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Precaecal digestibility of crude protein and amino acids from alfalfa (*Medicago sativa*) and red clover (*Trifolium pratense*) leaves and silages in broilers

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ABSTRACT

The aim of the present study was to determine the precaecal (pc) digestibility of crude protein and amino acids from organically produced alfalfa and red clover leaves and whole plant silages by a linear regression approach in slow-growing male Hubbard JA-757-broilers. Dried alfalfa leaves (AL; 219 g crude protein/kg dry matter (DM)), dried red clover leaves (RCL; 262 g crude protein/kg DM), alfalfa silage (AS; 240 g crude protein/kg DM) and red clover silage (RCS; 190 g crude protein/kg DM) were included in the diets at the levels of 100, 150 and 200 g/kg respectively at the expense of maize starch. Titanium dioxide was used as an indigestible marker for digestibility estimation. On day 41/42, digesta was sampled pen-wise from the terminal two thirds of the intestine section between Meckel's diverticulum up to 2 cm anterior to the ileocaeca-colonic junction. Feed intake did not differ significantly between feeding groups. All broilers grew during the experimental phase of digestibility estimation (day 30−41/42). The pc crude protein and amino acid digestibility of alfalfa and red clover leaves was lower than of alfalfa and red clover silages. Methionine was less digestible in AL (0.61) and RCL (0.73) than in AS (0.84) and RCS (0.99). This tendency was also observed for lysine (AL 0.49, RCL 0.61, AS 1.00, RCS 0.88) as well as for other amino acids. Anti-nutritional factors, *e.g.* saponins, were suspected of being responsible for the lower pc digestibility of the leaf products. The saponin analysis showed a higher content of medicagenic acid glycosides in AS than in AL, whereas a higher content of a zanhic acid glycoside was found in AL than in AS. Differences in saponin contents suggest that certain individual saponins, *e.g.* zanhic acid glycosides, might have a more negative effect on crude protein and amino acid digestibility than other saponins.

Abbreviations: AA, amino acid(s); AL, alfalfa leaves; AME_N, apparent metabolizable N-corrected energy; AS, alfalfa silage; CF, crude fiber; CP, crude protein; DM, dry matter; KOH, potassium hydroxide; Lys, Lysine; Met, Methionine; P1, phase 1; P2, phase 2; P3, phase 3; pc, precaecal; pcd, precaecal digestibility; RCL, red clover leaves; RCS, red clover silage.

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1. Introduction

The organic broiler nutrition is still challenged by legal requirements for home-grown and completely organic diets. The supply of protein and amino acids (AA), especially of the first limiting AA methionine (Met) and lysine (Lys), with home-grown organic protein sources is still difficult. EU guidelines also demand the addition of roughage to poultry (European Union, 2007, 2008). As part of the crop rotation in organic farming, forage legumes like alfalfa and red clover can provide high yields of crude protein (CP) and AA, especially Met. Due to their considerable CP and AA contents, alfalfa (in g/kg dry matter (DM); CP 244, Met 2.20, Lys 13.1) and red clover (in g/kg DM; CP 225, Met 1.91, Lys 11.2) could be valuable protein and AA sources in broiler nutrition (Hoischen-Taubner and Sundrum, 2016). Thus, these forage legumes could replace soybean cake, which is still the dominant protein source in organic broiler diets. However, whole plant material contains a high crude fiber (CF) content of 210 g/kg DM or more (Sauvant et al., 2004), which may have negative effects on CP digestibility. Hoischen-Taubner and Sundrum (2016) showed that the separation of leaves from stems results in higher CP contents (in g/kg DM; alfalfa: +40, red clover: +42) and lower CF contents (in g/kg DM; alfalfa: −48, red clover −42). Nevertheless, the high potential of alfalfa silage of the whole plant as home-grown CP and CF source has already been proved in the organic feeding of broilers (Wüstholz et al., 2016). The *in vitro* precaecal (pc) CP and AA digestibility of alfalfa and red clover was high in leaves and whole plants (Met 0.76−0.78, Lys 0.77−0.80) (Hoischen-Taubner and Sundrum, 2016). In an *in vivo* study with ISA JA 957 chickens, Ritteser and Grashorn (2015) found lower pc CP and AA digestibility results in clover silages (extruded/not extruded; Met 0.50/0.48, Lys 0.45/0.33) but very high digestibility values in dried alfalfa leaves (Met 0.93, Lys 0.87).

Apart from their valuable CP and AA concentrations, these forage legumes contain anti-nutritional factors such as trypsin inhibitors (Brown et al., 1985), phenols and polyphenol oxidase (red clover) (Winters et al., 2008) and saponins. Saponins especially are considered to be the main anti-nutritional substances in alfalfa (Sen et al., 1998). Reduced feed intake due to bitter taste, growth depression and an impaired nutrient digestion and absorption are biological effects described in this context (Cheeke, 1983, 1996). Szumacher-Strabel et al. (2019) reported that ensiling of alfalfa can lead to structural and quantitative changes of individual saponins. Thus, the nutrient digestibility of dried and ensiled material may vary.

The objective of this study was to determine the pc CP and AA digestibility of dried alfalfa leaves (AL), dried red clover leaves (RCL), alfalfa silage (AS; whole plant) and red clover silage (RCS; whole plant) in six-week-old broiler chickens by linear regression (Rodehutscord et al., 2004). Furthermore, this study aimed to evaluate differences in individual saponin contents between AL and AS.

Table 1
Analyzed nutritional composition (g/kg dry matter-DM) in the test feedstuffs alfalfa leaves (AL), red clover leaves (RCL), alfalfa silage (AS) and red clover silage (RCS).

Item	AL	RCL	AS	RCS
Dry matter (g/kg, as fed)	901	887	378	475
Crude ash	111	101	111	139
Crude protein	219	262	240	190
Crude fat	43.8	39.7	30.5	23.4
Crude fiber	174	125	273	182
Starch	68.3	66.6	11.5	39.8
Sugar	69.8	48.9	0.0	92.7
Lysine	13.1	14.2	11.7	9.8
Methionine	3.64	4.25	3.65	2.83
Cystine	2.85	2.21	2.12	1.56
Threonine	9.69	12.1	10.5	8.68
Tryptophan	3.31	3.83	2.62	2.38
Leucine	15.8	21.2	16.7	14.9
Isoleucine	8.66	10.9	10.1	8.11
Valine	11.0	14.3	12.7	10.6
Arginine	9.89	13.2	5.69	8.31
Histidine	5.13	5.46	4.62	4.00
Phenylalanine	10.3	13.8	10.9	9.67
Tyrosine	6.82	9.24	4.54	6.20
Alanine	12.1	15.3	12.8	10.7
Glycine	10.4	13.0	10.4	9.16
Serine	9.07	10.8	9.67	8.29
Proline	11.4	16.4	12.8	13.3
Aspartic acid	27.7	27.9	28.7	21.8
Glutamic acid	22.1	27.9	16.5	17.0
Calcium	29.1	13.1	17.1	14.7
Phosphorus	2.60	3.20	2.77	3.04
Sodium	0.11	0.06	0.12	0.07
Magnesium	2.30	3.70	3.21	2.81
Potassium	22.9	31.1	29.9	40.5
AME_N (MJ/kg DM)[1]	6.33	6.90	5.66	5.70
Coefficient of protein solubility	0.40	0.16	0.56	0.38

[1] AME_N: Apparent metabolizable N-corrected energy, calculated according to WPSA (1989).

2. Material and methods

2.1. Test feedstuffs

The test feedstuffs AL, RCL, AS and RCS formed the basis of this investigation (Table 1). All test feedstuffs were cultivated according to the current EU eco-directives Council Regulation (EC) No 834/2007 and Commission Regulation (EC) No 889/2008 (European Union, 2007; 2008). For the production of the AS (cultivar Plato, 3rd cut, bud stage), a population of alfalfa was mowed in July 2017, wilted for one day and chopped to a theoretical chopping size of 6 mm by a forage harvester (CLAAS, Jaguar 900 Speed Star; Germany). Afterwards, this material was compressed and ensiled using a round baler of the company GÖWEIL (Type LT-Master, Austria). The RCS (cultivar Titus, 2nd cut, just before the bloom stage) was harvested in July 2017 and provided as round bale silage by the Johann Heinrich von Thünen-Institute, Institute of Organic Farming, Germany. AL (cultivar Plato, 4th cut, middle of the bloom stage) and RCL (cultivar Titus, 4th cut, start of the bloom stage) were harvested using a special leaf-harvesting machine (Co. Trust'ing, France) in September 2017. Both leaf materials were dehydrated using hot air in a forage drying unit (Futtertrocknung Lamerdingen eG, Germany). Drying temperature ranged between 200−600 °C at the entrance and 100 °C at the end of the drying drum. Both leaf batches were finely ground to meal.

2.2. Experimental design and diets

The experiment was divided into three feeding phases: Phase 1 (P1, day 1–21), phase 2 (P2, day 22−28) and phase 3 (P3, day 29−41/42). In P1, the chickens were fed a commercial organic starter diet (per kg DM of diet: Apparent metabolizable N-corrected energy (AME_N) 12.8 MJ, CP 250 g, Met 4.43 g, Lys 12.2 g). Due to high CF contents and the possible presumption of anti-nutritional substances (*e.g.* saponins) in alfalfa and red clover, the experimental design differed from the common methodology of pc digestibility estimation (Rodehutscord et al., 2004; Kluth et al., 2005a). To avoid a sudden feed change from the starter to the experimental diets, an adaption period to the uncommon test feedstuffs was implemented. Therefore, diets of P2 (P2_AL, P2_RCL, P2_AS, P2_RCS) contained 150 g/kg alfalfa or red clover products, except the control group (P2_C) (Table 2). The pc digestibility was determined in P3.

Diets of P2 and P3 were produced in the facilities of the University of Applied Sciences Weihenstephan-Triesdorf (Germany). Silages were extruded (Bioextruder Lehmann Maschinenbau GmbH, Pöhl, Germany) and treated with propionic acid/water (99.5/0.5, v/v; BASF, Germany) according to the manufacturer's information to facilitate blending with the concentrates and to prolong the

Table 2
Ingredients (g/kg as fed basis) and analyzed nutritional composition (g/kg dry matter-DM) of the diets with the test feedstuffs alfalfa leaves (AL), red clover leaves (RCL), alfalfa silage (AS) and red clover silage (RCS) at a level of 150 g/kg in phase 2 (P2).

Ingredients	Feeding group				
	P2_C[1]	P2_AL	P2_RCL	P2_AS	P2_RCS
AL/RCL/AS[2]/RCS[2]*	–	150	150	150	150
Soybean cake*	120	60	70	75	80
Sunflower cake, dehulled*	155	170	160	160	180
Peas*	110	100	100	100	100
Maize*	310	270	275	271	280
Wheat*	271	173	168	160	121
Rapeseed oil*	–	47	45	54	57
Premix[3]	25	25	25	25	25
Calcium carbonate	7	1	3	1	3
Monocalcium phosphate	–	2	2	2	2
Sodium chloride	2	2	2	2	2
Analyzed nutritional composition					
Dry matter (g/kg, as fed)	875	891	890	746	792
Crude ash	65.5	73.3	74.1	71.3	84.3
Crude protein	220	228	221	214	205
Crude fat	59.9	112	104	112	108
Crude fiber	48.3	83.4	70.3	115	100
AME_N (MJ/kg DM)[4]	14.0	13.7	13.7	13.4	13.0
Lysine	10.2	10.6	10.5	10.2	9.28
Methionine	3.59	4.10	3.59	3.46	3.28
Lysine/AME_N (g/MJ)	0.73	0.78	0.77	0.76	0.71
Methionine/AME_N (g/MJ)	0.26	0.30	0.26	0.26	0.25
Coefficient of protein solubility	0.85	0.80	0.74	0.83	0.81

* organically produced.
[1] P2_C: Control group in P2.
[2] Silage calculation basis: 880 g/kg DM.
[3] Mineral and vitamin supplement per kilogram of diet was as follows: Ca 3.1 g, P 0.50 g, Na 0.75 g, Mg 0.50 g, Cl 0.88 g, Cu 5.0 mg, Zn 33 mg, Mn 53 mg, J 0.38 mg, Se 0.20 mg, vitamin A 2500 IU, vitamin D3 500 IU, vitamin E 25 mg, vitamin K 1.0 mg, vitamin B1 3.0 mg, vitamin B2 3.8 mg, vitamin B6 3.0 mg, vitamin B12 10 µg, niacinamide 40 mg, pantothenic acid 8.8 mg, folic acid 0.88 mg, biotin 175 µg, choline chloride 1250 mg.
[4] AME_N: Apparent metabolizable N-corrected energy, calculated according to WPSA (1984).

durability of the silage-concentrate mixtures. Diets of P2 were designed to be isocaloric and isonitrogenous (Table 2). In total, 12 diets (AL1−3, RCL1−3, AS1−3, RCS1−3) served for the estimation of the pc digestibility of CP and AA in P3. The basal diet was based on soybean cake, dehulled sunflower cake, peas, maize, wheat and maize starch (Table 3). Free AA were added to the basal diet to meet or exceed the recommendations of the GfE (GfE, 1999). All diets contained Titanium dioxide (TiO_2) as an indigestible marker at a level of 5 g TiO_2/kg. The respective alfalfa or red clover products were included in the diets at the levels of 100, 150 and 200 g/kg respectively, at the expense of maize starch. Hence, the difference in the CP and AA content of the diets resulted only from the test feeds (Table 4). All diets were pelleted without steam through a 3-mm die.

2.3. Animals and housing

The experiment was carried out at the Research Farm Zurnhausen of the University of Applied Sciences Weihenstephan-Triesdorf (Germany) and was approved by the University Animal Welfare Committee in accordance with the animal welfare legislation. Male broiler chickens (Hubbard JA-757, Brüterei Hölzl GmbH & Co. KG, Moosburg, Germany) were raised in floor pens on wood shavings and vaccinated against Infectious bursal disease, Marek's disease, Avian infectious bronchitis, Newcastle Disease and Coccidiosis. No coccidiostatics were used. Feed and water were offered *ad libitum*. The stable was thermostatically controlled, heat lamps were applied during the first 14 days. The temperature was set at 30 °C during the first two days before being reduced stepwise to 21 °C on day 21. Artificial lighting was continuously provided for the first 24 h and then reduced stepwise to 16 h per day.

At the beginning of the experiment, the average group weight was equal concerning mean value and standard deviation. To reduce the within-pen variation in body weight, chickens were weighed and the number of chickens per pen was reduced from 12 to 9 on day 14. During the first 21 days after hatching, the birds received a commercial organic starter diet. On day 21, chickens were weighed and diets of P2 (12 replicates for P2_AL, P2_RCL, P2_AS, P2_RCS, 8 replicates for P2_C; 9 chickens per pen) were provided. Group P2_C was removed from the experiment on day 28 as it should only function as a control group during the adaption period (P2). Four replicates of each of the remaining P2 feeding groups were assigned to the respective diets of P3 (P2_AL to AL1−3, P2_RCL to RCL1−3, P2_AS to AS1−3, P2_RCS to RCS1−3). On day 30, the bedding was removed and the birds were placed on plastic slats in order to avoid the intake of excrements or bedding during the experimental phase of digestibility estimation (day 30−41/42). During the experiment, animal losses were monitored daily. Feed intake (per pen) and individual body weights were recorded after each feeding phase. The average body weights per pen were determined on the basis of individual body weights. The calculation of feed conversion rate was based on body weights and feed intake in consideration of animal losses. On day 41 and 42 all broilers were killed using CO_2 asphyxiation (two

Table 3
Ingredients (g/kg as fed basis) of the diets with the test feedstuffs alfalfa leaves (AL), red clover leaves (RCL), alfalfa silage (AS) and red clover silage (RCS) at three inclusion levels in phase 3.

Ingredients	Feeding group		
	AL1/RCL1/ AS1/RCS1	AL2/RCL2/ AS2/RCS2	AL3/RCL3/ AS3/RCS3
AL/RCL/AS[1]/RCS[1]*	100	150	200
Maize starch	100	50.0	0.0
Soybean cake*		90.0	
Sunflower cake, dehulled*		88.0	
Peas*		100	
Maize*		300	
Wheat*		139	
Rapeseed oil*		40.0	
Premix[2]		25.0	
Calcium carbonate		4.0	
Monocalcium phosphate		2.0	
Sodium chloride		2.0	
TiO_2		5.0	
L-Lysine HCl[3]		2.0	
DL-Methionine[4]		1.0	
L-Threonine[5]		1.0	
L-Tryptophan[6]		1.0	

* organically produced.
[1] Silage calculation basis: 880 g/kg DM.
[2] Mineral and vitamin supplement per kilogram of diet was as follows: Ca 3.1 g, P 0.50 g, Na 0.75 g, Mg 0.50 g, Cl 0.88 g, Cu 5.0 mg, Zn 33 mg, Mn 53 mg, J 0.38 mg, Se 0.20 mg, vitamin A 2500 IU, vitamin D3 500 IU, vitamin E 25 mg, vitamin K 1.0 mg, vitamin B1 3.0 mg, vitamin B2 3.8 mg, vitamin B6 3.0 mg, vitamin B12 10 µg, niacinamide 40 mg, pantothenic acid 8.8 mg, folic acid 0.88 mg, biotin 175 µg, choline chloride 1250 mg.
[3] L-Lysine HCl, BESTAMINO, CJ CheilJedang Corp., Korea: Lysine content 790 mg/g (Minimum), DM 990 mg/g, CP 946 mg/g, purity 990 mg/g.
[4] MetAMINO, Evonik Nutrition & Care GmbH, Germany: Methionine content 990 mg/g, CP 581 mg/g, ash 5 mg/g.
[5] ThreAMINO, Evonik Nutrition & Care GmbH, Germany: Threonine content 985 mg/g, CP 724 mg/g, ash 5 mg/g.
[6] L-Tryptophan, Ajinomoto Animal Nutrition Europe, France: Tryptophan content 980 mg/g, DM 990 mg/g, CP 840 mg/g.

Table 4
Analyzed nutritional composition (g/kg dry matter-DM) of the diets with the test feedstuffs alfalfa leaves (AL), red clover leaves (RCL), alfalfa silage (AS) and red clover silage (RCS) at three inclusion levels (100/150/200 g/kg) in phase 3.

Item	AL1 100	AL2 150	AL3 200	RCL1 100	RCL2 150	RCL3 200	AS1 100	AS2 150	AS3 200	RCS1 100	RCS2 150	RCS3 200
Dry matter (g/kg, as fed)	889	884	886	885	886	883	820	780	747	838	808	782
Crude ash	68.6	74.9	78.2	69.3	73.6	78.6	70.0	73.4	78.8	77.2	81.7	86.9
Crude protein	172	190	199	182	192	206	179	196	204	177	180	190
Crude fat	86.3	92.5	95.9	94.8	90.8	92.7	89.7	92.3	93.9	87.7	85.9	89.5
Crude fiber	50.9	60.4	66.6	49.4	58.2	63.2	62.9	82.4	99.7	60.4	66.7	83.5
AME_N (MJ/kg DM)[1]	14.3	13.8	13.4	14.4	13.8	13.3	14.2	13.6	13.1	14.0	13.4	12.8
Lysine	10.2	11.5	11.8	11.1	11.2	11.8	11.0	11.1	11.7	10.4	10.6	10.8
Methionine	3.85	4.21	4.42	4.13	4.37	4.26	4.20	4.21	4.46	4.17	3.86	4.19
Cystine	3.06	3.25	3.34	3.03	3.18	3.18	3.11	3.16	3.22	3.06	2.93	2.99
Threonine	7.79	8.82	8.99	8.48	8.66	8.99	8.40	8.51	9.28	7.90	8.24	8.54
Tryptophan	2.75	3.01	3.22	2.97	3.02	3.10	3.17	3.12	3.16	3.01	2.97	2.93
Leucine	13.8	15.0	15.5	14.7	15.1	16.0	14.5	14.8	15.9	13.9	14.6	14.7
Isoleucine	7.00	7.62	8.03	7.69	7.68	8.28	7.62	7.83	8.35	7.14	7.59	7.60
Valine	8.40	9.12	9.60	9.09	9.16	10.1	9.04	9.27	9.96	8.46	9.16	9.13
Arginine	12.0	13.0	13.2	13.0	12.9	13.2	12.6	12.1	12.6	12.1	12.4	12.4
Histidine	5.12	5.79	5.73	5.46	5.63	5.85	5.45	5.31	5.60	5.11	5.34	5.33
Phenylalanine	8.74	9.50	9.84	9.47	9.69	10.29	9.20	9.46	10.11	8.82	9.31	9.37
Tyrosine	5.65	6.28	6.42	6.01	6.23	6.65	5.61	5.65	5.99	5.63	5.91	5.97
Alanine	8.66	9.64	9.92	9.20	9.77	10.51	9.17	9.46	10.3	8.77	9.32	9.72
Glycine	8.28	9.43	9.43	8.88	9.25	9.63	8.96	8.90	9.61	8.41	8.91	9.13
Serine	8.16	9.70	9.05	8.70	9.31	9.39	8.87	8.55	9.76	8.41	8.99	9.12
Proline	10.4	10.9	11.1	10.3	10.5	10.7	10.4	10.9	11.6	10.7	11.0	11.2
Aspartic acid	17.6	19.8	20.8	18.5	19.2	20.1	19.1	20.0	21.3	18.0	18.6	19.7
Glutamic acid	33.1	33.7	34.1	33.8	33.5	34.0	33.6	33.3	34.2	32.2	32.2	32.4
Coefficient of protein solubility	0.78	0.75	0.71	0.74	0.71	0.67	0.78	0.77	0.75	0.81	0.79	0.71

[1] AME_N: Apparent metabolizable N-corrected energy, calculated according to WPSA (1984).

replicates of each diet on each slaughter day). For the digesta sampling the intestine section between Meckel's diverticulum up to 2 cm anterior to the ileocaeca-colonic junction was isolated and cut into thirds (Kluth et al., 2005b). The terminal two thirds of this section were flushed with distilled water. The content was pooled for all broilers of one pen, immediately frozen and freeze-dried.

2.4. Chemical analyses

The analysis of DM and crude nutrients, including sugar and starch, in the test feedstuffs and diets were carried out according to Commission Regulation No 152/2009 (European Union, 2009). Therefore, the samples were ground through a 1.0 mm sieve. For analysis of AA, minerals, TiO_2 and saponins all samples were ground through a 0.5 mm sieve. The AA contents in the feedstuffs and diets were analyzed by HPLC according to Commission Regulation No 152/2009 (European Union, 2009) regarding sample preparation *via* oxidation and hydrolysis. The subsequent derivatization and chromatography were performed according to Cohen and Michaud (1993). The adapted analytical procedure was recently described in detail by Witten et al. (2019). Feedstuffs were additionally analyzed for minerals after microwave assisted digestion and determination *via* atomic absorption spectroscopy. However, the phosphorus content was examined photometrically according to Commission Regulation No 152/2009 (European Union, 2009). Protein solubility in potassium hydroxide (KOH) of the feedstuffs and diets was examined according to Araba and Dale (1990). The CP and AA analysis in digesta was carried out according to Commission Regulation No 152/2009 (European Union, 2009). Concentrations of TiO_2 in the diets and digesta were determined with the inductively coupled plasma optical emission spectrometry (ICP-OES) after acid hydrolysis.

Saponins in the test feedstuffs AL and AS were analyzed by ultra-high performance liquid chromatography-high resolution mass spectrometry (UPLC-HRMS) in the laboratory Twistaroma, Illkirch, France, as described recently by Pleger et al. (2020). In short, saponins were extracted, in triplicate, from 200 mg of dried samples in ethanol/water (80/20, v/v) containing 18 µg/mL of umbelliferone (Sigma-Aldrich, St. Louis, MO, USA) used to calculate the relevant content of each saponin. The relevant content of the major saponins was calculated based on the relative peak areas (saponin/umbelliferone) and expressed as equivalent umbelliferone (µg/g DM). Raw UPLC–HRMS data files (.mzXML) were processed in R statistical language (version 3.5.2, http://www.r-project.org/) using the open-free XCMS (Tautenhahn et al., 2008) and CAMERA R-packages. Saponins were putatively characterized by comparing the recorded exact mass with data from Twistaroma in-house database as well as with reference literature.

2.5. Calculations and statistics

Using the analyzed contents of CP, AA and TiO_2 in the diet and digesta, the apparent pc digestibility coefficients of CP and AA of the diets were calculated (on pen basis) according to the following equation:

Apparent pc digestibility coefficient = 1 - [($TiO_{2\ Diet}$*Item $_{Digesta}$)/($TiO_{2\ Digesta}$*Item $_{Diet}$)]

where TiO_{2Diet} and $TiO_{2Digesta}$ represent the respective concentrations of TiO_2 in the diet and the digesta samples, and $Item_{Diet}$ and $Item_{Digesta}$ represent the concentrations of CP or the respective AA in the diet and the digesta samples.

The daily intake of CP and AA (g/d) was determined as the feed intake (g DM/d; day 30−41/42) multiplied by the analyzed CP and AA concentrations in the diet. The amount of CP and AA (g/d) digested up to the terminal ileum was calculated as the product of CP and AA intake and the digestibility determined for the respective pen. A linear regression was applied between the daily intake of CP and AA and the digested amount of CP and AA to calculate the digestibility coefficients of CP and AA of the test feedstuffs (Rodehutscord et al., 2004). The slope of the regression was taken as a measure of the pc digestibility of CP and AA for the respective test feedstuff (AL, RCL, AS, RCS).

The broiler performance data (feed intake (g DM/d), body weight (g), daily weight gain (g), feed conversion rate (kg/kg)) were analyzed by analysis of variance, using the General Linear Model procedure of SPSS (2017). Significant differences between means were determined using the Tukey's multiple comparison test. Results are expressed as mean ± standard deviation. Probability values of ≤ 0.05 were considered statistically significant.

Linear regressions and differences between the slopes were tested for significance using the linear regression procedure of SPSS (2017).

3. Results

AL contained lower CP, Met and Lys concentrations (219, 3.64 and 13.1 g/kg DM respectively) than RCL (262, 4.25 and 14.2 g/kg DM respectively). CP, Met and Lys concentrations in AS (240, 3.65 and 11.7 g/kg DM respectively) were higher than in RCS (190, 2.83 and 9.8 g/kg DM respectively) (Table 1). Protein solubility in KOH was highest in AS (0.56), whereas AL and RCS showed lower results (0.40 and 0.38). The measured protein solubility of RCL was at a very low level (0.16). The occurence of 34 saponin compounds in the test feedstuffs AL and AS was proved in the saponin analysis *via* UPLC-HRMS. The relative contents of the nine major putatively identified saponins are presented in Table 5. In AS, particularly high contents were found for medicoside H, medicagenic acid 3-O-β-D-glucuronide and 3-Glc-Glc-28-Ara-Rha-Medicagenic acid (209, 242 and 138 equivalent umbelliferone μg/g DM respectively; saponin abbreviations see Table 5). Lower contents of these saponins were measured in AL (84.2, 89.3 and 101 equivalent umbelliferone μg/g DM respectively). In contrast, a high content of HexA-dHex-Pen-Pen-Pen-Zanhic acid was detected in AL, whereas low amounts of this saponin were found in AS (200 *versus* 12.4 equivalent umbelliferone μg/g DM respectively).

With a few exceptions, feed analysis showed a high compliance between the calculated and realized nutritional composition of the diets. Compared with the planned values (13.9 MJ/kg DM of diet) P2_AS and P2_RCS were lower in AME_N (13.4 and 13.0 MJ/kg DM of diet). The Lys/AME_N relation in P2_RCS was slightly lower in contrast to the other diets but still in agreement with the recommendations (0.72) of the GfE, 1999.

The animals showed a good health status throughout the experiment. Mortality was generally low (no losses in P2 and one loss during P3). There were no significant differences in feed intake between the feeding groups (Table 6 and 7). All feeding groups gained weight in P2 and P3.

The pc digestibility of CP from AS (0.88) and RCS (0.70) was higher than from AL (0.51) and RCL (0.50) (Table 8). AA were generally more digestible in AS (Met 0.84, Lys 1.00) and RCS (Met 0.99, Lys 0.88) than in AL (Met 0.61, Lys 0.49) and RCL (Met 0.73,

Table 5
Relative contents of nine major putatively identified saponins expressed as equivalent umbelliferone (μg/g dry matter-DM) in alfalfa leaves (AL) and alfalfa silage (AS).

Saponins*	Aglycone	Retention time (min)	Accurate mass measured	Mass error (ppm)	Saponin contents, umbelliferone equivalent (μg/g DM) (n = 3) (SEM[1])	
					AL	AS
Hex-Hederagenin	Hederagenin	21.93	633.39	16.19	11.3 (2.1)	65.5 (3.2)
3-Glc-medicagenic acid	Medicagenic acid	16.75	663.37	16.45	44.6 (4.9)	37.3 (1.3)
Medicagenic acid derived	Medicagenic acid	12.98	1104.53	4.88	51.4 (11.8)	62.6 (2.0)
Hex-HexA-Aglycone A	Unknown	16.45	823.40	14.25	61.4 (16.4)	23.4 (0.3)
Azukisaponin II	Azukisaponin	19.79	795.44	13.20	62.7 (12.9)	111 (3.2)
Medicoside H	Medicagenic acid	14.19	941.46	13.00	84.2 (9.7)	209 (5.4)
Medicagenic acid 3-O-β-D-glucuronide	Medicagenic acid	19.47	677.35	11.44	89.3 (21.8)	242 (4.0)
3-Glc-Glc-28-Ara-Rha-Medicagenic acid	Medicagenic acid	12.97	1103.52	4.80	101 (18.8)	138 (13.2)
HexA-dHex-Pen-Pen-Pen-Zanhic acid	Zanhic acid	13.98	1235.55	5.56	200 (34.5)	12.4 (0.5)

* Saponin abbreviations: Ara: arabinose; Glc: glucose; Hex: hexose; HexA: hexuronic acid; dHex: 6-deoxyhexose; Pen: pentose; Rha: rhamnose.
[1] SEM: Standard error of the mean of saponin concentration measured for three replicates.

Table 6
Growth performance of broilers fed diets containing 150 g/kg of the test feedstuffs alfalfa leaves (AL), red clover leaves (RCL), alfalfa silage (AS) and red clover silage (RCS) in phase 2 (P2).

Item		Feeding group					P-value
		P2_C[1]	P2_AL	P2_RCL	P2_AS	P2_RCS	
Daily feed intake (day 22−28)	g DM[2]/day	72.5	76.7	81.7	73.8	79.2	0.574
SEM		5.03	4.11	4.11	4.11	4.11	
Body weight (day 21)	g	406	401	410	403	406	0.632
SEM		5.31	4.34	4.34	4.34	4.34	
Body weight (day 28)	g	677^{b}	649^{c}	672^{bc}	704^{a}	653^{bc}	<0.001
SEM		8.39	6.85	6.85	6.85	6.85	
Daily weight gain (day 22−28)	g/day	38.8^{b}	35.3^{b}	37.4^{b}	43.0^{a}	35.2^{b}	<0.001
SEM		1.36	1.11	1.11	1.11	1.11	
Feed conversion rate (day 22−28)	kg/kg	1.87^{ab}	2.20^{a}	2.19^{a}	1.72^{b}	2.26^{a}	0.029
SEM		0.16	0.13	0.13	0.13	0.13	

$^{a–c}$ Different superscript letters indicate significant differences between the treatments (P≤0.05).
[1] P2_C: Control group in P2.
[2] DM, dry matter.

Lys 0.61). Within the group of essential AA, threonine and valine in AL were poorly digestible (both 0.40). The pc CP and AA digestibility ranged widely within all test feedstuffs, especially within AL (*e.g.* arginine 0.62, cystine 0.21) and RCL (*e.g.* arginine 0.78, valine 0.12). However, the coefficients of determination (r^2) of valine in RCL (0.03) and cystine in AL (0.15), RCL and AS (both 0.27) were low and, therefore, the estimated pc digestibility of these AA seems to be implausible or at least not very reliable.

4. Discussion

The CP and AA concentrations of AL and RCL (Table 1) were comparable to those described in literature (in g/kg DM; AL: CP 201–339, Met 2.76–3.10, Lys 10.0–17.4; RCL: CP 217–311, Met 2.45, Lys 15.3) (Ritteser and Grashorn, 2015; Hoischen-Taubner and Sundrum, 2016). The CP content of the tested AS was slightly higher compared to earlier reports of organically produced clover silages with 900 g/kg alfalfa and 100 g/kg white clover (in g/kg DM; CP 204–226, Met 2.90–3.60, Lys 10.5–11.0) (Ritteser and Grashorn, 2015; Wüstholz et al., 2016). The test feedstuff RCS showed a rather low CP content (190 g/kg DM) but fell within the range of reports of organically and conventionally produced RCS (158−212 g/kg DM) (Bayat et al., 2010; Presto Åkerfeldt et al., 2019). However, harvesting in an earlier stage can lead to higher CP and AA contents and should be aspired in the production of alfalfa and red clover products for monogastrics.

Despite there being no significant differences in feed intake, significant differences in body weights between the feeding groups were registered after P2. These differences might be explainable by differences in nutritional composition of the diets (*e.g.* lower AME_N and Lys/AME_N relation in P2_RCS) or in pc digestibility of CP and AA from test feeds. However, P2 diets were only fed for 7 days and, therefore, performance data have to be interpreted with care. Nevertheless, the introduction of 150 g/kg of the respective test feeds in the diets allowed the adaptation of the broilers to the uncommon feedstuffs. It also showed that weight losses were rather not to be expected during digestibility estimation in P3. As the results in Table 7 show, this expectation was met.

Higher pc CP and AA digestibility values were determined for AS and RCS than for AL and RCL (Table 8). Regarding the lower CF content in leaf products, higher pc digestibility results for AL and RCL than for AS and RCS were expected. However, the CF content of RCS was almost as low as of AL (182 *versus* 174 g/kg DM). Nevertheless, high pc CP and AA digestibility values were determined for AS, showing the highest CF content (273 g/kg DM). Whole plant silages as well as leaf products, containing leaves and remaining parts of stems, represent very heterogeneous feedstuffs. This heterogeneity is considerably higher compared to grain legumes and might explain, at least partly, the varying r^2-values.

Regarding the essential AA, particularly the low pc digestibility of threonine and valine of AL (both 0.40) has to be considered. In comparison, Ganzer et al. (2017) determined a pc digestibility of 0.79 for threonine and 0.87 for valine in organically produced soybean cake with slow-growing broilers (ISA J-257). With regard to the contents of the first limiting AA Met and Lys of AL (3.64 and 13.1 g/kg DM) (Table 1) and soybean cake (6.5 and 29.3 g/kg DM) (Ganzer et al., 2017), 2 g/kg DM AL could replace 1 g/kg DM soybean cake in diets. Thus, 2 g/kg DM AL would contribute the same amount of threonine and valine (19.4 and 22.0 g/kg DM) as 1 g/kg DM soybean cake (19.5 and 22.7 g/kg DM) to diets but only 7.7 and 8.8 g/kg DM digestible threonine and valine compared to 15.4 and 19.7 g/kg DM in soybean cake. Consequently, levels of up to 4 g/kg DM AL would be necessary to substitute 1 g/kg DM soybean cake in diets if threonine or valine contents were marginally present in diets. Hence, the low pc digestibility of threonine and valine in AL could lead to a restriction in the feeding of AL to broilers.

Information on the digestibility of alfalfa and red clover products in broilers is quite limited. Hoischen-Taubner and Sundrum (2016) evaluated the pc digestibility of alfalfa and red clover leaves and whole plants by an *in vitro* method. They found high estimates of pc CP and AA digestibility for all feedstuffs (Met 0.76−0.78, Lys 0.77−0.80). However, the applied enzymes pepsin and pancreatin originated from the gastrointestinal tract of pigs, which might result in divergent results between *in vivo* and *in vitro* studies (Hoischen-Taubner and Sundrum, 2016). In general, the utilization of an *in vitro* method adapted to chickens probably leads to a more precise simulation of chickens' digestion and to different digestibility estimates for such feedstuffs. Moreover, environmental

Table 7
Growth performance of broilers fed diets containing three different inclusion levels of the test feedstuffs alfalfa leaves (AL), red clover leaves (RCL), alfalfa silage (AS) and red clover silage (RCS) in phase 3.

		Feeding group													
Item		AL1	AL2	AL3	RCL1	RCL2	RCL3	AS1	AS2	AS3	RCS1	RCS2	RCS3	SEM	P-value
Inclusion level (g/kg)		100	150	200	100	150	200	100	150	200	100	150	200		
Daily feed intake (day 29−41/42)	g DM[1]/day	113	121	124	122	133	115	116	120	112	111	110	104	7.58	0.423
Daily feed intake (day 30−41/42)	g DM/day	119	132	133	131	144	123	120	128	117	114	115	107	8.48	0.178
Body weight (day 28)	g	650^{bc}	652^{bc}	644^{bc}	665^{bc}	682^{bc}	668^{bc}	738^{a}	679^{bc}	693^{b}	654^{bc}	632^{c}	672^{bc}	10.9	<0.001
Body weight (day 41/42)	g	1399^{c}	1418^{bc}	1341^{c}	1386^{c}	1372^{c}	1384^{c}	1615^{a}	1544^{ab}	1555^{a}	1375^{c}	1297c	1304^{c}	32.5	<0.001
Daily weight gain (day 29−41/42)	g/day	55.5^{bc}	56.8^{b}	51.5^{bcd}	53.2^{bcd}	51.1^{bcd}	53.0^{bcd}	64.9^{a}	64.0^{a}	63.8^{a}	53.5^{bcd}	49.2^{cd}	47.0^{d}	1.53	<0.001

[a–d] Different superscript letters indicate significant differences between the treatments ($P \leq 0.05$).
[1] DM, dry matter.

Table 8
Coefficients of precaecal digestibility (pcd) of crude protein and amino acids in organically produced alfalfa leaves (AL), red clover leaves (RCL), alfalfa silage (AS) and red clover silage (RCS) determined with a linear regression approach in 41/42-day-old broilers.

Item	AL			RCL			AS			RCS		
	pcd	SEM	r^2	pcd	SEM	r^2	pcd	SEM	r^2	pcd	SEM	r^2
Crude protein	0.51	0.108	0.69	0.50	0.115	0.65	0.88	0.111	0.86	0.70	0.195	0.56
Lysine	0.49	0.097	0.72	0.61	0.124	0.70	1.00	0.300	0.53	0.88	0.134	0.81
Methionine	0.61	0.097	0.80	0.73	0.100	0.84	0.84	0.144	0.77	0.99	0.133	0.85
Cystine	0.21	0.159	0.15	0.27	0.143	0.27	0.53	0.276	0.27	0.85	0.313	0.43
Threonine	0.40	0.106	0.59	0.48	0.144	0.53	0.78	0.154	0.72	0.65	0.183	0.56
Tryptophan	0.46	0.102	0.67	0.52	0.145	0.57	0.77	0.131	0.78	0.97	0.178	0.75
Leucine	0.45	0.118	0.60	0.50	0.127	0.61	0.81	0.097	0.87	0.79	0.132	0.78
Isoleucine	0.45	0.127	0.55	0.53	0.178	0.47	0.80	0.113	0.84	0.77	0.129	0.78
Valine	0.40	0.132	0.48	0.12	0.217	0.03	0.77	0.127	0.79	0.74	0.130	0.76
Arginine	0.62	0.082	0.85	0.78	0.086	0.89	0.79	0.100	0.86	0.90	0.090	0.91
Histidine	0.50	0.082	0.79	0.53	0.109	0.71	0.57	0.120	0.70	0.82	0.126	0.81
Phenylalanine	0.49	0.114	0.65	0.62	0.129	0.69	0.80	0.121	0.81	0.79	0.130	0.79
Tyrosine	0.44	0.113	0.61	0.52	0.122	0.65	0.72	0.136	0.74	0.77	0.149	0.73
Alanine	0.37	0.124	0.48	0.43	0.141	0.48	0.80	0.125	0.81	0.70	0.145	0.70
Glycine	0.30	0.105	0.46	0.36	0.147	0.38	0.61	0.145	0.64	0.63	0.179	0.56
Serine	0.32	0.089	0.56	0.48	0.122	0.61	0.74	0.181	0.62	0.66	0.177	0.58
Proline	0.40	0.102	0.61	0.52	0.138	0.58	0.77	0.091	0.88	0.72	0.156	0.68
Aspartic acid	0.49	0.102	0.70	0.52	0.115	0.67	0.81	0.097	0.87	0.74	0.134	0.75
Glutamic acid	0.52	0.112	0.69	0.69	0.110	0.80	0.80	0.089	0.89	0.92	0.115	0.87

influences and other processes in the living organism, such as the intestinal reaction to anti-nutritional substances, are not reflected in *in vitro* digestibility methods.

Ritteser and Grashorn (2015) determined the pc digestibility of dried AL and two clover silages of 900 g/kg alfalfa and 100 g/kg white clover (extruded/not extruded) by linear regression in ISA JA 957 chickens on day 42. The CP and AA of AL were highly digestible (Met 0.93, Lys 0.87), whereas the determined pc digestibility of CP and AA of the clover silages were lower (extruded/not extruded: Met 0.50/0.48, Lys 0.45/0.33). Ritteser and Grashorn (2015) suspected the high CF content of the clover silages (extruded/not extruded, in g/kg DM: 214/211) to be a possible reason for the lower pc digestibility values. However, AL showed similar CF contents (202 g/kg DM) but higher pc digestibility results. The feed intake of groups fed diets containing higher levels of AL decreased, which resulted in lower weight gains (100 *versus* 300 g/kg AL) and weight losses (500 g/kg AL). In our opinion, the very high CP and AA pc digestibility results determined for AL in the study by Ritteser and Grashorn (2015) have to be regarded carefully due to the reduced weight gains and weight losses during the feeding of AL. In the present study, no significant differences in feed intake were found and all feeding groups gained weight during P3 (Table 7). In contrast to the study from Ritteser and Grashorn (2015), we found higher pc CP and AA digestibility results for AS, containing high CF contents (273 g/kg DM), and lower pc CP and AA digestibility values for AL, having low CF contents (174 g/kg DM). This indicates that other factors than high CF contents must have influenced the pc digestibility of the tested AL and AS.

It is known that high amounts of dietary fiber in diets can reduce digestibility. Nevertheless, the inclusion of moderate amounts of dietary fiber could have positive effects on nutrient digestibility in poultry. As described by Mateos et al. (2012), this effect might be related to a well-developed gizzard that favors gastroduodenal refluxes and increases the secretion of digestive juices. However, the gastrointestinal development is affected by type and particle size of dietary fiber. In the present study, AL and RCL have been finely ground, whereas AS and RCS have been coarsely crushed during the extrusion and pelleting process. The larger particle size might have had positive effects on the gastrointestinal development of broilers fed diets containing AS and RCS and thus on the digestibility of CP and AA from the silages. However, this presumption cannot be proved because organ samples have not been taken in the study.

In linear regression approaches, feed-specific endogenous losses affected by anti-nutritional factors are also considered in the slopes and thus in the pc digestibility estimates (Rodehutscord et al., 2004). Various ANF in alfalfa and red clover were reported. Chang et al. (1978) and Brown et al. (1985) found trypsin inhibitors in AL and a trypsin inhibition activity in red clover has also been described by Maliar et al. (2011). Additionally, RCL contain phenols and polyphenol oxidase (Jones et al., 1995), which is responsible for a low protein degradation in RCS and red clover extracts possibly caused by the formation of protein-phenol complexes (Winters et al., 2008). However, trypsin inhibition and polyphenol oxidase activity have not been investigated in the present study. Therefore, possible effects on the pc CP and AA digestibility cannot be excluded.

Forage legumes contain saponins (Cheeke, 1983), which are considered as the main anti-nutritional substances in alfalfa (Sen et al., 1998). In earlier studies, particularly alfalfa saponins and to a lesser extent clover saponins have been of concern (Cheeke, 1996). Due to this, only the test feedstuffs AL and AS were analyzed for their specific saponin content. However, saponins can also be found in red clover (Oleszek and Jurzysta, 1986; Oleszek and Stochmal, 2002). Besides their throat-irritating effect and bitter taste (Oleszek et al., 1992), saponins influence digestion and absorption of nutrients. As reviewed by Francis et al. (2002), saponins reduce protein digestibility. Soybean saponins have been shown to affect the proteolytic activity of chymotrypsin and trypsin (Ishaaya and Birk, 1965). Furthermore, saponins can form complexes with proteins (Potter et al., 1993). It was shown that the *in vitro* digestibility of a complex between bovine serum albumin and soyasaponin was lower than that of free bovine serum albumin (Ikedo et al., 1996). As reported by Johnson et al. (1986), some saponins can reduce transmural potential difference, increase the permeability of intestinal mucosal cells

and thereby inhibit active nutrient transport. Based on these findings, it is hypothesized that saponins in the tested feedstuffs have adversely affected the pc CP and AA digestibility.

Saponins consist of one, two or three sugar side chains linked to a hydrophobic aglycone (mono-, bis- or tridesmosidic form) (Oleszek, 1996; Francis et al., 2002), resulting in a multitude of different saponins. Alfalfa saponins occur as triterpene glycosides of different aglycones like medicagenic acid, zanhic acid, soyasapogenol and hederagenin (Oleszek, 1996). Depending on their chemical structure of aglycone and sugar side chains, saponins reveal different biological activity. In a study on the intestinal membrane depolarizing activity of alfalfa saponins, zanhic acid glycosides showed a greater reduction of transmural potential difference than medicagenic acid glycosides in the small intestines of rats. The highest depolarizing activity was found in zanhic acid tridesmoside (Oleszek et al., 1994). In the present study, high amounts of a zanhic acid derived compound were found in the tested AL (Table 5), whereas AS contained low amounts of this compound. Conversely, AS showed higher contents of some medicagenic acid glycosides than AL. The concentration of saponins in leaves is higher than in stems (Livingston et al., 1977; Sen et al., 1998). Furthermore, Szumacher-Strabel et al. (2019) have demonstrated structural and quantitative changes of saponins during the ensiling process in ten alfalfa varieties. Three of five detected zanhic acid glycosides degraded substantially, including a decrease in zanhic acid tridesmoside level. In the present study, AL and AS were not gained in the same harvest. Nevertheless, the ensiling might have led to quantitative and structural changes in individual saponins of AS. Regarding the lower pc digestibility values in AL than in AS, especially the higher contents of zanhic acid glycosides in AL rather than medicagenic acid glycosides might have affected the pc digestibility. It might be possible that the pc CP and AA digestibility of RCL and RCS was also affected by saponins, if similiar saponin contents and changes occurred in these feedstuffs. However, these presumptions need to be demonstrated. The different biological activity of individual saponins reported in literature as well as our own findings demonstrate that the total saponin concentration is not sufficient to fully characterize the quality of alfalfa products as feedstuffs. Therefore, the analysis of individual saponins is highly recommended. Saponin concentrations are influenced by several factors like variety, growing season, year of growth and climate factors (Tava et al., 1999; Pecetti et al., 2006). Thus, variation in saponin concentrations of alfalfa and red clover products from different harvests may result in varying pc CP and AA digestibility values in broilers.

Besides that, another factor might have been responsible for the lower digestibility of AL and RCL. Protein solubility is an indicator of overprocessing (Araba and Dale, 1990). The low protein solubility of AL (0.40) and especially of RCL (0.16) therefore indicates a damage to proteins due to the strong heat treatment (100−600 °C) during the drying process. In contrast, proteins of the silages AS (0.56) and RCS (0.38) were more soluble. This might have contributed to the higher pc CP and AA digestibility of these feedstuffs. Contrary to that are the results from Pleger et al. (2020). The same lot of AL was compared to AL dried at low temperatures (ALLT; 45 °C) in a feeding trial with broilers. The ALLT showed a higher protein solubility (0.54) but no significant differences in the fattening and slaughtering performance of broilers.

5. Conclusions

Lower precaecal digestibility values of crude protein and amino acids were determined for alfalfa leaves and red clover leaves than for alfalfa silage and red clover silage, possibly due to higher concentrations of various anti-nutritional factors. The valuable crude protein and amino acid concentrations of alfalfa and red clover leaves could not be utilized in their full potential. It is likely that especially the occurrence of saponins have adversely affected the digestibility of the leaf products. Differences in saponin contents suggest that certain individual saponins, *e.g.* zanhic acid glycosides, might have a more negative effect on crude protein and amino acid digestibility than other saponins. For the establishment of alfalfa and red clover products as reliable protein sources in organic poultry nutrition, further investigations regarding processing methods (harvest, gentle drying, ensiling) and anti-nutritional factors such as phenols and polyphenol oxidase in red clover, trypsin inhibitors or saponins and their biological activity in the gastrointestinal tract are required.

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Declaration of Competing Interest

None.

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