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Acid hydrolysis of gelatin extracted from cow skin: properties and potential for use as a source of small peptides and free amino acids for broiler chickens

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Abstract

Context. Acid hydrolysis of animal and plant protein ingredients can generate beneficial and cost-effective peptides and free amino acids for use in livestock feed.

Aims. The aim was to determine whether a cow-skin gelatin (CSG) hydrolysate, rich in low-molecular-weight peptides and free amino acid and produced through acid (4 M HCl) hydrolysis, improved the digestibility of CSG and performance of broilers.

Methods. Day-old Ross 308 chicks ($n = 320$) were allocated to four treatments (control, maize–soy basal diet; CSG at 92 g/kg basal diet; acid-hydrolysed CSG (AHCSG) at 45 and 92 g/kg basal diet) in a completely randomised design with eight replicate pens per treatment and 10 chicks in each pen. Molecular weights of CSG and AHCSG were characterised by SDS–PAGE, and protein concentration and degree of hydrolysis of AHCSG determined. Broilers were assessed for performance measures and intestinal and serum characteristics.

Key results. The AHCSG had a protein concentration of 84.3%, with a degree of hydrolysis of 66.5%. Respective molecular weights of CSG and AHCSG were 20–128 kDa and 3.5–10 kDa. Bodyweight gain and feed intake were dramatically ($P \leq 0.001$) reduced and feed conversion ratio increased with inclusion of CSG and especially AHCSG in the diet. Inclusion of AHCSG reduced ($P < 0.05$) ileal digesta viscosity compared with CSG, and reduced ($P < 0.05$) plasma uric acid concentration, villi height and crypt depth compared with the CSG and control diets. AHCSG inclusion in the diet reduced protein digestibility by ~25% (at 45 g/kg) and 50% (at 92 g/kg) compared with the control; CSG also reduced protein digestibility by ~50%. The *Salmonella* population of ceca was reduced ($P = 0.05$) with the diet containing 92 g AHCSG/kg compared with the CSG and control diets. Trypsin activity was not affected by diet, but total alkaline protease activity was reduced ($P < 0.01$) at days 35 of age by inclusion of AHCSG in the diet. Carcass and breast relative weight, and breast fillet crude protein were lower ($P < 0.05$) for broilers fed diets containing CSG or AHCSG than the control.

Conclusions. Acid hydrolysis of CSG produced large quantities of free amino acids and small peptides, but this did not improve the performance of broiler chickens compared with CSG. Performance was similarly poor with dietary inclusion of AHCSG and CSG relative to the control diet without CSG products.

Implications. Although large quantities of free amino acids and small peptides were produced by acid hydrolysis of CSG, further research is needed to understand the possible problems with acid hydrolysis and why it negatively affects broiler performance.

Key words: acid, alkaline, carcass, digestibility, free amino acids, gelatin, hydrolysis, peptide.

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Introduction

Hydrolysis of animal and plant protein ingredients is widely used to produce easily digestible, high-quality peptides and free amino acids for human and animal consumption (Udenigwe and Aluko 2012; Álvarez *et al*. 2013; Agyei *et al*. 2015; Hou *et al*. 2017). Vijayalakshmi *et al*. (1986) suggested that protein hydrolysates, which contain low-molecular-weight peptides and free amino acids, could be valuable nutritional and therapeutic ingredients for diet

formulation. Álvarez *et al*. (2013) reported that alkaline hydrolysis of porcine blood proteins recovered from animal waste is an easy way to produce small peptides and free amino acids for animal feed. Bioactive peptides are small protein fragments that can be produced by using enzymatic, acid or alkaline hydrolysis (Chatterjee *et al*. 2018) or fermentation (Singh *et al*. 2014). These small peptides are suitable as a carrier for drugs and vitamins and are easily absorbed through the walls of the small intestine into the bloodstream (Aluko 2012; De Angelis *et al*. 2017; Chatterjee *et al*. 2018). Khalaji *et al*. (2016) indicated that the demand for protein and amino acids in poultry rations is increasing. They proposed that use of suitable alternative protein sources is fundamental to improving amino acid balance in broiler chicken diets and reducing the amount of synthetic amino acids required.

Byproducts from slaughterhouses account for almost one-third of the total liveweight of cattle, poultry and pigs (Rendueles *et al*. 1997; Apple *et al*. 1999), mostly as skin (Carpenter *et al*. 1961). Isolation and hydrolysis of proteins from slaughterhouse byproducts might enable production of profitable peptides and free amino acids for use as animal feed (Álvarez *et al*. 2009, 2013). One of the main, and valuable, proteins recovered from animal skin, bones, tendons and ligaments through acid or alkaline extraction is gelatin (Khiari *et al*. 2013), constituting ~66–79% of the protein (Khalaji *et al*. 2016; Beyranvand *et al*. 2019), which could be an alternative protein to soybean meal in broiler rations. However, previous trials have demonstrated that gelatin from cow skin contains considerable amounts of high-molecular-weight peptides. This limits its potential level of inclusion in chicken diets because of increased digesta viscosity and depression of nutrient digestion (Johnston-Banks 1990; Khalaji *et al*. 2016). Cross-links present in gelatin are found to correlate strongly with its viscosity (Sims and Bailey 1992). Processing the gelatin from skin to low-weight peptides and free amino acids by using a low-cost acid or alkaline hydrolysis might improve its functional and nutritional properties. However, both types of hydrolysis (acid or alkaline) may completely destroy or change the structure of several amino acids (Fountoulakis and Hans-Werner 1998; Ravindran and Bryden 2005). Álvarez *et al*. (2013) recommended performing alkaline and acid hydrolyses separately to an optimum pH as a way to collect large amounts of peptides and free amino acids after the hydrolysis process and to ensure protein and amino acid quality. The application of acid and alkaline processing to high-molecular-weight proteins has several beneficial effects such as obtaining profitable peptides and free amino acids (Álvarez *et al*. 2013), healthcare and cosmetics (Bautista *et al*. 2000; Clemente 2000), and nutritional and therapeutic values (Vijayalakshmi *et al*. 1986).

In view of the above, it is hypothesised that the hydrolysis of cow-skin gelatin (CSG) through an acid or alkaline process could cleave the cross-links of the high-molecular-weight proteins associated with viscosity, enhancing the production of low-weight peptides and free amino acids, to make the product suitable for inclusion in chicken diets. Based on previous work in our laboratory (Khalaji *et al*. 2016; Beyranvand *et al*. 2019; Barshan *et al*. 2019), the objective of the present study was to refine the acid hydrolysis of gelatin, and then evaluate its application in broiler chicken diets.

Materials and methods

Experiments were conducted based on procedures and guidelines approved by the Animal Care Committee of the Iranian Council of Animal Care (1995).

Gelatin extraction and hydrolysis

Fresh cow skin was collected from the slaughterhouse (dame Zanjan Morvarid Co. Zanjan, Iran) , and washed twice through a water system to remove contaminants, and then the gelatin was prepared as described by Ulfa *et al*. (2015).

The gelatin (100 g) was first processed in a Moulinex blender (Group SEB, Écully, France) into powder and mixed with distilled water at a ratio of 1 : 2 (w/v). The mixture was heated at 85°C for 20 min for inactivation of endogenous enzymes, and then samples were cooled to room temperature for 30 min before homogenisation in a centrifuge (D-7200; Andreas Hettich, Tuttlingen, Germany) at 10 000*g* for 20 min at 4°C, and collection of precipitate.

Acidic hydrolysis of cow-skin gelatin

The precipitate was mixed with 4 M HCl (240 mL) and boiled in a 500-mL closed glass bottle for 6 h at 110°C. The mixture was cooled at room temperature and neutralized to pH 6.5 with 4 M NaOH. The samples were centrifuged at 10 000g for 15 min at 4°C and the supernatant was collected as protein hydrolysate and freeze-dried. The method of protein hydrolysate production from CSG is given in Fig. 1.

Hydrolysate chemical characterisation

Degree of hydrolysis

The degree of hydrolysis (DH) after acidic treatment was defined as the percentage of peptide bonds cleaved with respect to the total number of peptide bonds. This was estimated according to the method described by Hoyle and Merritt (1994). Briefly, one volume of 20% trichloroacetic acid (TCA) was added to the supernatant, followed by centrifugation at 6000*g* at 4°C for 20 min to collect the 10% TCA-soluble material. The DH was calculated as:

$$\mathrm{DH}(\%) = (10\%\ \mathrm{TCA} - \text{soluble nitrogen in the sample}/\text{total nitrogen in the sample}) \times 100.$$

Proximate composition analysis

The composition of protein hydrolysate was determined according to standard procedures (AOAC International 2000). Moisture content was determined by drying samples in an oven at 105°C until a constant weight was achieved. The crude protein (CP) and lipid content of CSG and acid-hydrolysed CSG (AHCSG) were determined, respectively, by the Kjeldahl method (954.01), and by Soxhlet fat analysis after 3 N HCl acid hydrolysis (method 920.39) or oven drying (60°C for 72 h). Ash content was determined after incineration in a muffle furnace at 550°C for 5 h. Total phosphorus content of

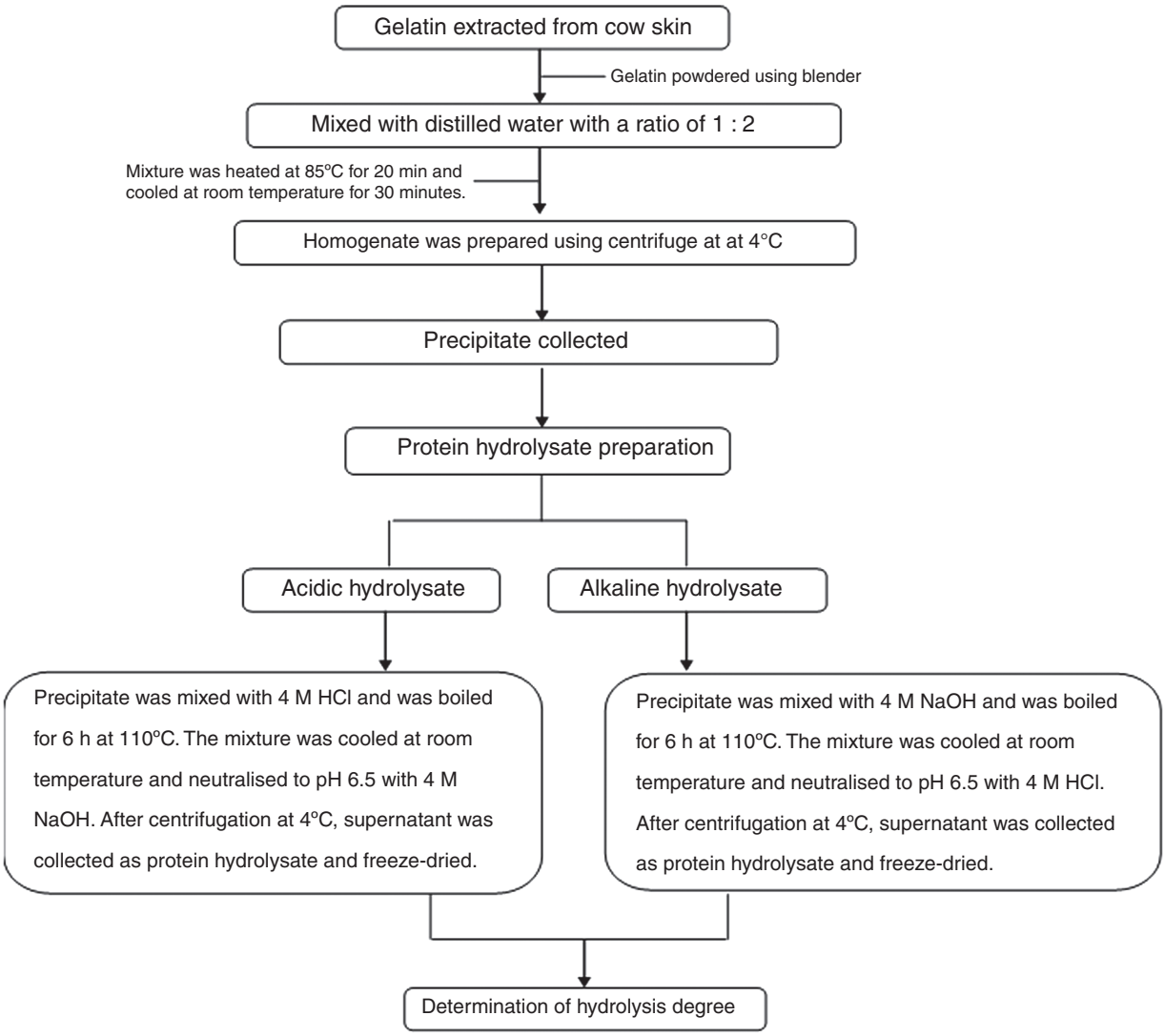

Fig. 1. Scheme of protein hydrolysate preparation from cow skin gelatin, using hydrochloric acid.

the samples was analysed by the ammonium molybdate method (method 946.06) and measured at 340 nm in a spectrophotometer (model 2150; UNICO, Dayton, NJ, USA). The calcium content of the samples was quantified by triethanolamine (50%) after digestion by perchloric acid. The nutrient compositions of CSG and AHCSG are given in Table 1.

Amino acid composition

Amino acid composition of samples, including CSG and AHCSG as well as formulated diets, were assayed using high performance liquid chromatography (HPLC) (method 982.30 E (a, b, c); AOAC International 2000) after hydrolysis with 6 M HCl at 110°C for 12 h and derivatisation using *o*-phthaldialdehyde. Total amino acids and free amino acids were analysed by using a Kinetex EVO C18 HPLC column (150 by 4.6 mm, particle size 5 µm, acetonitrile; Phenomenex, Torrance, CA, USA) at a flow rate of 1 mL/min with UV detection. Breeze 2 software was used for data analysis.

Electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with a 4% stacking gel and 12% separation gel was used to analyse protein composition of each protein hydrolysate as described by Laemmli (1970). Processed samples were mixed in Tris buffer (0.0625 M Tris HCl, pH 6.8, containing 2% SDS, 10% (v/v) glycerol and 0.5% (v/v) β-mercaptoethanol) at a ratio of 3 : 1 (v/v). Samples were heated at 100°C for 10 min before loading in the gel. SDS–PAGE was run at 15 mA, using a vertical electrophoresis system (Vertical Electrophoresis System; Bio-Rad Laboratories, Hercules, CA, USA). Proteins from the crude extract and acid hydrolysis were stained with 0.1% Coomassie Blue G-250 in

Table 1. Degree of hydrolysis and nutrient composition of cow-skin gelatin (CSG) before and after acid hydrolysis (AHCSG)

Analysed composition (%)	CSG	AHCSG
Degree of hydrolysis	–	66.50
Dry matter	95	98
Ash	24	17
Calcium	4.32	3.13
Total phosphorus	4.76	5.10
Crude protein	77.6	84.3
Amino acid:		
Aspartic acid	1.45	0.18
Glutamic acid	4.56	0.71
Hydroxyproline	5.31	2.27
Serine	0.13	2.44
Histidine	0	9.50
Glycine	21.25	21.02
Alanine	2.56	0.82
Arginine	1.92	0.10
Proline	28.30	11.09
Tyrosine	0.18	0.07
Valine	1.37	0.24
Methionine	1.62	0.25
Cysteine	0	2.13
Leucine	2.36	1.05
Isoleucine	3.80	1.19
Tryptophan	0	0.44
Phenylalanine	1.22	7.63
Lysine	3.00	22.88

35% methanol and 7.5% acetic acid. Destaining was performed with 35% methanol and 7.5% acetic acid. A molecular weight standard over the range 3.5–235 kDa was used to identify protein composition of the hydrolysates.

Husbandry and diet preparation

Day-old Ross 308 male broiler chicks (n = 320) of average weight 44 ± 2 g were used in the feeding experiment. Chicks were purchased from a breeder flock (age 45 weeks) and were weighed individually and by pen for equal weight distribution, and placed into 32 floor pens (1.2 m by 1 m) bedded with chick paper and provided with a pan feeder and a bell drinker. Chicks were vaccinated at the hatchery for infectious bronchitis (Nobilis IB 4-91) and Newcastle disease (Nobilis MA5 + Clone 30; Intervet International, Boxmeer, The Netherlands). Chicks were divided into four treatment groups, each consisting of eight replicates, with 10 chicks per pen, in a completely randomised design. Chickens were fed on mash diet *ad libitum* and had access to drinking water free from pathogens during the entire experimental period.

A chicken starter and grower diet was formulated based on maize and soybean meal (control), and three experimental treatment feeds were prepared from these basal diets by inclusion of AHCSG at 45 and 92 g/kg and CSG at 92 g/kg. Feeds based on starter diet were fed during 1–21 days of age, and those based on grower diet during 22–35 days of age to meet the nutritional requirements of the chicks (Table 2). The diet ingredients were ground with a hammer mill (horizontal rotor hammer mill; Changzhou Farthest Machinery, Liyang City, Jiangsu, China) fitted with a 2-mm screen for the starter diet and a 4-mm screen for grower diet. The particle-size distribution and geometric mean diameter of starter diet (1500 µm, range 720–3000 µm) and grower diet (2400 µm, range 755–4600 µm) were determined in triplicate according to the recommended procedure of ASABE (2008), using a sieve shaker (Analysette 3 Spartan; FRITSCH, Idar-Oberstein, Germany). Two batches of each experimental feed (150 kg for 1–21 days of age and 300 kg for 22–42 days of age) were mixed with a horizontal twin-shaft paddle mixer with 800 kg capacity. The formulated diets were analysed for CP (Kjeldahl; method 954.01), amino acids (HPLC; method 982.30 E (a, b, c); AOAC International 2000), and calcium and phosphorus as described above. Daily feed allocation for chicks in each pen was calculated as total amount of feed placed in the feeder minus the residual feed in the feeder.

Chicks were brooded by using two gas heaters, and litter surface temperature reached 28–31°C before arrival of the chicks; then the temperature was gradually decreased from 31°C to 24°C. The light schedule during the whole experimental period was set at cycle of 22 h light, 2 h dark. Feed intake and bodyweight (BW) were measured weekly and mortality recorded daily. Average feed intake and BW were corrected for mortality when calculating feed conversion ratio (FCR) for each pen.

Blood characteristics

At 14 and 35 days of age, blood samples were collected into heparinised tubes by puncturing the brachial vein of two randomly selected chicks from each pen. Samples were immediately centrifuged for 10 min at 3000*g* at 20°C, and then frozen at –20°C. The serum was analysed for uric acid (Fossati *et al*. 1980) and urea-nitrogen (Mathies 1964) by using commercial reagent kits (Pointe Scientific, Canton, MI, USA).

Ileal digesta viscosity

Ileal digesta viscosity was measured on Day 35 by collecting and pooling the ileal contents of two chicks from each pen. Two bird from each pen (16 birds per treatment) were randomly selected, weighed, and then humanely killed by cervical dislocation. The viscosity of samples was assayed according to the procedure suggested by Bedford and Classen (1993). A Brookfield digital viscometer (model DV2T; AMETEK Brookfield, Middleboro, MA, USA) at a speed rate of 200 rpm and shear rate 60 s^{-1} at 25°C was used for determining the viscosity. Data were collected using the optional Rheocalc T software (AMETEK Brookfield).

Ileal morphology

On Day 35, the two chicks from each pen that had been slaughtered so that ileal content could be collected were used for assessment of ileal morphology. A 2-cm segment of the middle of the ileum was washed in physiological saline solution, then fixed in 10% buffered formalin, and the

Table 2. Ingredients and composition of broiler diets without (control) or with cow-skin gelatin (CSG) or acid-hydrolysed CSG (AHCSG)
CP, crude protein

	Days 1–21				Days 22–35			
	Control	CSG	AHCSG		Control	CSG	AHCSG	
		92 g/kg	45 g/kg	92 g/kg		92 g/kg	45 g/kg	92 g/kg
Ingredients (g/kg)								
Maize	506.1	608	576	605	548.5	669	655	684
Soybean meal (44% crude protein)	417	0	0	0	375	0	0	0
Soybean oil	35	0	0	0	40	20	10	18
Maize gluten meal (50% crude protein)	0	170	263	185	0	125	215	130
Wheat bran	0	79	77.8	78.5	0	49	412	41
CSG	0	92	0	0	0	92	0	0
AHCSG	0	0	45	92	0	0	45	92
Dicalcium phosphate	18	18	18	18	16	16	16	16
Limestone	10.5	10.5	10.5	10.5	9	9	9	9
Sodium chloride	3.5	3.5	3.5	3.5	3.5	3.5	3.4	3.4
Vitamin and mineral premix[A]	5	5	5	5	5	5	5	5
DL-Methionine (99%)	3	2	0.8	1.5	2	2	0.4	1.2
L-Lysine HCl (78%)	1.4	11	0	0	0.5	9	0	0
L-Threonine	0.5	1	0.5	1	0.5	0.5	0	0.4
Calculated composition (g/kg)								
Metabolisable energy (MJ/kg)	11.84	12.23	12.13	12.21	12.96	12.93	12.88	12.97
Total phosphorus	7.5	7.5	7.5	7.5	6.8	6.5	6.5	6.2
Available phosphorus	5	5	5	5	4.5	4.3	4.3	4.3
Calcium	10	10	10	10	9.0	8.7	8.7	8.7
Analysed composition (g/kg)								
Crude protein	220	226	221	225	207	205	199	201
Lysine	14.82	14.67	14.84	24.84	12.99	12.61	13.11	24.21
Methionine	6.68	7.21	5.94	5.66	5.48	6.57	4.89	4.60
Methionine + cysteine	10.64	10.43	11.06	10.97	9.21	9.29	9.51	9.33
Threonine	9.32	6.71	8.05	7.01	7.28	5.78	6.61	5.32
Arginine	17.17	8.23	8.21	6.85	15.80	7.23	7.20	5.69
Tryptophan	2.93	1.4	1.50	1.49	2.69	0.86	1.32	1.28
Isoleucine	10.75	9.75	9.11	7.80	9.93	8.62	7.95	6.46
Leucine	20.64	26.13	33.57	26.46	19.50	21.85	29.16	21.31
Valine	11.74	8.06	10.48	8.48	10.92	6.76	9.16	6.93
Total phosphorus	8.17	7.75	6.94	7.12	6.64	6.41	7.20	6.93
Calcium	10.09	11.02	10.3	11.5	8.02	9.02	8.14	9.00

[A]Supplying the following (per kg diet): trans-retinol, 13 mg; cholecalciferol, 0.5 mg; α-tocopherol acetate, 80 mg; menadione, 3 mg; thiamine, 3 mg; riboflavin, 8 mg; pyridoxine, 5 mg; cyanocobalamin, 0.024 mg; nicotinic acid, 60 mg; folic acid, 2 mg; calcium pantothenate, 15 mg; choline, 1000 mg; manganese, 120 mg; zinc, 1100 mg; copper, 16 mg; selenium, 0.3 mg; iodine, 1 mg; iron, 40 mg.

fixing solution was changed twice at daily intervals for fixation. A single 0.5-cm sample was cut from each ileal section, dehydrated using increasing ethanol concentrations, cleared with xylene, and placed into PolyFin paraffin embedding wax (Polysciences, Warrington, PA, USA). Tissue sections (2 μm) were cut by microtome (EasyCUT 202; Milestone Medical, Sorisole, Italy), floated onto slides, and stained with hematoxylin solution (Gill No. 2) and eosin (Sigma-Aldrich, St. Louis, MO, USA). Villus height and crypt depth were measured on light-microscopy images (SMZ-140; Motic, HongKong) taken using a digital camera. Fifteen images from five tissue sections of each ileal section were taken, and measurements were made using imaging software. Measurements for villus heights were taken from the tip of the villus to the valley adjacent to villus–crypt junction and average height of villi was used for final analysis.

Total-tract protein digestibility

On Day 29, two chicks from each pen were randomly selected and transferred to the corner of the pen (floor space 50 cm^2) separated from the rest of the pen by a net fence, and were provided with a tube feeder and an automatic bell drinker. Chicks acclimated for 2 days in the new environment and then feed was removed from feeders for 12 h to allow intestinal emptying. The experimental diets were offered to the birds *ad libitum* for 48 h and total excreta output was collected in trays over 72 h, with output recorded and sampled every 24 h. Non-excreta material such as feathers was removed before drying excreta at 50°C inside an incubator (Memmert GmbH + Co, Schwabach, Germany). Feed intake was calculated as the total amount of feed placed in feeder minus residual feed. Excreta and feed samples were ground through a

1-mm screen. Samples were then analysed for dry matter content following drying at 105°C for 24 h and CP as described above. Digestibility was calculated using feed intake, protein content of the diet, and excreta output as follows:

$$\text{Digestibility}(\%) = ((\text{CP}_{\text{diet}} - \text{CP}_{\text{excreta}})/\text{CP}_{\text{diet}}) \times 100.$$

Cecal microflora population

On Day 35, two other birds from each pen were slaughtered by using the same euthanasia procedure and 1-g samples of cecal contents were pooled. Microbial populations were determined by serial dilution (10^{-5}–10^{-6}) of cecal samples in anaerobic diluents (saline solution 9%) before inoculation in Petri dishes with sterile agar as described by Bryant and Burkey (1953). *Salmonella Shigella* agar was used to culture *Salmonella*, and MacConkey agar to culture coliforms and *E. coli* (Liofilchem, Abruzzi, Italy). Plates were incubated at 37°C and counts were performed at 24–48 h after inoculation. Colony forming units were counted immediately after removal from the incubator (Memmert).

Digestive enzyme activity

Digestive enzyme activities were determined at 14 and 28 days of age. Two chicks from each pen were chosen randomly and weighed, then feed was removed from feeders for 6 h to permit intestinal emptying. The chicks were then slaughtered by cervical dislocation. The duodenum and a 10-cm segment of the jejunum adjacent to the distal pancreas, free of residual food, were removed and frozen in liquid nitrogen, until preparation for assay. The frozen intestine was partially thawed in a refrigerator at 4°C for 2 h. The samples were homogenised (dilution 1 : 5, w/v) in cold buffer (50 mM Tris HCl, pH 8.0, containing 10 mM $CaCl_2$) on ice at 11 000 rpm for 2 min. Thereafter, the homogenate was centrifuged at 14 000*g* for 45 min at 4°C. The supernatant was collected, and aliquots were stored at –80°C until analysis of digestive enzymes. Trypsin (EC 3.4.21.4) activity was determined by using *N*-alpha-benzoyl-L-arginine 4-nitroanilide hydrochloride as the substrate according to the method of Erlanger *et al.* (1961). One unit of activity was defined as the enzyme releasing 1 μmol *p*-nitroaniline/min (measured at 410 nm). Total alkaline protease activity was determined based on the casein hydrolysis assay of Kunitz (1947) as modified by Walter and Schutt (1974). One unit of activity was defined as the amount of enzyme needed to produce 1 μmol tyrosine/min (measured at 280 nm). The concentration of soluble protein in extracts was determined by the method of Lowry *et al.* (1951), using bovine serum albumin (0–1 mg/mL) as a standard at 750 nm. Enzyme activities are expressed in units/mg soluble protein.

Breast-fillet amino acid composition and carcass characteristics

Breast-fillet amino acid composition and relative weight of organs were determined on Day 35. Two birds from each pen (16 birds/treatment) were selected and killed by cervical dislocation; breast fillets (pectoralis major and minor, 50 g) were removed and placed in sterile plastic bags and stored at –20°C until the analysis. Amino acid composition of fillet was assayed with HPLC (method 982.30 E (a, b, c); AOAC International 2000) by hydrolysis with 6 M HCl at 110°C for 12 h and derivatisation using *o*-phthaldialdehyde as described above. The same birds were evaluated for intestinal length and relative organ weight.

Statistical analyses

Data were analysed in a completely randomised design, using the MIXED procedure of SAS Version 9.1 (SAS Institute, Cary, NC, USA) with dietary treatment ($n = 4$) as fixed effect. Each treatment had eight replicate pens with 10 chicks per pen. Normal distribution of the residual was tested by the UNIVARIATE procedure of SAS. When the effect was significant, differences among treatment means were tested using the Tukey's multiple comparison tests. Statistical significance was declared at $P \leq 0.05$.

Results

Gelatin and acid hydrolysates properties

With regard to degrees of hydrolysis and protein recovery after acid hydrolysis of gelatin, lysine and histidine greatly increased, and proline, hydroxyproline and glutamic acid greatly decreased (Table 1, Fig. 2). Protein bands and peptides in CSG samples appeared in the molecular size range ~20–128 kDa (Fig. 3). Peptide bands after acid hydrolysis appeared in a molecular size ranges ~3.5–10 kDa, suggesting that most CSG proteins were degraded to small peptides and free amino acids after acid hydrolysis.

Bird performance

Compared with chicks fed the control diet, BW gain and feed intake were significantly ($P \leq 0.001$) reduced and FCR increased greatly with inclusion of CSG and AHCSG in the diet (Table 3). This trend was apparent at all growth stages (14–35 days of age), except in the case of FCR during 1–7 days of age, when FCR was best for chicks fed AHCSG at 45 g/kg and worst for those fed CSG.

Blood characteristics

Blood urea and blood urea nitrogen (BUN) at 14 and 35 days of age were similar among birds fed all diets (Table 4). Dietary inclusion of AHCSG significantly ($P < 0.05$) decreased plasma uric acid concentration relative to the control or CSG diet at 14 days of age (AHCSG at 45 g/kg) and 35 days of age (both levels of AHCSG).

Ileal digesta viscosity and ileal morphology

Hydrolysis of CSG significantly ($P < 0.05$) reduced ileal digesta viscosity of chicks fed both levels of AHCSG compared with those fed CSG, to levels similar to chicks fed the control diet (Table 4).

Incorporation of AHCSG in the diet decreased ($P \leq 0.001$) villus height and crypt depth, but had no effect on villus height : crypt depth ratio, compared with those measures in birds fed control and CSG diets (Table 5); villus height and crypt depth were numerically greatest for birds fed the diet

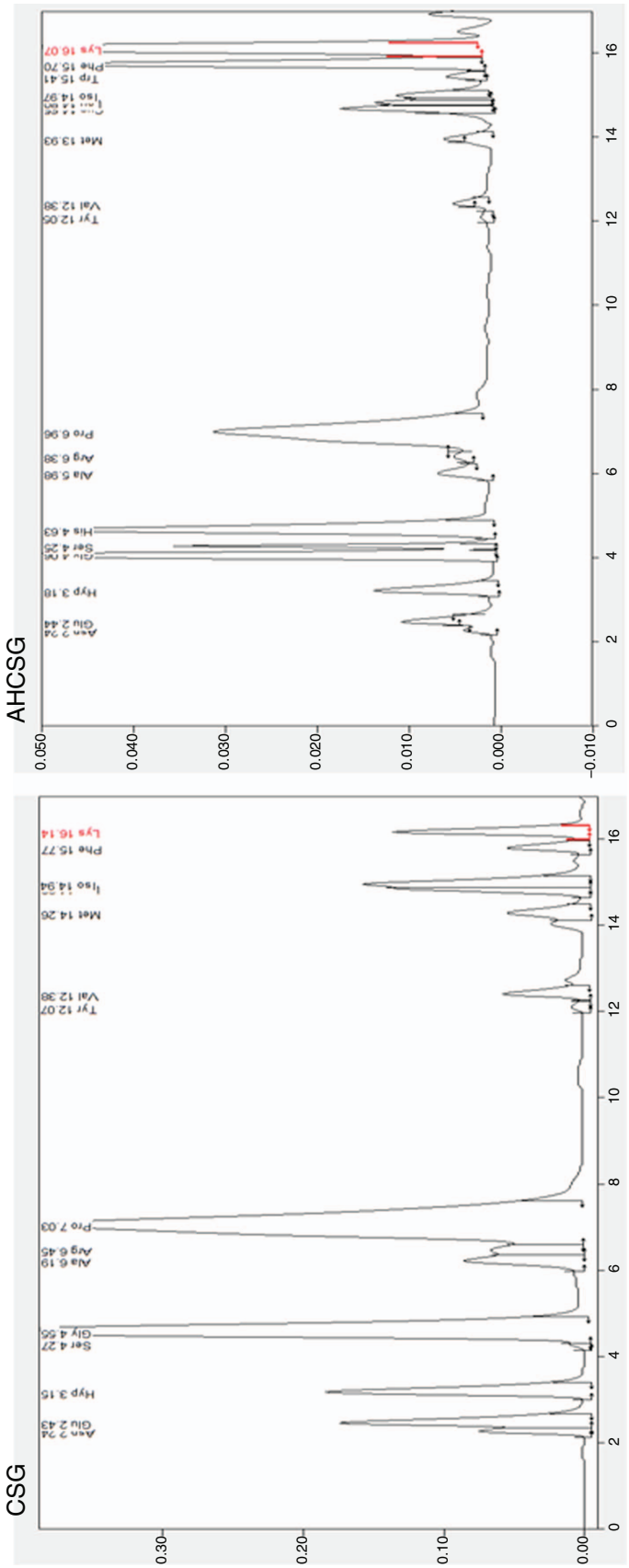

Fig. 2. HPLC graph of amino acid composition of cow skin gelatin (CSG) and acid-hydrolysed CSG (AHCSG). Differences in lysine content between CSG and AHCSG illustrated in red (right-hand peak).

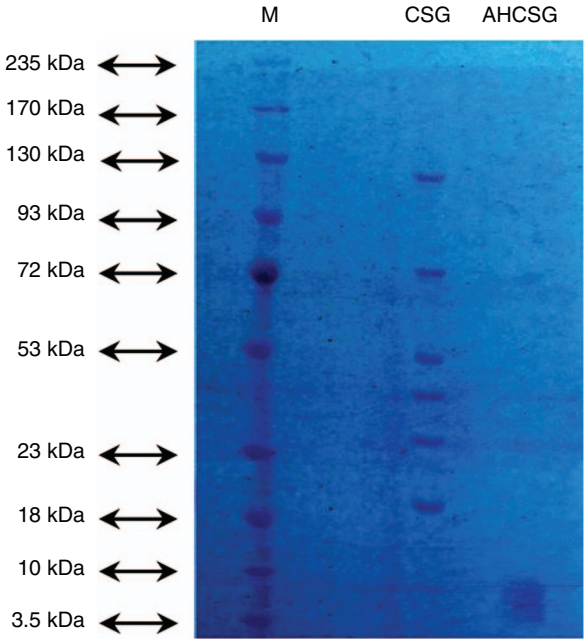

Fig. 3. Protein pattern of gelatin hydrolysate from cow-skin gelatin before (crude sample: CSG) and after (acid-hydrolysed: AHCSG) hydrolysis under acidic (4 M HCl) conditions for 6 h at 110°C. M, molecular weight marker.

containing CSG, although these values were not significantly different from the control.

Total tract protein digestibility

Protein digestibility was significantly ($P < 0.05$) reduced, by ~50%, in birds fed diets containing 92 g/kg of either AHCSG and CSG compared with birds fed the control diet (Table 5). Protein digestibility was intermediate in birds fed AHCSG at 45 g/kg, and not significantly ($P > 0.05$) different from the control or the two diets supplemented at 92 g/kg, although the calculated reduction relative to the control was ~25% (Table 5).

Cecal microflora population

Abundance of *E. coli* population in the caecum was similar in birds fed the four diets, whereas birds fed CSG had a greater ($P = 0.03$) abundance of coliforms than birds fed the control and AHCSG diets (Table 5). *Salmonella* was more abundant ($P < 0.05$) in birds fed the control and CSG diets than those fed AHCSG at 92 g/kg, with birds fed AHCSG at 45 g/kg intermediate ($P > 0.05$).

Digestive enzyme activity

Trypsin activity was similar in birds fed the four diets (Table 5). Total alkaline protease activity was similar in birds fed the four diets at Day 14, but at Day 35, alkaline protease

Table 3. Growth performance of broilers fed a diet without (control) or with cow-skin gelatin (CSG) or acid-hydrolysed CSG (AHSCS) from 1 to 35 days of age

Within a column, means followed by the same letter are not significantly different ($P > 0.05$); s.e.m., standard error of the mean; BW, bodyweight (g); FI, feed intake (g); FCR, feed conversion ratio (g FI/g BW gain). There were 10 broilers per pen and eight pens per diet. Pens means were used to calculate dietary averages

	Days 1–7			Days 1–14			Days 1–21			Days 1–28			Days 1–35		
	FCR	FI	BW	FCR	FI	BW	FCR	FI	BW	FCR	FI	BW	FCR	FI	BW
Control	1.19ab	165.06a	137.95a	1.19b	396.94a	332.84a	1.44b	932.06a	655.94a	1.56b	1659.14a	1073.13a	1.80b	2547.6a	1413.75a
AHCSG, 45 g/kg	0.95c	110.09c	115.00b	1.72a	210.98c	125.94b	2.11a	382.40c	219.08b	2.10ab	514.61c	264.46bc	2.60a	863.7c	331.67c
AHCSG, 92 g/kg	0.98bc	107.48c	111.46b	1.66ab	205.14c	127.28b	2.41a	364.83c	156.25b	2.65a	464.83c	181.25c	2.80a	627.3c	250.00c
CSG, 92 g/kg	1.25a	129.47c	103.45b	1.50ab	245.47b	163.15b	1.86ab	478.67b	259.24b	1.87ab	681.22b	365.70b	2.41ab	1118.9b	466.87b
s.e.m.	0.07	3.76	4.48	0.14	7.87	11.60	0.17	18.59	43.73	0.26	36.36	50.75	0.320	75.09	747.76
P-value	0.026	0.0001	0.001	0.02	0.0001	0.0001	0.38	0.0001	0.0001	0.04	0.001	0.0001	0.09	0.001	0.001

Table 4. Digesta viscosity at Day 14 and blood characteristics at Days 14 and 35 in broilers fed a diet without (control) or with cow-skin gelatin (CSG) or acid-hydrolysed CSG (AHSCS)

Within a column, means followed by the same letter are not significantly different ($P > 0.05$); s.e.m., standard error of the mean; BUN, blood urea nitrogen. Data are for two chicks per pen, and there were eight pens per diet

	Viscosity		Blood characteristics (mg/dL)					
	10 ps	30 ps	Day 14			Day 35		
			Urea	BUN	Uric acid	Urea	BUN	Uric acid
Control	12.60b	14.50b	3.33	1.53	11.17a	1.50	0.95	7.50a
AHCSG, 45 g/kg	11.33b	13.04b	2.66	1.20	5.23b	1.33	0.96	4.63b
AHCSG, 92 g/kg	7.50b	10.83b	4.00	1.83	8.25ab	1.00	1.00	2.70b
CSG, 92 g/kg	20.70a	23.78a	1.66	0.73	10.20a	1.00	1.00	10.50a
s.e.m.	2.29	2.64	0.82	0.38	1.11	2.13	0.028	0.28
P-value	0.01	0.02	0.29	0.28	0.02	0.46	0.46	0.05

Table 5. Ileal morphology, total tract crude protein (CP) digestibility coefficient, cecal microbial population, and intestinal enzyme activity in broilers fed a diet without (control) or with cow-skin gelatin (CSG) or acid-hydrolysed CSG (AHSCS)

Within a column, means followed by the same letter are not significantly different ($P > 0.05$); s.e.m., standard error of the mean; APA, total alkaline protease activity. Data are for two chicks per pen, and there were eight pens per diet

	Villus height (μm)	Crypt depth	Villus height : crypt depth	CP digestibility (%)	*E. coli* (log10 CFU/g DM)	Coliforms (log10 CFU/g DM)	*Salmonella* (log10 CFU/g DM)	Day 14 Trypsin	Day 14 APA (nmol/mg protein)	Day 35 Trypsin	Day 35 APA
Control	960a	145ab	6.89	54.52a	3.37	3.39b	3.38a	181.04	7250	197.17	6003a
AHCSG, 45 g/kg	712b	109c	6.82	40.95ab	3.26	3.49b	3.15ab	468.35	7353	101.17	4680b
AHCSG, 92 g/kg	720b	103c	7.01	27.51b	3.18	3.42b	3.04b	548.60	5440	125.08	3425c
CSG, 92 g/kg	1027a	169a	6.40	28.77b	3.62	4.18a	3.37a	276.50	6820	268.65	5372ab
s.e.m.	35	6.69	0.44	5.61	0.19	0.08	0.11	183.38	1289	62.32	215
P-value	0.001	0.001	0.641	0.04	0.52	0.03	0.05	0.55	0.71	0.31	0.007

Table 6. Breast fillet protein amino acid content in broilers fed a diet without (control) or with cow-skin gelatin (CSG) or acid-hydrolysed CSG (AHSCS)

Within a row, means followed by the same letter are not significantly different ($P > 0.05$); s.e.m., standard error of the mean. Data are for two chicks per pen, and there were eight pens per diet

	Control	CSG 92 g/kg	AHCSG 45 g/kg	AHCSG 92 g/kg	s.e.m.	*P*-value
Crude protein	18.813a	16.144b	16.536b	14.551c	0.366	0.003
Aspartic acid	0.523	0.151	0.482	0.545	0.241	0.64
Glutamic acid	3.791a	3.023ab	1.814b	2.765ab	0.421	0.05
Threonine	0.365	0.629	0.272	0.317	0.176	0.51
Serine	1.433	1.349	1.141	1.387	0.196	0.73
Histidine	1.021a	0.161c	0.723ab	0.592b	0.173	0.02
Glycine	1.961	1.977	1.907	2.443	0.413	0.78
Alanine	0.765	0.760	0.413	0.691	0.139	0.31
Arginine	0.383	0.332	0.449	0.599	0.198	0.79
Proline	0.702	0.688	0.489	0.815	0.143	0.48
Valine	0.735	0.648	0.530	0.737	0.155	0.76
Methionine	–	–	–	–	–	–
Cysteine	–	–	–	–	–	–
Leucine	0.779	0.739	0.580	0.821	0.238	0.89
Isoleucine	0.385	0.369	0.479	0.436	0.112	0.89
Tryptophan	–	–	–	–	–	–
Phenylalanine	4.438b	2.090d	5.503a	2.914d	0.619	0.04
Lysine	1.583	1.513	1.362	1.643	0.338	0.94

activity was lower ($P = 0.007$) in birds fed AHCSG at 92 g/kg than those fed AHCSG at 45 g/kg, which had significantly lower alkaline protease activity than birds fed the control diet; those fed CSG had values intermediate between those fed AHCSG at 45 g/kg and the control.

Breast-fillet amino acid composition and carcass characteristics

Breast-fillet protein content was significantly ($P = 0.003$) affected by dietary treatment, being reduced by 14% in birds fed CSG, 12% (AHCSG at 45 g/kg), and 23% (AHCSG at 92 g/kg) compared with the control diet (Table 6). Differences were observed among control and CSG- and AHCSG-supplemented diets in terms of amino acid composition of breast fillet. Both glutamic acid and histidine were significantly ($P < 0.05$) decreased by dietary inclusions: glutamic acid by AHCSG at 45 g/kg, and histidine by CSG and AHCSG 92 g/kg. Phenylalanine content of fillet from chicks fed CSG and AHCSG at 92 g/kg was a lower ($P < 0.05$) than from chicks fed the control diet and AHCSG at 45 g/kg. Other breast fillet-amino acids were similar among birds fed the four diets.

Carcass and breast yields were higher ($P < 0.005$) in control birds than those fed the three supplemented diets (Table 7). Birds fed the diet with AHCSG at 92 g/kg had lowest carcass and breast yield and the largest ($P = 0.02$) pancreas.

Intestinal organ characteristics

The intestine (duodenum, jejunum and ileum) was more than twice as long in birds fed diets with CSG and AHCSG than those fed the control diet ($P < 0.002$, Table 7).

Table 7. Carcass analysis of broilers fed a diet without (control) or with cow-skin gelatin (CSG) or acid-hydrolysed CSG (AHSCS)
Within a column, means followed by the same letter are not significantly different ($P > 0.05$); s.e.m., standard error of the mean. Data are for two chicks per pen, and there were eight pens per diet

	Carcass	Liver	Breast	Thigh	Pancreas	Duodenum	Jejunum	Ileum
	(g/100 g live bodyweight)					(cm/100 g live bodyweight)		
Control	59.50a	3.10	22.26a	19.62	0.254b	1.90b	5.18d	4.97c
AHCSG, 45 g/kg	47.58bc	2.84	14.52bc	16.04	0.273b	5.40a	13.75b	12.81ab
AHCSG, 92 g/kg	40.66c	3.06	10.11c	12.28	0.486a	6.28a	17.42a	15.42a
CSG, 92 g/kg	50.27b	2.94	16.04b	14.68	0.355b	4.23a	10.7d	11.01b
s.e.m.	1.89	0.19	1.24	1.51	0.02	0.45	0.62	0.88
P-value	0.001	0.773	0.004	0.124	0.018	0.002	0.001	0.001

Discussion

Non-enzymatic hydrolysis of animal waste product is a practical, low-cost method to produce large amounts of peptides suitable for industrial applications (Álvarez *et al*. 2013). The work described here was done to determine whether application of an appropriate hydrolysis procedure, using an acid, would improve the digestibility of CSG and produce gelatin rich in low-molecular-weight peptides, especially di- and tripeptides, with free amino acids, in order to qualify as having high nutritional value for broilers. The present experiment found that acid hydrolysis yielded low-molecular-weight peptides (<10 kDa) with a great degree of hydrolysis and no corresponding bands in electrophoresis compared with CSG.

Previous trials in our laboratory (Khalaji *et al*. 2016; Beyranvand *et al*. 2019) found that incorporating CSG into the diet of broiler chickens severely reduced BW gain and feed intake, owing to increased digesta viscosity and a reduction in protein digestibility. Johnston-Banks (1990) demonstrated that large molecules of peptide contribute most to increased digesta viscosity in gelatin extracted from cow skin. In the present study, feeding AHCSG greatly reduced digesta viscosity (to a level similar to the control) compared with CSG. Nonetheless, CP digestibility, feed intake and BW gain were greatly depressed in chicks fed both CSG- and AHCSG-containing diets relative to the control. Thus, hydrolysis of CSG did not ameliorate the negative effects on animal performance.

Álvarez *et al*. (2013) reported that hydrolysis with HCl produces large amounts of peptides appearing in a molecular-size range ~3–10 kDa in electrophoresis. Also, the protein recovery in the AHCSG in the present study was considerable. Because of the high protein and amino acid content, higher degree of hydrolysis and low peptide weight, the acid-hydrolysed gelatin was tested *in vivo* for elucidation of its suitability for poultry consumption.

As previously reported by Khalaji *et al*. (2016), in the present study, BW gain and feed intake of the birds were drastically reduced by incorporation of CSG and AHCSG into diets, with the hydrolysis of gelatin providing no amelioration. Regardless of the reduced ileal digesta viscosity with hydrolysis, the CP digestibility was severely decreased by inclusion of either CSG or AHCSG. These results agree with our previous study (Khalaji *et al*. 2016) in which CSG supplementation reduced protein digestibility. The reduced protein digestibility in broilers fed CSG and AHCSG might have reduced protein absorption for BW gain. Ozols (1990) indicated that the amino acid content and sequence of the substrate used greatly affects the outcome of hydrolysis. It has been reported that acid hydrolysis destroys serine, threonine, arginine and cysteine (Ravindran and Bryden 2005). Álvarez *et al*. (2013) reported that acid or alkali hydrolysis of proteins produces appreciable amounts of free amino acids and peptides <1 kDa. Di- and-tripeptides are quickly and efficiently absorbed by the intestine without initial pancreatic enzyme secretion (Zambonino Infante *et al*. 1997). Increased availability of free amino acid and small peptides after hydrolysis might therefore decrease protease secretion from pancreas, which in turn might adversely impact protein digestion in the intestinal lumen. At 35 days of age, alkaline protease activity was reduced in the intestine of birds fed AHCSG compared with those fed CSG or the control diet, whereas the relative weight of the pancreas was increased in birds fed both CSG and AHCSG diets. Furthermore, changes in intestinal growth were observed, including a reduction in villus height and crypt depth and an increase in intestinal organ length in birds fed CSG and AHCSG.

Hong *et al*. (2004) reported that breakdown of large-size peptides to small-size peptides can increase the activity of proteolytic enzymes in chicks. The lack of such effect in the present trial may be attributed to high levels of non-soluble protein in AHCSG. It has been reported that acid hydrolysis can promote intense polymerisation of protein groups, resulting in the formation of large insoluble polymers that are not visible by gel electrophoresis (Vaghefi *et al*. 2002; Liu *et al*. 2010; Álvarez *et al*. 2013). The acidity and molarity of HCl in hydrolysis solution should also be taken into account when evaluating the intensity of polymerisation and formation of insoluble polymers. Future trials are suggested to investigate suitable molarity of HCl in the hydrolysis process for removing insoluble polymers and to determine its biological value for broiler chicks. Kotzamanis *et al*. (2007) observed that inclusion of sardine byproduct hydrolysate (42% CP; main fraction of peptides 200–500 Da) in the diet reduced growth performance of fish owing to a large proportion of less soluble di- and tripeptides after hydrolysis. Furthermore,

Álvarez *et al*. (2013) indicated that acidic conditions may promote the formation of greater amounts of these polymers, which are much more stable and lower the digestibility and nutritive value of the products. However, comparison of findings of different studies is not straightforward because of the use of different protein sources, hydrolysis conditions and types (enzymatic or chemical hydrolysis), and dietary inclusion rate of hydrolysate (Gilbert *et al*. 2008). Broilers fed both levels of AHCSG had lower plasma uric acid concentrations than those fed CSG and the control diet, which might be due to reduction in protein digestibility and low utilisation of aggregates and free amino acid in birds fed AHCSG (Liu *et al*. 1996).

In a previous trial (Khalaji *et al*. 2016), the length and weight of the duodenum, jejunum and ileum were lower when CSG was included in the diets of broilers, but CSG did not affect jejunum or ileum mucosa villus height, crypt depth, or villus height : crypt depth ratio. The results for villus height and crypt depth for chicks fed the CSG diet in the present study are in accordance with the previous study, whereas, the duodenum, jejunum and ileum relative lengths increased with inclusion of both CSG and AHCSG, and villus height and crypt depth were reduced with inclusion of AHCSG in the diet. Frikha *et al*. (2014) found that including hydrolysed porcine mucosa in the diet of broilers did not affect villus histomorphology or nutrient retention in the small intestine. In pigs, Borda *et al*. (2005) found that inclusion of 5% hydrolysed porcine mucosa increased the villus height of the ileum measured 15 days after weaning. Vente-Spreeuwenberg *et al*. (2004) found no significant differences for villus height and crypt depth of the small intestine in weaned pigs when soybean was replaced with hydrolysed wheat gluten. Similarly, Corassa *et al*. (2007) reported that substitution of 5% spray-dried blood plasma with hydrolysed porcine mucosa did not affect villus morphology of the small intestine. Therefore, the effect of hydrolysis on small intestine morphology is inconsistent among reported studies.

There was a reduction in the cecal coliform and *Salmonella* populations in chicks fed diets with AHCSG. Similarly, Khalaji *et al*. (2016) reported a reduction in the cecal coliform population when high levels of CSG were included in the diet of broilers; the hydrolysates were tested by using an agar diffusion assay against 18 strains of bacteria (both Gram-positive and -negative), of which *Lactobacillus acidophilus* and *Bifidobacterium animalis* subsp. *lactis*, *Shewanella putrefaciens* and *Photobacterium phosphoreum* were found to be the most susceptible. Collagen has been described previously as a biomaterial with bactericidal and fungicidal properties when applied as a coating to wool-based materials (Jus *et al*. 2009). Gómez-Guillén *et al*. (2010) reported antimicrobial activity in peptide fractions from tuna- and squid-skin gelatin of 1–10 kDa and <1 kDa.

Carcass and breast-fillet yield were reduced in chicks fed AHCSG in the present study, and breast-fillet protein and amino acid composition was altered. Glutamic acid, histidine and phenylalanine content of breast fillet were reduced by inclusion of CSG and AHCSG in the diet. Previous studies have demonstrated that asparagine and glutamine are completely hydrolysed by acid hydrolysis and converted to aspartic acid and glutamic acid (Fountoulakis and Hans-Werner 1998; Álvarez *et al*. 2013). To our knowledge, no other studies with broilers have determined the potential benefits of free amino acids or small peptides from AHCSG, compared with feeding intact proteins, on protein retention and breast muscle growth. Some studies have reported the effects of feeding hydrolysed proteins to fish (Cahu *et al*. 1998; Kotzamanis *et al*. 2007), finding increased weight gain, total biomass, carcass weight and malformation rates of sea bass or carp larvae.

The present study demonstrated that hydrolysis of CSG with hydrochloric acid reduced the peptide size, increased lysine content and reduce the ileal digesta viscosity. However, broiler performance, protein digestibility, ileal morphology, enzyme activity, carcass and breast yield, and breast protein and amino acid content were still impaired after acid hydrolysis to at least a similar extent to the untreated CSG. This suggests that amino acids were destroyed and aggregates produced during acid hydrolysis. Further research is needed into understanding the possible problems with acid hydrolysis and developing alternative procedures such enzymatic hydrolysis, which may increase the application of CSG in broiler rations.

Conflicts of interest

The authors declare no conflict of interest.

Animal welfare statement

The authors confirm that the ethical policies of the journal, as noted in the journal's author guidelines, have been adhered to based on procedures and guidelines (Vol. 1. Isfahan University of Technology) approved by the Animal Care Committee of the Iranian Council of Animal Care.

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