Targeted metabolomic and nutritional analyses of a variety of protein-rich whole-food sources

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Article

Keywords: metabolomics, dietary protein, whole-foods, nutrients, GC-MS

Posted Date: October 27th, 2025

DOI: https://doi.org/10.21203/rs.3.rs-7694307/v1

Additional Declarations: No competing interests reported.

Abstract

Protein-rich whole-food sources may possess, as yet, unidentified non-protein components with anabolic potential. Targeted metabolomics can be applied to investigate candidate small compounds, an analytical approach not yet taken within diverse protein-rich whole-foods. We used targeted GC-MS based metabolomic analyses to determine the abundance of a range of small molecules implicated in the regulation of postprandial protein anabolism within a variety of protein-rich whole-foods (whole egg, pork, salmon, lentils and mycoprotein) and a less nutrient dense, more isolated source (egg whites). We aimed to establish the small metabolite profile across these conventionally consumed dietary protein sources and to investigate how they differ between their raw and cooked forms. Heatmap and principal component (PCA) analyses were conducted to identify differences between sources and to assess the effect of cooking. From the metabolites identified, 22 out of 24 (in the raw form; 92%) and 23 out of 24 metabolites (in the cooked form; 96%) differed between foods and the number of metabolites that either increased or decreased with cooking varied across foods. PCA analyses revealed the top three metabolites responsible for the variance between food sources were succinic-, arachidonic-, and myristic acids. Large differences at the nutritional and metabolite level between food sources indicate the diverse range of additional components, other than protein, within a whole-food matrix. The identification of potential anabolic non-protein components within a range of whole-foods provides the necessary step to examine why certain whole-foods may provide a more (or less) robust anabolic stimulus within human studies.

Introduction

The maintenance of human tissues is essential for sustaining normal physiological processes, and this relies heavily on the postprandial anabolic response to dietary protein intake [1]. This response is driven by the postprandial appearance of amino acids, which act both as substrates for protein synthesis and as nutritional signals that stimulate anabolic pathways, leading to tissue protein accretion. Skeletal muscle accounts for a substantial proportion of whole-body protein turnover and plays an important role in health and disease, thus, discussions of dietary protein quality often take a muscle-centric perspective [2]. Indeed, dose–response studies demonstrate that muscle protein synthesis (MPS) can be stimulated with as little as 10 g of isolated protein, with the maximal acute response reached at approximately 20–30 g [3]. The role of leucine is central in mediating this response via the mammalian target of rapamycin (mTOR) pathway [4,5].

Even when isonitrogenous and/or matched for leucine, the ingestion of more nutrient rich whole-food sources has reported to result in greater [6–8] and equivalent [9–11] acute muscle anabolism compared to isolated protein comparators. Such whole-foods, far more commonly consumed in real-life [12], generally comprise a multitude of other potentially bioactive nutrients contained within unique and complex food-matrices, leaving open the possibility that many other (small) molecules, or their nutrient-nutrient interactions may exert, as yet, undocumented anabolic effects. For example, *in vitro* data suggest that

vitamin D [13], omega-3 [14] and short chain fatty acids [15] may represent anabolic agents. It is worth noting, however, that these compounds may behave differently when interacting with other nutrients within a food matrix and metabolised within a physiological system. Additionally, components within a food source may undergo functionally relevant modifications during heat treatment (i.e. cooking) [16,17].

Human work investigating dietary proteins and whole-body and/or muscle anabolism tend to present macronutrient compositions, somewhat disregarding smaller metabolites/peptides, limiting insights into such potential nutrient anabolic signalling beyond amino acids (or other macronutrients), nor typically consider the translational aspect of cooking. Targeted metabolomics approaches are becoming more commonplace within the food industry to provide detailed readouts of small compounds of interest contained within foods [18,19]. A recent study from [20] reported striking differences in compounds related to anabolic pathways when comparing grass-fed meat and a plant-based meat alternative, despite ostensibly comparable macro- and micro- nutrient compositions. Further work is warranted to expand such nutrient profiling beyond what is displayed on nutritional labelling in diverse protein-rich food sources [20].

In the present work we selected a range of commonly consumed protein-rich whole-foods (e.g. meat, fish, plant, fungal) and a more isolated albumin protein (egg whites; presumably possessing a less varied nutrient profile). These foods were subjected to a targeted Gas Chromatography-Mass Spectrometry (GC-MS) metabolomic approach in parallel with 100 standards of (small) compounds specifically selected based on data existing that imply they may possess bioactive roles within myocellular (or related) anabolic pathways. We hypothesised albumin protein (i.e. egg whites) would show the narrower metabolite profile, whilst the whole-foods would be wider and disparate from one another. Furthermore, we hypothesised that the effect of cooking would alter the metabolite profile of all protein sources (e.g. breaking down some compounds to form new (smaller) compounds during the cooking process). This is the first study that has assessed a range of protein-rich foods to characterise a metabolite profile as it relates to human whole-body or muscle anabolism.

Material and Methods

Selection and preparation of food samples

Five different protein-rich whole-food sources were selected for analysis: eggs, pork, salmon, mycoprotein and red lentils. In addition, egg whites were separated manually from the same batch of eggs to use as a more isolated control food source. Prior to cooking, the more solid foods (pork and salmon) were manually minced. To cook the individual food samples, a water bath was used to maintain a constant temperature *('sous vide')* and time of cooking (see details in **Table 1**). For the macronutrient, micronutrient and full amino acid analyses, 100 g of each cooked food sample was frozen and sent to Premier Analytical Services for independent analysis. For each individual method of analysis applied, please see **Supplementary Material 1**. For the metabolomic analysis, three replicates (2 g each) of each

food source, raw and cooked (i.e. a total of 36 samples), were immediately frozen in liquid nitrogen and stored at -80 °C for subsequent analysis.

Table 1. Food sample cooking time and temperature for the water bath prior to sample extraction.

Food Samples	Cooking time (minutes)	Cooking temperature (°C)
Egg Whites	35	80
Whole egg	35	80
Pork	45	70
Salmon	45	70
Mycoprotein	45	60
Red Lentils	60	90

1.2.2 Sample extraction

Food samples were powdered under liquid nitrogen using a pestle and mortar and weighed to approximately 50 mg into bead beater tubes. One mL 50 % aqueous acetonitrile (to precipitate out the proteins) with 0.3 % formic acid was added to each replicate and then homogenised using a tissue lyser for 2 minutes. Two hundred µL of each sample homogenate were removed and added to a 1.5 mL tube. Eight-hundred µL LC-MS grade methanol spiked with 6.25 mg·L of internal standard (D27-deuterated myristic acid) were added to each tube and centrifuged (13.500 x G) for 5 minutes to precipitate out the proteins. Thereafter, 250 µL of the supernatant were then removed into 300 µL mass spec vials and completely dried down in a Speedvac evaporator. The latter step was repeated three times to achieve 750 µL as a total volume.

Sample derivatisation

Twenty-five µL of 18 mg·mL^{-1} methoxamine hydrochloride dissolved in pyridine were added to each sample and incubated at 50 ^{o}C for 30 minutes. Then, 75 µl of MSTFA were added to the samples and returned to 50 ^{o}C for a further 30 minutes.

Gas Chromatography – Mass Spectrometry (GC-MS)

Samples were analysed using an Agilent 7200 series accurate mass Q-TOF MS together with a 7890A GC system (Agilent Technologies, Santa Clara, USA), equipped with an EI (electron ionisation) ion source. An aliquot (0.6 µL) of each sample was injected into a non-deactivated, baffled glass liner with a 12:1 split ratio (14.448 mL· min^{-1} split flow) and the inlet temperature was maintained at 300 ^{o}C. A Zebron semi-volatiles (Phenomenex, Torrance, USA) column (30 m x 0.25 mm x 0.25 µm) coupled with a 10 m guard column was maintained at a constant helium flow of 1.2 mL·min^{-1}. The temperature gradient of the GC was ramped up at a rate of 15 ^{o}C·min^{-1}, from 70 ^{o}C to 310 ^{o}C over 16 minutes, and then held at

310 ^{O}C for a further 6 minutes. The total run time of 22 minutes was followed by a 7-minute backflush at 310 ^{O}C to clean the column at the end of every run. The MS emission current and emission voltages were held at 35 µA and 70 eV, respectively, and the MS was automatically calibrated after every run. The mass range was set from 50 to 600 atomic mass units (amu), with an acquisition rate of 5 spectra·s^{-1}, and a solvent delay of 4 minutes.

Data processing and analysis

Mass spectra data were analysed using Agilent technologies MassHunter qualitative (version B.07.00) and quantitative (for QTOF version B.08.00) software. Using the qualitative software, solvent blank subtractions were performed, and peaks were identified by comparison to the retention times and spectra of the 100 standards run. Metabolites were retained if detected in all three replicates. Of the 100 standards run, only 24 were above the level of detection and present in three replicates of at least one food source. Quantitative software was then used to extract and compare the peak areas (ion abundance) of the identified compounds between samples. Peak area integrations were manually checked by two of the investigators (F.A.D.H and D.S) to ensure that the correct compound peak was selected. Peak areas were then normalised to the internal standard to account for any extraction and instrument variations. The full list of annotated metabolites and their retention times are available as an excel spreadsheet (**Supplementary Material 2**).

Statistical analyses

Standard energy, macro- and micro- nutrient analyses were not performed on multiple replicates but rather conducted commercially by a third party and therefore no statistical tests were run with these data.

Within the metabolomic dataset we identified free cellular amino acids, which cannot be quantitatively directly compared with those determined commercially within the macronutrient analyses based on different extraction (i.e. metabolomics required free cellular amino acid not protein extraction) and measurement (e.g. determining grams vs. relative abundance) approaches. Rather than allowing for statistical comparison *between* metabolites, semi quantification of data as relative abundance is compound specific (depends on ionization energies and concentration), and therefore our primary statistical approach was to compare the abundance *within* the metabolite identified across the range of protein-rich whole-foods both in raw and cooked forms. We further sought to identify if the abundance of each metabolite differed within each source between the raw and cooked forms of each of the food sources, to assess the effect of cooking on the metabolite profile.

Individual metabolites were tested for normality using Kolmogorov-Smirnov tests (P<0.05) using GraphPad Prism (10.2.3). A one-way ANOVA was used to compare the abundance of each metabolite across food sources in the raw and cooked form (effect of metabolite). If an effect was detected (P<0.05), Tukey's multiple comparisons tests were applied to locate individual differences. Individual

paired *t*-tests were used to assess the abundance of each metabolite between the raw and cooked form of each food source (i.e. effect of cooking).

To visualise differences and identify top metabolites contributing to the nutritional disparity between food sources, the data were then analysed using MetaboAnalyst 6.0 (https://www.metaboanalyst.ca/). This software enabled the creation of a hierarchical clustering dendrogram (based on Spearman distance measure and Ward clustering algorithm) **(Figure 1)**, an unsupervised principal component analysis (PCA) (**Figure 2**) and a heatmap of the metabolites (based on the Pearson distance measure and Ward clustering algorithm), for raw (**Figure 3a**) and cooked forms (**Figure 3b**) separately and all combined (**Figure 3c**).

Results

Energy, macro- and micro- nutrient information

The energy, macro- and micro- nutrient contents per 100 g and the content of each amino acid of cooked egg whites, whole egg, pork, salmon, mycoprotein, and lentils, are reported in **Table 2**.

Table 2. Macro and micronutrient composition (per 100g) of food sources. All food sources were cooked sous vide (in a water bath).

Per 100g

Food source	Egg whites	Whole egg	Pork	Salmon	Myco-protein	Red Lentils
Macronutrients						
Energy, kJ	239	580	600	848	352	382
Energy, Kcal	56	139	143	204	77	91
Protein, g	10.6	14.5	18.7	20.3	11.6	9.0
Carbohydrate, g	2.9	<0.1	1.0	0.6	1.9	7.6
Fructose, g	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Glucose, g	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Sucrose, g	<0.1	<0.1	0.4	<0.1	<0.1	1.4
Maltose, g	<0.1	<0.1	<0.1	<0.1	0.2	<0.1
Lactose, g	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Total sugars, g	0.2	<0.1	0.4	<0.1	0.2	0.4
Fat, g	0.2	9.1	7.0	13.3	1.5	0.2
Saturates, g	<0.1	2.8	2.6	2.3	0.3	<0.1
Mono-unsaturates, g	<0.1	4.1	3.1	5.2	0.3	<0.1
Polyunsaturates, g	<0.01	1.8	0.9	5.2	0.9	0.1
Starch, g	2.7	<0.1	0.6	0.6	1.6	7.1
Dietary Fibre, g	<0.1	1.7	0.6	<0.1	4.9	11.5
Soluble Dietary Fibre, g	0.0	1.2	0.0	0.0	0.0	0.0
Insoluble Dietary Fibre, g	0.0	0.5	0.6	0.0	4.9	11.5
Micronutrients (vitamins and minerals)						
Niacin (Tot VitB3), mg	<0.1	<0.1	6.50	11.30	0.70	1.00
Nicotinamide, mg	<0.1	<0.1	6.10	10.50	0.30	0.80
Nicotinic acid, mg	<0.1	<0.1	0.40	0.80	0.40	0.20
Vitamin B2, mg	0.09	0.34	0.20	0.05	0.20	0.03
Vitamin B1, mg	<0.05	0.06	0.65	0.12	<0.05	0.17
Vitamin A, µg	<10	82.00	<10	<10	<10	<10

Retinol, µg	<10	78.00	<10	<10	<10	<10
β carotene, µg	<10	20.00	<10	<10	11.00	<10
alpha carotene, µg	<10	<10	<10	<10	<10	<10
Vitamin E, mg	<0.1	2.20	<0.1	3.28	0.42	0.24
Vitamin D3 / D2*, µg	<0.3	<0.3	<0.3	4.30	<0.3*	<0.3*
Vitamin C (mg/100g)	<0.1	<0.1	0.60	0.20	<0.1	<0.1
Vitamin B6 (mg/100g)	<0.05	0.07	0.27	0.34	0.08	<0.05
Vitamin B12, µg	<0.1	2.26	0.69	2.65	<0.1	<0.1
Cholesterol, mg	<0.5	<0.5	60.40	54.40	<0.5	<0.5
Omega-3, g	<0.01	0.1	0.1	3.0	0.1	0.0
Omega-6, g	<0.01	1.6	0.8	2.2	0.8	0.1
Salt (sodium), g	0.5	0.4	0.1	0.1	<0.03	<0.03
Calcium, mg	7.3	44.3	7.7	4.0	29.4	8.4
Iron, mg	<0.03	1.5	1.0	0.3	0.4	1.8
Magnesium, mg	13.6	12.4	22.9	27.8	36.6	22.5
Phosphorus, mg	12.7	199.0	208.0	270.0	213.0	112.0
Potassium, mg	127.6	119.9	358.6	410.9	56.7	277.0
Manganese, mg	<0.03	<0.03	<0.03	<0.03	4.8	0.3
Copper, mg	<0.03	<0.03	0.0	<0.03	0.4	0.2
Zinc, mg	<0.02	1.2	3.1	0.4	8.4	1.1
Essential amino acids (EAAs)						
Tryptophan, g	0.20	0.21	0.23	0.25	0.17	0.07
Histidine, g	0.20	0.21	0.23	0.25	0.17	0.07
Threonine, g	0.52	0.63	0.92	0.91	0.58	0.29
Valine, g	0.63	0.69	0.78	0.81	0.53	0.31
Methionine, g	0.40	0.41	0.48	0.55	0.20	0.06
Lysine, g	0.80	0.89	1.75	1.72	0.86	0.54
Iso-leucine, g	0.47	0.53	0.67	0.64	0.42	0.24
Leucine, g	0.90	1.02	1.50	1.39	0.79	0.54

Phenylalanine, g	0.65	0.57	0.81	0.77	0.53	0.38
Total EAAs, g	**4.77**	**5.16**	**7.37**	**7.29**	**4.25**	**2.50**
Non-essential amino acids (NEAAs)						
Aspartic acid, g	1.23	1.35	2.00	2.03	1.14	0.95
Serine, g	0.83	0.99	0.85	0.87	0.62	0.44
Glutamic acid, g	1.51	1.66	2.92	2.60	1.31	1.31
Alanine, g	0.67	0.71	1.15	1.17	0.69	0.33
Arginine, g	0.63	0.79	1.23	1.14	0.74	0.64
Proline, g	0.43	0.52	0.91	0.74	0.53	0.36
Cystine, g	0.25	0.27	0.18	0.17	0.09	0.07
Tyrosine, g	0.46	0.51	0.69	0.66	0.46	0.23
Glycine, g	0.38	0.41	0.94	0.87	0.50	0.30
Total NEAAs, g	**6.39**	**7.21**	**10.87**	**10.25**	**6.08**	**4.63**
Branched chain amino acids (BCAAs)						
Valine, g	0.63	0.69	0.78	0.81	0.53	0.31
Iso-leucine, g	0.47	0.53	0.67	0.64	0.42	0.24
Leucine, g	0.90	1.02	1.50	1.39	0.79	0.54
Total BCAAs, g	**2.00**	**2.24**	**2.95**	**2.84**	**1.74**	**1.09**

Consistency of individual food replicates

To test the consistency and similarity of our replicates, a hierarchical cluster analysis dendrogram was obtained using the metabolites detected in each of the six food sources (raw and cooked forms; Figure. 1**)**. We report good clustering (similarity) between the replicates and that the different food sources, whether they are raw or cooked, can be visually separated by their metabolomic profile. From the hierarchical cluster analysis, lentils display the most differential profile compared with the other foods, whether raw or cooked, typically showing a higher abundance of most metabolites compared with the other sources. As we demonstrated clear consistency and similarity between all three replicates of each food source (in the raw and cooked forms), a single value calculated as the average of the three replicates is visually presented hereafter.

Summary of the diversity of metabolites across food sources; raw form, cooked form and the effect of cooking

We found that out of all identified metabolites, 22 / 24 in the raw form (92%) and 23 / 24 metabolites in the cooked form (96%) differed between foods. When we determined the effect of cooking on each food individually, the number of metabolites that changed (either increased or decreased) with cooking varied across foods. In ascending order: 4 / 24 for whole egg; 5 / 24 for salmon; 6 / 24 for pork; 8 / 24 for egg whites; 12 / 24 for lentils; and 13 / 24 for mycoprotein. To illustrate the diversity of metabolites in the raw form, cooked form and from the effect of cooking, we first present a visual depiction of the data, using principal component analysis (PCA) and Heatmaps (shown in Figures 2 & 3). Second, we present a statistical analysis of each data set to detect significant differences in the abundance of a metabolite across food sources in the raw and cooked forms, and to assess if these were significantly altered with cooking **Supplementary Material Figure 1 to 5.**

Raw foods: abundance of metabolites between sources

The PCA analysis for raw food sources (Figure. 2a) revealed a distinct separation in nutritional components, with a larger proportion (74.1%) of the variance explained within principal component 1 (PC1), illustrating the large variability in metabolite profiles across the range of raw foods. The heatmap (Figure. 3a) provides a visual interpretation of the foods that are highest in a particular compound. As mentioned, lentils displayed the highest frequency of metabolite abundance.

Quantitative statistical analyses showed the abundance of amino acids differed between food sources (proline, threonine, asparagine, valine, lysine, aspartic acid, glutamic acid, β-alanine [all $P<0.0001$] and glycine *[*$P<0.001$]). Individual differences between food sources for each of these metabolites can be visualised in supplementary Figure 1a, with statistical analyses reported in **supplementary Table 1**. For all other metabolites identified, see supplementary material. The abundance of organic acids (malic acid, fumaric acid and threonic acid [all $P<0.0001$] and citric acid [$P<0.001$]) differed between sources, shown in detail in supplementary Figure 2a.

The abundance of fatty acids differed between sources (arachidonic acid, myristic acid, linoleic acid [all $P<0.001$]), depicted in supplementary Figure. 3a. However, palmitic acid ($P=0.678$) and heptadecanoic acid ($P=0.169$) did not differ between food sources. The abundance of sugar compounds (myo-inositol [$P=0.001$], glucose-6-phosphate [$P<0.0001$]) differed between sources (supplementary Figure. 4a). The abundance of the organic compound, putrescine ($P<0.0001$) differed between food sources (supplementary Figure 5a). The abundance of glyceric acid and succinic acid ($P<0.0001$, both) also differed between food sources (supplementary Figure 5a).

Cooked foods: abundance of metabolites between sources

The PCA analysis for cooked food sources (Figure 2b) revealed a distinct separation in nutritional components, with the larger part (95.9%) of the variance explained within PC1, further illustrating the large variability in metabolite profiles across the foods. The compounds that were the most influential are presented in **Table 3.** The heatmap (Figure 3b) provides a visual interpretation of the foods that are

highest in a particular compound. Generally, the metabolite abundance was greatest in cooked lentils and salmon.

Quantitative statistical analyses showed the abundance of amino acids differed between food sources (proline, threonine, asparagine, valine, lysine, β-alanine, glutamic acid [all $P<0.0001$]); however, there were no differences in tyrosine ($P=0.511$). Individual differences between food sources for each of these metabolites are shown in supplementary Figure 1b and for all other metabolites identified, see supplementary material. The abundance of organic acids (malic acid, citric acid, aspartic acid, fumaric acid and threonic acid [all $P<0.0001$]), differed between sources, supplementary Figure 2b.

The abundance of fatty acids differed between sources (palmitic acid [$P=0.002$], linoleic acid [$P<0.001$], heptadecanoic acid, arachidonic acid, myristic acid [all $P<0.0001$]), depicted in supplementary Figure 3b. The abundance of sugar compounds (myo-inositol [$P=0.001$], glucose-6-Phosphate [$P<0.0001$]) differed between sources (supplementary Figure 4b). The abundance of the organic compound, putrescine ($P<0.0001$) differed between food sources (supplementary Figure 5b). The abundance of glyceric acid and succinic acid ($P<0.0001$, both) differed between sources (supplementary Figure 5b).

The effect of cooking; the change in abundance of metabolites from their raw to cooked forms

The PCA analysis of raw and cooked food sources (**Figure 2c**) revealed a distinct separation in nutritional components with a larger proportion (85%) of the variance explained within PC1, further illustrating the clear effect of cooking on metabolite variability. The heatmap (**Figure 3c**) provides a visual interpretation of the foods (raw and cooked) that are highest in any given compound; specifically, despite being in raw form, the metabolite abundance is greatest in lentils and salmon.

Quantitative statistical analysis showed the abundance of metabolites changed with cooking, but not comparably so across food sources. For a more detailed analysis of the effect of cooking on the abundance of specific metabolites (i.e. increase/decrease/no change), please see **supplementary material Table 4**. The following summarises the statistical analyses only of the metabolites that either increased or decreased within each food source between raw and cooked forms. The cooking of egg whites resulted in a decrease (threonine [$P=0.005$], aspartic acid [$P=0.039$], tyrosine [$P=0.004$], fumaric acid [$P=0.048$], citric acid [$P<0.0001$], myristic acid [$P=0.031$], and increase (palmitic acid [$P=0.002$] and myo-inositol [$P<0.001$]) in the abundance of metabolites. The cooking of whole egg resulted in a decrease (lysine [$P=0.007$], and increase (palmitic [$P=0.012$], glucose-6-phosphate [$P=0.002$], myo-inositol [$P=0.001$]) in the abundance of metabolites. The cooking of salmon resulted in a decrease (glucose-6-phosphate [$P=0.049$] and increase (threonine [$P=0.002$], β-alanine [$P=0.014$], linoleic acid [$P=0.016$], myo-inositol [$P=0.002$] in the abundance of metabolites. The cooking of pork resulted in a decrease (valine [$P=0.018$], aspartic acid [$P=0.001$], arachidonic acid [$P=0.015$], glucose-6-phosphate [$P<0.0001$] and increase (palmitic acid [$P=0.013$], linoleic acid [$P=0.002$] in the abundance of metabolites. The cooking of lentils resulted in a decrease (glucose-6-phosphate [$P=0.006$]) and increase (proline [$P=0.013$], threonine [$P=0.001$], asparagine [$P=0.001$], valine [$P=0.001$], glycine [$P=0.008$], aspartic acid [$P=0.002$], β-alanine [$P=0.012$], tyrosine [$P=0.014$], arachidonic acid [$P=0.004$], putrescine

[*P*=0.002], succinic acid [*P*<0.001] in the abundance of metabolites. The cooking of mycoprotein resulted in a decrease (proline [*P*=0.001], threonine [*P*=0.011], valine [*P*=0.005], glutamic acid [*P*=0.007], aspartic acid [*P*=0.022], and tyrosine [*P*=0.037], fumaric [*P*=0.003], threonic [*P*=0.045], malic acid [*P*=0.021], arachidonic acid [*P*=0.015], succinic acid [*P*=0.010] and increase (palmitic acid [*P*=0.001], linoleic acid [*P*=0.023] in the abundance of metabolites.

Table 3. List of the top 10 most influential compounds from the PCA plot **Figure 2b**, Cooked food sources and references to support their potential influence / effect on muscle metabolism.

Compound	Loadings 1	References
Succinic Acid	0.93475	21
Arachidonic acid	0.28626	22–24
Myristic Acid	0.20601	25
Glyceric Acid	0.05501	26
Glycine	0.05119	27
Glucose-6-phosphate	0.03189	28
β-alanine	0.02572	29
Linoleic acid	0.01925	30,31
Threonine	0.01715	32
Putrescine	0.00775	33

Discussion

The present work determined the comparative abundance of a range of targeted nutrients and metabolites implicated in human cellular anabolic signalling pathways and other vital metabolic processes, within a range of protein-rich whole-foods in their raw and cooked forms. We show the abundance of virtually all selected metabolites varied markedly across all foods in their raw and cooked forms, with specific compounds unique in their content to each food (discussed below). We also report that cooking *per se* significantly modulates metabolite abundance, but divergently so across the differing food sources, with implications regarding the likely non-protein mediated anabolic or other metabolic roles of different whole-foods.

Habitual dietary protein intake can be obtained from various sources, with habits, cost and sustainability (among other considerations) all affecting the choice of individual foods. From a nutritional perspective, it is often viewed simply as prioritising the combination of protein content plus additional macro- and micro- nutrients, and overall energy intake. Per 100 g of mass, the foods included in this work contained a similar protein content (see Table 2), with salmon and lentils being the most and least protein dense,

respectively) (~ 20% and 9% of total mass, respectively). Therefore, a typically recommended protein serving of ~ 20 g [34] from the range of whole-foods studied would result in a similar energy intake (202 and 133 kcal in lentils and mycoprotein being the highest and lowest, respectively, a negligible difference of 69 kcal), with the variance due to differences in carbohydrate and fat content. Metrics of protein *quality* are largely driven by the preponderance of essential amino acids and specifically, leucine [35]. Within a 20 g protein serving of the present whole-foods, essential amino acid load would range from 6.1–8.3 g (pork > mycoprotein > salmon > whole egg > lentils) and leucine from 1.4–1.6 g (pork > whole egg > salmon > mycoprotein > lentils), implying all could be considered of high quality. Interpretation to applied nutrition here should be prudent, given we deliberately did not select lower protein density and/or quality foods. Pork and salmon, both primarily consisting of muscle fibres and connective tissue, unsurprisingly possessed the highest total essential, branched chain and leucine contents (per 100 g), suggesting they may have the greater capacity for stimulating tissue protein synthesis rates, prior to consideration of non-protein components within the food matrix [36,37]. These subtle differences in protein (quality), macronutrients and energy content highlight the need for human work to verify the anabolic effects of theoretically promising whole-foods, given this literature base has historically focussed on isolated protein sources, which comprise a much smaller proportion of real-life diets [38].

Another key consideration for highlighting the need to study the make-up of protein-rich whole-foods rather than isolates, is that more complex foods may contain structures or small molecules that play hitherto unknown anabolic effects [39,40] not characteristic of simpler, isolated proteins. A clear example highlighting how only comparing macro-nutrient level profiles of foods can lead to oversimplified comparisons was recently provided by the comparison of energy and protein matched boluses of grass-fed beef versus plant-based beef alternatives [20], where the authors reported drastically different small nutrient profiles. Extending on this observation, we report that despite being well matched on a protein level, egg whites, acting as a less nutrient dense, more isolated protein source, displayed a narrower, perhaps *simpler* metabolite profile from those targeted, compared to the whole-food sources. The poly-unsaturated fatty acids arachidonic acid and linoleic acid were amongst the compounds that differed most across (cooked) food sources (Table 3), and most abundant in salmon. Arachidonic acid is incorporated into cell membrane phospholipids which, in response to mechanical loading, is then released to the cytosol and promotes mTOR signalling to stimulate (muscle) protein synthesis [41]. This has been shown in cell culture models [42,43] and in human work with chronic supplementation [23]. Therefore, further (such as recent work by Paulussen [44]) human studies looking at the role of specific fatty acids and fish ingestion on measures of muscle anabolism are clearly warranted.

A principal component analysis (PCA) approach revealed that succinic acid was responsible for the largest variance between cooked food sources, with the greatest abundance found in salmon. Succinate, which is converted from succinic acid, has been identified to play a role in regulating skeletal muscle remodelling [21]. β-alanine was highly abundant within our dataset; a compound largely synthesised in the liver of living organisms, and therefore predominantly found in meat [45] which was in line with our data (most abundant in pork and salmon) (Fig. 3.). β-alanine is a well-established sports supplementation

strategy [45,46] by virtue of it being the rate-limiting precursor for the synthesis of carnosine, a key intracellular buffer permitting sustained high intensity contractions [47]. Together with various other compounds more abundant in muscle derived protein sources (i.e. animal), such as glucose-6-phosphate, there is rationale that such profiles may offer advantages to muscle metabolism when consumed habitually that have commonly been ascribed to protein [48].

Similarly, however, the non-animal derived foods we examined also have some characteristic profiles in common, which may also offer benefits to supporting tissue remodelling. It has been well-documented that non-animal based diets typically possess higher fibre and various bioactive (phyto)nutrients, all of which have been attributed to beneficial effects of such diets on cardio-metabolic health [49,50]. Here, we report that the non-animal foods were also higher in the polyamine putrescine (see Fig. 3), although salmon also represented an abundant source. Sufficient systemic polyamine levels have been implicated within the regulation of muscle mass in studies of atrophy [51] and hypertrophy [52], though the responsible mechanisms are not well understood. These examples highlight the complexity of how various compounds exert their effect, either directly or indirectly, to maintain physiological metabolic processes. Therefore, when comparing animal vs. non-animal diets, especially when high in protein, it is challenging to depict if or how these compounds are supporting adaptive responses observed in human trials [53,54]. This requires the need to investigate such metabolites in isolation to examine their influence on anabolic pathways through to phenotypic adaptations.

Human nutritional physiology research looking at (muscle) protein metabolism usually disregards the role of food processing and/or cooking; for example, by providing simple isolated protein drinks or detailing nutritional information of an uncooked version of the experimental food. However, the processing of food, such as high pressure extrusion [55], hydrolysis[56] or fortification[57] can all alter the structure and/or human bioavailability of protein within food. Heat treatment (during cooking)[58,59] is the most conspicuous example. To illustrate, cooking has been shown to improve the bioavailability of protein attributed towards a denaturation of larger proteins [60]; however, it remains to be investigated how the cooking affected the nutrient profile beyond total protein (amino acids). We were able to generically, and within each food source, investigate the impact of sous vide cooking on the small molecule profiles of the experimental foods. Interestingly, animal derived sources (whole egg; 4 / 24, salmon; 5 / 24, pork; 6 / 24) generally had the lowest number of metabolite changes with cooking compared with non-animal (lentils; 12 / 24 and mycoprotein; 13 / 24). This may be attributable to the rigid cell walls of non-animal sources that, when softened by cooking, can increase the permeability of the cell wall to improve the availability of essential nutrients that were previously contained within the raw matrix. In contrast, a potential major effect of cooking animal derived sources is the denaturing of complete proteins, rather than increasing the availability of metabolites within its structure. For example, one of the most abundant compounds, also found to increase ~ 2-fold in lentils with cooking was myo-inositol, a sugar alcohol, with a similar structural resemblance to glucose[61]. This provides a clear example of the metabolite changes that occur as a result of cooking, leading to an increased availability of certain nutrients. In combination with a sous-vide method of cooking, we can be confident that the changes

observed in metabolite abundance are predominantly from the effect of heat treatment rather than nutrient loss, which is a major advantage compared with alternative methods of cooking (i.e. boiling) [62].

Various other compounds implicated in the regulation of tissue mass were not identified within the current dataset which provides some limitations for our insight. For example, we wished to determine dileucine, a dipeptide suggested as a potent anabolic trigger in human muscle [63]; however, using our GC-MS approach this dipeptide method fragmented and therefore was not identifiable. Other profiling methods (e.g. [64], whether targeted or untargeted, may be able to overcome this obstacle for key compounds of future importance. However, worthy of note, the use of gas chromatography (as opposed to liquid chromatography) mass spectrometry was advantageous for this work due to the ability to detect fewer polar compounds (e.g. fatty acids; [65]. Another consideration is the pork used in this study was provided by an organic breeding system which, compared to conventional production, produces pork meat cuts with a greater content of amino acids [66]. Hence, factors such as the type of farming [67–69], time of year food is harvested[70] and other differences across food sources may have influenced the outcomes of this work.

Within the present investigation we have identified compounds of interest that have been recognised to contribute to metabolic processes such as the regulation of human tissue protein mass. The data presented displays diverse metabolite profiles between food sources, alongside demonstrating the effect cooking (sous-vide) on the metabolite profile. This work moves beyond typical food labelling, with a deeper dive into potential anabolic metabolites within a variety of animal and non-animal derived whole-food sources that could be investigated in future work in relation to their anabolic potential *in vivo* in humans.

Abbreviations

muscle protein synthesis (MPS), muscle protein breakdown (MPB), mammalian target of rapamycin (mTOR), gas chromatography – mass spectrometry (GC-MS)

Declarations

Acknowledgments

Firstly, the authors would like to acknowledge Premier Analytical Services for analysing the nutritional composition of the experimental foods.

Contributions

The authors' contributions were as follows: Conceptualisation, F.A.D.H and B.T.W; methodology, D.S and J.B; formal analysis and data curation, F.A.D.H and D.S; Statistical analysis, F.A.D.H.

T.J.F and H.E.T were employees at Marlow Foods, financial funders of F.A.D.H Ph.D studentship. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. All authors reviewed the manuscript.

Data availability

All data generated or analysed during this study are included in this published article and its supplementary information files. Correspondence and requests for additional materials should be addressed to F.A.D.H

Funding declaration

This work was supported by the financial funders of F.A.D.H Ph.D studentship; Marlow Foods, Stokesley, UK.

Competing interests

The authors declare no competing interests.

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Figures

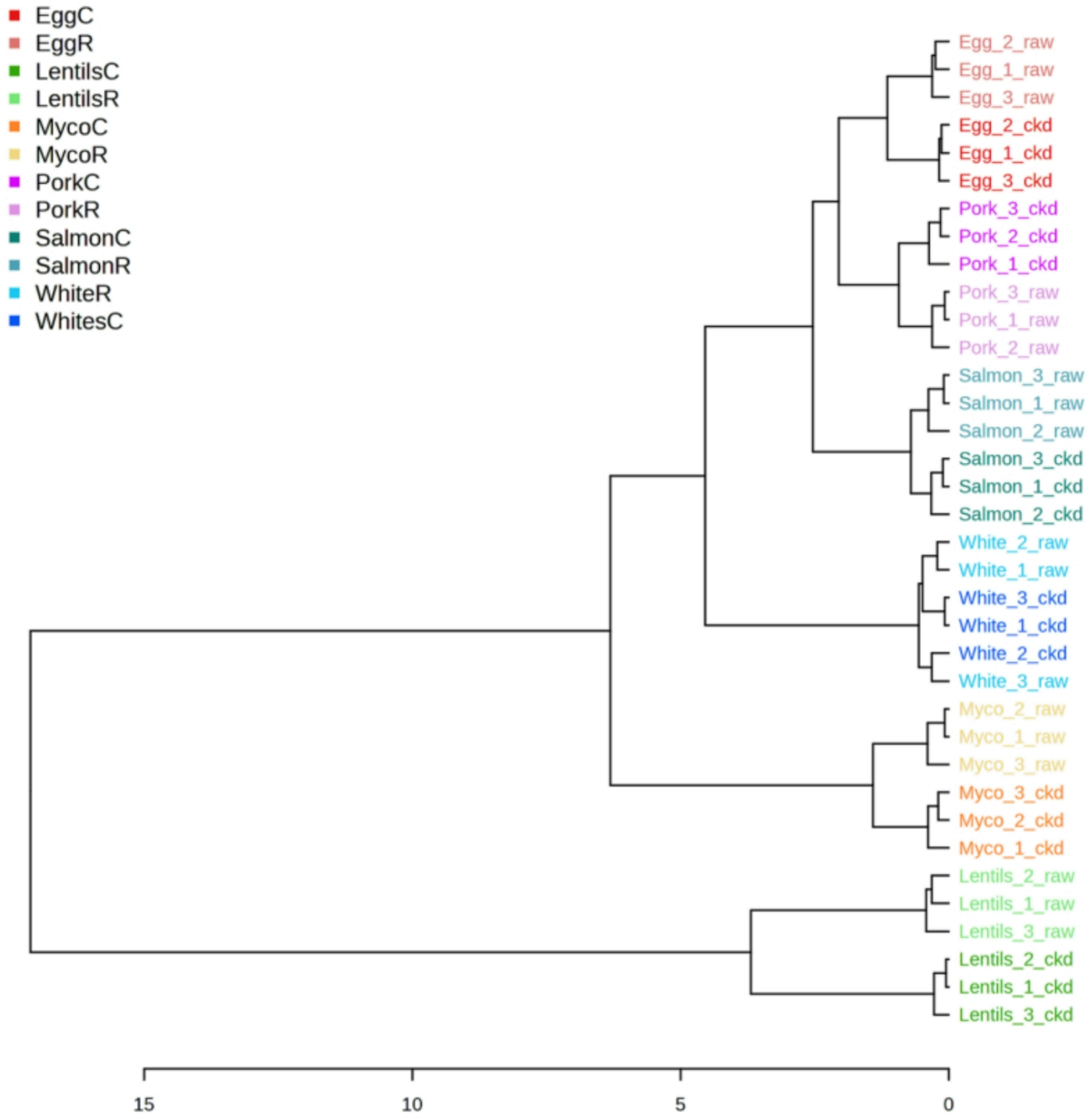

Figure 1

Hierarchical clustering dendrogram of raw and cooked food sources (distance measure using spearman, and ward clustering algorithm). Ckd, cooked, 1,2,3 represents each replicate. 0 – 15 scale is a representation of relatedness.

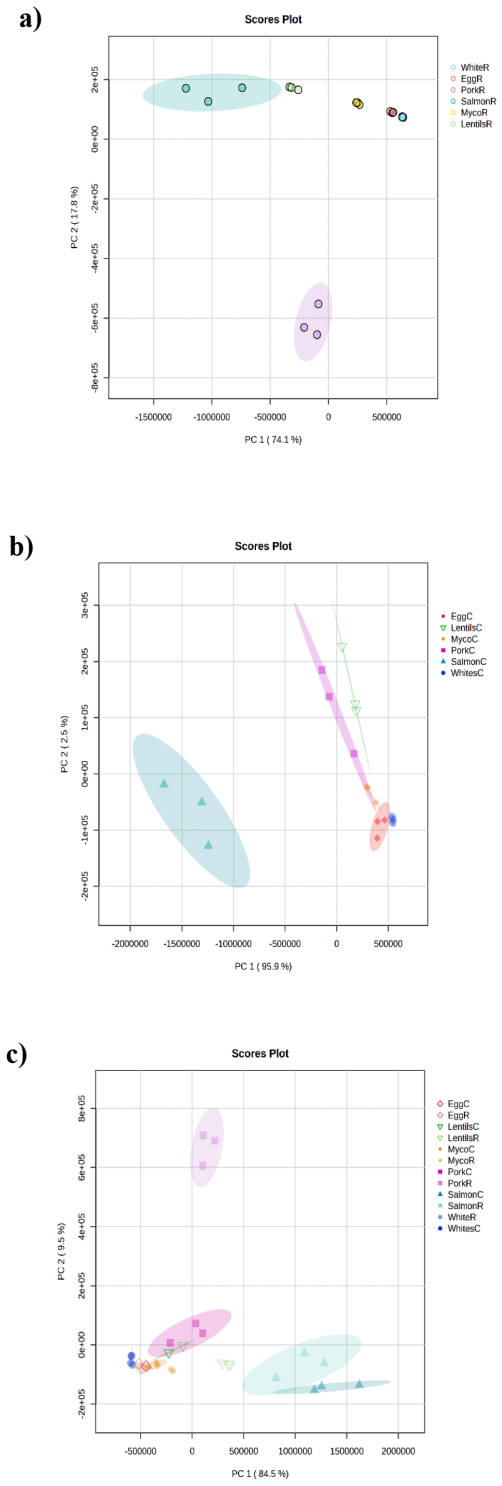

Figure 2

Plots of principal component analysis results for GC-MS data obtained for egg whites, egg, mycoprotein, lentils, salmon and pork. **a)** raw food sources: a distinct difference in salmon and pork compared to the other raw sources, with 74.1% of the variance explained within the first principal component one (PC1). **b)** cooked food sources: the metabolite composition of salmon, lentils and pork in the cooked form showed differences to egg whites, whole egg and mycoprotein, with 95.9% of the variance explained

within PC1. **c)** raw and cooked food sources: a distinct difference in metabolite composition between the raw and cooked form of pork, lentils, and salmon, but not for egg whites, whole egg and mycoprotein, with 84.5% of the variance explained within the PC1. In **Fig2c**, Open shapes show raw (R) form of food, filled shapes show cooked (C) forms of food. PC1 reveals the most variation, whilst principal component two (PC2) reveals the second most variation. Differences among clusters along PC1 are larger than similar looking distances along PC2.

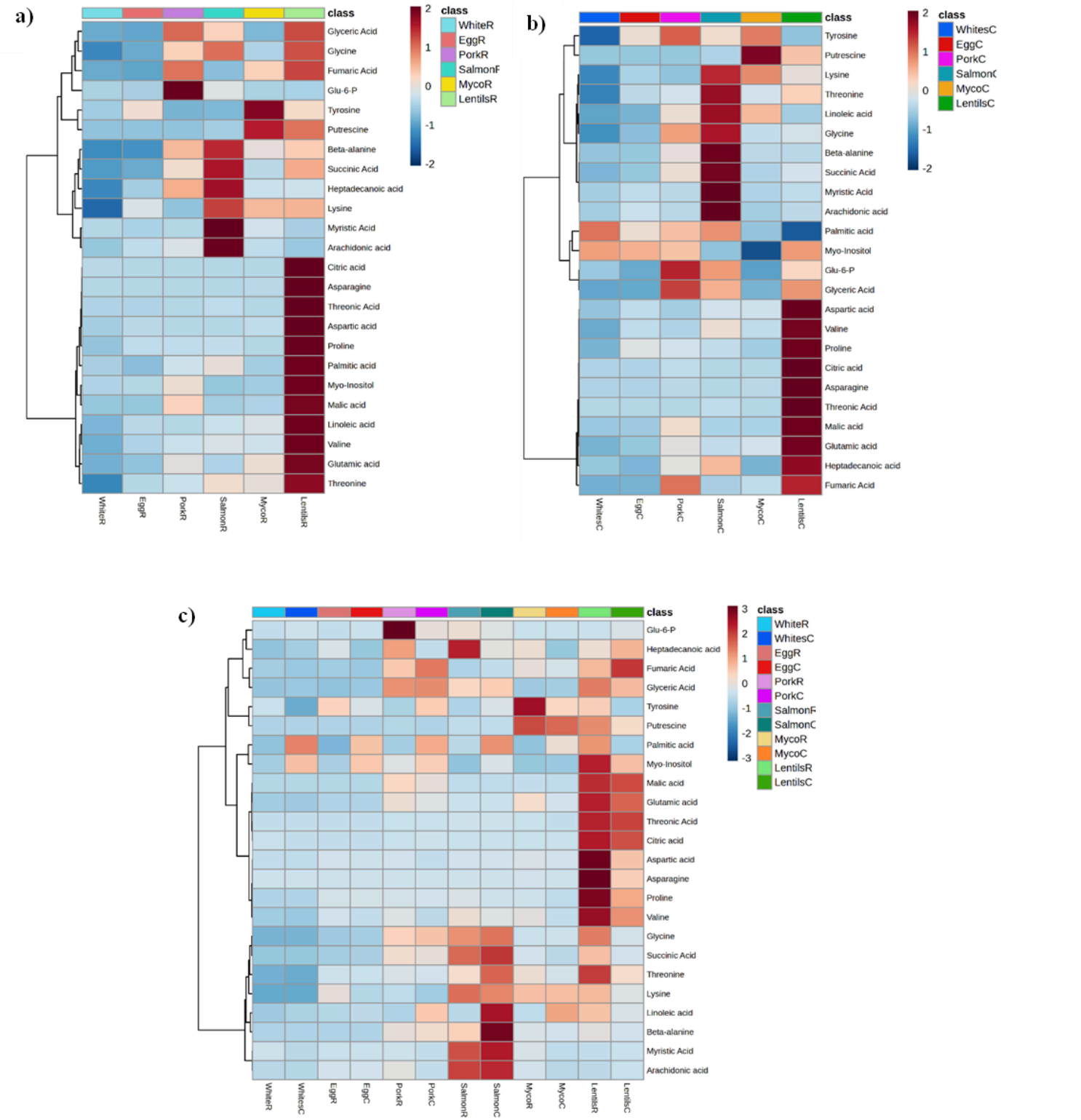

Figure 3

Metabolomics revealed distinct differences in metabolite profiles between all food sources. **a)**Heatmap of GC-MS metabolomic analyses of raw foods, **b)** cooked foods, **c)**raw and cooked foods. Red (intensity ranges from 0 to 2 (a+b) and 0 to 3 (c) means higher abundance of the corresponding metabolite, whereas blue means lower abundance (intensity ranges from 0 to -2 (a+b) and 0 to -3 (c). The food sources below the heatmap represent the of average of the three replicates shown in the dendrogram in Figure 1 for raw (R) and cooked (C) forms. A list of the top 10 most influential metabolites are reported in Table 2.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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- SupplementaryMaterial2RetentionTimes.xlsx
- SupplementaryMaterial3DifferencesbetweenSources.xlsx