**Title**: Cooking affects pork proteins *In vitro* rate of digestion due to different structural and chemical modifications

**Authors**: Bhaskar Mitra a; Lars Kristensen b; Rene Lametsch a; Jorge Ruiz-Carrascal a, c \*

*a Department of Food Science, Faculty of Science, University of Copenhagen, Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark*

*b Danish Meat Research Institute, Gregersensvej 9, 2630 Taastrup, Denmark*

c *Institute of Meat and Meat Products, University of Extremadura, Av. Ciencias s/n, 10003 Caceres, Spain*

\* Author to whom the correspondence should be sent:

Jorge Ruiz Carrascal, Department of Food Science, Faculty of Science, University of Copenhagen, Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark

Phone: +45 35 33 32 22

E-mail: jorgeruiz@food.ku.dk

**Abstract**

The effect of thermal processing on the in vitro digestibility of pork proteins was studied. Raw samples were considered the control group, while the thermal treatments included 58, 80, 98 and 160°C for 72 min, 118°C for 8 min and 58°C for 17 hours, resembling a range of different cooking procedures. Samples were subsequently subjected to pepsin digestion at pH 3.00 in the gastric phase followed by trypsin and α-chymotrypsin at pH 8.00 in the intestinal phase. Pork cooked at 58ºC for 72 min had a significantly higher pepsin digestibility rate than meat cooked at 80ºC or 160ºC. The trend was similar in the intestinal phase, with samples cooked at 58ºC for 72 min having enhanced digestion rate over other treatments after 120 min of digestion. A PLS model pointed out to an inverse relationship between *in vitro* proteolysis rate and variables like Maillard reaction compounds or protein structural changes.

**Keywords**: pork; protein oxidation; AGEs; protein structural modifications; *in vitro* digestibility; proteolysis rate.

**HIGHLIGHTS**

- In vitro protein digestibility in pork under different heat treatments was studied.

- Protein digestion rate decreased with higher cooking temperature and time

- Structural and chemical modifications of pork proteins were related to change in digestion rate

- Maillard reactions, protein aggregation and % beta sheet negatively affected protein digestion

1. **Introduction**

When meat proteins are subjected to various degrees of processing, they undergo changes in their structure and physico-chemical status (Mitra et al., 2017; Tornberg, 2005). With higher temperatures and longer times during heat treatment, meat might undergo oxidative processes leading to the formation of oxidative radicals (Gallego et al., 2015). Oxidative reactions lead to the formation of protein carbonyls, disulphide and dityrosine bridges, that could in turn accelerate mechanisms like fragmentation, polymerization and aggregation in actin, myosin heavy chains and sarcoplasmic proteins (Utrera, Morcuende, & Estévez, 2014; Yu, Morton, Clerens, & Dyer, 2017; Zhang, Xiao, & Ahn, 2013). Lipid and protein carbonyls, and also endogenous reducing sugars, like ribose, may promote the initiation of Maillard reactions, leading to the formation of compounds such as dicarbonyls or Strecker aldehydes, and eventually advanced glycation end products (AGEs) such as carboxymethylysine (Mitra, Lametsch, Greco, et al., 2018; Villaverde & Estévez, 2013). Such reactions might reduce protein extractability, solubility and enhance denaturation and aggregation (Soladoye et al., 2015).

Some of those modifications have been shown to influence digestibility, nutrient uptake, bio-value and functionality (Orlien et al., 2021; Promeyrat et al., 2011; Santé-Lhoutellier et al., 2008). In fact, recent studies have shown how casein processing modulates post-prandial plasma amino acid responses *in vivo* in humans (Trommelen et al., 2020), and beef cooking conditions have shown to affect ileal digestible amino acid content and digestible indispensable amino acid score (DIAAS) in growing pigs (Hodgkinson et al., 2018). While mild heating treatments might improve digestibility by partial unfolding of proteins (Zhang et al., 2020), strict heat regimes possibly can make dietary proteins less susceptible to enzyme proteolysis *via* formation of aggregates, crosslinking, change in digestive enzyme recognition sites or production of reactive carbonyl species (Filgueras et al., 2011; Morzel et al., 2006; Rysman et al., 2014; Singh et al., 2014). All these changes have been shown to affect to some extent proteolysis rate during in vitro digestibility tests (Orlien et al., 2021). This is in accordance with (Santé-Lhoutellier et al., 2008), who stated that a dramatic decrease in digestibility of myofibrillar fraction was noted when temperature-time combination was 100° C for 45 min opposed to a combination of 100° C for 5 min or 270° C for 1 min. Interestingly, other authors have suggested that protein digestibility is not influenced by cooking temperature, but their rate of digestion was altered (Bax et al., 2013). In this sense, a negative and highly significant correlation between *in vitro* pepsin activity and carbonyl formation in beef and no correlation between oxidative parameters and *in vitro* pancrease activity have been found (Santé-Lhoutellier et al., 2008). However, the specific conditions leading to a better digestion and absorption of muscle proteins are far from being clarified, and some authors have recently shown that cooked meat is better assimilated than raw meat in the elderly (Buffière et al., 2017). Moreover, it has not been fully elucidated to which level the extent and rate of *in vitro* pork protein digestion are caused either by Maillard reactions, protein oxidation or to changes in protein structure, in terms of proportion of 𝛼-helix, 𝛽-sheets or other random structures.

The same samples considered in this study were analyzed for structural, oxidative and Maillard modifications; discussions of such results as affected by thermal treatment have been published elsewhere (Mitra et al., 2017; Mitra, Lametsch, Akcan, et al., 2018; Mitra, Lametsch, Greco, et al., 2018). In summary, cooking time and temperature strongly influenced the occurrence of structural changes, such as the relative proportion of secondary structures (α-helix and β-sheet) present in meat proteins and also surface hydrophobicity and indicators of protein aggregation. Moreover, undergone chemical changes were also induced by the intensity of the thermal treatment, higher temperature and longer cooking times leading to more intense protein oxidation and higher levels of furosine or Nε-carboxymethyl lysine.

In the present paper authors aimed to address the impact of structural and chemical modifications on *in vitro* proteolysis rates in relation to cooking treatments. These *in vitro* models do offer an alternate approach in order to simulate digestion rates that cannot be measured by the complexity of in vivo systems. With respect to previous studies, a more complete approach was followed, including structural, oxidative, crosslinking and Maillard modifications, and the weight of each of those was ascertained through a multivariate method. Such results could provide knowledge and act as a guide in the future development of new meat formulations that have an enhanced digestibility and/or digestion rate.

1. **Materials and methods**
	1. *Chemicals (Analytical grade)*

Reagents used in this experiment are enlisted below: Phosphate buffered saline (PBS) and Sodium hydroxide (NaOH), gastric pepsin (porcine gastric mucosa, lyophilized powder, 3200-4500 U/mg), pancreatic trypsin (porcine pancreas, lyophilized powder, 1000-2000 U/mg), α-chymotrypsin (bovine pancreas, lyophilized powder, ≥ 40 U/mg), bile extract porcineand Trichloroacetic Acid were from Sigma Aldrich (Missouri, USA). Other chemicals like Sodium dodecyl sulphate (SDS) and DL-Dithiothreitol (DTT) were obtained from AppliChem GmbH, (Darmstadt, Germany). Urea (CH4N2O) and Hydrochloric Acid (HCl) were purchased from Merck (Darmstadt, Germany).

* 1. *Design of Experiments*

Seven female pigs weighing in between 83 to 86 kg were selected at slaughter. Alongside CO2 stunning and dressing operations, lean percentage of the carcass was measured, ranging between 59 and 63 %. After slaughtering, meat carcasses were chilled-stored at 4°C for 24 hours and pH was measured thereafter (5.5-5.6). Longissimus lumborum muscles from both sides of the carcass were selected. A total of 21 steaks of 2 cm thickness were chopped from each pig (11 from the left loin and 10 from the right), labelled and packed in vacuum bags (LogiCon EM-628824 -Vacuumpose 200 270 0,09 mm3, Kolding, Denmark) and kept at−80ºC. 3 randomly chosen steaks from each pig were assigned to each cooking method. Thus, the study design included 7 pigs 7 cooking methods x 3 steaks, for a total of 147 steaks. Sample replicates were then thawed and cooked.

* 1. *Thermal treatments*

Pork chops were cooked in various ways, trying to reflect the most common heat treatments and cooking methods for meat. Treatments were RAW (control), SV5872 (*sous vide* treatment at 58°C for 72 min in a thermostatic water bath), SV5817 (*sous vide* treatment at 58°C for 17 hours, in the same water bath), SV8072 (*sous vide* treatment at 80°C for 72 min, also in the same water bath), B9872 (braised vacuum packaged in simmering water at 98°C for 72 min), AC1188 (autoclave treatment at 118°C for 8 min, with an F-value = 2.56) and OV16072 (roasted in convective oven at 160°C for 72 min). Those chops to be cooked under 100°C, were coded and vacuum-packed in Cryovac CN 300 bags (Sealed Air Corporation, North Carolina, USA); those aiming for cooking at 118°C were put in LogiCon vacuum thermal resistant bags (EM-62890, Kolding, Denmark) while oven samples were roasted strictly without bags. Type T external probes (fitted to a Testo 176 T4 data logger) were attached to a dummy sample in each batch to track the time-temperature profile of the meat core and surface. After cooking, samples were submerged under ice cold water at 4°C. Replicates were then packed again, coded and stored at -80 °C freezer to avoid oxidation. These samples were analyzed for structural, oxidative and Maillard protein modifications, and results for those have been published elsewhere (Mitra et al., 2017; Mitra, Lametsch, Akcan, et al., 2018; Mitra, Lametsch, Greco, et al., 2018). In the present paper no results or discussion are devoted to how the cooking methods influenced such values, but only to their relationship with digestibility.

* 1. *Protein concentration*

Protein determination of steak samples was performed after they were measured at a wavelength of 280 nm (Mitra et al., 2017). With 20 mL of 0.01 M PBS (pH 7.4), 2 g meat were homogenized by Ultra Turrax T25 equipped with a S25N-18 G dispersing element (Ika Labortechnik, Staufen, Germany) for 30 sec at a speed of 20,500 rpm. Separate aliquots of 30 µL were taken in Eppendorf tubes, and 5 % (w/v) SDS (1148 µL) in 50mM Tris HCl (pH 8), Urea (8M, 20 µL) and DTT (1M, 2 µL) were added to achieve a dilution of 40 times. They were then vortexed and incubated at 80 °C for 30 min onto a microplate incubator (Provocell, Model No.PV-PVC-1, Esco technologies, Centurion, South Africa) prior to analysis. The absorbance of this solution at 280 nm was measured (SpectraMax i3x Multi-Mode Detection Platform, Molecular Devices, Inc., Silicon Valley, USA) and

used to estimate protein concentration.

* 1. *In vitro digestibility rate*
		1. *Sample preparation*

Protein digestibility studies were carried out according to the consensus method by (Minekus et al., 2014) with some adaptations in the protocol as previously explained in Kehlet et al., (2017). From each cooking treatment, 7 different samples (corresponding to 7 different animals, as explained in the experimental design) were run in duplicates. Frozen meat samples were minced with a coffee grinder for 30 secs, and 2 g were homogenized with an Ultra Turrax T25 in 20 mL of 0.01 M phosphate buffer (pH = 7.4) for 30 sec with a speed of 20,500 rpm. Sample protein concentration was measured as mentioned above and the solutions were further diluted to 1 mg/mL proteins.

* + 1. *Gastric Phase*

10 mL of the diluted meat homogenate were mixed with 7.5 mL of simulated gastric fluid (SGF Electrolyte Stock Solution) in a 50 mL glass beaker, and the mix was incubated in a water bath while stirred with a magnetic stirrer. All the components in the reaction vessel were preheated to 37° C so that it could resemble the normal body temperature from the very first moment. To this mixture, 5 µL of 0.3 M CaCl2, 0.2 mL of 1 M HCl and 0.695 µL of Millipore water were added to balance the ionic conductivity and resonate with the micro-environment conditions in the stomach lining. The pH was adjusted to 3.00 for the activation of pepsin, and this was checked regularly (aprox. every 2 mins). 1.6 mL of gastric pepsin (2000 U/mL, EC 3.4.23.1) was then added to achieve a final volume of 20 mL. After the initiation of the reaction, termination and protein precipitation procedures were evaluated according to (Bax et al., 2012; Sun et al., 2011) with subtle adjustments. The progression of protein digestion was checked at various time points: 0, 10, 20, 30 and 60 min. (0 point here means the time immediately after addition of the enzyme). An aliquot of 400 µL of the digestion mixture was taken from each reaction vessel at each time point and was immediately mixed with 800 µL of 20 % trichloroacetic acid (TCA, 1.22 N). Samples were always placed on ice during the termination reaction. After centrifugation at 10.000g for 15 min at 4° C, 200 µL of the supernatant, containing the hydrolyzed peptide fraction, was taken and pipetted into a quartz microtiter plate. The hydrolyzed peptide content expressed as optical density (OD) was measured at 280 nm by using SpectraMax i3x Platform (Molecular Devices, Inc., Danaher Corporation, Sunnyvale, CA, USA). Initial optical density (ODg0), proteolytic activity measured as the OD at different digestion times (ODg10, ODg20, ODg30, ODg60 corresponding to 10, 20, 30, 60 min and 50% proteolytic degradation, respectively) and the rate of proteolysis, expressed in optical density unites by hour (∆OD/h) at different digestion times (∆ODg10/h, ∆ODg20/h, ∆ODg30/h, ∆ODg60/h corresponding to 10, 20, 30, 60 min, respectively), were assessed as explained elsewhere (Bax et al., 2012). All the sample and instrument analyses were performed in duplicate.

* + 1. *Intestinal Phase*

From the remaining volume in the reaction vessel after the gastric phase, 10 mL of acidic chyme was mixed with 4.25 mL of simulated intestinal fluid (SIF Electrolyte Stock Solution) in a 50 mL beaker and put on a magnetic stirrer into the thermostatic water bath at 37° C. To this gastric chime, 1.25 mL of fresh bile (160mM), 20 µL of 0.3 M CaCl2, 40 µL of 1 M NaOH and 0.695 µL of Millipore water were added to impersonate the micro conditions in the small intestinal lining as the food passes from the stomach to the gastrointestinal duct (duodenum). The pH was fixed continuously to reach 8.00 for the activation of pancreatic trypsin and α-chymotrypsin. 2.5 mL of trypsin (100 U/mL, EC 3.4.21.4) and 1.25 mL of α-chymotrypsin (25 U/mL, EC 3.4.21.1) were then added simultaneously to make a final volume of 20 mL. Digestion was stopped by addition of 20 % trichloroacetic acid (TCA, 1.22 N) at various time points 0, 10, 20, 30, 60 and 120 min. Similarly to gastric phase, 400 µL of the sample were taken and added to 800 µL of 20 % TCA that were already kept in Eppendorf tubes which were placed on ice, and further vortexed for some seconds. Tubes were centrifuged subsequently at 10.000g for 15 min at 4° C and 200 µL of supernatant were estimated at 280 nm. Initial optical density (ODi0), proteolytic activity measured as the OD at different digestion times (ODi10, ODi20, ODi30, ODi60, ODi120 and ODi1/2 corresponding to 10, 20, 30, 60, 120 min and 50% proteolytic degradation, respectively) and the rate of proteolysis, expressed in optical density unites by hour (∆OD/h) at different digestion times (∆ODi10/h, ∆ODi20/h, ∆ODi30/h, ∆ODi60/h, ∆ODi120/h corresponding to 10, 20, 30, 60 and 120 min, respectively), were assessed as explained above. All the sample and instrument analyses have been performed in duplicate.

* 1. *Statistical Analysis*

Data were analyzed using a one-way ANOVA with the SPSS 22.0 package (SPSS 22.0, IBM, USA). A general linear model was used to evaluate the effect of cooking treatment on the different in vitro digestibility variables, with the cooking treatment as the fixed effect and animals as a random term. When the effect was significant (*P* < 0.05), Tukey's test was used at 5% level to make pair wise comparisons between sample means. All the values represented were denoted as mean ± standard error.

A partial least squares model (PLS) was also constructed to evaluate the connection between structural and chemical modifications to various proteolysis rates. The PLS model was generated by using LatentiX V 2.12 (Latent5, Copenhagen, Denmark). Two matrices were created namely Y being the dependent one and X being the independent one. While X contained 49 samples x 20 variables, Y had 49 samples x 9 variables. In short, the Y matrix comprised all the proteolysis rates for both the phases under different time points and the X matrix contained all structural and chemical modifications that were previously studied (Mitra et al., 2017; Mitra, Lametsch, Akcan, et al., 2018; Mitra, Lametsch, Greco, et al., 2018) and are listed and explained in Table 1. Both X and Y were auto-scaled and PLS model was calibrated.

1. **Results and discussion**
	1. *Gastric phase protein digestibility*

The effect of cooking treatments on the digestibility parameters during the gastric phase were studied and the digestion parameters with mean values and standard deviation for gastric phase are enlisted in Table 2. Previous studies had shown that heat treatment of meat proteins affects the structural and functional attributes either by denaturation or by subsequent cleavage, and these changes seems to influence digestibility (Sørensen & Bukhave, 2010; M. Zhang et al., 2020). In our study, OD at time 0 for all the sample groups was similar, except for, SV8072 and OV16072 groups that showed significantly lower in-vitro degradation of muscle proteins than the AC1188 group, perhaps due to the shorter treatment time duration of 8 min in these later autoclaved samples, although these differences might also be a consequence of protein degradation prior to the digestibility experiment.

Similar values for all samples were also identified in OD after 10min, except for the fact that SV5872 showed a significantly higher proteolytic activity than SV8072 and OV16072 groups. A possible explanation for this fact could be the slight increase in hydrophobicity in SV5872, which might enable a faster proteolytic activity compared to the other two groups, in which due to the more intense heat treatment, aggregation mechanisms triggered, thereby leading to higher particle size, more beta sheet interaction and possibly an impairment in accessibility by the gastrointestinal enzymes (Mitra et al., 2017). A very similar observation also holds true for ODg20, where SV5872 group, with lower cooking temperature, favored higher proteolytic susceptibility. Between SV5872 and SV5817 groups, no differences were noted, suggesting that digestion rate and extent of digestion in pork proteins could be mainly influenced by increasing thermal treatments, rather than by longer cooking periods. Here we also noticed that SV5872 samples had higher degradation than B9872 group. This is also in accordance with Li et al., (2017), who detected a decrease in pepsin digestibility of stewed pork, probably because of higher protein oxidation and initiation of aggregation resulting in modification of cleavage sites, hence a delay or false recognition in accessibility by digestion enzymes. In conjugation with the above report, it is also seen that for stewed pork samples, band intensities were lower than in raw samples via SDS-PAGE even before digestion and it could be because of formation of cross-links and polymerization induced by higher temperature and longer time cooking (Li et al., 2017). For ODg30, low temperature- short time cooking still showed higher activity than RAW and OV16072, hence indicating that with slight protein unfolding proteolysis could be enhanced. As per as ODg60 and ODg1/2 is concerned, only RAW group had lower activity than all the other treatment samples indicating lower accessibility of enzymes. But at maximal time of digestion, nearly all values were similar which tells us that it is mainly digestion rate what seems to be affected by the heating treatment, but with increasing digestion time, enzymes finally make themselves accessible to nearly all protein conformations. According to reports stated by Kondjoyan, Daudin, & Santé-Lhoutellier (2015), highest digestibility was found at the termination point of 4 h and at a heating temperature of 70 to 90° C and this is a bit inconsistent with the results we obtained. Additionally, when residence time dropped from 4 h to 2 h, raw meat was most digestible, followed by sample subjected to 50 and then other treatment groups with higher heat treatment (Kondjoyan et al., 2015). This also indicates that bioavailability probably depends on the residence time in the gastric phase.

Rate of proteolysis during pepsin digestion was also taken into further consideration (Table 2). In general, throughout all digestion, samples cooked at 58ºC for 72 min showed the highest digestion rates, although values were not significantly different to those cooked at the same temperature for 17h. On the other hand, samples cooked in the harshest conditions (oven at 160ºC for 72 min) or raw ones, tended to show the lowest digestion rates. More specifically, at ∆ODg10/h, only SV5872 had a significantly higher activity than SV8072, with all other groups not showing significant differences to either of these two. For ∆ODg20/h, as we go from SV5872 to other treatment groups, it is quite clear that rate of proteolysis is indeed enhanced compared to all other samples except for SV5817. As in for ∆ODg30/h, situation still remains the same with some groups like SV8072 and B9872 behaving almost similarly with SV5872. With respect to ∆ODg60/h, RAW and OV16072 groups were still significantly lower than other treatment groups which possibly implies a deterioration in the nutritional quality of meat proteins. A similar tendency has been recently found in different types of meat and aquatic dietary proteins cooked in different ways by Luo, Taylor, Nebl, Ng, & Bennett (2018). Therefore, considering these trends, it seems that cooking at low temperature could influence higher proteolysis rate, which in turn could influence appetite regulation, satiety, curbing hunger issues and faster absorption of amino acids in the tract for muscle maintenance. Such derivations might hold true as faster absorption of amino acids potentially can modify aminoacidemia response thereby leading to more rapid satiety response (Kehlet et al., 2017).

* 1. *Protein digestibility in small intestine*

The digestion parameters for the experimental groups of pork cooked under different heating regimes during the intestinal phase are enlisted in Table 3. For ODi0, RAW and SV5872 groups had significantly lower activity values than others and this possibly could be an artefact while transferring from gastric phase to intestinal phase. For ODi10, ODi20, ODi30, ODi60, ODi120 and ODi1/2, values for proteolytic activity were similar in all groups and had no significant differences in between them. This could be a result of the fact that most proteins were already hydrolyzed by pepsin during the gastric digestion, and as a result the trypsin-chymotrypsin enzyme combination had very little to cleave. This was also in accordance with Laguna, Picouet, Guàrdia, Renard, & Sarkar (2017) who reported similar findings. In fact, the aforementioned outcomes were also very similar to ∆ODi10/h, ∆ODi20/h, ∆ODi30/h and ∆ODi60/h proteolytic rates. Surprisingly for ∆ODi120/h, we could detect that SV5872 group had a significantly higher proteolytic rate than OV16072 samples, in line with what was shown for digestibility rates during the gastric phase. This makes sense considering the partially unfolded protein structure in SV5872 compared with the aggregated one in OV16072 (Mitra et al., 2017). While during the intestinal digestion differences were not significant, the trend was similar throughout all sampling times. So, globally, it seems there is a trend to a faster digestion when pork is cooked to lower temperatures, while harsh cooking conditions, as in the oven at 160ºC would lead to slower digestion rates.

In our experiment, samples were homogenized using an ultraturrax before starting the digestion process (see Material & Method section). This leads to a major disruption of muscle structure, much more intense than it occurs during chewing. This might have influenced the overall digestion process, eliminating the potential effect of meat structure on digestion rate. In fact, other authors have shown a different rate of digestion for different meat proteins depending on the chewing capacity (Rémond et al., 2007) and

* 1. *Influence of various factors on in vitro protein digestibility*

In order to address in which extent different factors related to the chemical and structural modification of pork proteins may affect their rate of *in vitro* digestibility, a PLS model was built with Y dependent matrix comprising gastric and intestinal proteolysis rates for all tested cooking conditions and X matrix including variables related to structural and chemical modifications of pork proteins, which analyses have been previously published elsewhere (Mitra et al., 2017; Mitra, Lametsch, Akcan, et al., 2018; Mitra, Lametsch, Greco, et al., 2018). PLS scores and loading plots are shown in Fig 1. It is interesting to mention that the thermal treatment conditions considered in these three papers conduced to strong modifications in measured variables, and thus, to wide ranges of variations for all them, enabling the study of the potential relationships between such variables and digestion rate.

In the score plot (Fig 1.A), there was a clear distribution of the different batches according to the heat treatment. Raw samples were placed in the positive values of the PC2 while most of the different heat treated samples were in the negative area of this PC. Moreover, the different cooked samples were distributed along the PC2 with a clear distribution: samples cooked in the harshest conditions (roasted in the oven at 160ºC for 72 minutes) showed the lowest negative values in the PC1, followed by those autoclaved at 118ºC for 8 min, then those braised at 98ºC for 72 min, then those sous-vide cooked at 80ºC for 72 min (mostly in the positive values of PC1), and then those sous-vide cooked at 58ºC for 17h, with sous-vide cooked samples at 58ºC for 72 min showing the highest positive values in the PC1. That is, from higher to lower temperature along the PC1.

If this score plot is superimposed on the loading plot (Fig. 1B), samples cooked at lower temperature are placed with higher values for intestinal and gastric *in vitro* digestion rates, while those cooked at harshest condition are closer to variables indicating protein aggregation (span and SSA), variables related to protein structural modifications (percentage of beta sheet and ratio between beta sheet and alpha helix assessed through Raman spectroscopy) and variables related to formation of AGEs (furosine and CML levels).

If we focus on the scoreplot itself, it seems obvious an inverse relationship of gastric and intestinal in vitro proteolysis rate of pork proteins with chemical modifications such as Maillard type compounds (Furosine and CML), with structural modification ratio (β/α or proportion of β sheet) and with particle and protein aggregation indicators (specific surface area –SSA- and Span). In first place, this points out to a negative influence of the development of Maillard reactions on digestibility rates. Such occurrence could be ascribed to the modification of protein active sites for proteolytic enzymes (Lapolla et al., 2004), but also to the induction of crosslinking through either Maillard reactions or other mechanisms (i.e.: formation of protein radicals), leading to aggregation (Zhao et al., 2019). Similarly to our study, impaired digestibility of milk proteins in infant formula as a consequence of Maillard reactions has been recently evidenced (Zenker et al., 2020). This negative influence of formation of AGEs on protein digestion could lead to unabsorbed digest reaching the large intestine, which could further have an influence on gut microbiota (Corzo-Martínez et al., 2013)

With reference to β/α ratio, we already know that more the beta sheets, higher the aggregations as well (P. Yu, 2005), hence lower accessibility by gastrointestinal enzymes (Calabrò & Magazù, 2012). This is also further supported by the denaturation pattern of the protein in relation to the time-temperature combinations as explained by Mitra et al., (2017). Surprisingly and contrary to previous studies (Sante-Lhoutellier et al., 2007), protein oxidation levels did not have that much of an impact on neither gastric rates nor intestinal rates. Additionally, protein crosslinks like LAN and all the particle size parameters also had a negligible influence on the digestibility rates.

As far as our knowledge, this is the first study in which all these types of chemical and structural modifications are considered together for addressing their potential influence on *in vitro* protein digestion rate. Much of previous research tried to address this kind of modifications separately. In fact, a number of studies have previously ascertained how protein oxidative modifications are behind the detrimental effect of meat cooking and/or processing on *in vitro* protein digestion rate values (Sante-Lhoutellier et al., 2007). Our study does not exactly contradict this kind of relationship but put some perspective in the vast number of changes affecting proteins during meat cooking. In fact, some of the connections found in our study between particle size and digestion rates could potentially be also related to protein radical formation (Santé-Lhoutellier et al., 2008).

1. **Conclusion**

In view of the results of the present study, it seems that there is a negative influence of cooking temperature on pork proteins digestion rates. Thar way, low temperature cooking treatment appears as an interesting strategy to improve pork protein functionality. These findings perhaps will have implications in designing new process parameters and in formulating novel meat products for elderlies who have less digestive power.

Among the factors related to heat treatment that may affect *in vitro* pork protein digestion rates, it seems that the degree of protein aggregation, the intensity of protein structural changes and the development of protein glycation reactions are the more detrimental ones.

**Acknowledgements**

The Danish Pig Levy Fund and University of Copenhagen are being thanked for funding this project as part of a doctoral thesis. Our sincerest appreciation for Ms. Linda de Sparra, senior lab technician, for her assistance in carrying out daily laboratory activities.

The authors declare no conflict of interest.

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Table 1.- Summary of measurements used for the PLS analysis in the same samples considered in this study, published in previous publications (A: Mitra et al., 2017b; B: Mitra, Lametsch, Akcan, et al., 2018; C: Mitra, Lametsch, Greco, et al., 2018a).

|  |  |  |  |
| --- | --- | --- | --- |
| Parameter | Type of modification | Meaning (and methodology) | Reference |
| (D4, 3) | 1 | Mean diameter in volume (light scattering) | A |
| (D3,2) | 1 | Mean diameter in surface (light scattering) | A |
| D (v, 0.1) | 1 | Threshold upper size for 10 % of sample particles (light scattering) | A |
| D (v, 0.5) | 1 | Threshold upper size for 50 % of sample particles (light scattering) | A |
| D (v, 0.9) | 1 | Threshold upper size for 90 % of sample particles (light scattering) | A |
| Span | 1 | Dispersion Index (light scattering) | A |
| SSA | 1 | Specific Surface Area (light scattering) | A |
| TRYP | 2 | Tryptophan content (fluorescence spectra) | B |
| FPO | 2 | Protein oxidation products (fluorescence spectra) | B |
| AAS | 2 | Alpha aminoadipic semialdehyde (HPLC) | B |
| GGS | 2 | Gamma-glutamic semialdehyde (HPLC) | B |
| BPB | 3 | Bromo phenol blue (light spectroscopy) | A |
| CL Perc | 4 | Cooking loss (%) | A |
| Thiols | 2 | Protein thiol groups (light spectroscopy) | B |
| Alpha Per  | 3 | % of Alpha helix (FTIR) | A |
| Beta Perc | 3 | % of Beta sheet (FTIR) | A |
| Beta/alph | 3 | Beta/alpha ratio (FTIR) | A |
| Furosine  | 5 | Furosine (HPLC) | C |
| CML | 5 | Nε-carboxymethyl lysine (HPLC/MS) | C |
| LAN | 5 | Lanthionine (HPLC/MS) | C |

1: Particle aggregation and size distribution

2: Protein oxidation

3: Protein structure

4: Technological

5: Protein glycation

Table 2. Digestion parameters for Gastric Phase - ODg0 (Initial optical density: see Material & methods section), ODg10, ODg20, ODg30, ODg60 (ODgmax) and ODg1/2 represents proteolytic activity at 0, 10, 20, 30, 60 min and 50% proteolytic degradation reached respectively. ∆ODg0/h, ∆ODg10/h, ∆ODg20/h, ∆ODg30/h and ∆ODg60/h denotes rate of digestion at 0, 10, 20, 30 and 60 min respectively. Data shown is represented as mean ± standard error. Significant differences in the Tukey’s test (*P* <0.05) are ascribed with different letters.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Parameters | RAW | Sous vide58ºC72 min | Sous vide80ºC72 min | Braised98ºC72 min | Autoclave118ºC8 min | Oven160ºC72 min | Sous vide58ºC17 hours |
|  |  |  |  |  |  |  |  |
| ODg0  | 0.016±0.003ab | 0.019±0.003ab | 0.012±0.002b | 0.016±0.001ab | 0.027±0.003a | 0.013±0.003b | 0.015±0.004ab |
| ODg10 | 0.042±0.008ab | 0.053±0.003a | 0.022±0.005b | 0.039±0.003ab | 0.043±0.006ab | 0.028±0.004b | 0.039±0.007ab |
| ODg20 | 0.044±0.005b | 0.078±0.005a | 0.045±0.005b | 0.048±0.003b | 0.060±0.006ab | 0.040±0.003b | 0.058±0.008ab |
| ODg30 | 0.044±0.005b | 0.079±0.006a | 0.055±0.006ab | 0.059±0.004ab | 0.063±0.006ab | 0.042±0.002b | 0.059±0.008ab |
| ODg60 | 0.047±0.004c | 0.083±0.004ab | 0.069±0.006abc | 0.077±0.005ab | 0.087±0.009a | 0.057±0.003bc | 0.070±0.009abc |
| ODg1/2 | 0.024±0.002c | 0.041±0.002ab | 0.035±0.003abc | 0.039±0.002ab | 0.043±0.004a | 0.029±0.002bc | 0.035±0.005abc |
| ∆ODg0/h | ---- | ---- | ---- | ---- | ---- | ---- | ---- |
| ∆ODg10/h | 0.158±0.040ab | 0.205±0.008a | 0.060±0.032b | 0.142±0.014ab | 0.100±0.022ab | 0.094±0.024ab | 0.143±0.025ab |
| ∆ODg20/h | 0.084±0.011b | 0.174±0.009a | 0.099±0.014b | 0.096±0.010b | 0.098±0.012b | 0.081±0.009b | 0.128±0.018ab |
| ∆ODg30/h | 0.057±0.006b | 0.119±0.009a | 0.086±0.011ab | 0.087±0.008ab | 0.074±0.008b | 0.059±0.005b | 0.087±0.011ab |
| ∆ODg60/h | 0.031±0.003b | 0.063±0.004a | 0.057±0.006a | 0.062±0.005a | 0.060±0.006a | 0.045±0.002b | 0.055±0.007a |

Table 3. Digestion parameters for Intestinal Phase - ODi0 (Initial OD), ODi10, ODi20, ODi30, ODi60, ODi120 (ODimax) and ODi1/2 represents proteolytic activity at 0, 10, 20, 30, 60, 120 min and 50 percent proteolytic degradation reached respectively. ∆ODi0/h, ∆ODi10/h, ∆ODi20/h, ∆ODi30/h, ∆ODi60/h and ∆ODi120/h denotes rate of digestion at 0, 10, 20, 30, 60 and 120 min respectively. Data shown is represented as mean ± standard error. Significant differences in the Tukey’s test (*P* <0.05) are ascribed with different letters.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Parameters | RAW | Sous vide58ºC72 min | Sous vide80ºC72 min | Braised98ºC72 min | Autoclave118ºC8 min | Oven160ºC72 min | Sous vide58ºC17 hours |
|  |  |  |  |  |  |  |  |
| ODi0 | 0.202±0.006c | 0.210±0.002c | 0.228±0.005a | 0.208±0.005bc | 0.227±0.003ab | 0.221±0.004ab | 0.216±0.005abc |
| ODi10 | 0.225±0.007 | 0.245±0.006 | 0.242±0.006 | 0.232±0.005 | 0.246±0.004 | 0.249±0.005 | 0.237±0.011 |
| ODi20 | 0.236±0.005b | 0.249±0.003ab | 0.256±0.006ab | 0.238±0.005b | 0.261±0.004a | 0.254±0.005ab | 0.241±0.005ab |
| ODi30 | 0.245±0.004b | 0.261±0.007ab | 0.268±0.006ab | 0.260±0.009ab | 0.272±0.005a | 0.255±0.005ab | 0.267±0.005ab |
| ODi60 | 0.257±0.008b | 0.277±0.005ab | 0.291±0.004a | 0.278±0.005ab | 0.291±0.009a | 0.283±0.006ab | 0.282±0.007ab |
| ODi120 | 0.280±0.005 | 0.312±0.007 | 0.305±0.008 | 0.287±0.009 | 0.303±0.009 | 0.283±0.008 | 0.300±0.007 |
| ODi1/2 | 0.140±0.002 | 0.157±0.003 | 0.153±0.004 | 0.143±0.004 | 0.152±0.004 | 0.142±0.004 | 0.150±0.003 |
| ∆ODi0/h | ---- | ---- | ---- | ---- | ---- | ---- | ---- |
| ∆ODi10/h | 0.137±0.039 | 0.212±0.040 | 0.099±0.037 | 0.145±0.029 | 0.118±0.019 | 0.167±0.026 | 0.150±0.051 |
| ∆ODi20/h | 0.101±0.022 | 0.117±0.013 | 0.084±0.018 | 0.089±0.013 | 0.104±0.005 | 0.101±0.014 | 0.076±0.011 |
| ∆ODi30/h | 0.087±0.015 | 0.102±0.010 | 0.078±0.011 | 0.104±0.018 | 0.091±0.011 | 0.069±0.009 | 0.102±0.009 |
| ∆ODi60/h | 0.055±0.010 | 0.067±0.004 | 0.063±0.006 | 0.069±0.003 | 0.065±0.010 | 0.063±0.005 | 0.066±0.007 |
| ∆ODi120/h | 0.039±0.002ab | 0.051±0.003a | 0.038±0.005ab | 0.039±0.003ab | 0.038±0.004ab | 0.031±0.004b | 0.042±0.003ab |

Figure 1. Score plot (A) and loading plot (B) of a PLS Model showing the relationship between chemical and structural modifications to *in vitro* rate of digestion.



Protein structural and chemical variables (X variables) are listed, defined, and explained in table 1. Particle aggregation and size distribution variables (green characters): mean diameter in volume (D4, 3), mean diameter in surface “Sauter diameter” (D3, 2), mean threshold size for which 10 % of the sample particles have a lower size (Dv.0.1), mean threshold size for which 50 % of the sample particles have a lower size (Dv.0.5), mean threshold size for which 90 % of the sample particles have a lower size (Dv.0.9), dispersion index (Span) and specific surface area (SSA). Protein oxidation variables (Orange characters): tryptophan content (TRYP), fluorescent protein oxidation markers (FPO), alpha-aminoadipic semialdehyde (AAS), gamma-glutamic semialdehyde (GGS) and total protein thiols (thiols). Protein structural variables (pink characters): surface hydrophobicity by bromophenol blue (BPB), alpha helix content (Alpha Per), beta sheet content (Beta Per) and ratio between alpha helix and beta sheet (Beta/alph). Technological variable (black characters): cooking loss percentage (CL Perc). Protein glycation variables (purple characters): Furosine, Nε-carboxymethyl lysine (CML) and Lanthionine (LAN). Protein digestion variables (Y variables: in red characters): GP10, GP20, GP30, GP 60 are referred to as rate of proteolysis in gastric phase for 10, 20, 30 and 60 min. IP10, IP20, IP30, IP60, IP120 are referred to as rate of proteolysis in intestinal phase for 10, 20, 30 ,60, 120 min.