Untargeted metabolomics to reveal red versus white meat-associated gut metabolites in a prudent and Western dietary context

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Abstract

Scope: To improve understanding of the epidemiological link between red and processed meat consumption and chronic diseases, more insight in the formation of metabolites during meat digestion is warranted.

Methods and results: Untargeted MS-based metabolomics was applied to explore the impact of red and processed meat consumption (compared to chicken), combined with a prudent or Western dietary pattern. A pig feeding study (n=32), as a sentinel for humans, was conducted in a 2×2 factorial design for four weeks. The luminal content of the small intestine and colon of the pigs were collected to determine their metabolic fingerprints. Seventy-six unique metabolites (38 in small intestine, 32 in colon, and 6 in both intestinal compartments) contributing to the distinct gut metabolic profiles of pigs fed either chicken or red and processed meat were (tentatively) identified. Consumption of red and processed meat resulted in higher levels of short- and medium-chain acylcarnitines and 3-dehydroxycarnitine, irrespective of dietary context, whereas long-chain acylcarnitines and monoacylglycerols were specifically associated with the red and processed-Western diet.

Conclusion: The identification of red and processed meat-associated gut metabolites in this study contributes to the understanding of meat digestion in a complex but controlled dietary context and its potential health effects.

1. Introduction

Epidemiological studies show that a high consumption of red and especially processed meat is a risk factor for various chronic diseases such as colorectal cancer, type 2 diabetes and cardiovascular diseases,[1] whereas no such associations have been reported for white meat.[2] Although possible mechanisms explaining this association have been hypothesized, a complete understanding is still lacking. The proposed factors involve intrinsic meat characteristics (e.g. heme-Fe, N-glycolylneuraminic acid content and fatty acid composition) and compounds formed during meat processing and/or gastrointestinal digestion (e.g. N-nitroso-compounds, heterocyclic aromatic amines, polycyclic aromatic hydrocarbons, trimethylamine-N-oxide, lipid and protein oxidation products).[3] Untargeted metabolomics of biofluids collected in interventions with meat is therefore a promising approach to gain more insight in the metabolic effects of meat digestion. Up until recently, metabolomics studies investigating putative biomarkers related to exposure and/or effect of meat intake mainly focused on urine and plasma. In these studies, creatinine, creatine, carnitine, acylcarnitines, carnosine, taurine, 1-methylhistidine, 3-methylhistidine and trimethylamine-N-oxide (TMAO) were frequently revealed as important metabolites linked to meat intake.[4-9] In addition to urine and blood, the use of feces as a matrix for metabolomics is increasing as the fecal metabolome provides a powerful snapshot of the reciprocal interaction between diet, host and gut microbiome.[10] The high metabolite coverage[11] and the direct contact between intestinal tissue and its content are additional arguments to include intestinal fluids as a matrix in metabolomics research when investigating dietary related gastrointestinal disorders. In this regard, Rombouts et al. applied metabolomics on *in vitro* colonic digests of beef and chicken, and identified 3-dehydroxycarnitine, tryptophan-derived metabolites and dityrosine as red meat related metabolites that could potentially be involved in red meat-associated diseases.[12]

The human diet contains many interacting compounds that may affect health in a multi-causal fashion. For example, fruits, vegetables, whole grains and dairy products show protective effects against several chronic diseases.[1] Studying the effects of isolated nutrients or food items may disregard overall effects caused by substitution, synergistic and/or antagonistic effects between nutrients within a complex diet.[13] In this regard, a prospective cohort study demonstrated that the association between red meat intake and colorectal cancer depends on the dietary antioxidant capacity.[14] Therefore, not only the differences between red and processed meat and white meat, but also the dietary context should be considered to gain more insight in the association between red and processed meat consumption and chronic diseases. The interest in metabolomics in nutritional epidemiology is growing as recent studies also show the potential of metabolomics to evaluate dietary patterns and relationships between the latter and diseases.[15, 16] Most metabolomic studies distinguish between a Western (or unhealthy) dietary pattern, high in refined sugars and fat, and low in fibers and antioxidants, and a prudent (or healthy, plant-based) one, low in refined sugars and fat, and high in fibers and antioxidants.[17-19]

Using pigs in nutrition studies offers the advantage of feeding strictly controlled diets to young piglets, thereby minimizing variation inherent to genetics, microbial community and environmental conditions. Several studies demonstrated the potential of pigs as a model for human metabolic studies in food research, as very similar postprandial responses in blood metabolome[20] and similar changes in microbial composition between the two species were found upon dietary intervention.[21] In this study, metabolomic fingerprinting of small intestinal and colon digest samples of pigs fed human diets, using an untargeted mass spectrometry based approach (UHPLC-HR-Q-Orbitrap-MS), was applied to explore the impact of red and processed meat versus chicken meat consumption in the context of a

prudent and Western dietary pattern. We hypothesized that the dietary context can affect the formation and/or consequence of potentially harmful red meat-associated metabolites.

2. Experimental section

2.1 Study design

The animal study was approved by the Ghent University Ethical Committee at the Faculty of Veterinary Medicine (EC 2016/26) and was previously described in detail.[22] Briefly, thirty-two piglets (8 piglets per treatment) of 5 weeks old (7.00 ± 0.88 kg) were subjected to a feeding experiment in a 2×2 factorial design with four dietary treatments: 'chicken-prudent', 'red&processed-prudent', 'chicken-Western' and 'red&processed-Western' (Table 1). The diets were formulated to mimic realistic human diets and to provide an equal meat intake per day. The red and processed meat mixture contained 62% fresh meat (mainly pork and beef) and 38% red processed meat (mainly cooked ham, filet de sax, salami and smoked bacon), whereas chicken meat was a combination of chicken thighs, breasts and chicken skin. The Western background diet was characterized by high amounts of refined grains, desserts and sweets, whereas the prudent background diet had high amounts of fruits, vegetables, whole grains and dairy products. The piglets were fed three meals per day (8, 12, 18h) during 30 min *ad libitum* using an individual feeding system and were weighed twice a week. The average feed intake and body weight during the experimental feeding period are illustrated in Supporting Information Figure S1. The average daily energy intake of the pigs did not differ significantly between the four treatment groups, but due to the lower energy density of the prudent background diet, the average daily feed intake of the prudent diets (1268 ± 204 g/day) was higher compared to the Western diets (905 ± 81 g/day). At the end of the 4-week experiment, piglets were euthanized 2 hours after receiving a last meal, and the luminal contents of the small intestine (duodenum, jejunum and ileum together) and colon were

collected separately. Weight and dry matter of the collected luminal contents are provided in Supporting Information Table S1. The collected samples were gently homogenized, lyophilized and stored at -80 °C until analysis. It should be noted that most piglets, regardless of dietary treatment, suffered from diarrhea during the second week of the experimental feeding period, but recovered quickly. Yet, two piglets fed the chicken-Western diet still presented diarrhea during sampling, and therefore, those colon samples were not included in the analysis.

2.2 Dosage information

The dosage and the equivalent in humans, and administration details of the diets were described in Goethals et al.[22] The amount of meat in the diets corresponded with a daily intake of 290 g meat for humans. This amount of meat was based on the results from the Belgian Food Consumption Survey 2004,[23] that reported a daily intake of 291 g of 'meat, fish, eggs and meat alternatives' at the 97.5 percentile of the Belgian population older than 15 years. Combined with the other food items, this fraction of meat corresponded to 21.5% of the total energy of the diets (Table 1).

2.3 Extraction and polar metabolomic profiling with UHPLC-Orbitrap-HRMS

The extraction protocol and subsequent UHPLC-Orbitrap-HRMS analysis procedure was developed and validated by Vanden Bussche et al.[24] First, 100 mg of the lyophilized and homogenized luminal content of the small intestine and colon were resolved in 2 mL ultrapure water and 12.5 µL internal standard (valine-d8, 100 ng/µl) was added. After mixing, 0.5 mL of an ice-cold methanol/ultrapure water mixture (80/20, v/v) was added. This mixture was vortexed, centrifuged (13300g, 10 min) and the supernatant was filtered through a polyamide filter (0.45 µm). Finally, the filtrates of the luminal content of the small intestine and colon were diluted with ultrapure water using a 1/9 and 1/1 dilution (filtrate/water, v/v),

respectively. The dilution factors were determined in a preliminary dilution experiment with 9 dilution factors ranging from undiluted to 1/500 on pooled samples from small intestine and colonic digests separately. Metabolite coverage using an untargeted approach as well as linearity and peak shape of 25 targeted metabolites from an in-house database were assessed to select the optimal dilution factor. An Accela UHPLC system of Thermo Fisher Scientific (San Jose, CA, USA) equipped with an Acquity HSS T3 C18 column (1.8 µm, 150 mm × 2.1 mm, Waters) at 45 °C and a vanguard precolumn (1.8 µm, 5 mm × 2.1 mm, Waters) was used for chromatographic separation. A binary solvent system with ultrapure water (A) and acetonitrile (B) both acidified with 0.1% formic acid was used at a flow rate of 0.4 mL/min. A gradient profile with the following proportions (v/v) of solvent A was applied: 0−1.5 min at 98%, 1.5−7.0 min from 98% to 75%, 7.0−8.0 min from 75% to 40%, 8.0−12.0 min from 40% to 5%, 12.0−14.0 min at 5%, 14.0− 14.1 min from 5% to 98%, followed by 4.0 min of equilibration at initial conditions. MS analysis was performed on the Exactive stand-alone benchtop Orbitrap MS (Thermo Fisher Scientific, San Jose, CA, USA), preceded by heated electrospray ionization (HESI), operating in polarity switching mode. Ionization source working parameters were set to a sheath, auxiliary, and sweep gas of 50, 25, and 5 arbitrary units (au), respectively, heater and capillary temperature of 350 and 250 °C, and tube lens, skimmer, capillary, and spray voltage of 60 V, 20 V, 90 V, and 5 kV (±), respectively. A scan range of m/z 50−800 was chosen, and the resolution was set at 100 000 full width at half maximum at 1 Hz. The automatic gain control (AGC) target was set at balanced (1 × 106 ions) with a maximum injection time of 50 ms. Quality control (QC) samples were prepared by combining small aliquots of the samples of each piglet. Column conditioning was performed by injecting the QC samples 6 times prior to analysis of the analytical samples, which were analyzed in random order. Following a series of 10 analytical samples, 2 QC samples and a blank (100% ACN) were injected to assess instrument performance. Also, an

external standard mixture of ca. 300 gastrointestinal metabolites was injected at the beginning and end of the analytical batch to monitor instrument performance.[24] Analyses of the luminal content of the small intestine and colon were performed in two batches.

2.4 Untargeted chemometric data analysis

HRMS data, obtained in polarity switching mode, were processed with Compound Discoverer™ 2.0 (Thermo Scientific) to perform noise filtering, baseline correction, spectra alignment, peak detection and quantification, and spectral deconvolution. A blank sample (100% ACN) was used for background subtraction and noise removal. The following parameter settings were applied: retention time window: 0.5 - 16 min; *m/z* range: 53.4 – 800 Da; peak intensity threshold: 500,000 arbitrary units; maximum retention time shift: 0.5 min; *m/z* width: 5 ppm. The CV of the QC samples injected after a series of 10 analytical samples were calculated for each metabolite and only those metabolites with a CV below 30% were retained.

Subsequently, regression analysis and predictive modelling were performed, thereby using principal component analysis (PCA) and (orthogonal) partial least-squares discriminant analysis ((O)PLS-DA) by means of R packages *pcaMethods* and *ropls*. Log-transformation and Pareto scaling were first implemented to induce data normality and standardize the features' intensity range. PCA models were created to visualize trends and possible outliers, whereas PLS-DA and OPLS-DA models were used to construct a prediction model that could explain and predict one Y-variable (dietary treatment) from the X-matrix (abundances of gut metabolites). PLS-DA models were constructed to compare the four treatment groups, whereas OPLS-DA models were used to retain discriminating metabolites between two groups. The number of predictive compounds in the latter two models were extracted applying autofit. PLS-DA and OPLS-DA models were evaluated by inspection of the R^2X

and R^2Y (goodness-of-fit) and Q^2Y (goodness-of-prediction, based on cross-validation with seven segments) model characteristics and by comparison of the root mean squared error of estimation (RMSEE) of the permuted (n=100) and the real model.

Since OPLS-DA models are not suited for multiple comparisons or interaction effects, the R package *limma* was applied to reveal statistical interaction effects between the type of meat and background diet.[25] The lmFit function in *limma* was used to fit linear models. Empirical Bayes moderated t-statistics and associated Benjamini Hochberg adjusted p-values (=q-values) and (non-adjusted) p-values were computed for the contrasts of interest, namely the main effects meat and background diet and the interaction effect meat×background diet. Metabolites differing between two groups (chicken versus red and processed meat, and prudent versus Western background diet) with variable importance in projection (VIP) scores >1 (obtained from the OPLS-DA models) and q-values <0.05 (obtained from *limma*) were retained. For the interaction effect meat×background, a less stringent selection criterium, based on the (non-adjusted) p-values in *limma*, was used ($p < 0.05$). A workflow illustrating the selection of the metabolites was provided as Supporting Information Figure S2.

2.5 Metabolite identification

Identification was performed for those metabolites differentiating between the two meat sources and metabolites with meat×background diet interaction effects. Tentative identification was based on accurate mass (molecular ion and C-isotope profile) and MS/MS fragmentation patterns. Experimental MS/MS fragmentation spectra of the metabolites of interest were generated by Q-Orbitrap Exactive™ MS (applying full-scan and parallel reaction monitoring scan events). Inclusion lists of parent ions with their associated accurate masses of $[M+H]^+$ or $[M-H]^-$ and expected retention times were constructed. Data-dependent fragmentation of the selected parent ions used the following MS/MS settings: resolution of

17,500 FWHM; AGC target of $2e^4$; maximum injection time of 40 ms; isolation window of 2.0 *m/z* and normalized collision energy at 20, 30 or 40 eV. More details on the instrumental settings of the UHPLC-Quadrupole-Orbitrap HRMS analysis are described in De Paepe et al.[10] The experimental fragmentation patterns were implemented in open-source SIRIUS (CSI:FingerID)[26] and Metfrag (http://msbi.ipb-halle.de/Metfrag) software to generate information about the identity of the metabolites by computing fragmentation trees and by matching the experimental fragmentation patterns with reference spectra of databases, notably the Human Metabolome Database (http://www.hmdb.ca/). Subsequently, fragmentation patterns and retention times of the metabolites were compared against commercial standards if available (Supporting Information Table S2). The recommendations for standard metabolite identification from the Chemical Analysis Working Group were followed.[27] Therefore, metabolites were described as identified metabolites by matching masses and retention times with authentic standards (Tier 1), putatively annotated metabolites by matching MS/MS spectra with library spectrum data (Tier 2), or putatively characterized metabolite class by spectral similarities to a similar compound class and knowledge from previous literature (Tier 3).

Next to the metabolites that were revealed by the untargeted chemometric analysis, some additional metabolites based on literature and the generated results were examined for their presence and significance, and were included in the list of (tentatively) characterized metabolites. All (tentatively) characterized metabolites were manually processed with XCalibur 2.1 and area ratios were obtained through normalisation of the peak intensities based on the associated QC samples. These area ratios were used to construct heat maps with R package *made4* and subjected to univariate analysis. Mixed model ANOVA with meat, background diet and meat×background diet as fixed effects, and litter, pen and euthanization

day as random effects was applied with SAS Enterprise Guide 7. P-values <0.05 were considered statistically significant.

2.6 Pathway analysis

The *mummichog* algorithm, [28] which bypasses the bottleneck of metabolite identification, was used to evaluate altered biochemical pathways associated with meat intake. Based on *m/z*, p-values and statistical scores obtained in *limma* comparing chicken versus red and processed meat of all metabolites of the small intestine and colon digests, a likelihood list of affected pathways (p-value <0.05) was deduced. The following settings were applied: mass accuracy: 5 ppm; analytical mode: positive; p-value cutoff: 0.05; library: Homo sapiens [MFN].

3. Results and discussion

3.1 Dietary induced changes in the gut metabolome

Data processing with Compound Discoverer™ resulted in the detection of 1851 and 1990 metabolites for the small intestine and colon digests, respectively. The PCA-X score plots (Figure S3, Supporting Information) confirmed an accurate instrumental performance by the close clustering of the QC samples and showed a clear separation of the piglets' samples according to background diet but not meat type. Score plots of PLS-DA models of small intestine and colon comparing the four dietary treatment groups are presented in Figure 1. In both PLS-DA models, PC1 discriminates between the background diets (explaining 17.1% and 15.7% of the variance), whereas PC2 discriminates between meat type (explaining 7.1% and 9.6% of the variance). The PCA-X and PLS-DA models and the number of differentiating metabolites (Table 2) indicate a higher impact of the different background diets in comparison with the meat types on the intestinal metabolome of the pigs. About 16%

of the measured metabolome was significantly different comparing the two background diets, whereas only 3.5% to 5% was influenced by the type of meat. This could be expected because of the more contrasting composition of the background diets compared to the two meat types. Based on the same selection criteria (q-value <0.05), no significant metabolites were found for the interaction effect meat×background diet. However, by using a less stringent criterium (non-adjusted p-value <0.05), 260 metabolites (95 in the small intestine and 165 in the colon metabolome) were retained for this interaction effect (Figure S2, Supporting Information). Nonetheless, many of these metabolites (n = 159) showed an overlap with the already retained meat- or background diet-associated metabolites. Retention time and monoisotopic mass of metabolites resulting from multivariate statistics and differing between chicken versus red and processed meat, and of metabolites with an interaction effect meat×background, are provided in Table S3-S6, Supporting Information.

3.2 Identification of differentiating metabolites

In digests of small intestine and colon respectively, 44 and 38 meat-associated metabolites mainly resulting from multivariate statistics, but also few originating from literature and results obtained in one of the two intestinal compartments were subjected to univariate statistics and (tentatively) identified (Tables 3 and 4). Six of those metabolites namely 3- (or 1-)methylhistidine, L-carnitine, C3-carnitine, C4-DC-carnitine, 3-dehydroxycarnitine and hydroxyprolyl-leucine, were present (and modulated by meat intake) in both matrices, so 76 unique metabolites were (tentatively) characterized. Additional information on the metabolites such as retention time, scores from multivariate statistics, most abundant fragment ions, CSI:FingerID similarity and Metfrag scores is available in Supporting Information Tables S7 and S8. The identity of 17 of the 76 metabolites could be confirmed with authentic reference standards (Tier 1). Retention times, most abundant fragment ions

and suppliers of the standards are available in Supporting Information Table S2. The other 59 metabolites were retained as putatively characterized metabolites based on good CSI:FingerID and Metfrag scores, previous annotation of certain metabolites in relation to meat in literature, and/or the putative identification of multiple compounds of the same classes.

Heat maps of the (tentatively) characterized metabolites in small intestinal and colon digests of the piglets are presented in Supporting Information Figures S4 and S5.

3.2.1 Metabolites associated with red and processed meat intake

L-carnitine, short- and medium-chain acylcarnitines, lysophosphatidylcholines, carnosine, 3-dehydroxycarnitine, hydroxyprolyl-leucine, and some linoleic acid derivatives contributed to the distinct metabolic profile related to red and processed meat consumption. Interestingly, higher abundances of long-chain acylcarnitines and monoacylglycerols were only found following consumption of red and processed meat in combination with the Western background diet. Potential involvement of the (putatively) identified red and processed meat-associated metabolites in chronic diseases is further discussed below.

Acylcarnitines are formed during mitochondrial fatty acid oxidation. Plasma acylcarnitine profiles are therefore indicative of metabolic state, and disturbances in the relative composition have been linked with mitochondrial dysfunction and tissue damage,[29] as well as with insulin resistance, even though the causative link is not clear yet.[30] For example, it was demonstrated that intraperitoneal injection of C6-carnitine and C8-carnitine in mice impaired glucose tolerance, insulin tolerance and insulin secretion,[31] and that acylcarnitine profiles were characteristic for different diabetic states in mice,[32] whereas other studies also reported positive effects such as increasing insulin sensitivity upon oral supplementation with acetyl-L-carnitine in insulin-resistant subjects.[33] Several studies

identified altered acylcarnitine concentrations in plasma or urine following consumption of (red) meat,[4, 6, 8] or a high fat diet.[19, 34] Although most studies reported elevated levels of acylcarnitines, different responses can be observed for short-, medium and long-chain acylcarnitines. However, caution in the interpretation of acylcarnitine profiles in biofluids is warranted, as the study of Schooneman et al. demonstrated that plasma acylcarnitine levels do not reflect tissue levels.[35] In addition, the exact physiological role of acylcarnitines ingested through the diet or formed during gastrointestinal digestion, should be further investigated. Present acylcarnitine profiles reflect dietary intake of carnitine, originating from red meat, and dietary fatty acid composition. Pigs fed the red&processed-Western diet contained higher levels of medium- and especially long-chain acylcarnitines in their small intestines, presumably explained by their concurrent high presence of medium- and especially long-chain fatty acids and carnitine in their diets (Supporting Information Table S9). Acylcarnitines in the intestinal lumen have been hypothesized to originate from intestinal tissue release, intraluminal esterification, or microbial production,[36] or through the involvement of the enterohepatic cycle.[37] Whereas most acylcarnitines were detected in the luminal content of the small intestine and no longer in the colon, 3-dehydroxycarnitine was present in both gastrointestinal compartments, but more abundant in the colon. 3-Dehydroxycarnitine is a known fecal metabolite produced from carnitine by gut microbes,[12, 38] whereas TMAO is the urinary counterpart of carnitine degradation originating from microbial conversion of carnitine to trimethylamine (TMA), either or not through 3-dehydroxycarnitine formation, followed by the hepatic conversion into TMAO.[39]

Lysophosphatidylcholines, detected at higher abundances following red and processed meat intake in the present study, can also serve as precursors for TMAO formation.[40] Although TMAO is also found at elevated levels following fish consumption,[5] which is generally associated with lower cardiometabolic risk, the formation of TMAO is one of the

proposed mechanisms by which red meat is linked with cardiovascular diseases through the acceleration of atherosclerosis.[39, 41] It remains unclear however if TMAO comprises a causal agent or merely serves as a biomarker for an underlying phenomenon, since circulating TMAO can be confounded by many factors, among which kidney function and colonic microbial composition.[42] Besides a link with atherosclerosis, the levels of TMAO and lysophoshatidylcholines have also been linked to colorectal cancer development, as plasma TMAO was positively associated with rectal cancer risk in postmenopausal women in a case-control study,[43] and lysophoshatidylcholines were found to be increased in colorectal tumor tissue.[44] However, the favorable health effects of the conditionally essential nutrient carnitine should be emphasized as well. Indeed, carnitine supplements have been shown to exert beneficial health effects in patients with severe cardiovascular disorders[45] and to improve insulin resistance as carnitine might prevent the accumulation of intracellular lipids, enhance glucose metabolism and exert antioxidant effects.[46]

Monoacylglycerols originate from hydrolysis of triacylglycerols. Because of the higher fat content and/or differences in lipolysis rate, more monoacylglycerols could be expected in pigs consuming the Western diets. However, it is unclear why particularly pigs fed the red&processed-Western diet had higher levels of monoacylglycerols compared to pigs fed the chicken-Western diet. Slightly higher levels could be expected in red and processed meat compared to chicken meat since monoacylglycerols may be used as emulsifier in meat products[47] and since hydrolysis also occurs during storage and processing of meat (and especially in meat products) by microbial and endogenous lipases.[48] Nevertheless, this does not fully explain the outcome as no substantial differences in monoacylglycerols were observed between pigs consuming the red&processed-prudent and chicken-prudent diets.

Carnosine is a well-known dipeptide present in meat and has been described as a urinary marker for meat intake with higher concentrations found in response to the

consumption of pork followed by beef and chicken.[5, 9] The higher levels of hydroxyprolyl peptides (hydroxyprolyl-leucine and hydroxyprolyl-asparagine) observed in this study can likely be attributed to the higher collagen content of meat products in the red and processed meat mixture.[9]

3.2.2 Metabolites associated with chicken meat intake

Chicken meat consumption mainly resulted in higher abundances of di- and oligopeptides, many of them containing an imidazole moiety and a range of fatty acids. In combination with the Western and prudent background diet respectively, cyclodipeptides and linoleic acid derivatives were putatively characterized.

Anserine, a dipeptide with diverse biological activities (including antioxidant activity) is generally present in large amounts in chicken and was detected in the small intestine. Its hydrolysis product 3-methylhistidine is frequently suggested as a candidate urinary biomarker for chicken intake. On the other hand, 1-methylhistidine, with the same accurate mass and highly similar fragmentation pattern, reflects general meat intake but is also influenced by endogenous muscle catabolism and muscle mass.[4, 5, 49] Based on its higher abundance, particularly following chicken meat consumption, the metabolite detected both in the small intestine and colon in this study was tentatively ascribed as 3-methylhistidine.

Cadaverine and agmatine are biogenic amines that originate from food directly and/or are produced in the gut by microbial decarboxylation of respectively lysine and arginine, with many physiological functions.[50] Lithocholyltaurine is a conjugated bile salt formed in the liver from lithocholic acid and taurine, whereas taurine is an abundant amino acid present in dark meat of chicken that may play a protective role in cardiovascular[51] and gut health.[52] The putatively characterized metabolite N-(4,5-dihydro-1-methyl-4-oxo-1H-imidazol-2-yl)alanine is the keto tautomer of N-(1-methyl-4-hydroxy-3-imidazolin-2,2-ylidene)alanine

and was recently described as potential urinary marker for general meat intake.[9] Notably, urinary excretion was higher for chicken and beef compared to pork, which is in agreement with the present results since pork represented about 70% of the red and processed meat. Whereas guanidinoacetic acid has been reported as a urinary marker for chicken intake,[53] less is known about guanidinovaleric acid found in the present study. Higher abundances of cyclic dipeptides were mainly detected following consumption of the chicken-Western diet. Cyclic dipeptides can be produced by bacteria and yeasts or during thermal processing of foods, have been identified in chicken essence and beef, and exert a wide variety of biological activities including antiviral, antibacterial and antioxidant functions.[54]

3.2.3 Oxygenated lipids in the digestion metabolome of the colon

Two other classes of metabolites that were detected upon the consumption of chicken or red and processed meat, either or not modulated by the background diets, were putatively characterized as dicarboxylic acids (undecanedioic acid and dodecanedioic acid) and oxidized linoleic acid metabolites. Linoleic acid is a direct precursor of the bioactive hydroxy-octadecadienoic (HODEs) and oxo-octadecadienoic acids (oxoODEs), which have been mechanistically linked with inflammation and pathological conditions.[55] These linoleic acid derivatives can occur in food or be formed during digestion through oxidation of linoleic acid via the intermediate hydroperoxyl-octadecadienoic acid (HPODE). HPODE on its turn can be reduced to HODE but other reaction pathways also occur, resulting in the formation of among others 4-hydroxy-2-nonenal, dioxododecenoic acid (DODE) or trihydroxyoctadecenoic acid (TriHOME).[56,57] The exact identity of the oxidized linoleic acid metabolites is difficult to determine as many potential fatty acid derivatives with identical accurate mass, and very similar fragmentation profiles exist. In this study, the exact identity of the fatty acid derivatives could not be confirmed and is therefore not discussed in detail.

However, more research is warranted with respect to the dietary contribution and biological potency of these oxidized fatty acids as these may possess important health implications.

3.3 Pathway analysis

The *mummichog* algorithm uses the accurate masses of significant metabolites to investigate the probability to correspond with metabolites on a given pathway, based on *a priori* pathway and network knowledge from the Homo sapiens MFN library. The significance of the pathways is assessed using Fisher's exact test and compared with random sampling of metabolites from the total metabolite list to obtain the p-values for all pathways. Of course, the results depend on the quality of the input data and of the *a priori* pathway and network knowledge. The algorithm identified nine metabolic pathways that were significantly altered in the gut digestome comparing chicken versus red and processed meat consumption (Figure 2). The carnitine shuttle was the most significantly altered pathway followed by several amino acid pathways. The linoleate metabolism pathway was nearly significant. Most of the (tentatively) identified metabolites in the present study were related to the altered pathways proposed by this algorithm.

3.4 Study limitations

Studying meat-associated metabolites within a complex but controlled diet brings along some limitations. All food items were mixed to obtain homogenous diets in order to avoid selection of particular food items by the piglets and to obtain digest samples of a whole diet instead of one particular meal. This implicates (i) a loss of food structure, hereby also influencing digestibility and bioavailability, (ii) potential increased chemical interaction between the food

items already during preparation and storage of the experimental diets and (iii) the repeated concomitant intake of about 90 food items instead of variable and distinct meals.

The explorative study design allows to investigate the alterations in the metabolite profiles of the luminal content of the small intestine and colon following consumption of chicken versus red and processed meat across different diets. However, more research is needed to reveal if the differentiating metabolites mainly result from the food itself, or from the digestion and transformation of food-derived compounds, or from differences in physiological response. The differentiating metabolites are not necessarily specific to chicken or red meat intake and do not necessarily serve as dietary intake biomarkers.

The focus on the gut metabolome in an *in vivo* model is unique in this research area and could be responsible for the detection of the high number of acylcarnitines as differentiating metabolites following different types of meat intake. It is possible that these differences observed in the luminal content of the small intestine diminish after further digestion and absorption and have therefore not been detected to a same extent in common metabolomics matrices such as urine and blood. A multi-matrix approach including blood, urine, intestinal and tissue samples in future nutrimetabolomic studies could generate more insights in metabolite distribution, transformation, absorption and clearance effects.

Concluding remarks

This study revealed a range of gut metabolites discriminating between chicken versus red and processed meat intake across different background diets and confirmed the presence of several metabolites that had been reported to occur in urine or blood following meat intake. The metabolites were mainly related to protein degradation and lipid metabolism, pointing towards the use of proteomics/peptidomics and lipidomics platforms as promising

complementary approaches in this line of work. The differences in metabolic profiles may on the one hand be linked to compositional differences between red and processed meat and chicken meat, but may also be explained by induced changes in host metabolism or microbial composition/activity. Although the abundance of most meat-associated metabolites did not depend on the dietary context, long-chain acylcarnitines and monoacylglycerols only occurred at higher levels following the consumption of red and processed meat in a Western dietary pattern. As several of the digestion metabolites can be linked to red meat-associated diseases as reported in literature, these findings advocate in-depth research on the relevance of these metabolites in the relationship between meat consumption in its dietary context and human health.

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Conflict of interest

The authors declare no conflict of interest.

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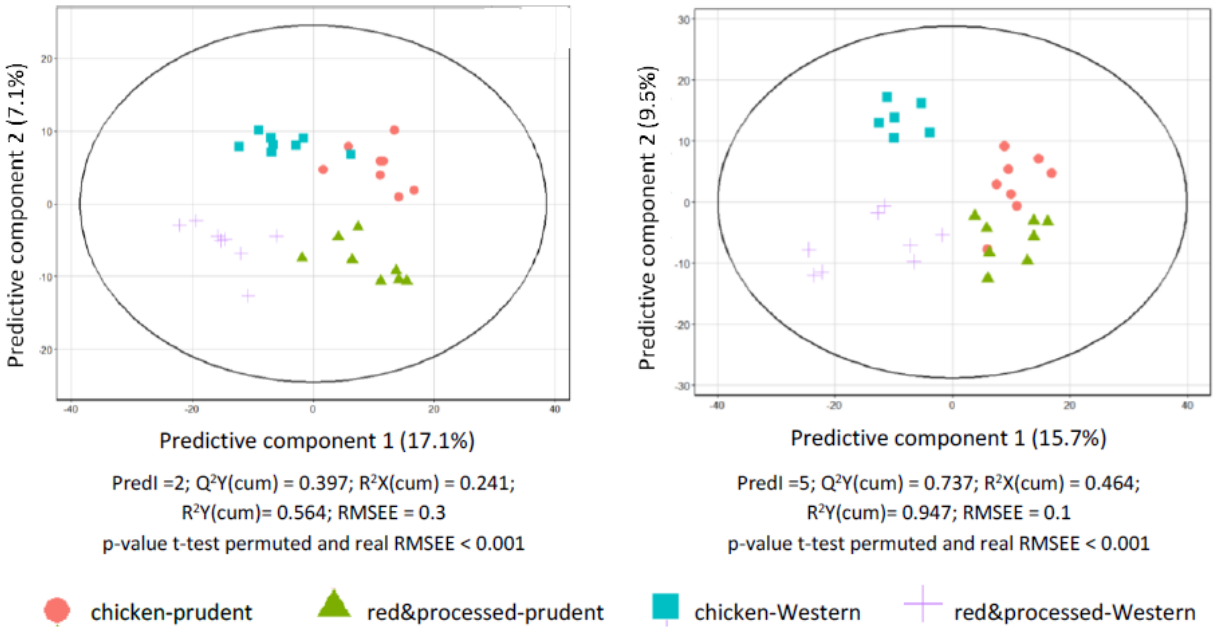

Figure 1. PLS-DA score plots demonstrating the diverging effects of the four diets on the small intestinal (left) and colon (right) metabolome. The number of predictive compounds (predI) was extracted applying autofit.

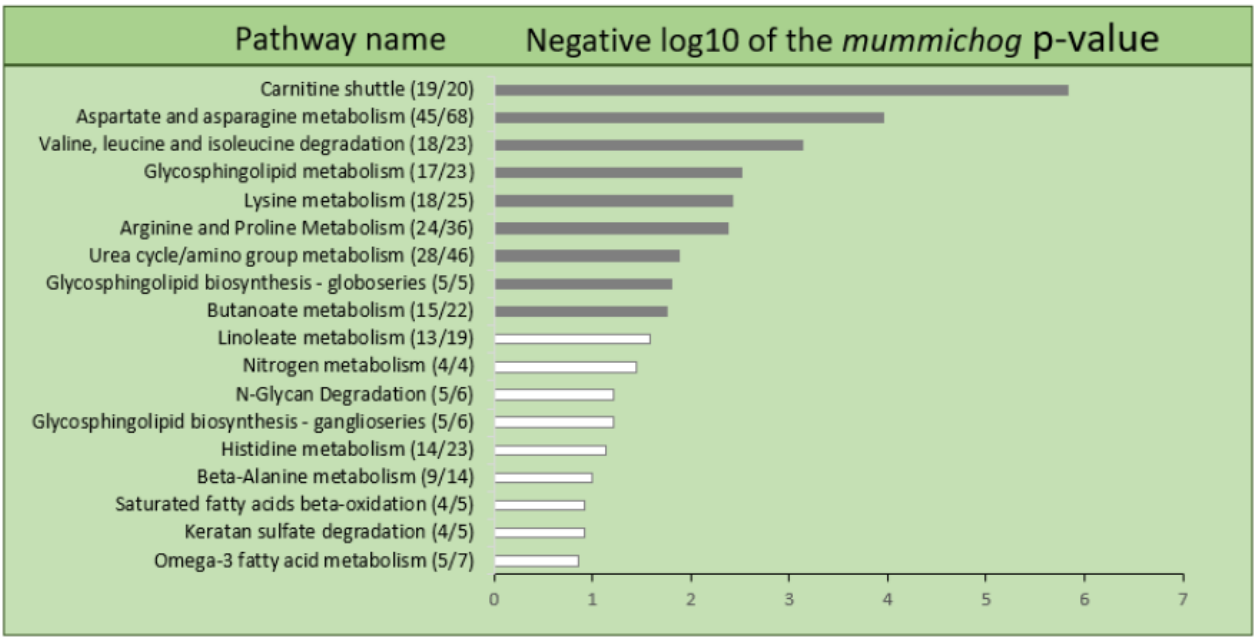

Figure 2. Nine metabolic pathways were significantly altered in the small intestinal and/or colon metabolome after the consumption of chicken versus red and processed meat (Fisher's exact test p-value ≤0.05, grey filled bars). The ratio in brackets represents the number of significant matches to the total number of matched metabolites in the pathway.

Table 1. Formulation of the four diets expressed in g/kg (fresh weight basis) and energy percentage (E%)

	prudent		Western		prudent		Western	
	chicken	R&P	chicken	R&P	chicken	R&P	chicken	R&P
	g/kg				E%			
red (62%) & red processed meat (38%)		145		197		21.5		21.5
chicken meat	145		197		21.5		21.5	
cereal products and potatoes	304	304	199	199	45.6	45.6	23.4	23.4
fruits and vegetables	299	299	173	173	10.4	10.4	4.50	4.50
dairy products and eggs	243	243	143	143	15.1	15.1	12.7	12.7
butter and cooking fats	8.62	8.62	14.3	14.3	7.06	7.06	8.54	8.54
fats and sweets	0.51	0.51	275	275	0.33	0.33	29.4	29.4

R&P = red & processed meat

Table 2. OPLS-DA model parameters and number of differentiating metabolites.

	Model parameters					Number of differentiating metabolites			
	R^2X	R^2Y	Q^2	RMSEE [a)]	p-value [b)]	chicken	red& processed	prudent	Western
Small intestine chicken *vs.* red&processed	0.379	0.993	0.749	0.044	< 0.001	26	38	-	-
Small intestine prudent *vs.* Western	0.317	0.980	0.772	0.075	< 0.001	-	-	233	66
Colon chicken *vs.* red&processed	0.270	0.992	0.780	0.049	< 0.001	82	17	-	-
Colon prudent *vs.* Western	0.412	0.996	0.859	0.035	< 0.001	-	-	193	128

[a)] RMSEE, Root Mean Squared Error of Estimation.
[b)] p-value corresponds with the t-test comparing the mean of the permuted RMSEEs with the RMSEE value of the real model.

Table 3. List of (tentatively) characterized metabolites influenced by meat intake in the small intestine (n = 8 piglets per dietary treatment).

			Average peak area ratio (SD)				p-values mixed model univariate statistics		
m/z	tentative metabolite identity	ID level	chicken-prudent	red&processed-prudent	chicken-Western	red&processed-Western	background diet	meat	meat × background diet
Chicken									
103.123	Cadaverine[b]	1	1.56(0.87)	1.07(0.84)	1.80(1.57)	0.43(0.59)	0.604	0.022	0.247
170.092	3- (or 1-)Methylhistidine[a]	3	1.83(0.78)	0.59(0.11)	1.39(0.46)	0.47(0.14)	0.103	<0.001	0.341
173.139	Acetylagmatine	3	2.53(1.52)	0.15(0.13)	2.29(2.06)	0.03(0.04)	0.725	<0.001	0.906
215.139	Valyl-Proline	3	1.71(0.39)	0.61(0.16)	1.72(0.58)	0.60(0.16)	0.999	<0.001	0.936
241.129	Anserine[b]	1	2.27(1.73)	0.27(0.08)	2.11(1.18)	0.26(0.10)	0.821	<0.001	0.840
265.085	Aspartyl-Methionine	3	1.69(0.40)	0.94(0.43)	1.47(0.50)	0.66(0.21)	0.096	<0.001	0.823
281.113	Aspartylphenylalanine	3	1.41(0.25)	0.92(0.32)	1.68(0.37)	0.93(0.10)	0.143	<0.001	0.169
304.150	DL-alpha-Asp-Gly-DL-leu	3	1.36(0.33)	0.76(0.19)	1.44(0.27)	0.82(0.11)	0.440	<0.001	0.973
362.155	H-Asp-Asp-Leu-OH	3	1.17(0.27)	0.73(0.30)	1.50(0.36)	1.10(0.33)	0.004	0.001	0.843
401.202	H-Gly-DL-Asp-DL-Pro-DL-Leu-OH	3	1.76(0.51)	0.29(0.12)	2.35(0.40)	0.36(0.15)	0.015	<0.001	0.047
520.224	H-Val-Glu-Thr-Asp-Gly-OH	3	1.07(0.32)	0.77(0.17)	1.52(0.49)	1.01(0.28)	0.063	0.035	0.561
484.308	Litocholyltaurine	3	1.31(0.81)	0.62(0.29)	1.64(1.48)	0.49(0.22)	0.959	0.042	0.674
Red&processed meat									
162.112	L-carnitine[b]	1	0.17(0.05)	2.15(0.36)	0.11(0.04)	1.93(0.67)	0.517	<0.001	0.704
204.123	C2-carnitine[b]	1	0.23(0.05)	2.01(0.34)	0.15(0.04)	1.79(0.55)	0.347	<0.001	0.653
218.138	C3-carnitine[b]	1	0.52(0.20)	1.77(0.57)	0.33(0.14)	2.08(0.59)	0.661	<0.001	0.097
232.154	C4-carnitine[b]	1	0.30(0.13)	1.89(0.53)	0.25(0.04)	2.17(0.78)	0.501	<0.001	0.332
246.169	C5-carnitine[b]	1	0.43(0.17)	1.88(1.11)	0.37(0.15)	1.87(0.95)	0.907	<0.001	0.945
248.149	C4-3-OH-carnitine	3	0.34(0.16)	2.14(0.42)	0.24(0.11)	1.96(0.54)	0.390	<0.001	0.784
260.185	C6-carnitine[b]	1	0.36(0.21)	1.69(0.59)	0.34(0.07)	1.96(0.78)	0.549	<0.001	0.492
262.128	C4-DC-carnitine	3	0.14(0.03)	2.36(0.59)	0.20(0.12)	2.14(0.60)	0.590	<0.001	0.337
146.117	3-Dehydroxycarnitine	3	0.49(0.15)	1.83(0.59)	0.27(0.09)	1.97(0.67)	0.805	<0.001	0.263
227.113	Carnosine[b]	1	0.80(0.66)	1.58(0.51)	0.66(0.37)	1.46(0.56)	0.503	0.001	0.964
245.149	Hydroxyprolyl-Leucine	3	0.00(0.00)	2.53(0.85)	0.04(0.10)	2.28(0.69)	0.562	<0.001	0.415
452.313	LysoPC(O-14:1)	3	0.60(0.43)	1.36(0.46)	0.57(0.18)	1.44(0.55)	0.848	<0.001	0.722
480.344	LysoPC(P-16:0)	3	0.58(0.46)	1.17(0.63)	0.78(0.20)	1.60(0.97)	0.175	0.006	0.617
508.375	LysoPC(P-18:0)	3	0.38(0.25)	1.59(0.74)	0.53(0.13)	1.82(1.05)	0.425	<0.001	0.875
510.391	LysoPC(O-18:0)	3	0.54(0.35)	1.20(0.59)	0.97(0.27)	1.36(0.64)	0.103	0.009	0.456
Red&processed meat (× Western background diet)									
314.232	C10:1-carnitine	3	0.28(0.09)	1.26(0.42)	0.65(0.27)	2.42(1.02)	<0.001	<0.001	0.033
316.247	C10-carnitine[b]	1	0.36(0.14)	1.50(0.69)	0.43(0.20)	2.50(1.43)	0.070	<0.001	0.107

342.263	C12:1-carnitine	3	0.53(0.39)	1.10(0.39)	0.62(0.40)	1.97(0.99)	0.096	0.003	0.170
344.279	C12-carnitine	3	0.42(0.25)	0.89(0.37)	0.75(0.34)	2.35(1.57)	0.008	0.003	0.075
368.279	C14:2-carnitine	3	0.32(0.22)	0.87(0.35)	0.84(0.62)	2.24(1.75)	0.093	0.086	0.433
370.294	C14:1-carnitine	3	0.34(0.24)	0.97(0.37)	0.69(0.41)	2.22(1.47)	0.081	0.023	0.309
372.310	C14-carnitine[b]	1	0.38(0.22)	0.96(0.39)	0.78(0.39)	2.46(1.39)	0.008	0.003	0.102
398.325	C16:1-carnitine[c]	3	0.43(0.27)	1.19(0.55)	0.76(0.41)	2.36(1.50)	0.115	0.018	0.359
400.341	C16-carnitine[b]	1	0.42(0.27)	0.88(0.29)	0.97(0.52)	2.08(1.30)	0.002	0.004	0.172
424.341	C18:2-carnitine	3	0.47(0.30)	0.93(0.38)	0.98(0.43)	2.26(1.21)	0.009	0.012	0.195
426.357	C18:1-carnitine[b]	1	0.54(0.41)	1.18(0.48)	0.81(0.36)	1.99(1.10)	0.027	0.001	0.239
428.372	C18-carnitine[b]	1	0.31(0.17)	1.10(0.35)	0.85(0.52)	2.25(1.17)	0.001	<0.001	0.179
329.268	Palmitoleoylglycerol	3	0.19(0.22)	0.22(0.24)	0.84(0.88)	3.42(2.63)	0.001	0.009	0.011
331.283	Palmitoylglycerol[c]	3	0.16(0.06)	0.41(0.26)	0.84(0.61)	2.66(1.43)	<0.001	0.001	0.006
353.268	Linolenoylglycerol	3	0.51(0.65)	0.50(0.56)	0.87(0.74)	2.77(2.00)	0.002	0.017	0.015
357.299	Oleoylglycerol[b]	1	0.37(0.27)	0.31(0.22)	0.78(0.54)	2.13(1.33)	<0.001	0.014	0.008
359.315	Stearoylglycerol[c]	3	0.29(0.11)	0.41(0.21)	0.94(0.51)	1.83(0.89)	<0.001	0.011	0.041

a) Metabolites did not result from untargeted analysis, but were added based on literature or obtained results in the other gastrointestinal compartment. b) Identification was confirmed with commercial standards. c) The identity of the detected metabolite does not correspond with the available specific standard, but is likely an isomer of the available standard, which is covered by the nomenclature used in the list. *.m/z* corresponds with $[M+H]^+$ mass of the parent ion. ID level = level of identification according to Sumner et al.[27] with Tier 1 representing identified metabolites and Tier 3 representing putatively characterized metabolite classes. SD = standard deviation

Table 4. List of (tentatively) characterized metabolites influenced by meat intake in the colon (n = 8 piglets per dietary treatment, except chicken-Western, where n = 6).

			Average peak area ratio (SD)				p-values mixed model univariate statistics		
m/z	tentative metabolite identity	ID level	chicken-prudent	red&processed-prudent	chicken-Western	red&processed-Western	background diet	meat	meat × background diet
Chicken									
127.087	2-(4-Methylimidazol-1-yl)ethanol	3	1.65(1.04)	0.14(0.05)	2.38(1.09)	0.23(0.23)	0.099	<0.001	0.188
157.061	4-Imidazolone-5-propionic acid	3	1.41(1.11)	0.43(0.14)	2.77(1.37)	0.54(0.34)	0.024	<0.001	0.050
160.108	Guanidinovaleric acid	3	1.08(1.12)	0.20(0.12)	3.01(4.13)	0.21(0.11)	0.188	0.021	0.194
162.076	Methylglutamate	3	1.81(0.48)	0.43(0.13)	1.87(0.73)	0.32(0.11)	0.848	<0.001	0.587
170.092	3- (or 1-)Methylhistidine	3	1.31(0.72)	0.30(0.31)	2.69(2.39)	0.25(0.17)	0.092	<0.001	0.071
186.087	N-(4,5-Dihydro-1-methyl-4-oxo-1H-imidazol-2-yl)alanine	3	1.20(0.38)	0.85(0.26)	1.61(0.46)	0.68(0.40)	0.434	<0.001	0.044
203.103	Prolylserine	3	1.85(0.79)	0.13(0.08)	2.35(0.77)	0.16(0.13)	0.200	<0.001	0.257
212.103	Acetyl-3-(or 1-)methylhistidine	3	2.04(1.11)	0.33(0.11)	1.58(1.24)	0.26(0.15)	0.478	0.001	0.601
257.124	2-(3-Carboxy-3-aminopropyl)-L-histidine	3	1.61(0.39)	0.07(0.04)	2.46(1.02)	0.08(0.06)	0.212	<0.001	0.229
314.207	H-Pro-Val-Val-OH	3	1.73(0.77)	0.71(0.34)	1.52(0.39)	0.43(0.25)	0.220	<0.001	0.879
403.234	deamino-hPhe-Ala-Ala-Pro-NH2	3	1.30(1.06)	0.11(0.07)	1.96(1.42)	0.16(0.12)	0.266	<0.001	0.346
175.097	Suberic acid	3	1.15(0.39)	0.98(0.45)	1.21(0.32)	0.54(0.26)	0.078	0.012	0.131
217.143	$C_{11}H_{20}O_4$ (Undecanedioic acid)[b]	1	1.53(0.50)	0.81(0.18)	1.12(0.47)	0.55(0.16)	0.014	<0.001	0.518
227.128	$C_{12}H_{18}O_4$ (Dioxo-dodecenoic acid)	3	1.20(0.35)	0.95(0.29)	1.20(0.19)	0.57(0.22)	0.020	<0.001	0.043
247.154	$C_{12}H_{22}O_5$ (Hydroxydodecanedioic acid)	3	1.25(0.37)	1.03(0.22)	1.34(0.15)	0.53(0.21)	0.008	<0.001	0.002
279.232	$C_{18}H_{30}O_2$ (Octadecatrienoic acid)	3	1.07(0.59)	0.50(0.21)	1.94(1.42)	0.62(0.17)	0.099	0.004	0.196
343.211	$C_{18}H_{30}O_6$ ((-)-11-Hydroxy-9,15,16-trioxooctadecanoic acid)	3	1.26(0.39)	0.92(0.35)	1.39(0.28)	0.47(0.26)	0.107	<0.001	0.020
433.245	Glucosyl (2E,6E,10X)-10,11-dihydroxy-2,6-farnesadienoate	3	1.16(0.36)	0.70(0.15)	1.57(0.22)	0.70(0.26)	0.047	<0.001	0.055
Chicken (× Western background diet)									
318.166	H-DL-Leu-Gly-DL-Glu-OH	3	0.97(0.43)	1.14(0.48)	1.68(0.42)	0.72(0.22)	0.352	0.019	0.002
213.160	Cyclo(L-Valyl-L-Leucyl)	3	0.30(0.33)	0.65(0.75)	3.31(3.31)	0.24(0.24)	0.036	0.029	0.008
247.144	Cyclo(L-Valyl-L-Phenylalanyl)	3	0.17(0.11)	0.37(0.35)	3.00(1.50)	1.14(0.70)	<0.001	0.010	0.002
261.160	Cyclo(L-Leucyl-L-Phenylalanyl)	3	0.50(0.27)	0.79(0.41)	2.41(1.12)	1.02(0.26)	<0.001	0.014	0.001
Chicken (× prudent background diet)									
295.228	$C_{18}H_{32}O_3$ (HODE)[c]	3	2.12(1.81)	0.42(0.09)	0.48(0.20)	0.75(0.54)	0.094	0.070	0.018
299.258	$C_{18}H_{34}O_3$ Hydroxyoctadecenoic acid	3	3.09(3.36)	0.28(0.17)	0.34(0.13)	0.69(0.65)	0.098	0.084	0.031
317.268	$C_{18}H_{36}O_4$ Dihydroxyoctadecanoic acid	3	3.13(3.25)	0.28(0.17)	0.32(0.13)	0.67(0.62)	0.078	0.070	0.026
Red&processed meat									
146.117	3-Dehydroxycarnitine[a]	3	0.70(0.18)	1.33(0.36)	0.95(0.31)	1.10(0.53)	0.890	0.017	0.094
162.112	L-carnitine[a]	1	0.98(0.44)	1.51(0.88)	0.78(0.19)	1.12(0.35)	0.112	0.028	0.657
218.139	C3-carnitine	1	0.48(0.24)	1.49(0.49)	0.69(0.18)	1.05(0.80)	0.255	<0.001	0.140
262.128	C4-DC-carnitine	3	0.82(0.40)	1.25(0.38)	0.82(0.24)	0.86(0.45)	0.131	0.079	0.195

311.223	$C_{18}H_{32}O_4$ (HPODE)[c]	3	1.00(1.17)	2.25(1.79)	0.31(0.17)	0.77(0.53)	0.040	0.093	0.419
329.234	$C_{18}H_{34}O_5$ (TriHOME)	3	0.96(0.64)	1.63(0.62)	0.28(0.17)	0.70(0.39)	<0.001	0.008	0.413
295.226	$C_{18}H_{30}O_3$ (15(16)-epODE)[a]	3	0.82(0.59)	1.28(0.50)	0.40(0.14)	0.70(0.30)	0.007	0.031	0.594
130.086	D-pipecolic acid[b]	1	0.53(0.29)	1.24(0.68)	0.54(0.20)	1.47(1.20)	0.788	0.054	0.760
148.097	2-amino-6-Hydroxyhexanoic acid	3	0.81(0.46)	1.28(0.30)	0.54(0.09)	1.15(0.36)	0.230	0.007	0.616
243.195	$C_{14}H_{26}O_3$ (Oxotetradecanoic acid)	3	0.35(0.13)	0.76(0.30)	0.57(0.30)	2.13(1.69)	0.024	0.010	0.105
181.122	Norecasantalic acid or Jasmolone	3	0.90(0.32)	1.32(0.22)	0.68(0.12)	0.89(0.15)	0.001	0.001	0.107
245.149	Hydroxyprolyl-Leucine	3	0.00(0.00)	1.99(0.81)	0.00(0.00)	1.95(0.89)	0.960	<0.001	0.957
246.108	Hydroxyprolyl-Asparagine	3	0.59(0.18)	1.34(0.44)	0.79(0.21)	1.20(0.64)	0.970	0.004	0.417

a) Metabolites did not result from untargeted analysis, but were added based on literature or obtained results in the other gastrointestinal compartment. b) Identification was confirmed with commercial standards. c) The identity of the detected metabolite does not correspond with the available specific standard, but is likely an isomer of the available standard, which is covered by the nomenclature used in the list. *m/z* corresponds with $[M+H]^+$ mass of the parent ion, except for $C_{18}H_{32}O_3$ (HODE), $C_{18}H_{32}O_4$ (HPODE) and $C_{18}H_{34}O_5$ (TriHOME), where *m/z* corresponds with $[M-H]^-$. ID level = level of identification according to Sumner et al.[27] with Tier 1 representing identified metabolites and Tier 3 representing putatively characterized metabolite classes. SD = standard deviation

Graphical abstract

A pig feeding trial with chicken or red and processed meat on the one hand, and a prudent or Western background diet on the other hand, was conducted to investigate the effects of meat consumption in a complex dietary context. Alterations in the digestion metabolome of the small intestine and the colon content of the pigs were investigated by an untargeted UHPLC-HRMS-based polar metabolomics platform. The identification of meat-associated gut metabolites could contribute to the understanding of the epidemiological link between high red and processed meat consumption and chronic diseases.