.4 ± 43.2^{b}	396.0 ± 33.5^{b}	15.7 ± 1.3^{a}
Threonine*	488.2 ± 34.6^{b}	5.2 ± 0.3^{a}	2.3 ± 0.3^{a}	575.4 ± 40.9^{c}	475.9 ± 8.0^{b}	17.4 ± 0.7^{a}
Tryptophan*	474.2 ± 48.8^{b}	5.5 ± 0.5^{a}	1.2 ± 0.0^{a}	573.0 ± 12.9^{b}	742.1 ± 7.4^{c}	31.6 ± 1.0^{a}
Tyrosine*	679.0 ± 45.2^{b}	61.7 ± 2.9^{a}	30.8 ± 2.1^{a}	3.5 ± 0.3^{a}	4355.1 ± 11.2^{c}	366.3 ± 27.4^{b}
Valine*	2911.0 ± 59.4^{b}	48.0 ± 2.9^{a}	1.9 ± 0.1^{a}	808.9 ± 38.7^{c}	1.7 ± 0.1^{a}	86.9 ± 5.4^{b}

Values are expressed as mean ± standard deviation. For each amino acid and digestion condition, differences among protein sources were assessed by one-way ANOVA followed by Tukey's post hoc test. Different superscript lowercase letters (a-c) within the same row, separately for crude and digested proteins, indicate statistically significant differences among protein sources ($p < 0.05$). Amino acids marked with an asterisk (*) are essential amino acids.

anti-nutritive factors in their composition and the effect on the gut microbiome. Additionally, safety concerns may arise from the possible presence of allergens, heavy metals, pesticides, mycotoxins, and microbial contaminants[11]. Insect farming needs good manufacturing practice in order to ensure safety and quality[15]. In the European Union, few edible insects are classified as novel foods and are regulated under Regulation (EU) 2015/2283 and its implementation. To date, four insect species have been authorized as novel foods, including *Tenebrio molitor*, *Locusta migratoria*, *Alphitobius diaperinus larvae*, and *Acheta domesticus* (EU 2023/5). Among them, *Acheta domesticus* (the common house cricket) is one of the most studied, for its high content in essential amino acids[16].

Recently, was observed that cricket protein intake in healthy volunteers was associated with reduced systemic inflammation and TNF-α, and improved gut health with increased abundance of *Bifidobacterium*[17].

The lack of in vivo data on the effects of insects or their derivatives on humans in Western countries may be partly due to food neophobia, which represents a significant barrier to the design and implementation of dietary intervention trials. Protein-based fortified products, such as protein beverage are continuously growing in popularity, especially for athletes, since can be a convenient option to supply a high dose of protein[18]. These drinks involve just the dissolution of crude isolate protein powders, typically in water, before consumption. From a future perspective, evaluating insect-derived proteins within familiar food matrices is essential not only to better reflect real-life dietary exposure, but also to improve consumer acceptance and mitigate food neophobia, which currently represents a major barrier to the adoption of insect-based foods[19].

The search for alternative protein sources is becoming increasingly crucial in the face of global food security challenges and environmental sustainability concerns. Among these alternatives, insect-derived proteins stand out due to their high nutritional value, low ecological footprint, and potential to promote gut health. The aim of this study was to investigate the effects on gut microbiome of a single donor of three isolate proteins: whey, pea, and *Acheta domesticus* pure water-soluble to simulate a daily ingestion using the Simulator of the Human Intestinal Microbial Ecosystem or SHIME®[20–22]. The impact of these proteins on microbiome was assessed through multiomics approach (metataxonomic, metagenomics, and metabolomics analyses).

Results

Aminoacid composition of isolate proteins

The total free aminoacid (TFAA) concentration before the in vitro digestion reached 17561 mg/kg for cricket, 784 mg/kg for pea and 355 mg/kg for whey and increased after digestion to 26037, 36425 and 2478 mg/kg for cricket, pea and whey protein isolate, respectively (Table 1). Although characterized by the initial highest concentration, TFAA of cricket powder was subjected to the lowest increase after digestion (about 40%).

Cricket proteins showed the highest concentrations of several amino acids, in some cases up to 1000 times higher than in pea and whey. The most abundant were valine, proline, alanine, ornithine, glutamic acid, glycine, and cysteine. Lower concentrations were observed for arginine, lysine, and methionine. Pea protein included high levels of ornithine, tyrosine, valine, cysteine, γ-aminobutyric acid, phenylalanine, histidine, and lysine.

Whey powder primarily contained ornithine, followed by tyrosine and methionine, with generally lower amino acid concentrations compared to the other sources.

After digestion, the concentration of all amino acids increased regardless of the protein used (Table 1). Although cricket protein showed the highest total amino acid content, it exhibited the lowest relative increase

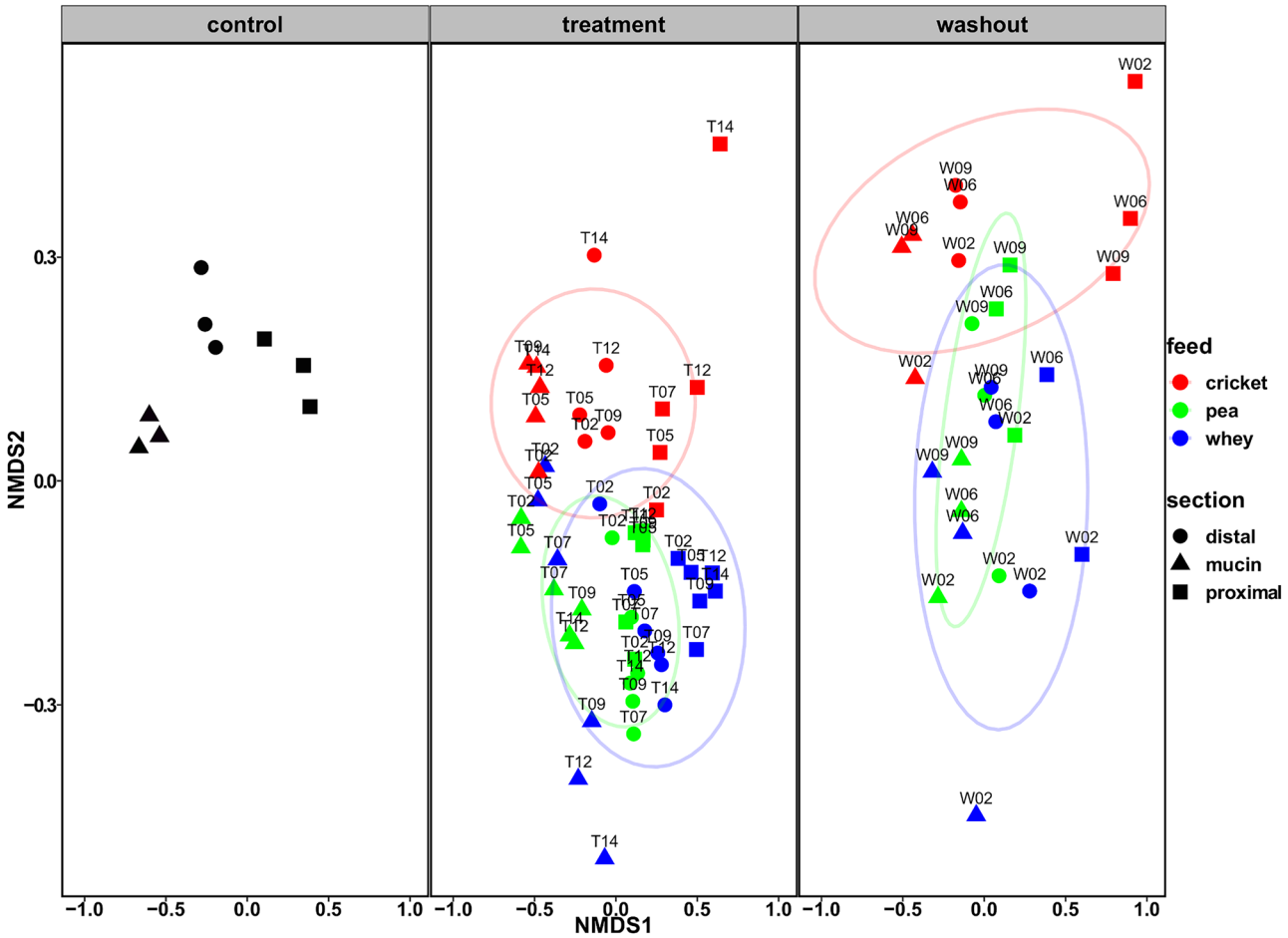

Fig. 1 | Non-metric Multi-Dimensional Scaling (NMDS) of microbiota assessed by amplicon sequencing, based on Bray-Curtis distance. Samples are colour-coded according to the protein intake (cricket, pea and whey), while the shape according to the SHIME® section (proximal, distal or mucin), numbers represent the sampling day. Each panel showed an experimental period (control, treatment and washout).

after digestion, approximately 40%. Nine out of 21 amino acids exceeded 1000 mg/kg.

Among these, the highest concentration was observed for proline (5107 mg/kg), followed by tyrosine (3569 mg/kg), histidine (2915 mg/kg), glycine (2001 mg/kg), phenylalanine (2626 mg/kg), ornithine (2270 mg/kg), γ-aminobutyric acid (1342 mg/kg), alanine (1746 mg/kg), and glutamic acid (1214 mg/kg). Pea protein was also rich in amino acids after digestion, with eight out of 21 showing concentrations above 1000 mg/kg. The amino acid with the highest concentration was proline (8303 mg/kg), followed by histidine (5408 mg/kg), tyrosine (4375 mg/kg), leucine (3364 mg/kg), phenylalanine (2857 mg/kg), ornithine (2784 mg/kg), γ-aminobutyric acid (2539 mg/kg), valine (1802 mg/kg), and arginine (1163 mg/kg). Whey protein showed the lowest overall amino acid content and included the histidine (4844 mg/kg), followed by tyrosine (369 mg/kg), phenylalanine (220 mg/kg), ornithine (215 mg/kg), proline (242 mg/kg), alanine (180 mg/kg), γ-aminobutyric acid (199 mg/kg), glycine (152 mg/kg), and arginine (104 mg/kg) (Table 1).

By comparing the concentration of amino acids between the three types of protein after digestion, cricket displays significantly higher concentrations of alanine, glutamic acid, glycine, methionine, serine, threonine, and valine, while pea shows higher concentrations of arginine, cysteine, γ-aminobutyric acid, histidine, leucine, lysine, ornithine, phenylalanine, proline, tryptophan, and tyrosine (Table 1, ANOVA, $P < 0.05$).

Donor characteristic

From questionnaire analysis, the subject demonstrated high adherence to the MDS with a score of 10 out of 14, as well as a high level of physical activity, achieving the criteria of "vigorous intensity activity on at least 3 days and accumulating at least 1500 MET minutes/week"[23]. In addition, the 3-day food record was used for assessing energy and macronutrient intakes during 2 weekdays and one weekend day prior to sample collection. In particular, the median individual macronutrient intake showed that 44% of energy intake came from carbohydrates, 19% from protein and 37% from fat. The use of a single donor allowed the evaluation of intra-individual microbiome responses to different protein intake under controlled conditions.

Microbiota dynamics during the experiment

Metataxonomic approach was used to evaluate the microbial dynamics during the experiment. Only Simpson index was significantly highest ($P < 0.05$) in proximal colon with pea intake, while in the distal section, the highest value was observed with cricket intake (Supplementary Fig. 1).

PERMANOVA analysis and ANOSIM tests showed that microbiota variation was primarily influenced by colonic section (distal or proximal, $R^2 = 19.6\%$, $P < 0.01$), followed by sample type (lumen or mucin-associated microbiota ($R^2 = 15.6\%$, $P < 0.01$) and then sampling time ($R^2 = 15.6\%$, $P < 0.01$). Protein intake accounts for 13.5% of the variance, confirming a dietary impact, though less pronounced than section or sample type. Period (control, treatment, washout) was also significant but explains a smaller portion of the variance (9.3%, $P < 0.01$). Non-metric multi-dimensional scaling (NMDS) analyses based on ASVs at genus level showed that cricket intake was able to modify the structure of the microbiota since pea and whey clustered together and were well separated from cricket in both period (treatment or washout) or sample type (lumen or mucin) ($P < 0.05$, Fig. 1).

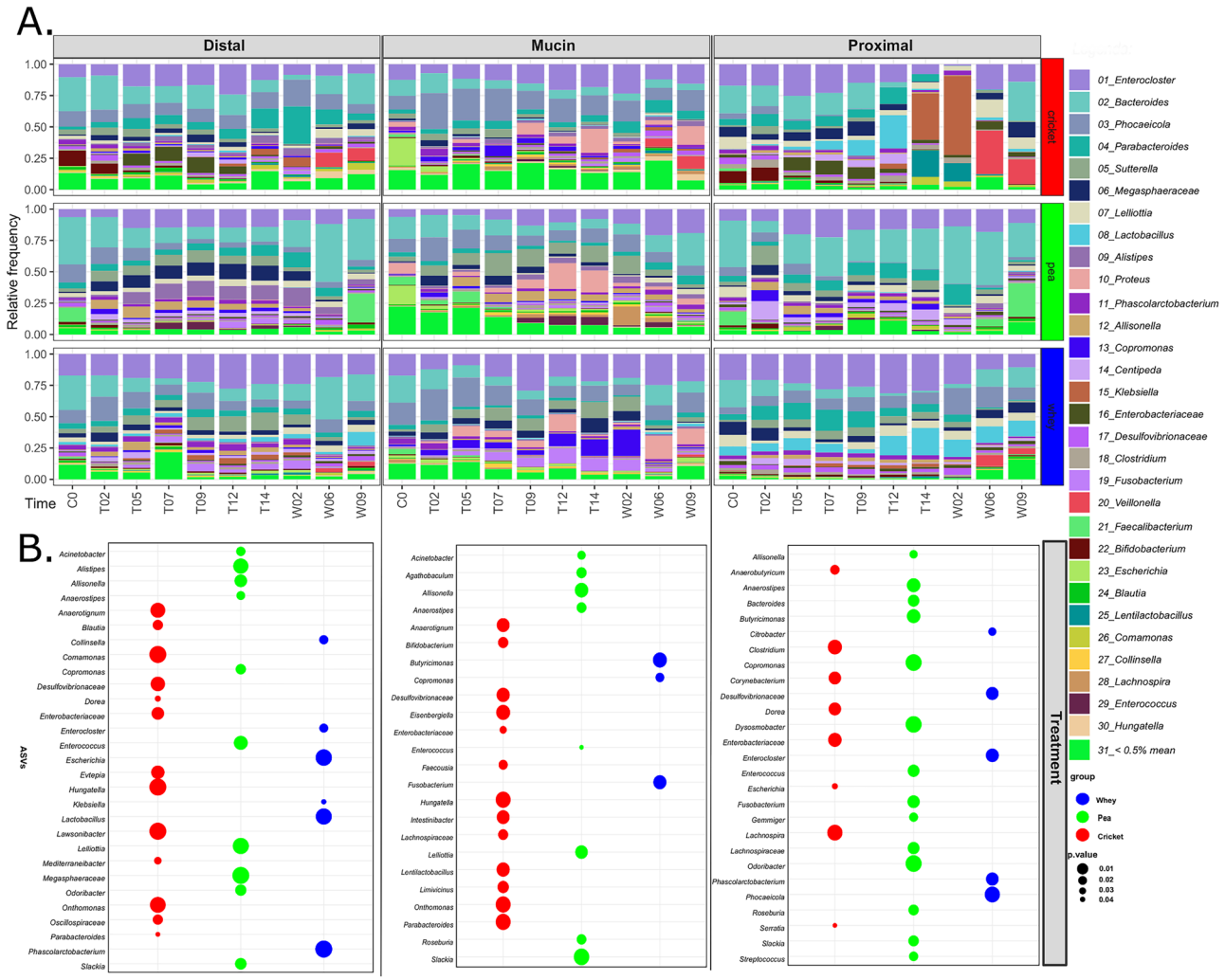

Fig. 2 | Global composition of microbiota at genus level assessed by 16S rRNA gene sequencing during the experiment. A Bar plot illustrating the microbiota composition according to protein intake (cricket, pea, and whey) and SHIME® section (proximal, distal, or mucin), with sampling points indicated along the *x*-axis. Only Amplicon Sequence Variants (ASVs) with a relative frequency > 0.5% in at least 10% of the samples are shown. **B** Bubble plot showing the ASVs significantly associated with specific protein intakes across the three SHIME® sections, as determined by the indicator species analysis. Bubbles are color-coded according to protein intake (cricket, pea, and whey), and the size of each bubble corresponds to the p-value of the association. Larger bubbles indicate a lower p-value, reflecting a stronger association between an ASVs and a specific protein intake.

At genus level, we observed that *Enterocloster* and *Bacteroides* were the most abundant ASVs, followed by *Phocaeicola, Parabacteroides* and *Sutterella* (Fig. 2A). In general, we observed an increase in frequency of the minor ASVs during the treatments followed by a gradual decrease during wash out (Fig. 2A). This trend was observed for *Lachnospira* and *Fusobacterium* in mucin-whey, *Lactobacillus* in proximal colon-whey samples, *Alistipes* and *Enterococcus* in distal colon-pea samples. Cricket intake increased the presence of *Proteus* in mucosa samples during the second week of treatment, while *Veillonella* in the washout (Fig. 2A). *Bifidobacterium* was mainly present in lumen samples (distal and proximal) with cricket intake (Fig. 2A). *Collinsella* increased its relative frequency in the mucin layer samples especially with whey intake, while *Megasphaeraceae* showed an increase with pea intake in distal section (Fig. 2A).

The indicator species analyses performed for each colonic region showed in distal tract that cricket intake was associated with the predominance of *Blautia*, *Dorea*, *Lawsonibacter*, and *Comamonas* among others (Fig. 2B, FDR < 0.05). Pea intake with *Alistipes*, *Enterococcus*, *Megasphaeraceae* and *Slackia,* while whey intake with *Collinsella*, *Escherichia* and *Lactobacillus* (Fig. 2B, FDR < 0.05). In solid layer simulated by the mucin, we observed that *Bifidobacterium*, *Intestinibacter*, *Lachnospiraceae*, *Lentilactobacillus* and *Parabacteroides* were associated with cricket intake, *Acinetobacter*, *Enterococcus*, *Roseburia* and *Slackia* with pea, *Butyricimonas* and *Fusobacterium* with whey intake (Fig. 2B, *FDR* < 0.05). In the proximal section, cricket intake was associated with *Clostridium, Lachnospira, Corynebacterium, Dorea*, and *Enterobacteriaceae.*

To identify differentially features across protein intake, we performed a pairwise comparisons between each treatment. A linear model was constructed to account for variables such as feed type, period, section, and time.

From the model, we observed that *Bifidobacterium*, *Blautia*, *Clostridium*, *Dorea*, *Intestinibacter*, *Lachnospira*, *Lactiplantibacillus*, *Lactobacillus*, and *Lentilactobacillus* were significantly more abundant with cricket intake when compared with pea or whey (Fig. 3A, FDR < 0.01).

In contrast, *Alistipes*, *Enterococcus*, *Faecalibacterium*, *Fusobacterium*, *Slackia*, and *Sutterella* were linked to pea intake, while *Butyricimonas*, *Collinsella*, *Escherichia*, *Fusobacterium*, *Lactobacillus*, and *Sutterella* were associated with whey when compared to cricket intake (Fig. 3A, FDR < 0.01).

By comparing whey and pea intake, we observed an association between *Alistipes*, *Enterococcus*, *Bacteroides*, *Faecalibacterium*, *Blautia* and *Slackia* with pea intake and *Acinetobacter*, *Clostridium*, *Lentilactobacillus* and *Lactobacillus* with whey (Fig. 3A, FDR < 0.01).

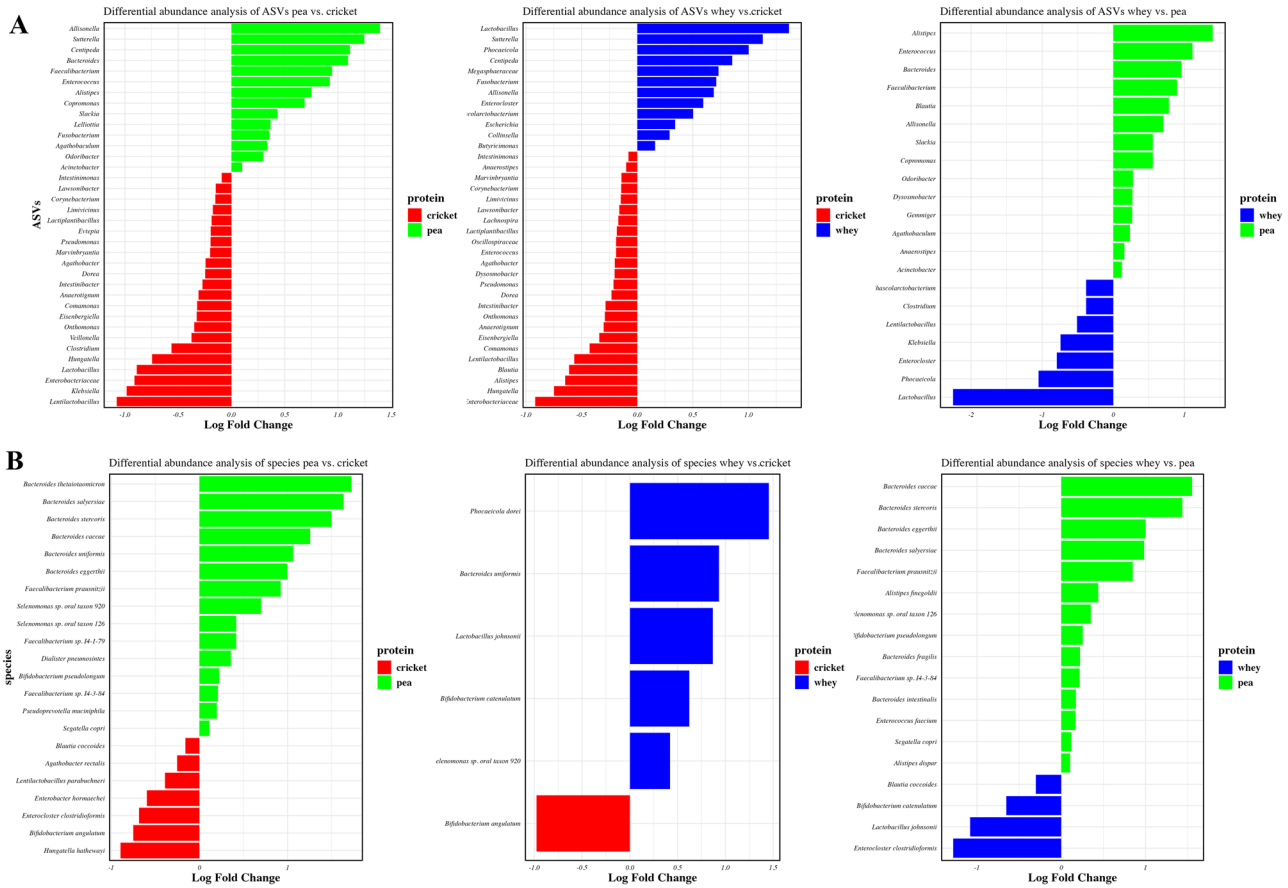

Fig. 3 | Differential microbial abundance across protein sources based on amplicon sequencing and shotgun metagenomics analyses. Plot A: bar plots shows the differential abundance of Amplicon Sequence Variants (ASVs) for three protein intake comparisons: Pea vs. Cricket, Whey vs. Cricket, and Whey vs. Pea. The fill color of each bar represents the protein: red for cricket, green for pea, and blue for whey. The *x*-axis represents different ASVs, reordered by log fold change (log2), which was calculated using a linear modeling approach applied with the lmFit function from the limma package in *R*. *P*-values were adjusted for multiple comparisons using the Benjamini–Hochberg procedure to control the False Discovery Rate (FDR). The *y*-axis shows the log fold change values, where bars above the *x*-axis indicate higher abundance in the first protein listed in the comparison, and bars below the *x*-axis indicate higher abundance in the second protein. **Plot B** shows the differential abundance of species detected by shotgun metagenomics for the same three protein intake comparisons.

For all the comparison *Intestinimonas*, *Lactiplantibacillus*, *Pseudomonas*, *Intestinibacter* and *Lentilactobacillus* were always associated with cricket intake (Fig. 3A, FDR < 0.01).

Protein intake modulates the microbiome at strain level

The microbiome composition variation conducted using Kraken was consistent with the results from metataxonomic 16S rRNA gene sequencing. The statistical linear models confirmed the association of *Blautia coccoides*, *Lentilactobacillus parabuchneri*, and *Bifidobacterium angulatum* with cricket intake (Fig. 3B, FDR < 0.01). Pea intake was linked to several *Bacteroides* (including *B. uniformis* and *B. thetaiotaomicron*) and *Faecalibacterium* species, while whey intake was associated with *Phocaeicola dorei*, *Bacteroides uniformis* and *Bifidobacterium catenulatum* (Fig. 3B, FDR < 0.01). A total of 64 metagenome-assembled genomes (MAGs) were reconstructed, all with completeness greater than 90% and contamination below 5%. The distribution of these MAGs varied depending on the type of food intake (Supplementary Fig. 2). Cricket intake was associated with several species-level genome bins (SGBs) such as *Clostridium bolteae*, *Parabacteroides distasonis*, *Intestinimonas butyriciproducens*, and *Bifidobacterium angulatum*, with *Lactiplantibacillus plantarum* and *Lactobacillus buchneri* being reconstructed exclusively whit cricket. In the pea-fed group, *Desulfovibrio vulgaris*, *Faecalibacterium prausnitzii*, *Selenomonas* sp., and *Enterococcus faecium* were the most prevalent. Whey protein intake was primarily characterized by *Bacteroides dorei*, *Bacteroides fragilis*, and *Lactobacillus johnsonii*. Core taxa such as *Lachnoclostridium* sp. and *Alistipes finegoldii* were consistently abundant across all dietary treatments (Supplementary Fig. 2).

Protein intake modulates the potential function of the microbiome

Shotgun data were used to discover the functional potential of microbiome due to the different protein intake.

Once comparing cricket *vs.* pea protein intake, the functional gene clusters at level 2 showed that cricket intake boosted an increase of genes involved in protein secretion systems, as well as those involved in metabolism of aromatic compounds, proteolysis and amino acid metabolism, such as peptidases and aminotransferases (Fig. 4A, FDR < 0.01). Pea intake busted the genes involved in protein degradation and biosynthesis, alongside those related to histidine metabolism, DNA replication, and membrane transport.

Comparing whey vs. cricket, the latter displays the highest abundance of cluster genes related to organic acids and branched-chain amino acids, while whey displays the highest abundance of genes clusters for fermentation and protein processing and modification (Fig. 4A, FDR < 0.01).

Comparing pea *vs.* whey intake, the latter boosted gens for osmotic stress, ABC transporters, fermentation, and tricarboxylate transporters. Pea intake boosted genes involved in membrane transport, branched-chain amino acid metabolism, and protein folding (Fig. 4A, FDR < 0.01)

At function level, when we compared cricket-protein with pea protein intake, we found that cricket intake was associated with a significantly higher

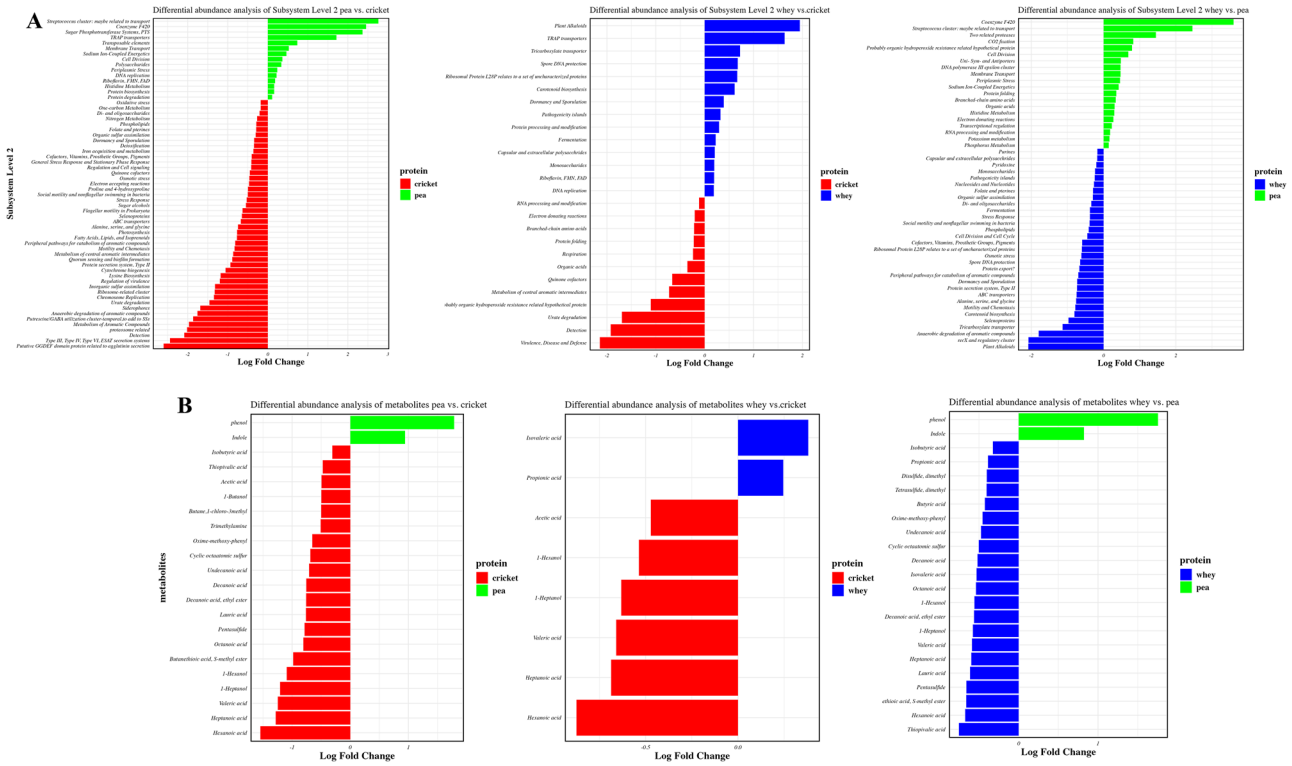

Fig. 4 | Differential abundance analysis of functional genes (Subsystem Level 2) and metabolites across protein intake comparisons. Plot A: bar plots show the differential abundance of genes at Subsystem Level 2 for three protein intake comparisons: Pea vs. Cricket, Whey vs. Cricket, and Whey vs. Pea. The fill color of each bar represents the protein: red for cricket, green for pea, and blue for whey. The *x*-axis represents different genes at Subsystem Level 2, reordered by log fold change (log2), which was calculated using a linear modeling approach applied with the *lmFit* function from the *limma* package in *R*. *P*-values were adjusted for multiple comparisons using the Benjamini–Hochberg procedure to control the False Discovery Rate (FDR). The y-axis shows the log fold change values, where bars above the *x*-axis indicate higher abundance in the first protein listed in the comparison, and bars below the *x*-axis indicate higher abundance in the second protein. **Plot B** shows the differential abundance of metabolites for the same three protein intake comparisons.

abundance of several genes ($n = 334$), including branched-chain amino-acid (BCAA) transporters genes (LivF, LivH, LivM and DppC), involved in the degradation of aromatic and other amino acids, including aspartate racemase, asparagine synthase, methionine synthase, glutamate-N-acetyltransferase, and enzymes for glycine/serine metabolism (Supplementary Data 2A, FDR < 0.01). Cricket-protein intake samples also boosted several genes for bacteriocin transport (e.g., lantacin F, lactacin F) and for vitamin biosynthesis (thiamine, biotin, and riboflavin pathways). Additional signatures included universal stress proteins, starvation sensors such as RspA, and redox-active genes like putative sulfite reductase, anaerobic selenate reductase, and ferredoxin reductase (Supplementary Data 2A, FDR < 0.01). By contrast, pea protein displayed few differentially abundant genes ($n = 132$), including those for lysine degradation (L-β-lysine 5,6-aminomutase and D-lysine 5,6-aminomutase) and chorismate mutase II (EC 5.4.99.5/AroHII) alongside clusters linked to oxidative and nitrosative stress (heat-shock dnaK operon, periplasmic stress response). When cricket and whey protein intake were compared, whey samples were enriched in genes ($n = 62$) belonging to the serine–glyoxylate cycle, succinate dehydrogenase, aldehyde-lyases, and the acetoin–2,3-butanediol pathway, together with genes for bacteriocins (Supplementary Data 2B, FDR < 0.01). Cricket intake ($n = 22$) was characterized by glycine/sarcosine reductase, oxaloacetate decarboxylase, DMSO reductase, and enzymes involved in glutathione utilization, sulphite reduction, and sulfolactate lyase, underscoring their greater reliance on amino-acid fermentation and sulphur metabolism (Supplementary Data 2B, FDR < 0.01). Comparing whey intake versus pea, we observed that whey boosted several genes ($n = 501$), including transporter genes for oligo or dipeptide and bacteriocins along with genes involved in acetoin, butanediol metabolism. Pea intake significantly increased genes ($n = 174$) associated with proteolysis and amino acid metabolism along with peptidase and aminotransferases (Supplementary Data 2C, FDR < 0.01).

Metabolomic composition

Before treatment, the content of SCFAs did not significantly differ ($P > 0.05$) between the three SHIME units. The variable section explains the most variance ($R^2 = 20.2\%$, $P = 0.001$), followed by time ($R^2 = 46.8\%$, $P = 0.001$), period ($R^2 = 40.4\%$, $P = 0.001$) and feed ($R^2 = 12.1\%$, $P = 0.013$).

SCFAs composition varied according to the protein intake (Supplementary Data 3). Cricket intake led to an increase in acetic acid production, especially in distal colon. Whey intake led to a butyrogenic and propionogenic effect at the end of the first week of treatment, with the highest amount in the distal colon (Fig. 5 and Supplementary Data 3, $P < 0.05$). Acetic, propionic and butyric acids increased in their concentration during pea intake in both colon section at the same way. At the end of the washout period, SCFAs concentration decreased in every SHIME unit, reaching the initial concentrations (Fig. 5 and Supplementary Data 3, $P < 0.05$). A biplot based on the metabolomic composition was used to analyse samples from three treatments. The biplot revealed that for the SCFAs in the distal colon, the cricket samples were distinctly separated from the other groups (ANOSIM statistic R = 0.26, P = 0.001). This separation was primarily driven by the influence of the following compounds: acetic acid, hexanoic acid, heptanoic acid, valeric acid, and octanoic acid (Supplementary Fig. 3).

The linear models showed distinct metabolomic profiles linked to each protein intake. Cricket intake was significantly associated with a wide range of metabolites, including various short-chain fatty acids such as propionic acid, isobutyric acid, acetic acid, valeric acid, and hexanoic acid, as well as alcohols like hexan-1-ol and heptan-1-ol (Fig. 4B, FDR < 0.01). Several sulphur-containing compounds, including (methyltrisulfanyl)methane, 2,2-dimethylpropanethioic S-acid, and octathiocane, were also enriched. Pea intake showed a simpler profile, with the microbial metabolites phenol and 1H-indole being most prominent when compared with cricket or whey (Fig. 4B, FDR < 0.01). In contrast, whey intake was mainly associated with

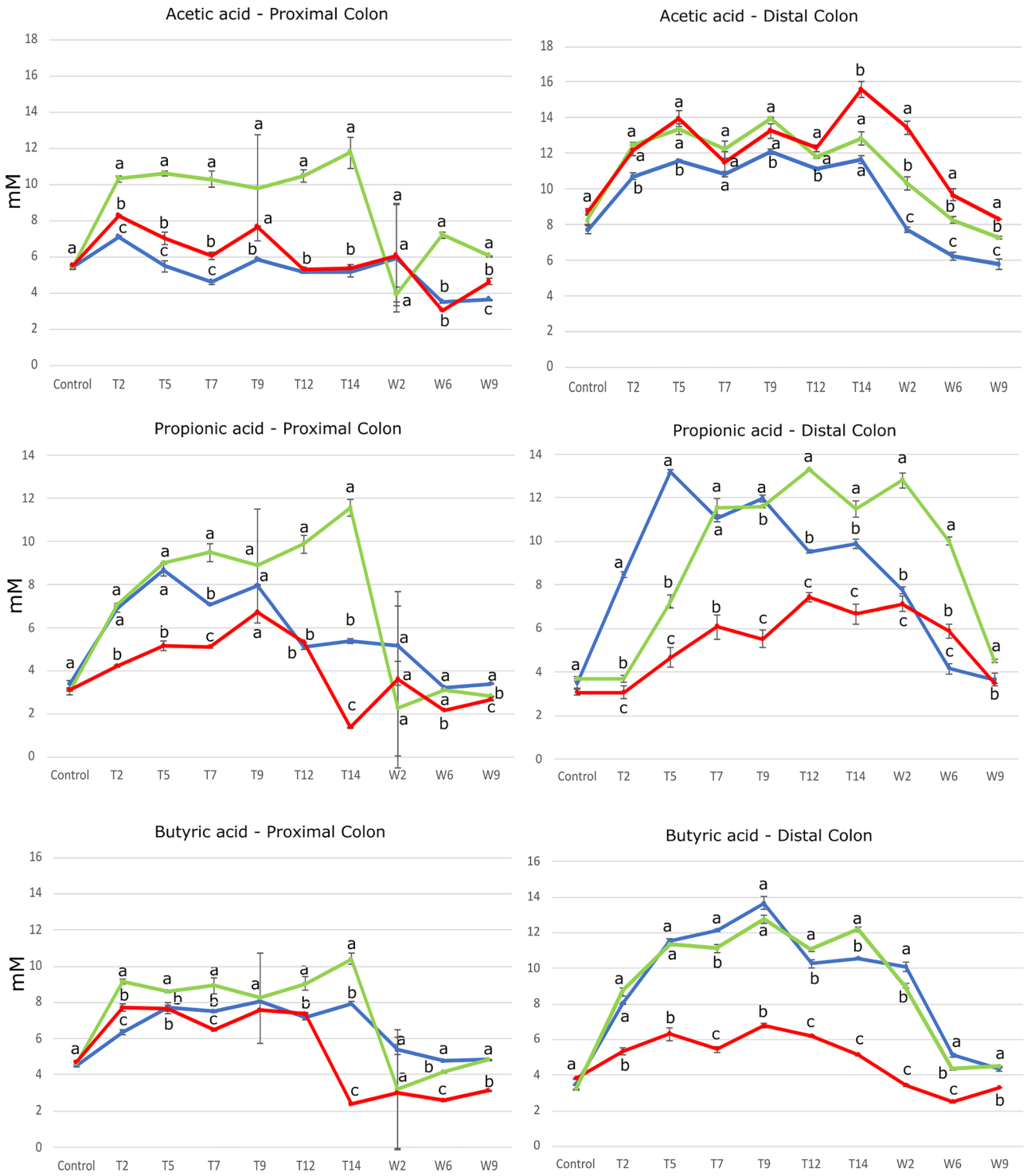

Fig. 5 | Concentration of SCFAs (acetic, propionic, and butyric acid) over time in each SHIME® section (proximal and distal colon), according to protein intake (red for cricket, green for pea, and blue for whey). For each sampling point, the bars represent the standard deviation, and the letters indicate statistical significance based on a one-way ANOVA between protein intake. Different letters denote significant differences between protein intake, as determined by Tukey's Honest Significant Difference (Tukey HSD) post-hoc test.

isovaleric acid, 2,2-dimethylpropanethioic S-acid, and propionic acid (Fig. 4B, FDR < 0.01).

Metabolome and microbiome correlations

The putative correlation analysis between microbiota (ASVs) and the metabolome (SCFAs and VOCs) reveals distinct relationships between gut microbial genera and various metabolites.

Alistipes shows a broad spectrum of positive associations, being directly correlated with several microbial metabolites including acetic acid, valeric acid, isobutyric acid, phenol, butan-1-ol, 1H-indole, and lauric acid (Supplementary Fig. 4, FDR < 0.01). *Bacteroides* exhibits consistently negative associations with a wide range of sulfur-compounds. *Enterococcus* displays multiple positive associations across both SCFAs (e.g., acetic, butyric, isobutyric, isovaleric acids) and sulfur-containing metabolites (e.g.,

(methyldisulfanyl)methane, (methyltetrasulfanyl)methane. *Bifidobacterium* showed positive associations with lipid-related metabolites such as undecanoic acid and dimethyl pentasulfide, while showing a negative association with phenol (Supplementary Fig. 4, FDR < 0.01). *Blautia* and *Lachnospiraceae* are positively linked to medium-chain fatty acids and alcohols, including valeric acid, heptanoic acid, heptan-1-ol, and hexan-1-ol. *Lactiplantibacillus* and *Lactobacillus* are largely negatively associated with a range of metabolites, including butyric acid, acetic acid, and phenol. Finally, *Lentilactobacillus* shows a positive association with octanoic acid, while being negatively associated with phenol and butan-1-ol (Supplementary Fig. 4, FDR < 0.01).

To elucidate multi-omic signatures associated with different feed protein sources, we performed a supervised multiblock sparse Partial Least Squares Discriminant Analysis (sPLS-DA) using the mixOmics R package. The analysis simultaneously integrated metabolomic, microbiome, and network-derived pathway data obtained from the same set of samples, enabling the identification of complementary features that collectively discriminate between cricket-, pea-, and whey-based diets (Supplementary Fig. 5). Predictive performance was further assessed via ROC analysis on the combined feature set. The integrated signature achieved high classification performance (AUC = 1.00) for cricket-fed samples and high discrimination for the remaining groups (AUC = 0.98 and AUC = 0.97 for pea and whey, respectively).

Discussion

Gut intestinal microbiome plays a crucial role in human health, with diet being one of the main factors influencing its composition and function. High-protein diets are often recommended for weight management, elderly populations, and athletes; however, the effects of elevated protein intake that reach the colon on microbiome composition and function are often contradictory and unexplored[24]. In the present study, we investigated how the colonic microbiome responds to an overexposure of dietary proteins (since the majority of dietary proteins are absorbed in the small intestine) and how animal, plant or insect-based protein influences the microbiome using a gastrointestinal simulator.

Cricket are high-protein source, providing essential amino acids, fats, minerals[25] with digestibility and metabolism similar to pea and whey proteins as already assessed[26]. Recent studies have shown that cricket-derived compounds could modulate microbiota composition, reducing inflammation and promoting metabolic benefits[27]. However controlled study is required to verify the effect of novel food intake.

The three proteins exhibited a significant increase in free amino acids after in vitro digestion with whey and pea that confirmed a remarkable ability to release a large amount of free amino acids, highlighting their great digestive efficiency[24]. Although the amount of free amino acids released after cricket protein digestion was lower compared to the other proteins, cricket protein contained a notably high proportion of amino acids that were already free or easily accessible even before digestion. This suggests that cricket protein had higher early bioavailability of amino acids, which may be beneficial for conditions requiring fast nutrient uptake, such as post-exercise recovery or compromised digestion[28,29]. Specifically, crude cricket proteins showed the highest levels of free phenylalanine. As previously shown, phenylalanine is more rapidly absorbed when ingested as free amino acids, compared to its absorption from protein-derived phenylalanine[30]. This feature could be particularly beneficial for older adults, in whom the postprandial release of dietary protein-derived phenylalanine into circulation is often attenuated[31]. Furthermore, phenylalanine is an essential amino acid that plays a crucial role in the biosynthesis of dopamine, epinephrine, and norepinephrine, all of which are involved in the gut-brain axis[32]. When comparing the concentration of free amino acids after in vitro digestion, cricket protein displayed significantly higher concentrations of alanine, methionine, and valine. These amino acids are associated with antioxidant activity due to their ability to donate electrons and neutralize free radicals. This unique amino acid profile could contribute to the potential health-promoting properties of cricket-derived proteins, including their antioxidant capacity[14].

After cricket protein digestion, significantly higher concentrations of essential amino acids such as methionine and valine and non-essential like alanine, glutamic acid, glycine, serine and threonine were observed. These AA are associated with the production of key short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate[33], as well as sulphur-containing metabolites like methanethiol and hydrogen sulphide[34]. In contrast, pea protein was enriched in both essential amino acids (such as arginine, histidine, leucine, lysine, and tryptophan) and non-essential amino acids (such as cysteine, γ-aminobutyric acid (GABA), ornithine, proline, and tyrosine). Many of these amino acids are precursors for bioactive compounds, including polyamines, aromatic and indolic compounds, SCFAs, and neurotransmitter-like molecules (e.g., tyramine, histamine)[34]. We observed a direct association between cricket intake and several beneficial taxa. These include: *Bifidobacterium*, an anti-inflammatory and pro-homeostatic immunomodulatory taxon[35]; *Lactiplantibacillus* and *Lactobacillus*, known for their protective effects on intestinal epithelial cells[36], neuroprotective properties[37], and positive impact on colonic function[38]; *Blautia* and *Lachnospira*, known for their beneficial effects on gut health[39–42].

Additionally, taxa with multifaceted roles, such as *Clostridium*[43], *Intestinibacter*[44], and *Dorea*[45,46], were also associated with cricket intake. As is already known, the human gut microbiota exhibits taxonomic variations throughout different sections of the gastrointestinal tract[6]. Notably, in the simulated mucosal layer, a significant presence of *Bifidobacterium, Lentilactobacillus* and *Parabacteroides,* which are well known as potential probiotics[47,48] was observed. Pea intake was associated with beneficial *Faecalibacterium*[44,49], as well as equol-producing taxa like *Slackia*[50,51]. However, potentially detrimental taxa such as *Enterococcus*[52,53], *Sutterella*, which has been positively correlated with HOMA-IR[54,55], *Fusobacterium*[56,57] and *Alistipes* were also associated with pea consumption. In particular, *Alistipes* has a controversial role due to its ability to produce harmful metabolites, including phenol and indole, as previously reported[58].

Whey intake was associated with *Butyricimonas*, a SCFAs-producing bacterium[59], as well as *Lactobacillus*. Moreover, whey was linked to the controversial genus *Collinsella*, which has been associated with insulin resistance and obesity[60,61], as well as with *Fusobacterium* and *Sutterella*.

At species level, cricket intake enriched the prevalence of *Bifidobacterium angulatum*, which is capable of producing gamma-aminobutyric acid (GABA), playing a role in the functioning of the gut-brain axis[62]. It has already been observed that cricket intake increases the abundance of *Bifidobacterium*[17].

Additionally, it increased the presence of histamine-producing *Lentilactobacillus parabuchneri*[63]. Under normal conditions, histamine helps modulate immune responses, gut motility, and other physiological functions[64,65]. For pea intake, we observed that several *Bacteroides* species, including *B. uniformis* and *B. thetaiotaomicron*, were associated with this protein. These species play a potential role in alleviating obesity[66], while *Bifidobacterium catenulatum* has been shown to confer liver protection[67]. Regarding the predominance of *Bacteroides,* it was already reported that protein would increase substrate availability for these bacteria, due to their proteolytic activity and supporting their growth over other bacteria belonging to Firmicutes[68].

The shift in microbiota composition also reflects a shift in the metagenomic function. In our experiment, cricket intake led to an increase in genes involved in the biosynthesis of B-vitamins, highlighting a potential stimulatory effect of insect-based proteins on microbiome for vitamin production. Previous studies have shown that microbiome-based vitamin production can vary depending on the host's health status and dietary habits[69]. Furthermore, the gut microbiota possesses a remarkable ability to synthesize and utilize B-vitamins when dietary carbohydrates are insufficient and protein levels are high, favouring proteolytic bacteria that metabolize amino acids to support B-vitamin production[70]. This suggests that the high protein content in cricket-based diets may create an environment that enhances B-vitamin biosynthesis by modulating the microbiota.

Additionally, vitamins, especially B-vitamins, play a significant gastroprotective role due to their antioxidant, anti-aging, and anti-inflammatory effects[71]. Cricket intake increase the BCAA transport genes suggesting an adaptive response of the microbiome to efficiently manage the availability of these amino acids. Highest level of circulating BCAA was reported to be associated with increased of insulin resistance[72,73], however, the highest abundance of BCAA transport genes suggests that, through this pathway, the gut microbiome could contribute to lower BCAA levels in circulation[74]. We then observed that insect proteins increased the abundance of genes involved in bacteriocin transport that can offer a competitive advantage to lactic acid bacteria by reducing microbial competition and inhibiting the growth of harmful microbes[75], thereby potentially contributing to the enrichment of beneficial microbes in the gut. By comparing cricket and pea protein intake, the latter ones display the highest abundance of lysins, and as a consequence, several genes involved in its degradation, with the consequent production of SCFAs[76] were observed. In line with literature, protein intake enriched in the entire dataset several microbial proteases as well as transporters, indicated a highest aminoacid fermentations. As a consequence, we then observed a variety of metabolites of protein fermentation including, including SCFAs, phenols, ammonia, amines and indoles.

Due to the aminoacidic composition, cricket protein may thus promote higher acetate production[33]. Acetate can inhibit the growth of pathogens[77,78] and acts as a co-substrate to support the growth of other beneficial microbes, such as *Lactobacillus*[79], through cross-feeding mechanisms. Furthermore, acetate contributes to lipid biosynthesis[79] and has been associated with appetite regulation[80]. We then observed higher levels of propionic acid due to protein intake when comparing to pea intake. It has been demonstrated that propionic acid lowers fatty acid content in liver and plasma, reduces food intake, exerts immunosuppressive actions[81], and may improve tissue insulin sensitivity[82], while also helping to control intestinal inflammation[80,83]. Cricket protein supplementation boosted the production of medium-chain fatty acids (MCFAs), including hexanoic, heptanoic, octanoic, and lauric acid. These MCFAs are more rapidly absorbed from the gut and transported directly to the liver via the portal vein and play a role in regulating glucose and lipid metabolism[84,85]. The SCFA-enhancing effects of cricket-derived amino acids are functionally reflected in the correlations observed for *Blautia* and *Lachnospiraceae* with MCFAs and SCFAs, supporting a microbiota-mediated mechanism through which dietary proteins modulate host metabolism. Lauric acid, in particular, exhibits a broad spectrum of antimicrobial activities against and helping to maintain the balance of gut microbiota[86]. However, the metabolism of cricket protein also results in the production of trimethylamine, which has been associated with negative health outcomes for the host[24]. Pea protein intake was primarily characterized by the highest levels of tyrosine and tryptophan, which subsequently led to elevated concentrations of phenol and indole, as already reported[87]. The potentially detrimental effects of indole, phenol, and their derivatives are acknowledged, including their association with oxidative stress and the reduction of gut epithelial barrier function[24,88–90]. The putative correlation analysis between microbiota and metabolite profiles (SCFAs and VOCs) supports and expands upon the functional differences observed among protein intake. The correlation analyses between the microbiome and metabolome across the treatments revealed the association between the pea associated *Alistipes* with indole and phenol, as well as acetic acid, reinforced its controversial role in human gut[58]. *Enterococcus*, enriched by pea protein intake, was positively correlated with SCFAs and sulphur compounds. Its ability to decarboxylate tyrosine, abundant in pea protein, may contribute to the production of bioactive metabolites like tyramine, with potential neuromodulatory effects[34]. Cricket protein promoted elevated levels of SCFAs (e.g., acetate and propionate) and medium-chain fatty acids (e.g., octanoic and lauric acid), which were associations with *Lentilactobacillus* (with octanoic acid). The enrichment of *Lactiplantibacillus* and *Lactobacillus* after cricket consumption, together with their negative correlations with acetic acid and phenol, suggests a role in modulating excessive fermentation or potential harmful aromatic compounds. *Bifidobacterium*, stimulated by cricket intake as already observed[17] was correlated with lipid-derived compounds such as undecanoic acid, and its negative correlation with phenol, reinforces its protective function, possibly aided by its ability to metabolize accessible amino acids into less harmful products.

In conclusion, the results of this study indicate that cricket protein does not exhibit any detrimental effects compared to pea and whey proteins. Each protein intake has its own unique characteristics that may contribute positively to gut health. In the context of a single-donor model and in the absence of the whole food matrix, exposure to cricket protein was associated with changes in the gut microbiome, including an increased abundance of potentially beneficial microbial taxa and enhanced short-chain fatty acid production. Moreover, pea and whey proteins also show positive microbiome modulation, cricket protein's impact is notably favourable. The use of a single donor allowed the evaluation of intra-individual microbiome dynamics under controlled experimental conditions; however, inter-individual variability was not addressed and represents an important limitation of the study. However, it is important to note that in our simulated SHIME® system, the food matrix was not present, meaning potential synergistic effects from compounds in the whole food form, such as for pea protein, may not be fully represented. Nonprotein dietary components can influence the availability of protein and offer additional substrates to the microbiota, as already discussed[24]. Despite SHIME® providing critical insights, it does not replicate systemic and host-level responses in living organisms, making in vivo validation necessary for a comprehensive understanding of gut microbiota-related interventions[22].

Since the gut microbiome can regulate the host's status, significant inter-personal variation within the human population may mask the dietary effects observed in vitro[91]. Therefore, while these findings suggest that cricket protein is a promising alternative protein source, they should be considered donor-specific, highlighting the need for further studies involving multiple donors and in vivo validation to fully assess long-term effects on gut health.

Future research should evaluate insect-derived proteins within real and familiar food matrices in order to assess their overall nutritional and microbiome-related effects. This approach may also improve consumer acceptance in Western countries and help overcome food neophobia while better reflecting real-life dietary exposure.

Methods

Chemicals and reagents

Chemicals used in this study were of analytical grade. Diethyl ether and sodium hydroxide were purchased from Carlo Erba (Milan, Italy), while 1-octanol, 4-pentenoic acid, sulfuric acid (95-97%), and sodium chloride were acquired from Merck (Darmstadt, Germany). All the fatty acids used in the study were obtained from Sigma Aldrich (St. Louis MO, USA). Double distilled water was obtained with a Milli-Q filter system (Millipore, Milan, Italy). Ringer' solution was purchased from VWR (Milan, Italy). The reagents (Feed media, hydrochloric acid 0.5 M, sodium hydroxide 0.5 M, mucin powder, pancreatin powder) were acquired from ProDigest® (Gent, Belgium), while sodium hydrogen carbonate was supplied from Fisher Chemical (Milan, Italy) and Oxgall dehydrated fresh bile from BD (Milan, Italy). All the reagents and enzymes for the INFOGEST protocol were obtained from Sigma (Milan, Italy). Reagents for amino acid analysis were obtained from ERRECI S.r.l. (Milan). Hydrochloric acid (HCl), Trizma® base ($NH_2C(CH_2OH)_3$), 5-Sulfosalicylic acid dihydrate ($C_7H_6O_6S$) used for the water/salt soluble extract were purchased from Merck (St. Louis, Missouri, USA). Reagents for the DNA extraction were supplied by QIAGEN (Milan, Italy), while the reagents for the PCR and library preparation from Roche (Milan, Italy).

Raw ingredients and in vitro digestion

Three commercial isolate proteins: pea protein isolate unflavoured (Pea protein isolate, Bulk®); whey protein isolate unflavoured (Whey protein, Decathlon), and defatted *Acheta domesticus* isolate protein (Nutri, Italian Cricket farm srl.) were selected and used in the experiment. To mimic the

use levels of the European Regulation in foods for insects, an intake of 10 g of each pure protein per day was considered. Proteins were pre-digest following the INFOGEST static in vitro simulation of gastrointestinal digestion protocol[92].

Amino acidic profile of pure protein proteins

Water/salt-soluble extracts from protein isolate prior to and after the INFOGEST were prepared according to the method described by Weiss et al.[93]. Briefly, a 1:4 ratio of sample to Tris–HCl buffer (50 mM, pH 8.8) was used. Samples were resuspended in the buffer and held at 4 °C for 1 hour under stirring conditions (150 rpm), followed by centrifugation at 20,000 × *g* for 20 min. The obtained supernatants were used for the determination of total free amino acids (TFAA) by using a Biochrom 30+ series Automatic Amino Acid Analyzer (Biochrom Ltd., Cambridge Science Park, United Kingdom), equipped with a Li-cation-exchange column (4.6 × 200 mm internal diameter)[94]. Results are reported as mean ± standard deviation of three independent analyses and expressed as mg/kg of protein isolate.

Participant and ethical aspects

A 34-year-old healthy individual with a normal weight (Body Mass Index = 22 kg/m^2) who was following an omnivorous diet was selected after conducting a detailed interview to assess suitability for the study. The subject, voluntarily recruited in February 2024, had no medical conditions, was not taking chronic medications or prebiotics and had no history of gastrointestinal diseases, eating disorders, chronic or psychiatric illnesses, recent antibiotics, or surgery. To ensure the participant meets the health and activity criteria required for the study, the individual completed the 14-item Mediterranean Diet Score (MDS)[95], which was used to assess the adherence to the Mediterranean-type regimen. Physical activity levels were calculated using the International Physical Activity Questionnaire (IPAQ) Short Form[23,96]. Additionally, dietary habits over the 3-day food record[97], was completed in the days prior to sample collection. Stool samples were self-collected by volunteers[98] immediately kept at 4 °C in anaerobic conditions, and reached the laboratory approximal within 1 hour. The volunteer gave written informed consent to the study, and the study protocol was approved by the Ethics Committee of the University of Turin (approval n° 0416051 12/07/2024) in accordance with the Declaration of Helsinki.

Simulator of the human intestinal microbial ecosystem

A Triple M-SHIME® (ProDigest, Ghent, Belgium) configuration was design. Each parallel SHIME® unit includes three consecutive bioreactors that simulate: stomach and small intestine (ST/SI), proximal colon (PC) and distal colon (DC). Bioreactors temperature was maintained at 37 °C under anaerobic conditions by flushing sterile N_2[99,100]. PC and DC bioreactors were filled with 500 and 800 mL of adult M-SHIME growth medium with starch (PD-NM002B Prodigest, Belgium), and the pH was maintained constant through computer-controlled addition of 0.5 M HCl and 0.5 M NaOH within the following ranges: 5.75–5.95 and 6.6–6.9, respectively[22]. Each DC bioreactor has an artificial mucosal layer, consisting in two polyethylene netting containing 30 mucin agar-covered plastic media[101]. All PC and DC bioreactors were inoculated with the selected donor fresh stool slurry (5% v/v)[20]. The inoculated microbiota had around 16 h[100] of adaptation by a static incubation in the bioreactors following by 2 weeks of stabilization, allowed the evolution of the stable colonic microbiota within colon bioreactors, and then 2 weeks of steady state, which representing the experiment baseline. During these weeks, the bioreactors were fed three times per day with the appropriate M-SHIME media with pH adjusted to 2 (PD-NM002B). During the fourteen days of treatment, each PC vessels were supplemented with 10 g/day of each protein once a day. At the end of the treatment, nine days of washout were carried out under the same feeding conditions of the control state. Samples from lumen (PC and DC bioreactors) and mucosa (DC bioreactors) were collected from each colon vessels the day before the beginning of the treatment (C0), after 2, 5, 7, 9, 12 and 14 days of treatment (T02, T05, T07, T09, T12 and T14, respectively), and after 2, 6 and 9 days of wash out (W02, W06 and W09, respectively). Mucin agar-covered microcosms were washed two times by dipping them five times in sterile Ringer' solution to remove the luminal microbiota, collected in 50 ml tubes and processed following the ProDigest® guideline. The resulting pellet was stored at −20 °C until the DNA extraction. Lumen samples were immediately analysed for the volatile and SCFAs composition. An aliquot of lumen samples was stored at −20 °C until DNA extraction (Supplementary Data 1).

DNA extraction, metataxonomic and metagenomic analyses

Total DNA was extracted from lumen (1 ml) and mucosal samples (~ 250 mg of the pellet) using the DNeasy PowerSoil Pro with the QIAcube HT workstation according to the manufacturer's instructions. Metataxonomic analysis was performed in duplicate. DNA amplification (V3-V4 region of the 16S rRNA gene) and library preparation were performed with the automatic liquid handling Hamilton Microlab® STAR™ following the Illumina Sequencing Library Preparation instruction. Amplicon sequencing (2X250 bp) and whole metagenomics shotgun (2X150bp) were performed by the Novogene company (Munich, Germany). For metataxonomic analysis, raw reads were denoised by using QIIME2 software[102] and DADA2 plug[103]. A total of 13.772.008 denoised reads (average 75.670 reads/sample) were used for taxonomic assignment against the Greengenes2 database[104]. Amplicon sequence variants (ASVs) were averaged between the two replicates and normalized to the lowest number of sequences per sample. The following selected sampling points for both lumen and mucosal from the Triple-M-SHIME, were analysed by shotgun metagenomics: C0, T07, T14, and W09 (Supplementary Data 1). Shotgun data were first filtered for low quality reads, adapters, and for reads shorter than 50bp using adapter removal[105]. The draft genome of *H. sapiens*, GRCh38 was used for filtering out human contaminants reads with Bowtie2 v.2.4.4[106] in end-to-end sensitive mode. A total of 478 Gbp (Q30 > 99%) of reads were then used for downstream analysis.

Taxonomic assignment and abundance estimation were performed using the Kraken 2 algorithm[107], and further refined with Bracken (Bayesian Re-estimation of Abundance with Kraken) following the pipeline presented in ref. 108. Raw reads were normalized using DeSeq2 R package[109]. Low-abundant hits with a relative abundance lower than 0.001% were filtered out, and all bacteria phyla identified by Kraken2 were cross-referenced with the scientific literature to retain only those with substantial reference support and detect possible contaminants or false positives.

Short reads were then used for functional characterization with Superfocus[110] with default paraments. Functional table was then normalized for Copies Per Million (CPM) with Superfocus. Reads were then assembled in contigs with MetaSPAdes v. 3.11.0[111]. Reads assembled into 863.676 contigs longer than 1000 bp. The total contig length per sample in average was 139.49 Mbp with an average N50 length of 18.136 bp. MAGs (Metagenome-assembled genomes) were obtained from contigs using MetaBat2[112], and quality was assessed with CheckM[113]. Only medium and high-quality MAGs (completeness > 50% and contamination < 10%)[114,115] were used for the downstream analysis. Taxonomic assignment in species-level genome bin (SGBs) was performed with Kraken2[107]. Pangenome calculation and marker gene identification were performed by Tormes[116].

Short and medium chain free fatty acids analysis

Short and medium chain free fatty acids were determined by means of capillary gas chromatography coupled with a flame ionization detector (GC/FID), according to the method suggested by ProDigest®[117] with slight modifications. Specifically, 1 mL of lumen sample (Supplementary Data 1) was added with 500 µL of a H_2SO_4:H_2O solution (1:1; v/v), a small amount of NaCl (small layer on the back of a glass Pasteur pipette), and 400 µL of NaOH 0.1 M. After vortexing, 4 mL of diethyl ether and 50 µL of internal standard (4-pentenoic acid, 100 mM) were immediately added, and the mixture vortexed again. After centrifugation (1400 × *g* for 3 min), the top layer was collected and transferred into a GC vial. One microliter was injected in splitless mode (1 min) into a GC/FID Shimadzu GC-2010

equipped with a Stabilwax fused silica capillary column (20 m × 0.18 mm ID × 0.18 µm film thickness; Restek, Bellafonte, PA, USA). The separation was achieved with an oven temperature set up from 40 °C to 248 °C at a rate of 35 °C/min, and held for 8 min. Inlet temperature was 248 °C. Helium was used as carrier gas at a constant linear velocity of 41.0 cm/s. The recognition of the short and medium fatty acids was performed by comparing their retention times with those of the corresponding commercial standards under identical analytical conditions. For quantification, calibration curves for each SCFAs were built in the range 0.1–25.0 mM for the more abundant analytes and a range 0.1–200.0 µM for the less abundant. Results were expressed as millimolar (mM). All analyses were performed in duplicate.

Volatilome profile

Volatile organic compounds (VOCs) were analysed through headspace solid-phase microextraction gas chromatography coupled with mass spectrometry (HS-SPME-GC/MS), following to the procedure described by Ossola et al.[118] with some modifications. Briefly, 2 mL of each lumen samples (Supplementary Data 1) were added of 10 µL of 1-octanol (350 µg/mL) as internal standard (IS) in a 20 mL headspace vials sealed with aluminium caps and PTFE silicone septa. The analyses were performed in a GCMS-QP2010 Plus (Shimadzu, Japan), interfaced with a computerized system for data acquisition (Software GC–MS Solution V. 2.5, Shimadzu, Japan). A Combi Pal system was used to incubate each sample at 40 °C for 10 min, where after which a DVB/CAR/PDMS-coated fused silica fibre (10 mm length, df 50/30 µm; Supelco, Bellafonte, PA, USA) was exposed at the same temperature for 40 min. The same fibre was then desorbed in the GC/MS inlet at 260 °C for 5 min (split ratio 1:30). VOCs were separated in a RTX-5 fused silica capillary column (20 m × 0.10 mm ID × 0.10 µm film thickness; Restek, Bellafonte, PA, USA) with the following oven temperature program: from 40 °C (held for 0.70 min) to 220 °C (rate 7 °C/min), and then to 260 °C (rate 20 °C/min), held for 4 min. A constant linear velocity equal to 34.0 cm/s of helium was used (carrier gas). The temperatures of the ion source and the interface were, respectively, 200 °C and 245 °C. Acquisition was performed in scan mode in a *m/z* range of 50–400 (scan speed 2000 amu/s). Identification of VOCs was achieved with the support of the NIST08s library from the National Institute of Standards and Technology. Blank injections were carried out to exclude environmental contamination. The internal standard method was used to quantify the VOCs, and results were expressed as µg/mL. All analyses were performed in duplicate.

Statistical analyses

To assess differences in total free amino acid (TFAA) concentrations before and after digestion, a one-way Analysis of Variance (ANOVA) was performed using the *aov* function in R (version 4.4.2). Following ANOVA, Tukey's Honest Significant Difference (Tukey HSD) post-hoc test was conducted to identify pairwise differences between protein sources. For microbiota analysis, α-diversity and non-metric multidimensional scaling (NMDS) were calculated using the *vegan* and *cmdscale* packages in R, respectively. PERMANOVA (999 permutations) and ANOSIM tests were also conducted in R to evaluate differences in community composition. A multi-level indicator species analysis[119] was used to identify significant associations between microbial taxa and protein intake across different colonic regions. To explore associations between Amplicon Sequence Variants (ASVs), microbial species, genes, and metabolites, a linear modeling approach was applied using the *lmFit* function from the *limma* package in R. A design matrix was created to incorporate protein type, experimental period, gut section, and sampling time as factors. Protein intake was the main variable of interest, with "cricket" set as the reference category. P-values were adjusted for multiple comparisons using the Benjamini–Hochberg procedure to control the False Discovery Rate (FDR). Principal Coordinates Analysis (PCoA) was performed to assess sample clustering based on short-chain fatty acids (SCFAs) and volatile organic compounds (VOCs) profiles. A one-way between-group analysis of Variance (ANOVA) was conducted for acetic, propionic and butyrric acid using the *aov* function with Tukey's Honest Significant Difference (Tukey HSD) post-hoc test.

Correlation analysis between log2 normalized data of microbiota and metabolome was conducted using Spearman correlation coefficients on via the *corr.test* function in R. Only correlations with FDR < 0.01 were visualized. To integrate multiple omics datasets—specifically, taxonomic profiles, metabolomic data, and molecular interaction networks—we employed the R package mixOmics, which is designed for N-integration of heterogeneous data types measured on the same set of samples. Integration was performed using the Data Integration Analysis for Biomarker discovery using Latent Components (DIABLO) framework, also known as multiblock PLS-DA (Partial Least Squares Discriminant Analysis). DIABLO enables simultaneous integration of multiple datasets while modeling their associations with a categorical outcome variable.

Before integration, each dataset was pre-processed through normalization using the scale() function in R, followed by the removal of near-zero variance predictors using the nearZeroVar() function from the caret package. This ensured the stability and interpretability of the multivariate analysis. The R version is 4.4.3, the mixOmics library version is 6.30.0.

Bar plots, boxplots, bubble plots, biplots, corrplot and stacked plots were generated using the 'ggplot2' package in R.

Data availability

The metataxonomic and metagenome sequences are available at the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) under the BioProject accession numbers PRJNA1280763and PRJNA1299316, respectively.

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Competing interests

The authors declare no competing interests.

Additional information

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