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Non Ruminant Nutrition

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Impact of dietary oxidized protein on oxidative status and performance in growing pigs

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Abstract

Rendered products from the meat industry can provide economical quality sources of proteins to the animal and feed industry. Similar to lipids, rendered proteins are susceptible to oxidation, yet the stability of these proteins is unclear. In addition, interest in understanding how oxidative stress can impact efficiency in production animals is increasing. Recent studies show that consumption of oxidized lipids can lead to a change in the oxidative status of the animal as well as decreases in production efficiency. To date, little is known about how consumption of oxidized proteins impacts oxidative status and growth performance. The objectives of this study were to determine if feeding diets high in oxidized protein to growing pigs would: 1) impact growth performance and 2) induce oxidative stress. Thirty pigs (42 d old; initial body weight [**BW**] 12.49 ± 1.45 kg) were randomly assigned to one of three dietary treatments with increasing levels of oxidized protein. Spray-dried bovine plasma was used as the protein source and was either unheated upon arrival, heated at 45 °C for 4 d, or heated at 100 °C for 3 d. Diets were fed for 19 d and growth performance was measured. Blood plasma (days 0 and 18), jejunum, colon, and liver tissues (day 19) were collected to analyze for markers of oxidative stress (e.g., protein oxidation, lipid oxidation, DNA damage, and glutathione peroxidase activity). Average daily gain (**ADG;** $P < 0.01$) and average daily feed intake (**ADFI;** $P < 0.01$) had a positive linear relationship to increased protein oxidation, but there was no effect on gain to feed ratio. Furthermore, protein ($P = 0.03$) and fat ($P < 0.01$) digestibility were reduced with increased protein oxidation in the diet. Crypt depth showed a positive linear relationship with dietary protein oxidation levels ($P = 0.02$). A trend was observed in liver samples where pigs fed the plasma heated to 45 °C had increased lipid oxidation compared with pigs fed the plasma either unheated or heated to 100 °C ($P = 0.09$). DNA damage in the jejunum tended to have a linear relationship with the dietary protein oxidation level ($P = 0.07$). Even though results suggest dietary oxidized protein did not induce oxidative stress during short-term feeding, differences in performance, gut morphology, and digestibility are likely a result of reduced protein availability.

Key words: digestibility, growth, oxidative stress, pigs, protein oxidation

Introduction

Oxidative stress has implications for the health of both animals and humans. Oxidative stress is defined as an imbalance between prooxidants and antioxidants (Berlett and Stadtman, 1997). Within an organism, this imbalance can cause oxidation and damage of lipids, proteins, and DNA. In livestock species, oxidative stress can impact the efficiency of growth and, therefore, production costs (Dibner et al., 1996; DeRouchey et al., 2004). In companion animals or humans where longevity is the overall goal, oxidative stress can lead to certain health issues.

Abbreviations

ADFI	average daily feed intake
ADG	average daily gain
BW	body weight
DM	dry matter
DNPH	2,4-Dinitrophenylhydrazine
EDTA	Ethylenediaminetetraacetic acid
GPx	glutathione peroxidise
PBS	phosphate-buffered saline
ROS	reactive oxygen species
TBARS	thiobarbituric acid reactive substances

For example, in humans, it is believed that protein oxidation plays a part in certain age-related diseases, such as Alzheimer's disease, Parkinson's disease, inflammatory bowel disease, muscular dystrophy, and diabetes (Estévez and Luna, 2017).

From a nutritional aspect, the consumption of oxidized products could further damage lipids, proteins, or DNA, resulting in an increase of oxidative stress. Primarily because of potential objectionable odors associated with oxidized lipids, these sources are commonly treated with antioxidants. However, protein sources are commonly thought to be relatively stable to oxidation. It is known, however, that when exposed to heat, proteins can oxidize and create reactive oxygen species (**ROS**) as do lipids (Roldan et al., 2014; Feng et al., 2015; Zhang et al., 2017). These ROS are small molecules, such as superoxide ion, peroxide, hydroxyl radical, or hydroxyl ion, that can cause further oxidation of proteins, lipids, or DNA.

Research in nutrition has primarily focused on dietary lipid oxidation in livestock species (Boler et al., 2012; Lindblom et al., 2017). Since proteins can be oxidized to form free radicals and initiate oxidative stress, it is hypothesized that dietary oxidized protein can initiate oxidative stress similar to oxidized lipids. Rendered protein sources are widely used in both the livestock and companion animal feeds due to their high protein content and relatively low cost. Through the rendering process, these proteins are concentrated while being exposed to grinding and to high temperatures (i.e., <115 °C; Anderson, 2006), all of which can accelerate protein oxidation. Additionally, these ingredients are stored in a nonthermally controlled environment, potentially leading to oxidation of proteins. Rendered protein sources, therefore, are an ingredient category of interest and a potential model to understand dietary sources of oxidized protein. Therefore, the objectives of this study were to determine if feeding diets containing oxidized protein to growing pigs would: 1) result in the reduction of growth performance and 2) induce oxidative stress.

Materials and Methods

The protocol for this experiment was reviewed and approved by The Institutional Animal Care and Use Committee at Iowa State University (Ames, IA).

Animal and housing

Thirty female pigs (42 d old) with an average initial body weight (**BW**) of 12.49 ± 1.45 kg were individually housed in raised pens at the Swine Nutrition Farm at Iowa State University (Ames, IA) for 19 d. Pens allowed for free movement and provided individual feeders and water. Each pig was assigned to one of three dietary treatments and allowed ad libitum access to feed and water.

Diets and feeding

A basal diet consisting of corn, soybean meal, and spray-dried bovine plasma (10% inclusion) from a commercial source (APC Inc., Ankeny, IA) was formulated to meet NRC (2012) energy and nutrient requirements. Spray-dried bovine plasma was chosen as the main protein source due to its high protein content (84%) as well as very low lipid content (0.5%), thus allowing for minimal contribution to oxidation from lipid sources when heated. Two additional diets also containing 10% bovine plasma that had been previously exposed to heat treatment (45 °C for 4 d or 100 °C for 3 d) were also formulated (Table 1). Spray-dried bovine plasma across all treatments originated from the same production lot. Heating at 45 °C for 4 d was to simulate the maximum plausible temperature these feed ingredients could be exposed to during storage. This could be in the heat of summer, being stored in a silo or on a rail car before being mixed into a diet. Heating at 100 °C for 3 d was to induce as much protein oxidation as possible while still maintaining the solubility of the plasma. Spray-dried bovine plasma had little impact on the overall lipid oxidation of diets because lipid originating from the plasma would be minimal (0.5%). This is important because dietary oxidized lipids have been shown to impart damaging effects on growth performance and induce oxidative stress (Dibner et al., 1996; DeRouchey et al., 2004; Boler et al., 2012; Liu et al., 2014; Rosero et al., 2015; Lindblom et al., 2017). By selecting a protein source with minimal levels of lipids (0.5 %), the effect of protein oxidation with minimal effect of lipid oxidation could be evaluated.

Table 1. Diet formulation and analyzed composition of diets, DM basis

	Treatment[1]		
	20 °C	45 °C	100 °C
Diet formulation			
Corn	79.2	79.2	79.2
Soybean meal	8.2	8.2	8.2
Bovine spray-dried plasma	8.5	8.5	8.5
Soybean oil	1.8	1.8	1.8
Limestone	1.2	1.2	1.2
DL-Methionine	0.11	0.11	0.11
L-Lysine·HCl	0.22	0.22	0.22
Mineral premix[2]	0.14	0.14	0.14
Vitamin premix[3]	0.06	0.06	0.06
Titanium dioxide[4]	0.44	0.44	0.44
Analyzed composition			
DM, %	86.9	86.8	87.2
Ash, %	4.7	4.4	4.6
Protein, %	23.5	22.1	22.5
Gross energy, %	4,562	4,570	4,564
Fat, %	6.1	4.9	4.6
Carbonyl in spray-dried plasma[5], nmol/mg	2.7	5.2	8.1
Carbonyl in final mixed diet[5], nmol/mg	6.2	7.3	13.5

[1]Difference in dietary treatments were created by either no heat treatment to spray-dried bovine plasma (20 °C), heating plasma at 45 °C for 3 d (45 °C), or heating at 100 °C for 3 d (100 °C).
[2]Mineral premix supplied per kg of complete diet: 9 ppm Cu, 120 ppm Fe, 120 ppm Zn, 7 ppm Mn, 0.2 ppm I, and 0.2 ppm Se.
[3]Vitamin premix supplied per kg of complete diet: 2,143 IU vitamin A, 245 IU vitamin D3, 17.5 IU vitamin E, 1.1 IU vitamin K, 3.9 mg riboflavin, 19.6 mg niacin, 9.5 mg pantothenic acid, and 18 μg vitamin B_{12}.
[4]Titanium dioxide was used as an indigestible marker for determining the apparent total tract digestibility.
[5]Carbonyls were analyzed as a measure of protein oxidation in the plasma after heating as well as in a complete diet after mixing. Values are reported as nmol of carbonyl/mg of protein.

When proteins become oxidized they can further oxidize lipids and vice versa (Nielsen et al., 1985). This synergistic effect should be considered when evaluating data or studies examining oxidation in the diet. Consequently, it should be expected that when either lipid or protein oxidation is present, the other can also be impacted (Nielsen et al., 1985). Other factors in the diet can act as antioxidants such as vitamin E. To reduce the antioxidant impact on the overall diet in the current experiment, vitamin E levels for all dietary treatments were formulated only to NRC (2012) requirements.

Vitamins and minerals were included in concentrations that met or exceeded the requirement for growing pigs; no antioxidants were included in the diet formulation. Titanium dioxide was added as an indigestible marker to diets to allow for apparent total tract digestibility calculation using a grab-sample fecal collection. After mixing, feed was stored at 20 °C until fed.

Sample collection

Pig and feeder weights were recorded on days 0 and 19 to determine the growth performance data. Feces were collected from day 15 to 19, pooled, and stored at −20 °C until freeze-dried. Fecal samples were subsequently ground through a 1-mm screen prior to the analysis. Blood (15 mL) was collected via jugular venipuncture on days 0 and 18. After collection, vials were centrifuged at 1,000 × *g* for 10 min at 4 °C. Plasma was collected and stored at −80 °C for further analysis. On day 19, pigs were euthanized by captive bolt followed by exsanguination for the harvesting of various tissues. Liver, jejunum, and colon were washed with phosphate-buffered saline (**PBS**), snap-frozen in liquid nitrogen, and stored at −80 °C until further analysis. Jejunum samples were placed in 10% w/v formalin to be stained with hematoxylin and eosin at Iowa State University Veterinary Diagnostic Laboratory (ISUVDL, Ames, IA). The resulting slides were analyzed for crypt depth and villi height (OLYMPUS BX 53/43 microscope with an attached DP80 Olympus camera, Waltham, MA). Fifteen villus and crypt pairs with proper orientation were measured per pig (OLYMPUS cellSens Dimension 1.16 software), averaged, and reported as 1 value per pig.

Chemical analysis

Ingredients, diets, and fecal samples were analyzed in duplicate for DM (AOAC 930.01), ash (AOAC 942.05), CP (AOAC 992.15), and acid hydrolyzed ether extract (AOAC 2003.06). Gross energy was determined using a bomb calorimeter (model 6200; Parr Instrument Co., Moline, IL) with benzoic acid (6,318 kcal GE/kg; Parr Instrument Co.) for calibration. Diets and spray-dried plasma were analyzed for carbonyls by solubilizing 1 g of sample in 50 mL of 5% w/v sodium dodecyl sulfate (Soglia et al., 2016) followed by centrifugation for 10 min at 3,000 × *g* at 4 °C to remove any remaining insoluble particles. This solution was used to analyze carbonyls (Levine et al., 1990).

Oxidative stress assays

Tissue and blood plasma samples were analyzed for carbonyls (protein oxidation), thiobarbituric acid reactive substances (**TBARS**; lipid oxidation), 8-OH-2-deoxyguanosine (DNA damage), and glutathione peroxidase (**GPx**) activity (antioxidant enzyme). Kits for TBARS, 8-OH-2- deoxyguanosine, and GPx activity were obtained from a commercial company (Cayman Chemical, Ann Arbor, MI) and performed according to the manufacturer's instructions.

Blood plasma at days 0 and 18, along with jejunum, colon, and liver samples, were analyzed for carbonyls following a modified protocol (Levine et al., 1990). A 300 mg sample of tissue was first rinsed with PBS solution then homogenized in 2 mL of 50 mM phosphate buffer, 1 mM Ethylenediaminetetraacetic acid (**EDTA**), pH 6.7. After, samples were centrifuged at 10,000 × *g* for 15 min at 4 °C, supernatant and blood plasma were collected and stored at −80 °C until analyzed. Supernatant or blood plasma was thawed and 200 µL was transferred to a 2-mL microtube with 800 µL of 10 mM 2,4-Dinitrophenylhydrazine (**DNPH**) in 2.5 M HCl. A duplicate sample where DNPH was replaced with 2.5 M HCl was run to standardize for final protein. Both tubes were incubated and vortexed every 15 min at 20 °C in the dark for 1 h; 1 mL of 20% trichloroacetic acid was added to each tube, then vortexed and placed on ice for 5 min. Tubes were centrifuged at 10,000 × *g* for 10 min at 4 °C. Once the supernatant was discarded, the pellet was resuspended in 1 mL of 10% trichloroacetic acid. Tubes were again centrifuged at 10,000 × *g* for 10 min at 4 °C, the supernatant was discarded, and the pellet was resuspended in a 1:1 solution of ethanol:ethyl acetate. Tubes were centrifuged and washed with ethanol:ethyl acetate solution again then resuspended in 500 µL 6 M guanidine HCl. After the pellet was fully dissolved, samples with DNPH were read at 365 nm, and the protein concentration was determined on corresponding tubes without DNPH. Values of carbonyls were then corrected for protein by dividing the concentration of carbonyls by protein concentration using the following equation:

$$Corrected\ \ carbonyl\ \ \left(\frac{nmol}{mg}\right) = \frac{Carbonyl\ \ concentration\ \left(\frac{nmol}{ml}\right)}{Protein\ \ concentration\ \left(\frac{mg}{ml}\right)}$$

The TBARS assay was performed on jejunum and liver tissues. Samples were prepared according to the manufacturer's instructions by sonicating a 25-mg sample of tissue in a 1.5-mL centrifuge tube with 250 µL of Radioimmunoprecipitation buffer containing 1 mM EDTA. Samples were then centrifuged at 1,600 × *g* for 10 min at 4 °C. Supernatant was collected and stored at −80 °C until assay. The assay was conducted following the manufacturer's instructions using a colorimetric procedure (Cayman Chemical, Ann Arbor, MI).

The 8-OH-2-deoxyguanosine assay was performed on jejunum, colon, and liver samples. Sample preparation was performed according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI). A 1 g sample of tissues was first homogenized in 5 mL of 0.1 M phosphate buffer, pH 7.4 with 1 mM EDTA. Samples were then centrifuged at 1,000 × *g* for 10 min at 4 °C. Supernatant was collected and stored at −80 °C. DNA was then purified using a commercial kit (Qiagen DNeasy kit, Venlo, the Netherlands). First 200 µL of thawed sample was added to a 1.5-mL centrifuge tube with 20 µL of protease K. The solution was used to purify DNA following the manufacturer's instruction. After repeating elution of the spin column, 10 µL of nuclease P1 was added to each sample of 400 µL. Nuclease P1 was prepared by adding 1.4 mL of 20 mM sodium acetate, 5 mM ZnCl, 50 mM NaCl, and pH 5.3 solution to 1.4 mg of lyophilized powder enzyme. This solution containing 1 mg/mL of enzyme was diluted 1:20 to achieve a final dilution of 50 µg/mL. The sample solution was adjusted to a pH of 8.0 using 1 M tris, then incubated at 37 °C for 10 min. Next, 10 µL of alkaline phosphatase was added to each tube then incubated for another 30 min at 37 °C. Samples were then boiled for 10 min. This sample solution was then used following the manufacturer's instructions to determine the 8-OH-2- deoxyguanosine content of each sample.

Glutathione peroxidase activity was measured in both plasma and jejunum samples. To prepare jejunum samples, 1 g of tissue was washed with PBS then homogenized in 5 mL of

50 mM tris-HCL pH 7.5, 5 mM EDTA, and 1 mM Dithiothreitol. Samples were then centrifuged at 10,000 × *g* for 15 min at 4 °C. The supernatant was collected and stored at –80 °C until assayed. Following thawing, the assay was conducted following the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI).

Calculations and statistical analysis

After chemical analyses were completed on diets and fecal samples, digestibility was calculated. This was accomplished by correcting the difference in output from input with the increase in TiO_2 concentration. The following equation was used for the calculation of digestibility:

$$\% \text{ Digestibility} = 100 - \left[100 \times \left(\frac{TiO_{2(diet)}}{TiO_{2(feces)}}\right) \times \left(\frac{Nutrient_{(feces)}}{Nutrient_{(diet)}}\right)\right]$$

Pigs were randomly assigned to one of the three dietary treatments (n = 10 pigs per treatment), resulting in 30 pigs total. Pigs were individually housed and fed, resulting in pig being the experimental unit. Therefore, a completely randomized design was used to analyze the data. Data were analyzed as a completely random design using the Proc GLM procedure of SAS (version 9.4; SAS) with means reported using LSMEANS using a Tukey post hoc test to compare means. Additionally, an unprotected *F*-test was used with contrast statements to analyze for a linear relationship between response variables with carbonyl values in the diet. *P*-values ≤ 0.05 were considered significantly different, with *P*-value of 0.05 ≤ *P* ≤ 0.10 considered trends.

Results and Discussion

Diets and composition

To achieve differences in protein oxidation levels, the spray-dried bovine plasma was unheated or heated at either 45 °C for 4 d or 100 °C for 3 d. The process of heating the spray-dried plasma resulted in carbonyl values of 2.7, 5.2, and 8.1 nM carbonyl/mg of protein for the 20 °, 45 °, and 100 °C processed spray-dried plasma, respectively (Table 1). Final mixed diets contained 6.1, 7.3, and 13.5 nM of carbonyl/mg of protein in 20 °, 45 °, and 100 °C treatments, respectively. It is assumed that due to the low lipid content of spray-dried plasma, extremely limited products of lipid oxidation were included in the diet. Additionally, mixed diets were only stored 1 d prior to the start of the study. This short storage time also limited the extent for protein oxidation in the spray-dried plasma to initiate lipid oxidation in the diet. Therefore, lipid oxidation in the diet was not quantified. We acknowledge that products of protein oxidation may initiate lipid oxidation. However, due to the low lipid content of spray-dried plasma as well as the short time period between heating and feeding, the likelihood of this occurring is low and would have minimal impacts on the extent of lipid oxidation in the diet.

Growth performance and gut morphology

One potential outcome of animals consuming dietary oxidized proteins in their diet is an induction of oxidative stress. With respect to livestock species, the increase in oxidative status can impact production efficiency, thus growth performance data were collected in the current study, even though pigs were housed individually. Over the 19-d trial, a significant positive linear relationship (Table 2) between protein oxidation in the diet and average daily gain (**ADG**) was observed ($P < 0.01$). A similar positive linear relationship was also observed between protein oxidation in the diet and average daily feed intake (**ADFI**; $P < 0.01$). However, no difference for gain to feed ratio was noted ($P = 0.87$). This is consistent with findings from other studies where no difference was detected for feed efficiency in broiler chickens fed oxidized soy protein isolate (Wu et al., 2014; Chen et al., 2015) or pigs fed oxidized lipids (DeRouchey et al., 2004; Boler et al., 2012; Liu et al., 2014; Rosero et al., 2015). However, poultry appear to be more susceptible to the effects of oxidation. Studies examining the effect of dietary lipid oxidation on poultry have observed a decrease in feed efficiency as dietary lipid oxidation increased (Takahashi and Akiba, 1999; Tavárez et al., 2011). Contrary to the current study where pigs fed oxidized spray-dried plasma had increased ADG and ADFI, other studies have not observed differences in ADG or ADFI when pigs were fed oxidized lipids (Boler et al., 2012; Wu et al., 2014). ADG and ADFI have been shown to decrease in pigs fed diets with greater concentrations of oxidized lipids (DeRouchey et al., 2004; Liu et al., 2014; Chen et al., 2015; Rosero et al., 2015). Reduced ADFI when feeding oxidized lipids is likely due to decreased palatability (Rosero et al., 2015). This in conjunction with decreased digestibility would result in reduced ADG. However, in the current study, lipid oxidation was minimized, thus palatability was likely not a factor. It is understood that pigs will consume to meet their energy requirement (Patience et al., 2015). In this study, even though gross energy digestibility was not impacted, reduced digestibility of protein could result in decreased net energy, thus resulting in the increased ADFI and subsequent ADG.

Table 2. Growth performance data for pigs fed diets containing increasing levels of dietary oxidized protein

	Treatment[1]				*P*-value[3]	
	20 °C	45 °C	100 °C			
Carbonyl in diet, nmol/mg	6.2	7.3	13.5	SEM	Treatment	Linear
Growth performance[2]						
ADG, kg	0.54[b]	0.54[b]	0.61[a]	0.02	0.03	<0.01
ADFI, kg	0.83[b]	0.84[b]	0.95[a]	0.03	0.02	<0.01
Gain:feed	0.65	0.65	0.65	0.01	0.95	0.87

[1]Difference in dietary treatments were created by either no heat treatment to spray-dried plasma (20 °C), heating plasma at 45 °C for 3 d (45 °C), or heating at 100 °C for 3 d (100 °C).
[2]There were 10 individually penned gilts per treatment with an initial BW of 12.5 ± 1.5 kg. The trial lasted 19 d. GF, gain:feed ratio.
[3]In addition to analyzing as individual treatments, a contrast was analyzed and the resulting *P*-values for a linear relation between response variables and carbonyl values in the diet are reported.
[a,b]Difference in superscripts within row indicate significant differences between treatments.

Table 3. Histology data for pigs fed diets containing increasing levels of dietary oxidized protein

	Treatment[1]				*P*-value[2]	
	20 °C	45 °C	100 °C			
Carbonyl in diet, nmol/mg	6.2	7.3	13.5	SEM	Treatment	Linear
Histology						
Villi height, µm	378.5	375.4	414.1	27.1	0.55	0.28
Crypt depth, µm	163.0[y]	163.3[y]	193.3[z]	9.5	0.06	0.02
Villi:crypt ratio	2.60	2.58	2.36	0.18	0.60	0.32

[1]Difference in dietary treatments were created by either no heat treatment to spray-dried plasma (20 °C), heating plasma at 45 °C for 3 d (45 °C), or heating at 100 °C for 3 d (100 °C). There were 10 individually penned gilts per treatment with an initial BW of 12.5 ± 1.5 kg.
[2]In addition to analyzing as individual treatments, a contrast was analyzed and the resulting *P*-values for linear relation between response variables and carbonyl values in the diet are reported.
[y,z]Difference in superscripts within row indicate a tendency between treatments.

Table 4. Digestibility of diets containing increasing levels of dietary oxidized protein

	Treatment[1]				*P*-value[2]	
	20 °C	45 °C	100 °C			
Carbonyl in diet, nmol/mg	6.2	7.3	13.5	SEM	Treatment	Linear
Dry matter, %	71.7[b]	77.1[a]	71.4[b]	1.2	<0.01	0.08
Ash, %	15.4[b]	21.5[a]	14.7[b]	2.0	0.05	0.18
Protein, %	69.5[ab]	73.8[a]	66.9[b]	1.6	0.02	0.03
Gross energy, %	73.0	75.0	76.1	1.4	0.31	0.21
Ether extract, %	36.3[a]	33.7[a]	20.1[b]	2.2	<0.01	<0.01

[1]Difference in dietary treatments were created by either no heat treatment to spray-dried plasma (20 °C), heating plasma at 45 °C for 3 d (45 °C), or heating at 100 °C for 3 d (100 °C). There were 10 individually penned gilts per treatment with an initial BW of 12.5 ± 1.5 kg.
[2]In addition to analyzing as individual treatments, a contrast was analyzed and the resulting *P*-values for a linear relation between response variables and carbonyl values in the diet are reported.
[a,b]Difference in superscripts within row indicate significant differences between treatments.

In examining histology samples from the jejunum (Table 3), a linear relationship was observed for deeper crypt depths ($P = 0.02$) with increased oxidation. Previously, Rosero et al. (2015) reported that villus height and crypt depth increase in pigs consuming oxidized lipids for 35 d. Additionally, as peroxidation in the diet increased, abortive capacity measured by mannitol and D-xylose decrease, while lipid damage in the jejunum measured by Malondialdehyde concentration increased (Rosero et al., 2015). It was proposed that lipid oxidation stimulated enterocyte proliferation as a result of oxidative stress in the small intestine, thus leading to changes in morphology (Rosero et al., 2015). However, in the current study, no significant difference in the oxidative status of the jejunum existed. Therefore, based on our results of this short-term feeding trial, it is suggested that the observed trend for greater surface area is a result of the body responding to a decrease in protein and lipid apparent total tract digestibility in order to capture more nutrients.

Digestibility

Results for apparent total tract digestibility of DM, ash, crude protein, gross energy, and lipid are summarized in Table 4. Protein digestibility had a negative linear relationship to carbonyl levels in the diet ($P = 0.03$). Additionally, fat digestibility also had a negative linear relationship to carbonyl levels in the diet ($P < 0.01$).

In examining the effect of dietary oxidized protein on digestibility, Chen et al. (2015) concluded that as diets increased in oxidation, DM, protein, and lipid digestibility all decreased. In general, the results are consistent with the data reported herein comparing increased oxidation treatments with digestibility. It appears that in general, as feeds increase in the content of oxidized protein, there is a reduction in available nutrients, such as lipids, and protein. It has been shown that as proteins become oxidized, structural changes occur increasing their aggregation (Promeyrat et al., 2010). Furthermore, it has been proposed that oxidation of proteins decreases their susceptibility to proteolysis, again resulting in decreased digestibility (Sante-Lhoutellier et al., 2007; Wu et al., 2014). This reduction in protein and lipid digestibility could explain an observed increase in surface area of the jejunum. As less proteins and lipids are absorbed, the gastrointestinal tract responds by increasing the surface area in order to capture more nutrients. Furthermore, the reduction in protein and lipid digestibility may contribute to the increase in intake as the pig consumes more to meet their energy requirement (Patience et al., 2015).

Measures of oxidative stress

Markers of oxidative stress were analyzed to observe damage to lipids, protein, and DNA as well as antioxidant enzyme activity in tissues and blood plasma due to increased intake of oxidized proteins (Table 5). Plasma was collected at day 0 for baseline levels and was analyzed for carbonyls. Values of 2.25, 1.88, and 1.89 nmol of carbonyls/mg of protein in 20 °, 45 °, and 100 °C treatments, respectively, were observed. These blood plasma

Table 5. Measures of oxidative stress in pig plasma, jejunum, colon, and liver tissues fed increasing levels of dietary oxidized protein

	Treatment[1]				P-value[2]	
	20 °C	45 °C	100 °C			
Carbonyl in diet, nmol/mg	6.2	7.3	13.5	SEM	Treatment	Linear
Blood plasma						
Day 0 carbonyl, nmol/mg	2.25	1.88	1.89	0.25	0.52	0.56
Day 18 carbonyl, nmol/mg	3.18	3.02	3.07	0.23	0.89	0.94
Change in carbonyl[3]	0.92	1.14	1.20	0.36	0.85	0.69
GPx activity, nmol/min/mL[4]	68.1	70.9	76.4	10.1	0.84	0.57
Jejunum						
Carbonyl, nmol/mg	9.52	10.71	10.76	3.01	0.95	0.85
TBARS, μM	0.84	0.68	0.57	0.21	0.64	0.45
8-OH-2-deoxyguanasine, pg/mL)	655.0	695.1	790.1	50.6	0.17	0.07
GPx activity, nmol/min/mL[4]	17.9	23.6	33.5	9.9	0.54	0.29
Colon						
Carbonyl, nmol/mg	5.97	7.55	7.93	1.04	0.38	0.36
8-OH-2-deoxyguanasine, pg/mL	132.6	116.1	127.8	15.5	0.74	0.89
Liver						
Carbonyl, nmol/mg	3.73	2.90	3.94	0.52	0.35	0.37
TBARS, μM	9.72[y]	12.78[z]	10.48[yz]	0.98	0.09	0.62
8-OH-2-deoxyguanasine, pg/mL	69.4	62.7	60.6	3.2	0.15	0.15

[1]Difference in dietary treatments were created by either no heat treatment to spray-dried plasma (20 °C), heating plasma at 45 °C for 3 d (45 °C), or heating at 100 °C for 3 d (100 °C). There were 10 individually penned gilts per treatment with an initial BW of 12.5 ± 1.5 kg.
[2]In addition to analyzing as individual treatments, a contrast was analyzed and the resulting *P*-values for a linear relation between response variables and carbonyl values in the diet are reported.
[3]Change in carbonyl was calculated by subtracting day 0 values by day 18 for each pig.
[4]GPx activity is indirectly measured by coupling the reaction of GPx with glutathione reductase. Units are in nmol/min/mL of NADPH converted to $NADP^+$ by glutathione reductase.
[y,z]Difference in superscripts within row indicate a tendency between treatments.

carbonyl values did not have any significant relationship to diet carbonyl values ($P = 0.56$). After 18 d, these blood plasma carbonyl values increased to 3.18, 3.02, and 3.07 for 20 °, 45 °, and 100 °C treatments, respectively. These values were found to not have any relationship to diet carbonyl levels ($P = 0.94$). Additionally, the change from baseline values of carbonyl concentration in the blood plasma for pigs fed plasma unheated, heated to 45 °C, and heated to 100 °C was nonsignificant. No significant results were observed in the plasma suggesting either products of oxidation are not stored in the plasma or that sufficient antioxidant capacity was available to clear the products of oxidation.

In the jejunum, no relationship between diet carbonyl values and TBARS, carbonyls, or GPx activity ($P \geq 0.29$) was observed. When analyzed as a linear contrast with diet carbonyl values, a positive linear trend was observed for DNA damage in the jejunum ($P = 0.07$). As the main site of protein absorption, it would be expected that jejunum would have the greatest exposure to dietary oxidized protein. However, these results suggest that either oxidized products did not have an effect on the tissue or it had enough antioxidant capacity to maintain homeostasis during this relatively short feeding period.

In colon tissue, there was no relationship between oxidation in the diet, colon carbonyls, and DNA damage in the colon ($P \geq 0.36$). It was hypothesized that as a result of the oxidized protein being less digestible, the prooxidants would pass the small intestine and accumulate in the large intestine. However, these results suggest that even though dietary oxidized protein is less digestible, this did not result in an increase in oxidative status in the colon. It is possible that end products of protein oxidation do not impact the oxidative status of tissues during this short trial. Furthermore, oxidized products may be utilized by microbes in the large intestine, therefore limiting the tissues' exposure.

Liver samples from pigs fed diets containing plasma heated to 45 °C had a tendency for increased lipid oxidation compared with pigs fed the plasma either unheated or heated to 100 °C ($P = 0.09$). For carbonyls in the liver, no relationship was observed with diet carbonyls ($P = 0.37$). Additionally, no relationship was observed for DNA damage in the liver ($P = 0.15$).

In summary, during this short feeding trial, no difference was observed in DNA damage, lipid damage, or antioxidant enzyme activity in the plasma, colon, or liver. Results from this study indicate that dietary oxidized protein did not induce oxidative stress. However, crypt depth increased with increased inclusion of oxidized protein in the diet, resulting in a greater surface area. This could have been a response to decreased protein and lipid digestibility. Additionally, pigs fed diets with increased inclusion of oxidized protein in the diet consumed more and gained more per day, but the feed efficiency was unaffected.

Conclusion

Dietary oxidized protein in the form of heated spray-dried plasma fed during a short feeding period did not induce oxidative status in key tissues of pigs fed those diets. However, oxidation of proteins is known to increase the aggregation and oxidative modification of amino acids and this could impact digestibility. This study did observe decreased protein digestibility, changes in gut morphology, and increased ADFI and ADG. While spray-dried plasma is produced under mild conditions that would not induce large amounts of protein oxidation, other protein sources that undergo extreme temperatures during processing or

storage have the potential to have much greater levels of protein oxidation. This leads to the need to understand the potential impact of oxidation on other protein sources. Additionally, most protein sources would be fed for the duration of the animal's lifespan, not just the very short period used here. While this study showed the response in growing pigs to a very short-term exposure (19 d) to dietary oxidized protein, future research investigating the long-term effects of the consumption of these products is warranted.

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Conflict of interest statement

The authors disclose that there was no conflict of interest.

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