Dietary inclusion of nitrite-containing frankfurter exacerbates colorectal cancer pathology, alters metabolism and causes gut dybiosis in APC^{min} mice.

Authors: William Crowe, Xiaobei Pan, James Mackle, Adam Harris, Gary Hardiman, Chris T Elliott, Brian D Green*

Institute for Global Food Security, School of Biological Sciences, Queens University Belfast, Biological Sciences Building, Chlorine Gardens, Belfast BT9 5DL, Northern Ireland, UK.

*Correspondence to: b.green@qub.ac.uk

Abstract

Colorectal cancer (CRC) is the second most prevelant malignancy in Europe and diet is an important modifiable risk factor. Processed meat consumption, including meats with preservative salts such as sodium nitrite, have been implicated in CRC pathogenesis. This study investigated how the CRC pathology and metabolic status of adenomatous polyposis coli (APC) multiple intestinal neoplasia *(min*) mice was perturbed following 8 weeks of pork meat consumption. Dietary inclusions (15%) of either nitrite-free pork, nitrite-free sausage or nitrite-containing sausage (frankfurter) were compared against a parallel control group (100% chow). Comprehensive studies investigated: gastrointestinal tract histology (tumours, aberrant crypt foci (ACF) and mucin deplin foci (MDF), lipid peroxidation (urine and serum), faecal microbiota and serum metabolomics (599 metabolites). After 8 weeks mice consuming the frankfurter diet had 53% more (P=0.014) gastrointestinal tumours than control, although ACF and MDF did not differ. Urine and serum lipid peroxidation markers were 59% (P=0.001) and 108% (P=0.001) higher, respectively in the frankfurter group. Gut dysbiosis was evident in these mice with comparably fewer *Bacteriodes* and more *Firmicutes*. Fasting serum levels of trimethylamine N-oxide (TMAO) and numerous triglycerides were elevated. Various serum phosphotidylcholine species were decreased. These results demonstrate that nitrite-containing sausages may exaccerbate the development of CRC pathology in APC^{Min} mice to a greater extent than nitrite-free sausages, and this is associated with greater lipid peroxidation, wide-ranging metabolic alternation and gut dysbiosis.

Keywords
Processed meat, sausages, colorectal cancer, microbiome, metabolism, metabolomics, gastrointestinal tumours.

Introduction

Colorectal cancer (CRC) is the third most prevalent malignancy worldwide. Over 1.9 million people were diagnosed with CRC in 2020 [1]. CRC always begins in the form of abnormal growths called polyps in the colon or rectum. The majority of colorectal tumours are adenocarcinomas that originate from polyps. Polyps are pedunculated masses of tissue that may be pre-cancerous. Around 5% of CRC is attributable to familial adenomatous polyposis[2]. The remaining cases of CRC are believed to be influenced by environmental factors. One modifiable risk factor that has been proposed as a leading candidate in the search for causes of CRC is dietary pattern. A diet high in processed meat is suggested as a major risk factor for CRC development. The International agency for Reseach on Cancer (IARC) have advised consumers to avoid consuming processed meat and limit the quantity of red meat consumed. Processed meat is classed as a group 1 carcinogen, and therefore carcinogenic to humans. This conclusion is based on 9 case-control studies and 18 cohort studies and of these 6 and 12, respectively, linked processed meat consumption to CRC 3[3]. However, these studies only represent 10% of the existing body of evidence, the remainder of which was deemed uninformative due to methodological limitations. IARC list examples of processed meat as hot dogs (frankfurters), ham, sausages, corned beef, and biltong or beef jerky as well as canned meat and meat-based preparations and sauces. Processed meat is often preserved with nitrite salts, and recent scientific evidence suggests that it is this component that is responsible for

68 processed meats unfavourable link with CRC [4]. Sodium nitrite uniquely prevents the
69 growth of *Clostridium Botulinum*, it also contributes to the colour and taste of
70 processed meat. Ingested nitrite has the potential to form N-Nitroso compounds
71 (NOC) including nitrosamines, some of which are known to be carcinogenic. NOCs
72 have been proposed to be involved in the aetiology of several types of cancer,
73 particularly those in the gastrointestinal tract [5].

74

75 The composition of gut microbiota (consisting of more than 10^{13} bacteria), is
76 modulated by exposure to environmental factors such as diet and medication. These
77 microbes protect the host by inhibiting the growth of pathogens and contributing to
78 the production of short chain fatty acids (SCFA). SCFAs include acetate, butyrate,
79 and propionate are used an energy source and participate in the proliferation of
80 intestinal epithelial cells[6]. Dysbiosis has been implicated in the pathogenesis of
81 numerous health conditions, including CRC [7]. It has been reported in CRC that there
82 is a depletion of *Bacteroidetes* and *Firmicutes*, and at the order level there is a
83 reduction in *Clostridiales* [8]. Sodium nitrite is also known to prevent the colonisation
84 of *Clostridiales*.

85 A total of ten animal studies have investigated the effect of nitrite consumption on
86 CRC, 4 of these studies found that nitrite consumption had no effect on CRC, 5
87 studies found that nitrite consumption significantly increased the risk of CRC, and 1
88 study found that nitrite containing processed meat significantly decreased the risk of

89 CRC[4]. All studies reporting a significant causative effect supplied ≥50% processed
90 meat in the diet. This does not reflect typical human consumption and therefore
91 further studies employing more realistic levels of processed meat are required.

92 Current public health advice treats all processed meat equally, when in reality there
93 are many differences in their composition. Most notably there is the addition of
94 sodium nitrite as a preservative which is present in some products and not in others.
95 For example, nitrites are not traditionally an ingredient in the manufacture of
96 traditional British/Irish sausages but it is more common in continental European
97 sausages. Therefore, the primary aim of this investigation was to examine whether
98 cancer progression in a mouse model of CRC pathology differs when fed a moderate
99 amount of either nitrite-containing frankfurter sausage or nitrite-free sausage. A
100 secondary aim was to examine whether CRC development was impacted by the
101 consumption of either processed (nitrite-free sausage) or unprocessed (pork) meat. A
102 number of additional analyses were undertaken to ascertain what the wider
103 metabolic consequences of these meat diets.

104

105 **Results**

106 *Intestinal pathology*

107 In the duodenum the mean tumour count for mice fed a 15% frankfurter diet was 4.2
108 (Figure 1b). This was 75% higher than the mean tumour count of the control group

109 (p=0.022). No other inter-group differences were detected for duodenal tumours. The
110 quantity of tumours in the jejunum did not differ between any groups. Frankfurter
111 fed animals had 88.2% more tumours in their ilieum than the control (p=0.011), and
112 190.9% more than pork fed mice (p=0.001), but did not significantly different from
113 the nitrite-free sausage group. Sausage fed mice had 154.6% more tumours in their
114 ilieum than the pork fed mice (p=0.050). In the colon, the only significant difference
115 was that pork fed mice had 81.8% more tumours than the control mice (p=0.047). In
116 terms of the overall number of GI tumours, the frankfurter group had the highest
117 with a mean of 11.2 and this was significantly more (p=0.002) than the control which
118 had a mean of 7.3 tumours, it was also significantly higher (p=0.029) than the
119 sausage group which had a mean of 8.6 tumours, and significantly higher (p=0.019)
120 than the pork group which had a mean of 8.3 tumours.

121 As shown in Table 1 no significant differences were found in ACF or MDF between
122 any dietary group. There was a trend for pork-fed mice to have the highest quantities
123 of ACF (not significant), and for sausage-fed mice to have the highest quantities of
124 MDF (not significant).

125

126 *Lipid peroxidation*

127 Frankfurter feeding consistently led to the highest concentrations of urinary DHN-
128 MA (Figure 1c). By week 3 these were 54.30% higher than control (p=0.001), 47.39%
129 higher than sausage-fed (p=<0.001), and 65.66% higher than pork-fed (p=<0.001). By

130 week 3 Pork-fed mice had 72.17% higher urinary DHN-MA than sausage-fed
131 (p=0.048). By week 6 the frankfurter-fed group had DHN-MA levels that were
132 83.05% higher than the control (p=0.030), 80.26% higher than sausage-fed (p=0.009),
133 and 80.66% higher than pork-fed (p=0.013). At week 8 the frankfurter group had
134 DHN-MA levels that were 59.18% higher than the control (p=0.001), 67.92% higher
135 than sausage-fed (p=<0.001), and 69.07% higher than pork-fed (p=0.001).

136 Similar results were found when comparing serum MDA concentrations between
137 groups. Frankfurter-fed mice had the highest mean concentration (928.89 pg/ml) and
138 this was 48.07% higher than the control (p=<0.001), 42.90% higher than pork-fed
139 (p=<0.001), and 52.56% higher than sausage-fed (p=0.001). These were all
140 significantly different after controlling for mouse baseline values.

141 *Bodyweight and food intake*

142 As shown in Figure 1d, mice fed pork or frankfurter diet had significantly higher
143 body weight than the control and sausage groups at week 1. At almost every
144 timepoint the frankfurter group had the highest mean bodyweight and this was
145 significantly higher than the control. At weeks 6, 7, and 8 the sausage group had
146 significantly lower mean bodyweights than the control. At weeks 2-6 the pork group
147 had significantly higher mean bodyweights than the control, but not at weeks 7 and
148 8.

149

150 *Altered concentrations of serum metabolites*

151 Unsupervised Principal Component Analysis (PCA) of serum metabolomic data
152 explained 89.4% of the total variation (76.9%, 8.0% and 4.5% for PC1, PC2 and PC3,
153 respectively; Figure 2a). There was visible separation with a tendency for frankfurter
154 group profiles to separate from the other three groups along the PC1 axis. Profiles
155 from control, pork and sausages groups were more closely clustered together with
156 variation more restricted to the PC2 axis.

157 Serum metabolites were ranked by false-discovery rate (FDR) corrected p-values
158 based on one-way ANOVA. Out of 599 measured metabolites 232 significantly
159 differed between two or more groups. When each of the dietary interventions was
160 compared with Control it was the frankfurter diet which caused by far the greatest
161 number of metabolite alternations (Figure 2b). In total 218 metabolite alternations
162 were observed for frankfurter (165 of which were unique), whereas comparably
163 fewer were observed for pork and sausage, 52 and 42, respectively. Hierarchical
164 clustering analysis (HCA) was conducted by selecting the 50 most statistically
165 significant metabolites from the ANOVA found that the greatest distinction in
166 profiles was between the control and frankfurter-fed mice (Figure 2c). Metabolic
167 profiles obtained for pork-fed and sausage-fed mice were similar to one another and
168 were clustered together by HCA. From this it was evident that many triglyceride
169 species were significantly higher in the frankfurter group, and several certain
170 cholestrol esters and phosphotidylcholines were significantly lower.

171 Figure 3 shows that serum TMAO (trimethylamine N-oxide) levels were 184%
172 higher in mice receiving frankfurter diet. However, the levels of TMAO precursor
173 metabolites (choline, betaine, carnitine, acetylcarnitine propionylcarnitine) are not
174 similarly affected. Levels of choline and betaine did not differ between groups. Mice
175 consuming any of the meat-containing diets had similarly higher levels of carnitine
176 (71-119%), acetylcarnitine (114-217%) or propionylcarnitine (97-155%). In no cases
177 were these carnitine metabolites the highest overall in mice consuming the
178 frankfurter diet.

179

180 *Faecal microbiota*

181
182 As Figure 4 shows, there are clear differences between in the profiles (Figures 4a and
183 4c) and the diversity (Figure 4b) of the frankfurter fed mices microbiota compared
184 with all other groups. These differences exist on all phylogenetic levels (phylum,
185 class, order, family, genus, and species). An analysis of covariance (ANCOVA) was
186 used to compare the mean OTU levels of each phylum from post study between
187 dietary groups, whilst controlling for baseline levels of each phylum. There was no
188 significant difference in *Bacteroidetes* from the terminal sample between any groups,
189 when controlling for baseline. The highest levels of *Firmicutes* were seen in the
190 frankfurter consuming group, and the lowest levels were seen in the sausage
191 consuming group, however when controlling for baseline, the there was no
192 significant differences between any groups. The highest levels of *Actinobacteria* were

193 seen in the frankfurter consuming group, and this was significantly higher than the
194 control (p=0.002), sausage (0.001), and pork (<0.001) consuming groups. Similarly,
195 the highest levels of *Proteobacteria* were seen in the frankfurter group and these were
196 significantly higher than the pork (P=0.014), and sausage (P=0.011), but not the
197 control (P=0.551). The control group had significantly higher levels of *Tenericutes*
198 than the pork (P=0.001), sausage (P=0.001), and frankfurter (P=0.002) fed groups. The
199 control group had significantly lower levels of *Verrucomicrobia* than the pork
200 (P=0.551), sausage (P=0.551), and frankfurter groups (P=0.551).

201 The most abundant bacteria at the species level in the control, sausage and pork
202 group was *Bacteriodes thetaiotaomicron*, the gut microbiome consisted of a mean (SD)
203 relative abundance of 16.6 (9.37), 14.7 (9.07), and 19.7 (3.71)% respectively. The
204 frankfurter group tended to have lower abundance than all 3 other groups at 7.3
205 (2.9)% (not significant). *Duboisiella newyorkenesis* was the most abundant species in
206 the frankfurter group, making up a total of 28.8 (1.37)% of all species. This species
207 was not detectable in any other group. All four groups displayed a high abundance
208 of bacteria from the *Lachnospiraceae* family, which was largely attributed to higher
209 levels of bacteria from the *Blautia* genus (see Supplementary Table 1). The sausage
210 group was the only group with measureable *alistipes*, which was the 3rd most
211 abundant species 11.7 (5.5)%.

212 The most abundant bacteria at the family level in all four dietary groups is *Clostridiales*,
213 the next most abundant in the control, sausage, and pork groups is *Bacteroidales*, whilst
214 *Erysipelotrichales* is the second most abundant in the frankfurter group.

215

216 **Discussion**

217 This study found that a modest inclusion of a sodium-nitrite containing pork
218 frankfurter in the diet of APC^{min} mice significantly increased the number of intestinal
219 tumours present. A similar inclusion of either nitrite-free pork sausage or minced
220 pork did not increase tumour numbers. However, the numbers of intestinal foci
221 (ACF and MDF) did not differ between any groups. Frankfurter consumption was
222 also associated with higher levels of oxidative stress, as indicated by serum MDA
223 and urinary DHN-MA, than any other dietary group. Unbiased PCA analysis of
224 serum metabolomics data showed a distinct clustering of the frankfurter group
225 which distinguished this diet from all others. This metabolic shift resulted from
226 vastly more serum metabolites being significantly altered in Frankfurter-fed mice
227 than any other group. When hierarchical clustering was performed on a shortlist of
228 the 50 most affect metabolites across all groups it was demonstrated that the
229 metabolic status of mice fed minced pork and those fed pork sausage were most
230 similar to one another. It also illustrated the widerspread metabolic divergance of
231 frankfurter-fed mice. Of the many metabolites profiled trimethylamine N-oxide

232 (TMAO) stood out, since it has previously has linked to CRC development [9], and
233 here it was uniquely elevated in the frankfurter group.

234 Intestinal microbiota play a significant role in TMAO production and
235 precursor molecules of TMAO found in red meat, eggs, milk and fish. However, the
236 increases in circulating TMAO here most likely result from alterations in the gut
237 microbiome. It is clear that all dietary interventions in this study impacted the
238 composition of faecal microbiota to some extent. However, the reduced microbial
239 diversity (Shannon index) of the frankfurter group suggested that this diet resulted
240 in gut dysbiosis. The most remarkable observation was the sheer dominance of *D.*
241 *newyorkenesis* in the frankfurter group. The levels of this organism in all other groups
242 fell well below threshold cut-off values. *D. newyorkenesis* is a poorly characterised
243 organism and we questioned whether *D. newyorkenesis* is a driver of TMAO
244 formation which is subsequently detected in the blood circulation. The genome of
245 this recently identified bactierial species [10] has was sequenced recently (but is not yet
246 fully annotated). We utilized the makeblastdb application and produced a
247 searchable BLAST databases from FASTA files obtained from D. newyorkenesis and
248 carried out pairwise alignments to search for gene sequences encoding enzmyes
249 involved in TMA-synthesis pathways (including choline TMA-lyase (cutC) and
250 carnitine oxygenase (cntA)) [11] but we did not find any significant alignments, which
251 would suggest that this organism is not involved in TMAO production.

252 Nonetheless, at the genus level *Dubosiella* is positively associated with obesity in
253 mice [12] and at the 'family' level, *Erysipeltrchaceae,* is positively associated with CRC in
254 murine models and humans [13,14]. It is not however clear if predominance of
255 *Erysipeltrchaceae* is causative of CRC or if colonisation occurs in response to CRC. The
256 *Clostridiales* family, although present in all four groups, was noticeably less prevalent
257 in the frankfurter group. It may be that *Erysipeltrchaceae* to some extent displaced
258 *Clostridiales*, but another explanation could be the known ability of nitrites to prevent
259 the growth of organisms from the *clostridium* genus.

260 An in-depth literature search reveals that ten pre-clinical studies have thusfar
261 evaluated the effect of nitrite consumption on the development of CRC. The
262 deficiency of most of these studies is that cancer was chemically induced and not
263 spontaneous. None-the-less, five studies reported nitrite exposure to increase cancer
264 risk [15-19]. Three studies reported that nitrite treatment made no difference [20-22], and
265 one study reported nitrite to actually be beneficial [23]. Our study has several
266 advantages over previous studies in this area. Firstly, it is the only study to provide
267 a nitrite-containing meat matrix to $Apc^{Min+/+}$ mice, which could be described as a
268 'gold-standard' model of spontaneous CRC development. This minimises the known
269 unpredictability of cancer development (especially in location and timing) of
270 chemically-induced CRC models. Secondly, all previous pre-clinical studies linking
271 nitrite to CRC development, provided a minimium of 50% processed meat in the
272 diet. Not only are such levels unrealistic for humans, they disproportionately alter

273 the overall nutritional profile of the diet. Our study has detected a measurable effect
274 with just 15% dietary inclusion, and although this still represents a relatively high
275 intake of processed meat it clearly demonstrates that lower dietary quantities can
276 exacerbate the disease. Thirdly, this study has investigated other relatively
277 unexplored mechanisms through which diets impacts CRC. The application of both
278 metabolome and microbiome data makes this study particularly unique. Lipid
279 peroxidation (i.e. the oxidative destruction of membrane lipids and production of
280 compounds impairing cells and tissues) was evident and this is in keeping with
281 other studies [16,18]. The aformentioned rise in TMAO in the frankfurter group could
282 also be linked linked to oxidative stress [24]. TMAO is generated from the metabolism
283 of a number of nutrients including carnitine and choline which are present in pork
284 meat. Increased TMAO in the circulation apparently induces superoxide production,
285 a reactive oxygen species linked to oxidative stress [24]. TMAO's production of reactive
286 oxygen species has been confirmed in cells *in vitro* [25]. The association between
287 TMAO, oxidative stress and cancer still needs to be verified, but mounting evidence
288 suggests that TMAO could play a role in chronic inflammation as a cause of
289 carcinogenesis in CRC [26]. Furthermore, *in silico* studies suggest that TMAO
290 metabolism and CRC share many similar genetic pathways [27].

291 In sharp contrast to the frankfurter group, the minced pork and pork sausage
292 group did not display elevated serum TMAO. The TMAO responses did not mirror
293 those of common TMAO precusors. Choline levels were the same in all groups. As

294 would be expected carnitine (and several acylcarnitines) were high in all three pork
295 diet interventions. Another TMAO precursor betaine (found typically in plants but
296 not in meat) did not differ in any groups either. Therefore, in these experiments the
297 supply of precursors does not appear to be important factor in TMAO production,
298 and the suspicion would be that altered microbiota are more pivotal. A limitation of
299 this study is the fact that the microbial profiling undertaken involved the analysis of
300 16S rRNA amplicon rather than a whole-metagenomics approach, thus it was not
301 possible to evaluate whether there was an abundance of bacterial species with genes
302 encoding the enzymatic machinery involved in TMAO production.

303 An obvious advantage of the present study design is that it reflects real-world
304 human food choices. To this end it directly compared commercially available pork
305 products on an equivalent gram-for-gram basis. The unfortunate consequence of this
306 approach is that the nutritional properties of the pelleted diets cannot be precisely-
307 matched, or be isocaloric, which is a potential limitation. Another limitation is that is
308 was not possible to record food intake, and we cannot pinpoint the underlying
309 reasons for the higher bodyweights of mice in the frankfurter group. It is possible
310 that mice found this pelleted diet more palatable and consumed more, but there are
311 also differences in the levels of fibre, and the types of fat in the diets which could
312 have contributed also to this effect. It is not known if raised bodyweight exacerbated
313 CRC pathology in this group, but this is very questionable given that the pork
314 sausage group had the lowest body weight, but also the second highest number of

315 tumours overall. There were metabolic differences evident in the frankfurter group,
316 for instance, the significantly elevated diglycerides and triglycerides, which were
317 likely explained by the higher saturated fat content of this diet. The decreased
318 phosphatidylcholine and lysophosphatidylcholine (lysoPC) levels may also be a
319 consequence of this, but equally, similar observations have been noted in CRC
320 patients [28]. Increased lysoPC metabolism in tumour cells *in vitro* is one explanation
321 for a decline in their blood concentrations.

322 The results from this study clearly show that not all processed meats carry the same
323 risk of CRC development, and that the consumption of nitrite-containing processed
324 meat exacerbates CRC pathology in a genetically predisposed murine model.
325 However, higher dietary fat and increased body weight cannot completely be ruled
326 out as potential confounding factors. The metabolomic and gut microbiome data
327 demonstrate the systemic alterations that can occur as result of simple dietary
328 choices. These also provide important mechanistic data. Further dose-response
329 studies should be performed, perhaps using humanised or colon-specific CRC
330 mouse models, and employing frankfurter as a model food matrix. Such work is
331 required to determine the levels at which nitrite inclusion becomes harmful.

332 **Methods**

333 **Animal models**

334 Forty female $Apc^{Min/+}$ mice were purchased from Charles River and delivered to Axis
335 Bioservices (Coleraine, Northern Ireland) where the study was conducted. All mice

336 were four weeks old on delivery. The mice were all fed an ad lib maintenance diet of
337 AIN76 for a two week acclimitization period (Figure 1a). Mice were caged in groups
338 of 3 or 4 in a 12 hour light, 12 hour dark cycle. Mice had unlimited access to their
339 respective diets and water for the duration of the study which was 12 weeks, with
340 the exception of 12 hours prior to blood collection, when they were fasted. Body
341 weight was measured every 3–4 days. We attempted to measure food intake in a
342 similar manner, but since diets were required (for welfare reasons) to be placed in
343 dishes in home cages this led to extremely erroneous data and was subsequently
344 abandoned. Mice were randomly assigned to one of four treatment groups,
345 described below. Studies in mice underwent Ethical Review and were performed in
346 accordance with the UK Animals (Scientific Procedures) Act 1986.

347 **Diets**

348 Each treatment group received one of four diets for the duration of the study, the
349 dietary groups included; Control, which consisted of 100% AIN76 (Altromin,
350 Germany). Sausage meat (15%)/AIN76 (85%), Frankfurter (15%)/AIN76 (85%),
351 unprocessed pork (15%)/AIN76 (85%). Sausage meat was manufactured and
352 provided by Finnebrogue Artisan (Downpatrick, UK) as was the unprocessed pork.
353 Nitrite-containing Frankfurter was manufactured by Herta (Illkirch, France) and
354 purchased from a local supermarket. All meat was freeze dried by European Freeze
355 Dry (UK), and powderized. Altromin then incorporated this into pellet composed of

356 15% meat powder and 85% AIN76. The nutritional properties of each diet were
357 analysed after pelleting (Supplementary Table 2).

358 **Biological samples**

359 Blood samples were collected from the tails of all animals at 3 time points, week 0,
360 week 3, week 6, a final cardiac puncture blood was taken at the termination of the
361 study (week 8). Urine was collected from individual animals at baseline and week 8.
362 Faecal samples at the same timepoints but were collected from each cage.

363 **Tumor scoring in the gastrointestinal tract**

364 Animals were sacrificed using CO_2 asphyxiation and their organs harvested. As
365 described previously[29], the intestinal tract was removed washed in phosphate
366 buffered saline and sectioned into the duodenum, jejunum, ileum, and colon. The
367 sections were opened longitudinally and fixed in 10% formalin, colons were stained
368 for 5 minutes in 0.05% methylene blue solution (Sigma), and small intestine were
369 stained for 48 hours in 0.05% methylene blue. Using x25 magnification all tumors
370 present were counted by one researcher in a blinded manner. The minimum size for
371 accepting and counting a tumor was 0.5mm diameter.

372 **Identification of aberrant crypt foci and mucin depleted foci**

373 Aberrant crypt foci (ACF) were measured in the colon by a well described method [30].
374 The colons were washed in saline, and stained with 0.2% methylene blue for 5 mins.
375 They were placed on a glass slide and visualised using a magnification of 32x on a

376 light microscope. Mucin depleted foci (MDF) were also measured in the colon, the
377 sections were washed in saline, and stained with 1% high-iron diamine alcian blue
378 procedure for 30 mins. All scoring was completed by one researcher and verified by
379 a second researcher in a blinded manner.

380 **Lipid peroxidation**

381 Serum samples from every time point were analysed for malondialdehyde (MDA)
382 using ELISA methodologies (Abcam, Cambridge UK). In brief, thiobarbituric acid,
383 serum and standards were added to 96-well microtiter plate and incubated at 95°C
384 for 60 mins and cooled on ice. An aliquot from each well was added to the
385 colorimetric plate and measured at OD532 nm using a plate reader. Urine samples
386 from baseline and week 8 were analysed for 1,4-dihydroxynonane mercapturic acid
387 (DHN-MA) using a Bertin Bioreagent enzyme immunoassay (Wolverhampton UK).
388 Briefly, buffer, standards and urine were added to each well, the detection antibody
389 was then added, and the plate was incubated overnight at 4°C. Ellman's reagent was
390 added, and the absorbance was measured at 414nm.

391 **Metabolite profiling**

392 The MxP Quant 500 kit (Biocrates, Life science AG, Innsbruck, Austria) was used to
393 quantify amino acids, acylcarnitines, bile acids, biogenic amines, carboxylic acids,
394 ceramides, cholesterol esters, diacylglycerols, fatty acids, glycerophospholipids,
395 glycosylceramides, sphingolipids, and triacylglycerols in serum samples from
396 baseline and week 8. Serum (10μL) samples were analysed using a triple-quadrupole

397 mass spectrometer (Xevo TQ-S, Waters Corporation, Milford, CT, USA). Samples
398 were dispensed into a 96-well plate, dried at room temperature for 30 min and then
399 50 μL of phenylisothiocyanate (PITC) was added. After a further 20 min incubation
400 at room temperature samples were dried under nitrogen for 1 h. Ammonium acetate
401 (3 mM) was added, and the plate was shaken at room temperature for 30 min and
402 centrifuged for 2 min at 500 *g*. Each well then had 150 μL transferred from it to a 96
403 deep well plate along with and equal volume of dH_2O. A 96-well plate containing
404 490 μL of FIA solvent is shaken along with 10 μL of extract at room temperature for
405 5 mins. From each well 5 μL of sample Samples were injected through the MxP
406 Quant 500 kit column system for amino acids and biogenic amines analysis. Other
407 metabolites were semi-quantified using the same mass spectrometer without column
408 separation by the flow injection analysis (FIA). Metabolite concentrations were
409 calculated using the Analyst/MetIDQ software and expressed as μM in serum.

410 **Microbial profiling**

411 A QIAamp DNA stool mini kit (Qiagen, Manchester, UK) was used to extract DNA
412 from faecal samples. All reagents, unless otherwise stated were provided by Qiagen.
413 Faecal samples were weighed (220mg) and added to a 2ml Eppendorf tube along
414 with 1.4 ml of ASL buffer, which was vortexed and heated for 5 mins at 95°C. The
415 contents were added to a 2 ml screw capped tube containing 0.5 g of both 0.1 mm
416 and 0.5 mm zirconium beads. The tube was then added to a FastPrep 24 g5 bead
417 beater (MP biomedicals, Eschwege, Germany) for 5 x 20 s bursts. The suspension

was subsequently spun at 14,000 *g* for 3 mins. The supernatant (1.2 ml) was collected and transferred into a QIAamp silica-gel membrane tube and spun for 3 min, and DNA extracted in accordance with the manufacturer's instructions. Amplicon sequencing was carried out on an Illumina MiSeq. Variable regions V4-V5 of the bacterial 16S ribosomal RNA gene were amplified from extracted DNA using PCR conditions and custom primers as described in the Microbiome Helper protocol [31]. The forward (515FB = GTGYCAGCMGCCGCGGTAA) and reverse (926R = CCGYCAATTYMTTTRAGTTT) primers used Nextera Illumina index tags and sequencing adapters fused to the 16S sequences. Each sample was amplified with a different combination of index barcodes to allow for sample identification after multiplex sequencing. After amplification, paired-end 300 + 300 bp v3 sequencing was performed for all samples on an Illumina MiSeq. Raw sequencing reads were trimmed as previously described [32].

Analysis of 16S sequencing data

Analysis of 16S sequencing data was carried out on the Hardiman Laboratory GRANDE server. Primer sequences were removed from the sequencing reads using cutadapt (v 1.14) [33], and primer-trimmed files were imported into QIIME2 (v. 2019.10.0) [34]. Forward and reverse paired-end reads were joined using VSEARCH (v 2.9.0) [35] and inputted to Deblur [36] to correct reads and obtain amplicon sequence variants (ASVs). Taxonomy was assigned to ASVs using the SILVA rRNA gene database [37] and the "feature-classifier" option in QIIME2, sklearn. Rare sequences were removed from the feature table. Any sequences below an arbitrary cut-off frequency of 100 were also removed. Estimates of alpha-diversity (Observed ASVs,

440 Shannon Diversity), beta-diversity (weighted UniFrac), and relative abundance of
441 ASVs were obtained using QIIME2 (v 2019.10.0). Differential abundance tests were
442 done using the Analysis of compositions of microbiomes (ANCOM)plugin [38].

443 **Statistical analysis**

444 All data were tested for normality using Kologorov-Smirnoff and where appropriate
445 data was transformed. Group comparisons for tumour numbers, ACF and MDF
446 were performed by a One-way ANOVA with LSD post-hoc test (two-tailed; SPSS
447 version 21). Lipid peroixdation markers DHN-MA and MDA at week 8 were
448 compared between groups whilst controlling for baseline concentrations by an
449 ANCOVA. Metabolomic data were cube-root transformed, underwent mean-
450 centered scaling and compared using univariate (ANOVA), multivariate and
451 hierarchial clustering tools within MetaboAnalyst 4.0 (http://metaboanalyst.ca).

452

453 **Data Availability Statement**

454 Raw mass spectrometry data is available on the Metabolomics Workbench
455 (https://www.metabolomicsworkbench.org/) under study ST002313,
456 [http://dx.doi.org/doi: 10.21228/M81M72]. The 16S rRNA Seq data have been
457 submitted to the European Nucleotide Archive (ENA), accession number
458 PRJEB56215.

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Competing Interests

W.C., X.P., G.H., J.M., A.H, and B.D.G declare no competing interests. C.T.E. declares that he is an editor for npj Science of Food, but was not involved in the journal's review of, or decisions related to, this manuscript.

Author Contributions

W.C. organised, managed and conducted laboratory work. X.P. conducted metabolomic studies. G.H., J.M. and A.H. performed metagomic sequencing analysis. W.C. and B.D.G. wrote the manuscript. B.D.G, G.H. and C.T.E edited the manuscript. All authors approved the final text of the manuscript.

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588

589 **Figure Legends**

590 **Figure 1: APC^{Min} mice consuming 15% frankfurter (85% chow) for 8 weeks have**
591 **significantly more gastrointestinal tumours, higher levels of lipid peroxidation,**
592 **and greater body weight than APC^{Min} mice consuming a control diet (100% chow).**
593 **a)** after acclimatisation mice were fed one of four chow diets which were either
594 Control (n=9; standard chow (red)), or chow with a 15% incorporation of pork mince
595 (n=9; blue), pork sausage (n=10; light blue), or nitrite-containing frankfurter sausage
596 (n=10; green). **b)** numbers of tumours did not differ between any groups in any of
597 the specific regions of the GI tract. However, compared with control signifcantly
598 more tumours were displayed by the frankfurter group alone. **c)** terminal serum
599 MDA concentrations of mice consuming frankfurter were elevated compared to that
600 of control terminal samples, after controlling for baseline using an ANCOVA. Urine
601 DHN-MA concentrations were compared between groups at the same timepoint
602 using an ANOVA. Levels in mice consuming frankfurter supplemented chow had
603 higher levels at 28 and 56 days. **d)** bodyweights of frankfurter-fed mice were
604 consistently higher than control mice. Error bars shown represent s.e.m.

605

606 **Figure 2 Serum metabolite profiles of frankfurter-fed mice deviate most from**
607 **control, whereas those fed pork or sausage align more closely.** Serum collected at
608 56 days under went metabolomic profiling: **a)** shows scores plots of unsupervised
609 PCA based on the first three principle components (PCs) for Control (n=9; standard
610 chow (red)), or chow with a 15% incorporation of pork mince (n=9; blue), pork
611 sausage (n=10; light blue), or nitrite-containing frankfurter sausage (n=10; green).
612 Metabolomic data explained 89.4% of the total variation (76.9%, 8.0% and 4.5% for
613 PC1, PC2 and PC3, respectively). There was a tendency for profiles from the
614 frankfurter group to separate along the PC1 axis. Profiles from control, pork and
615 sausages groups clustered more closely with variation more restricted to PC2. **b)** is a
616 Venn diagram showing the number of the 599 metabolites measured which
617 significantly differed in group comparisons with control. c) heat map with
618 hierarchical clustering based on the top 50 metabolites ranked by FDR –corrected P-

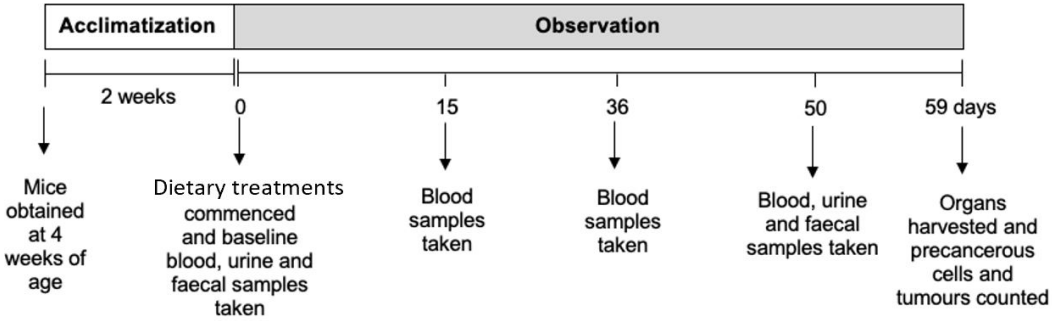
619 value (ANOVA). Each row represents a metabolite and each column a sample. Red
620 (high) and blue (low) indicates relative concentrations. Dendrograms indicating
621 similarity are shown at top (samples) on left side (metabolites). Serum metabolite
622 measurements readily clustered mice by diet with pork and nitrite-free sausage
623 being most similar to one another. Frankfurter consumption was characterised by
624 significantly higher levels of certain species of diglycerides (DG), triglycerides (TG)
625 and acylcarnitines (C), and by lower levels of certain phosphatidylcholines
626 (PC/LysoPC) and cholesterol esters (CE). Other notable observations included the
627 differing bile acid responses of taurocholic acid (TCA) and cholic acid (CA). The
628 profound variability of trimethylamine N-oxide (TMAO) responses in frankfurters
629 was also very evident. Control samples are shown in red, minced pork in dark blue,
630 nitrite-free sausage in light blue, and frankfurter (nitrite-containing) in green.
631
632 **Figure 3 Serum TMAO (trimethylamine N-oxide) is higher and more variable in**
633 **mice receiving frankfurter diet, yet TMAO precursor metabolites are not similarly**
634 **affected.** Serum levels of TMAO (at 56 days), typically formed from choline, betaine
635 and carnitines were significantly higher in mice receiving frankfurter-containing
636 chow. Levels of choline and betaine did not differ between groups. Mice consuming
637 any meat-containing chow had higher levels of carnitine, acetylcarnitine, and
638 propionylcarnitine. Control samples are shown in red (n=9), minced pork in dark
639 blue (n=10), nitrite-free sausage in light blue (n=9), and frankfurter (nitrite-
640 containing) in green (n=10). Data shown are violin plots containing individual
641 samples values with the median and quartile values represented as dotted lines.
642
643 **Figure 4 Faecal microbiome profiles differ most in frankfurter-fed mice with**
644 **reduced micribial diversity and a profound shift in abundance of taxa between**
645 **feeding groups.** Faecal samples were collected at 56 days from each mouse cage for
646 metagenomic sequencing providing cage replicates (n=3) for each treatment. **a)**
647 shows scores plots of unsupervised PCA based on the first three principle
648 components (PCs). Species level microbiome data explained 73.4% of the total
649 variation (42.9%, 15.5% and 15.0% for PC1, PC2 and PC3, respectively). **b)** the
650 Shannon diversity index as calculated by Qiime 2 indicates a reduction in microbial
651 diversity in the frankfurter-fed animals. Individual values are shown along with
652 means (dotted lines), and the error bars shown represent s.e.m. **c)** Relative
653 abundance of microbial taxa is shown at the level of the phylum, the family, and the
654 species. The taxa differed most profoundly in the frankfurter-fed animals. Bacterial
655 species with an abundance of <1% were aggregated and referred to as 'All others'
656 (white bars in figure). At the species level the dominance of *Dubiosiella Newyorkenesis*
657 in the frankfurter group was a particularly striking observation. Control samples are
658 shown in red, minced pork in dark blue, nitrite-free sausage in light blue, and
659 frankfurter (nitrite-containing) in green. uncult. – uncultured bacteria; ambig. –
660 ambiguous taxa.

Table 1. Numbers of aberrant crypt foci and mucin depleted foci did not significantly differ between any groups

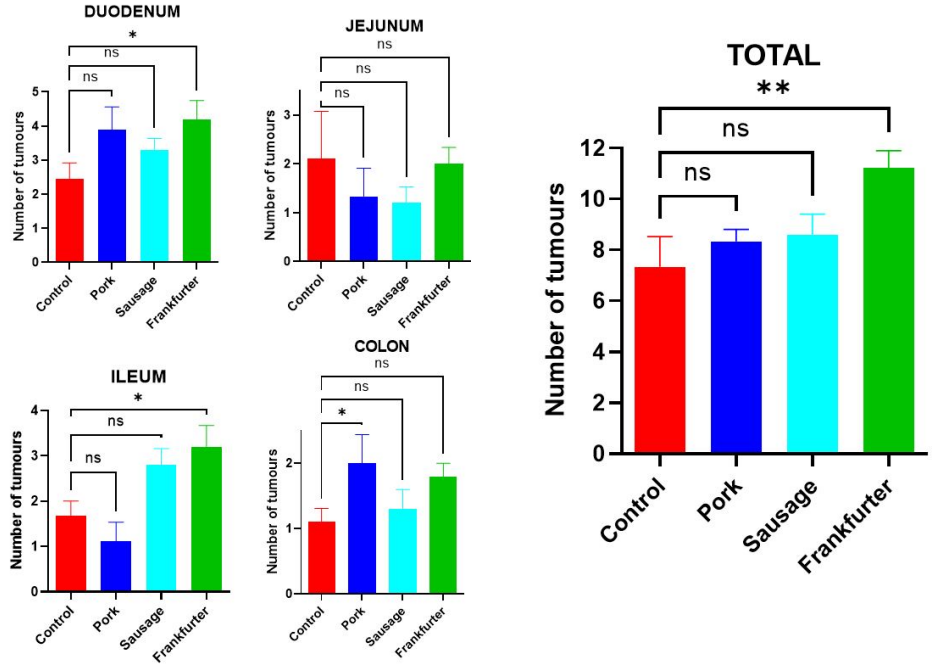
	Control	Pork	Sausage	Frankfurter
ACF	102.44 (10.86)	115.11 (17.03)	101.80 (10.71)	101.90 (15.18)
MDF	6.11 (2.76)	5.98 (2.67)	6.5 (2.59)	5.08 (3.12)

ACF- aberrant crypt foci; MDF- mucin depleted foci. Mean values were compared between groups by analysis of variance, alpha value of less than 0.05 was accepted as statistically significant. Values in parenthesis are standard deviations for the corresponding mean.

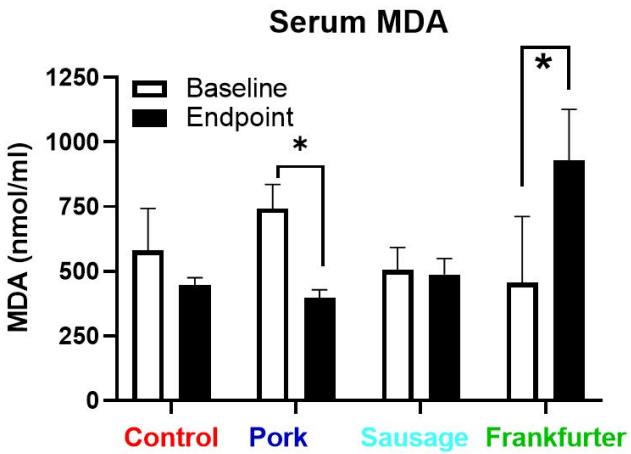
(a) Study plan

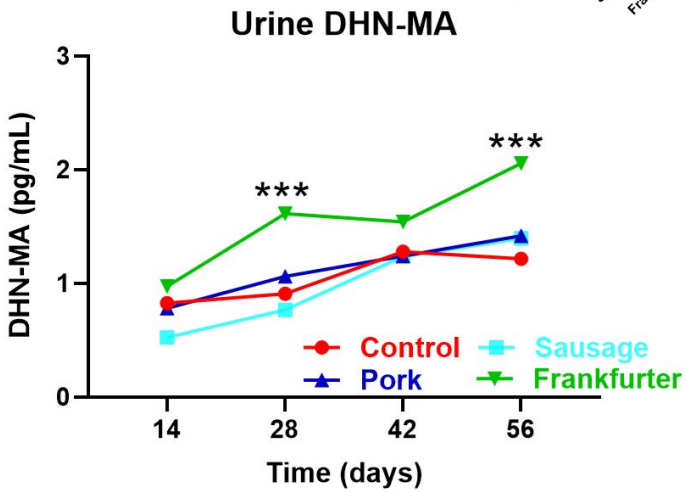


(b) Gastrointestinal tumours

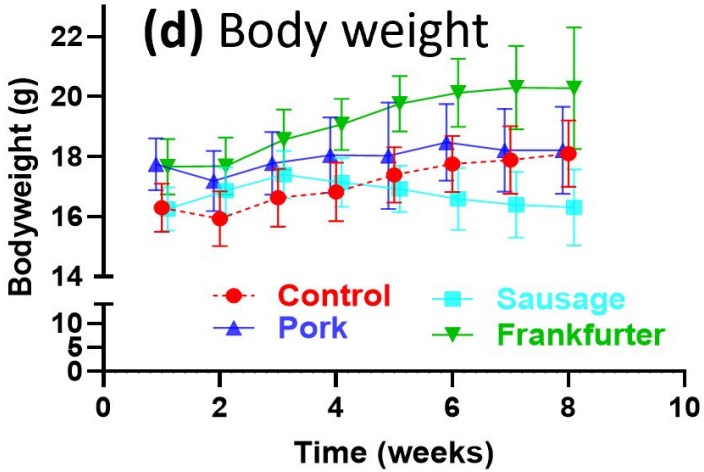


(c) Oxidative stress

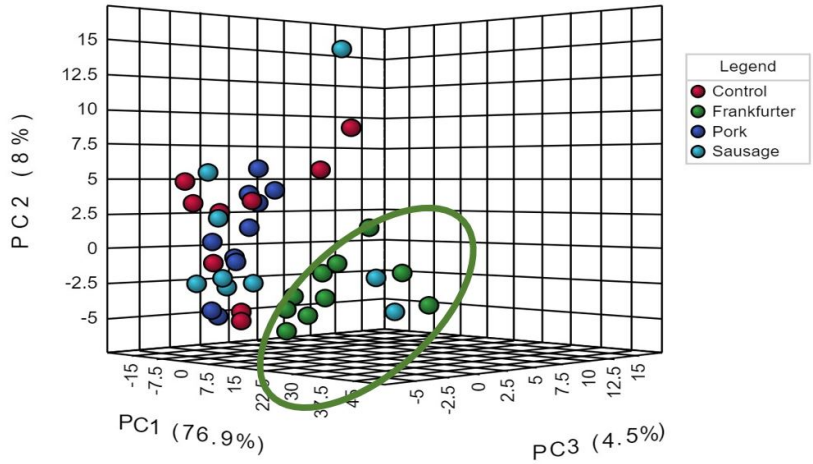




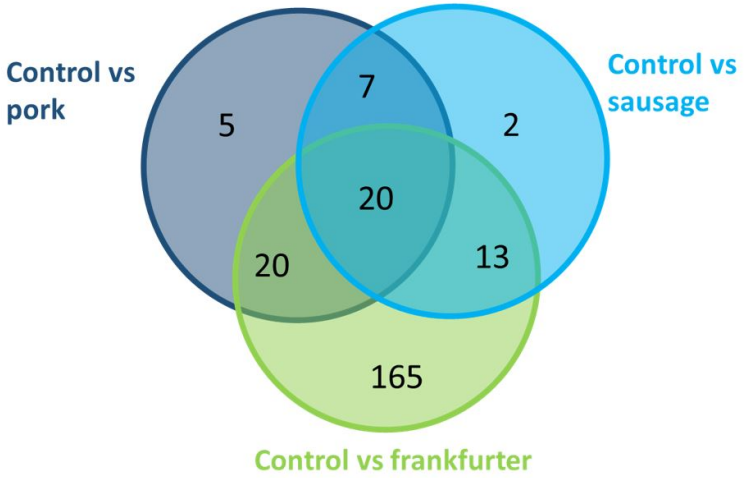
(d) Body weight



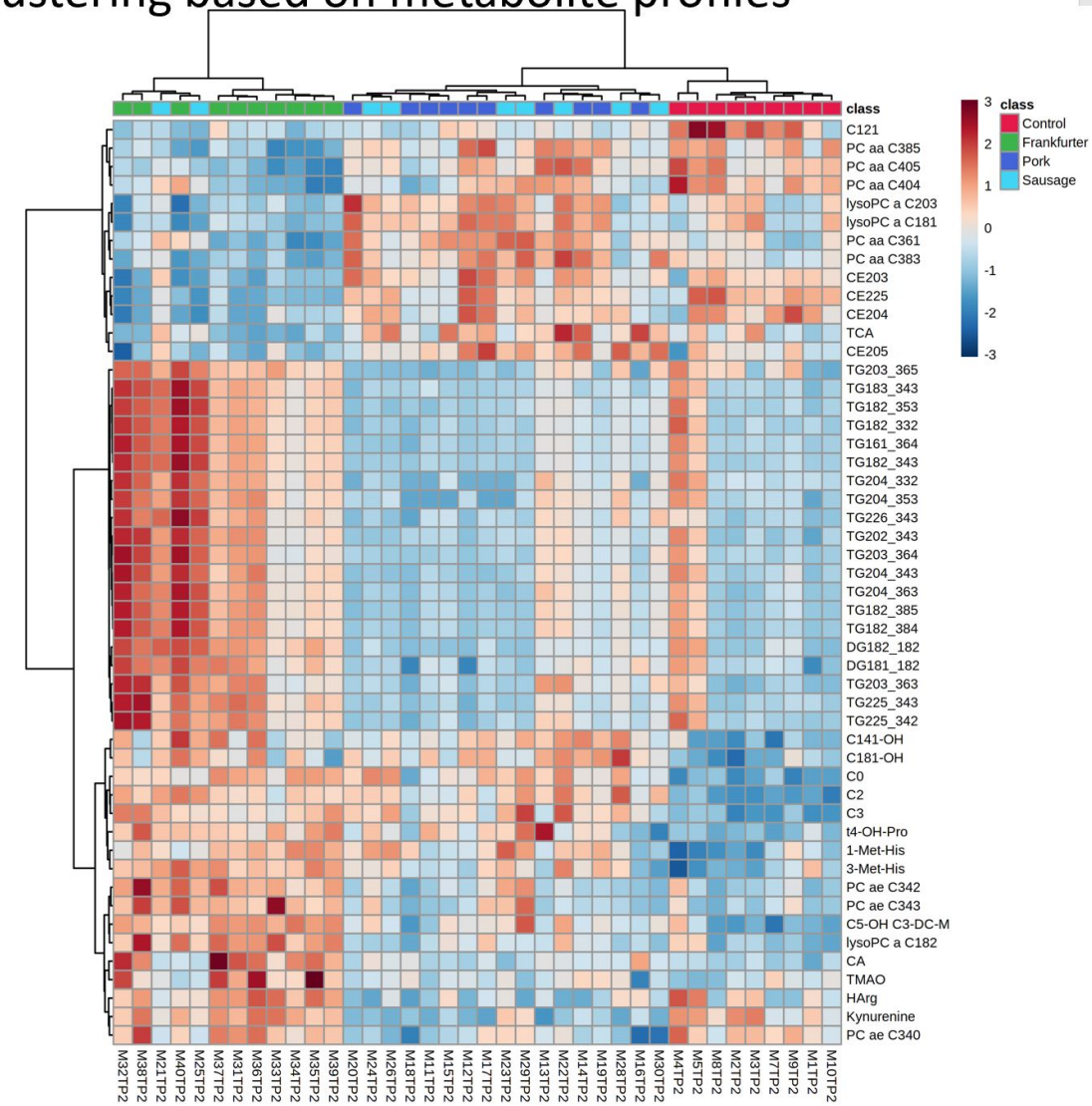
(a) Perturbed metabolite profiles

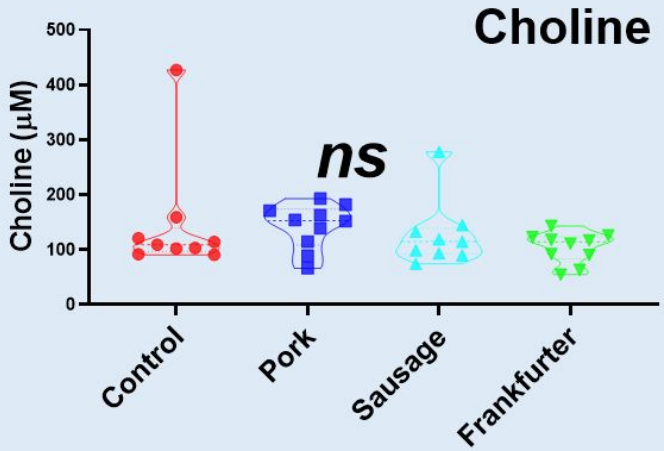


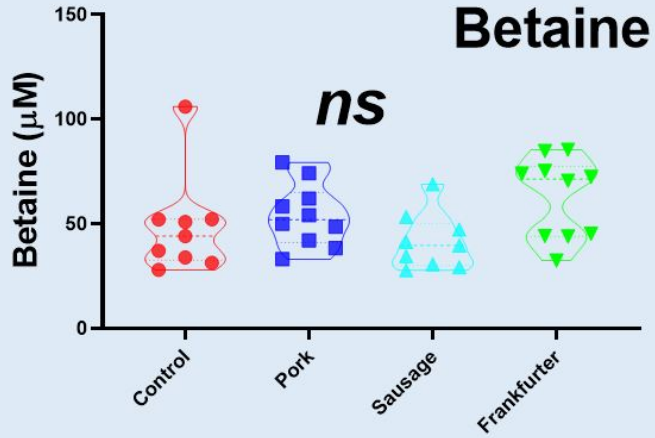
(b) no. of metabolites affected

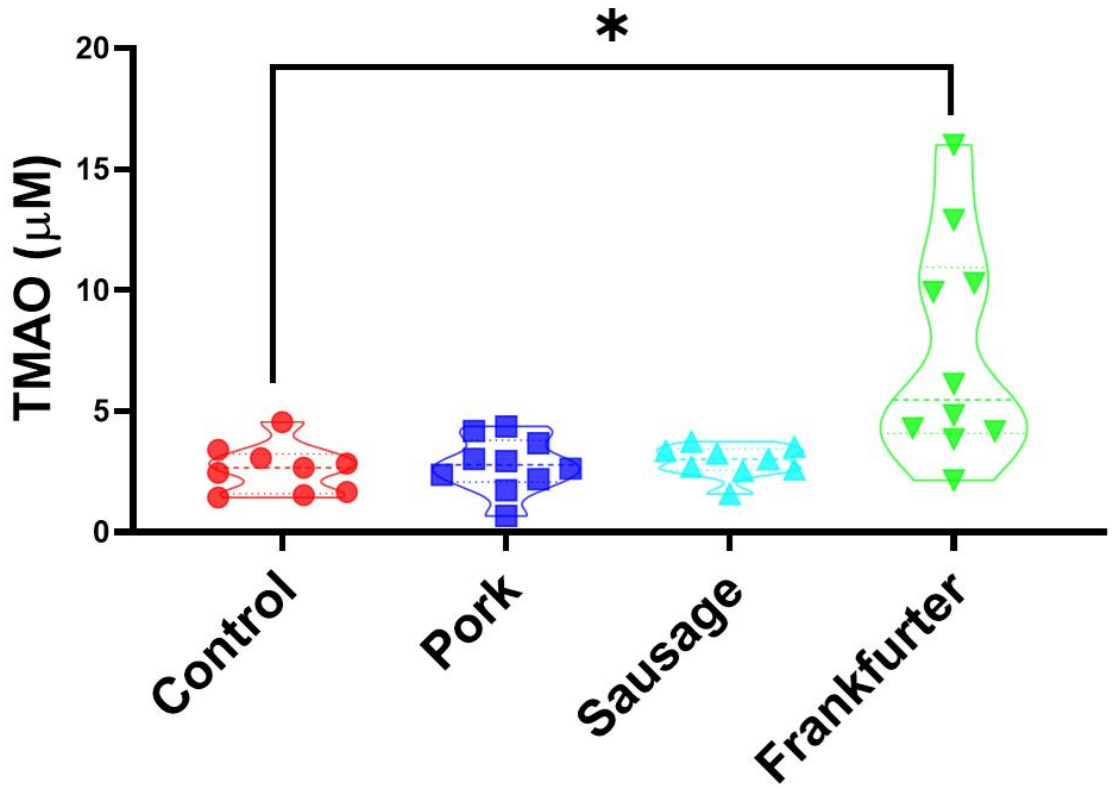


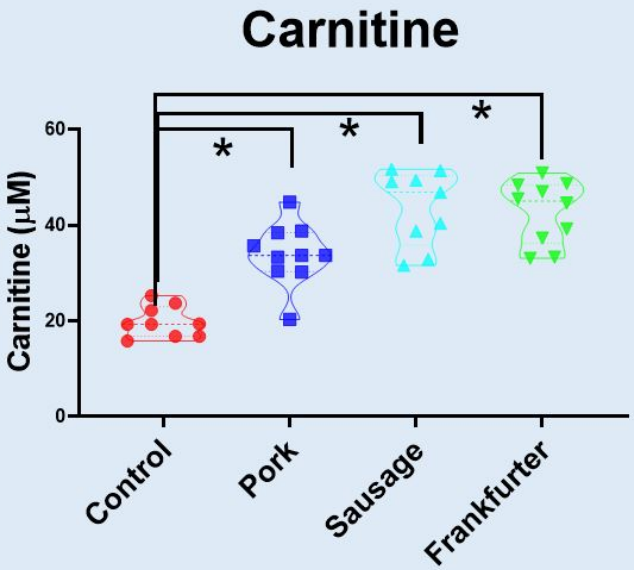
(c) Clustering based on metabolite profiles

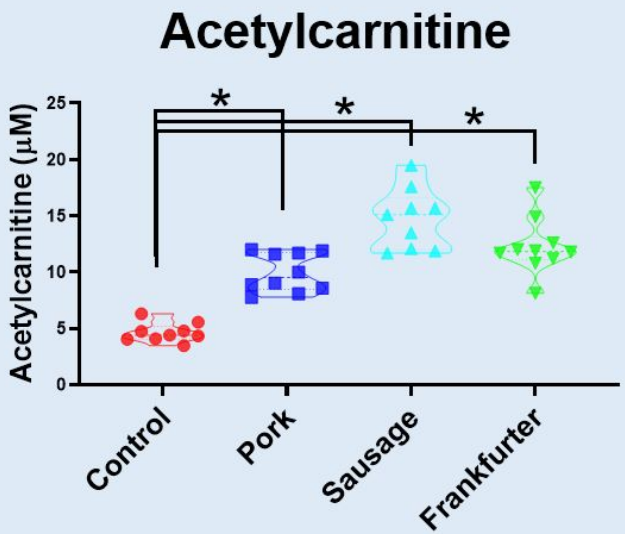


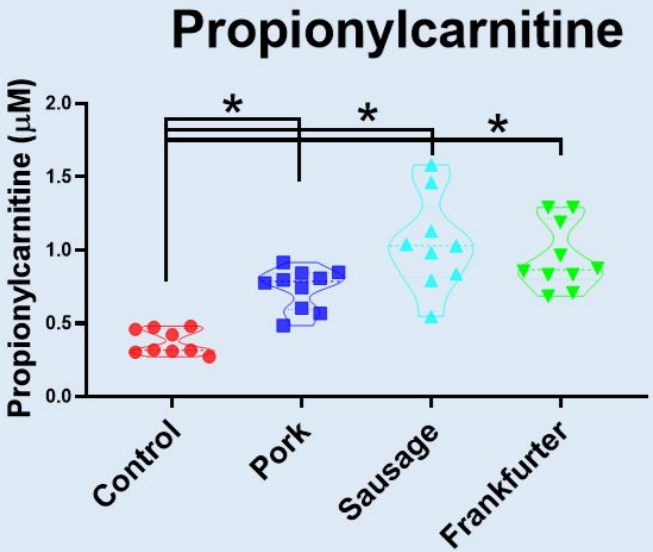

Choline
Choline (μM)
500
400
300
200
100
0
ns
Control
Pork
Sausage
Frankfurter


Betaine
Betaine (μM)
150
100
50
0
ns
Control
Pork
Sausage
Frankfurter

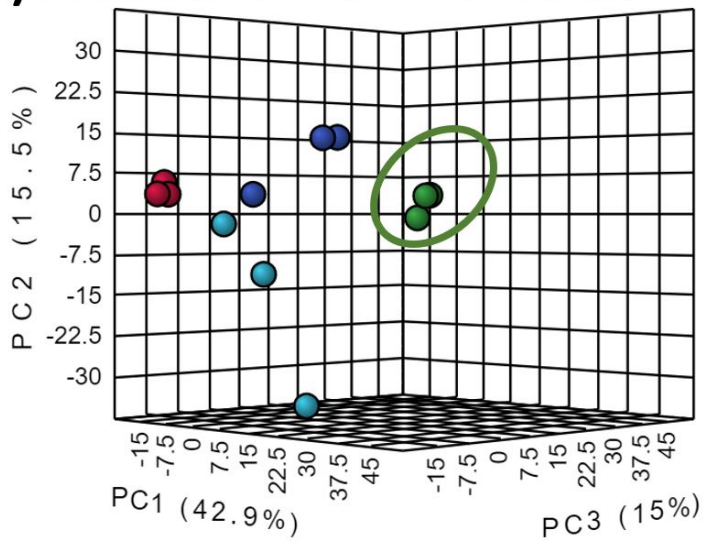

TMAO (μM)
20
15
10
5
0
*
Control
Pork
Sausage
Frankfurter


Carnitine
Carnitine (μM)
60
40
20
0
*
*
*
Control
Pork
Sausage
Frankfurter

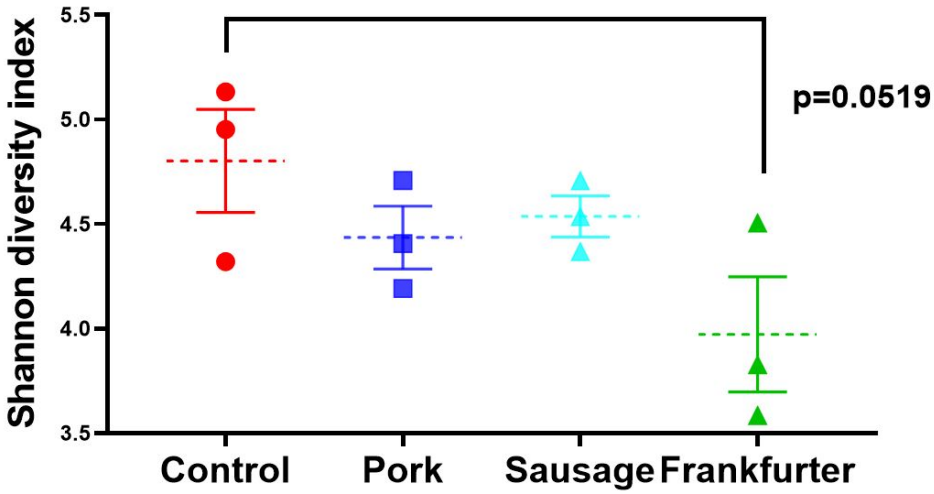
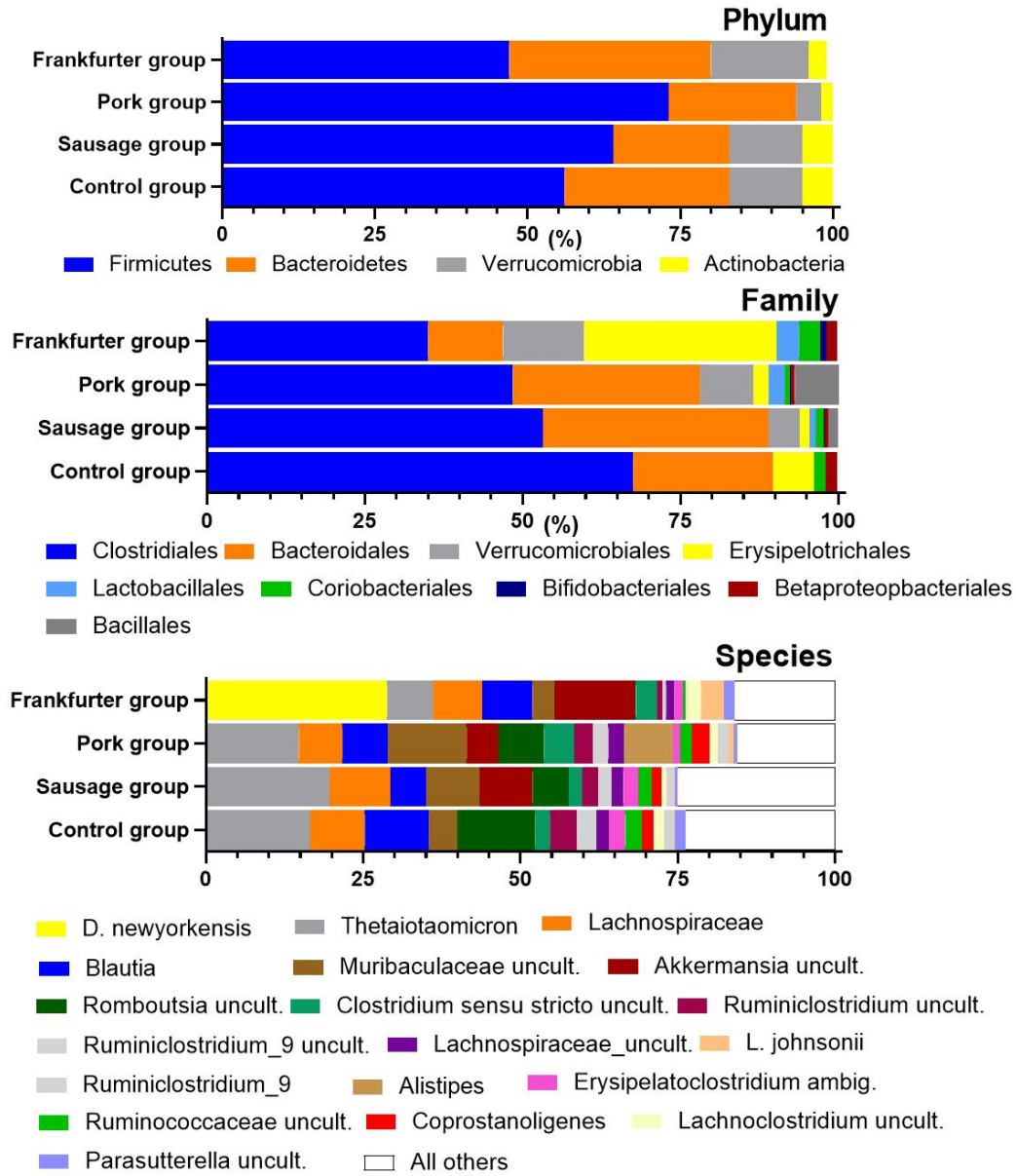

Acetylcarnitine
Acetylcarnitine (μM)
25
20
15
10
5
0
*
*
*
Control
Pork
Sausage
Frankfurter


Propionylcarnitine
Propionylcarnitine (μM)
2.0
1.5
1.0
0.5
0.0
*
*
*
Control
Pork
Sausage
Frankfurter

(a) Altered faecal microbiota



(b) Reduced microbial diversity



(c) Profound changes in microbial composition