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Inulin-fortification of a processed meat product attenuates formation of nitroso compounds in the gut of healthy rats

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Abbreviations

ATNC, apparent total N-nitroso compounds; CRC, colorectal cancer; DM, dry matter; GSH-Px, glutathione peroxidase; HEX, hexanal; MDA, malondialdehyde; NOC, N-nitroso compounds; O⁶ MeG, O⁶-methyl-2-deoxyguanosine; OPLS-DA, orthogonal projections to latent structures discriminant analysis; PCA, principal component analysis; PCC, protein carbonyl compounds; RSNO, nitrosothiols; FeNO, nitrosyl iron compounds; SCFA, short chain fatty acid; TSP, 3-(trimethylsilyl)-propionate; 4-HNE, 4-hydroxynonenal.

20

21 **Abstract**

22 Intake of red and processed meat has been suspected to increase colorectal cancer risk potentially via
23 endogenous formation of carcinogenic *N*-nitroso compounds or increased lipid- and protein oxidation.
24 Here we investigated the effect of inulin fortification of a pork sausage on these parameters. During
25 four weeks, healthy Sprague-Dawley rats ($n = 30$) were fed one of three diets; inulin-fortified pork
26 sausage, control pork sausage or a standard chow diet. Fecal content of apparent total N-nitroso
27 compounds (ATNC), nitrosothiols and nitrosyl iron compounds (FeNO) were analyzed in addition to
28 liver metabolism and oxidation products formed in liver, plasma and diets. Intriguingly, inulin
29 fortification reduced fecal ATNC ($p = 0.03$) and FeNO ($p = 0.04$) concentration. The study revealed
30 that inulin fortification of processed meat could be a strategy to reduce nitroso compounds formed
31 endogenously after consumption.

32

33 **Key words** Fiber-fortification, inulin, processed meat, nitroso compounds, oxidation

34

35

36 **1. Introduction**

37 Consumption of red and particularly processed meat has been associated with a possible increased risk
38 of colorectal cancer (CRC) (Chan et al., 2011). Among the major hypotheses explaining this possible
39 association, endogenous formation of *N*-nitroso compounds (NOCs) following red or processed meat
40 ingestion has been suggested (Hughes, Cross, Pollock and Bingham, 2001). Many NOCs are suspected
41 to be carcinogenic and red meat consumption has been shown to dose-dependently increase the fecal
42 excretion of NOCs (Hughes et al., 2001). NOCs can lead to alkylation of DNA, resulting in the
43 formation of pro-mutagenic DNA adducts. This can induce G:C→ A:T mutations, which might
44 eventually initiate carcinogenesis (Gottschalg, Scott, Burns and Shuker, 2007, Mirvish, 1995). Intake of
45 red meat has in fact been shown to increase DNA adduct formation in mice and human, including the
46 pro-mutagenic DNA adduct O⁶-methyl-2-deoxyguanosine (O⁶ Meg) (Le Leu et al., 2015, Winter et al.,
47 2011). Heme iron, a component of red and processed meat, has been suggested to stimulate NOC
48 formation following red or processed meat ingestion (Cross, Pollock and Bingham, 2003). Endogenous
49 NOCs are likely formed via various routes throughout the gastrointestinal tract, including acid and
50 bacterial catalyzed reactions, generally as a result of the reaction between nitrosating agents and
51 nitrosable substrates (Hughes, Magee and Bingham, 2000). Acid catalyzed nitroso compound
52 formation occurs mainly in the stomach, where nitrosating agents, such as dietary nitrite reaching the
53 stomach, result in the formation of various nitroso compounds (Kobayashi, 2018). The acidic
54 environment of the stomach has been found to favor the formation of nitrosothiols, which has been
55 suggested to be the initial step in the formation of nitroso compounds in the gastrointestinal tract
56 (Kuhnle et al., 2007). The nitrosothiols formed in the stomach might be precursors for NOCs formed
57 further down the GI tract, as the increasing pH favors their release of NO (Kuhnle and Bingham, 2007).

58 In the small intestine, it has been suggested that heme might be nitrosylated by nitrite or the NO
59 released from nitrosothiols, making it possible for the nitrosylated heme to act as a nitrosating agent
60 increasing the formation of NOC (Kuhnle et al., 2007).

61 Bacterial catalyzed NOC formation has been found to require the presence of bacteria with nitrite and
62 nitrate reductase enzymes activity (Calmels, Ohshima and Bartsch, 1988, Calmels, Ohshima, Henry
63 and Bartsch, 1996). Intriguingly, a study investigating the formation of NOCs in germ free rats found
64 that the presence of a colonic flora was necessary for NOC formation to occur (Massey, Key, Mallett
65 and Rowland, 1988). In the large intestine, nitrosable substrates formed via protein degradation as well
66 as nitrosating agents are available, providing a site for bacterial catalyzed NOC formation (Hughes et
67 al., 2000).

68 Besides the suggested role of heme in NOC formation, heme iron in red meat has been suggested to
69 stimulate lipid and protein oxidation (Bastide, Pierre and Corpet, 2011, Van Hecke, Vanden Bussche,
70 Vanhaecke, Vossen, Van Camp and De Smet, 2014). In particular, lipid oxidation may result in the
71 formation of potentially toxic end-products including malondialdehyde (MDA) and 4-hydroxynonenal
72 (4-HNE) formed via peroxidation particularly of polyunsaturated fatty acids. Both MDA and 4-HNE
73 have been found to be able to react with DNA to form DNA adducts, whereas 4-HNE also has shown
74 cytotoxic effects (Bastide et al., 2011, Nair, Bartsch and Nair, 2007).

75 Previous investigations have indicated that dietary fiber consumption has a protective effect against
76 CRC development (Bingham et al., 2003). In fact, earlier studies found that dietary fibers consumed in
77 combination with red meat attenuated meat-induced DNA damage and potential harmful protein
78 fermentation products, whereas fecal short chain fatty acids (SCFAs) concentrations were increased
79 (Le Leu et al., 2015, Toden, Bird, Topping and Conlon, 2007, Winter et al., 2011). Moreover, fiber
80 addition to meat products has shown a lowering effect on lipid oxidation following *in vitro* digestion

81 (Hur, Lee and Lee, 2014). Thus, we recently demonstrated that inulin fortification of a pork sausage
82 product increased fecal content of SCFAs when fed to healthy rats during a 4-week intervention
83 (Thogersen et al., 2018). Based on the same experimental study, we here investigated whether the
84 incorporation of inulin into a pork sausage product also had a protective effect on the formation of
85 apparent total N-nitroso compounds (ATNC) upon consumption. Compounds-specific denitrosation
86 prior to analysis was used in order to investigate the types of nitroso compounds formed. The effect of
87 inulin fortification on protein and lipid oxidation was examined by measuring oxidation markers in
88 plasma and liver. Furthermore, as the liver is a key metabolic organ, possible hepatic metabolic
89 changes were studied using ^1H nuclear magnetic resonance spectroscopy.

90

91 **2. Materials and methods**

92 *2.1 Sausage diets*

93 Two different sausage batches were manufactured for the study, and Table S1 shows the nutritional
94 composition of the experimental diets. The sausages were made from a sausage emulsion of pork meat,
95 pork backfat, which was prepared with a bowl cutter using a standard procedure for frankfurter
96 sausages. After mincing of pork meat and backfat, the remaining ingredients provided in Supporting
97 Information, Table S2, were added to the minced meat. For the inulin-enriched sausages, inulin was
98 added to a fiber content approximating the content in the chow diet (5.6 % compared to 6.05 %). The
99 inulin used was Orafit® HP (Beneo-Orafit, Oreye, Belgium), a long-chain chicory inulin product
100 containing 99.5 % inulin with an average degree of polymerization of 23 ranging from 5 to 60. Inulin
101 fibers were added in their dry form without any pre-treatment. Casings (22/24 lamb casings) were filled
102 with 82 g meat batter to reach a final weight of 75 g after heat treatment. After heat treatment the
103 sausages were frozen at stored at -18 °C until further use.

104

105 *2.2. Rat intervention and sample collection*

106 Thirty healthy Sprague-Dawley rats (NTac:SD) at the age of four weeks (Taconic, L1. Skensved,
107 Denmark) were used in this study. The rats were housed in our Association for Assessment and
108 Accreditation of Laboratory Animal Care (AAALAC) accredited facility, and randomly housed into ten
109 U1400 cages (Tecniplast, Buguggiate, Italy) on aspen bedding and with enrichment (Tapyei, Harjumaa,
110 Estonia) in groups of three rats per cage following weighing and ear-marking. The facility was health
111 monitored according to FELASA guidelines (2014) revealing none of the infections listed. The rats

112 were allowed a seven-day adaptation period during which they were fed a standard chow diet *ad*
113 *libitum* (Altromin 1324, Brogaarden, Denmark) with free access of water. After adaptation, the rats
114 were randomly divided into three groups receiving one of the following diets during an experimental
115 period of four weeks; 1) Pork sausages enriched with 5.6 % chicory inulin ($n = 12$), 2) Pork sausages
116 without enrichment ($n = 12$), 3) standard chow diet (Altromin 1324), ($n = 6$). Body weight, food and
117 water intake have been published elsewhere (Thogersen et al., 2018).

118 After the intervention period, fecal samples were collected and the rats were sacrificed according to
119 previously described procedures (Thogersen et al., 2018). After anesthesia by hypnorm/midazolam
120 (diluted 1:1 with sterile water prior to mixing; 0.2 mL/g body weight), heart blood was collected
121 followed by decapitation.. Liver samples were collected by carefully removing the liver. Samples of
122 approximately 2x2 cm were subsequently transferred to Cryotubes and snap frozen in liquid nitrogen.
123 Samples were stored at -80 °C until analysis.

124 The study was in accordance with Directive 2010/63/EU of the European Parliament and of the
125 Council of 22 September 2010 on the protection of animals used for scientific purposes and the Danish
126 Animal Experimentation Act (LBK 474 15/05/2014). Specific approval was granted by the Animal
127 Experiments Inspectorate under the Ministry of Environment and Food in Denmark (License No 2012-
128 15-2934-00256).

129

130 2.3 Nitroso compounds

131 Prior to analysis, fecal samples were disrupted using a TissueLyser LT (Qiagen). Approximately 200
132 mg fecal sample, 500 μ L 1.0 mm glass beads (Sigma-Aldrich, St. Louis, MO, USA) and 1 mL HPLC

grade water per 200 mg feces were added a 2 mL Eppendorf Tube and sample disruption was conducted for 10 min oscillating at 50 1/s. The samples were centrifuged at 14,000 x g for 15 min at 4 °C and the collected supernatant was stored at -80 °C until further analysis. Nitroso compound determination was based on a previously described method (Kuhnle et al., 2007) with modifications using chemiluminescence detection with an Ecomedics CLD 88 Exhalyzer (Ecomedics, Dürnten, Switzerland). A purge vessel containing 15 mL of a tri-iodide solution (2 g potassium iodide, 1.3 g iodine 40 mL water and 140 mL glacial acetic acid) and heated to 60 °C was connected via a condenser to a wash bottle containing 1 M NaOH. The wash bottle was connected to the Ecomedics CLD 88 Exhalyzer via a polypropylene filter (0.2 µm, Whatman, USA). The NaOH wash bottle and condenser were kept at 0 °C. For mixing injected sample and transferring released NO to the analyzer, nitrogen gas was bubbled through the system and the signal obtained was processed using instrument software Chart v5.5.8 (eDAQ, Australia). Quantification was based on the injection of sodium nitrite (Sigma-Aldrich, Steinheim, Germany) in a concentration range of 1.22-19.5 µM. For the determination of ATNC, 100 µL fecal supernatant were combined with 100 µL 0.1 M N-ethylmaleimide (NEM) and 0.01 M diethylene triamine pentaacetic acid (DTPA) in water to chelate metal iron and preserve nitroso thiols, and 500 µL sulfamic acid solution (50 g/L in 1 M HCl, Fisher Scientific, Loughborough, UK) to remove nitrite, vortex mixed and incubated for 2 min. Subsequently, the solution was injected into the reaction vessel. Nitrosothiol (RSNO) determination was conducted using the procedure prior to injection for ATNC determination followed by the addition of 100 µL aqueous HgCl₂ (10 mM). After vortex mixing and 2 min of incubation, the solution was injected into the purge vessel. Likewise, nitrosyl iron compound (FeNO) determination was conducted using the procedure prior to injection for nitrosothiol determination followed by the addition of 100 µL K₃Fe(CN)₆ (10 mM). After vortex mixing and 2 min of incubation, the solution was injected into the purge vessel. The difference between

156 mercury(II) stable and unstable compounds was used as a measure of nitrosothiols and the difference
157 between ferricyanide stable and unstable compounds as a measure of nitrosyl iron (Kuhnle et al., 2007).
158 A possible protective effect of inulin against NOC formation was investigated under *in vitro* acidic
159 conditions. Bovine hemoglobin (Sigma Aldrich, St Louis, USA), hydrochloric acid and chicory inulin
160 (Beneo GmbH, Mannheim, Germany) were mixed resulting in final concentrations of 100 μ M, 7 mM
161 and 740 μ M for the three constituents, respectively. Sodium nitrite (Sigma-Aldrich, Steinheim,
162 Germany) was added in a final concentration range of 2.5-50 μ M for initiation of the reaction.
163 Following incubation for 15 minutes, ATCN determination in 100 μ L was conducted using the
164 procedure described above. Incubation of corresponding solutions without addition of inulin was used
165 as control.

166

167

168 2.4 ¹H nuclear magnetic resonance spectroscopy (NMR spectroscopy)

169 Intact liver tissue was analyzed by ¹H NMR spectroscopy using high-resolution-magic-angle spinning
170 (HR-MAS) analysis. Approximately 10 mg of liver sample was added to 30 μ L HRMAS disposable
171 inserts (Bruker BioSpin, GmbH, Rheinstetten, Germany) containing 10 μ L D₂O with 0.05 % 3-
172 (trimethylsilyl)-propionate (TSP) and subsequently kept at -80 °C until analysis. ¹H NMR spectroscopy
173 was conducted using a Bruker Avance III 600 MHz spectrometer operating at a ¹H frequency of 600.13
174 MHz equipped with an HR-MAS probe (Bruker BioSpin, Rheinstetten, Germany). A one-dimensional
175 (1D) Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence with pre-saturation to suppress the water
176 resonance was used. The following parameters were used: number of scans (NS) = 128, spectral width

177 (SW) = 10417 Hz, data points (TD) = 32768, target temperature = 278 K and a spinning speed of 4200
178 Hz. Prior to Fourier transformation, the free inductions decays (FIDs) were multiplied by a line-
179 broadening function of 0.3 Hz. The obtained spectra were baseline- and phase corrected and referenced
180 to TSP (0.0 ppm) using Topspin 3.0 (Bruker BioSpin). Data were loaded into MATLAB R2017b
181 (Mathworks Inc., Natick, USA) and spectral regions above 9.8 ppm, below 0.5 ppm and the water
182 signal region (4.9-5.15 ppm) were removed. Spectra were normalized to total area of the spectrum and
183 subdivided into regions of 0.01 ppm. Chenomx NMR Suite 8.13 (Chenomx Inc., Edmonton, Canada)
184 and literature (Beckonert et al., 2010) was used for metabolite assignment. The following multivariate
185 data analysis was conducted using SIMCA 15.0 (Sartorius Stedim Data Analytics AB, Umeå, Sweden).
186 Data were pareto-scaled and principal component analysis (PCA) was conducted followed by
187 construction of an orthogonal projections to latent structures discriminant analysis (OPLS-DA) model
188 using cross validation with seven segments. An OPLS-DA S-line plot was created in order to reveal
189 metabolites important for the separation between dietary treatment groups.

190

191 *2.5 In vitro digestion of experimental diets*

192 The *in vitro* digestions were performed in triplicate according to a previously described protocol,
193 specific for studying oxidation processes during passage in the gastrointestinal system (Van Hecke et
194 al., 2014). In brief, 4.5 g of the experimental diets were sequentially incubated at 37°C for 5 minutes
195 with 6 mL saliva, 2 hours with 12 mL gastric juice, and 2 hours with 2 mL bicarbonate buffer (1 M, pH
196 8.0), 12 mL duodenal juice and 6 mL bile juice. After completion, samples were homogenized with an
197 ultraturrax (9500 rpm) and aliquots were stored at -80°C until analysis of lipid and protein oxidation
198 markers.

199

200 2.6 Lipid- and protein oxidation

201 Oxidation parameters were measured in liver, plasma, experimental diets and *in vitro* digests of the
202 diets. Liver extracts were prepared by homogenizing 1 g of liver tissue in 10 mL 1 % Triton-X-100
203 phosphate buffer (pH 7; 50 mM) for 45 seconds using an ultraturrax homogenizer, followed by
204 centrifugation (15 min, 15,000 g, 4 °C), after which the supernatant was filtered through glass wool.
205 Supernatants were immediately analyzed for malondialdehyde (MDA) and activity of glutathione
206 peroxidase (GSH-Px). The measurement of total (unbound and bound) MDA was based on a
207 previously described method (Van Hecke, Ho, Goethals and De Smet, 2017) with few modifications
208 and was based on the formation of TBARS from the reaction of MDA with 2-thiobarbituric acid
209 (TBA). The absorbance at 532 nm was measured following 1-butanol extraction, and a 1,1,3,3-
210 tetramethoxypropane standard curve was used for quantification of MDA. The activity of GSH-Px in
211 plasma and liver extracts was determined by measuring the oxidation of NADPH whereby one unit of
212 GSH-Px activity was defined as the amount of extract needed to oxidize 1 μ mol of NADPH per min at
213 25 °C (Hernández, Zomeño, Ariño and Blasco, 2004). Measurement of protein carbonyl compounds
214 (PCC) was based on a previously described method (Ganhão, Morcuende and Estévez, 2010) and based
215 on the formation of a stable 2,4 dinitrophenylhydrazone product as a result of carbonyl groups reacting
216 with 2,4-dinitrophenylhydrazine (2,4-DNPH). Unbound reactive 4-HNE and HEX were measured in
217 diets and *in vitro* digests by HPLC following their derivatization with cyclohexanedione as previously
218 described (Van Hecke et al., 2017).

219

220 2.7 Statistical analysis

221 Values are given as mean \pm SEM. For determination of statistical differences between mean values of
222 the three dietary treatment groups, one-way ANOVA were performed followed by Tukey's honest
223 significant different (HSD) test when significant differences were found. For comparison of nitroso
224 compound formation between the two sausage-based diet groups as well as ATNC formation under
225 acidic conditions, two-sample t-test was conducted. For the two sausage-based diet groups, nitroso
226 compound formation below the detection limit was set to zero. P-values < 0.05 were considered
227 significant. Pearson correlations with Bonferroni-Holm corrections and p -value < 0.05 were calculated
228 to investigate possible correlations between relative abundance of fecal bacteria and NOC
229 concentrations. Statistical analyses were conducted using MATLAB R2017b (Mathworks Inc., Natick,
230 USA).

231 3. Results

232 In the present study, 30 healthy rats were fed three different diets; inulin-fortified pork sausage product,
233 control pork sausage product or a standard chow diet, during an intervention period of four weeks. We
234 have formerly reported metabolomics analyses of fecal and blood samples collected from the rats
235 (Thogersen et al., 2018). Here nitroso compounds excretion, the liver metabolome and lipid and protein
236 oxidation markers were measured to examine a possible beneficial effect of inulin-fortification on these
237 parameters. Body weight did not differ between dietary treatment groups by the end of the study
238 (Thogersen et al., 2018).

239

240 3.1 Nitroso compounds

241 For all of the measured nitroso compounds, i.e. ATNC, nitroso thiols and nitrosyl haem, the fecal
242 concentration was below the detection limit following chow diet consumption. In general, the highest
243 concentrations were observed upon consumption of the control sausage. The concentration of ATNC
244 was found to be significantly reduced after consumption of inulin-fortified sausage ($1.39 \pm 0.15 \mu\text{M}$)
245 compared to the control sausage diet ($2.13 \pm 0.28 \mu\text{M}$) ($p = 0.03$) (Figure 1). Selective denitrosation
246 prior to analysis revealed no significant difference in RSNO ($p = 0.11$) or other unspecified nitroso
247 compounds ($p = 0.29$) after consumption of the two sausage-based diets. A significant reduction in
248 FeNO ($p = 0.04$) was found after consumption of the inulin-fortified sausage ($0.79 \pm 0.06 \mu\text{M}$)
249 compared to control ($1.29 \pm 0.22 \mu\text{M}$). Concentrations for each individual rat can be found in
250 supplementary material (Table S3). A complementary experiment with incubation of bovine
251 hemoglobin and sodium nitrite under acidic conditions with or with the addition of inulin showed no

252 effect of inulin on ATCN formation (Table 1). Calculations of Pearson correlation coefficients with
253 Bonferroni-Holm correction and significance level $p < 0.05$ found no positive correlations between
254 relative abundance of gut bacteria (published elsewhere (Thogersen et al., 2018)) and concentration of
255 nitroso compounds (data not shown).

256

257

258 3.2 Lipid- and protein oxidation

259 Oxidation analyses of the experimental diets revealed that the chow diet contained higher
260 concentrations of MDA, 4-HNE and HEX compared to the two sausage-based diets, and protein
261 oxidation was increasing in the order control sausages, inulin-enriched sausages and chow diet (Table
262 2). Analysis of *in vitro* digests of experimental diets revealed higher MDA, 4-HNE, HEX and PCC
263 concentrations in *in vitro* digests of the chow diet compared to the two sausages-based diets. Analyses
264 of the anti-oxidative enzyme system of rat samples revealed higher GSH-Px activity in liver samples
265 from rats fed the two sausage-based diets compared to chow diet ($p < 0.001$), whereas a near-
266 significant ($p = 0.069$) increased GSH-Px activity was observed in plasma samples from rats fed the
267 sausage-based diets compared to standard chow diet (Table 2).

268

269 3.3 Liver metabolome

270 PCA scores plot of spectral data obtained from HRMAS analysis of liver tissue revealed a clear
271 grouping of the rats receiving the standard chow diet in the first component explaining 74.3 % of the
272 variation (Figure 2). No clear separation between the two sausage-based diets could be observed. An

273 OPLS-DA model comparing rats fed the standard chow diet and rats fed the two sausage-based diets
274 was constructed ($Q^2 = 0.79$) (Figure S1, Supplementary material) and S-line plot revealed that glucose
275 and lipids were among the main drivers of the separation (Figure 3). Chow diet consumption was
276 characterized by higher hepatic glucose levels, whereas consumption of the sausage-based diets was
277 characterized by higher lipid levels in the liver (Figure 3). In addition, a peak at 3.26 ppm appeared
278 important for the separation between the chow diet group and the rats fed the sausage-based diets. The
279 3.26 ppm peak is most likely arising from betaine and has its highest intensity in the chow diet group.
280 Multivariate data analysis did not show any separation between the two sausage-based diets.

281 4. Discussion

282 4.1 Inulin fortification reduces fecal nitroso compound excretion

283 Red and processed meat intake has been suspected to cause harmful effects on colon health (Chan et
284 al., 2011), whereas dietary fiber consumption has been associated with colonic health benefits
285 (Bingham et al., 2003). Therefore, the inclusion of dietary fibers into processed meat products might be
286 a strategic tool in reducing the potential meat-associated harmful effects on colon homeostasis. We
287 have previously shown that inulin fortification of a pork sausage product positively affected the
288 metabolome and gut microbiota of healthy rats by increasing the fecal concentration of SCFAs as well
289 as the relative abundance of *Bifidobacteria* compared to a corresponding non-enriched sausage
290 (Thogersen et al., 2018). Here we examined the effect of the same inulin fortification of a pork sausage
291 product on the formation of nitroso compounds, the liver metabolome as well as markers of lipid and
292 protein oxidation using a rat model.

293 Intriguingly, our study demonstrated that inulin fortification reduced the fecal concentration of ATNC
294 compared to the consumption of control sausages without fortification. Compound-specific
295 denitrosation indicated that this was partly ascribed to a reduction in nitrosyl iron compounds. Based on
296 *in vitro* studies, it has previously been proposed that fermentation of non-digestible carbohydrates
297 could lead to a reduced availability of NOC precursors in the form of amines (Allison and Macfarlane,
298 1989, Silvester, Bingham, Pollock, Cummings and O'Neill, 1997). In addition, under simulated gastric
299 conditions, wheat bran has been demonstrated to act as a nitrite scavenger (Møller, Dahl and Bøckman,
300 1988). However, human studies investigating the effect of consuming resistant starch or wheat bran in
301 combination with red meat showed no effect on fecal NOC excretion (Bingham et al., 1996, Silvester et
302 al., 1997). NOC can be formed from the reaction of nitrosating agents and nitrosable substrates such as

amines formed via fermentation of protein residues reaching the colon (Kobayashi, 2018). This reaction can be catalyzed by colonic bacteria with nitrate- or nitrite reductase enzyme activity (Calmels et al., 1988, Calmels et al., 1996). Hence, the reducing effect that inulin fortification exerts on fecal nitroso compound excretion may be ascribed to a reduction in substrate availability or changes in catalysis of the reaction. Increasing the availability of fermentable carbohydrates in the colon upon high red meat intake might attenuate the formation of protein fermentation products by switching the bacterial fermentation of proteins towards carbohydrate fermentation (Toden et al., 2007, Winter et al., 2011), thereby reducing the availability of substrates for nitroso compound formation. The reducing effect of inulin on ATNC formation might be a result of a high colonic fermentability of inulin compared to other fermentable carbohydrates previously examined in human studies (Bingham et al., 1996, Silvester et al., 1997). We previously demonstrated a strong effect of diet on the gut microbial composition of the rats included in the present study (Thogersen et al., 2018). Hence, the reduced ATNC excretion observed after inulin-fortified sausage consumption may also be associated with changes in abundance of colonic bacteria with nitrate- or nitrite reducing activity. Alternatively, it may be caused by a reduced nitrate reductase activity, as earlier studies have shown a reducing effect of wheat bran and cellulose on this enzyme activity (Mallett, Rowland and Bearne, 1986, Mallett, Wise and Rowland, 1983). The fact that no suppressing effect of inulin on ATNC formation was found after incubation of bovine hemoglobin under *in vitro* acidic conditions suggests that the presence of inulin did not affect an acid-catalyzed ATNC formation expected to take place in the stomach. Thus, the reducing effect of inulin on ATNC formation appears to result from mechanisms taking place further down the gastrointestinal system.

324 In the literature, the carcinogenicity of the different types of nitroso compounds has been discussed.
325 Hogg 2007 argued that S-nitrosothiols and nitrosyl iron species, in contrast to N-nitroso species, are not
326 tumorigenic and even suggested a possible protective effect of S-nitrosothiol and nitrosyl iron
327 formation, reducing the formation of DNA adduct alkylating agents and increasing excretion (Hogg,
328 2007). However, others argue that both nitrosothiols and nitrosyl heme may promote the formation of
329 nitroso-compound-specific DNA-adducts (Kuhnle et al., 2007) and *in vitro* studies have shown the
330 ability of nitrosyl heme and nitrosothiols to act as a nitrosating agents (Alkaabi, Williams, Bonnett and
331 Ooi, 1982, Bonnett, Charalambides, Martin, Sales and Fitzsimmons, 1975).

332 Fecal concentration of all of the measured nitroso compounds for rats fed the standard chow diet were
333 below the detection limit. The lower fecal concentration of nitroso compounds after chow diet
334 consumption compared to the two sausage-based diets is in accordance with earlier findings showing
335 lower fecal concentrations of nitroso compounds after consumption of a vegetarian diet compared to
336 high red meat diet (Kuhnle et al., 2007). This is likely caused by an expected higher heme iron content
337 in the sausage based diets compared to the chow diet (Cross et al., 2003) and the addition of sodium
338 nitrite salt to the sausages.

339

340 4.2 *Effect of diet on liver metabolome and oxidation products*

341 Liver metabolism is crucial to the organism, making it a key metabolic organ. HRMAS analysis of
342 intact liver tissue revealed a clear separation of the chow diet group from the two sausage-based diet
343 groups when multivariate data analysis of ¹H NMR spectral data was conducted. Liver tissue from rats
344 fed the sausage-based diets was characterized by higher amounts of lipids, whereas the chow diet group

345 was characterized by higher hepatic glucose levels. This finding can likely be ascribed to the higher
346 dietary fat and carbohydrate intake for the sausage-based diet groups and chow diet group, respectively.
347 The results are consistent with earlier findings that the liver metabolome is influenced by metabolic
348 status and can be modified by diet, revealing increased hepatic glucose and lipid content after
349 consumption of high-carbohydrate or high-fat diet, respectively (Bertram, Larsen, Chen and Jeppesen,
350 2012).

351 No effect of inulin fortification of the pork sausage product was found on lipid and protein oxidation
352 end products in plasma or liver samples nor in *in vitro* digests of experimental diets. Previous studies
353 found a lowering effect of fiber addition to meat products on lipid oxidation after *in vitro* digestion,
354 probably explained by a lowering effect of fibers on lipid digestion (Hur et al., 2014, Hur, Lim, Park
355 and Joo, 2009). In addition, Toden et al., 2010 found a reducing effect of high amylose maize starch
356 (HAMS) on plasma MDA concentrations in plasma samples of rats fed chicken or beef with or without
357 HAMS (Toden, Belobrajdic, Bird, Topping and Conlon, 2010). Differences in physicochemical
358 properties characterizing different dietary fibers might affect the ability of a specific fiber to reduce
359 lipid digestion in meats in addition to differences in lipid content and lipid size of the meat as suggested
360 by Hur et al. 2009 (Hur et al., 2009).

361 Intriguingly, oxidation analysis of the experimental diets and *in vitro* digests of diets revealed higher
362 concentrations of oxidation products in the chow diets compared to the two sausage-based diets. It has
363 previously been shown that nitrite curing of pork meat reduced the formation of oxidation products
364 compared to corresponding uncured meat after *in vitro* digestion (Van Hecke et al., 2014), which could
365 explain why the two sausage-based diets show lower oxidation compared to the chow diet. The higher
366 oxidation products in chow diet and *in vitro* digests of the chow diet could also be caused by a higher

367 content of reducing sugars in the chow diet, since reducing sugars might to be able to accelerate
368 oxidation (Yamauchi, Goto, Kato and Ueno, 1984). The dry characteristics of the chow diet as well as a
369 longer storage time and higher storage temperature of the chow diet compared to the sausage-based diet
370 might also contribute to the observed increased oxidation (Lin, Hsieh and Huff, 1998).

371 The analysis of oxidation products of experimental diets and *in vitro* digests of diets were conducted on
372 equal amounts of fresh matter. However, the dry matter content of the chow diet is lower than that of
373 the sausage-based diets being 89 %, 43 % and 42 % for chow diet, inulin-enriched sausage and control
374 sausage, respectively. Therefore, lipid oxidation products per gram dry matter of experimental diets
375 were calculated and are given in supplementary material, Table S4. According to the calculations, 4-
376 HNE and HEX were still significantly higher in the chow diet, but for MDA, the calculations showed
377 the highest concentration in the inulin-fortified sausages.

378 The higher degree of oxidation in the chow diet compared to the sausage-based diets prior to ingestion
379 was not reflected in plasma or liver samples of the rats, where no differences in oxidation products
380 between diets groups were found. Intriguingly, despite the chow diet being more oxidized prior to
381 ingestion, an increased GSH-Px activity was observed in liver as well as a near-significant increase in
382 plasma from rats fed the sausage-based diets compared to the chow diet. A high GSH-Px activity can
383 be an indication of a higher level of oxidative stress, since GSH-Px reduces lipid hydroperoxides
384 formed via oxidation of unsaturated fatty acids, thereby functioning as a defense mechanism against the
385 formation of toxic oxidation end-products (Bastide et al., 2011). Thus, the higher fat content, including
386 polyunsaturated fatty acids, in the sausage-based diets could potentially give a higher oxidative stress
387 during digestion compared to that of the chow diet resulting in increased GSH-Px activity.

388 In conclusion, inulin fortification of a pork sausage product reduced fecal content of ATNC and FeNO
389 compared to a non-enriched sausage in healthy rats, indicating a protective effect of inulin against
390 nitroso compound formation. Although no effect of fiber fortification was found on oxidation products,
391 our results indicate a potential of using inulin fortification of processed meat products as an approach to
392 reduce the formation of potentially carcinogenic nitroso compounds.

393

394

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400

401 **Conflict of interest**

402 The authors declare no conflict of interest.

403

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547 Figure 1. Concentration of fecal nitroso compounds after 4 weeks of intervention in rats, mean \pm SEM
548 (Chow, n = 6; Sausage + inulin, n = 12; control sausage, n = 12). Different letters within each
549 compound class indicate significant differences between control sausage and inulin sausage. ATNC,
550 apparent total N-nitroso compounds; RSNO, nitrosothiols; FeNO, nitrosyl iron compounds; other,
551 remaining unspecified nitroso compounds. Concentrations for each individual rat can be found in
552 supplementary material (Table S3). For the chow diet group, all nitroso compounds analyzed were
553 below the detection limit.

554

555

556 Figure 2. PCA scores plot of NMR metabolite profiles obtained for liver samples from rats fed inulin-
557 enriched sausages (yellow), control sausages (red) or chow diet (blue) for 4 weeks.

558

559 Figure 3. OPLS-DA S-line plot of liver samples from rats fed either of the two sausage-based diets (n =
560 24) versus standard chow diet for 4 weeks (n = 6), $Q^2 = 0.79$.

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NaNO₂ concentration (μM)	Control (AUC)	+ inulin (AUC)	p-value
50	240.33 ± 6.35	229.54 ± 1.21	0.28
40	179.56 ± 2.69	167.91 ± 5.18	0.12
30	88.52 ± 9.39	104.88 ± 9.42	0.29
20	60.86 ± 2.11	60.87 ± 0.58	1.00
10	20.47 ± 1.10	21.60 ± 0.75	0.44
5	8.25 ± 0.48	8.01 ± 1.27	0.87
2.5	3.58 ± 0.29	3.30 ± 0.55	0.67

Table 1. ATNC formation expressed as area under the curve (AUC) (mean ± SEM, n = 3) for incubation of bovine hemoglobin and varying amounts of sodium nitrite under acidic conditions with or without (control) the addition of inulin.

		Chow	Sausage + inulin	Control sausage	p- value
Liver	MDA (nmol/g liver)	419.70 ± 15.09	395.61 ± 9.52	399.09 ± 8.60	0.33
	GSH-Px (U/g)	51.3 ± 2.7 ^a	73.5 ± 1.7 ^b	77.6 ± 2.9 ^b	<0.001
	PCC (nmol DNPH/mg protein)	4.41 ± 0.19	4.28 ± 0.23	4.83 ± 0.33	0.31
Plasma	MDA (nmol/mL)	9.51 ± 0.61	9.83 ± 0.30	9.83 ± 0.21	0.81
	GSH-Px (U/mL)	1.96 ± 0.01	2.32 ± 0.15	2.41 ± 0.08	0.07
Diets	MDA (nmol/g diet)	65.5 ± 4.2 ^a	39.4 ± 0.35 ^b	32.0 ± 1.0 ^b	<0.001
	4-HNE (ng/g diet)	64.1 ± 7.5 ^a	4.2 ± 0.91 ^b	3.7 ± 0. ^b	<0.001
	HEX (ng/g diet)	508.9 ± 26.1 ^a	7.4 ± 0.2 ^b	8.4 ± 2.1 ^b	<0.001
	PCC (nmol DNPH/mg protein)	13.6 ± 0.5 ^a	9.5 ± 0.5 ^c	7.4 ± 0.2 ^b	<0.001
In vitro digest	MDA (nmol/g digest)	76.4 ± 4.0 ^a	25.8 ± 0.9 ^b	24.1 ± 0.2 ^b	<0.001
	4-HNE (ng/g digest)	20.4 ± 0.9 ^a	3.4 ± 0.3 ^b	4.2 ± 0.1 ^b	<0.001
	HEX (ng/g digest)	87.4 ± 1.0 ^a	6.4 ± 0.4 ^b	7.4 ± 0.5 ^b	<0.001
	PCC (nmol DNPH/mg protein)	13.7 ± 0.2 ^a	8.3 ± 0.2 ^b	8.0 ± 0.4 ^b	<0.001

570 Table 2. Determination of oxidation parameters of rat liver, plasma, experimental diets and in vitro digestion of experimental diets,
571 mean ± SEM (For liver and plasma: chow, n = 6; Sausage + inulin, n = 12; control sausage, n = 12, except for MDA in plasma: chow, n = 4;
572 Sausage + inulin, n = 10; control sausage, n = 10 and PCC in liver: chow, n = 6; Sausage + inulin, n = 12; control sausage, n = 10. For diets
573 and in vitro digest, n = 3 for each diet group). MDA, malondialdehyde; GSH-Px, Glutathione peroxidase; 4-HNE, 4-hydroxy-2-neonenal;
574 HEX, hexanal; PCC, protein carbonyl compounds.

Declaration of interests

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

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Figure 1
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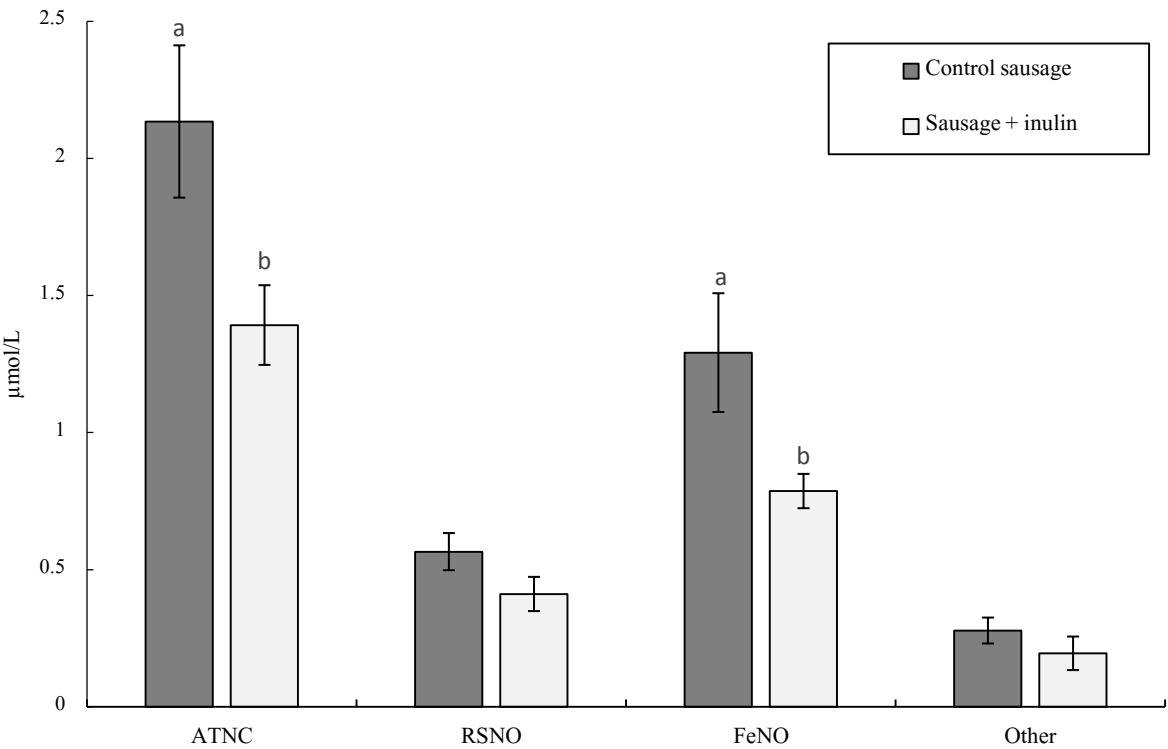


Figure 2
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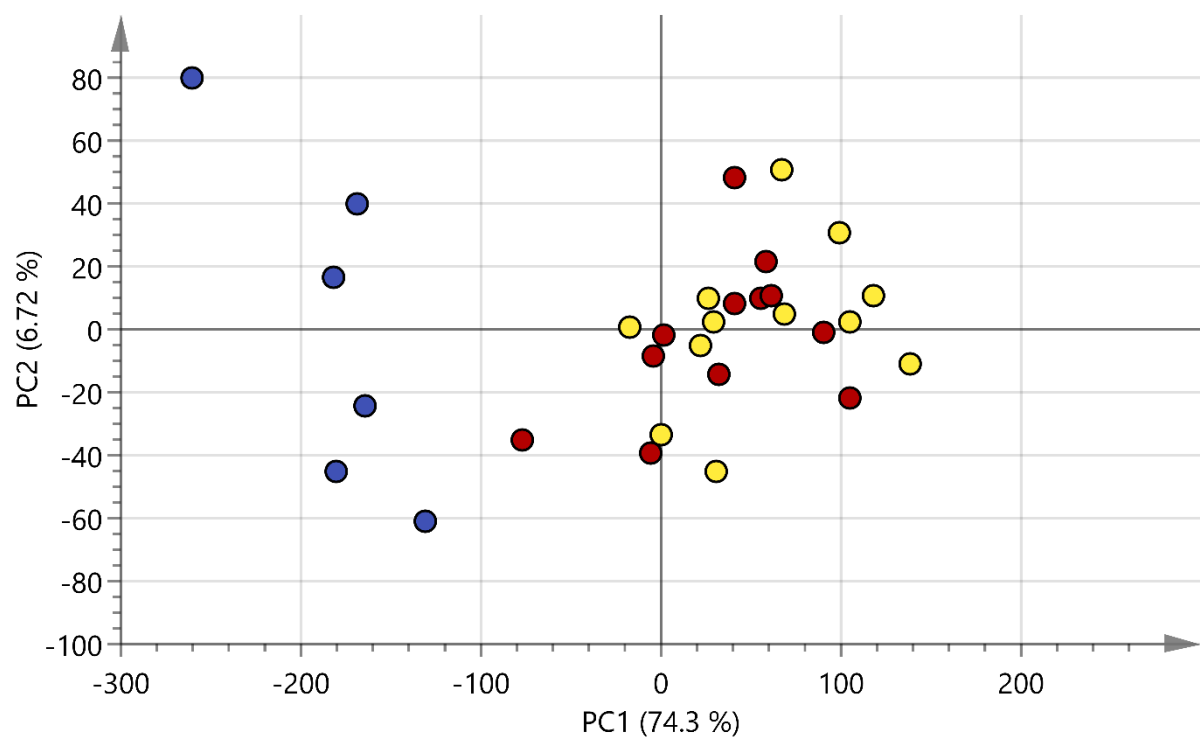


Figure 3
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Supplementary material

per 100 g	Control sausage	Inulin-enriched sausage	Chow
Energy (kcal)	245.0	259.0	318.9
Fat (g)	19.00	20.00	2.80
- Saturated	7.20	7.30	0.47
- Monounsaturated	8.30	8.40	0.63
- Polyunsaturated	2.60	3.10	1.69
Carbohydrate (g)	6.40	7.80	40.83
Protein (g)	12.00	12.00	19.19
Dietary fiber* (g)	0.00	5.60	6.05
NaCl (g)	2.50	2.50	0.54

Table S1. Nutritional content of diets.

*Calculated values

Ingredient (% w/w)	Inulin-enriched sausage	Control sausage
Inulin	6.0	-
Salt (with 0.3nitrite)	2.0	2.0
Spices	2.0	2.0
AIN76 mineral mix: TD79055	2.0	2.0
AIN76 vitamin mix: CA40077	0.6	0.6
Choline bitartrate: CA30190	0.12	0.12

Sunflower oil	2.0	2.0
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Table S2. Ingredients added to emulsion of minced pork meat and pork back fat during manufacturing of the two sausage-based diets; pork sausages enriched with inulin and control pork sausages.

Rat ID	Concentration [umol/L]			
	ATNC	RSNO	FeNO	Other
Control sausage				
24	1.54	0.66	0.69	0.20
6	1.21	0.33	0.89	0.00
12	1.64	0.47	0.77	0.40
4	1.78	0.50	0.94	0.34
10	1.47	0.41	1.06	0.00
2	1.45	0.32	0.78	0.34
17	2.18	0.73	1.30	0.15
25	3.88	1.06	2.61	0.21
3	2.50	0.85	1.23	0.43
29	2.11	0.44	1.32	0.35
11	4.20	0.70	3.03	0.47
8	1.64	0.32	0.88	0.44
Sausage + inulin				
26	1.18	0.10	0.92	0.17
9	0.96	0.24	0.71	0.00
30	1.38	0.50	0.88	0.00
16	1.84	0.69	0.86	0.29
23	0.70	0.14	0.56	0.00
7	2.38	0.76	1.05	0.57
18	0.95	0.24	0.27	0.44
14	1.01	0.25	0.72	0.04
28	1.27	0.51	0.73	0.03
27	1.65	0.47	0.80	0.38
19	1.30	0.42	0.88	0.00
5	2.08	0.61	1.05	0.41

Table S3. Concentration of fecal nitroso compounds after 4 weeks of intervention in rats. ATNC, apparent total N-nitroso compounds; RSNO, nitrosothiols; FeNO, nitrosyl iron compounds; other, remaining unspecified nitroso compounds. For the chow diet group, all nitroso compounds analyzed were below the detection limit.

	Chow	Sausage + inulin	Control sausage	p- value
MDA (nmol/g DM)	73.9 ± 4.7 ^a	91.0 ± 0.8 ^b	76.5 ± 2.4 ^a	0.016
4-HNE (ng/g DM)	72.3 ± 8.4 ^a	9.7 ± 2.1 ^b	8.8 ± 1.9 ^b	<0.001
HEX (ng/g DM)	573.7 ± 29.5 ^a	17.0 ± 0.6 ^b	20.1 ± 4.9 ^b	<0.001

Table S4. Lipid oxidation of experimental diets expressed per gram dry matter, mean ± SEM (n = 3 for each diet group). MDA, malondialdehyde; 4-HNE, 4-hydroxy-2-nonenal; HEX, hexanal; DM, dry matter.

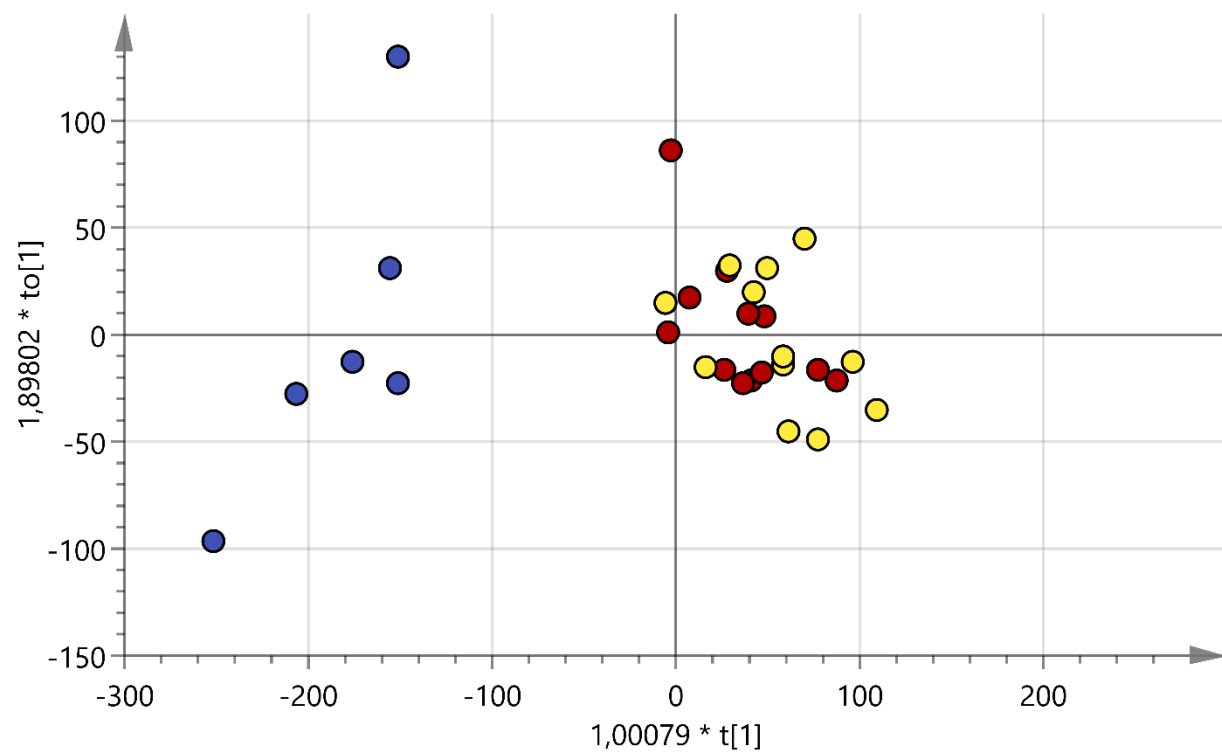


Figure S1. OPLS-DA scores plot of metabolite profiles obtained for liver samples from rats fed standard chow diet (blue), inulin-enriched sausage (yellow) or control sausage (red), $Q^2 = 0.79$.