THE EFFECT OF NITRITE-CURING OF CHICKEN, PORK AND BEEF ON OXIDATION AND NOC-SPECIFIC DNA ADDUCT FORMATION DURING *IN VITRO* DIGESTION

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Abstract – This study investigated the catalyzing effects of haem on the formation of oxidation products and N-nitroso compounds (NOCs) during digestion. Literature data on the role of nitrite-curing of meat in this mechanism are not univocal. For this purpose, uncured and nitrite-cured chicken, pork and beef were exposed to an *in vitro* digestion model simulating mouth, stomach, duodenum and colon. Three different fecal microbiota originating from three individuals were used to simulate colonic fermentation. Haem pigment and total Fe^{2+} were highest in beef, followed by pork and chicken. Following duodenum and colon digestion, uncured beef resulted in significantly higher formation of the fat oxidation product malondialdehyde (MDA) and protein carbonyl compounds (PCC). Digestion of nitrite-cured meats resulted in significantly lower fat and protein oxidation compared to its uncured equivalents. Detection of the NOC-specific DNA adduct O^6-Carboxy-Methylguanine (O^6-C-MeG) depended strongly on the investigated microbiota. Digestion of beef resulted in significantly higher O^6-C-MeG formation compared to pork and chicken. No significant influence of nitrite-curing on O^6-C-MeG could be observed.

Key Words – Haem, Health, Colon

I. INTRODUCTION

A significant epidemiologic association between high red meat consumption and colorectal cancer was reported in several meta-analyses while consumption of poultry was not [1,2]. Corpet [3] suggested that haem-Fe in meat has a catalytic effect on the formation of geno- and cytotoxic oxidation products and carcinogenic N-nitroso compounds (NOCs). Nitrite-curing of meat is suggested to increase NOC formation [3]. Therefore, the present study investigated the formation of these suggested harmful products during digestion of uncured and nitrite-cured chicken, pork and beef in an *in vitro* model. After duodenum and colon digestion, samples were collected and analyzed for the oxidation products MDA and PCC and the NOC-specific DNA adduct O^6-C-MeG.

II. MATERIALS AND METHODS

Fresh, lean meat samples were collected from the *m. Pectoralis profundus* from chicken, *m. Longissimus dorsi* from pig and *m. Biceps femoris* from beef. Subcutaneous pork fat from one batch was added to the sampled meat to a total fat content of 5% to exclude a possible confounding effect of a different fat profile. Nitrite salt (0.6% nitrite) was added at 20 g/kg meat, corresponding to an amount of 120 mg nitrite/kg meat. All meat samples were heated in a warm water bath for 15 minutes after the core temperature had reached 65°C. After manufacturing, all meat samples were minced, vacuum packed and stored at -20°C until start of the incubation.

For the *in vitro* digestion, the protocol described by Versantfoort *et al*. [4] was adapted by adding oxidants and antioxidants that are normally present in digestive juices. Hence, saliva also contained peroxidase [5] and $NaNO_2$ [6] while ascorbic acid [7], H_2O_2 [8] and $FeSO_4$ [8] were added to the gastric juice. During each incubation, 4 replicates of each meat sample (4.5g) were incubated 5 minutes with 6 ml saliva, 2 hours with 12 ml gastric juice, 2 hours with 2 ml bicarbonate buffer (1M), 12 ml duodenal juice and 6 ml bile. After duodenum digestion, 2 replicates of each meat sample were diluted with 44 ml H_2O to obtain the same solid/liquid ratio as in colon (see further). Duodenum samples were stored at -20°C in aliquots after homogenizing with an ultraturrax. The 2 remaining replicates of each meat sample entered colon digestion. SHIME medium (22 ml)

[9] and human fecal microbiota (22 ml) were added to the digesta. Closed vessels were flushed with N_2 for 30 minutes to guarantee an anaerobic environment. Consequently, vessels were incubated for 72 hours while stirring at 37°C. Each incubation run was repeated three times with microbiota originating from three different individuals. Undigested meat samples were obtained by homogenizing 4.5g meat in 82 ml H_2O, mimicking the liquid/solid ratio in duodenum and colon.

Concentrations of MDA in digesta were measured colorimetrically in accordance with Grotto *et al.* [10]. The measurement of PCC following their covalent reaction with 2,4-dinitrophenyl-hydrazine (DNPH) was done according to Ganhão *et al.* [11]. O^6-C-MeG was quantified by U-HPLC-MS/MS analysis after 182 µL of the filter sterilized sample was incubated for 18 hours at 37°C with 100 µg CT-DNA and internal standard (50 µL, 20 ng/ml O^6-D3-MeG) [12].

Data on MDA and PCC were analyzed per digestion stage using a linear mixed model with the fixed effects of meat type, nitrite and meat type × nitrite and the random effect of incubation run (SAS Enterprise Guide 5). Data on O^6-C-MeG were analyzed separately per run (microbiota) using a linear model with the fixed effects of meat type, nitrite and meat type × nitrite.

III. RESULTS AND DISCUSSION

Haem pigment and total Fe^{2+} were lowest in chicken (16,0 ± 0,4 and 4,3 ± 0,2 mg/kg respectively), followed by pork (31,7 ± 4,3; 5,5 ± 0,3) and beef (151,7 ± 1,5; 13,9 ± 1,9). Residual nitrite in cured meat samples were clearly lower when higher concentrations of haem pigment and total Fe^{2+} were present (84,5 (n=1); 36,4 ± 0,2 and 27,0 ± 1,2 mg/kg for beef, pork and chicken respectively), which may be explained by the reaction of nitrite with haem and Fe^{2+} to nitroso-haem and nitrosyl-Fe^{2+}.

MDA concentrations in uncured beef samples before digestion were significantly higher than for chicken and pork (Table 1). Since all meats were sampled fresh and MDA concentrations in fresh meat are generally negligible, the higher MDA concentrations in uncured beef must have been formed during processing (mincing, adding fat and heating). Undigested nitrite-cured meats showed significantly lower MDA concentrations due to the antioxidant properties of nitrite. After duodenum digestion, a clear increase in MDA was observed for all meat types in accordance to Hur *et al.* [13] who digested beef patties using a similar *in vitro* digestion model. After duodenum and colon digestion, uncured beef had significantly higher MDA values compared to pork and chicken. Digested cured meats had significantly lower MDA levels compared to their uncured equivalents. Adding nitrite (1g/l) in drinking water also significantly reduced lipid oxidation in colon of rats on a haem-diet [14].

High standard deviations for MDA values were found after colon digestion, which were related to high and variable MDA concentrations in the applied microbiota (5,0 ± 0,1, 17,0 ± 0,2 and 18,3 ± 1,1 nmol/ml for microbiota 1, 2 and 3 respectively). After subtraction of MDA originating from the microbiota, the MDA concentrations in colon were lower compared to duodenum. This might be explained by reaction between MDA and bacterial DNA, resulting in the formation of the MDA-specific DNA adduct M1dG [15].

Table 1: Malondialdehyde formation (nmol/ml digesta) in uncured and nitrite-cured chicken, pork and beef during *in vitro* digestion (mean ± SD)

	Uncured		Nitrite-cured	
Undigested				
Chicken	5.7 ± 0.2	b,x	2.8 ± 0.4	b,y
Pork	5.4 ± 0.0	b,x	2.0 ± 0.0	b,y
Beef	15.0 ± 0.9	a,x	7.4 ± 0.1	a,y
Duodenum				
Chicken	9.8 ± 1.8	c	8.7 ± 1.6	b
Pork	14.8 ± 2.0	b,x	9.2 ± 1.4	b,y
Beef	28.1 ± 1.4	a,x	11.0 ± 1.5	a,y
Colon				
Chicken	13.9 ± 7.3	c	12.8 ± 8.4	b
Pork	17.7 ± 9.3	b,x	13.0 ± 8.6	b,y
Beef	26.3 ± 10.2	a,x	15.3 ± 8.2	a,y

a,b,c = means for chicken, pork and beef with different superscripts are significantly different ($P < 0.05$); x,y = means for uncured and nitrite-cured with different superscripts are significantly different ($P < 0.05$)

Uncured beef before digestion had significantly higher PCC concentrations than chicken (Table 2). For beef, a clear increase in PCC was observed after duodenum digestion compared to uncured beef before digestion. This increase was less distinctive for chicken and pork. PCC of uncured beef was significantly higher than uncured chicken

and pork in duodenum and colon. Other authors reported higher PCC formation when myofibrils were incubated with higher concentrations of Fe^{2+} / H_2O_2 [16]. The higher concentrations of haem pigment and Fe^{2+} in beef could thus explain the higher PCC formation during digestion.

Cured meats before digestion had significantly lower PCC than uncured equivalents. PCC concentrations of cured meat before digestion were in accordance to Santé-Lhoutellier *et al.* [16], who reported 1.4 nmol DNPH/mg protein in non-oxidized myofibrils. Similarly, significantly lower protein oxidation was observed in cured meats after duodenum and colon digestion compared to uncured meats.

Table 2 Protein carbonyl compounds formation (nmol DNPH/mg protein) in uncured and nitrite-cured chicken, pork and beef during *in vitro* digestion (mean ± SD)

	Uncured	Nitrite-cured
Undigested		
Chicken	2.6 ± 0.3 ^{b,x}	1.1 ± 0.1 ^{y}
Pork	3.0 ± 0.6 ^{ab,x}	1.2 ± 0.3 ^{y}
Beef	3.5 ± 0.7 ^{a,x}	1.4 ± 0.4 ^{y}
Duodenum		
Chicken	2.8 ± 1.4 ^{b}	2.1 ± 0.5
Pork	3.3 ± 1.3 ^{b,x}	2.0 ± 0.6 ^{y}
Beef	6.6 ± 1.6 ^{a,x}	2.3 ± 0.2 ^{y}
Colon		
Chicken	4.7 ± 0.6 ^{b,x}	3.6 ± 0.6 ^{y}
Pork	4.6 ± 0.3 ^{b,x}	3.8 ± 0.5 ^{y}
Beef	5.7 ± 0.7 ^{a,x}	4.4 ± 0.4 ^{y}

a,b,c = means for chicken, pork and beef with different superscripts are significantly different ($P < 0.05$); x,y = means for uncured and nitrite-cured with different superscripts are significantly different ($P < 0.05$)

Digesta in the present study were fermented by microbiota originating from three different persons, resulting in a highly variable formation of the NOC-specific DNA adduct O^6-C-MeG. DNA adducts were not detected when microbiota 1 was used in colon fermentation. A low response was found for microbiota 2 and a high response for microbiota 3. Enzyme activity of microbiota affecting the mutagenicity of NOCs can greatly vary depending on the bacterial composition [17]. It is likely that differences in the microbial enzyme activity in the applied microbiota result in varying potency of microbiota to activate NOCs to its carcinogenic metabolites.

In both microbiota positive for O^6-C-MeG, digestion of beef led to significantly higher values than chicken (microbiota 2) or pork and chicken (microbiota 3). These findings are in agreement with Bingham *et al.* [18] who showed that consumption of high amounts of white meat did not influence fecal ATNC while a dose-response increase in ATNC was observed with consumption of red meat.

Curing of meats did not increase O^6-C-MeG formation in the present study. Previously, nitrite-curing of meat was suggested to increase endogenous NOC formation [3]. However, Joosen *et al.* [19] found that consumption of uncured and nitrite-cured meat had the same effect on endogenous nitrosation. Increased ATNC in the colon of rats was found when given a haem diet enriched by nitrite in drinking water [14]. However, these authors reported that nitrosyl-Fe was the main contributor of the increased ATNC, which is probably not associated with increased CRC risk.

Table 3 O^6-C-MeG formation (ng/ml digesta) in uncured and nitrite-cured chicken, pork and beef after colon fermentation (mean ± SD)

	Uncured	Nitrite-cured
Colon1	*nd*	*nd*
Colon2		
Chicken	10.8 ± 1.1 ^{b}	11.9 ± 0.0
Pork	13.3 ± 1.0 ^{a}	12.0 ± 0.5
Beef	14.4 ± 1.3 ^{a,x}	11.9 ± 0.8 ^{y}
Colon3		
Chicken	540 ± 39 ^{b}	558 ± 30 ^{b}
Pork	504 ± 15 ^{b,y}	620 ± 23 ^{b,x}
Beef	762 ± 6 ^{a}	760 ± 96 ^{a}

a,b,c = means for chicken, pork and beef with different superscripts are significantly different ($P < 0.05$); x,y = means for uncured and nitrite-cured with different superscripts are significantly different ($P < 0.05$)

IV. CONCLUSION

Our results confirm the catalyzing effect of haem on the formation of oxidation products and NOCs. However, the hypothesized stimulating effect of nitrite-curing on NOC formation was not supported. Nitrite-curing of meats significantly reduced fat and protein oxidation during digestion and no effect on NOC formation was observed.

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