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Combined consumption of beef-based cooked mince and sucrose stimulates oxidative stress, cardiac hypertrophy, and colonic outgrowth of *Desulfovibrionaceae* in rats

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ABSTRACT

Scope

High consumption of red meat and sucrose increases the epidemiological risk for chronic diseases. Mechanistic hypotheses include alterations in oxidative status, gut microbial composition, fat deposition and low-grade inflammation.

Methods and results

For two weeks, 40 rats consumed a diet high in white or red meat (chicken-based or beef-based cooked mince respectively), and containing corn starch or sucrose in a 2×2 factorial design. Lard was mixed with lean chicken or beef to obtain comparable dietary fatty acid profiles. Beef (*vs*. chicken)-fed rats had higher lipid oxidation products (malondialdehyde, 4-hydroxy-2-nonenal and hexanal) in stomach content and blood, and lower blood glutathione. Sucrose (*vs*. corn starch)-fed rats showed increased blood lipid oxidation products and glutathione peroxidase activity, higher liver weight and malondialdehyde concentrations, and mesenterial and retroperitoneal fat accumulation. Beef-sucrose-fed rats had increased cardiac weight, suggesting pathophysiological effects on the cardiovascular system. The colonic microbiome of beef-sucrose-fed rats showed an outgrowth of the sulfate-reducing family of the *Desulfovibrionaceae*, partly in expense of the *Lactobacillus* genus, indicating intestinal dysbiosis. Blood C-reactive protein, a marker for inflammation, was not different among groups.

Conclusions

Consumption of a cooked beef-based meat product with sucrose increased oxidative stress parameters and promoted cardiac hypertrophy and intestinal dysbiosis.

Key words: chronic disease, colonic microbiota, digestion, oxidation, red meat

INTRODUCTION

A 'Western' dietary pattern is, amongst others, characterized by a high intake of red/processed meats and sweets, and low intake of fruits and vegetables.[1] Large-scale meta-analyses of epidemiologic studies have shown that a high consumption of red and/or processed meat is associated with a higher risk of developing various chronic diseases, such as colorectal cancer,[2] cardiovascular diseases, and diabetes mellitus type 2.[3] A high intake of sucrose (disaccharide of glucose and fructose) and sugar-sweetened beverages is associated with a higher epidemiologic risk of developing cardiovascular diseases,[4,5] and diabetes mellitus type 2.[6] An estimated 8.2% of cardiometabolic deaths in the USA was recently related to high processed meat consumption and 7.4% to high consumption of sugar-sweetened beverages.[7]

The mechanisms behind these epidemiologic associations are not yet fully understood, but, amongst others, may include alterations in oxidative status, inflammation, fat deposition, and/or alterations in the colonic microbiota composition and their metabolic capacity. Heme-Fe, present in higher concentrations in red meat than in white meat, is known to oxidize polyunsaturated fatty acids (PUFA) and generate lipid oxidation products (LOPs) during gastrointestinal digestion, when antioxidant supply is deficient.[8-11] These LOPs include 4-hydroxy-2-nonenal (4-HNE) and hexanal (HEX), originating from the oxidation of *n*-6 PUFAs, and malondialdehyde (MDA), originating from the oxidation of both *n*-3 and *n*-6 PUFAs. When the formation of these oxidation products exceeds the capacity of dietary and cellular antioxidants to neutralize these compounds, a condition known as 'oxidative stress' is promoted. Oxidative stress is also known to be induced by a high consumption of sucrose.[12,13] Consumption of sucrose-sweetened soft drinks, compared to diet soft drinks, milk or water, also promoted hepatic and muscular fat accumulation in human volunteers,[14] whereas *L*-carnitine, a compound present in red meat, ameliorates the negative influence of high-fat diets on the lipid profile in rats.[15] Sucrose consumption promoted inflammation in healthy young men,[16] whereas rather inconsistent effects of red meat on inflammation are reported.[8]

High red meat consumption may change the colonic microbiota composition and its metabolic capacity, yet limited studies are available. In a Western dietary model, heme-Fe decreased the relative abundance of Firmicutes and increased the Bacteroidetes in the murine colon microbiota, hereby decreasing the ratio of Gram-positive to Gram-negative bacteria, along with colonic epithelial cell hyperproliferation.[17] Heme-Fe supplementation was also reported to induce an 8-fold increase of *Akkermansia muciniphila* in the colon microbiota of mice. This was accompanied by a degradation of the mucus barrier, which the authors attributed to the sulfide-producing and mucin-degrading nature of *A. muciniphila*.[18] Rats on a diet of extracted chicken (*vs.* red meat) protein had higher relative abundances of cecal *Lactobacillus*.[19] In contrast, no changes were found in selected fecal bacterial groups, among which *Lactobacillus*, following a dietary intervention in which healthy human volunteers consumed 300 g lean red meat per day for 4 weeks.[20] More studies are necessary to fully elucidate the changes in the colonic microbiome following the consumption of white and red meat, especially in interaction with other dietary compounds.

In the present study, we aimed at elucidating the effects of red meat and sucrose consumption on oxidative stress, the colonic microbial community, and inflammation. Therefore, rats were subjected for 2 weeks to a diet high in white meat (chicken) or red meat (beef), in combination with either corn starch or sucrose in a 2 × 2 factorial design. Beef and chicken meat have intrinsic differences in fatty acid profile, however this profile is also variable and strongly influenced by the diet of animals.[21] Because the focus of the present study was on the effect of differing heme-Fe content between chicken and beef, we aimed to obtain comparable dietary fatty acid profiles and exclude confounding effects thereof. For this purpose, lard was mixed with the lean chicken or beef muscle, and safflower oil was added to all experimental diets to standardize the dietary fatty acid profile. Since heme-Fe is a known pro-oxidant,[9-11] it was expected that the beef-based *vs*. chicken-based cooked mince would be more sensitive to oxidation during its preparation and gastrointestinal digestion. Hence, the prepared chicken-based and beef-based product were model meat products with low or high sensitivity to oxidation respectively.

MATERIALS AND METHODS

1. Manufacturing of meat samples and animal diets

Lean samples from the *m. pectoralis profundus* of chicken and beef were purchased, and manually chopped into cubes of approximately 20-30 cm^3. Lard was added to the muscle at a proportion of 15 % on the final weight to equalize the fatty acid profile. Subsequently, the mixture was minced in a grinder (Omega T-12), equipped with a 10 mm plate, followed by grinding through a 3.5 mm plate, vacuum packing in plastic bags, and heating for 70 min in a water bath at 70°C. The cooked samples were then homogenized using a food processor. The diets were formulated to contain 65% of the cooked chicken or beef product (w/w), and 29% of corn starch or sucrose, along with other ingredients (Table 1). The experimental diets were vacuum packed in daily portions (± 100 g) and stored at -20°C.

2. Rat experiment and sample preparation

The rat experiment was conducted following the principles of laboratory animal care and the Belgian law on the protection of animals. The experimental protocol was approved by the Ghent University Ethical Committee (ECD 14/58). Forty male Sprague-Dawley rats (± 200 g) (Janvier laboratories, France) were given an adaptation period of 10 days, and were housed per group of four rats during which a standard laboratory diet (Ssniff R/M-N pellets) (Ssniff, Soest, Germany) and water were provided *ad libitum*. Thereafter, rats were housed by two, and every other day, 8 rats were randomly assigned to each of the four experimental diets, which were offered *ad libitum*, and refreshed daily. Body weight and daily food intake was monitored every 2 days. After 10 days on the experimental diets, feces were collected in the cage during 24 hours (n=5 per treatment). Following 14 days on the diets, rats were anesthetized by 5% isoflurane gas, and blood was collected from the abdominal aorta into heparin tubes until death occurred. For each of the euthanasia days, a rat of each dietary treatment was sampled in random order. Plasma and red blood cells (RBC) were separated by low speed centrifugation, and immediately stored at -80°C in different aliquots. Organs (brain, colon, duodenum, heart, kidney, liver,

pancreas, prostate) were removed, carefully rinsed with 0.9% NaCl solution and weighed, after which 1% triton-x-100 phosphate buffer (pH 7; 50 mM) was added to the heart, kidney, and liver in a 1/10 ratio (w/v), and to the brain, colon, duodenum, pancreas and prostate in a 1/5 ratio (w/v). Solutions were homogenized with an ultraturrax, and centrifuged (15 min, 15.000g, 4°C) after which the supernatant was filtered through glass wool and stored at -80°C in different aliquots. The weight of mesenterial and retroperitoneal fat was measured. Samples of the liver and the contents of stomach, cecum and colon were stored as such at -80°C. Intestinal segments (2 cm) from the mid-colon were collected and fixed in 10% formaldehyde buffer for intestinal morphology analysis.

3. Chemical composition of the experimental diets

The diets were analyzed for dry matter, crude protein and crude fat content according to the ISO 1442–1973, ISO 937–1978 and ISO 1444–1973 methods, respectively. Lipids were extracted using chloroform/methanol (2/1; v/v), and fatty acids were methylated and analyzed by gas chromatography (HP6890, Brussels, Belgium).[22] Hematin was determined colorimetrically,[23] and converted to heme-Fe using the formula heme-Fe = hematin × atomic weight Fe/molecular weight hematin. Alpha-tocopherol was measured by reverse phased HPLC (Agilent Technologies, 1200 series, Degasser, Germany).[24] All analyses were performed in duplicate.

4. Oxidation and oxidative stress parameters

MDA in the diets, stomach content, feces, blood and organ tissues was measured as TBARS (thiobarbituric acid reactive substances) through a colorimetrical assay.[25] 4-HNE and HEX in the diets, stomach content, feces and plasma were measured by HPLC following their derivatization with cyclohexanedione.[26] Alfa-tocopherol was measured in the liver by reversed-phase HPLC,[24] and in plasma by HPLC following saponification and hexane extraction.[27] The concentrations of glutathione (GSH) and oxidized glutathione (GSSG) in the RBC fraction were determined by HPLC using γ-glutamyl glutamate as internal standard.[28] The activity of gluthatione peroxidase (GSH-Px) in plasma was determined by measuring the oxidation of NADPH, whereby one unit of GSH-Px activity was defined as the amount of extract needed to oxidize 1 μmol NADPH/min at 25 °C.[29] All parameters were analyzed in duplicate.

5. C-reactive protein

Quantification of C-reactive protein (CRP) in plasma was performed in duplicate using a commercial ELISA kit (Promokine, Heidelberg, Germany).

6. Colonic microbial composition

Following DNA extraction,[30] DNA quality was validated by means of agarose gel electrophoresis and via PCR, as previously described,[31] with the bacterial primers P338 and P518r.[32] Real-time PCR (qPCR) was performed as previously described.[33] The quality of the PCR product was determined with agarose gel electrophoresis to ensure that no inhibition of the PCR took place. The DNA extracts were sent to LGC Genomics GmbH (Berlin, Germany) for Illumina sequencing on the Miseq platform. The amplicon sequencing and data processing were carried out as described in the Supporting information (1). A table containing the relative

abundance of different OTU (operational taxonomic units), together with their taxonomic assignments for each sample was generated (Supporting information 2). The raw fastq files that were used to create the OTU table that served as a basis for the microbial community analysis, have been deposited in the National Center for Biotechnology Information (NCBI) database (Accession number SRP145578).

7. Cecal volatile fatty acids

Volatile fatty acids in cecal contents were measured in duplicate by gas chromatography (HP 7890A, Agilent Technologies, Diegem, Belgium), using a FID detector and a Supelco Nukol capillary column (30 m × 0·25 mm × 0·25 µm, Sigma-Aldrich, Diegem, Belgium).[34]

8. Colon crypt depth

Intestinal tissue samples were processed under standard conditions in an automatic tissue processor (Shandon, Pittsburgh, PA, USA), after which they were cut using a microtome into 5 µm slices, and stained using standard H&E coloring. Image acquisition was performed using an inverted widefield microscope (Nikon Ti, Nikon Instruments, Paris, France), equipped with an automated stage and Perfect Focus System. Separate tiles were acquired with a 20x (NA=0.75) lens and stitched into large mosaic images that covered the entire tissue section. Well-oriented crypts (circa 30 per animal) were measured, and the average crypt depth was calculated per rat for colon tissues using ImageJ image processing freeware.

9. Statistics

For all data, with exception of the microbial data, normality of distribution and homogeneity of variance were analyzed by the Kolmogorov-Smirnov test and Levene's test respectively. When appropriate, a mixed model ANOVA procedure (SAS Enterprise Guide 7) was used with the fixed effects of meat source, carbohydrate source and their interaction term, and the random effect of euthanasia day. Tukey-adjusted post hoc tests were performed for all pairwise comparisons with $P \leq 0.05$ considered significant. Data points higher than Q3 + 1.5 IQR or lower than Q1 - 1.5 IQR were evaluated for their influence in the model by the internally and externally studentized residuals, Cook's D, DFFITS and the restricted likelihood distance. In case these data points were highly influential, P-values were reported, both with and without these data points. When data were not normally distributed or heterogeneity of variance was present, an independent samples Kruskal-Wallis test with pairwise comparisons was performed (SPSS Statistics 25) using either dietary treatment, meat source or carbohydrate source as independent variables. For the microbial composition, statistical analyses were performed in R, version 3.3.1. (http://www.r-project.org)[35] using the packages vegan,[36] and phyloseq[37] for community analysis. Differences in order-based Hill's numbers[38] between the different treatments were defined via ANOVA and the post-hoc Dunn's Test of Multiple Comparisons (dunn.test package) with Benjamini-Hochberg correction. Significant differences in community composition between different treatments were identified with pairwise permutational ANOVA (PERMANOVA) with Bonferroni correction, using the *adonis* function (vegan). Differences between dietary treatments were identified by using LEfSe (linear discriminant analysis effect size)[39] (http://huttenhower.sph.harvard.edu/galaxy). The LEfSe analysis conditions were as follows: (1) alpha values for the factorial Kruskal-Wallis test among classes, and for the pairwise Wilcoxon test among subclasses were less than 0.05; (2) the threshold on the

logarithmic LDA score for discriminative features was set to 2.0, and (3) the strategy for multi-class analysis was all-against-all (more strict).

RESULTS

Experimental diets

Diets had a similar proximate and fatty acid composition (Table 1). Heme-Fe concentrations were 10–fold higher in the beef diets, compared to the chicken diets. The beef-sucrose diet contained 30% less vitamin E compared to the other diets.

Animals

Figure 1 presents body weight and feed intake of rats throughout the experimental period, and the weight of heart, liver, mesenterial and retroperitoneal fat at the day of euthanasia. All parameters were statistically analyzed with the mixed model procedure, except for liver weight, which was analyzed using the non-parametric procedure. Body weight was not significantly different throughout the feeding period, with exception of a trend for a higher body weight in sucrose-fed rats on day 7, 9 and 11. There was also a trend ($P=0.081$) for a lower average daily weight gain in rats on the beef-starch diet (8.2 ± 1.6 g/day) compared to rats on the chicken-sucrose diet (9.6 ± 1.3 g/day). Feed intake was not significantly different during the first week, but the starch-fed rats consumed 11% more feed compared to the sucrose-fed rats on days 9, 11 and 13 (all $P<0.012$). Sucrose and beef consumption both independently increased hearth weight, with the heart of the beef-sucrose fed rats weighing significantly more (+10%) than the one of the chicken-starch fed rats. When one highly influential data point was removed from the analysis, the heart weight of the beef-sucrose fed rats was also significantly higher than the beef-starch fed rats. When expressed relatively to the total body weight, the hearts of beef-starch and beef-sucrose fed rats (both 0.31%) weighed more than the heart of chicken-starch fed rats (0.28%, both $P<0.005$), but not more than chicken-sucrose fed rats (0.29%, both $P>0.103$). The sucrose diets significantly increased liver weight, fat deposition in the mesenterium and retroperitoneum, and tended to increase kidney weights ($P=0.094$). Prostate and pancreas weight was not significantly different among treatments. When highly influential data points were removed, the effect of sucrose consumption on mesenterial fat remained significant ($P=0.033$).

Lipid oxidation products throughout digestion

Figure 2 presents the contents of TBARS, 4-HNE and HEX in the diet, stomach content and feces of rats. All parameters were statistically analyzed using the mixed model procedure. Already before digestion, the beef-sucrose diet contained the highest amounts of TBARS, 4-HNE and HEX. The beef-starch diet also contained 12-fold higher 4-HNE and 4-fold higher HEX concentrations compared to the chicken diets, whereas its TBARS content was 40% lower than the chicken-sucrose diet. In the stomach content, all oxidation products were present in higher concentrations, compared to their respective undigested diets. Stomach TBARS were significantly different among all dietary treatments, with the highest levels in rats on the beef-sucrose diets, followed by rats on the beef-starch diets, chicken-sucrose diets, and rats on the chicken-starch diets having the lowest concentrations. The 4-HNE and HEX concentrations in the stomach contents of rats on the beef diets were 16- and 17-fold higher, compared to the

chicken diets, with no significant effects of sucrose. The feces contained higher TBARS concentrations than the stomach content, with the exception of the beef-sucrose rats, whose fecal TBARS concentrations were approximately equal when compared to their stomach content. In contrast, fecal 4-HNE and HEX concentrations were clearly lower compared to the stomach, with fecal 4-HNE concentrations up to 23-fold lower than stomach 4-HNE in the beef-starch rats. Beef (*vs.* chicken)-fed rats contained significantly higher fecal TBARS concentrations, whereas sucrose (*vs*. starch)-fed rats contained significantly higher fecal HEX concentrations.

Blood oxidative stress parameters

Figure 3 presents oxidative stress parameters in the blood of the rats following 2 weeks on the experimental diets. All parameters were statistically analyzed using the mixed model procedure, except for GSH-Px and the GSSG:GSH ratio, which were analyzed by the non-parametric procedure. Sucrose-fed rats had significantly higher concentrations of TBARS, 4-HNE and HEX, lower concentrations of GSSG, and increased activities of GSH-Px compared to starch-fed rats. The beef-fed rats had significantly higher concentrations of 4-HNE and HEX, and significantly lower concentrations of GSH and GSSG, compared to chicken-fed rats, with no interaction term being significant for any parameter. The GSSG:GSH ratio was not significantly different among diets. On individual dietary treatment level, rats on the beef-sucrose diets had significantly higher concentrations of TBARS, 4-HNE and HEX, lower concentrations of GSH and GSSG, and tendency for higher GSH-Px concentrations (P=0.052), compared to the chicken-starch rats. Removal of highly influential data points further decreased the already significant P-values for 4-HNE and GSSG, whereas the P-value for the GSSG:GSH ratio remained unaffected. In addition, the effect of sucrose-feeding on GSH concentrations became significant (P=0.041), and GSH concentrations became significantly lower in beef-starch (*vs*. chicken-starch) fed rats (P=0.021).

TBARS in organ tissues

Tissue TBARS were statistically analyzed by the non-parametric procedure as previously described. Sucrose-fed rats had significantly higher hepatic TBARS concentrations, compared to the starch-fed rats (Supplementary Figure 1). Beef-fed rats tended to have higher colonic TBARS concentrations compared to the chicken-fed rats. No significant differences were present in the other organs.

A-tocopherol

The non-parametric procedure was used for α-TOC in plasma, whereas the mixed model procedure was used to analyze the hepatic α-TOC concentrations. Highest hepatic α-TOC concentrations were found in rats on the chicken-starch diet, which were significantly lower in the chicken-sucrose and beef-starch rats (both -16%), and lowest concentrations were found in the beef-sucrose rats (-33%). In plasma, no significant differences were found among treatments (Supplementary Figure 2).

C-reactive protein

Plasma CRP was statistically analyzed using the mixed model procedure, and was not influenced by meat type (P=0.401), carbohydrate (P=0.130), nor their interaction term (P=0.189) (Supplementary Figure 3).

Colon microbiota composition

No significant differences were found in the three Hill orders, indicating no differences in α-diversity in the colon microbiota of rats among dietary treatments (Supplementary Figure 4). Real-time PCR analysis showed significantly more 16S rRNA gene copies in the colon content of beef (*vs*. chicken)-fed rats (7.9 *vs*. 4.1 × 10^8 copies/µL, P=0.002). The Bonferroni-corrected PERMANOVA indicated that rats on the beef-sucrose diets had a different colonic microbial community composition compared to rats on the chicken-starch diets (P=0.013), whereas no other treatments were significantly different. The colonic microbiota composition at phylum and family level is shown in Figure 4. The phyla of the Firmicutes and Bacteroides were the most present with respective median relative abundances of 43.9% (range 8.8 to 72.4%) and 37.9% (range 7.2 to 83.7%), followed by the phyla of the Proteobacteria (median of 15.1%, range 1.1 to 51.5%), Verrucomicrobia (median of 0.8%, range 0.0 to 27.8%) and Actinobacteria (median of 0.3%, range 0.0 to 4.6%). Statistical analysis using LEfSe (Table 2, condition A; main class diet) showed that the family of the *Desulfovibrionaceae* was the most discriminant feature for the beef-sucrose group (LDA score 4.96, P=0.026), whereas the *Lactobacillus* genus was the most discriminative for the chicken-starch group (LDA score 4.60, P=0.024). Changes were observed in various low-abundance groups (median ≤0.1%), with the genera *Enterococcus* (P<0.001) and *Pseudoflavonifractor* (P=0.001) discriminating for the chicken-starch rats, and the *Micrococcaceae* (P=0.038) and *Clostridium*_XVIII (P=0.004) discriminating for the chicken-sucrose rats. By changing LEfSe conditions (Table 2, condition B; main class meat, subclass carbohydrate), the genera *Escherichia/Shigella* (P=0.006), *Roseburia* (P=0.039), *Anaerofilum* (P=0.008), and *Barnesiella* (P=0.038), and the order of *Actinomycetales* (P=0.037) were discriminative for the chicken-fed rats. When carbohydrate was used as main class and meat type as subclass in LEfSe conditions (Table 2, condition C; main class carbohydrate, subclass meat), only *Lactobacillus* (P=0.042) was discriminative for the starch-fed rats.

Cecal volatile fatty acids

Cecal volatile fatty acids were statistically analyzed using the non-parametric procedure. Total cecal VFA were significantly higher in starch-fed rats (+18%), compared to sucrose-fed rats (Supplementary Table 1). Cecal VFA in starch-fed rats had a lower proportion of acetate (-5%, P=0.012), and higher proportions of propionate (+8%, P=0.079), butyrate (+15%, P=0.013) and valerate (+16%, P=0.005), compared to the sucrose-fed rats. Chicken or beef consumption had no significant effect on the VFA profile. No significant differences were found in the branched-chain fatty acids.

Colonic crypt depth

Colonic crypt depth was statistically analyzed using the mixed model procedure, and was not influenced by meat type (P=0.112), carbohydrate source (P=0.561), nor their interaction term (P=0.589) (Supplementary Figure 5).

DISCUSSION

This study showed that the combined consumption of beef-based cooked mince and sucrose stimulated oxidative stress, induced cardiac hypertrophy, and altered the colonic microbial composition, with an outgrowth of the sulfate-reducing family of the *Desulfovibrionaceae* as one of the most prominent changes. These observations may shed light on the mechanisms underlying the epidemiological associations between the high consumption of red meat or sucrose and the higher risk of developing various chronic diseases.[2-7]

To obtain similar dietary fatty acid profiles in the different dietary treatments, lard was added to lean muscle from chicken or beef. The beef-based meat product contained higher LOPs, explained by the catalyzing effects of heme-Fe on oxidation during cooking of red meat, as previously observed in similarly prepared model meat products.[9,40] Following its preparation, the beef-sucrose diet contained more LOPs and lower α-tocopherol concentrations compared to the beef-starch diet, despite its similar preparation conditions. This may be explained by metal-induced oxidation by reducing sugars in neutral pH conditions, as previously described in an *in vitro* oxidation system,[41,42] or during *in vitro* oxidation of meat protein.[43] On the other hand, in acidic conditions (pH 3), ferrous iron-induced oxidation was equally high in an *in vitro* oxidation system with or without fructose.[42] This could explain the observation that both the acid stomach digested beef diets with starch or sucrose were oxidized to a similar high extent. Whereas food in the stomach was more oxidized than the undigested diets, as previously reported,[44] fecal contents of 4-HNE and HEX were negligible compared to the stomach content. This can be explained by their absorption and/or metabolism in the gastrointestinal tract, or because the applied analytical method only measures unbound reactive 4-HNE and HEX, and not their adducts with protein or other compounds. This finding may raise questions concerning the involvement of reactive dietary LOPs in the association between red/processed meat consumption and colorectal cancer, as previously proposed.[45] Compared to the stomach, higher fecal TBARS contents were observed, whereas in a previous rat feeding study conducted by our group, TBARS in the colonic content were lower.[40] Hence, additional artefactual TBARS formation may occur before collection of the feces in the cage, compared to collection of colon content.

Both the consumption of the beef-based cooked mince and sucrose independently increased oxidative stress parameters in blood, as demonstrated by increased concentrations of TBARS, 4-HNE and HEX, increased GSH-Px activity and decreased GSH concentrations, in spite of no difference in the GSH:GSSG ratio. In accordance, blood MDA increased in rats consuming red turkey meat.[44] Decreased GSH concentrations in red blood cells, and increased urinary 4-HNE metabolites were previously observed following the consumption of a lean beef diet for 2 weeks.[40] This fall in GSH and increase in urinary 4-HNE may be explained by the formation of a mercapturic acid conjugate of 4-HNE with GSH, after which it can be excreted in the urine.[45] High sucrose consumption also stimulated systemic oxidative stress in rats.[12,13] Since there was no increased 4-HNE and HEX formation in the stomach of sucrose-fed compared to starch-fed rats, the observed oxidative stress in sucrose-fed rats is likely not a consequence of enhanced lipid oxidation during digestion. An alternative explanation may be the metabolic conversion of fructose to uric acid in the liver, which is accompanied by mitochondrial

oxidative stress and dysfunction, and hepatic steatosis in rats.[47,48] Indeed, in the present study, hepatic oxidative stress was observed in rats on the sucrose diets, indicated by the increased hepatic TBARS concentrations, along with increased liver weight and fat accumulation in the mesenterium and retroperitoneum. Central obesity is described to be associated with increased CRP levels as a marker for inflammation,[49] however, no such changes were observed in the present study.

Diets with the beef-based meat products or sucrose independently increased cardiac weight, along with increased parameters of oxidative stress in the blood. Oxidative stress has a causative role in the development of cardiac hypertrophy.[50] During oxidative stress conditions, nitric oxide (•NO) in the blood is oxidized to peroxynitrite ($ONOO^-$). Whereas controlled expression of •NO is considered to be beneficial, due to its vasodilating and cardioprotective properties, the formation of $ONOO^-$ is a crucial pathogenic mechanism in many diseases.[51] In a similar way, upon its metabolic fate in the liver, fructose increases plasma uric acid, which can irreversibly react with •NO, resulting in the formation of 6-aminouracil and depletion of the vasodilating •NO.[52] Furthermore, renin is released following oxidative stress in the kidneys, contributing to an elevated blood pressure.[53] Fructose-fed rats were previously observed to suffer from left ventricular hypertrophy and elevated blood pressure.[54] In a previous study from our research group, rats on a high fat-beef diet had increased cardiac MDA concentrations, compared to rats on a lean-chicken diet, and increased renal MDA, compared to rats on a diet of lean-chicken, lean-beef, or fat-chicken.[40] No such changes were however observed in the present study.

Next to an influence of the dietary treatments on oxidative stress parameters, shifts were also observed in the colonic bacterial community composition. As indicated by both LEfSe and PERMANOVA analyses, these bacterial communities were especially different between rats on the chicken-starch diets and beef-sucrose diets, the latter characterized by a bloom of *Proteobacteria*, due to an outgrowth of members of the *Desulfovibrionaceae*, and decreased *Lactobacillaceae*. A bloom of *Proteobacteria* is considered to be a signature of intestinal dysbiosis.[55] Similarly, increased *Desulfovibrionaceae* and decreased *Lactobacillaceae* were previously observed when mice were fed a high-fat-sucrose 'Western' diet, compared to a low-fat standard chow diet.[56] Hildebrandt et al.[57] reported an outgrowth of *Desulfovibrionaceae* in mice on high-fat diets. The shifts in the present study were quite clear, since the median relative abundance of *Desulfovibrionaceae* was 23% in the beef-sucrose group, and 3% in rats on the chicken-starch diet, hence, alterations in the bacterial metabolic capacities are expected. *Desulfovibrionaceae* are known for their sulfate-reducing capacities, hereby producing toxic hydrogen sulfide (H_2S). As Ijssennagger et al.[58] explained, H_2S may reduce disulfide bonds of the intestinal mucus layer, hereby breaking the mucus barrier, resulting in exposure of the epithelium to bacteria and toxins, potentially leading to inflammation. The present study showed no differences in blood CRP as a measurement of inflammation, however the presence of inflammation in the intestinal mucosa cannot be excluded solely based on this parameter. E.g., Guéraud et al.[11] found no differences in plasma CRP, however, myeloperoxidase activity, as a marker for neutrophil infiltration, was increased in colonic mucosa of rats following the consumption of heme-Fe/PUFA diets. Ijssennagger et al.[58] also reviewed that in patients with

'inflammatory bowel disease', the mucus barrier is compromised, along with increased abundances of sulfate-reducing bacteria and increased fecal sulfide concentrations. The consumption of red meat,[59] or hemin[60] has been shown to aggravate chemically-induced colitis in mice. In the latter study, this was also accompanied with changes in the colonic microbial community, such as decreased abundances of *Lactobacillus* and increased *Proteobacteria*, due to increases in members of the *Enterobacteriaceae*, along with functional changes, such as increased nitrogen and sulfur metabolism.[60] The colonic crypt depth in the present study was not significantly altered, however it was previously reported that heme-Fe induces hyperproliferation of the colonic crypts.[18] This different outcome may be explained by the 30-fold higher concentrations of heme-Fe in the diets of the latter study, compared to the heme-Fe levels in the beef diets of the present study.

In conclusion, this rat feeding study showed red meat and sucrose consumption to increase blood oxidative stress parameters, which may have contributed to the observed cardiac hypertrophy, and especially its combined consumption stimulated intestinal dysbiosis.

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CONFLICT OF INTEREST

The authors declare no competing financial or personal interests.

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Figure 1: Body weight, feed intake and weight of heart, liver, mesenterial fat and retroperitoneal fat (n=10). All parameters were statistically analyzed using the mixed model procedure, except for the liver weight which was analyzed using the non-parametric procedure. Different superscripts indicate significant differences ($P<0.05$) among diets. P-values of the fixed factors meat source (P_m), carbohydrate source (P_c) and their interaction term ($P_{m\times c}$) in the mixed model procedure, and P-values of the fixed factors dietary treatment (P_d), meat source (P_m), carbohydrate source (P_c) in the non-parametric procedure are highlighted in bold when $P< 0.05$.

Figure 2: Oxidation products in the diet (n=3 technical replicates), stomach content (n=10) and feces (n=5); thiobarbituric acid reactive substances (TBARS), 4-hydroxy-2-nonenal (4-HNE), hexanal (HEX). All parameters were statistically analyzed using the mixed model procedure. Error bars represent standard deviations. No statistical analysis was performed on the dietary oxidation products. Different superscripts indicate significant differences ($P<0.05$) among diets. P-values of the fixed factors meat source (P_m), carbohydrate source (P_c) and their interaction term ($P_{m\times c}$) are highlighted in bold when $P< 0.05$.

Figure 3: Oxidative parameters in plasma or red blood cells (RBC) following 2 weeks on the diets (n=10); malondialdehyde (MDA), 4-hydroxy-2-nonenal (4-HNE), hexanal (HEX), glutathione (GSH), oxidized glutathione (GSSG), and glutathione peroxidase (GSH-Px) activity. All parameters were statistically analyzed using the mixed model procedure, except for GSH-Px which was analyzed using the non-parametric procedure. P-values of the fixed factors meat source (P_m), carbohydrate source (P_c) and their interaction term ($P_{m\times c}$) in the mixed model procedure, and P-values of the fixed factors dietary treatment (P_d), meat source (P_m), carbohydrate source (P_c) in the non-parametric procedure are highlighted in bold when $P< 0.05$.

Figure 4: Composition of colonic bacterial community at the phylum (A) and family (B) level. Each bar represents an animal. Since the colon of three rats had no colonic contents at the moment of euthanasia, the number of rats in the group of chicken-starch, chicken-sucrose, beef-starch and beef-sucrose was 10, 9, 9, and 9, respectively.