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Gastric protein hydrolysis of raw and roasted almonds in the growing pig



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ABSTRACT

Gastric protein hydrolysis may influence gastric emptying rate and subsequent protein digestibility in the small intestine. This study examined the gastric hydrolysis of dietary protein from raw and roasted almonds in the growing pig as a model for the adult human. The gastric hydrolysis of almond proteins was quantified by performing tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequent image analysis. There was an interaction between digestion time, stomach region, and almond type for gastric protein hydrolysis ($p < 0.05$). Gastric emptying rate of protein was a significant ($p < 0.05$) covariate in the gastric protein hydrolysis. In general, greater gastric protein hydrolysis was observed in raw almonds (compared to roasted almonds), hypothesized to be related to structural changes in almond proteins during roasting. Greater gastric protein hydrolysis was observed in the distal stomach (compared to the proximal stomach), likely related to the lower pH in the distal stomach.

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1. Introduction

Almonds are a dietary protein source consumed worldwide as both a single food and as an ingredient in many food products. In California alone, the market production of almonds was greater than 848 million kg in 2014–2015 (Almond Board of California, 2015). In addition to being a source of dietary protein, almonds also contain lipids, fiber, potassium, magnesium, and α -tocopherol (Yada, Lapsley, & Huang, 2011).

Almonds may be consumed as raw, dry roasted, or in other forms. It has been previously observed that the roasting process damages the inner parenchyma of plant cell walls, allowing the contents to be open to the extracellular space (Altan, McCarthy, Tikekar, McCarthy, & Nitin, 2011; Perren & Escher, 2013; Varela, Aguilera, & Fiszman, 2008). The high process temperatures used during dry roasting of almonds (~ 130 – 154 °C) are likely to result in modification of secondary and tertiary protein structure, as these structures are modified at temperatures greater than 70 °C (Davis & Williams, 1998).

Additionally, protein aggregation may occur, promoting chemical reactions with amino acid side chains, e.g. Maillard reaction (Davis & Williams, 1998). The protein structural modifications that occur during roasting may alter the rate at which almond dietary proteins are released from the food matrix and hydrolyzed by digestive enzymes in the gastrointestinal tract.

In the stomach, dietary proteins are chemically hydrolyzed by pepsin and hydrochloric acid as the food matrix completes its mechanical breakdown by peristaltic contractions of the stomach wall. The stomach may be separated into two regions: the proximal region, consisting of the fundus and body, and the distal region, consisting of the antrum and pylorus (Fig. 1). Previous studies have indicated that functional differences in stomach contents exist between the two regions. Emptying of gastric contents (Collins et al., 1991; Guerin et al., 2001), mixing of gastric contents (Holdsworth, Johnson, Mascal, Roulston, & Tomlinson, 1980), pH (Bornhorst et al., 2014), and physical properties (Bornhorst, Ferrua, Rutherford, Heldman, & Singh, 2013; Bornhorst, Roman, Dreschler, & Singh, 2013) have been reported to vary based on stomach region. The differences in emptying and properties may arise due to physiological differences in functionality (e.g. peristaltic contractions that occur in the distal stomach region) and/or variations in biochemical environment (e.g. pH and enzymes) facilitated through separation of gastric contents, emptying, and mixing. For example, previous studies have shown that after meals of raw or roasted, diced almonds, large pH gradients exist, both as a

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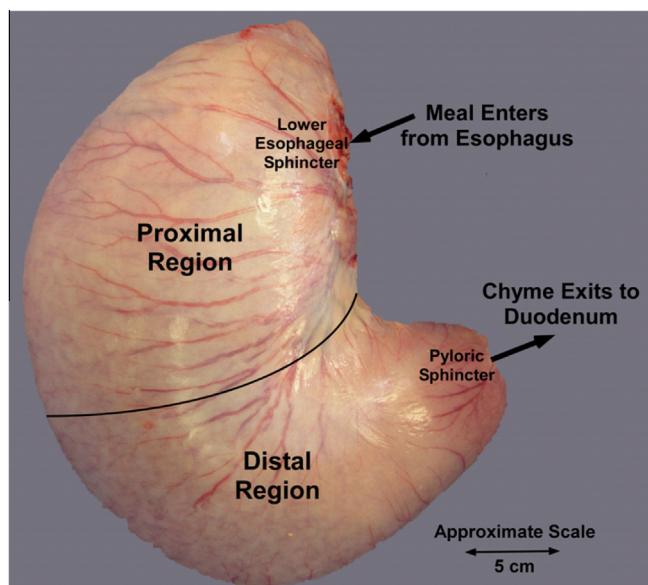


Fig. 1. Image of a full pig stomach showing the approximate location of the proximal and distal stomach regions. The lower esophageal sphincter and pyloric sphincter are denoted with arrows to show where the meal entered and chyme exited the stomach, respectively (Adapted and modified from Bornhorst, Ferrua et al. (2013)).

function of intragastric location (e.g. proximal or distal stomach), and as a function of digestion time. After 20 min of digestion, the intragastric pH values varied between 1.8 and 7.2 (maximum and minimum values) in different locations in the stomach of pigs that consumed meals of raw or roasted, diced almonds. The pH gradient was similar after 720 min of digestion, where the pH values varied between 1.1 and 6.9 in different locations of the stomach (Bornhorst et al., 2013; Bornhorst et al., 2014).

The goal of the present study was to investigate the gastric hydrolysis of intact protein in raw and roasted almonds, using the pig as a model for the adult human (Moughan & Rowan, 1989). It was hypothesized that changes in almond protein structure and matrix structure as a result of roasting would result in modifications to gastric protein hydrolysis. In addition, it was hypothesized that the non-uniform pH in the stomach would produce variations in dietary protein hydrolysis between the proximal and distal stomach that would increase during the gastric digestion period due to the longer time spent in different pH environments.

2. Materials and methods

2.1. Experimental diets and animal trial protocols

The raw and roasted almond diets and animal trial protocols have been previously described (Bornhorst, Roman, Rutherford et al., 2013; Bornhorst et al., 2013). Briefly, all animal protocols were approved by the Massey University Animal Ethics Committee (Protocol 11/30). Seventy-two male growing pigs (23 ± 0.2 kg bodyweight [mean \pm SE], PIC Camborough 46 sow \times PIC boar 356L, PIC New Zealand, Christchurch, New Zealand) were acclimatized for a seven day period. On the final day of the study (day 8), they were fed a single meal of either medium diced raw or dry roasted almonds (5% of metabolic bodyweight; half of the daily food portion) with a small quantity of water (1.25% of metabolic bodyweight). Pigs were then euthanized at 20, 60, 180, 300, 480, or 720 min postprandial ($n = 6$ pigs per time point for each almond type). The stomach was dissected out and chyme samples were taken from the proximal (i.e. fundus) and distal (i.e. pyloric)

stomach region (Fig. 1) and were immediately frozen at -20°C . The samples were then freeze-dried and stored at -20°C until analysis.

2.2. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Tricine-SDS-PAGE was performed on defatted samples of raw and roasted almonds as well as the defatted samples of proximal and distal gastric chyme. Chyme or diet sample proteins were extracted as previously described (Rutherford et al., 2011). Briefly, samples (1:100 w:v sample:buffer) were mixed with tricine sample buffer containing β -mercaptoethanol (19:1 v:v) for protein extraction. The wells were loaded with $15 \mu\text{L}$ of the protein extract. Electrophoresis was completed as previously described (Rutherford et al., 2011). All reagents were purchased from Bio-Rad (Bio-Rad Laboratories, Richmond, CA, USA). Gels were scanned after the final destaining step. Samples from the proximal and distal stomach region of each pig (6 pigs per time point for each almond type) were used for the peptide band analysis.

2.3. Peptide band analysis

The intensity of selected peptide bands of the scanned gels was quantified in arbitrary density units (ADU) using ImageJ (National Institutes of Health, Bethesda, MD, USA). The selected bands were 50, 45, 42, 34, 28, 26, and 22 kDa. These bands were selected as they were assumed to be the major (45, 42, and 22 kDa bands) and minor (50, 34, 28, 26 kDa bands) polypeptides of amandin (Sathe et al., 2002).

Briefly, the gel images were converted into 8 bit images and the image background was subtracted to allow for better visualization of protein bands. On each gel, the wells were individually selected and converted into intensity plots using the Gel Analyzer toolbox in ImageJ. The intensity plots for each gel will show a peak for each protein band that varies in size depending on the height and intensity of the protein band. For each peptide band analyzed, a straight line was drawn across the intensity plots of all wells to delineate each side of the band, according to the original gel image. The area under the curve for each band was calculated in ImageJ in ADU.

2.4. Calculations and statistical analysis

The gastric hydrolysis of the whole SDS-PAGE lane (referred to as total soluble protein) or individual protein bands was calculated as follows (Rutherford et al., 2011):

$$\text{Gastric hydrolysis (\%)} = \frac{\left[\left(\frac{\text{band intensity}}{\text{mg sample}} \right)_{\text{diet}} - \left(\frac{\text{band intensity}}{\text{mg sample}} \right)_{\text{chyme (t)}} \right]}{\left(\frac{\text{band intensity}}{\text{mg sample}} \right)_{\text{diet}}} * 100 \quad (1)$$

The band intensity (ADU) was taken from the image analysis of the SDS-PAGE gels. The band intensity for each chyme sample was compared to the diet (raw or roasted almonds) on the same gel to account for any differences between staining or imaging between gels. The band intensity was normalized by the sample amount for each sample. The calculations were completed without correction for indigestible marker in the sample, as the marker concentration in the gastric samples were not different from those in the diets (Bornhorst, Roman, Rutherford et al., 2013).

Statistical analyses were performed using the Mixed Model procedure of SAS (version 9.4, 2013; SAS Institute Inc., Cary, NC, USA). An analysis of repeated measures in a completely randomized

Table 1

Gastric emptying of protein of raw and roasted almonds over a 720 min postprandial period. Values are averages (n = 6 pigs/time point) ± standard error and indicate the percentage of the original amount of protein remaining in the stomach. Data adapted from Bornhorst, Roman, Rutherford et al. (2013). Almond type and digestion time significantly influenced the almond protein gastric emptying rate (p < 0.05).

Digestion time (min)	Protein remaining in the stomach (%)	
	Raw almonds	Roasted almonds
20	98.8 ^a ± 2.4	101.7 ^a ± 2.4
60	94.2 ^{ab} ± 2.7	96.2 ^a ± 2.1
180	74.0 ^{bc} ± 5.3	88.0 ^a ± 2.8
300	60.4 ^{cd} ± 6.1	80.9 ^{ab} ± 2.1
480	54.7 ^{cd} ± 6.9	69.9 ^{bc} ± 3.2
720	46.0 ^d ± 3.0	50.0 ^c ± 3.9

Values with different letters in each column (abcd) represent significant differences (p < 0.05) within each almond type across digestion times.

design was performed with the pig as the experimental unit, and the stomach region (proximal or distal) as the repeated measure. The full statistical model included almond type, gastric digestion time, stomach region, and all their interactions as fixed effects and stomach emptying rate of protein as a covariate. The values of stomach emptying rate of protein have been presented elsewhere, but are shown in Table 1 to facilitate data interpretation (Bornhorst, Roman, Rutherford et al., 2013). The most appropriate covariance structure of the mixed model for each response variable was selected after fitting the models by the Restricted Maximum Likelihood method and comparing them using the log-likelihood ratio test. The final selected model for each response variable was chosen by comparing complete models versus reduced models (i.e. removing predictors that did not influence the response variable) using the log-likelihood ratio test.

The model diagnostics of each fitted response variable were tested after combining the PROC UNIVARIATE and the ODS GRAPHICS procedures of SAS (SAS, 2009) before comparing the means. When the model assumptions were not fulfilled for an individual variable, a transformation of the raw data was conducted to achieve those assumptions. Finally, when the F-value of the analysis of variance was significant (p < 0.05), the least square means of selected treatments were compared using the Student's *t*-test.

3. Results

All variables were influenced by the covariate of protein gastric emptying (p < 0.05; data not shown), although the specific influence differed between variables. The 34 kDa band was significantly influenced (p < 0.05) by gastric emptying, while total soluble protein and the 50, 42, and 26 kDa protein bands were significantly influenced (p < 0.05) by the interaction between gastric emptying, stomach region, and almond type. The interaction between gastric emptying and stomach region significantly (p < 0.05) influenced the 45 and 22 kDa protein bands, and the interaction between gastric emptying and almond type significantly influenced (p < 0.05) the 45, 28 and 22 protein bands.

For the gastric protein hydrolysis of the total soluble protein and the 45, 42, 34, 28, and 22 kDa protein bands, there were significant (p < 0.05) interactions between almond type, stomach region, and digestion time (Table 2, Fig. 2). For the 50 and 26 kDa protein bands, there was no interaction between almond type, stomach region, and digestion time, but there was an interaction between stomach region and digestion time as well as an interaction between stomach region and almond type (p < 0.05, Table 2).

The gastric hydrolysis for the total soluble protein and for the 45, 42, 34, and 22 kDa protein bands significantly increased over time in both stomach regions, although the degree of increase varied between the proximal and distal stomach regions and between

Table 2

Statistical significance of the main factors and their interactions for gastric hydrolysis of the total soluble protein and individual peptide bands in growing pigs fed raw and roasted almonds during 720 min of gastric digestion.

Effect ^b	Response variables ^a							
	Band (kDa)	50	45	42	34	28	26	22
	Total soluble protein ^c							
Stomach region (R)	***	NS	***	***	NS	***	NS	***
Digestion time (T)	*	**	***	***	**	NS	*	***
Almond type (A)	***	*	***	***	NS	***	***	***
R × T	***	***	***	***	NS	***	*	***
R × A	**	***	***	***	***	***	***	***
A × T	NS	NI	NS	NS	NS	NS	NI	NS
R × A × T	*	NI	***	**	*	*	NI	*

NS = not significant; p > 0.05; *0.05 > p > 0.01; **0.01 > p > 0.001; ***p < 0.001.

^a NI = factor not included in model. Factors were not included in the model when they did not influence the response variable, based on the log-likelihood ratio test. This test was completed for all variables.

^b The stomach region (R) was either proximal or distal; the digestion time (T) was either 20, 60, 180, 300, 480, or 720 min; the almond type (A) was either raw or roasted.

^c Total soluble protein was estimated as the amount of protein that was soluble in the Tricine extraction buffer; values presented were based on the arbitrary density units for the whole lane.

raw and roasted almonds (p < 0.05; Table 3 and Fig. 2). For example, in the 45 kDa protein band, the raw almond gastric hydrolysis in the distal region increased from 19.6 to 87.4% between 20 and 720 min of digestion. In contrast, the roasted almond gastric hydrolysis in the distal region only increased from 20.1 to 55.9% between 20 and 720 min of digestion.

In addition, the gastric hydrolysis of the total soluble protein, 45, 42, 34, 28 and 22 kDa protein bands after 720 min of digestion in raw almonds was significantly greater (p < 0.05) in the distal region of the stomach compared to the proximal region. However, differences between the proximal and distal stomach after 720 min of digestion were not observed in roasted almonds (p > 0.05). For example, after 720 min digestion, raw almonds had an 11.4% gastric hydrolysis of total soluble protein in the proximal region that was significantly lower (p < 0.05) than the gastric hydrolysis in the distal region (55.2%). In contrast, roasted almonds had similar (p > 0.05) values of gastric hydrolysis of total soluble protein (29.3 and 33.3%, respectively) in the proximal and distal stomach regions after 720 min digestion. Similarly, raw almonds in the distal stomach had a significantly (p < 0.05) greater gastric protein hydrolysis of the 45 kDa band (87.4%) compared to raw almonds in the proximal stomach (64.3%) and roasted almonds in either stomach region (57.6 and 55.9%, respectively). Similar trends were observed for the 42, 34, and 22 kDa protein bands (Table 3).

The gastric hydrolysis of the 50 and 26 kDa protein bands (Table 4) increased over time in both the proximal and distal stomach regions (p < 0.05). For both 50 and 26 kDa protein bands in the distal stomach, the gastric protein hydrolysis after 20 min (2.8 and 10.0%, respectively) significantly increased (p < 0.05) during digestion to values of 75.9 and 72.2%, respectively, after 720 min. Gastric protein hydrolysis of the 50 and 26 kDa protein bands in the proximal stomach significantly increased during digestion, although to a lesser magnitude (−10.1 to 18.3% for the 50 kDa protein band, and −1.3 to 45.2% for the 26 kDa protein band). For both the 50 and 26 kDa protein bands, raw almonds had greater gastric protein hydrolysis in the distal stomach compared to the proximal stomach (p < 0.05). Roasted almonds had greater gastric protein hydrolysis of the 50 kDa protein band in the distal stomach (p < 0.05), but similar gastric protein hydrolysis in both stomach regions for the 26 kDa protein band.

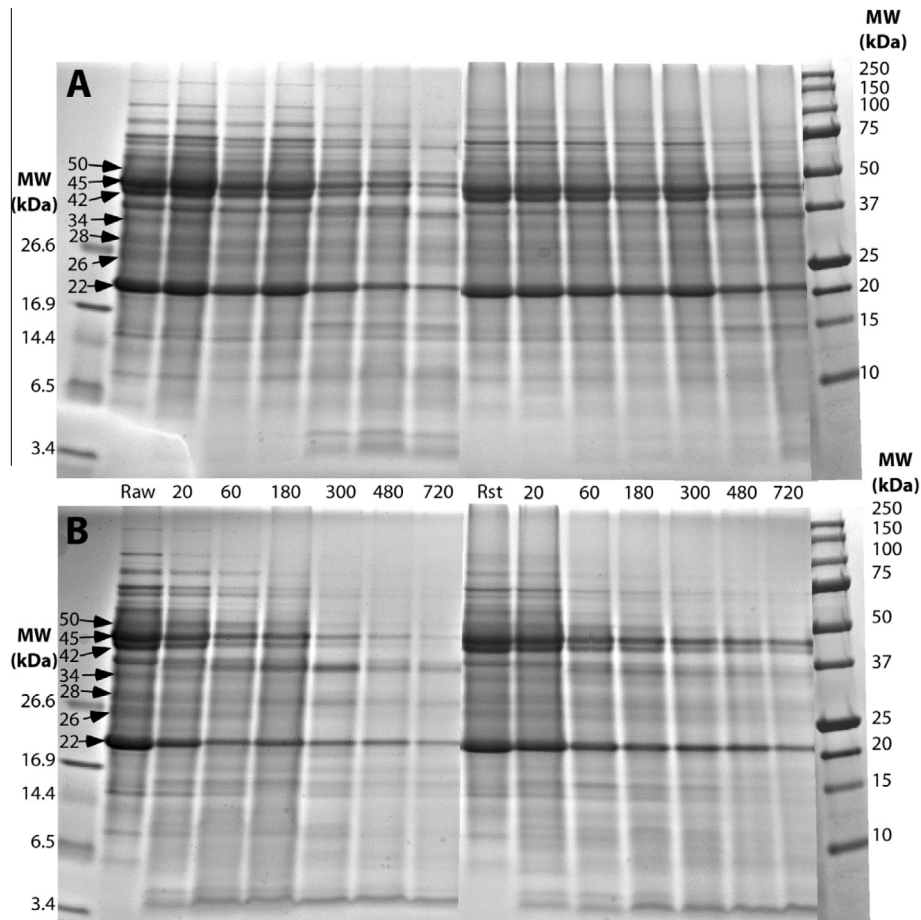


Fig. 2. SDS-PAGE patterns from proximal (A) and distal (B) gastric chyme for pigs fed raw (Raw) or roasted (Rst) almonds. Values on each edge are the molecular weight standards (kDa). The values listed between the gels are the digestion times (minutes) for each lane (both above and below). Arrows represent bands analyzed for gastric hydrolysis: 50 kDa, 45 kDa, 42 kDa, 34 kDa, 28 kDa, 26 kDa, and 22 kDa. The gel shown here is a representative example; six gels each for the proximal and distal stomach regions were completed for image analysis.

When comparing raw and roasted almonds, for both the 50 and 26 kDa protein bands, roasted almonds had a greater protein hydrolysis in the proximal stomach compared to raw almonds, while raw almonds had a greater protein hydrolysis in the distal stomach compared to roasted almonds ($p < 0.05$, Table 4). For example, in the 26 kDa protein band, the gastric protein hydrolysis of raw and roasted almonds was 8.9% (raw almonds) and 27.3% (roasted almonds) in the proximal stomach. This trend was reversed in the distal stomach, where raw almonds had 67.9% protein hydrolysis, while roasted almonds had 17.7% protein hydrolysis.

4. Discussion

The almond SDS-PAGE protein bands obtained in the present study for undigested raw and roasted almonds were similar to those reported in previous studies (Gallier & Singh, 2012; Sathe et al., 2002; Wolf & Sathe, 1998). It has been observed that almonds contain 188 different proteins, where approximately 117 of these proteins are in the range of 20–25 kDa. The dominant protein in almonds is amandin, which accounts for about 65% of the total almond protein (by mass) and has a monomeric molecular weight of 63 kDa (Mandalari et al., 2014; Wolf and Sathe, 1998). Amandin is composed of at least two major polypeptides, with an acidic α chain of 42–46 kDa and a basic β chain of 20–22 kDa linked via

disulfide bonds. Under reducing conditions, amandin is mainly composed of three major polypeptides, 45–46, 42–43 and 22 kDa (Sathe et al., 2002), which have the same molecular weight of three bands identified in Fig. 2 (45, 42, and 22 kDa). Amandin also contains several additional minor polypeptides such as 52, 36, 28 and 24 kDa (Sathe et al., 2002), which could be the other four bands identified in Fig. 2 (50, 34, 28, 26 kDa).

It is expected that the exposure time to the biochemical and physical conditions in the stomach will increase dietary protein hydrolysis. This would result in an increase in gastric hydrolysis of proteins over time, as was observed in the current study (Fig. 2, Tables 3 and 4). Similarly, other *in vitro* (Gallier and Singh, 2012; Kaur, Rutherford, Moughan, Drummond, & Boland, 2010; Mandalari et al., 2014; Zhang & Vardhanabhuti, 2014) and *in vivo* (Bouzerzour et al., 2012; Montoya et al., 2014) studies using SDS-PAGE to study dietary protein hydrolysis during gastric digestion have shown increases in protein hydrolysis as a result of increased gastric digestion time.

However, for some protein bands and the total soluble protein, negative gastric hydrolysis values were observed (Table 3), indicating that the ADU for the specific band was greater in the chyme compared to the diet sample. For the total soluble protein, the negative values may be indicative of proteins that had an initial molecular weight greater than 150 kDa (which did not appear on the SDS-PAGE gel used) that were broken down into peptides of <150 kDa, causing an increase in the total soluble proteins

Table 3

Least squares means (n = 6) of gastric hydrolysis (%) of the total soluble protein or individual protein bands in the proximal and distal region of pigs fed raw and roasted almonds during 720 min of gastric digestion.

Band ID (kDa)	Digestion time (min)	Raw		Roasted		SEM ^a
		Proximal	Distal	Proximal	Distal	
Total soluble protein ^b						7.7
	20	-19.3 ^b	-5.4 ^c	1.2 ^b	-5.3 ^c	
	60	-4.0 ^{ab}	-6.0 ^c	5.3 ^b	8.1 ^{bc}	
	180	12.5 ^a	20.4 ^b	2.4 ^b	18.0 ^{abc}	
	300	9.4 ^{ab,y}	43.3 ^{a,z}	12.4 ^{ab,y}	21.9 ^{ab,y}	
	480	8.0 ^{ab,y}	53.2 ^{a,z}	22.3 ^{ab,y}	25.3 ^{ab,y}	
	720	11.4 ^{ax}	55.2 ^{a,z}	29.3 ^{ayx}	33.3 ^{ay}	
45						5.7
	20	-3.9 ^d	19.6 ^d	7.4 ^c	20.1 ^c	
	60	6.9 ^{d,y}	20.9 ^{d,zy}	12.6 ^{c,y}	31.9 ^{bc,z}	
	180	34.6 ^{c,zy}	49.9 ^{c,z}	20.2 ^{c,y}	40.7 ^{b,z}	
	300	39.4 ^{bc,yx}	75.2 ^{b,z}	26.0 ^{bc,x}	45.1 ^{ab,y}	
	480	53.7 ^{ab,y}	84.8 ^{ab,z}	43.8 ^{ab,y}	45.7 ^{ab,y}	
	720	64.3 ^{ay}	87.4 ^{a,z}	57.6 ^{ay}	55.9 ^{ay}	
42						6.8
	20	-9.7 ^{c,zy}	11.3 ^{d,z}	-0.8 ^{c,zy}	-12.8 ^{c,y}	
	60	6.2 ^{bc}	8.0 ^d	-1.8 ^c	3.0 ^{bc}	
	180	20.7 ^{b,zy}	37.7 ^{c,z}	0.4 ^{bc,y}	15.2 ^{ab,y}	
	300	21.2 ^{b,y}	70.7 ^{b,z}	3.8 ^{bc,y}	19.7 ^{ab,y}	
	480	27.2 ^{ab,y}	83.1 ^{a,z}	24.7 ^{ab,y}	15.1 ^{ab,y}	
	720	44.2 ^{ay}	86.1 ^{a,z}	41.7 ^{ay}	29.8 ^{ay}	
34						10.6
	20	-16.0 ^b	15.0 ^d	0.4 ^b	18.7 ^b	
	60	-0.1 ^{ab}	17.0 ^d	10.6 ^b	34.1 ^{ab}	
	180	17.6 ^{ab}	43.5 ^{cd}	15.0 ^b	37.2 ^{ab}	
	300	17.9 ^{ab,y}	58.0 ^{bc,z}	22.8 ^{b,y}	40.3 ^{ab,zy}	
	480	26.4 ^{ay}	71.2 ^{ab,z}	57.6 ^{a,z}	48.2 ^{ab,zy}	
	720	37.1 ^{ay}	82.9 ^{a,z}	62.5 ^{ay}	58.9 ^{ay}	
28						9.8
	20	-5.7	12.4 ^c	0.6	6.9 ^b	
	60	8.4	9.4 ^c	10.8	11.2 ^b	
	180	18.1 ^y	56.7 ^{b,z}	19.8 ^y	23.3 ^{b,y}	
	300	12.4 ^y	74.1 ^{a,z}	14.6 ^y	27.2 ^{b,y}	
	480	-0.6 ^x	77.4 ^{a,z}	1.9 ^{yx}	33.1 ^{ab,y}	
	720	-2.9 ^x	76.0 ^{a,z}	4.7 ^x	51.7 ^{ay}	
22						6.0
	20	-6.0 ^d	21.7 ^d	4.8 ^c	15.9 ^c	
	60	12.1 ^d	26.2 ^d	10.5 ^c	27.5 ^{bc}	
	180	36.2 ^{c,zy}	49.5 ^{c,z}	16.8 ^{c,y}	43.2 ^{ab,z}	
	300	38.7 ^{bc,yx}	59.7 ^{bc,z}	23.3 ^{bc,x}	47.7 ^{a,zy}	
	480	54.7 ^{ab,y}	69.8 ^{ab,z}	43.2 ^{ab,y}	46.9 ^{ay}	
	720	61.8 ^{ay}	79.7 ^{a,z}	49.8 ^{ay}	52.2 ^{ay}	

Values with different letters in each column (abcd) represent significant differences ($p < 0.05$) within each stomach region across the digestion times. Values with different letters in the same row (zyx) represent significant differences ($p < 0.05$) within each postprandial time across each stomach region and almond type interaction.

^a SEM: pooled standard error of the mean.

^b Total soluble protein was estimated as the amount of protein that was soluble in the Tricine extraction buffer; values presented were based on arbitrary density units for the whole lane.

analyzed. In the other protein bands, negative values could be indicative of larger proteins being broken down, resulting in peptides of similar molecular weight, and increasing the band intensity. However, the negative values of gastric hydrolysis were not observed for all treatments, and were primarily observed after 20 min of digestion. Furthermore, some of the negative values may have been a result of measurement error; for example, -3.9% gastric hydrolysis (45 kDa, raw almonds, proximal stomach, 20 min) with an SEM of 5.7 is not significantly different from zero.

In addition to hydrolysis, proteins are also leaving the stomach via gastric emptying into the small intestine. It has been previously reported by Bornhorst, Roman, Rutherford et al. (2013) that after

Table 4

Least squares means (n = 6) of gastric hydrolysis (%) of 50 and 26 kDa protein bands in the proximal and distal region of pigs fed raw and roasted almonds during 720 min of gastric digestion. The values presented are overall means for each two-way interaction; the three-way interaction between the stomach region-digestion time-almond type was not significant for these protein bands.

Stomach region	50 kDa		26 kDa		
	Proximal	Distal	Proximal	Distal	
Digestion time (min)					
	20	-10.1 ^b	2.8 ^d	-1.3 ^b	10.0 ^d
	60	-0.3 ^{ab,y}	8.3 ^{d,z}	6.9 ^b	18.8 ^{cd}
	180	6.7 ^{ab,y}	38.3 ^{c,z}	11.6 ^{b,y}	42.4 ^{bc,z}
	300	2.9 ^{ab,y}	59.0 ^{b,z}	15.0 ^{b,y}	52.1 ^{b,z}
	480	15.5 ^{ab,y}	68.1 ^{ab,z}	26.7 ^{ab,y}	64.3 ^{ab,z}
	720	18.3 ^{ay}	75.9 ^{a,z}	45.2 ^{ay}	72.7 ^{a,z}
SEM ^a		1.9		2.8	
Almond type					
Raw		-6.7 ^{b,y}	54.0 ^{a,z}	8.9 ^{b,y}	67.9 ^{a,z}
Roasted		17.2 ^{ay}	37.1 ^{b,z}	27.3 ^a	17.7 ^b
SEM ^a		1.1		1.3	

Values with different letters in each column (abcd) represent significant differences ($p < 0.05$) within each stomach region across the digestion times. Values with different letters in the same row (zyx) represent significant differences ($p < 0.05$) within each postprandial time across each stomach region for each band.

^a SEM: pooled standard error of the mean.

consuming the same meals of raw or roasted, diced almonds considered in the present study, raw almonds had significantly greater gastric emptying of protein (54 vs. 50% protein emptied after 12 h digestion for raw and roasted almonds, respectively; Table 1). All variables analyzed in the current study were significantly influenced by the covariate of protein gastric emptying ($p < 0.05$). Overall, raw almonds had greater gastric protein hydrolysis (Table 3) and a faster rate of gastric protein emptying (Table 1), suggesting that faster protein hydrolysis may lead to increased gastric emptying rate. It has been previously reported by Montoya et al. (2014) that there was a significant negative correlation between the gastric digestion of dietary protein and dry matter retained in the stomach in rats after consumption of meals with several protein sources (e.g. beef muscle protein, gelatin, soy). This may be related to the fact that dietary proteins are a potent stimulus of cholecystokinin, as well as other hormones that will cause a reduction in gastric emptying rate. Both single amino acids and peptides of varying sizes are capable of inducing gut hormone secretion through different sensory pathways (Psichas, Reimann, & Gribble, 2015). The results from this study suggest that there is a relationship between gastric protein hydrolysis and gastric emptying of protein that merits future investigation, as this relationship may vary based on food structure and composition.

In addition to the residence time (e.g. gastric emptying rate), the specific biochemical environment (e.g. amount of acid and enzymes) that almonds are exposed to in the stomach will be a controlling factor in their hydrolysis. However, the biochemical environment in the stomach is neither constant nor uniform throughout the stomach or over digestion time. It has been previously reported that for the same meals of raw or roasted diced almonds, almond type had no significant influence on gastric pH, but the pH was significantly influenced by gastric region (proximal vs. distal; Fig. 1). The average pH of raw and roasted almonds ranged from 6.5 to 4.0 in the proximal stomach region and from 4.8 to 2.0 in the distal stomach region between 20 min and 720 min of gastric digestion, respectively (Bornhorst, Roman, Rutherford et al., 2013).

The lower pH in the distal stomach may have influenced the significantly greater gastric protein hydrolysis values observed in the distal stomach compared to the proximal stomach, especially in raw almonds. For example, after 720 min of digestion, raw

almonds had 44% greater gastric hydrolysis of total soluble protein in the distal region compared to the proximal region. When comparing the 45, 42, 34, 28, and 22 kDa protein bands, raw almonds had an average of 42% greater gastric hydrolysis in the distal region compared to the proximal region after 720 min of digestion. The greater gastric hydrolysis observed in the distal region may have been a result of increased pepsin activity, in addition to the lower pH conditions. Pepsin works most effectively at a pH of 2 and its activity drops to less than 50% at pH values greater than 3, and it is rapidly inactivated above pH 5–6 (Kondjoyan, Daudin, & Santé-Lhoutellier, 2015; Piper & Fenton, 1965; Pletschke, Naudé, & Oelofsen, 1995).

Although there were differences in the biochemical environment between the proximal and distal stomach regions after consumption of almond meals, similar pH profiles were observed in each region between raw and roasted almonds (Bornhorst, Roman, Rutherford et al., 2013). However, almond type significantly influenced gastric protein hydrolysis for the total soluble protein and all protein bands in the distal stomach, except 34 kDa. This suggests that there are factors other than the biochemical environment that influence the gastric protein hydrolysis in raw and roasted almonds.

Raw and roasted almonds had the same SDS-PAGE protein profile prior to digestion (Fig. 2), but in general, raw almonds had a greater amount of gastric protein hydrolysis compared to roasted almonds. For example, differences between raw and roasted gastric protein hydrolysis can be observed after 720 min of gastric digestion in the distal stomach, where the gastric hydrolysis of the 45 kDa protein band was 87.4% for raw almonds compared to 55.9% for roasted almonds. Similar trends were observed for the other protein bands and the total soluble protein (Table 3, Fig. 2). The differences in gastric protein hydrolysis observed between the raw and roasted almond meals are likely due to protein structural modifications that occur in almonds during roasting.

Removal of moisture from almonds during roasting results in modifications to their macro- and micro-structure. As moisture is removed from almond kernels, cells detach and the pore volume and porosity increases (Perren and Escher, 2013). Previous studies of almond microstructure have indicated that after roasting (with hot air), there are significant damages to the epidermis and the almond tissue, resulting in cell separation, loss of cellular shape, destruction of the endoplasmic network, distortion of protein bodies, and increases in number and size of oil bodies (Altan et al., 2011; Perren and Escher, 2013; Varela, Chen, Fiszman, & Povey, 2006). In addition, thermal treatment will impact the structure of the almond proteins. At heating temperatures greater than 80 °C, the secondary and tertiary structure of almond proteins is irreversibly modified. These modifications may include intermolecular interactions, which may lead to aggregation as well as reactions with reducing sugars and amine groups (Davis & Williams, 1998). Previous studies have indicated significantly greater amounts of advanced glycation end products in roasted almonds compared to raw almonds (Zhang, Huang, Xiao, & Mitchell, 2011), suggesting that the thermal treatment during roasting modifies the protein structure, allowing for such reactions to take place.

These studies suggest that modifications in almond macro-, micro-, and protein structure may partially explain the differences in gastric protein hydrolysis observed between raw and roasted almonds. Therefore, it is likely that the decreased gastric protein hydrolysis in roasted almonds was the result of decreased penetration into the almond matrix by gastric secretions and resistance to hydrolysis by almond proteins, both as a result of structural disruption and modification across various structural length scales. A systematic study regarding changes in macro-, micro-, and protein structure in relation to protein hydrolysis during gastric digestion is warranted to explain the presently observed results.

5. Conclusions

The gastric hydrolysis of almond proteins increased as a result of gastric digestion time in growing pigs that consumed a meal of raw or roasted almonds. The gastric emptying rate of protein was a significant covariate in the gastric hydrolysis of almond proteins. The lower pH conditions in the distal stomach resulted in faster gastric protein hydrolysis, particularly in raw almonds. In general, consumption of raw almonds resulted in greater gastric protein hydrolysis for all analyzed protein bands compared to roasted almonds. Decreases in roasted almond gastric protein hydrolysis were likely related to protein structural changes that occurred during roasting.

Conflict of interest

The authors declare no conflicts of interest in the completion and analysis of the presently reported work.

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