Red and processed meat consumption versus chicken meat within two different dietary patterns: modulation of the colon microbial community and volatile metabolites

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Abstract

Diet has a major role in shaping the gut microbial community. Meat is an important component of many diets, however, studies investigating the effect of meat type and the interaction with other food components on the colonic microbial community and metabolites are scarce.

In present study, 32 pigs were fed red&processed meat or chicken meat within either a prudent or a Western dietary pattern for four weeks (2×2 full factorial design). After euthanasia, the colon microbial community was investigated by sequencing the V4-V5 region of 16S ribosomal RNA gene. In addition, the volatile organic compounds in colon contents were assessed using solid phase micro-extraction followed by GC-MS (either quantified or based on their presence/absence).

Characteristic bacterial groups could be identified using linear discriminant analysis effective size, with more differences between the dietary patterns than between the meat types. Amongst others, *Lactobacilli* were characteristic for the chicken × prudent dietary pattern treatment and *Paraprevotella* for the red&processed meat × prudent dietary pattern treatment. Enterobacteriaceae and *Desulfovibrio* were characteristic for the chicken × Western dietary pattern treatment and *Butyrivibrio* for the red&processed meat × Western dietary pattern treatment. Campylobacteraceae and *Clostridium XIVa* were characteristic for respectively chicken and red&processed meat intake, irrespective of the dietary pattern.

The concentration of butyrate was significantly higher when the prudent dietary pattern was given compared to the Western dietary pattern, but no differences for the volatile fatty acids, protein fermentation products, methane thiol and dimethyldisulfide were observed. Ethylene glycol was present in half of the pigs fed red&processed meat within a Western dietary pattern, whereas it was not observed in the other treatments. The volatiles 1-methylthio-propane and ethyl valerate were observed more frequently in pigs fed red&processed meat compared to chicken meat. The prevalence of 3-methylbutanal was >80% for pigs receiving a Western dietary pattern, whereas for the pigs fed a prudent dietary pattern the prevalence was <35%. These volatile metabolites could originate from the diet, however, it cannot be excluded that also biosynthesis by microorganisms or other reactions in the gut occurred.

1. Introduction

Understanding which metabolic effects are induced by meat consumption is important as, in observational epidemiological studies, high red and/or processed meat consumption has been associated with a higher risk to develop chronic diseases, such as colorectal cancer, cardiovascular diseases, and type 2 diabetes mellitus (Ekmekcioglu et al., 2018). Yet, until now there is insufficient knowledge regarding the responses and pathways that are involved in these associations (Ekmekcioglu et al., 2018). In addition, several epidemiological studies have shown that consumption of chicken is not associated with these diseases, however, no definite explanation has so far been found (Demeyer, Mertens, De Smet, & Ulens, 2016). Moreover and importantly, it is not clear to what extent the dietary pattern has impact on these findings. Since foods and diets have a complex composition, it is difficult to attribute health effects to one or a limited number of specific compounds (Jacobs, 2014). Consequently, we should not only focus on the role of individual compounds, but rather on the matrix and interaction effects. Most animal feeding studies investigating the effect of meat on health parameters used high levels of meat (Van Hecke et al., 2018) or focused on isolated meat compounds, such as heme-iron (Ijssennagger et al., 2015). Some studies used semi-purified diets designed to mimic the nutrient loads in

current Westernized diets, but potential biologically active whole foods are seldom included (Turner & Lloyd, 2017).

The dietary pattern has a major role in shaping the composition and activity of the gut microbial community and accumulating evidence supports a role for the gut microbial community in the development of several chronic diseases, including those associated with high red meat consumption (Knip & Siljander, 2016; Niederreiter, Adolph, & Tilg, 2018; Tang & Hazen, 2014). Despite the knowledge that exists on the effects of carbohydrates, there are still many unanswered questions on the impact of dietary fats and proteins on the gut microbial community (Conlon & Bird, 2015; Danneskiold-Samsøe et al., 2018). Although it is known that differences are present between the fecal microbial community of vegetarian versus omnivorous humans (Graf et al., 2015), insight about the effect of animal protein and fats is rather limited. Zhu et al. (2015) demonstrated differences between the cecal microbial community of rats fed diets based on poultry versus a diet containing pork and beef, with, among others, a higher abundance of *Lactobacillus* in rats fed poultry. Similarly, a higher abundance of *Lactobacillus* was observed in the colon of rats fed a diet containing chicken and starch compared to a diet high in beef and sucrose (Van Hecke et al., 2018). It has been hypothesized that meat, high in sulfur-containing amino acids, promotes the growth of sulfur-reducing bacteria (Vipperla & O'Keefe, 2013). Indeed, Desulfovibrionaceae were abundantly found in pigs fed a diet high in cooked red meat (30 %) and in rats receiving 65% beef and sucrose (Belobrajdic, King, Christophersen, & Bird, 2012; Van Hecke et al., 2018). Intriguingly, the opposing diets were respectively red meat with wheat fibre components and red or chicken meat with corn starch instead of sucrose, showing the importance of other food compounds in the diet. Yet, Le Leu et al. (2015) observed no alterations in certain selected fecal bacterial groups after a dietary intervention with healthy human volunteers consuming 300 g lean red meat per day for four weeks.

Fermentation of undigested foods in the colon results in the formation of several metabolites including volatile organic compounds. The assessment of these small endogenous molecules is important for metabolome analysis (Zhang & Davies, 2016) and are complementary with other

metabolomic techniques to assess the impact of nutrition (Vernocchi et al., 2012). The assessment of volatile metabolites has been proposed as one of the approaches for investigating the biochemical characterization of metabolic changes triggered by the gut microbial community and dietary variation (Di Cagno et al., 2011; Vitali et al., 2010). Many microbial volatile organic compounds have biogenic actions on mammalian cells, including cytotoxic and inflammatory effects (Thorn & Greenman, 2012). For example, protein fermentation yields intrinsically toxic compounds, such as phenol, cresol, indole and skatole, that affect the epithelial cell metabolism and barrier function. The bacterial metabolism of tryptophan in the colon results in the production of a wide range of indolic compounds, whereas phenolic compounds such as phenol and cresol are generated by the partial breakdown of tyrosine and phenylalanine by a wide range of intestinal obligate or facultative anaerobes (Evenepoel, Meijers, Bammens, & Verbeke, 2009). However, the effects of long-term exposure of the colonic mucosa to these metabolites remain unclear and it is possible that the impact of protein fermentation is overshadowed by other dietary or lifestyle factors (Windey, De Preter, & Verbeke, 2012). Next, volatile sulfur compounds are a family of gases that can be highly toxic to tissues. Yet, it has also been reported that some of these compounds, such as dimethyl sulfide, methanethiol and dimethyldisulfide, may be produced by methylation of hydrogen sulfide as a detoxification mechanism by mucosal enzymes (Garner et al., 2007). Likewise, carbon disulfide may be produced by carbonation of hydrogen sulfide as a detoxification mechanism exerted by colonic bacteria (Vitali et al., 2010). On the other hand, bacterial carbohydrate fermentation results in the generating of short-chain fatty acids, such as acetate, propionate, butyrate, which are known for their health-promoting effects on the colonic mucosa (Kau, Ahern, Griffin, Goodman, & Gordon, 2011).

The purpose of this study is to investigate the effect of meat, consumed in representative quantities and combined with existent dietary patterns, on the microbial community and colonic volatile organic compounds using a pig model. The pig is an omnivorous animal with comparable nutritional requirements to humans. Despite some anatomic differences, the physiology of digestion and associated metabolic processes are analogous between humans and pigs (Patterson et al., 2008). The pigs in present study received complex human diets in which chicken or fresh

and processed red meat was consumed in combination with a diet based on either a Western dietary pattern (high in refined sugars and fat, low in fibers and antioxidants) or a prudent dietary pattern, based on the Flemish dietary recommendations (low in refined sugars, high in fiber and antioxidants).

2. Materials & Methods

Animal trial

Briefly, 32 castrated male pigs (Topigs × Piétrain) of three weeks old and originating from eight litters (i.e. four pigs per litter) were purchased from a local farm and allocated to eight pens. Each dietary treatment was repeated in two pens ($n = 8$ pigs per treatment) and each treatment was given to one of the pigs of each litter. On arrival, all pigs were fed a commercial pre-starter diet *ad libitum* for one week. Thereafter, the dietary treatments were gradually introduced during one week, after which the pigs were subjected to the dietary treatments for four weeks. The pigs were weighed twice a week and the average body weight at the start of the experimental feeding period did not differ significantly among the dietary treatments (7.00 ± 0.88 kg). During the experimental feeding period, the pigs were fed fresh meals at room temperature three times a day (8, 12, 18 h) during 30 min *ad libitum*. An individual feeding system was used to record individual feed intake per meal. Water was always available *ad libitum*. Due to practical reasons, euthanasia and sampling was spread over four consecutive days. Each day, two pigs from each dietary treatment were slaughtered, starting with the heaviest pigs. Two hours after receiving the last meal, the pigs were euthanized by intramuscular injection of xylazine (20 mg/ml, 0.1 ml/kg body weight) and intraperitoneal injection of nembutal (60 mg/ml, 0.8 ml/kg body weight) followed by exsanguination. For this particular study, the large intestinal content was collected and homogenized, pH was measured and the samples were stored in aliquots at -80°C until further analysis. Eight animals were not included in the present trial (i.e. two, one, three and two pigs fed respectively chicken × prudent, red&processed × prudent, chicken × Western and red&processed × Western), because the colon digest was watery (less than 15 g dry matter/100 g colon contents), which could indicate that these pigs suffered from diarrhea. The experimental protocol was approved by the Ghent University Ethical Committee (EC 2016/26).

The four diets (Table 1) were formulated to provide similar daily intakes of metabolizable energy and an equal meat intake per day. More than 90 food items were used to compose the dietary patterns to mimic the diversity and nutrient levels of complex human diets. Items such as fruits, vegetables, bread, potatoes, vegetable oils, nuts, rice, pasta, cheese and milk were included in both dietary patterns and for the Western dietary pattern additionally chocolate, mayonnaise, lemonade, cakes and fries (among others) were included. The red meat contained 62% fresh meat (mainly pork and beef) and 38% processed red meat (mainly cooked ham, filet de sax, salami and smoked bacon), whereas the chicken meat consisted of thighs and breasts. A similar animal fat percentage was obtained by adding chicken skin to the chicken meat. A detailed list of food items and the crude composition of the four diets are reported in the supporting information 1.

Microbial community analysis

The DNA extraction was carried out as described by Vilchez-Vargas et al. (2013). The quality of the DNA extracts was validated by means of agarose gel electrophoresis and via PCR, using the protocol of Boon, De Windt, Verstraete, and Top (2002) with the bacterial primers P338 and P518r (Muyzer, De Waal, & Uitterlinden, 1993). 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 2. A table containing the relative abundance of different OTU (operational taxonomic units), together with their taxonomic assignments for each sample was generated (Supporting information 3). 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 database (Accession number XXXX). The total bacterial count in the colon was analyzed by real-time PCR, as previously described (De Vrieze et al., 2015).

Statistical analyses of the microbial community

The R Studio version 3.2.3. software (<http://www.r-project.org>, R Development Core Team, 2013) was used for statistical analysis. The packages phyloseq (McMurdie & Holmes, 2013) and vegan (Oksanen et al., 2016) were used for microbial community analysis. The community composition of the biological replicates was statistically compared by means of analysis of variance (ANOVA, aov function) to validate that the bacterial community did not significantly differ between biological replicates. Differences in order-based Hill's numbers (Hill, 1973) between the four treatments were defined via ANOVA and the post-hoc Dunn's Test of Multiple Comparisons (dunn.test package) with Benjamini-Hochberg correction. Separate non-metric distance scaling (NMDS) plots of the bacterial community data were created based on the (un)weighted Unifrac and Bray-Curtis distance measures. Significant differences in community composition between treatments were identified by means of pair-wise Permutational ANOVA (PERMANOVA) with Bonferroni correction, using the adonis function (vegan). The effect of the dietary treatments on the total bacterial counts in the colon were analyzed using one-way ANOVA. Results were found significantly different when $p < 0.05$.

Linear discriminant analysis (LDA) effective size (LEfSe) was performed using the online interface for the method, provided at <http://huttenhower.sph.harvard.edu/lefse/> (Segata et al., 2011). Analyses were performed at different taxonomic levels combining the corresponding OTU. The factorial Kruskal-Wallis test and the pairwise Wilcoxon test were run three times, to investigate the effect of 'dietary pattern', 'meat type' and 'dietary treatment' and the alpha-values among subclasses was set at 0.05. The non-negative threshold for the logarithmic LDA score for discriminative features was set on 2 and the strategy for multi-class analysis was set as 'all against all'.

Colonic volatile organic compounds analyses

The volatile organic compounds were analysed following Di Cagno et al. (2011) with minor modifications. The volatiles were extracted from the headspace of the colon samples using solid phase micro-extraction (SPME) with a carboxen-polydimethylsiloxane coated fiber (85 μm) and the Combi PAL autosampler (CTC Analytics, Zwingen, Switzerland). The fibre was conditioned prior to analysis following the instructions of the manufacturer. Before sampling, the fiber was

exposed to the GC inlet for 20 min for thermal desorption at 250°C. One gram of colonic sample was placed into 10 ml glass vials and 4-methyl-2-pentanone (final concentration of 2 µg/g colon) was added as the internal standard. The SPME fiber was exposed to each sample for 40 min at 50°C. The fiber was then inserted into the injection port of the GC for sample desorption for 20 min. As control, an empty glass vial was stored under similar conditions to the sample vials and analyzed using the same method. The GC-MS analyses were carried out on a Trace DSQ II (Thermo, Finnigan) that operated at 70 eV (EI+ mode) with Xcalibur software (version 1.4 SR1) for data acquisition and processing. A fused silica capillary column (SLB-IL60 column) of 30 m × 0.25 mm × 0.2 µm was used (Supelco, Bellefonte, PA, USA). The temperature program was: 40°C for 5 min, 4.5°C/min to 65°C for 1 min and 10°C/min to 250°C which was held for 10 min. Interface and ion source temperatures were 250 and 230°C, respectively. The mass-to-charge ratio interval was 35-350 a.m.u. at 3.0 scans per second. Injections were carried out in splitless mode and helium (1 ml/min) was used as carrier gas. Compounds were putatively identified by comparison with the National Institute of Standards and Technology (version 2005) mass spectral library followed by manual visual inspection and retention time matching of selected standards (ethanol, propanoic acid, methylphenol, acetic acid, nonanal, heptanal, 1-propanol, methyl acetate, ethyl acetate, 1-butanol). A dichotomous variable was assigned based on the presence and absence of the volatile compound: i.e. 1 if the volatile was present in both analytical replicates with a 50% or greater probability of a match to library standards and 0 if the volatile was absent in at least one analytical replicate.

Semi-quantitative data of three protein degradation products and two methylated sulfides (cresol, indole, x-methyl-indole, methanethiol and dimethyldisulfide, present in all pigs) were obtained by expressing the relative area of specific quantification ions (respectively m/z 108, 117, 130, 48 and 94) versus the internal standard area (quantification ion m/z 100).

Short chain fatty acids were quantified by gas chromatography (HP 7890A, Agilent Technologies, Diegem, Belgium) equipped with a FID detector and a Supelco Nukol capillary column (30 m × 0.25 mm × 0.25 µm, Sigma-Aldrich, Diegem, Belgium) using an established method of Gadeyne et al. (2016). Ammonia was determined spectrophotometrically (Gadeyne et al., 2016).

Statistical analysis of the colonic volatile organic compounds

For the (semi-)quantified metabolites, normality was checked by the Kolmogorov-Smirnov and Shapiro-Wilk tests and the homogeneity of variance was verified by the Levene's test. For all parameters, with exception of acetic acid and propionic acid, at least one of the conditions was not fulfilled. Therefore, the non-parametric Kruskal-Wallis test was used. The fixed effects 'treatment', 'diet' or 'meat' were analysed separately. The binomial data were analyzed using Firth's logistic regression at a confidence level of 95% with 'diet', 'meat' or 'treatment' as the fixed effect. Statistical analyses were performed using SPSS Statistics version 24 (IBM SPSS, Armonk, NY) with 'pig' considered the experimental unit. Results were found significantly different when $p < 0.05$.

3. Results

The average daily energy intake of the pigs did not differ significantly among the four treatments and the pigs consumed an equal amount of meat per day (Supporting information 4). The pH of the intestinal digest samples varied between 5.72 and 6.77, but did not differ significantly among the dietary treatments (data not shown).

The total bacterial counts in the colon varied between 7.87 and 9.26 log copies 16S rRNA gene/ μ l, with a mean ($\pm$ standard error of the mean) of 8.72 ($\pm$ 0.09). No significant differences among treatments were found for meat type and the interaction meat type $\times$ dietary pattern using ANOVA, whereas significantly higher bacterial counts were observed in the treatments having a prudent diet compared to those having a Western diet (respectively 8.88 and 8.53 log copies 16S rRNA gene/ μ l.).

After 16S rRNA sequencing, a total of 285332 usable raw reads were obtained from all samples with an average of 11888 ± 6856 (standard deviation) reads per sample. OTU were delineated at the 97% similarity level. The total number of OTU was 11422 with an average of 476 ± 101 (standard deviation) per sample.

No significant differences could be observed for H_0 and H_1 Hill diversity orders between the four treatments (Figure 1). The H_2 diversity of the pigs receiving the prudent diet with red and processed meat was significantly lower compared to the pigs receiving a Western diet with either

chicken or red and processed meat ($p = 0.033$ and 0.032 respectively). As shown in Figure 2, the clustering between the dietary patterns was more pronounced than between chicken and red and processed meat. A significant difference in community composition was observed between the treatment combining red and processed meat with a Western dietary pattern and the treatment combining red and processed meat with a prudent dietary pattern ($p = 0.002$, using PERMANOVA). No significant differences were observed among the other treatments.

At the phylum level, Firmicutes and Bacteroidetes were the most predominant phyla in all four treatments (Figure 3A), with their relative abundance varying among treatments between 15 and 74% for Firmicutes and 17 and 83% for Bacteroidetes. The phyla Proteobacteria, Tenericutes and Spirocheates were present to a lesser extent ($<5\%$). No significant differences at the phylum level were observed. The relative abundance of the colonic bacteria at the family level are presented in figure 3B and to identify bacteria that are characteristic for either the dietary pattern, meat type or whole dietary treatment, linear discriminant analysis effect size was applied (Figure 4).

As depicted in figure 4A, several differential features with an LDA score higher than 2.0 were observed between the prudent and Western dietary pattern. Listed in order of LDA score, higher relative abundances were observed for Prevotellaceae, *Prevotella*, *Butyrivibrio*, *Anaerovibrio*, Sutterellaceae, *Desulfovibrio*, Burkholderiales, Clostridiaceae_1, Betaproteobacteria, *Dorea*, Bacteroides, Bacteroidaceae, *Clostridium_sensu_stricto*, Enterobacteriales, *Escherichia/Shigella*, *Pyramidobacter*, Synergistales, Synergistaceae, Synergistia, Synergistetes and Enterobacteriaceae in the colon of animals fed a Western dietary pattern compared to the prudent dietary pattern. On the other hand, *Paraprevotella*, *Mitsuokella*, *Megasphaera* and *Dialister* were relatively more abundant in the colon of animals fed a prudent dietary pattern compared to the Western dietary pattern.

As can be observed in Figure 4B, *Campylobacter* and Campylobacteraceae were characteristic for chicken, whereas *Clostridium* cluster XIVa was found to be characteristic for red and processed meat.

Figure 4C presents the differential features for the four dietary treatments separately. *Butyrivibrio* was characteristic for the treatment combining red and processed meat with a Western dietary pattern. Regarding the treatment with chicken combined with a Western dietary

pattern, Enterobacteriaceae, Bacteroides, Bacteroidaceae, *Escherichia/Shigella*, Enterobacteriales, *Anaerobaculum*, and *Desulfovibrio* were characteristic. The treatment with red and processed meat combined with the prudent dietary pattern had significantly higher relative abundances for *Paraprevotella* compared to the others. The treatment with chicken and a prudent dietary pattern had higher relative abundances of Lactobacillales, Lactobacillaceae, *Lactobacillus*, Bacilli and *Mitsuokella* compared to the other three treatments. Note that the relative abundances of *Lactobacillus* were more than tenfold higher compared to those found for the treatment combining red and processed meat with a Western dietary pattern.

The quantified volatile organic compounds are presented in Table 2. The factor 'meat type' and 'treatment' had no effect (all $p > 0.05$), whereas for the dietary pattern significantly higher concentrations were only observed for butyrate with higher concentration when the prudent dietary pattern was fed compared to the Western dietary pattern.

In addition to the volatile compounds reported in Table 2, another 54 volatile organic compounds were detected (Table 3). Although some compounds were ubiquitously or commonly present, most of them were found infrequently. Compared to the prudent dietary pattern, phenol was significantly less common in pigs receiving a Western dietary pattern, whereas 3-methylbutanal was observed more frequently in the latter, regardless of the meat type. Ethyl valerate was detected significantly more in pigs fed red and processed meat compared to chicken, regardless of the dietary pattern. The compound 1-methylthio-propane was observed in certain pigs fed red and processed meat, whereas this compound was absent when chicken was fed. Ethylene glycol was present in half of the pigs fed red and processed meat within a Western dietary pattern, whereas it was not observed in the other three treatments.

4. Discussion

This study investigated the impact of meat type on the colon microbial community and volatile organic compounds within two different dietary patterns. A higher number of significant differences was found between the dietary patterns than between meat types. This outcome could be expected as the composition of the dietary patterns is more contrasting compared to the two animal protein sources. It was on the other hand rather surprising that the H₂ diversity was greater when a Western dietary pattern was fed, since a healthier dietary pattern is usually associated with a higher microbial community diversity (Doré & Blottière, 2015). Like in humans, the pigs' colon was dominated by a mix of Bacteroidetes and Firmicutes, however, the expected increase of Firmicutes with a prudent dietary pattern or an increase of Bacteroidetes due to the high fat content of the Western dietary pattern was not observed (Winglee & Fodor, 2015). In a Western dietary model, heme-iron supplementation increased the relative abundances of Bacteroidetes and decreased Firmicutes in colonic contents of mice (Ijssennagger et al., 2015), however this finding was also not observed in present study. Still, higher concentrations of butyrate, which is associated with individuals consuming a healthy, plant-rich diet (Kau et al., 2011), were measured in the pigs receiving a prudent dietary pattern.

Butyrate is produced predominantly by *Clostridium* clusters XIVa and IV of the phylum Firmicutes (Vipperla & O'Keefe, 2013), yet, it has also been reported that bacteria from the *Clostridium* cluster XIVa are significantly more abundant in feces of omnivores compared to vegetarians (Kabeerdoss, Devi, Mary, & Ramakrishna, 2012; Kim, Hwang, Park, & Bae, 2013). Present study demonstrates that (processed) red meat rather than chicken meat is responsible for this difference. *Butyrivibrio*, belonging to the *Clostridium* cluster XIVa (Devillard, McIntosh, Duncan, & Wallace, 2007), are also predominantly present in the rumen of beef cattle (Durso, Wells, & Kim, 2015) and *Campylobacter* is frequently found in poultry (Newell & Fearnley, 2003). Hence, it seems that the bacterial differences between the chicken and red and processed meat treatments originated directly from the meat source. Similarly, three bacteria associated with cheese and cured meats (*Lactococcus lactis*, *Pediococcus acidilactici* and *Staphylococcus*) became significantly more prevalent in human fecal samples after consumption of an animal-based diet (David et al.,

2014). However, it cannot be excluded that endogenous changes of the gut microbial community also occurred.

Interestingly, Lactobacillaceae were characteristic for a prudent dietary pattern combined with chicken, whereas the presence of this family was lower in the diet combining red and processed meat with a Western dietary pattern. Likewise, Van Hecke et al. (2018) reported that the *Lactobacillus* genus was the most discriminative for rats fed a diet containing chicken and starch compared to, amongst others, a diet with beef and sucrose. Zhu et al. (2015) found that *Lactobacillus* was higher in rats fed white meat proteins (chicken and fish) compared to red meat (beef and pork) or non-meat protein (soy and casein) groups. Reductions in *Lactobacillus* were also reported in different mouse studies fed high fat diets (Tachon, Lee, & Marco, 2014). Several species of *Lactobacillus* genus have been shown to play a role in the gut mucosal immune and barrier function (Miranda et al., 2018) and a greater abundance of *Lactobacillus* may reduce the antigen load from gut bacteria to the host and alleviate certain inflammation responses and metabolic syndromes (Zhu et al., 2015).

Prevotella are frequently associated with plant-rich diets (Ley, 2016), but in present study were found to be characteristic for the Western dietary pattern. Yet, *Prevotella* are commonly encountered in the Western gut microbiome (Johnson, Heaver, Walters, & Ley, 2017) and have also been linked with inflammatory conditions (Ley, 2016). *Paraprevotella*, characteristic for the prudent dietary pattern, has been recognized relatively recently and little is known about its clinical significance (Tan et al., 2018). It has been postulated that the shifts in microbial populations driven by fat and the associated increases in gut bacteria, such as *Escherichia coli* and Enterobacteriaceae (characteristic for present Western dietary pattern), are linked with poor health outcomes (Conlon & Bird, 2015). Of course, many characteristic bacterial groups identified in this study are commonly present in the intestinal tract and not *per se* harmful, which is why additional insight on the presence of specific metabolites are needed. For example, members of the Sutterellaceae family are frequently found in the intestinal tract of humans, but have also been reported to cause gastrointestinal infections (Morotomi, 2014). The phylum Synergistetes consists of anaerobic Gram-negative bacteria and although the significance of *Pyramidobacter* in fecal microbial community is yet unknown, the oral isolate *Pyramidobacter piscolens* is known to

produce hydrogen sulfide, which is implied in compromising gut barrier function (Lankelma et al., 2017). In addition, hydrogen sulfide may induce direct radical-associated DNA damage (Attene-Ramos, Wagner, Gaskins, & Plewa, 2007). Likewise, *Desulfovibrio* are known for their sulfate-reducing capacities, hereby producing hydrogen sulfide, which may subsequently break the mucus barrier, resulting in exposure of the epithelium to bacteria and toxins (Ijssennagger, van der Meer, & van Mil, 2016). In a previous study, red meat consumption has been associated with an outgrowth of *Desulfovibrio* in rats, with median relative abundancies of 23% (Van Hecke et al., 2018). In contrast, present study showed more than two-hundred fold lower abundancies and no alterations in the volatile sulfuric compounds. Also no distinction between meat types and, except for phenol, dietary pattern could be made in present study for the investigated protein degradation products. The diets consisted of comparable crude protein levels and the muscle tissues of poultry and mammals do not differ a lot in their amino acid patterns (Beach, Munks, & Robinson, 1943), which may be the reason why little effect of meat type and dietary pattern on the protein degradation products was observed.

According to Raman et al. (2013) there is a core group of volatile organic compounds, which reflect essential functions of the resident colonic microbial community irrespective of day-to-day dietary influences. Some volatiles may originate directly from dietary sources and variations in the remaining volatile organic compounds are most likely owing to individual dietary practices and distinctions of the microbial community. Most of the volatile organic compounds observed in present study have been described in the colon of humans (de Lacy Costello et al., 2014; De Preter, Van Staeyen, Esser, Rutgeerts, & Verbeke, 2009; el Manouni el Hassani et al., 2018; Garner et al., 2007; Raman et al., 2013). Therefore, only the compounds of which the prevalence significantly differed between treatments will be discussed here.

The organic ester ethyl valerate was more present when red and processed meat was consumed, regardless of the dietary pattern. Ester syntheses, using different alcohols and fatty acids as substrates, may be mediated by bacteria such as is present in *E. coli* or by enzymes derived from the intestinal mucosa (Garner et al., 2007). Ethyl valerate has been reported in feces of both healthy and unhealthy subjects (Garner et al., 2007; Raman et al., 2013; Saa et al., 2014). Studies

have demonstrated that fatty acid ethyl esters may be involved in organ injury (Garner et al., 2007) and more in general, ester volatile organic compounds may have injurious effects on barrier or local immune functions in the gut (Raman et al., 2013). However, no differences among the other esters were observed in present study and ethyl valerate is commonly present in fruits and approved as a flavour additive by the Joint FAO/WHO Expert Committee on Food additives (JECFA), so little harmful effect of this specific compound is expected. Likewise for 3-methylbutanal, 'no safety concern at current levels of intake when used as a flavouring agent' has been reported by JECFA. This aldehyde has been found mainly in the treatments with a Western dietary pattern in present study. It is a key flavour component in semi-hard cheeses and commonly found in (dry cured) meat (Ruiz, Ventanas, Cava, Andrés, & García, 1999). Studies have shown that aldehydes are produced during inflammatory processes as a result of lipid peroxidation and oxidative stress (Fritz, Petersen, & Medicine, 2013), but for 3-methylbutanal there is no certainty about its reactivity (Estévez, 2011). The compound 3-methylbutanal can either be formed by Strecker degradation of leucine or during cholesterol thermoxidation (Cardenia, Olivero, & Rodriguez-Estrada, 2015). Additionally, Cardenia et al. (2015) showed that 3-methylbutanal can be used as volatile marker of cholesterol oxidation in egg yolk, which is in accordance with the high cholesterol content of the Western dietary pattern. Cholesterol oxidation products are known to be potentially involved in the initiation and progression of major chronic diseases (including atherosclerosis, neurodegenerative processes, diabetes, kidney failure, and osteoporosis), as well as to be pro-inflammatory, pro-apoptotic, cytotoxic, carcinogenic and mutagenic (Otaegui-Arrazola, Menendez-Carreño, Ansorena, & Astiasarán, 2010). It is therefore not clear whether the occurrence of 3-methylbutanal is mainly a result of the dietary composition or because of reactions that occurred during digestion.

The compound 1-(methylthio)-propane, in present study characteristic for red and processed meat and especially when combined with a prudent dietary pattern, has not been evaluated by JECFA. It is found in dry cured loin and fresh beef as being a potent aroma compound originating from sulfur containing amino acids (Muriel, Antequera, Petrón, Andrés, & Ruiz, 2004; Raes et al., 2003) and *Microbacterium foliorum* was able to produce this compound during ripening in a cheese model medium (Deetae, Spinnler, Bonnarme, & Helinck, 2009). This fits with the

composition of the red and processed meat with a prudent dietary pattern containing fresh beef, dry cured meat products and considerable amounts of cheese. The compound 1-(methylthio)-propane has been detected in feces of healthy volunteers, but not in patients with ulcerative colitis, *Clostridium difficile* or *Campylobacter jejuni* infections (Garner et al., 2007). To the best of our knowledge, this compound has not yet been associated with specific bacteria in the colon or with the detoxification of hydrogen sulfide and its importance in this context should be further explored.

Ethylene glycol is a colorless, odourless and soluble chemical, mainly used in automobile anti-freeze, brake solution and as an industrial solvent. Metabolites of ethylene glycol formed in the liver and kidneys such as glycoaldehyde, glycolic acid, glyoxylic acid and oxalic acid are toxic compounds which can cause cardio-pulmonary failure, metabolic acidosis, central nervous system depression and kidney injury (Leth & Gregersen, 2005; Seo et al., 2012). Khattab (2007) showed histological changes in the cells of the gastric mucosa, including disruption of surface epithelium or mucosal erosions in rats administered a sublethal dose of ethylene glycol. Also, Aye et al. (2010) reported that ethylene glycol induces DNA strand-breaks and chromosome mutations in Chinese Hamster Ovary cell lines combined with an exogenous activation system. Furthermore, this compound has been reported in culture media and/or human specimens infected with *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Salmonella typhimurium* (Sohrabi, Zhang, Zhang, Ahmetagic, & Wei, 2014). As ethylene glycol was solely detected in the colon of pigs fed a Western dietary pattern combined with red and processed meat, it could be an important compound and deserves further investigation.

In conclusion, the consumption of either chicken or fresh and processed red meat combined with two different dietary patterns resulted in distinct modifications of the colon microbial community of the pigs. Characteristic bacterial groups could be identified, with more differences between the dietary patterns than between the meat types. Besides for butyrate, no effect of dietary treatments was observed for the quantified volatile organic compounds. Differences among treatments regarding the presence or absence of specific volatile organic compounds were rather

limited. Most of the volatiles could be traced back to a dietary source, however, also biosynthesis by microorganisms or other reactions in the gut may have occurred.

To what extent characteristic microbial groups and volatile metabolites originate directly from the diet or are influenced by the digestion process deserves further investigation.

Acknowledgements

E.V., T.V.H. and J.D.V. were supported as postdoctoral fellows from the Research Foundation Flanders (FWO Vlaanderen). This work was funded by the FWO research project G011615N and benefitted from a statistical consult with Ghent University FIRE (Fostering Innovative Research based on Evidence). The authors thank S. Coolsaet, D. Baeyens, C. Melis, T. Van Der Eecken, B. Vlaeminck and E. Claeys for their practical assistance during the preparation of the diets, sampling and laboratory analyses. J. Michiels is thanked for his assistance in the design of the animal trial.

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