Effect of a "diluted" diet containing 10% lignocellulose on the gastrointestinal tract, intestinal microbiota, and excreta characteristics of dual purpose laying hens

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ABSTRACT Low performing dual purpose hens have different nutritional requirements compared to conventional hybrid hens. Lignocellulose is a low fermentable polymer, acting as a diet diluent and may influence physiological and digestive processes. This study investigated the effect of a 10% dietary lignocellulose dilution on the development of gastrointestinal organs, intestinal morphology, intestinal microbiota, and excreta characteristics of dual purpose hens. One-day-old female Lohmann Dual chicks were allocated to 12 pens and fed two different diets: A standard control diet (**CON**) and a treatment diet (**LC**), based on CON but diluted with 10% lignocellulose (ARBOCEL®). At 52 wk of age, gastrointestinal organs were extracted and weights determined. Colorectal tissue samples were chemically fixed and stained for histomorphological examinations. Cecal digesta samples were analyzed for bacterial metabolites and composition using gas chromatography, HPLC, photometry, and PCR. Excreta dry matter and viscosity was consistently assessed during the trial. LC-fed hens showed increased weights of the gizzard ($P = 0.003$), small ($P < 0.001$), and large intestine ($P = 0.048$) compared to hens fed CON. LC-fed hens had a larger colorectal villus area ($P = 0.049$), a higher mucosal enlargement factor of villi ($P = 0.016$) and crypts ($P = 0.030$) than CON-fed hens. The concentration of short-chain fatty acids (**SCFAs**) ($P = 0.017$) and ammonia ($P = 0.013$) was higher in CON-fed hens compared to LC-fed hens. Bacterial composition and activity was generally not affected by feeding the different diets. LC-fed hens had a higher excreta dry matter content than hens fed CON at 10 ($P < 0.001$), 17 ($P < 0.001$), and 22 ($P = 0.002$) wk of age. Correlation analyses revealed a negative relationship between the concentration of SCFAs in the cecum and the colorectal villus surface area ($P < 0.01$). In conclusion, the feeding of high levels of lignocellulose increased gastrointestinal organ weights and colorectal surface area in dual purpose laying hens. A higher intestinal surface area in combination with lower concentrations of SCFAs might indicate a compensatory reaction of hens fed LC enhancing the absorption of bacterial metabolites by increasing the intestinal mucosal surface.

Key words: dual purpose chicken, lignocellulose, digestive tract, gastrointestinal morphology, microbiota

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INTRODUCTION

New approaches to avoid the killing of day-old male chicks of the layer type are necessary. The use of dual purpose chicken might be one possible solution using both sexes, the male for meat and the female for egg production. Studies showed that dual purpose hens fed with standard layer diets developed higher bodyweights and an increased body fat percentage accompanied with lower productivity in comparison with commercial hybrid hens (Rizzi et al., 2002; Rizzi et al., 2007; Rizzi and Chiericato, 2010; Steenfeldt and Hammershøj, 2015). From this, it can be concluded that low performing hens have different nutritional requirements compared to conventional hybrid laying hens. Recently published data showed that the feeding of an energy- and nutrient-reduced diet containing 10% lignocellulose reduced body fat content and improved laying performance in dual purpose laying hens (Röhe et al., 2019). The question arose whether a high concentration of dietary fiber might be accompanied with alterations of the chickens intestinal tract and microbiota. It is well known that the feeding of dietary fiber could affect the digestive tract development, intestinal morphology, and gut microbiota in poultry. Known changes depend on the used fiber source, inclusion

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level, and its chemical and physical characteristics such as particle size, solubility, and degree of lignification (Hetland and Svihus, 2001; Montagne et al., 2003; De Vries et al., 2012). The term "dietary fiber" underwent different definitions, one is that it includes any polysaccharide reaching the large intestine such as resistant starch, lignin, soluble and insoluble non-starch polysaccharides (**NSP**) (Montagne et al., 2003). Lignocellulose, a constituent of plant cell walls, is mainly composed of the insoluble NSP cellulose (40 to 47 wt%) and hemicellulose (25 to 35 wt%) as well as the biopolymer lignin (16 to 31 wt%) (Liu et al., 2014). Lignocellulose is the most abundant and bio-renewable biomass on earth and has gained particular attention as potential resource for sustainable production of chemicals and fuels (Zhou et al., 2011). Moreover, research has been focused on the use of lignocellulose as a dietary component for livestock and companion animals with potential effects on digestive physiology and function. Studies showed that dietary lignocellulose at low inclusion levels up to 0.8% might stimulate the development of the digestive tract in pullets and laying hens (Yokhana et al., 2015) and enhance mucosal development in broilers (Sarikhan et al., 2010; Makivic et al., 2019). In general, chickens show a low bacterial capacity to ferment insoluble NSP because they have a high feed passage rate, a short digestive tract and limited microbial cellulolytic activity in the hindgut (McNab, 1973; Carré et al., 1990; Jørgensen et al., 1996; De Vries et al., 2012; Waite and Taylor, 2014). However, it was reported that the dietary inclusion of low concentrations of lignocellulose might modulate bacterial populations and metabolites in the small and large intestine of broilers (Sarikhan et al., 2010; Bogusławska-Tryk et al., 2015; Kheravii et al., 2017; Makivic et al., 2019). Moreover, some studies showed that dietary lignocellulose at low inclusion levels might have a beneficial effect on litter quality by lowering the excreta moisture content (Farran et al., 2013; Kheravii et al., 2017; Makivic et al., 2019).

The goal of the current study was to investigate the effect of feeding diets containing high levels of lignocellulose on the development of gastrointestinal organs, intestinal histomorphology, intestinal microbiota, and excreta characteristics in dual purpose laying hens. We hypothesized that the diet dilution by 10% lignocellulose (ARBOCEL® R, J. Rettenmaier & Söhne GmbH + Co KG, Rosenberg, Germany) would have a clear impact on the digestive tract traits, the bacterial composition and activity in the hindgut ,and excreta characteristics of dual purpose laying hens.

MATERIAL AND METHODS

All procedures involving handling and treatments of animals were approved by the local state office of occupational health and technical safety (Landesamt für Gesundheit und Soziales, Berlin, Germany, LaGeSo G 0171/16).

Animals, Rearing Conditions, and Experimental Diets

In total, one hundred thirty two 1-day-old female chicks of a dual purpose breed (Lohmann Dual, Lohmann Tierzucht, Cuxhaven) were randomly allocated to 12 pens. The birds were kept on litter-floor pens (*Miscanthus* shavings) and had *ad libitum* access to feed and water. The ambient temperature was adjusted as follows: for the first 2 d of age the ambient temperature was 35°C and was then gradually decreased to 19 ± 1°C by 35 d of age and maintained at a constant to the end of the experiment. The lighting regime was 24 h during the first 2 d, followed by a gradually reduction to 9 h of light per d until 17 wk and followed by an increase to 14 h of light per d until the end of the trial. Two different experimental diets were offered in mash form resulting in 6 replicates per feeding group: the basal control diet (**CON**) and a treatment diet (**LC**), based on CON but diluted with 10% lignocellulose (ARBOCEL® R, J. Rettenmaier & Söhne GmbH + Co KG, Rosenberg, Germany). The used lignocellulose source was produced by means of mechanical processing of fresh natural dried wood and had an average fiber length of 200 to 300 μm and a bulk density of 60 to 105 g/l, per supplier information. Detailed information on the feed composition, nutrient content, and feeding schedule are described in Röhe et al. (2019). Feed composition and analyzed nutrient content of a grower and a layer diet are displayed in Table 1. In addition, the feed discrete mean particle size (**dMean**) is indicated based on the results of the conducted dry-sieve analysis.

Sampling and Analyses

Dry-Sieve Analyses Analyses of the feed particle sizes of diets were conducted with a grower diet (6 to 12 wk of age) and a layer diet (32 to 42 wk of age). A representative 100-g sample of each diet was passed through a sieve stack situated on sieve shaker (Analysette 3, Fritsch, Idar Oberstein, Germany) for 10 min at an amplitude of 7. The sieve stack (Analysensiebe, Retsch GmbH, Haan, Germany) was composed of 9 sieves with screens of different mesh sizes (4, 2.5, 2.0, 1.6, 1.25, 1.0, 0.63, 0.40, and 0.15 mm). After the shaking process, the amount of particles retained on each screen was determined by subtracting the weight of the sieve and the retained feed from the blank weight of the sieve. The dMean was calculated as described earlier (Fritz et al., 2012).

Determination of Organ Weights The proventriculus, gizzard, small intestine, large intestine, and the liver were extracted from the chickens carcass and subsequently intestinal content, adhering fat, and mesenteries removed. The organs were weighed, and organ-to-BW ratios calculated. The total gastrointestinal tract weight was determined by summing the single intestinal segments.

Table 1. Feed composition (%) and analyzed nutrient content of grower (6 to 12 wk of age) and layer diets (32 to 42 wk of age).

	CON[1]		LC[1]	
	Grower	Layer	Grower	Layer
Ingredient (%)				
Wheat	22.56	39.75	20.30	35.78
Maize	30.09	21.58	27.08	19.42
Soybean meal. extracted	5.00	5.00	4.50	4.50
Rapeseed meal. extracted	4.87	4.50	4.38	4.05
Rapeseed expeller		5.50	0.00	4.95
Sunflower meal. extracted	10.00	9.80	9.00	8.82
Triticale	5.00		4.50	0.00
Barley	5.00		4.50	0.00
Wheat bran	10.00		9.00	0.00
Oat bran	3.00	1.00	2.70	0.90
ARBOCEL®R[2]			10.00	10.00
Calcium carbonate	1.39	8.51	1.25	7.66
Sodium bicarbonate	0.27	0.15	0.24	0.14
Common salt	0.18	0.23	0.16	0.21
Monocalcium phosphate	0.10	0.10	0.09	0.09
Choline chloride	0.05	0.05	0.05	0.05
Premix[3]	0.30[1]	0.30[2]	0.27	0.27
L-Lysin HCL	0.48	0.23	0.43	0.21
DL-Methionine	0.13	0.11	0.12	0.10
L-Threonin	0.08		0.07	0.00
Plant oil	1.50	3.19	1.35	2.87
Analyzed Nutrients (g/kg)				
Crude Protein	186	182	170	165
Crude Fat	39.9	64.4	35.5	56.1
NDF	134	104	195	198
ADF	67.3	62.1	154	120
ADL	14.6	20.8	45.2	37.7
Starch	430	372	375	326
Crude Ash	46.3	120	39.7	109
Calcium	6.98	33.5	6.33	29.3
Phosphorus	5.09	4.74	4.66	4.41
Sodium	1.80	1.76	1.59	1.97
Potassium	5.82	4.92	5.37	4.51
Calculated				
AME_N (MJ/kg)[4]	13.15	12.70	11.65	11.15
dMean[5]	1.47	1.54	1.36	1,37

[1]CON = control-fed birds; LC = lignocellulose-fed birds.
[2] J. Rettenmaier & Söhne GmbH+CO. KG, Rosenberg, Germany.
[3]provided per kg grower (layer): 10,000 (9,000) IU vitamin A; 2,500 (2,500) IU vitamin D3; 40.0 (20.0) mg vitamin E (α-tocopherol acetate); 1.50 (2.00) mg vitamin K3; 2.50 (1.00) mg vitamin B1; 5.00 (4.00) mg vitamin B2; 25.0 (20.0) mg nicotinic acid; 3.00 (2.00) mg vitamin B6; 25.0 (25.0) μg vitamin B12; 75 (100) μg biotin; 8.00 (6.52) mg calcium pantothenic acid; 0.80 (0.50) mg folic acid; 80.0 (50.0) mg Zn (zinc oxide); 40.0 (5.00) mg Fe (iron carbonate); 80.0 (50.0) mg Mn (manganese oxide); 15.0 (12.0) mg Cu (copper sulfate-pentahydrate); 1.00 (1.00) mg I (calcium iodate); 0.25 (0.20) mg Se (sodium selenite).
[4]AME_N (MJ/kg) = nitrogen-corrected apparent metabolizable energy estimated according to WPSA (1984).
[5]dMean = discrete mean particle size (based on dry-sieve analysis) according to equation of Fritz et al. (2012).

Histomorphological Analyses Tissue sections from the colorectum were cut open longitudinally and placed on cork boards by using hedgehog spines and fixed in a 4% phosphate-buffered formaldehyde solution for 24 h. After dehydration and infiltration with solidified paraffin wax, the samples were embedded. The paraffin blocks were cut at 4 μm with a sledge microtome (Typ SM 2000 R, Leica, Nussloch, Germany). Obtained sections were mounted on glass slides. Tissue slides were stained with AB/PAS (Chroma, Waldeck, Germany) at pH 2.5 and analyzed with a light microscope (Photomicroscope III, Zeiss, Germany), which was equipped with a digital camera (DP72, Olympus, Germany). Histomorphometric parameters were measured by using an image analysis software (CellSense software, Olympus, Germany). In total, 15 vertically oriented villi and crypts per section were analyzed. The villus length (measured from the tip of the villi to the villus crypt junction) and crypt depth (defined as the depth of the invagination between adjacent villi) was measured and based on that the villus length-to-crypt depth ratio calculated. Furthermore, villus and crypt area was assessed by multiplying the individual villus respectively crypt area by the number of villi respectively crypts per 1,000 μm intestinal cross-section. In order to estimate the enlargement of the intestinal surface epithelium by villi and crypts, the mucosal enlargement factor of the villus and crypt was determined by dividing the total villus respectively crypt surface length by the length of corresponding lamina muscularis mucosae) as described earlier (Wiese et al., 2003; Rieger et al., 2015). Furthermore, the absolute number of goblet cells (total number of goblet cells per villus respectively crypt) and the relative number of goblet cells (goblet cells per 100 μm basement membrane of villus respectively crypt) were counted. Moreover, the absolute mucin staining area (total mucin staining area per villus respectively crypt in mm^2) and the relative mucin staining area (mucin staining area per villus area respectively crypt area in %) was determined for the assessment of the intestinal mucus layer thickness (Röhe et al., 2018).

Determination of Bacterial Metabolites in the Cecum Digesta After sampling, cecal digesta was frozen in liquid nitrogen and stored at −80°C. The determination of bacterial metabolites was conducted as described by Kröger et al. (2017). Short-chain fatty acids (**SCFAs**) were analyzed by gas chromatography (Agilent Technologies 6890 N, auto sampler G2614A, and injection tower G2613A; Network GC Systems, Böblingen, Germany) equipped with a flame ionization detector. D- and L-lactate was measured by HPLC (Agilent 1100; Agilent Technologies, Böblingen, Germany) with a pre-column (Phenomenex C18 4.0 4.0 × 2.0 mm; Phenomenex Ltd., Aschaffenburg, Germany) and an analytical column (Phenomenex Chirex 3126 (D)-penicillamine 150 × 4.6 mm; Phenomenex Ltd.) Ammonia was analyzed calorimetrically by the Berthelot reaction in microtitration plates using a Tecan Sunrise microplate reader (Tecan Austria GmbH, Grödig, Austria).

Analyses of Bacterial Cell Concentration and Activity in the Cecum Digesta Digesta samples were taken from the cecum, instantly frozen in liquid nitrogen and stored at −80°C. The quantification and qualification of selected representatives of the microbiota were carried out from DNA and RNA extracts in order to assess bacterial concentration and activity. The bacterial groups were examined by seven group primers: clostridial cluster I, IV, and XIVa,

Table 2. Primers used for quantification of bacterial 16S copy numbers in cecal contents.

Specificity	Primer	Primer sequences (5′ to 3′)	Product (bp)	A_T[1]	Reference
Clostridial Cluster XIVa	g-Ccoc-F	AAATGACGGTACCTGACTAA	440	60	(Matsuki et al., 2002)
	g-Ccoc-R	CTTTGAGTTTCATTCTTGCGAA			(Matsuki et al., 2002)
Clostridial Cluster I	CI-F1	TACCHRAGGAGGAAGCCAC	231	63	(Song et al., 2004)
	CI-R2	GTTCTTCCTAATCTCTACGCAT			(Song et al., 2004)
Clostridial Cluster IV	sg-Clept-F	GCACAAGCAGTGGAGT	239	60	(Matsuki et al., 2002)
	sg-Clept-R	CTTCCTCCGTTTTGTCAA			(Matsuki et al., 2002)
Lactobacillus spp.	Lac-1	AGCAGTAGGGAATCTTCCA	341	58	(Walter et al., 2001)
	Lac-2	CACCGCTACACATGGAG			(Heilig et al., 2002)
Bifidobacterium spp.	g-BIFID-F	TCGCGTCYGGTGTGAAAG	243	58	(Rinttilä et al., 2004)
	g-BIFID-R	CCACATCCAGCRTCCAC			(Rinttilä et al., 2004)
Bacteroides-Prevotella-Porphyromonas Cluster	BPP1	GGTGTCGGCTTAAGTGCCAT	140	55	(Rinttilä et al., 2004)
	BPP2	CGGAYGTAAGGGCCGTGC			(Rinttilä et al., 2004)
E. coli/Hafnia/Shigella group	Entero-F	GTTAATACCTTTGCTCATTGA	340	55	(Malinen et al., 2003)
	Entero-R	ACCAGGGTATCTAATCCTGTT			(Malinen et al., 2003)

[1]A_T = annealing temperature (°C).

Lactobacillus spp., *Bifidobacterium* spp., the *Bacteroides-Prevotella-Porphyromonas* cluster and the E. coli/ Hafnia/ Shigella group (Table 2). DNA and RNA Extraction was performed with a commercial NucleoSpin® RNA Kit (REF 740955, Macherey-Nagel GmbH & Co. KG, Düren, Germany) in combination with the NucleoSpin® RNA/DNA Buffer Set (REF 740944, Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer, except for the use of 100 mg sample. Quantification of bacterial DNA and rRNA was performed with a Stratagene MX3000p (Stratagene, Amsterdam, The Netherlands) using a commercial master mix (Brilliant II SYBR® Green QPCR Master Mix with Low ROX (Stratagene, Amsterdam, Netherlands). Primer sequences and annealing temperatures are given in Table 2. All primers were purchased from MWG Biotech (Straubing, Germany). A calibration series of PCR products with known copy numbers per ng DNA was used to calculate copy numbers/ g sample. With respect to the quantification of bacterial 16S rRNA, total RNA was transcribed into cDNA using a commercial kit (Superscript III, ThermoFisher Scientific, Berlin, Germany) and subsequently amplified as described before.

Analyses of Dry Matter and Viscosity in Excreta

Excreta dry matter and viscosity were measured from samples taken at different time points (at weeks 10, 17, 22, and 52 of the trial). With respect to dry matter analyses, fresh excreta was weighed into aluminum jars of known weight. Samples were dried in an incubator at 103°C and weighed again after weight constancy to detect loss of water.

Viscosity was determined by adding 10 ml of water to 5 g of excreta. Samples were continuously stirred for 30 min at 30°C, followed by centrifugation for 15 min at 1854 × g at 4°C. In total, 2 ml of the supernatant was centrifuged for further 10 min at 17500 × g. Afterwards 532 μl of that supernatant was used for viscosity analysis (DV-II Viscometer, Brookfield Eng Labs inc., Stoughton, MA, USA).

Statistical Analyses

Statistical analyses were conducted using SPSS (version 25.0, Chicago, IL). Results are reported as means and standard error of the means (mean ± SEM). The normally distributed data were analyzed by using Students t test. Spearman correlation analyses were performed displaying correlations between the mucosal enlargement factor of colorectal villi and the relative weight of the gastrointestinal organs as well as between the concentration of SCFA in the cecum and the mucosal enlargement factor of colorectal villi. Non-normally distributed data from microbiological data was analyzed via Kruskal-Wallis test and subsequent Mann-Whitney-U test, where appropriate. Differences were considered significant at $P < 0.05$.

RESULTS

With respect to the particle size distribution of the experimental diets results of the dry-sieve analyses showed that the inclusion of lignocellulose led to an increase of the proportion of smaller particles resulting in a lower dMean of the LC diet compared to CON diet (Table 1). During the whole feeding trial, birds were healthy and showed no clinical evidence of disease. Results on the animal performance of dual purpose hens are display in Röhe et al. (2019).

Gastrointestinal Organ Weights

The inclusion of dietary lignocellulose affected the relative weight of gastrointestinal organs (Table 3). LC-fed hens showed increased relative weights of the gizzard ($P = 0.003$), small intestine ($P < 0.001$) and large intestine ($P = 0.048$) resulting in a higher weight of the total gastrointestinal organs ($P = 0.002$) compared to hens fed CON.

Table 3. Impact of dietary lignocellulose on the relative weight of the gastrointestinal organs (%) and liver weight (%) of dual purpose hens.[1]

Item	CON[2]	LC[2]	SEM[3]	*P*-Value[4]
Proventriculus	0.32	0.35	0.01	0.264
Gizzard	2.36	3.51	0.22	0.003
Small intestine	1.95	2.29	0.06	<0.001
Large intestine	0.61	0.74	0.03	0.048
Total gastrointestinal tract	5.25	6.84	0.30	0.002
Liver	2.20	2.45	0.12	0.313

[1]Data are means of six replicate pens.
[2]CON = control-fed birds; LC = lignocellulose-fed birds.
[3]Results are reported as means ± SEM.
[4]Statistical analyses are based on Students t test.

Table 4. Impact of dietary lignocellulose on the villus length (VL), crypt depth (CD), villus length-to-crypt depth ratio (VL/CD), villus area (VA), villi mucosal enlargement factor (VMEF), crypt area (CA), crypts mucosal enlargement factor (CMEV) and ratio between enlargement factors (EFV/EVC) in the colorectum of dual purpose hens.[1]

Item	CON[2]	LC[2]	SEM[3]	*P*-Value[4]
VL (μm)	302	331	14.5	0.329
CD (μm)	65.0	76.1	3.35	0.101
VL/CD	4.94	4.66	0.22	0.535
VA (mm^2)	0.21	0.28	0.02	0.049
VMEF	3.86	5.52	0.37	0.016
CA (mm^2)	0.036	0.043	0.002	0.214
CMEF	1.57	2.14	0.14	0.030
VMEF/CMEF	2.51	2.62	0.14	0.718

[1]Data are means of six replicate pens.
[2]CON = control-fed birds; LC = lignocellulose-fed birds.
[3]Results are reported as means ± SEM.
[4]Statistical analyses are based on Students *t* test.

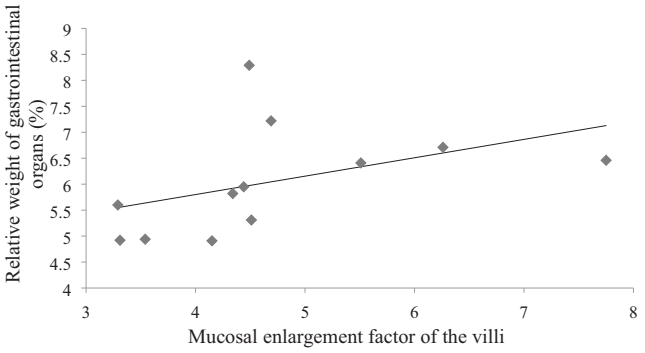

Figure 1. Correlation analyses of the mucosal enlargement factor of colorectal villi and the relative weight of gastrointestinal organs of dual purpose hens (Spearman coefficient: 0.699; $P = 0.011$).

Histomorphological Analyses

LC-fed hens had a larger colorectal villus area ($P = 0.049$) than CON-fed hens (Table 4). The mucosal enlargement factor of villi ($P = 0.016$) and crypts ($P = 0.030$) were higher in LC-fed hens compared to those fed CON. Correlation analyses revealed that the mucosal enlargement factor of colorectal villi was positively related to the relative weight of the gastrointestinal tract ($P = 0.011$; Figure 1). No differences in the number of goblet cells and the detected mucin staining area of the colorectum could be detected among the feeding groups (Table 5).

Bacterial Metabolites and Cell Counts

Analyses of bacterial metabolites in the cecum showed that the absolute concentration of acetic acid ($P = 0.018$), propionic acid ($P = 0.010$), n-valeric acid ($P = 0.001$), and the total amount of the SCFA ($P = 0.017$) were higher in CON-fed hens compared to those receiving LC (Table 6). With respect to the molar ratio, the proportion of SCFAs in the cecum was not influenced by feeding the different diets. The cecal concentration of ammonia was higher in CON-fed hens than in LC-fed hens ($P = 0.013$). Correlation analyses showed that the mucosal enlargement factor of colorectal villi ($P = 0.022$) was negatively related to the absolute concentration of SCFA in the cecum (Figure 2). Bacterial copy numbers of 16S rDNA as well as bacterial activity as measured via 16S rRNA were similar among the feeding groups (Table 7 and 8), except that the 16S rRNA of *Lactobacillus* spp. was significantly higher in CON-fed hens compared to LC-fed hens ($P = 0.002$).

Excreta Dry Matter and Viscosity

Analyses of the excreta dry matter content showed that LC-fed hens had a lower excreta water content than hens fed CON at 10 ($P < 0.001$), 17 ($P < 0.001$), and 22 ($P = 0.002$) wk of age while viscosity of excreta samples of both feeding groups were comparable during the trial (Table 9).

DISCUSSION

The bodyweight and body fat percentage of dual purpose hens was reduced by feeding a nutrient reduced diet containing a 10% lignocellulose which was accompanied with an increased laying performance (Röhe et al. 2019). The aim of the current study was to examine whether the dietary addition of high levels of lignocellulose might also influence gastrointestinal organ weights, intestinal morphology and microbiota as well as excreta characteristics of dual purpose laying hens.

Gizzard Development

The results showed that hens fed with lignocellulose had increased weights of the gizzard compared to those fed the basal diet. Several studies investigated the effect of dietary fiber on the gizzard development of chicken (Jørgensen et al., 1996; Hetland and Svihus, 2001; González-Alvarado et al., 2007; Jiménez-Moreno et al., 2009). However, only a few studies used the non-fermentable fiber source lignocellulose as feed ingredient. In line with the results of the present study, increased relative weights of the gizzard were observed in pullets fed diets containing 1% lignocellulose

Table 5. Impact of dietary lignocellulose on the absolute and relative number of goblet cells as well as the absolute and relative mucin staining area in the colorectum of dual purpose hens.[1]

Item	Region	CON[2]	LC[2]	SEM[3]	*P*-Value[4]
Absolute goblet cell number	Villus	49.3	47.0	1.51	0.468
Relative goblet cell number	Villus	16.4	14.4	0.62	0.111
Absolute goblet cell number	Crypt	11.9	12.5	0.64	0.668
Relative goblet cell number	Crypt	18.2	16.6	0.85	0.367
Absolute mucin staining area (mm^2)	Villus	0.60	0.80	0.006	0.102
Relative mucin staining area (%)	Villus	28.2	28.2	1.09	0.982
Absolute mucin staining area (mm^2)	Crypt	0.012	0.011	0.001	0.879
Relative mucin staining area (%)	Crypt	31.6	26.1	1.54	0.070

[1]Data are means of six replicate pens.
[2]CON = control-fed birds; LC = lignocellulose-fed birds.
[3]Results are reported as means ± SEM.
[4]Statistical analyses are based on Students t test.

Table 6. Impact of dietary lignocellulose on the absolute and relative concentration of bacterial metabolites in the cecum digesta of dual purpose hens.[1]

Item	CON[2]	LC[2]	SEM[3]	*P*-Value[4]
Acetic acid (μmol/g)	37.5	23.2	3.22	0.018
Propionic acid (μmol/g)	3.79	2.19	0.34	0.010
i-butyric acid (μmol/g)	0.29	0.34	0.09	0.759
n-butyric acid (μmol/g)	4.83	2.89	0.53	0.061
i-valeric acid (μmol/g)	0.51	0.28	0.12	0.355
n-valeric acid (μmol/g)	0.61	0.31	0.05	0.001
Total SCFA (μmol/g)	47.5	29.2	4.11	0.017
Acetic acid (mol. %)	78.8	80	0.85	0.530
Propionic acid (mol. %)	8.15	7.76	0.46	0.692
i-butyric acid (mol. %)	0.64	1.05	0.22	0.372
n-butyric acid (mol. %)	9.95	9.28	0.68	0.644
i-valeric acid (mol. %)	1.19	0.91	0.25	0.604
n-valeric acid (mol. %)	1.32	1.10	0.06	0.066
D-Lactate (μmol/g)	0.51	0.57	0.11	0.813
L-Lactate (μmol/g)	2.87	4.23	0.41	0.099
Ammonia (μmol/g)	8.38	4.54	0.84	0.013

[1]Data are means of six replicate pens.
[2]CON = control-fed birds; LC = lignocellulose-fed birds.
[3]Results are reported as means ± SEM.
[4]Statistical analyses are based on Students *t* test.

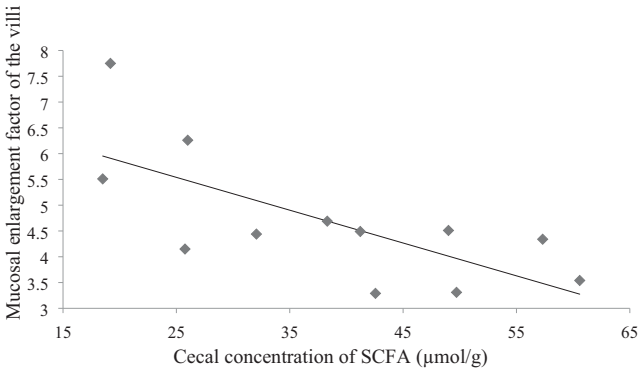

Figure 2. Correlation analyses of the concentration of short-chain fatty acids (SCFA) in the cecum and the mucosal enlargement factor of colorectal villi of dual purpose hens (Spearman coefficient: −0.650; $P = 0.022$).

(ARBOCEL® RC FINE) over a period of 10 wk (Yokhana et al., 2015). Similarly, laying hens, aged 31 wk, developed heavier gizzards when fed diets diluted with 0.8% lignocellulose (ARBOCEL® RC FINE) compared to hens fed a control diet after 12 wk of feeding (Yokhana et al., 2015). Interestingly, no effects on gizzard weight development were found in younger hens fed those diets for a shorter period of 3, 6, and 9 wk (Yokhana et al., 2015) which suggests that the length of the feeding period of lignocellulose might be relevant for the development of effects in the digestive tract. In contrast, feeding diets supplemented with 0.4 or 0.6% lignocellulose (ARBOCEL® R) did not affect gizzard weights in 42 d old broilers (Makivic et al., 2019). In another study, relative gizzard weights of broilers were also not influenced by feeding diets supplemented with 1 or 2% lignocellulose (OptiCell®, Agromed Austria GmbH) over a period of 35 d (Kheravii et al., 2017).

Several studies showed that the feeding of dietary fiber or so-called "structural components" could stimulate gizzard development in chickens. The feeding of mostly insoluble NSP sources, such as hulls of pea, oat, and soy or wood shavings, increased the gizzard weight of broilers (Jørgensen et al., 1996; González-Alvarado et al., 2007; Amerah et al., 2009; Jiménez-Moreno et al., 2009). Furthermore, insoluble NSP stimulated the gizzard function as indicated by a lower gizzard digesta pH (González-Alvarado et al., 2007; Jiménez-Moreno et al., 2009; Jiménez-Moreno et al., 2011; Makivic et al., 2019). An increase of dietary fiber was often accompanied with an increase in the proportion of coarser particles in the diets (Amerah et al., 2009; Jiménez-Moreno et al., 2009). It is well known that the feeding of coarsely ground as well as mash diets can increase the relative gizzard weights of broilers and laying hens compared to feeding finer particles and thermally processed diets (Engberg et al., 2002; Peron et al., 2005; Amerah et al., 2007; Rougière et al., 2009; Röhe et al., 2014). Consequently, it might be difficult to distinguish between the effect of fiber inclusion and that of the feed particle size. In the present study, the inclusion of lignocellulose did not increase the proportion of coarser particles in the diet, but on the contrary increased the fraction of smaller feed particles. Thus, observed effects regarding an enhanced gizzard development seem to be not connected with an increase in feed particle size. Independent of the particle size, fiber particles are harder to grind and thus accumulate in the gizzard lumen (Hetland et al., 2003), which in

Table 7. Impact of dietary lignocellulose on bacterial cell count (log10 16S rDNA copy number/g) in cecal digesta of dual purpose hens.[1]

Item	CON[2]	LC[2]	SEM[3]	*P*-Value[4]
Clostridial cluster I	10.1	9.66	0.15	0.180
Clostridial cluster IV	10.3	10.5	0.05	0.093
Clostridial cluster XIVa	10.2	10.2	0.05	0.394
Lactobacillus spp.	8.48	8.25	0.07	0.093
Bifidobacterium spp.	8.72	8.98	0.11	0.485
Bacteroides/Prevotella/Porphyromonas-Cluster	9.92	10.1	0.08	0.310
E. coli/ Hafnia/ Shigella group	7.44	7.97	0.14	0.132

[1]Data are means of six replicate pens.
[2]CON = control-fed birds; LC = lignocellulose-fed birds.
[3]Results are reported as means ± SEM.
[4]Statistical analyses are based on Mann-Whitney-U-Test.

Table 8. Impact of dietary lignocellulose on the bacterial activity (log10 copy number 16S rRNA/g) in cecal digesta of dual purpose hens.[1]

Item	CON[2]	LC[2]	SEM[3]	*P*-Value[4]
Clostridial cluster I	10.2	9.79	0.15	0.093
Clostridial cluster IV	12.8	12.8	0.05	0.818
Clostridial cluster XIVa	13.4	13.6	0.06	0.132
Lactobacillus spp.	10.2	9.58	0.11	0.002
Bifidobacterium spp.	9.87	10.0	0.11	0.589
Bacteroides/Prevotella/Porphyromonas Cluster	11.7	11.35	0.08	0.937
E. coli/ Hafnia/ Shigella group	6.95	7.15	0.13	0.905

[1]Data are means of six replicate pens.
[2]CON = control-fed birds; LC = lignocellulose-fed birds.
[3]Results are reported as means ± SEM.
[4]Statistical analyses are based on Mann-Whitney-U-Test.

Table 9. Impact of dietary lignocellulose on the excreta dry matter (DM) and -viscosity of dual purpose hens at different time points of the trial.[1]

Weeks of age	CON[2]	LC[2]	SEM[3]	*P*-Value[4]
Excreta DM (%)				
Week 10	19.5	22.7	0.53	<0.001
Week 17	22.1	25.5	0.59	<0.001
Week 22	21.1	25.5	0.85	0.002
Week 52	22.1	22.7	0.50	0.596
Viscosity (mPas)				
Week 10	2.05	1.76	0.13	0.287
Week 17	1.55	1.35	0.06	0.095
Week 22	1.28	1.30	0.05	0.815
Week 52	1.33	1.36	0.07	0.862

[1]Data are means of six replicate pens.
[2]CON = control-fed birds; LC = lignocellulose-fed birds.
[3]Results are reported as means ± SEM.
[4]Statistical analyses are based on Students *t* test.

turn might stimulate organ development and function (Hetland et al., 2005; Mateos et al., 2012). Moreover, as average daily feed intake of LC-fed hens was higher during the laying period (Röhe et al., 2019), it would be also conceivable that an increased dry matter intake stimulated gizzard activity leading to an increased gizzard weight.

Intestinal Tract and Histomorphology

In the present study, relative weights of the small and large intestine were higher in lignocellulose fed hens compared to those receiving the control diet. Similarly, pullets showed increased small intestinal weights after feeding diets containing 1% lignocellulose (ARBOCEL® RC FINE) over a period of 10 wk compared to pullets fed the control (Yokhana et al., 2015). By contrast, feeding diets supplemented with 0.4 or 0.6% lignocellulose (ARBOCEL® R) did not affect relative weights of the intestine in 42 d old broilers (Makivic et al., 2019). Several studies showed that dietary inclusion of insoluble fiber sources such as oat hulls and sawdust was accompanied with an increase in length and weight of the small and large intestine in chicken (Welch et al., 1988; Jørgensen et al., 1996; Hetland and Svihus, 2001; Sklan et al., 2003; Oke and Oke, 2007; Jiménez-Moreno et al., 2009). In general, it is suggested that an increase in the intestinal size and length but also an enlargement of the intestinal mucosa contributes to a higher intestinal weight (Uni et al., 2003). In this study, correlation analyses proved that an increase of the gastrointestinal weight was related to the enlargement of the intestinal mucosa.

Furthermore, the results showed that dietary lignocellulose inclusion enhanced the mucosal development of the large intestine indicated by a greater villus area and a higher villus and crypt mucosal enlargement factor in the colorectum. However, the number of mucus producing goblet cells and the relative mucin staining area were not affected by feeding lignocellulose. Studies on the effect of feeding lignocellulose on intestinal histomorphology and intestinal mucus production are

scarce but are generally in line with the results of this study. Broilers fed 0.6% lignocellulose (ARBOCEL® R) at the expense of soybean meal showed an increased villus height and width as well as crypt depth in the duodenum, jejunum and ileum compared to those receiving the control diet (Makivic et al., 2019). Similarly, an increase of dietary insoluble fiber by adding lignocellulose up to 0.75% (ARBOCEL®) led to an increase of the villus height and crypt depth in the ileum of broilers after 42 d of feeding (Sarikhan et al., 2010). The dietary inclusion of 1.25% lignin resulted in an increased jejunal villus length in 42-day-old broilers while a higher inclusion level of 2.5% lignin (Alcell®, Alcell Technologies Inc., Canada) decreased villus length (Baurhoo et al., 2007). In the same study, the number of jejunal goblet cells per villus were not affected by feeding the different lignin inclusion levels (Baurhoo et al., 2007). Apart from lignocellulose or lignin as fiber source, only few studies exist investigating the effect of other insoluble fiber sources on intestinal morphology in chickens providing contradictory results. An elevation of the dietary crude fiber content from 1.61 to 4.44% by adding pea hulls, mainly consisting of insoluble fiber, tended to reduce linearly villus height and significantly lowered crypt depth in the jejunum of broilers (Jiménez-Moreno et al., 2011). In contrast to that but in line with our findings, the jejunal villus height and the villus surface area of 98-day-old turkeys increased as the concentration of dietary crude fiber was heightened from 3 to 9% (Sklan et al., 2003). It was speculated that an increase in the digestive tract weight accompanied with an enlargement of the intestinal mucosa displays a compensatory reaction of chickens due to the feeding of high fiber, low nutrient diets (Bedford, 2000). Thus, nutrient absorption might be enhanced by increasing the digestive capacity (Brenes et al., 1993; Bedford, 2000). On the other hand, Amerah et al. (2009) reported a decrease in weight and length of the small intestine of broilers fed increasing levels of dietary fiber. It was suggested that the lower nutrient density in the intestine of birds fed diets containing insoluble fiber might reduce the intestinal surface area although histomorphometric parameters were not determined (Amerah et al., 2009).

Bacterial Metabolites and Microbiota

The hypothesis of Bedford (2000) that chickens fed with nutrient reduced diets enhance the absorption of nutrients by increasing the intestinal mucosal surface area is supported by the results of this study. Hens fed the nutrient reduced diet had lower cecal concentrations of SCFAs, particularly lower levels of acetic acid, propionic acid and n-valeric acid, compared to hens fed with the control diet. Coincidentally, those hens had a higher colorectal mucosal enlargement factor, a histomorphometric parameter reflecting the mucosal surface (Wiese et al., 2003). This might indicate a compensatory reaction of birds fed lignocellulose enhancing the absorption of bacterial metabolites by developing a higher intestinal mucosal surface area. Accordingly, correlation analyses in this study have shown that the concentration of SCFAs in the cecum of hens was negatively correlated with the colorectal mucosal enlargement factor of the villi, in other words: the lower the concentration of SCFA in the gut lumen, the higher the absorptive villus surface area. Thus, the hypothesis on a compensatory reaction to increase resorption of energy yielding SCFA may hold true.

In chickens, the bacterial fermentation of insoluble fiber sources and lignified material such as lignocellulose is low (McNab, 1973; Carré et al., 1990; Jørgensen et al., 1996; Montagne et al., 2003; De Vries et al., 2012; Waite and Taylor, 2014). Lower concentrations of SCFAs and ammonia were also detected in the cecum of LC-fed hens compared to those fed CON suggesting a diet dilution effect of the lignocellulose inclusion. However, the results also showed that generally neither the number nor the activity of detected bacterial populations differed between hens of both feeding groups, which favors the idea that the reduced SCFA concentrations are due to an increase in the villus surface accompanied with a higher SCFA absorption. Uniquely, the activity of *Lactobacillus* spp. was significantly higher in CON-fed hens compared to LC-fed hens. There is a lack of studies investigating the impact of feeding lignocellulose on the microbiota in laying hens. Some studies on broilers showed that the feeding of lower inclusion levels of lignocellulose might modulate bacterial populations and metabolites in the small and large intestine (Sarikhan et al., 2010; Bogusławska-Tryk et al., 2015; Kheravii et al., 2017; Makivic et al., 2019). The feeding of broilers with diets containing lignocellulose up to 1% (ARBOCEL® RC) increased counts of *Lactobacillus* spp. in the ileum and *Bifidobacterium* spp. in the ileum and caeca, and decreased counts of ileal and cecal *Escherichia coli* and *Clostridium* spp. (Bogusławska-Tryk et al., 2015). The concentration of ileal and cecal SCFAs was higher in broilers fed 0.5% lignocellulose compared to those fed the control diet. Interestingly, a higher dietary inclusion level of 1% lignocellulose showed no effect on cecal SCFAs concentration (Bogusławska-Tryk et al., 2015). As lignocellulose was included in the diet as an expense of wheat it could be speculated that observed effects on intestinal microbiota and metabolites could be also attributed to a varying nutrient composition of the feed. Diets diluted with 1 and 2% lignocellulose had in general no effect on detected bacteria counts except that *Clostridium* spp. counts were reduced in the cecum of broilers fed 2% lignocellulose (OptiCell®) (Kheravii et al., 2017).

Excreta Characteristics

Analyses of the excreta revealed that hens fed LC had generally a higher dry matter content than hens fed CON, although hens of both feeding groups showed a comparable excreta dry matter content at 52 wk

of age. Few studies display that dietary lignocellulose inclusion might have a positive effect on litter quality observed during broiler trials. Broilers fed with diets supplemented with 0.6, 0.8, 1, and 2% lignocellulose had a lower moisture content in the litter compared to broilers fed control diets (Farran et al., 2013; Kheravii et al., 2017; Makivic et al., 2019). It was hypothesized that the dietary inclusion of lignocellulose might increase digesta retention time and water holding capacity, which might enhance the water absorption in the digestive tract and thus elevates the excreta dry matter content (Kheravii et al., 2017). With respect to the excreta viscosity, differences could be not observed between LC- and CON-fed hens. It is well known that dietary soluble fiber can increase gut viscosity resulting in a reduced feed passage rate while an opposite effect is supposed for dietary insoluble fiber (Van der Klis and Van Voorst, 1993; Almirall and Esteve-Garcia, 1994; Choct et al., 1996).

In conclusion, the results of this study show that feeding of high levels of lignocellulose increased the weights of gastrointestinal organs of dual purpose laying hens, which was accompanied with the development of an increased colorectal mucosal surface. The amount of cecal SCFAs and ammonia was reduced in lignocellulose-fed hens compared to those fed the basal diet. Interestingly, the concentration of SCFAs in the cecum of hens was negatively correlated with the colorectal villus surface. This might indicate a compensatory reaction of birds fed lignocellulose enhancing the absorption of energy yielding bacterial metabolites by increasing the intestinal mucosal surface. In order to prove this, further studies are needed including experiments in metabolism cages investigating the energy requirements for maintenance and production of dual purpose hens in relation to feeding diets with varying energy- and nutrient levels. Cecal microbial composition and activity was generally not influenced by feeding the different diets supporting the hypothesis that the mucosal absorption rate of cecal SCFAs was increased in lignocellulose-fed hens due to an increased mucosal surface area. Moreover, a lower excreta moisture content could be detected in lignocellulose fed hens, which might have positive effects on litter quality under practical conditions. In connection with recently published data on animal performance and body composition (Röhe et al., 2019) results of this study suggest that the feeding of nutrient reduced diets containing high levels of fibre might be an interesting possibility to feed dual purpose chickens, maintain animal health and simultaneously improve economic viability.

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