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Original Research Article

Influence of feeding crimped kernel maize silage on the course of subclinical necrotic enteritis in a broiler disease model

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ABSTRACT

This experiment was carried out with 375 male broilers (Ross 308) from days 1 to 28 to evaluate the influence of crimped kernel maize silage (CKMS) on the manifestation of subclinical necrotic enteritis, microbiota counts, organic acid production and relative weights of gastrointestinal segments. A necrotic enteritis disease model was applied. Birds were allocated into 3 different dietary treatments: a maize-based feed (MBF, control diet), and 2 diets supplemented with 15% (CKMS15) or 30% (CKMS30) of crimped ensiled kernel maize. The disease model involved a 10-time overdose of an attenuated live vaccine against coccidiosis given orally on day 17, followed by oral inoculation of *Clostridium perfringens* Type A (S48, 10^8 to 10^9 bacteria/bird) twice daily on days 18, 19, 20 and 21. Scoring of intestinal lesions was performed on days 22, 23, 25 and 28. Ileal and caecal digesta samples were collected for the quantification of selected bacterial groups and organic acids. The results showed that there was no effect of dietary treatments on small intestinal lesion scores ($P > 0.05$). Lesions scores peaked on days 23 and 25 and decreased again on day 28 ($P = 0.001$). No effect of age on microbiota counts was observed, but feeding of CKMS30 reduced the number of coliforms in ileal contents ($P = 0.01$). Dietary treatments did not affect organic acid concentrations in ileum and caeca, but there was an effect of age; butyric acid was higher on days 22, 23 and 25 than on day 28 ($P = 0.04$). Acetic acid and propionic acid concentrations in caeca were the highest on days 22 and 28 but the lowest on days 23 and 25. Relative gizzard and caeca weights were increased, and relative ileum weights were decreased when birds were fed CKMS30 ($P < 0.05$). In conclusion, the inclusion of CKMS in broiler diets had no effects on the course of necrotic enteritis but had potential benefits in terms of inhibition of potentially harmful microorganisms.

1. Introduction

After the ban of in-feed antibiotics, strong concerns regarding an increased emergence of necrotic enteritis induced by *Clostridium perfringens* (Cp) Type A have been raised. In the EU, the use of ionophore coccidiostats with antibiotic effect on Cp is currently under discussion. This will enhance the challenge due to disease re-emergence, which results in increased mortality, reduced bird welfare and higher contamination risk of poultry products (Timbermont et al., 2011). Various dietary strategies including the use of probiotics and prebiotics as dietary supplements have been implemented as an attempt to prevent the disease with variable rates of success (Caly et al., 2015). Besides these, wet fermented compound feed has been found to provide some benefits for poultry. It was found to reduce feed and gastrointestinal pH and to improve gut health thus supporting the barrier function of the stomach against colonization of acid sensitive bacteria, e.g., *Escherichia coli, Salmonella* and *Campylobacter* in broilers (Bjerrum et al., 2005; Rehman et al., 2009; Heres, 2003; Heres et al., 2003a,b). Increased lactic acid bacteria counts and growth inhibition of acid sensitive pathogenic and zoonotic bacteria such as *E. coli*, *Campylobacter* and *Salmonella* have been found to be related

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to the use of fermented feed in pigs and layers (Van Winsen et al., 2001; Engberg et al., 2009). These changes have been assumed to be due to an acidification of the upper digestive tract. However, fermentation of compound feed has some disadvantages including degradation of supplemented synthetic amino acids (Canibe and Jensen, 2012) and occurrence of wet litter related to high dietary water intake (Forbes, 2003; Engberg et al., 2009). Supplementation of concentrated compound feed with a fermented energy-rich component such as crimped kernel maize silage (CKMS) may be a way to avoid these negative effects of fermentation whilst taking advantage of its benefits on gastrointestinal health. Crimped kernel maize silage is characterized by high concentration of acetic and lactic acid, low pH, and high numbers of lactic acid bacteria. In a production trial with broilers fed CKMS supplemented diets, the mortality was low (2% to 3%) and did not differ between treatments, but CKMS improved litter quality and footpad health (Ranjitkar et al., 2016a,b). However, scientific literature regarding the influence of CKMS on the course of necrotic enteritis is lacking. Therefore, the objective of this experiment was to study the effects of adding different levels of CKMS (0, 15% and 30%) on necrotic enteritis, microbiota counts, organic acid production and relative weights of gastrointestinal segments when applying a subclinical necrotic enteritis disease model.

2. Material and methods

2.1. Bird husbandry, experimental design and diets

The experiment was carried out according to the guidelines of the Danish Animal Experiments Inspectorate, Ministry of Environment and Food, Danish Veterinary and Food Administration with respect to animal experimentation and care of animals under study.

A total of 375 day-old male broiler chickens (Ross 308) were purchased from a commercial Danish Hatchery (DanHatch A/S, Sønderborg, Denmark). Chickens (average weight 47.4 ± 1 g) were wing-tagged and allocated to 15 pens (25 birds per pen) each providing a floor area of 1.7 m^2, covered with wood shavings as bedding material. Birds were grown in the same house equipped with automatic control of temperature, light and humidity. Room temperature was 33 °C during the first 3 days and was gradually decreased by 0.6 °C per day until 21 °C was reached. This temperature was then maintained throughout the experiment. Relative humidity was 45% in week 1, 50% in week 2, 55% in week 3 and 60% in week 4. Light was provided for 24 h on day 1, 23 h on days 2 to 5, 16 h on days 6 to 13 and 19.5 h during the remaining period. Birds were allocated to 3 dietary treatments each with 5 replications in a completely randomized design. Treatments were maize-based feed (MBF) serving as a control without addition of ensiled maize, CKMS15 providing 15% ensiled kernel maize and CKMS30 providing 30% ensiled kernel maize. Ingredients and chemical composition of the experimental diets are presented in Table 1. Ensiled maize was stored frozen at −20 °C. Every day, an appropriate portion of CKMS was taken out of the freezer and thawed for feeding the next day. The CKMS was mixed with the compound feed to reach levels of 15% and 30%, for the groups CKMS15 and CKMS30, respectively. All grower diets were formulated taking dry matter (DM) content of CKMS into account to obtain the same nutritional composition after mixing. Birds had *ad libitum* access to feed and water. Until day 5, all birds were fed a common starter diet providing 265.9 g protein and 13.5 MJ/kg DM. From day 6, CKMS15 and CKMS30 groups were gradually adapted to the respective diets to reach a dietary inclusion level of 15% and 30%, respectively at day 10. Samples of each diet were analysed for DM, crude ash, nitrogen, fat, sugar, starch and crude fibre as per the methods described before (Table 1). Individual body weights of the birds in each pen were registered on days 14 (25 birds/pen) and 28 (10 birds/pen).

2.2. Preparation and analysis of crimped kernel maize silage

The CKMS was prepared as described by Ranjitkar et al. (2016a,b). In short, crimped kernel maize was ensiled for 8 weeks by adding a mixture of organic acids including formic acid, propionic acid, benzoic acid and ammonium formate (Kemira AIV Pro, Helsinki, Finland). The CKMS was vacuum-packed and stored at −20 °C. Concentration of DM, pH and microbial counts (coliforms, lactose negative enterobacteria and lactic acid bacteria [LAB]) were analysed as described by Engberg et al. (2004). In short, DM was analysed following freeze-drying of samples at −50 °C, coliforms and lactose negative enterobacteria were enumerated on MacConkey agar (Merck KGaA Darmstadt, Germany) incubated at 38 °C for 24 h, and LAB were counted on MRS agar (Merk KGaA, Germany) incubated in an anaerobic cabinet at 38 °C for 48 h. Crude protein (N × 6.25) in CKMS was determined using the Dumas combustion method (Helrich, 1990). Ash was analysed according to the method described by Helrich (1990) and fat was extracted with diethyl ether after acid hydrolysis (Stoldt, 1952). Short chain fatty acids (SCFA) and lactic acid were analysed as described by Canibe et al. (2007). Starch, non-starch polysaccharides (NSP) and lignin were analysed using the methods described by Bach Knudsen (1997).

2.3. Necrotic enteritis infection model

The applied necrotic enteritis disease model involved oral inoculation of all birds with an attenuated live vaccine against coccidiosis (Paracox-5, MSD Animal Health) given at a 10 times overdose on day 17. A Cp Type A strain (S48) which had been confirmed positive for the netB gene (Abildgaard et al., 2010a) was grown overnight in anaerobic broth (Oxoid CM0957) at 38 °C to a final concentration of approximately 10^9 cfu/mL. All birds were orally inoculated with 0.5 mL of the overnight culture corresponding to 10^8 to 10^9 cfu/bird. Cp challenge was performed twice daily in the morning and afternoon on 4 consecutive days from days 18 to 21.

2.4. Lesion scoring, bacterial enumeration and weights of gastrointestinal segments

On days 22, 23, 25 and 28, 5 birds were randomly selected per pen and euthanized by cervical dislocation. Contents from ileum and caeca were collected, pooled separately for each segment and stored at −80 °C for analysis of SCFA and bacterial quantification. On each day, severity of small intestinal lesions related to necrotic enteritis was scored macroscopically according to the method described by Shojadoost et al. (2012). In short, lesions were scored by the same person and scores were given in a range from 0 to 6 increasing with increased severity of lesions. Empty intestinal segments (gizzard, jejunum, ileum and caeca) of 5 birds per pen were collected and weighed, and relative segment weights were calculated taking bird weights into consideration. Lactic acid bacteria, Cp, coliforms, and lactose negative enterobacteria were cultured from fresh ileal and caecal contents and enumerated as described by Engberg et al. (2004).

2.5. Quantification of Cp by qPCR

Concentration of netB positive Cp in contents of ileum and caeca was determined using a real time PCR method as described by Abildgaard et al. (2010b). DNA was extracted from 0.4 g ileal and

Table 1
Composition of experimental starter and grower diets.

Item	Starter feed (days 1 to 5)	Grower feed[1] (days 6 to 28)		
	All groups	MBF	CKMS15	CKMS30
Ingredients, g/kg				
Wheat	8.3	127.4	230	322.8
Maize	510.0	510	360	210
Oat	60			
Maize gluten (60%)	30	–	–	–
Rapeseeds milled	20.0	20	41.6	63.6
Soybean meal (de-hulled)	315.4	300.8	322.9	353.8
Soybean oil	15	10	10	10
$CaCO_3$	10.6	8.6	9.6	10.6
Monocalcium phosphate	12.3	37.9	8.6	9.6
NaCl	1.4	1.5	1.7	1.9
$NaHCO_3$	2.8	2.5	2.7	3
Lysine hydrochloride (100%)	4.6	2.9	3.4	4
DL-methionine (100%)	4.0	3.6	4	4.5
Threonine (98%)	1.6	1.3	1.6	1.8
Vitamin and mineral mixture[2]	4.0	3.5	3.9	4.4
Added prior to feeding, g/kg of concentrated grower feed				
CKMS	–	–	150	300
Analysed nutrients[3], g/kg DM				
Dry matter, g/kg	893.1	887.5	845.9	804.5
AME, MJ	13.5	14.0	13.6	14.0
Protein (N × 6.25)	265.9	236.6	233.4	236.3
Starch	393.2	458.9	437	447.8
Total sugar	51.5	57.5	54.8	51.7
Total NSP	115	104	112	108
Lignin	37	12	28	18
Fat	63.8	55.1	61.2	63.7
Ash	58.7	52	51.7	51.3
Phosphorus	7.8	6.4	6.5	6.5
Microbial count				
Lactobacilli, lg cfu/g	–	3.3	6.4	6.9
Organic acids, mmol/kg				
Acetic acid	–	6.3	13.8	19
Benzoic acid	–	0	0.5	0.5
Lactic acid	–	0	21.8	29.6

MBF = maize-based feed; CKMS = crimped kernel maize silage; AME = apparent metabolizable energy; NSP = non-starch polysaccharides.
[1] CKMS15- MBF with 15% CKMS; CKMS30- MBF with 30% CKMS.
[2] Vitamin and mineral mixture provided per kg of diet: retinol (retinyl acetate), 12,000 IU; cholecalciferol, 5,000 IU; vitamin E (DL-α-tocopheryl acetate), 50 IU; vitamin E (synthetic), 54.9 IU; menadione, 3 mg; thiamin, 2 mg; riboflavin, 6 mg; pyridoxine, 4 mg; D-pantothenic acid, 13 mg; niacin, 55 mg; betaine hydrochloride, 260 mg; folic acid, 2 mg; biotin, 200 μg; cyanocobalamin, 16 μg; calcium D-pantothenate, 1.08 g; $FeSO_4 \cdot 7H_2O$, 20 mg; ZnO, 100 mg; MnO,120 mg; $CuSO_4 \cdot 5H_2O$, 18 mg; KI, 560 μg; Na_2SeO_3, 300 μg; $CoCO_3$, 500 μg.
[3] Analysis was conducted on the complete feed after mixing with CKMS and whole wheat.

0.2 g caecal contents using the QIA amp fast DNA stool mini kit (Qiagen). Purity and concentration of DNA was measured using Nanodrop ND-1000 UV–visible spectrophotometer (NanoDrop Technologies Inc, Wilmington, DE, USA) and agar gel electrophoresis. Reaction set-up was done and maxima SYBR Green/ROX qPCR Master Mix (2X) was prepared using the protocol from the supplying company (Thermo Fisher Scientific) adding forward primer (netB KP 78-F) – GCT GGT GCT GGA, reverse primer (netB APK 79-R) – TCG CCA TTG AGT and nuclease-free water. Real time qPCR (Applied Biosystems ViiA 7 real time PCR system) was performed for quantification of bacteria. Data was analysed using ViiA 7 software of the Applied Biosystems real time PCR system. For converting PCR cycle threshold values (Ct) to bacterial cell numbers, a standard curve was constructed with the 10 folds dilutions of standard solution containing plasmid of netB gene.

2.6. Statistical analysis

Statistical analysis was performed using general linear models procedure of SAS software (SAS Institute, 1990) according to the following general model: $Yxy = \mu + \alpha x + \beta y + (\alpha\beta)\, xy + \varepsilon xy$, where Yxy was the observed dependent variable, μ was the overall mean, αx was the effect of treatment (MBF, CKMS15 and CKMS30), βy was the effect of bird age (day), $(\alpha\beta)\, xy$ was the interaction between treatment and days, and εxy was the random error. Results are given as least square means with a pooled standard error (SE). Probability values below or equal to 0.05 were accepted to indicate significant difference between means.

3. Results

3.1. Composition of CKMS

Composition of starter and grower diets is shown in Table 1. Concentrated grower diets were given from days 6 to 28. The DM content of CKMS was 59.3% and pH was 3.95 (Table 2). With respect to organic acids, CKMS contained high concentration of lactic acid (179.1 mmol/kg) and acetic acid (57.6 mmol/kg). Benzoic acid (2.7 mmol/kg), formic acid (1.6 mmol/kg) and traces of propionic acid were measured deriving from the initial addition of organic acids to support the ensiling process.

3.2. Intestinal lesions and quantification of C. perfringens

Different dietary treatments had no effect on small intestinal lesion scores. However, there was an effect of bird age (days) ($P < 0.01$, Table 3). Severity of intestinal lesions peaked on day 23 and decreased gradually until day 28 ($P < 0.01$, Table 3).

Concentration of netB toxin positive Cp in ileal and caecal contents (Table 3) measured by quantitative polymerase chain reaction (qPCR) was higher on days 22, 23 and 25 as compared to day 28 ($P < 0.001$ and $P = 0.002$ for ileal and caecal contents, respectively). Further, concentration of netB toxin positive Cp was the lowest in ileal contents of birds receiving the control diet ($P < 0.01$). However, a significant interaction between diet and day was observed ($P = 0.05$). Compared to birds receiving the control diet, Cp concentration was higher in ileal contents of birds fed CKMS15 on day 22 and in CKMS30-fed birds on day 28. Using the microbial plate count method, no differences between dietary treatments were observed with respect to caecal and ileal Cp concentration ($P > 0.05$). Similar to netB toxin positive Cp concentrations, the lowest Cp counts were observed on day 28 ($P < 0.001$). The quantity of netB positive Cp obtained by qPCR and plate count (Table 3) also followed the pattern of the lesion scores showing the highest Cp concentration on day 23. During the first 3 days of sampling, lesions were fresh, whereas on day 28 lesions, although still macroscopically visible, were already covered with new tissue indicating a healing process.

3.3. Counts of coliforms, lactose negative enterobacteria, lactic acid bacteria, and concentration of organic acids in ileal and caecal contents

Compared with MBF, CKMS30 reduced counts of coliforms (Table 4, $P < 0.05$) in ileal content. Further, counts of lactic acid bacteria in ileal and caecal contents were influenced by the diet ($P < 0.05$), where birds receiving CKM30 had the lowest counts in both intestinal segments (Table 4).

There was no effect of day on coliforms, lactose, negative enterobacteria and lactic acid bacteria ($P > 0.05$).

Table 2
Microbial and biochemical composition of crimped kernel maize silage (CKMS).

Item	CKMS
Dry matter, g/kg	593.9
pH	3.9
Microbial counts, lg cfu/g	
Lactic acid bacteria	7.8
Coliform bacteria	nd[1]
Yeasts	nd
Mould	nd
Short-chain fatty acids, mmol/kg	
Acetic acid	57.6
Benzoic acid	2.7
Propionic acid	0.5
Formic acid	1.6
Lactic acid	179.1
Analysed nutrients, g/kg DM	
Protein (N × 6.25)	93.7
Starch	724.7
Total NSP	77.0
Lignin	10.0
Fat	44.6
Ash	15.7

NSP = non-starch polysaccharides.
[1] Not detected.

Concentrations of acetic, butyric, proprionic, lactic and succinic acid in contents of ileum and caeca are shown in Table 5. There was no influence of the diet on the concentrations of these organic acids ($P > 0.05$). However, an effect of day was observed with respect to ileal butyric acid concentration ($P = 0.04$), which was the lowest on day 28. Further, caecal concentrations of acetate ($P = 0.001$) and propionate ($P = 0.01$) decreased from days 22 to days 23 and 25, whereafter they increased again on day 28 (Table 5).

3.4. Body weights and relative weights of gastrointestinal segments

Individual body weights were registered on days 14 (25 birds/pen) and 28 (10 birds/pen). On day 14, body weights of broilers receiving MBF, CKMS15 and CKMS30 were 486, 511 and 516 g, respectively, whereas on day 28, birds weighed 1,455, 1,419 and 1,346 g respectively. No difference between dietary groups was detected at neither time points ($P > 0.05$). Relative weights of gastrointestinal segments are presented in Table 6. Relative gizzard and caeca weights were higher ($P < 0.05$) whereas ileum weight was lower ($P < 0.05$) in birds fed CKMS30.

4. Discussion

C. perfringens is an environmental bacterium and belongs to the indigenous intestinal microbiota of poultry (Al-Sheikhly and Truscott, 1977). Several predisposing factors cause the normal numbers of Cp to multiply exponentially (Drew et al., 2004; Jia et al., 2009). Large numbers of Cp in the intestine is a co-factor for the development of clinical and sub clinical necrotic enteritis. In the present experiment, no differences between the dietary treatments were observed with respect to Cp counts. However, high dietary levels of indigestible, water-soluble non-starch polysaccharides (NSP) predispose to necrotic enteritis (Timbermont et al., 2011). The CKMS15 diet contained double the amount of wheat vs. MBF and CKMS30 contained 2.5 times the amount of wheat vs. MBF and more than 30% wheat inclusion. Dietary addition of α-xylanase would be advisable when feeding such high wheat levels. The absence of xylanase might possibly have masked the improvement brought about by use of CKMS.

C. perfringens counts above 5 to 6 log cells per gram digesta have been suggested to predict higher probability of necrotic enteritis-specific gut lesions (Kaldhusdal et al., 1999). Similar to results obtained by Abildgaard et al. (2010b), in the present study, concentration of Cp was estimated to be higher when plate counting was applied as compared to qPCR method. The reason for this may be that qPCR targeted only netB positive Cp (infection strain). Furthermore, DNA from Cp spores, which are prone to germinate and grow on TSC plates, may not have been extracted and subjected to amplification by PCR.

C. perfringens counts were high on the first 3 days after infection and decreased on day 28 (Table 3). These results indicate that birds were recovering from the disease, which is further supported by the lowest lesion score found at that time point (Table 3). Similarly, the lowest concentrations of butyric acid in ileal contents were observed on day 28 (Table 5) pointing in the same direction, as *Clostridium* spp. produce significant amounts of butyric acid (Wang et al., 2001). Further, reduced acetic acid and propionic acid concentrations in caecal contents on days 23 and 25 (Table 5) may indicate that caecal fermentation is modified in relation to the course of infection and is about to be re-established on day 28.

Birds fed CKMS30 had lower counts of coliforms in ileal content (Table 4). This agrees with results using fermented compound feed by Heres et al. (2003a,b) and Engberg et al. (2009); and solid state fermented feed by Naji et al. (2015). The coarse structure of CKMS might have increased the secretion of hydrochloric acid in the proventriculus reducing gizzard pH (Engberg et al., 2004) and increasing the passage rate of digesta (Hetland and Svihus, 2001). These factors act together to limit bacterial proliferation in intestinal contents. It has been shown that fermented liquid feed reduced level of enterobacteria in different parts of the gastrointestinal tract of pigs (Van Winsen et al., 2001; Canibe and Jensen, 2003). In agreement with findings in pigs (Canibe and Jensen, 2003) and in layers (Engberg et al., 2009), CKMS30 also reduced

Table 3
Intestinal lesion scores, *Clostridium perfringens* (Cp) counts (lg cfu/g digesta) and concentration of netB positive Cp (lg cfu/g digesta) in intestinal contents of broilers fed maize-based feed (MBF), CKMS15 or CKMS30 and at different time points post infection.[1]

Item[2]	Diets[3]				Bird age, day					*P*-value		
	MBF	CKMS15	CKMS30	SEM	22	23	25	28	SEM	Diet	Days	Diet × Days
Lesion score	1.97	1.76	1.87	0.154	1.26^{c}	2.28^{a}	2.09^{ab}	1.82^{bc}	0.178	0.63	0.001	0.84
Cp												
Ileum	7.63	7.67	7.42	0.153	7.84^{a}	8.11^{a}	7.84^{a}	6.51^{b}	0.172	0.47	<0.001	0.25
Caeca	7.59	7.69	7.46	0.162	7.67^{a}	8.06^{a}	8.07^{a}	6.52^{b}	0.193	0.70	<0.001	0.30
netB positive Cp												
Ileum	4.84^{b}	5.46^{a}	5.64^{a}	0.191	5.45^{b}	6.47^{a}	5.78^{b}	3.54^{c}	0.381	0.01	<0.001	0.05
Caeca	4.09	4.52	4.57	0.221	5.08^{b}	5.66^{ab}	5.72^{a}	3.92^{c}	0.63	0.634	0.002	0.96

CKMS = crimped kernel maize silage.
[a,b,c]Means in each row with different superscripts differ significantly ($P < 0.05$).
[1] Values are least square means.
[2] Lesion score, $n = 300$ in total, 25 birds per treatment per day; Cp concentrations, $n = 60$ in total, 5 pooled samples per treatment per day.
[3] CKMS15- MBF with 15% CKMS; CKMS30- MBF with 30% CKMS.

Table 4
Counts of coliform bacteria, lactose negative enterobacteria and lactic acid bacteria in ileal and caecal contents (lg cfu/g digesta) of broilers fed maize-based feed (MBF), CKMS15 or CKMS30 and at different time points post infection.[1]

Item[2]	Diets[3]				Bird age, day					*P*-value		
	MBF	CKMS15	CKMS30	SEM	22	23	25	28	SEM	Diet	Days	Diet × Days
Coliform bacteria												
Ileum	6.38^{ab}	6.61^{a}	5.94^{b}	0.317	6.15	6.39	6.43	6.27	0.317	0.01	0.84	0.20
Caecum	8.35	8.50	8.40	0.164	8.31	8.52	8.56	8.30	0.164	0.43	0.62	0.94
Lactose negative enterobacteria												
Ileum	4.20	4.30	4.00	0.236	4.13	4.15	4.35	4.21	0.136	0.12	0.55	0.99
Caecum	6.54	6.45	6.60	0.281	6.43	6.63	6.82	6.23	0.161	0.32	0.09	0.93
Lactic acid bacteria												
Ileum	8.75^{ab}	8.95^{a}	8.60^{b}	0.097	8.75	8.75	8.77	8.79	0.117	0.04	0.78	0.24
Caecum	9.50^{a}	9.30^{b}	9.17^{b}	0.071	9.24	9.52	9.35	9.19	0.069	0.003	0.10	0.37

CKMS = crimped kernel maize silage.
^{a,b}Means in each row with different superscripts differ significantly ($P < 0.05$).
[1] Values are least square means.
[2] $n = 60$ in total, 5 pooled samples per treatment per day.
[3] CKMS15- MBF with 15% CKMS; CKMS30- MBF with 30% CKMS.

Table 5
Concentration of organic acids (mol/kg) in ileal and caecal contents of broilers fed maize-based feed (MBF), CKMS15 or CKMS30 and at different time points post infection.[1]

Organic acids[2]	Diets[3]				Bird age, day					*P*-value		
	MBF	CKMS15	CKMS30	SEM	22	23	25	28	SEM	Diet	Days	Diet × days
Ileum												
Acetic acid	14.2	16.1	12.8	5.775	10.3	12.9	21.3	13.0	5.775	0.48	0.10	0.78
Butyric acid	1.5	1.7	0.8	0.546	1.0^{ab}	1.5^{a}	2.7^{a}	0.2^{b}	0.626	0.52	0.04	0.90
Propionic acid	0.6	0.6	0.6	0.846	0.4	0.4	1.2	0.4	0.846	0.99	0.50	0.41
Lactic acid	33.0	37.7	25.8	8.411	33.7	33.6	30.5	31.0	8.411	0.12	0.94	0.59
Succinic acid	0.3	0.2	0.1	0.321	–	0.1	1.2	0.9	0.172	0.72	0.13	0.90
Ceaca												
Acetic acid	76.2	81.9	81.7	4.090	86.3^{a}	71.0^{b}	72.1^{b}	90.46^{a}	4.09	0.43	0.001	0.43
Butyric acid	16.0	16.5	15.8	0.233	18.36	15.1	14.3	16.7	2.233	0.90	0.13	0.80
Propionic acid	5.4	6.1	5.5	0.283	6.21^{a}	5.01^{b}	5.22^{b}	6.06^{a}	0.283	0.09	0.01	0.51
Lactic acid	2.7	2.3	2.4	0.766	3.5	3.5	1.6	3.2	1.566	0.94	0.40	0.65
Succinic acid	3.7	3.2	4.0	1.308	3.1	3.1	4.3	2.8	1.308	0.62	0.33	0.21

CKMS = crimped kernel maize silage.
^{a,b}Means in each row with different superscripts differ significantly ($P < 0.05$).
[1] Values are least square means.
[2] $n = 60$ in total, 5 pooled samples per treatment per day.
[3] CKMS15- MBF with 15% CKMS; CKMS30- MBF with 30% CKMS.

Table 6
Relative weights of gastrointestinal segments (g/kg body weight) of broilers fed maize-based feed (MBF), CKMS15 or CKMS30 throughout the experimental period of 28 days.[1]

Organ weight[2]	Diets[3]				Bird age, day					*P*-value		
	MBF	CKMS15	CKMS30	SEM	22	23	25	28	SEM	Diet	Days	Diet × days
Gizzard	17.8^{b}	18.8^{b}	19.3^{a}	0.03	21.1^{a}	18.8^{b}	16.5^{c}	18.5^{b}	0.03	0.005	<0.001	0.99
Jejunum	17.2	20.8	19.1	0.12	21.6	19.1	18.2	17.4	0.14	0.11	0.160	0.52
Ileum	13.9^{a}	13.7^{a}	12.9^{b}	0.36	13.9^{a}	13.9^{a}	12.4^{b}	12.5^{b}	0.31	0.01	<0.001	0.30
Caeca	3.8^{b}	4.1^{b}	4.3^{a}	0.09	4.2^{b}	4.1^{ab}	4.5^{a}	3.8^{c}	0.11	<0.001	<0.001	0.07

CKMS = crimped kernel maize silage.
^{a,b,c}Means in each row with different superscripts differ significantly ($P < 0.05$).
[1] Values are least square means.
[2] $n = 300$ in total, 25 birds per treatment per day.
[3] CKMS15- MBF with 15% CKMS; CKMS30- MBF with 30% CKMS.

lactic acid bacteria counts in ileal and caecal contents (Table 4). The reason for the lower counts of both coliform bacteria and lactic acid bacteria may be the lower amount of available substrate present in the small intestine when CKMS30 is added to the concentrate. A slower release of feed from the gizzard due to increased structure of CKMS30 may limit the concentration of substrate available for bacterial growth. Engberg et al. (2009) found an improved nutrient digestibility of fermented feed, which may likewise contribute to a reduced substrate availability.

Gizzard weight is an indicator for feed structure (Hetland et al., 2002; Engberg et al., 2004; Bjerrum et al., 2005). The coarse structure of CKMS might have stimulated the mechanical activity of the gizzard which resulted in higher gizzard weight (Hetland et al., 2002; Engberg et al., 2004; Svihus, 2011). The CKMS diet is harder to grind and may stay in the gizzard for longer time before it is passed further to the small intestine. Further, increased caeca weights were observed when birds were fed CKMS30 (Table 6), which might indicate a higher caecal load with fermentable substrates due to reduced small intestinal digestion. Lower ileum weights found in birds receiving CKMS30 does point in that direction.

The necrotic enteritis challenge model applied in this experiment was a model for subclinical necrotic enteritis, which resulted

in the development of small intestinal lesions related to the disease without causing mortality. The model involved an overdose of attenuated live vaccine against coccidiosis (Paracox 5) followed by oral inoculation of a netB positive Cp strain twice a day for 4 consecutive days. As reviewed by Shojadoost et al. (2012), a number of necrotic enteritis challenge models suggest the use of certain dietary ingredients as predisposing factors for the disease in broilers. Diets with high protein content of animal origin, e.g., fishmeal have been used in different necrotic enteritis challenge models (Kaldhusdal, 2000; Wu et al., 2010; Gholamiandehkordi et al., 2007). Furthermore, cereals like barley, wheat and rye containing high concentration of water soluble NSP such as beta-glucans and arabinoxylans which increase digesta viscosity and predispose birds to necrotic enteritis have been identified as triggers to necrotic enteritis (Annett et al., 2002; Dahiya et al., 2006; McDevitt et al., 2006). In the present study, the CKMS30 group received wheat at dietary levels above 30% without addition of xylanase. In order to eliminate this confounding factor, exo-enzymes or any alternative should be used in future experiments. Due to its lower levels of water soluble NSP, maize is considered to be less detrimental to the intestinal tract of broilers than wheat and barley (Kaldhusdal and Skjerve, 1996; Kaldhusdal, 2000; Annett et al., 2002). However, in the present study, it was still possible to trigger necrotic enteritis related lesions in 80% of the birds. This indicates that this model can be used under conventional dietary conditions and may be particularly suitable for studies evaluating the effect of different dietary compositions or that of feed additives, e.g., enzymes, probiotics and prebiotics etc. to prevent necrotic enteritis.

5. Conclusion

The supplementation of crimped kernel maize silage with 15% or 30% did neither reduce the incidence of necrotic enteritis nor the course of the disease in broilers. However, maize silage supplementation at 30% inclusion level (CKMS30) lowered the proliferation of potentially pathogenic bacteria such as coliforms, increased gizzard and caeca weight but decreased ileum weight. The CKMS supplemented diets had no effect on intestinal lesion score. Lesion scores increased from day 22 to days 23 and 25 whereafter they decreased again on day 28. Ileal butyric acid concentrations decreased on day 28 as did Cp counts and netB positive Cp quantified by PCR. These results indicate that the disease is about to be resolved at that time point. The necrotic enteritis disease model used was successful to provoke sub-clinical necrotic enteritis without the use of predisposing dietary factors. Therefore, application of this model can be recommended when investigating the effect of different feed compositions or the efficacy of different feed additives, e.g., prebiotics, probiotics, plant-based extracts, against necrotic enteritis.

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