AARHUS UNIVERSITY

Coversheet

This is the accepted manuscript (post-print version) of the article.

Contentwise, the accepted manuscript version is identical to the final published version, but there may be differences in typography and layout.

How to cite this publication

Please cite the final published version:

Zahra Sattari Najafabadi, Tina Skau Nielsen & Mette Skou Hedemann (2019) Dietary protein source and butyrylated high-amylose maize starch included in a high-protein diet determines the urinary metabolome of rats, *International Journal of Food Sciences and Nutrition*, 70:3, 255-266, DOI: [10.1080/09637486.2018.1499711](https://doi.org/10.1080/09637486.2018.1499711)

Publication metadata

Title:	Dietary protein source and butyrylated high-amylose maize starch included in a high-protein diet determines the urinary metabolome of rats
Author(s):	Zahra Sattari Najafabadi, Tina Skau Nielsen & Mette Skou Hedemann
Journal:	International Journal of Food Sciences and Nutrition
DOI/Link:	https://doi.org/10.1080/09637486.2018.1499711
Document version:	Accepted manuscript (post-print)

General Rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognize and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

If the document is published under a Creative Commons license, this applies instead of the general rights.

PROOF COVER SHEET

Author(s): Zahra Sattari Najafabadi, Tina Skau Nielsen, and Mette Skou Hedemann

Article title: Dietary protein source and butyrylated high-amylose maize starch included in a high-protein diet determines the urinary metabolome of rats

Article no: IIJF_A_1499711

Enclosures: 1) Query sheet
2) Article proofs

Dear Author,

1. Please check these proofs carefully. It is the responsibility of the corresponding author to check these and approve or amend them. A second proof is not normally provided. Taylor & Francis cannot be held responsible for uncorrected errors, even if introduced during the production process. Once your corrections have been added to the article, it will be considered ready for publication.

Please limit changes at this stage to the correction of errors. You should not make trivial changes, improve prose style, add new material, or delete existing material at this stage. You may be charged if your corrections are excessive (we would not expect corrections to exceed 30 changes).

For detailed guidance on how to check your proofs, please paste this address into a new browser window: <http://journalauthors.tandf.co.uk/production/checkingproofs.asp>

Your PDF proof file has been enabled so that you can comment on the proof directly using Adobe Acrobat. If you wish to do this, please save the file to your hard disk first. For further information on marking corrections using Acrobat, please paste this address into a new browser window: <http://journalauthors.tandf.co.uk/production/acrobat.asp>

2. Please review the table of contributors below and confirm that the first and last names are structured correctly and that the authors are listed in the correct order of contribution. This check is to ensure that your name will appear correctly online and when the article is indexed.

Sequence	Prefix	Given name(s)	Surname	Suffix
1		Zahra	Sattari Najafabadi	
2		Tina	Skau Nielsen	
3		Mette	Skou Hedemann	

Queries are marked in the margins of the proofs, and you can also click the hyperlinks below.

General points:

- 1. Permissions:** You have warranted that you have secured the necessary written permission from the appropriate copyright owner for the reproduction of any text, illustration, or other material in your article. Please see <http://journalauthors.tandf.co.uk/permissions/usingThirdPartyMaterial.asp>.
- 2. Third-party content:** If there is third-party content in your article, please check that the rightsholder details for re-use are shown correctly.

3. **Affiliation:** The corresponding author is responsible for ensuring that address and email details are correct for all the co-authors. Affiliations given in the article should be the affiliation at the time the research was conducted. Please see <http://journalauthors.tandf.co.uk/preparation/writing.asp>.
4. **Funding:** Was your research for this article funded by a funding agency? If so, please insert 'This work was supported by <insert the name of the funding agency in full>', followed by the grant number in square brackets '[grant number xxxx]'
5. **Supplemental data and underlying research materials:** Do you wish to include the location of the underlying research materials (e.g. data, samples or models) for your article? If so, please insert this sentence before the reference section: 'The underlying research materials for this article can be accessed at <full link>/ description of location [author to complete]'. If your article includes supplemental data, the link will also be provided in this paragraph. See <<http://journalauthors.tandf.co.uk/preparation/multimedia.asp>> for further explanation of supplemental data and underlying research materials.
6. The **PubMed** (<http://www.ncbi.nlm.nih.gov/pubmed>) and **CrossRef databases** (www.crossref.org/) have been used to validate the references. Changes resulting from mismatches are tracked in red font.

AUTHOR QUERIES

- Q1: The year for “Jakobsen et al. 2016” has been changed to 2017 to year to match the entry in the references list. Please provide a revision if this is incorrect.
- Q2: Please provide the city for Merck Millipore.
- Q3: If the spelling of “hydroxyphenyllactate” is incorrect, please provide a correction.
- Q4: The year for “Cheng et al. 2011” has been changed to 2012 to match the entry in the references list. Please provide revisions if this is incorrect.
- Q5: Please provide complete details for Bresson et al. 2016 in the reference list or delete the citation from the text.
- Q6: Please provide names of all authors for reference ‘Cheng et al, 2012; Kieffer et al 2016; Liabeuf et al 2013; Melanson et al 2006, Sarosiek et al 2016; Stella et al 2006; Turck et al 2016; Yeung et al 1991 ’ (or only the first 10 authors and et al if there are more than 10), as per journal style
- Q7: Please confirm the change of year in ref Le Leu et al according to pubmed.
- Q8: Please provide missing publisher name and location for ref Milliken GA, Johnson DE. 1984.
- Q9: Please provide an English translation of the title in the reference “Stoldt W. 1952”, as per journal style
- Q10: There is no mention of Turck et al. 2016 in the text. Please insert a citation in the text or delete the reference as appropriate.
- Q11: Please provide missing volume and page range for ref Turck et al 2016
- Q12: Please provide missing volume and page range for ref Winter et al 2011.
- Q13: The ORCID details of the authors have been validated against ORCID registry. please check the ORCID ID details of the authors.
- Q14: Please check and resupply corresponding authors email id if it is inaccurate.
- Q15: Please provide appropriate missing symbol for ■

How to make corrections to your proofs using Adobe Acrobat/Reader

Taylor & Francis offers you a choice of options to help you make corrections to your proofs. Your PDF proof file has been enabled so that you can mark up the proof directly using Adobe Acrobat/Reader. This is the simplest and best way for you to ensure that your corrections will be incorporated. If you wish to do this, please follow these instructions:

1. Save the file to your hard disk.
2. Check which version of Adobe Acrobat/Reader you have on your computer. You can do this by clicking on the Help” tab, and then About”.

If Adobe Reader is not installed, you can get the latest version free from <http://get.adobe.com/reader/>.

3. If you have Adobe Acrobat/Reader 10 or a later version, click on the Comment” link at the right-hand side to view the Comments pane.

4. You can then select any text and mark it up for deletion or replacement, or insert new text as needed. Please note that these will clearly be displayed in the Comments pane and secondary annotation is not needed to draw attention to your corrections. If you need to include new sections of text, it is also possible to add a comment to the proofs. To do this, use the Sticky Note tool in the task bar. Please also see our FAQs here: <http://journalauthors.tandf.co.uk/production/index.asp>.

5. Make sure that you save the file when you close the document before uploading it to CATS using the Upload File” button on the online correction form. If you have more than one file, please zip them together and then upload the zip file. If you prefer, you can make your corrections using the CATS online correction form.

Troubleshooting

Acrobat help: <http://helpx.adobe.com/acrobat.html>

Reader help: <http://helpx.adobe.com/reader.html>

Please note that full user guides for earlier versions of these programs are available from the Adobe Help pages by clicking on the link Previous versions” under the Help and tutorials” heading from the relevant link above. Commenting functionality is available from Adobe Reader 8.0 onwards and from Adobe Acrobat 7.0 onwards.

Firefox users: Firefox's inbuilt PDF Viewer is set to the default; please see the following for instructions on how to use this and download the PDF to your hard drive: http://support.mozilla.org/en-US/kb/view-pdf-files-firefox-without-downloading-them#w_using-a-pdf-reader-plugin

RESEARCH ARTICLE

Dietary protein source and butyrylated high-amylose maize starch included in a high-protein diet determines the urinary metabolome of rats

Zahra Sattari Najafabadi , Tina Skau Nielsen and Mette Skou Hedemann

Department of Animal Science, Faculty of Science and Technology, Aarhus University, Tjele, Denmark

ABSTRACT

Intake of red and processed meat increases the risk of colorectal cancer (CRC), whereas dairy product consumption and the intake of dietary fibre are negatively associated with this risk. We investigated the effect of (i) low-protein diets with either whey or cooked meat (beef) as the protein source and (ii) high-protein diets with cooked meat (beef) either without or with the inclusion of 10% butyrylated high-amylose maize starch (HAMS), on the urinary metabolome of rats. Urine samples from rats were analysed using untargeted LC-MS metabolomics. The level and source of the dietary protein affected the urinary excretion of numerous metabolites indicating that several metabolic pathways were changed. The inclusion of HAMS in a high-protein diet caused significant alterations in the excretion of several metabolites. HAMS reduced urinary excretion of potentially harmful metabolites resulting from a high level of meat consumption.

ARTICLE HISTORY

Received 17 April 2018
Revised 27 June 2018
Accepted 9 July 2018

KEYWORDS

Rat; resistant starch; untargeted metabolomics; LC-MS; colon health

Introduction

Meat is an integral part of the human diet. It provides the highest amount of protein per unit energy and the best quality of protein including all essential amino acids in adequate proportions (Young et al. 2013). However, a dose-dependent relationship is established between the red and processed meat consumption and colorectal cancer (CRC) risk (Chan et al. 2011; O'keefe 2016). Our knowledge of the role of dietary proteins in CRC aetiology is in general poor and recognising the involved mechanisms, as well as the differences among the effects of protein types remains to

be established. For example, whey protein consumption is generally regarded to be protective against experimentally induced CRC compared with other protein types such as red meat (Toden et al. 2007).

There is convincing evidence that consumption of dietary fibre (DF) is protective against CRC, perhaps due to the anti-carcinogenic effects of their fermentation products (O'keefe 2016). One of the major sources of DF available for microbial fermentation is resistant starch (RS), however, the intake is relatively low in Westernized populations (Bajka et al. 2006). In addition, specific delivery of short-chain fatty acids

CONTACT Zahra Sattari Najafabadi zahra.sattari@anis.au.dk Department of Animal Science, Faculty of Science and Technology, Aarhus University, Tjele, P.O. Box 50, Tjele 8830, Denmark

Supplemental data for this article can be accessed [here](#).

© 2018 Informa UK Limited, trading as Taylor & Francis Group

(SCFA) to the large bowel offers considerable benefits in clinical and non-clinical applications and acylation of starch to a high degree of substitution (DS 0.2–0.3) with acetic, propionic or butyric acid has been shown to be an effective means of achieving this aim (Lopez-Rubio et al. 2009). Butyrylated high-amylose maize starch (HAMSB) included in a high-protein (meat) diet of both humans and animals have shown positive effects on colonic health parameters (Toden et al. 2007). However, to our knowledge, no studies have been performed on the changes induced on the metabolic profile following such treatments.

As a post-genomic tool metabolomics can identify dietary markers and gain mechanistic insight into nutrition (Jakobsen et al. 2017). Nutritional metabolomics studies are also conducted to determine a quantitative link between the intake of a particular nutrient or eating pattern to a particular metabolic profile (Rasmussen et al. 2012). An untargeted approach in metabolomics studies makes it a superior tool to reveal endogenous effects in nutritional research without any a priori knowledge. However, few studies have employed untargeted metabolomics to study the effect of meat intake (Jakobsen et al. 2017).

Q1 The aim of the present work was firstly, to investigate whether the urinary metabolome of rats was affected by the type of dietary protein; whey or red meat, and secondly, to explore if the urinary metabolome was affected by adding HAMSB to a high-protein (meat) diet. It was hypothesised that the choice of protein source and HAMSB can reduce the CRC risk by altering colonic cell and microbial metabolism reflected in the urinary metabolome. The aim of the study was not an absolute quantification of the metabolites but to compare relative differences, and pronounced effects of the diets observed on the urine profile. In addition, the amount of butyrate and total organic acids were measured in caecum and faeces in the four groups to verify the effect of the experimental diets on large bowel's butyrate content.

Materials and methods

Chemicals

Q2 Formic acid purchased from Fluka (Fluka; Sigma-Aldrich, St. Louis, MO), high-performance liquid chromatography grade acetonitrile from VWR (West Chester, PA) and distilled water from Milli-Q system (Merck Millipore, MA) were used for aqueous solvents and eluents. Glycocholic acid (glycine-1¹³C) monohydrate and p-chlorophenylalanine were purchased from Sigma Aldrich (St. Louis, MO), to be

used as internal standards. Ascorbic acid was used to preserve urine samples from microbial infection after collection. Standards for comparison while metabolite identification where all purchased from Sigma Aldrich.

Animals, experimental design and sampling

The experiment included forty 5-week old healthy, male Sprague–Dawley rats (Taconic Europe, Ry, Denmark) with an initial body weight (BW) of ~120 g. The rats were housed in groups (3–4 per cage) in standard 35 × 56 × 17 cm plastic cages and allowed to adapt to the experimental facilities for 5 days prior to the start of the experiment. A standard laboratory diet for growing rats (AIN93G) and water were provided *ad libitum*. The animal experiment was conducted according to the protocols approved by the Danish Animal Experiments Inspectorate and complied with the guidelines of the Ministry of Food, Agriculture and Fisheries concerning animal experiments and care of animals.

After acclimatisation, the rats were weighed and randomly assigned to the four dietary treatments (Table 1) based on the BW to reach the same average BW ± standard error in each treatment group.

The rats received the experimental diets *ad libitum* for the following four weeks. During the second week of the experiment, the rats were transferred to single housing in metabolic cages designed for the quantitative collection of faeces and urine as described by Eggum (1973). After a 3-day habituation period in the metabolism cages with *ad libitum* feeding of the experimental diet they received in the previous period, the rats received 20 g feed/day equivalent to *ad libitum* feeding for the following five days. Feed residues were recorded on a daily basis. Faeces were collected quantitatively every second day for four days and stored at –20 °C until further analysis. Urine was collected for 48 hours and ascorbic acid (1 ml) was added daily to the urine collection flask to prevent microbial activity. Urine samples were stored in the freezer (–80 °C) until further analysis. After the total of 7 days in the metabolism cages, rats were transferred back to group housing (2–3 rats per cage) receiving the same diet as in the balance period for the last week of the experiment. Rats were anaesthetized with 0.3 mL/100 g BW of Dormicum (5 mg/mL) and Hypnorm (1:1). Caecum digesta was collected and stored at –20 °C for SCFA analysis. Rats were euthanized by an injection of pentobarbital into the heart.

Table 1. Ingredients and chemical composition of the experimental diets.

	Diet			
	LPW 15% protein (whey)	LPM 15% protein (meat)	HPM 30% protein (meat)	HPM + HAMSB 30% protein (meat)
Ingredients (g/kg, as-fed basis)				
Corn starch ^a	436	450	283	183
Sugar	100	100	100	100
Cellulose	50	50	50	50
Lard	182.5	91	–	–
Lacprodan®-87 ^b	181	–	–	–
L-cysteine	3.0	3.0	3.0	3.0
AIN 93G Mineral Mix	35	35	35	35
AIN 93G Vitamin Mix	10	10	10	10
Choline bitartrate	2.5	2.5	2.5	2.5
t-butylhydroquinone	0.14	0.14	0.14	0.14
Meat ^c	–	259	517	517
HAMSB ^d	–	–	–	100
Chemical composition (% of DM)				
DM	96.5	96.7	96.5	96.5
Protein (N × 6.25)	15.9	18.1	32.5	33.7
Starch	40.9	43.5	30.9	18.5
Fat	19.3	19.4	20.8	19.9
Ash	3.1	3.3	3.9	4.1

^aLow amylose maize starch.^bCommercially available concentrated whey (83% whey protein).^cMeat: minced beef, cooked, dried and milled at 1 mm screen.^dHigh amylose maize starch butyrylated.

Experimental diets

The four experimental diets (Table 1) were formulated based on the open formula AIN93G growth diet for rats (Reeves et al. 1993) containing 20% protein, 7% fat and 64% carbohydrate. The experimental diets were a low-protein whey (LPW) diet, a low-protein meat (LPM) diet, a high-protein meat (HPM) diet and an HPM diet with 10% of butyrylated high-amylose maize starch (HPM + HAMSB) included. The diets were designed to be balanced in protein, 15% for LPW and LPM diets, and 30% for the HPM and HPM + HAMSB diets, and in fat (19%).

The minced, cooked, dried, ground beef, which was the only source of protein in LPM, HPM and HPM + HAMSB diets, contained 58% protein, 94.2% dry matter, 2.51% ashes and 35.3% fat on an as-is basis. The diets were mixed in the feed production unit at Aarhus University, Foulum and stored at –20 °C until fed.

The HAMSB incorporated into the HPM + HAMSB diet was produced according to the method described by Tupa et al. (2013). In brief, esterification of high-amylose maize starch (HAMS) by the use of butyric acid was performed by mixing 500 mL of butyric acid, 74 g of tartaric acid (as a catalyst), and 80 g of oven-dried HAMS in a round bottom 1000 mL glass bottle fitted with a stopper. The mixture was placed in a heating block and heated to

120 °C, with constant magnetic stirring. The reaction ran for 3 hours and the product was left to cool to room temperature before several washings with distilled water by vacuum filtration in a Buchner funnel. This procedure was repeated several times to produce enough HAMSB to be included in the experimental diet (10% feed). Determination of the acyl content and the DS was performed by heterogeneous saponification and back titration with HCl as described by Tupa et al. (2013). According to this method, the DS of the final product for incorporation into the rat diet was ~0.93.

Chemical analysis

All chemical analyses on diets were performed in duplicate on the freeze-dried material. The dry matter (DM) content was determined by drying the samples at 103 °C to constant weight, and ash was analysed according to the AOAC method (Helrich 1990). Nitrogen was measured by DUMAS (Hansen 1989) and protein calculated as $N \times 6.25$. Fat was determined using the Stoldt procedure (Stoldt 1952). Starch was analysed essentially as described by Knudsen (1997) except that acid hydrolysis was performed in 2 M H₂SO₄ for 1 h instead of 1 M H₂SO₄ for 2 h. Caecum digesta and faecal samples were analysed for SCFA concentration by gas chromatography (HP-6890 Series Gas Chromatograph; Hewlett Packard, Palo Alto, CA) using an SGE-BP1 column (30 m × 0.25 mm × 0.25 µm; Trajan Scientific, Ringwood, Australia) with 5% phenylpolysiloxane and 95% dimethylpolysiloxane and a flame ionisation detector, after submitting the samples to an acid–base treatment followed by ether extraction and derivatisation, as described by Canibe et al. (2007).

Untargeted LC-MS metabolomics analysis of urine

The urine samples (40 µl) were diluted with water (140 µl) and 20 µl of internal standard (glycocholic acid (Glycine-1¹³C) and *p*-chlorophenylalanine, final concentration 0.1 mg/ml) was added. The samples were left at 4 °C for 20 minutes for protein precipitation. After 10 minutes of centrifugation (13,200 rpm, 4 °C) the supernatant was transferred to vials with micro-inserts.

A high-performance liquid chromatography Ultimate 3000 (HPLC; Dionex, Sunnyvale, CA) system coupled to an Impact HD Q-TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) was used for the HPLC analyses.

The analytical column was a reversed-phase C18 columns, high-strength silica (HSS) T3 from Waters (Milford, MA) (2.1×100 mm; $1.8 \mu\text{m}$ particles) held at 30°C . The eluent was introduced into the mass spectrometer by electrospray ionisation, with capillary set in the positive and negative mode to 4500 and 3600 V, respectively. End-plate offset voltage was set to 500 V. Nitrogen was used as both nebuliser and drying gas with a gas pressure of 1.8 bar. Drying gas temperature and flow were 200°C and 8.0 L/min, respectively. Spectra were acquired over the scan range of 50–1000 m/z . A solution of lithium formate clusters (5 mM) (water/isopropanol/formic acid in a 50:50:0.2 v/v/v ratio) was injected prior to each chromatographic run as an external calibrant.

The mobile phases consisted of (A) Milli-Q water with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. Formic acid is added to the solutions as a buffer, to adjust the pH to 3. The column was equilibrated at 95% of solvent A for 2 minutes before injection. The gradient was 5% (B) for 0.11 minute, followed by a linear increase to 70% (B) within 8 minutes. Then it increased to 100% (B) in 0.5 minute. Isocratic conditions were kept for 1 minute before returning to 5% (B) in 30 seconds. The injection volume was $3 \mu\text{L}$, and the flow rate was 400 $\mu\text{L}/\text{min}$.

As quality controls (QC), blank samples (Milli-Q water) and pooled urine samples from all 40 rats were reinjected after every 4 samples to evaluate the analytical system performance for potential cross-contamination from samples, loss of sensitivity and system repeatability during the run. Argon gas was used as collision gas for the tandem mass spectrometry (MS/MS) analysis using collision energies from 15 to 22 eV. Bruker Daltonics software packages MicrOTOF control v.2.3, HyStar v.3.2 and Data Analysis v.4.0 were used for MS control and data calibration before further analyses.

Data processing and metabolite identification

Acquired mass spectra were calibrated and peak detection was performed using the Find Molecular Features (FMF) option in DataAnalysis version 4.2 (Bruker Daltonics GmbH, Bremen, Germany). The FMF parameters for compound detection were set to 5 S/N threshold, compound length set to 8 spectra and smoothing width of 2 for the peaks. The mass spectra were imported to Bruker Compass ProfileAnalysis 2.1 for the initial statistical evaluation. A data matrix was generated with retention time, m/z and respective

compound feature intensities. The data were normalised according to the peak intensity of the internal standard to compensate for variability in sample processing and analytical platform operation. This matrix was exported to LatentX 2.12 where analysis of the sample and variable variations patterns was performed using partial least square discriminant analysis (PLS-DA), a supervised method for pattern recognition. The PLS-DA models were built on data which was pareto scaled to reduce the relative importance of large values while still keeping the data structure partially intact (van den Berg et al. 2006). The models were validated using random cross validation with 3 segments and 1000 repetitions. The model robustness was evaluated using the model statistics and 'actual versus predicted' plots together with the regression coefficients (r^2) of each model. No outliers were identified by their residual variance and Hotelling's T^2 value. Root mean square error (RMSE) together with the explained variance of the Y matrix was used to select the optimum number of principal components in each PLS-DA model. The molecular features with the highest weight in differentiating the two diet groups were selected using scaled regression coefficients, which show the contribution of each variable to the score classification.

Compounds were identified based on queries in the METLIN (<http://metlin.scripps.edu/>) and Human Metabolome Database (<http://www.hmdb.ca/>) online databases for obtaining possible chemical structures using accurate mass and mass spectrometric fragmentation patterns. The identification of the annotated compounds was confirmed with standards, when available, on the same analytical system under the same conditions.

Analysis of variance was carried out on identified metabolites with the diet as the source of variation using the generalised linear models (GLM) procedure of SAS release 9.2. The analysis was carried out on LPW versus LPM and HPM versus HPM + HAMSB, respectively. Tests were made at the 5% significance level. Least squares means were compared using Fisher's least-significance-difference procedure (Milliken and Johnson 1984). Results are presented as least squares means (lsmeans) with their SEM. However, to obtain normality some of the metabolites had to be analysed on a logarithmic scale. As confidence intervals on the original scale are not symmetric around the parameter estimates, the confidence limits rather than the standard errors are presented for these metabolites.

Statistical analysis of butyrate and total organic acids concentrations

Butyrate and total organic acids concentrations in caecum digesta and faeces were analysed according to the following simple ANOVA model using the GLM procedure of SAS (SAS Institute, Inc, Cary, NC):

$$X_{(ij)} = \mu + \alpha_{(i)} + \beta_{(j)} + \alpha\beta_{(ij)} + \varepsilon_{(ij)},$$

where $\alpha_{(i)}$ is the diet (i =LPW, LPM, HPM or HPM + HAMSB); $\beta_{(j)}$ is the effect of block (j =1, 2 or 3); $\alpha\beta_{(ij)}$ is the interaction between diet and block and $\varepsilon_{(ij)}$ denotes the residual error. The residuals were assumed normally distributed and independent and their expectations were assumed zero.

Results

Experimental diets

The protein content of the LPM diet was higher (18.1%) than the intended 15%, and in the LPW diet, it was slightly higher (15.9%). The high-protein diets contained approximately twice the amount of the protein present in the LPW and LPM diets. The fat content of the four experimental diets was balanced around 19% of DM corresponding to a high-fat diet compared with the level in an AIN93G standard rodent diet (7%). The inclusion of the high levels of cooked beef in the HPM and HPM + HAMSB diets resulted in the high-fat content (~19%) and the two other diets were balanced according to that.

Butyrate and organic acids in caecum digesta and faeces

The concentration of butyrate in the caecum almost doubled going from the two low-protein diets to the HPM diet and again it almost doubled following the inclusion of HAMSB in the HPM diet. The concentration of total organic acids was similar for the LPW, HPM and HPM + HAMSB diets (average 75.5 $\mu\text{mol/g}$ wet weight) and significantly reduced by the LPM diet (Table 2). In faeces, the concentration of butyrate was more than five times higher (24.7 $\mu\text{mol/g}$ wet weight) following the HPM-HAMSB diet compared with the three other diets. Total organic acids in faeces were more than two times higher for the LPW and HPM-HAMSB fed rats compared with the LPM and HPM diets (Table 2).

Multivariate data analysis

The quality of the data was evaluated using the reinjection of blank samples and quality control samples

Table 2. Concentration of butyrate and total organic acids in caecum digesta and faeces of rats fed the four different diets for four weeks (least-square mean values with their standard errors, $n = 10$ per group).

	Diet				SEM	<i>p</i> diet
	LPW	LPM	HPM	HPM + HAMSB		
Caecum (μmol/g wet weight)						
Butyrate	7.2 ^c	6.4 ^c	12.1 ^b	23.0 ^a	0.83	<.001
Total organic acids	71.2 ^a	55.0 ^b	79.8 ^a	75.6 ^a	3.7	<.001
Faeces (μmol/g wet weight)						
Butyrate	5.2 ^b	5.2 ^b	0.5 ^b	24.7 ^a	2.7	<.001
Total organic acids	101.8 ^a	40.2 ^b	43.5 ^b	113.2 ^a	7.9	<.001

^{a,b,c}Mean values within a row with unlike superscripts were significantly different ($p < .05$).

after every 4 samples. The blank samples showed no addition of peaks throughout the run, suggesting that no cross contamination between samples and no carryover effects occurred. The chromatograms of the quality control samples were closely clustered in the PCA and PLS-DA plots and displayed no significant shifts in the retention time (data not shown).

After preprocessing of the data sets from the LPM and LPW diets, the numbers of molecular features (variables) in the positive and negative mode were 3145 and 1908, respectively. The PLS-DA score plots of the urine samples analysed in positive and negative ionisation modes are shown in Figures 1 and 2. In both ionisation modes, a clear grouping of the subjects according to the source of protein in the diet can be observed. The first two principal components (PCs) describe 59.7% of the variation in positive and 48.5% of the variation in negative mode, respectively.

To investigate which metabolites discriminate between HPM and HPM + HAMSB groups, PLS-DA plots and their corresponding regression coefficients plots were obtained (Figures 3 and 4). The numbers of molecular features (variables) in the positive and the negative mode were 583 and 1438, respectively. The figures showed a high predictive value for all models, no misclassification of the validation datasets was observed. In the obtained PLS-DA plots the first two PCs describe 38.42% of the variation in positive and 42.04% of the variation in negative mode, respectively. Metabolites were selected in the positive and negative mode for identification based on the regression coefficient plots.

Metabolites in urine

Based on the results from the PLS-DA analyses, the 40 metabolites having the highest influence on the discrimination were chosen in a positive and negative mode in each group and were subjected to further

Figure 1. PLS-DA score plot of urine samples from the LPW and LPM groups in positive mode.

Figure 2. PLS-DA score plot of urine samples from the LPW and LPM groups in negative mode.

Figure 3. PLS-DA score plot of urine samples from the HPM and HPM + HAMSB groups in positive mode.

analysis. Among them, a total of 51 metabolites identified in negative, positive or in some cases both modes were analysed by MSMS and then identified. Of these metabolites, the ones potentially influencing colorectal health and metabolism or indicating

Figure 4. PLS-DA score plot of urine samples from the HPM and HPM + HAMSB groups in negative mode.

alterations in the activity of gut microbiota, based on previous studies, are presented in Table 3. In case the change in the urinary excretion of a metabolite could be correlated to its content in the diet, the metabolite is presented in the Supplementary Table 1.

According to the results, the level and source of the dietary protein affected several metabolic pathways. Metabolites such as p-cresol glucuronide, indoxyl sulphate, hippuric acid, trimethylamine-N-oxide and phenylacetylglutamine can originate from microbial metabolism and the dietary protein source affected their urinary excretion. Furthermore, significant differences were observed in the level of derivatives of choline, branched-chain amino acids (BCAA), carnitine and aromatic amino acids metabolites, as well as metabolites related to the tricarboxylic acid (TCA) cycle.

In the HPM and HPM + HAMSB groups, significant differences existed also in the urinary excretion of metabolites related to microbial fermentation, TCA cycle and protein metabolism. The changes in the level of pyridoxic acid and riboflavin showed that HAMSB inclusion in a high-protein diet causes alterations in the metabolism of vitamins. In addition, the excretion of metabolites of carbohydrates in the urine was affected by the intake of HAMSB.

Discussion

In the present study, an untargeted metabolomics approach was applied with no prior selection of metabolites. Thereby, it was possible to make an explorative investigation of the influence of the dietary protein source and the inclusion of HAMSB in a HPM diet, on the urinary metabolic profiles. This approach combined with multivariate data analysis revealed discrimination in the metabolome of LPW

Table 3. List of urinary metabolites discriminating between LPW, LPM and HPM, HPM + HAMSB diets^{a,b}.

Metabolite	Group ^c	p Value (effect of diet)		Relative peak area		Variation ^d		Value (constant of diet)	Relative peak area		Variation ^d	
		LPW	LPM	LPW	LPM	LPW	LPM		HPM	HPM + HAMSB	HPM	HPM + HAMSB
Citric (Isocitric) acid ^{e,g}	1	.0008	2123	1606	86	.01	89	550	462	21	22	
Tartaric acid ^e	1	.05	1595	1306	97	<.0001	95	2061	3458	105	96	
Carnitine ^e	2	<.0001	12	1950	1581–2405	NS	10–15	2087	2091	1883–2312	1887–2316	
Acetylcarnitine ^e	2	<.0001	18	1889	1486–2402	.002	14–23	2212	3160	1828	1828	
p-Cresol sulphate ^e	3	<.0001	517	1534	111	NS	109	1932	1359	245	245	
p-Cresol glucuronide ^f	3	<.0001	1865	3846	183	.04	180	3192	2368	264	264	
Phenylacetylglucine ^e	3	<.0001	2703	3797	147	.008	144	3946	3248	163	163	
Kynurenic acid ^e	3	.02	471	305	44	NS	43	297	271	253–346	231–319	
Xanthurenic acid ^e	3	.0002	1850	960	124	NS	129	880	724	138	138	
Hippuric acid ^e	4	<.0001	1813	3010	107	NS	105	2098	2163	98	98	
Piperidine ^e	4	ND	–	–	–	.02	–	126	101	7	7	
Choline ^f	4	<.0001	450	169	145–197	NS	387–523	156	153	126–192	124–189	
Trimethylamine N-oxide ^e	4	<.0001	112	433	17	NS	17	591	636	55	60	
Creatine ^e	5	<.0001	17	1626	1391–1901	.005	15–20	2240	2606	80	80	
Creatinine ^e	5	<.0001	3129	4387	142	NS	139	4565	4457	85	93	
Imidazolepropionic acid ^e	5	ND	–	–	–	.02	–	150	187	11	11	
3-Methylhistidine ^e	5	ND	–	–	–	NS	–	104	45	33	33	
Spermine ^e	5	ND	–	–	–	.049	–	90	62	9	9	
N1-Acetylspermidine (N8-Acetylspermidine) ^{e,g}	5	ND	–	–	–	<.0001	–	93	71	3	3	
Riboflavin ^e	5	ND	–	–	–	.004	–	80	118	8	8	
Salicylic acid ^e	5	.01	9	6	0.6	ND	0.6	–	–	–	–	

NS: not statistically significant ($p > .05$); ND: not detected.^a $n = 10$ per group except for citric acid, xanthurenic acid, kynurenic acid and Trimethylamine N-oxide in LPW, tartaric acid in HPM and citric acid and creatinine in HPM + HAMSB group, where the number of observations was 9.^bRelative peak area and variation values are divided by 1000.^cGroup: Metabolites related to 1. TCA cycle, 2. Carnitine metabolism, 3. Aromatic amino acids metabolites 4. Metabolites suggesting a change in the activity of gut microbiota, 5. Metabolites with possible implications on colon health.^dThe variation is given as standard error or 95% confidence intervals.^eIdentified using commercially available standards.^fPutatively identified using MS/MS, and METLIN/Human Metabolome Data Base.^gCases with isomeric structure, where differentiation could not be performed using the available HPLC column.

and LPM groups, as well as the HPM and HPM + HAMSB groups.

Increase in butyrate concentration in the caecum after DF or resistant starch consumption has previously been reported in pigs and rats (Le Leu et al. 2007; Nielsen et al. 2014). Similarly, we observed an increase in butyrate and organic acid content of caecum and faeces in HPM + HAMSB group. Milk is known to be particularly rich in SCFA, therefore, an increase in the content of SCFA in the blood after consumption of the milk diet in humans was reported by Bertram et al. (2007). This is in line with the increase in faecal excretion of organic acids in LPW group in our study. A proportion of the pool SCFA in the large intestine is derived from amino acids fermentation (Lopez-Legarrea et al. 2014). Consequently, butyrate and organic acid content of caecum increased in high-protein groups.

The urinary excretion of citrate (as the main component of TCA cycle) is strongly influenced by the acid–base status of the organism, especially the intracellular pH. Consumption of meat has an acidifying effect in the body due to the high content of sulphuric amino acids (methionine and cysteine), which decreases the intracellular pH, meaning that the tubular reabsorption of citrate increases and its excretion decreases (Xu et al. 2010). This is likely to explain the lower excretion of citrate in the urine of LPM rats compared with the LPW. A similar trend was observed in the excretion of other TCA cycle metabolites, aconitic and also tartaric acid, an indirect part of TCA cycle produced from the conversion of oxaloacetate (Hedemann et al. 2017). We also observed a decreased excretion of TCA cycle metabolites in rats fed the HPM + HAMSB diet compared with the HPM. Cummings et al. (1979) previously reported that adding 29.8 g DF from wheat per day to a HPM diet reduces the urinary pH. A potential similar reduction of the urinary pH after feeding 10% HAMSB to the rats on a high-protein diet in our study could explain the lower urinary excretion of citric, succinic and malic acid. However, tartaric acid as an indirect part of the TCA cycle was not following this pattern. This compound was excreted more from rats receiving the HPM + HAMSB diet compared with the HPM group. Tartaric acid was included in the synthesis of the HAMSB as a catalyst (Tupa et al. 2013), and our results could indicate that tartaric acid may have reacted directly with the starch, whereby it has been added to the starch polymer along with butyrate. The titration method applied to evaluate the DS of the esterified HAMS only accounts for the

number of double bindings generated in the esterification process and not for the exact molecules associated with the double bond. Therefore, it is likely that tartaric acid accounts for some of the substitutions (DS 0.93) measured in the HAMSB and this may explain the higher urinary excretion following intake of the HPM + HAMSB diet.

Carnitine is cited in the literature as a marker of meat ingestion (Stella et al. 2006). Excretion of this metabolite and its acyl forms was significantly increased in rats receiving meat as the source of protein compared with whey. HAMSB inclusion in the diet of rats receiving the high level of meat protein caused the excretion of acetylcarnitine and 2-ethylacrylcarnitine to increase significantly. An explanation for this increase requires further investigation.

Sulphate is added to various compounds in the liver to ease their excretion and hence is present in the urine as esters of phenol, indole and p-cresol. These compounds are derived from bacterial degradation of aromatic amino acids in the large intestine (Florin et al. 1991). Tyrosine from dietary protein is considered the parent compound from which p-cresol, hydroxyphenyllactate and p-cresol glucuronide are formed (Booth et al. 1960; Stella et al. 2006; Liabeuf et al. 2013). Tyrosine and phenylalanine are present in relatively similar amounts in whey and meat (Cribb 2005; Gilbert et al. 2011), however, their absorption is reduced in the presence of BCAAs (Matthews 2005). The content of BCAAs is high in concentrated whey compared with meat (Cribb 2005; Gilbert et al. 2011), and this may explain the lower urinary excretion of different aromatic acid metabolites such as p-cresol glucuronide and p-cresol sulphate in LPW rats compared with LPM rats. In addition, our results show that inclusion of HAMSB in a high-protein diet, causes a significant decrease in p-cresol glucuronide excretion, in line with previous reports on reduced urinary p-cresol after HAMS consumption in rats (Kieffer et al. 2016). In general, protein metabolites are known to be toxic, but relevance to CRC is debateable. p-Cresol has resulted in dose-dependent formation of various DNA adducts *in vitro* (Winter et al. 2011).

The level of urinary phenylacetylglutamine was previously found to be associated with the level of fat intake. A higher excretion of phenylacetylglutamine has been correlated to increased levels of its precursors, such as phenylacetate, produced by the gut microbiota after high dietary fat intake (Delaney et al. 2004; Kim et al. 2009). The experimental diets in our study contained the same high level of fat, however, we

902
903
904
905
906
907
908
909
910
911
912
913
914
915
916
917
918
919
920
921
922
923
924
925
926
927
928
929
930
931
932
933
934
935
936
937
938
939
940
941
942
943
944
945
946
947
948
949
950
951
952
953
954

observed lower excretion of phenylacetylglycine from the LPW and HPM + HAMSB groups. This may be an indicator of a change in the gut microbiota composition induced by whey or inclusion of HAMSB in favour of reducing this metabolite's precursors. Kynurenic and xanthurenic acid are other aromatic amino acid metabolites present in the urine which are produced by the gut microbiota (Cheng et al. 2012; Shibata et al. 2013). A rat study showed a significant decrease in the activity of tryptophan catabolizing enzyme following a Western-style diet containing 28% protein and 33% fat (Ohland et al. 2016). Similarly, receiving meat as the protein source compared with whey in our study resulted in a reduction in the amounts of these metabolites. This change may be interpreted as an altered metabolism by the large intestinal microflora due to the diet (Ohland et al. 2016).

Hippurate is one of the most widely detected urinary metabolites of host-microbial origin in humans and rodents and is an important biomarker of gut microbial activity (Zhang et al. 2015). After providing 10-year-old boys with animal protein as either milk or meat for one week, Bertram et al. (2007) observed reduced urinary excretion of hippurate after milk consumption. Similarly, we found a lower excretion of hippurate following the LPW diet compared with the LPM diet, suggesting alterations in the microfloral metabolism. The level of this metabolite was not significantly different between the two high-protein groups, which can indicate a lack of alteration in the overall activity of gut microbiota after dietary HAMSB inclusion.

Piperidine is a secondary amine produced by colonic bacteria (Shephard et al. 1987; Hughes et al. 2000). It can later be subjected to N-nitrosation in the large intestine and produce N-nitroso compounds known to exert carcinogenic/mutagenic effects (Hughes et al. 2000). Piperidine was present in the urine of rats on high-protein diets and the inclusion of HAMSB caused a significant decrease in its excretion. This can be interpreted as a change in the gut microflora activity in favour of reduced production of this amine as a result of receiving HAMSB in the diet.

The choline content of beef is more than five times higher than that of whole milk (Zeisel et al. 2003), but we observed a higher urinary excretion of choline in the LPW group, which could indicate lower intestinal absorption of choline in the LPM fed rats. This is in line with our results for trimethylamine-N-oxide (TMAO), a product of modulations on unabsorbed choline by the gut microbiota (Cheng et al. 2012;

Bresson et al. 2016). A higher excretion of TMAO was observed in the urine of rats in the LPM group. Nevertheless, TMAO can also be produced by microbial fermentation of carnitine in the colon (Jakobsen et al. 2017), therefore, both unabsorbed choline and carnitine content of the meat may have contributed to the higher excretion of TMAO in the LPM group. TMAO is generally regarded a non-toxic substance, however, it is of clinical interest because of its potential to form the carcinogen N-nitrosodimethylamine (Cheng et al. 2012).

Higher excretion of creatine/creatinine in LPM-fed rats compared with LPW-fed rats was observed. Normally, creatine, which functions to increase the availability of cellular ATP, is acquired through the regular dietary intake of foods such as meat and fish (Stella et al. 2006). However, creatine and creatinine as guanidines are nitrosatable and considered as N-nitroso precursors (Shephard et al. 1987). HAMSB inclusion in a high-protein diet resulted in an increased excretion of creatine, suggesting a lower possibility of N-nitroso compounds production due to higher excretion of their precursor from the body.

The source of imidazole propionate remains unclear, but it shares a very similar structure with urocanate and is believed to be a histidine catabolite (Moriya et al. 2017). Histidine can be produced by the gut microbiota. Changes in the level of imidazole propionic acid has been reported as a sign of alterations in the gut microbiota (Sarosiek et al. 2016). The HPM + HAMSB rats excreted higher amounts of this metabolite. 3-Methylhistidine is another histidine metabolite present in the urine and it is correlated with a higher risk of CRC due to an increased level of fat and protein consumption (Yeung et al. 1991). This metabolite was identified in the urine of rats on high-protein diets.

Spermine and an N-acylated form of spermidine are biogenic polyamines. In our study, urinary excretion of these compounds was reduced as a result of HAMSB inclusion in a high-protein diet. Exogenous polyamines are obtained through the diet or produced by the gut microbiota. Red meat intake has been correlated with urinary polyamines (Vargas et al. 2014). These compounds are considered to be involved in almost all the steps of colorectal tumorigenesis (Linsalata et al. 2014). Lower urinary excretion of polyamines after the inclusion of HAMSB in a high-protein diet suggests a change in their production process potentially leading to improvement of the gut health.

Meat is a rich source of riboflavin (Cosgrove et al. 2005) and in our study, the inclusion of HAMSB in a high-protein diet caused higher urinary excretion of riboflavin and pyridoxic acid, a metabolite of vitamin B₆. A diet including fibre-rich whole-grain cereals has been previously reported to increase the intake of B-vitamins (Melanson et al. 2006). Higher concentrations of vitamin B₂ and vitamin B₆ in the plasma are associated with a lower risk of CRC (Eussen et al. 2010) and urinary riboflavin excretion can be used as a biomarker for riboflavin status in the body (Choi et al. 2005). Therefore, a higher excretion of water-soluble vitamin metabolites following the HPM + HAMSB diet can be interpreted as the capability of HAMSB to improve colorectal health.

The existence of salicyluric acid has been reported in milk (Booth et al. 1962). It can be metabolised to salicylic acid by intestinal microorganisms (Nakamura et al. 1991), which has been shown to have CRC preventive traits (Paterson and Lawrence 2001). In our study, more salicylate was excreted in the urine of rats on LPW diet compared with LPM. This can be interpreted as a higher capacity of whey as a protein source to contribute to colonic health compared with meat.

Conclusions

We identified several metabolites that occur in significantly different amounts in the urine of rats from the four experimental groups. According to our results, HAMSB and whey increase butyrate and organic acid content of the caecum and faeces. Furthermore, choosing whey as protein source leads to a lower production of potentially harmful metabolites such as TMAO, creatine and creatinine in the colon, compared with meat and thereby a lower urinary excretion. In addition, the inclusion of HAMSB showed to have the potential to improve colon health by increasing riboflavin and pyridoxic acid and decreasing the level of polyamines, 3-methylhistidine and piperidine with potential negative effect on colonic health. Interpreting the dietary effects on urinary excretion of some metabolites, especially the ones resulted from the large intestinal microbial activity requires further investigation. This will help provide a better picture of the interaction between the diet and intestinal microbiota and consequently their effect on colorectal health.

Acknowledgements

The authors acknowledge Mihai Victor Curtasu and Kasper Vrangstrup Poulsen for their technical assistance.

Disclosure statement

No potential conflict of interest is reported by the authors.

Funding

The study was financially supported by the Independent Research Fund Denmark, project "Starch based nutraceuticals – Can starch improve colonic health?" [project no. DFF-4005-00258].

ORCID

Zahra Sattari Najafabadi <http://orcid.org/0000-0002-2102-4031>

References

- Bajka BH, Topping DL, Cobiac L, Clarke JM. 2006. Butyrylated starch is less susceptible to enzymic hydrolysis and increases large-bowel butyrate more than high-amylose maize starch in the rat. *Br J Nutr.* 96:276–282.
- Bertram HC, Hoppe C, Petersen BO, Duus JØ, Mølgaard C, Michaelsen KF. 2007. An NMR-based metabonomic investigation on effects of milk and meat protein diets given to 8-year-old boys. *Br J Nutr.* 97:758–763.
- Booth A, Robbins D, Dunkley W. 1962. Occurrence of salicyluric acid in milk. *Nature.* 194:290–291.
- Booth AN, Masri MS, Robbins DJ, Emerson OH, Jones FT, Deeds F. 1960. Urinary phenolic acid metabolites of tyrosine. *J Biol Chem.* 235:2649–2652.
- Canibe N, Højberg O, Badsberg JH, Jensen BB. 2007. Effect of feeding fermented liquid feed and fermented grain on gastrointestinal ecology and growth performance in piglets. *Animal Sci J.* 85:2959–2971.
- Chan DS, Lau R, Aune D, Vieira R, Greenwood DC, Kampman E, Norat T. 2011. Red and processed meat and colorectal cancer incidence: meta-analysis of prospective studies. *PloS One.* 6:e20456.
- Cheng Y, Xie G, Chen T, Qiu Y, Zou X, Zheng M, Tan B, et al. 2012. Distinct urinary metabolic profile of human colorectal cancer. *J Proteome Res.* 11:1354–1363.
- Choi HK, Liu S, Curhan G. 2005. Intake of purine-rich foods, protein, and dairy products and relationship to serum levels of uric acid: the Third National Health and Nutrition Examination Survey. *Arthritis Rheumatol.* 52:283–289.
- Cosgrove M, Flynn A, Kiely M. 2005. Consumption of red meat, white meat and processed meat in Irish adults in relation to dietary quality. *Br J Nutr.* 93:933–942.
- Cribb PJ. 2005. US whey proteins in sports nutrition. *USDEC.* 3:1–12.
- Cummings J, Hill M, Bone E, Branch W, Jenkins D. 1979. The effect of meat protein and dietary fiber on colonic function and metabolism. II. Bacterial metabolites in feces and urine. *Am J Clin Nutr.* 32:2094–2101.
- Delaney J, Neville WA, Swain A, Miles A, Leonard MS, Waterfield CJ. 2004. Phenylacetylglutamine, a putative biomarker of phospholipidosis: its origins and relevance to

Q6

- phospholipid accumulation using amiodarone treated rats as a model. *Biomarkers*. 9:271–290.
- Eggum B. 1973. 406th Report from the National Institute of Animal Science. Copenhagen.
- Eussen SJ, Vollset SE, Hustad S, Meyer K, Ueland PM, Jenab M, Slimani N, et al. 2010. Plasma vitamins B2, B6, B12, and related genetic variants as predictors of colorectal cancer risk. *Cancer Epidemiol Biomark Prev*. 19:2549–2561.
- Florin T, Neale G, Gibson G, Christl S, Cummings J. 1991. Metabolism of dietary sulphate: absorption and excretion in humans. *Gut*. 32:766–773.
- Gilbert J-A, Bendtsen NT, Tremblay A, Astrup A. 2011. Effect of proteins from different sources on body composition. *Nutr Metab Cardiovasc Dis*. 21:B16–B31.
- Hansen B. 1989. Determination of nitrogen as elementary N, an alternative to Kjeldahl. *Acta Agric Scand B*. 39:113–118.
- Hedemann MS, Hermansen K, Pedersen S, Knudsen KEB. 2017. Resistant starch but not enzymatically modified waxy maize delays development of diabetes in Zucker diabetic fatty rats. *J Nutr*. 147:825–834.
- Helrich K. 1990. Official method of analysis of the association of official analytical chemists. Washington, DC, USA: AOAC.
- Hughes R, Magee E, Bingham S. 2000. Protein degradation in the large intestine: relevance to colorectal cancer. *Curr Issues Intest Microbiol*. 1:51–58.
- Jakobsen L, Yde CC, Hecke T, Jessen R, Young JF, Smet S, Bertram HC. 2017. Impact of red meat consumption on the metabolome of rats. *Mol Nutr Food Res*. 61:1600387.
- Kieffer DA, Piccolo BD, Vaziri ND, Liu S, Lau WL, Khazaeli M, Nazertehrani S, et al. 2016. Resistant starch alters gut microbiome and metabolomics profiles concurrent with amelioration of chronic kidney disease in rats. *Am J Physiol Renal Physiol*. 310:F857–F871.
- Kim S-H, Yang S-O, Kim H-S, Kim Y, Park T, Choi H-K. 2009. 1H-nuclear magnetic resonance spectroscopy-based metabolic assessment in a rat model of obesity induced by a high-fat diet. *Anal Bioanal Chem*. 395:1117–1124.
- Knudsen KEB. 1997. Carbohydrate and lignin contents of plant materials used in animal feeding. *Anim Feed Sci Technol*. 67:319–338.
- Le Leu RK, Brown IL, Hu Y, Morita T, Esterman A, Young GP. 2007. Effect of dietary resistant starch and protein on colonic fermentation and intestinal tumourigenesis in rats. *J Carcinog*. 28:240–245.
- Liabeuf S, Glorieux G, Lenglet A, Diouf M, Schepers E, Desjardins L, Choukroun G, et al. 2013. Does p-cresylglucuronide have the same impact on mortality as other protein-bound uremic toxins? *PLoS One*. 8:e67168.
- Linsalata M, Orlando A, Russo F. 2014. Pharmacological and dietary agents for colorectal cancer chemoprevention: effects on polyamine metabolism (review). *Int J Oncol*. 45:1802–1812.
- Lopez-Legarrea P, Fuller NR, Zulet MA, Martinez JA, Caterson ID. 2014. The influence of Mediterranean, carbohydrate and high protein diets on gut microbiota composition in the treatment of obesity and associated inflammatory state. *Asia Pac J Clin Nutr*. 23:360–368.
- Lopez-Rubio A, Clarke JM, Scherer B, Topping DL, Gilbert EP. 2009. Structural modifications of granular starch upon acylation with short-chain fatty acids. *Food Hydrocoll*. 23:1940–1946.
- Matthews DE. 2005. Observations of branched-chain amino acid administration in humans. *J Nutr*. 135:1580S–1584S.
- Melanson KJ, Angelopoulos TJ, Nguyen VT, Martini M, Zukley L, Lowndes J, Dube TJ, et al. 2006. Consumption of whole-grain cereals during weight loss: effects on dietary quality, dietary fiber, magnesium, vitamin B-6, and obesity. *J Am Diet Assoc*. 106:1380–1388.
- Milliken GA, Johnson DE. 1984. Analysis of Messy Data (vol. I).
- Moriya T, Satomi Y, Murata S, Sawada H, Kobayashi H. 2017. Effect of gut microbiota on host whole metabolome. *Metabolomics*. 13:101.
- Nakamura J, Katayama M, Kido M, Nishida K, Sasaki H. 1991. Decrease of gastrointestinal mucosal damage by salicylic acid compared with salicylic acid in rabbits. *J Pharm Pharmacol*. 43:766–773.
- Nielsen TS, Laerke HN, Theil PK, Sørensen JF, Saarinen M, Forssten S, Knudsen KEB. 2014. Diets high in resistant starch and arabinoxylan modulate digestion processes and SCFA pool size in the large intestine and faecal microbial composition in pigs. *Br J Nutr*. 112:1837–1849.
- O'keefe SJ. 2016. Diet, microorganisms and their metabolites, and colon cancer. *Nat Rev Gastroenterol Hepatol*. 13:691–706.
- Ohland CL, Pankiv E, Baker G, Madsen KL. 2016. Western diet-induced anxiolytic effects in mice are associated with alterations in tryptophan metabolism. *Nutr Neurosci*. 19:337–345.
- Paterson J, Lawrence J. 2001. Salicylic acid: a link between aspirin, diet and the prevention of colorectal cancer. *QJM*. 94:445–448.
- Rasmussen LG, Winning H, Savorani F, Toft H, Larsen TM, Dragsted LO, Astrup A, Engelsen SB. 2012. Assessment of the effect of high or low protein diet on the human urine metabolome as measured by NMR. *Nutrients*. 4:112–131.
- Reeves PG, Nielsen FH, Fahey GC. 1993. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J Nutr*. 123:1939–1951.
- Sarosiek K, Pappan KL, Gandhi AV, Saxena S, Kang CY, McMahon H, Chipitsyna GI, et al. 2016. Conserved metabolic changes in nondiabetic and type 2 diabetic bariatric surgery patients: global metabolomic pilot study. *J Diabetes Res*. 2016:1. ID 3467403.
- Shephard S, Schlatter C, Lutz WK. 1987. Assessment of the risk of formation of carcinogenic N-nitroso compounds from dietary precursors in the stomach. *Food Chem Toxicol*. 25:91–108.
- Shibata K, Inomoto K, Nakata C, Fukuwatari T. 2013. Pantothenic acid deficiency may increase the urinary excretion of 2-Oxo acids and nicotinamide catabolites in rats. *J Nutr Sci Vitaminol*. 59:509–515.
- Stella C, Beckwith-Hall B, Cloarec O, Holmes E, Lindon JC, Powell J, Van Der Ouderaa F, et al. 2006. Susceptibility of human metabolic phenotypes to dietary modulation. *J Proteome Res*. 5:2780–2788.

- Stoldt W. 1952. Vorschlag zur vereinheitlichung der fettbestimmung in lebensmitteln. *Eur J Lipid Sci Technol.* 54:206–207.
- Toden S, Bird AR, Topping DL, Conlon MA. 2007. Differential effects of dietary whey, casein and soya on colonic DNA damage and large bowel SCFA in rats fed diets low and high in resistant starch. *Br. J. Nutr.* 97:535–543.
- Tupa M, Maldonado L, Vázquez A, Foresti ML. 2013. Simple organocatalytic route for the synthesis of starch esters. *Carbohydr Polym.* 98:349–357.
- Turck D, Bresson J-L, Burlingame B, Fairweather-Tait S, Heinonen M, Hirsch-Ernst KI, Mangelsdorf I, et al. 2016. Dietary reference values for choline EFSA panel on dietetic products, Nutrition and Allergies (NDA). *EFSA J.* 14.
- Van Den Berg RA, Hoefsloot HC, Westerhuis JA, Smilde AK, Van Der Werf MJ. 2006. Centering, scaling, and transformations: improving the biological information content of metabolomics data. *BMC Genom.* 7:142.
- Vargas AJ, Ashbeck EL, Thomson CA, Gerner EW, Thompson PA. 2014. Dietary polyamine intake and polyamines measured in urine. *Nutr Cancer.* 66:1144–1153.
- Winter J, Nyskohus L, Young GP, Hu Y, Conlon MA, Bird AR, Topping DL, Le Leu RK. 2011. Inhibition by

- resistant starch of red meat-induced promutagenic adducts in mouse colon. *Cancer Prev Res.* doi: 10.1158/1940-6207.
- Xu J, Yang S, Cai S, Dong J, Li X, Chen Z. 2010. Identification of biochemical changes in lactovegetarian urine using ¹H NMR spectroscopy and pattern recognition. *Anal Bioanal Chem.* 396:1451–1463.
- Yeung K, Eyssen GM, Li G, Glazer E, Hay K, Child P, Gurgin V, et al. 1991. Comparisons of diet and biochemical characteristics of stool and urine between Chinese populations with low and high colorectal cancer rates. *J Natl Cancer Inst.* 83:46–50.
- Young JF, Therkildsen M, Ekstrand B, Che BN, Larsen MK, Oksbjerg N, Stagsted J. 2013. Novel aspects of health promoting compounds in meat. *Meat Sci.* 95:904–911.
- Zeisel SH, Mar M-H, Howe JC, Holden JM. 2003. Concentrations of choline-containing compounds and betaine in common foods. *J Nutr.* 133:1302–1307.
- Zhang Y, Zhao F, Deng Y, Zhao Y, Ren H. 2015. Metagenomic and metabolomic analysis of the toxic effects of trichloroacetamide-induced gut microbiome and urine metabolome perturbations in mice. *J Proteome Res.* 14:1752–1761.

PROOF