



Short Communication

Volatile basic nitrogen measurement in digesta using a Berthelot reaction in automated Skalar instrumentation

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ABSTRACT

The undigested nitrogenous fraction entering the hindgut of chickens is further metabolized by microbiota present producing volatile basic metabolites including amines and ammonia (NH₃). Ammonia increases pH and may result in overgrowth of *Clostridium perfringens* further producing toxic metabolites that cause dysbacteriosis or necrotic enteritis (NE). There are few reports in chickens examining the production and concentration of nitrogenous metabolites in the hindgut. A Berthelot reaction using an automated flow, reaction and spectrophotometric instrumentation to detect volatile basic nitrogen in the form of NH₃ from cecal contents was carried out. In the Berthelot reaction, NH₃ in the sample is chlorinated to monochloramine using dichloroisocyanuric acid that then reacts with salicylate to form 5-aminosalicylate which is stable under alkaline conditions (pH 12 to 13). After oxidation and oxidative coupling, a coloured complex is formed that can be measured at 660 nm. Cecal contents were collected and pooled from 2 birds per pen with 48 pens total in each of 3 experiments and stored in sealed containers at –20 °C prior to analysis. Experiment 1 compared samples collected from birds fed either no meat and bone meal (MBM) or 6% MBM from d 0 to 14, and samples collected from birds fed either no MBM or 5% MBM from d 14 to 42. All birds were challenged with *Eimeria* on d 9 and *C. perfringens* on d 14 and 15. Experiment 2 compared cecal contents from birds fed either 0.5% or 0.9% calcium (Ca), and Exp. 3 compared unchallenged with NE challenge on d 16 and 29. Results demonstrated an increase ($P < 0.05$) in cecal NH₃ as a result of feeding MBM or high dietary Ca with a corresponding increase in pH. Birds experimentally challenged with NE had lower cecal NH₃ and lower pH on d 16 but not d 29 compared to unchallenged controls. It was concluded that the Berthelot method using Skalar instrumentation equipment is suitable to measure the concentration of volatile nitrogen as NH₃ in cecal contents of chickens.

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1. Introduction

There is currently heightened interest in gut health status as a result of changes in regulations related to the use of in-feed antibiotics. Supermarkets and fast-food chain consumers demand products produced without the use of antibiotic growth promoters (AGP).

Necrotic enteritis (NE) is an enterotoxemia of chickens mainly caused by the Gram-positive, obligate, anaerobic bacterium *Clostridium perfringens* that has been widely researched in the past few years (Broom, 2017; Jayaraman et al., 2013; Sokale et al., 2019; Xue et al., 2018). Inducement of NE challenge has been studied in the quest to find effective replacements for AGP and to determine the effect of dietary protein level and source (Palliyeguru et al., 2010), and fiber (Barekatin et al., 2013; McReynolds et al., 2009). Factors that favor the proliferation of *C. perfringens* in the hindgut include high pH usually caused by the presence of volatile basic nitrogen including ammonia (NH₃).

Ammonia production is a major issue in chicken production. It presents health and welfare risks in rearing sheds. It is also detrimental to the health and welfare of humans. Volatile nitrogen (N) increases air pollution, eutrophication of waters and increases odor

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(Antezana et al., 2015; Gbotosho and Burt, 2013; Sharma et al., 2017; Wen et al., 2014). Ammonia in the ceca is the by-product of the putrefactive deamination of by-pass proteins and other nitrogenous substrates by bacteria. Many cecal bacteria such as *Bacteroides*, *Bifidobacteria* and *Clostridia* possess putrefactive activity. *C. perfringens* is mainly responsible for the build-up of NH_3 and other odorous metabolites in digesta of NE challenged chickens (Sharma et al., 2017).

Rapid measurement of ammonia in the digesta or excreta may provide a way to determine the susceptibility of birds to NE infection. A high level of undigested material in the hindgut is an indication of nutrients bypassing host digestion (Apajalahti and Vienola, 2016). Metabolism of bypassed nutrients produces short-chain fatty acids (M'Sadeq et al., 2015) in the case of saccharolytic fermentation or total volatile nitrogen consisting of trimethylamine (TMA) and NH_3 (He et al., 2015) in the case of nitrogenous putrefactive fermentation. Ammonia increases the pH of the intestinal contents and favors the growth of *C. perfringens* (Allison and Macfarlane, 1989; Paiva and McElroy, 2014; Qaisrani et al., 2015). Total volatile basic nitrogen has been used as a quality indicator for raw fish during transport to market and also used to assess the quality of fishmeal. Ammonia is typically present at higher concentrations than TMA in decomposing fish (Bechtel, 2008). In chickens, it is possible that part of the growth suppression observed during NE challenge is caused by the adverse effects of the toxic metabolites TMA and NH_3 in the cecum. The determination of the level of volatile basic nitrogen in digesta of chickens is lacking in literature and hereby proposed. The Berthelot reaction using Skalar instrumentation has been used for the determination of NH_3 in soils, plants and water (sea, brackish water and river). In this method, NH_3 present in the sample is chlorinated using dichloroisocyanuric acid to monochloramine and then reacted with salicylate to form 5-aminosalicylate. After oxidation and oxidative coupling, a colored indo-phenolic complex is formed that can be easily measured spectrophotometrically at 660 nm. Further details of the chemistry of the reaction are described by Krom (1980).

The objective of this work was to report a first description of the use of Berthelot procedure using automated Skalar instrumentation equipment (Skalar Analytical B.V., Breda, The Netherlands) to measure volatile basic nitrogen as NH_3 in digesta of broilers fed diets containing MBM, over-processed (OP), phytase and high calcium (Ca) during subclinical NE.

2. Materials and methods

2.1. Samples from necrotic enteritis challenged broilers

All experimental procedures were reviewed and approved by the University of New England Animal Ethics Committee (AEC 17-009, AEC 17-117, AEC 18-031). All the experiments used Ross 308 male broiler chicks, 672 in Exp. 1 and 768 in Exp. 2 and 3. The chicks were reared and housed in an environmentally controlled room bedded with fresh softwood shavings, with ad libitum access to feed and water. The chicks of similar range weight were weighed and randomly allocated to 48-floor pens (0.85 m²) of 6 replicate pens per treatment, each with 14 birds in Exp. 1 and 16 birds in Exp. 2 and 3. The lighting and temperature program for the experimental period followed the breeder's recommendations.

Eight diets were formulated in accordance with Ross 308 nutrient specifications in each trial and have been reported elsewhere (Zanu et al., 2019a, 2019b, 2019c). Treatments were arranged in a 2 × 2 × 2 factorial arrangement. Factors were: Exp. 1, meat and bone meal (MBM) (no, or yes 60 g/kg in starter [S; d 0 to 14], and 50 g/kg in grower [G; d 15 to 28] and finisher [F; d 29 to 42]), antibiotic (zinc bacitracin, 100 mg/kg in S, G and 50 mg/kg in F with salinomycin, 60 mg/kg in S, G, F), phytase (500 or 1,500 FTU/kg;

Quantum Blue, AB Vista, Malborough, UK); Exp. 2, NE challenge (no or yes); phytase (500 or 1,500 FTU/kg; Quantum Blue, AB Vista, Malborough, UK) and Ca (0.6% or 1.0% in S, 0.5% or 0.9% in G and 0.4% or 0.8% in F), and diets were formulated to a constant level of available phosphorus (P) (0.40% in S, 0.35% in G and 0.35% in F) irrespective of the Ca level; Exp. 3, NE challenge (no or yes), MBM (as-received [AR] or over-processed [OP]), phytase (500 or 5000 FTU/kg; Quantum Blue, AB Vista, Malborough, UK). The phytase matrix values for 500 FTU/kg were applied in both the 500 and 5,000 FTU/kg phytase groups in all the experiments. The diets were offered ad libitum throughout the S, G and F phases in each trial.

2.1.1. Necrotic enteritis challenge

In Exp. 1, all birds were given 5,000 sporulated oocysts each of field strains of *Eimeria acervulina*, *Eimeria brunetti* and *Eimeria maxima* (Eimeria Pty Ltd, Glenorie, NSW, Australia) on d 9, and 10⁸ CFU of *C. perfringens* Strain EHE-NE18 known to express NetB toxin (Commonwealth Scientific and Industrial Research Organization, Geelong, Australia) on d 14 and 15. In Exp. 2 and 3, half of the birds (384) were challenged following the procedure in Exp. 1 and the other half were unchallenged but rather gavaged with sterile buffer (d 9) and thioglycollate broth (d 14 and 15) so as to undergo the same stress as the challenged group. This has been previously described (Rodgers et al., 2015; Stanley et al., 2014). In all experiments, cecal samples were taken on d 16 post-challenge.

2.1.2. Digesta sample collection

On d 16 of all the experiments, sampled birds were electrically stunned (Mitchell Cat 44N Poultry Stunner, Clontarf, QLD, Australia) and euthanized by cervical dislocation. Cecal samples were collected from 2 birds per pen and pooled. In Exp. 3, further samples were taken on d 29. The cecal pH was measured with a spear tip piercing pH electrode (Sensorex, California, USA) fitted to a digital pH meter (Mettler-Toledo, UK) then placed into containers and stored at −20 °C until NH_3 analysis.

2.2. Cecal NH_3 determination procedure

2.2.1. Reagents

Sulfuric acid, selenium (Se), hydrogen peroxide (30 %).

Sulfuric acid, Se mixture stock solution: 3.5 g of Se powder was weighed onto a watch glass. In a fume hood, it was carefully transferred into 1.0 L of concentrated sulfuric acid (95% to 97%) in a 2.0-L beaker. The Se powder was dissolved by heating the beaker at 300 °C until the original black color of the solution turned from deep blue into a clear light yellow. This could take up to 3 h. The solution was left to cool down to room temperature.

2.3. Digestion mixture

A 10.80-g amount of salicylic acid was weighed and added to 150 mL of the sulfuric acid, Se mixture stock solution. It was dissolved by stirring with a glass rod. The solution was stable for 48 h.

2.4. Procedure

A 0.100-g amount (accuracy 0.001 g) of the sample was weighed into a 75-mL digestion tube ensuring that as little as possible of the digesta adhered to the sides of the tube. Two standard samples (IPE 148 Lucerne - 91/*Medicago sativum*) and 2 blanks were included in each series of 50 samples. These were pre-digested in 2.5 mL of the digestion mixture (which was added with an automatic pipette) and allowed to stand overnight. A hollow glass bubble was placed on top to avoid contamination and aid in refluxing. After standing overnight, the tubes were inserted into a digestion block and

heated at 100 °C for 2 h to obtain a complete reduction of the nitrosalicylic acid. The digestion tubes were removed from the block and allowed to cool to room temperature. Slowly and carefully, 1 mL of hydrogen peroxide (30%) was added and mixed thoroughly with the contents of the tube by gently shaking. After the reaction had ceased, 1 mL of hydrogen peroxide (30%) was again added. The digestion tubes were placed back into the digestion block and heated to 330 °C for 1 h. The tubes were removed from the digestion block and cooled to room temperature. Again, a further 1 mL of hydrogen peroxide (30%) was added. The contents of the tube were mixed thoroughly by gently shaking. The digestion tubes were placed back into the digestion block and heated to 330 °C for 2 h when they were finally removed and cooled to room temperature. The volume of each sample was increased to 75 mL with deionized water, sealed with parafilm and mixed by inverting completely 3 to 4 times. The samples were permitted to stand overnight, and an aliquot taken for analysis on the Skalar.

2.5. Skalar San2^{plus} analyser reagents

Stock A solution of 1,000 mg of N/L was prepared by dissolving 3.8190 g of ammonium chloride (NH₄Cl) in 800 mL of deionized water. This solution was then made to 1 L with deionized water and mixed well.

Stock B solution was used to prepare working standards. It contained 200 mg of N/L and was prepared by taking 40 mL of Stock A into a 200-mL beaker. A 150-mL amount of deionized water was added along with 6.7 mL of concentrated sulfuric acid (95% to 97%). Once cooled the solution was made to 200 mL total volume with deionized water and mixed well. Working standards of 1, 2, 4, 6, 8, 10, 15, and 20 mg of N/L were prepared from stock solution B and liquid sampler rinse described below.

2.6. Reagents

2.6.1. Liquid sampler rinse

A 33.3-mL amount of sulfuric acid (95% to 97%) was added slowly and with much stirring to 800 mL deionized water. Once cooled the solution was made to 1 L with deionized water and mixed well.

2.6.2. Sodium hydroxide solution

This was made by dissolving 21 g of NaOH in 800 mL deionized water and once dissolved and cooled, made to 1 L. This is 7 g more of NaOH than that used on the existing ammonia manifold to compensate for the increased acid concentration in the digested samples.

2.6.3. Salicylate reagent

This was made by dissolving 85 g sodium salicylate and 0.6 g sodium nitroprusside in 800 mL of deionized water and once cool made to 1 L.

2.6.4. Cyanuric acid reagent

This was made by dissolving 5-g dichlorisocyanuric acid sodium salt and 24-g sodium hydroxide pellets in 800 mL of water. The mixture was allowed to cool and then made to 1 L with deionized water.

The working standards were prepared fresh daily.

2.7. Skalar equipment

This consists of a 140-compartment auto-sampler that feeds into the Skalar San2+ segmented auto-analyzer (Skalar Analytical B.V., Breda, The Netherlands). The system has 2 multi-channel peristaltic pumps for feeding of test reagents and samples. Samples are first sent through a membrane dialyzer (7 to 9 kDa) for clean-up. The sample

material is then reacted with the test reagents in spiral reaction chambers for appropriate lengths of time before being sent to a visible light spectrophotometer with detector set at 660 nm. The entire system is maintained at 37 °C. Absorbance is measured and data are sent to an automated computer system to determine concentration against standards. The initial Pre-dilution P4 step was not used in this methodology as the sample was introduced straight onto the analytical manifold. The sampling time was 50 s and the rinse time between samples was 115 s. The software used was Windows-based Flow-Access for data acquisition and instrument control. A schematic of the flow rate is shown in Fig. 1. Figures 2 and 3 present linearity data for the standard curve and recovery of ammonia respectively.

2.8. Statistical analysis of data

The data in all studies were analyzed as a 2 × 2 × 2 factorial arrangement of treatments using the PROC General Linear Models (GLM) procedure of SAS 9.3 package SAS Institute Inc., 2010 to assess the main effects and 2- or 3-way interactions, with the factors as Exp. 1, MBM (no or yes), phytase (500 or 1,500 FTU/kg) and antibiotics (no or yes); Exp. 2, NE challenge (no or yes), phytase (500 or 1,500 FTU/kg) and Ca (low or high); and Exp. 3, NE challenge (no or yes), MBM (AR or OP) and phytase (500 or 5,000 FTU/kg). Tukey's mean separation test was used to make pairwise comparisons between treatment means ($P < 0.05$).

3. Results and discussion

Results from Exp. 1 (d 16, post-challenge) indicated that the inclusion of dietary MBM resulted in an increase in cecal digesta NH₃ ($P < 0.01$) with a corresponding increase in pH ($P < 0.05$) as shown in Table 1. In Exp. 2 (d 16, post-challenge), increasing dietary Ca from 0.38% to 0.78% resulted in an increase ($P < 0.05$) in cecal digesta NH₃ with a corresponding increase in pH ($P < 0.05$) as shown in Table 2. In Exp. 3, the challenge with NE decreased cecal NH₃ ($P = 0.059$) with a corresponding decrease in pH ($P < 0.05$) compared to samples from birds not infected with NE as shown in Table 3. No effect of NE challenged on NH₃ or pH was observed in cecal contents after recovery on d 29 (post-challenge). Performance and other details of these experiments are reported elsewhere (Zanu et al., 2019a, 2019b, 2019c).

Attempts at determining NH₃ concentration in chicken digesta and excreta have been reported previously using a modified Kjeldahl procedure (Khemphaka et al., 2011). In that work, birds fed diets with high levels of shrimp meal (up to 15%) or purified chitin (up to 3.8%) had reduced levels of NH₃ in cecal digesta and excreta compared to birds fed control diets. The authors suggested that NH₃ forming bacteria may be inhibited by chitin or chitin-containing feed ingredients. The current study provides a procedure that can be used to measure volatile nitrogen as NH₃ in digesta of chickens using the Berthelot reaction in automated Skalar equipment. The pH of the content of the ceca only correlated significantly with the NH₃ concentration in Exp. 3 on d 16 (Pearson correlation; $r = 0.432$; $P = 0.002$).

Since cecal volatile nitrogen (TMA and NH₃) levels have not been previously reported in induced necrotic enteritis experiments, it was not possible to compare the result of this study to others. However, a study by Endo and Nakano (1999) indicated that probiotics decrease both pH and NH₃ in the cecal contents of 49-d-old broiler chickens using a colorimetric determination with a commercial kit (Ammonia Test Wako, Wako Pure Chemical Industries, Ltd., Tokyo, Japan). This confirms the current report that an increase in NH₃ in ceca leads to an increase in pH. The increase in NH₃ and pH by feeding MBM could be due to aromatic amino acids (tyrosine and tryptophan) in MBM (Suloma et al., 2013) being anaerobically fermented by putrefactive *C. perfringens* leading to the formation of

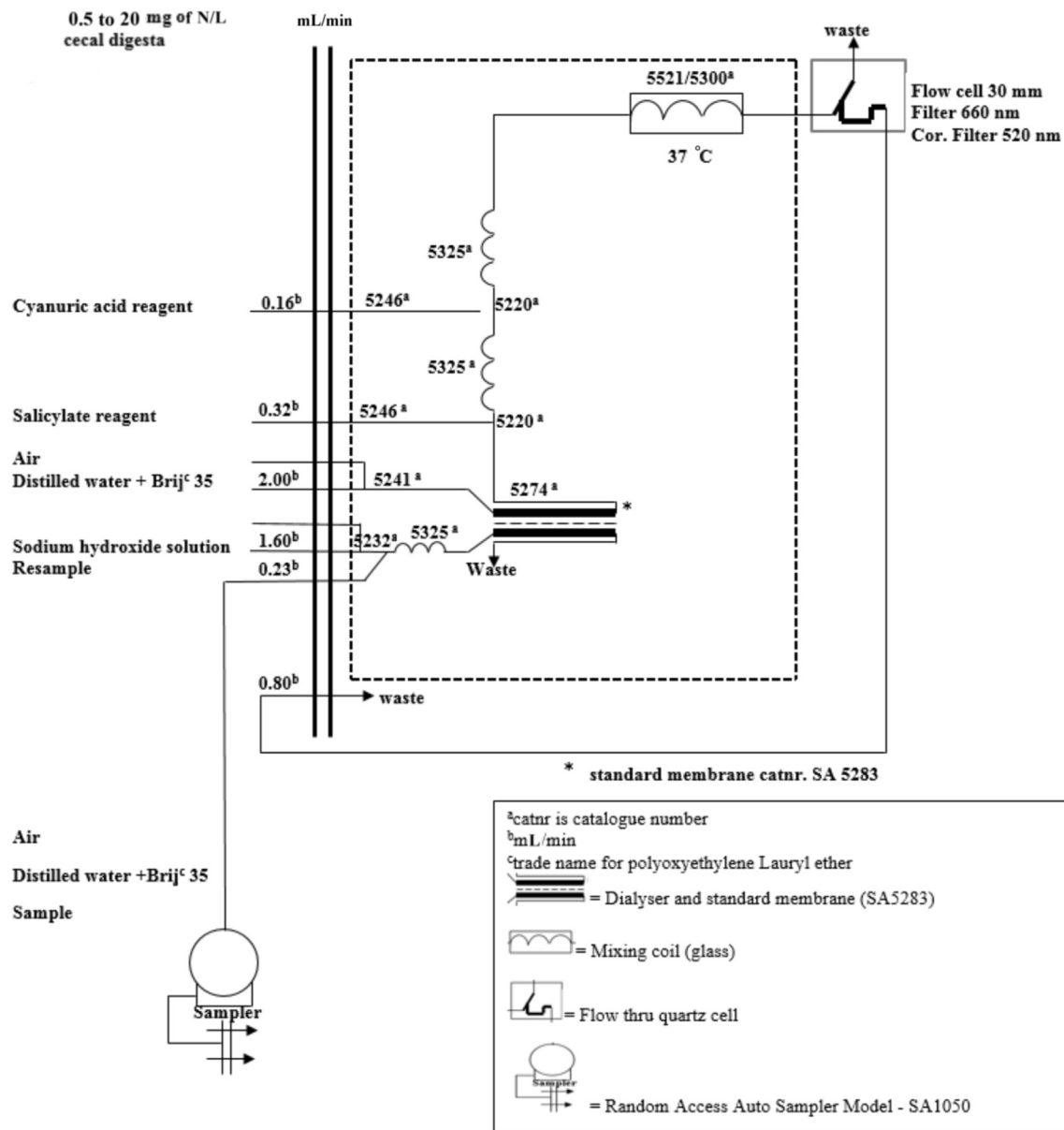


Fig. 1. Manifold flow diagram (0 to 20 mg of N/L cecal digesta). N = nitrogen; SA = sampler assembly; Cor = correction filter; Brij = polyoxyethylene lauryl ether (Henan Daken Chemical, Zheng Zhou, China).

NH₃ as a result of deamination and decarboxylation. These metabolites tend to increase the pH providing a favorable environment for the proliferation of the otherwise acid-sensitive *C. perfringens* (Rinttilä and Apajalahti, 2013). In pigs, an increase in the level of NH₃ in the colonic digesta as a result of feeding different non-conventional dietary fiber sources and indigestible crude protein was also reported to have an impact on pH (Jha and Leterme, 2012). These authors also reported a correlation between NH₃ and crude protein in the diets. An association between cecal NH₃ and pH was also observed in a study by Poeikhampha and Bunchasak (2010). Those authors reported that at 8 and 24 h of fermentation of porcine cecal digesta, a higher sodium gluconate supplementation (7,500 mg/kg) reduced pH and NH₃.

In the case of high NH₃ and pH recorded in Exp. 2 in the current work, Ca with a high acid-binding capacity might have interfered with gizzard pH (Angel et al., 2002; Selle et al., 2009) and hence

decreased pepsin digestion. Additionally, higher Ca levels might have bound with phytate to form insoluble Ca–phytate complex that was less accessible to phytase. This might have resulted in a possible reaction of phytate with dietary and endogenous proteins such as pepsin and pepsinogen thereby reducing the activity of these enzymes to digest the protein (Woyengo et al., 2010; Yu et al., 2012). Interestingly, the low pH recorded in Exp. 3 of this study as a result of the challenge was rather unexpected, but perhaps not unusual as a similar finding has been reported in a previous challenge study (Barekatain et al., 2013). But the fact that it correlated with the level of NH₃ gives credence to this method. This observation suggests that endogenous proteins were either putrefied prior to exit from the ileum, or never entered the ceca because they were particulate, or if they did enter into the ceca, the infection might have suppressed other putrefactive bacteria. The 2-way phytase × MBM interaction observed on d 16 of Exp. 3 where only

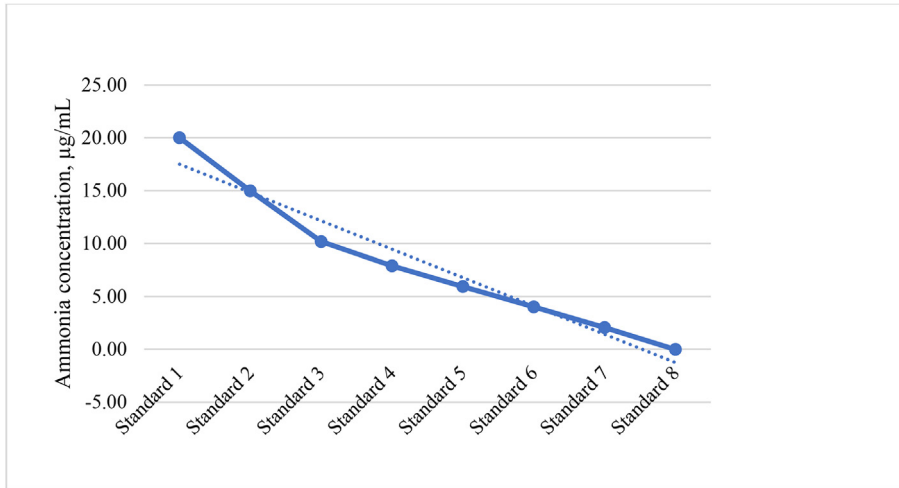


Fig. 2. Typical standard curve relating ammonia concentration (x) to standard (y), $y = 2.6798x + 20.184; 0.9517$ (random residual error).

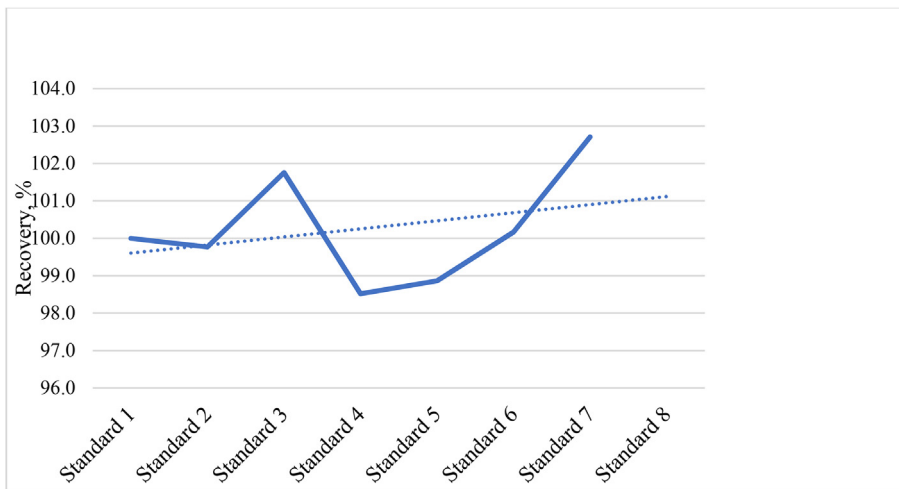


Fig. 3. Typical standard curve relating recovery (x) to standard (y), $y = 0.2159x + 99.391; 0.0964$ (random residual error).

Table 1

Experiment 1, effect of diet MBM, Phy¹ and AB² on cecal ammonia and pH on d 16, (as-is).

Item	Cecal NH ₃ , mg/g	Cecal pH
Diet with no MBM	22.53 ^b	6.10 ^b
Diet with 5.0% MBM	40.44 ^a	6.40 ^a
Percentage of change	44.29	4.69
SEM	2.95	0.04
<i>P</i> > <i>f</i>		
MBM	0.003	0.046
Phy	0.306	0.305
AB	0.673	0.067
MBM × Phy	0.479	0.072
MBM × AB	0.415	0.601
Phy × AB	0.703	0.431
MBM × Phy × AB	0.465	0.287

MBM = meat and bone meal; Phy = phytase; AB = antibiotics.

P > *f*, where the *P* is a probability, and the *f* ratio is a test statistic.

^{a,b} Within a column of the main effect, means without a common superscript are different (*P* < 0.05).

¹ Phy, Phytase, 500 vs. 1,500 FTU/kg (Quantum Blue 5G, AB Vista, Malborough, UK).

² AB, Salinomycin 60 mg/kg in starter (S; d 0 to 14), grower (G; d 15 to 28), and finisher (F; d 29 to 42); zinc bacitracin 100 mg/kg in S, G and 50 mg/kg in F.

Table 2

Experiment 2, the effect of dietary Ca on cecal ammonia and pH on d 16, (as-is).

Item	Cecal NH ₃ , mg/g	Cecal pH
Dietary Ca (0.5%)	18.70 ^b	5.96 ^b
Dietary Ca (0.9%)	23.02 ^a	6.20 ^a
Percentage of change	18.77	3.87
SEM	1.04	0.05
<i>P</i> > <i>f</i>		
NE	0.692	0.961
Phy	0.715	0.208
Ca	0.040	0.023
NE × Phy	0.178	0.925
NE × Ca	0.298	0.303
Phy × Ca	0.952	0.051
NE × Phy × Ca	0.264	0.704

P > *f*, where the *P* is a probability, and the *f* ratio is a test statistic. Ca = calcium; NE = necrotic enteritis; Phy = phytase, 500 vs. 1,500 FTU/kg (Quantum Blue 5G, AB Vista, Malborough, UK).

^{a,b} Within a column of the main effect, means without a common superscript are different (*P* < 0.05).

Table 3
Experiment 3, effect of NE on cecal ammonia and pH on d 16 and 29, (as-is).

Item	NE Phy	MBM	NH ₃ , mg/g d 16	pH d 16	NH ₃ , mg/g d 29	pH d 29
Main effects	–		15.77	6.26	16.30	5.89
	+		13.47	6.01	15.59	5.89
2-way interaction	500	AR	13.71	5.93 ^b	16.64	6.05
	500	OP	15.07	6.14 ^{ab}	15.39	5.92
	5,000	AR	14.03	6.43 ^a	15.23	5.85
	5,000	OP	15.67	6.03 ^b	16.51	5.75
SEM			0.60	0.06	0.410	0.05
<i>P</i> > <i>f</i>						
NE			0.059	0.018	0.410	0.974
Phy			0.907	0.061	0.990	0.103
MBM			0.697	0.356	0.863	0.309
NE × Phy			0.154	0.168	0.812	0.236
NE × MBM			0.916	0.269	0.692	0.506
Phy × MBM			0.213	0.005	0.143	0.847
NE × Phy × MBM			0.729	0.592	0.382	0.192

P > *f*, where the *P* is a probability, and the *f* ratio is a test statistic. NE = necrotic enteritis; Phy = phytase, 500 vs. 5,000 FTU/kg (Quantum Blue 5G, AB Vista, Malborough, UK); MBM = meat and bone meal; AR = as-received; OP = over-processed. The 2-way interaction was separated by Tukey.

^{a,b} Within within a column of the 2-way interaction, means without a common superscript are different (*P* < 0.05).

in birds fed high phytase (5,000 FTU/kg) and OP MBM compared to those fed high phytase (5,000 FTU/kg) and AR MBM was cecal pH reduced is also worth noting. It is possible that the over-processing of the MBM might have reduced the CaCO₃ content through the process of calcination (Silva et al., 2019) or the Ca solubility of OP MBM might have been decreased agreeing with the report of Kim et al. (2018) that highly soluble Ca sources increase gut pH.

Overall, a precise and suitable method was demonstrated to evaluate the total basic volatile nitrogen (NH₃) concentration in cecal digesta of chickens. The results were found to be correlated to pH. This method may also be applied to digesta collected from other locations across the gut in addition to fresh excreta. Such measurement may be useful to diagnostically predict broiler flocks that are susceptible to NE.

Conflicts of interest

The authors declare there is no conflict of interest.

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