Poultry Science®

Bromelain can reduce the negative effects of a subclinical necrotic enteritis in broiler chickens

Kosar Gharib-Naseri, Sarbast K. Kheravii, Hong Thi Nguyen, and Shu-Biao Wu*

School of Environmental and Rural Science, University of New England, Armidale, New South Wales 2351, Australia

ABSTRACT This study was conducted to examine the efficacy of a bromelain-based supplementation coded ANR-pf on growth performance and intestinal lesion of broiler chickens under necrotic enteritis (**NE**) challenge. A total of 540 Ross 308 day-old male chicks were randomly allocated into 6 treatments of 6 replicates. The bromelain formulation was delivered to chickens through gavaging or in drinking water method twice, on d 8 and 13. Nonchallenged groups included 1) without or 2) with the specific bromelain formulation gavaged at 0.8 mL/kg. NE-challenged groups included 3) without the specific bromelain formulation; 4) gavaged with 0.4 mL/kg; 5) gavaged with 0.8 mL/kg and 6) supplemented with 0.8 mL/kg via drinking water. Birds were challenged with *Eimeria spp.* on d 9 and *Clostridium perfringens* (NE-18 strain) on d 14 and 15. On d 14 and 19, fresh faecal contents were collected for the determination of oocyst counts. Intestinal lesion scores were determined on d16. Performance and mortality were recorded throughout the entire experiment. Among challenged groups, birds received additive via drinking water had higher weight gain (**WG**) compared to the remaining groups ($P < 0.001$) in the grower phase and had lower FCR compared to 0.4 mL/kg inoculated group in the grower and finisher phases ($P < 0.001$). Bromelain supplementation via drinking water improved the WG of challenged birds, similar to that of the nonchallenged birds ($P < 0.001$), and lowered FCR compared to other challenged groups ($P < 0.001$). Nonchallenged birds and birds that received bromelain formulation in drinking water did not have lesions throughout the small intestine whereas challenged birds, either un-supplemented or supplemented with bromelain via inoculation route recorded similar lesion score levels in the jejunum. At d 19, birds received bromelain in drinking water had lower fecal oocyst numbers compared to challenged birds without additive ($P < 0.001$). In conclusion, bromelain administration via drinking water could ameliorate the negative impacts of NE-infection in broilers by improving performance, lowering the oocyst numbers and lesion scores.

Key words: bromelain, necrotic enteritis, *Eimeria*, broiler chicken, performance

2024 Poultry Science 103:103560
https://doi.org/10.1016/j.psj.2024.103560

INTRODUCTION

Necrotic enteritis (**NE**) in broiler chickens is a common disease found in all poultry-growing areas of the world which can be controlled by antibiotics. However, increasing concerns regarding antibiotic resistance and the presence of drug residues in animal products have led many countries to ban the use of antibiotic growth promoters in animal feed, which has led to rising incidence of *Clostridium* infections and the development of NE in commercial chickens (Immerseel et al., 2004). Most economic losses are associated with the presence of the subclinical forms of this disease, as the birds show no clinical signs and the poor performance will be noticed at the end of the rearing period (Shojadoost et al., 2012). This has triggered a search for viable alternatives to antibiotics in the animal industry due to more frequent outbreaks of enteric diseases such as necrotic enteritis.

Phytogenic feed additives (often also called phytobiotics or botanicals) are commonly defined as plant-derived compounds incorporated into diets and have shown to be effective compounds to improve the productivity of livestock (Windisch et al., 2008). Bromelain is a mixture of natural proteinases, rich in cysteine proteases, extracted from different parts of the pineapple (*Ananas comosus*) (Hale, 2004). It is rich in cysteine proteases which have considerable commercial importance (Feijoo-Siota and Villa, 2011) and its biological effects are linked to the proteolytic activity (Mazorra-Manzano et al., 2018). Dietary protease is an enzyme that hydrolyses high molecular weight polypeptides into oligopeptides with lower molecular weight for further digestion by endogenous proteases or as single amino

Received September 2, 2023.
Accepted February 12, 2024.

*Corresponding author: swu3@une.edu.au

acids. It plays an important role in improving protein digestion because of being capable to hydrolyze the less digestible proteins in animal feeds into more usable peptides and amino acids. However, the effects of protease supplementation are not only limited to the hydrolysis of dietary proteins in pigs and broilers. Indeed, proteases are reported to improve nutrient digestibility (Freitas et al., 2011; Yin et al., 2018), and this improvement can indirectly result in the reduction of potentially fermentable protein substrates favoring pathogenic bacterial growth in the hindgut (Yin et al., 2018), including *C. perfringens* (Wilkie et al., 2005) and *Campylobacter spp.* (Wise and Siragusa, 2007). The change in the intestinal population with the over-flourishing of protein-degrading bacteria is generally considered detrimental to health (Qaisrani et al., 2015; Lin and Olukosi, 2021). Protease supplementation, therefore, can be a potential solution for necrotic enteritis as a preventative measure.

Apart from acting as a proteolytic enzyme for stimulating protein digestion, numerous studies have ascertained further beneficial properties of bromelain to humans and animals, such as anti-inflammatory (Sahbaz et al., 2015), anti-oedematous and debridement (Hu et al., 2011), antioxidant (Yenice et al., 2019), and antibacterial (Begum et al., 2015; Hossain et al., 2015) properties. Additionally, Hale et al. (2005) have shown that the proteolytic activity of bromelain solution can remain stable for at least 1 wk at room temperature, and this can be an attractive characteristic for in-water delivery of this compound. In addition, bromelain is stable and can maintain its proteolytic activity under a broad range of temperatures (37−70°C) and pH (3−8), where most enzymes are destroyed or denatured. Therefore, it is also a safe and versatile agent for prolonged and high-dose use (Chobotova et al., 2010; Pavan et al., 2012; Arefin et al., 2020). Evidence in humans demonstrated that active components of bromelain can be absorbed in the intestinal wall without degradation whilst maintaining biological activity (Castell et al., 1997; Chobotova et al., 2010). For these reasons, bromelain can be a potential alternative to antibiotic and microbiota-derived proteases that is worth investigating.

A growing number of studies in pigs and other farm animals have demonstrated bromelain as a potential additive to improve nutrient utilization, performance, and immune potency (Chandler and Mynott, 1998; Begum et al., 2015; Nguyen et al., 2018; Wiszniewski et al., 2019; Daiba et al. 2023); however, reported data in poultry is very limited and little is known about these effects on broiler chickens. Thus, the objective of the present study was to assess the effectiveness of a bromelain-containing product (ANR-pf®) to determine if different dose and administration methods (gavaged or in-water application) can ameliorate the negative effects of a subclinical necrotic enteritis challenge in broiler chickens.

MATERIAL AND METHODS

All procedures of this study were reviewed and approved by the Animal Ethics Committee of the University of New England (20/065). All procedures involving the birds, including health, care, and use of laboratory animals, were fulfilled within the Code of Practice for the Use of Animals for Scientific Purposes issued by the Australian Bureau of Animal Health (NHMRC, 2013).

Experimental Procedures, Design, and Diets

A total of 540 Ross-308 male line parental chicks were obtained on the hatching day from a hatchery in Goulburn NSW. Birds were weighed and randomly assigned to 48-floor pens, with hardwood shavings used as bedding materials, contributing to 6 treatments with 6 replicate pens, each with 15 birds. The treatments were: nonchallenged groups included 1) without **(NC)** or 2) with the specific bromelain formulation gavaged at 0.8 mL/kg **(NC+0.8 G)**; NE-challenged groups included (3) without the specific bromelain formulation **(CC)**; (4) gavaged with 0.4 mL/kg **(CC+0.4 G)**; (5) gavaged with 0.8 mL/kg **(CC+0.8 G)** and (6) supplemented with 0.8 mL/kg via drinking water (**CC+0.8 W**). The experiment used a completely randomized design and treatments were based on different dose and application method of the product. The treatment details are presented in Table 1.

Feed was prepared in pellet form and fed in 3 phases, starter (d 1−10), grower (d 11−24), and finisher (d 25−35). The diet was wheat and soybean meal based and formulated to meet the nutrient requirements recommended for Ross-308 broilers. Diet composition and the

Table 1. Experimental treatments.

Group[1]	Challenge	ANR-pf dose[2] (mL/kg)	Application type	Administered days
NC	No	-	-	-
NC + 0.8 G	No	0.8	Gavage	d 8 and 13
CC	Yes	-	-	-
CC + 0.4 G	Yes	0.4	Gavage	d 8 and 13
CC + 0.8 G	Yes	0.8	Gavage	d 8 and 13
CC + 0.8 D	Yes	0.8	In drinking water	d 8 and 13

[1]NC: negative control without both NE-challenge and bromelain supplementation; NC+0.8 G: negative control without NE-challenge and supplemented with bromelain at 0.8 mL/kg using gavaging method; CC: NE-challenged group without bromelain supplementation; CC+0.4 G: NE-challenged group supplemented with bromelain at 0.4 mL/kg via gavaging method; CC+0.8 G: NE-challenged group supplemented with bromelain at 0.8 mL/kg through gavaging method; CC+0.8 D: NE-challenge group supplemented with bromelain at 0.8mL/kg via drinking water route.

[2]The proprietary supplement, ANR-pf, contains 670 mg/g of bromelain in powder form before mixed with water.

Table 2. Composition and nutrient content of wheat-based diets (%) for all growth phases.

Ingredient (%)	Starter (d 0-10)	Grower (d 10-24)	Finisher (d 24-35)
Wheat	48	44	49
Soybean meal	31	30	24.7
Sorghum	15	20	20
Canola oil	1.5	2.57	3.20
Limestone	1.24	1.13	1.04
Dicalcium phosphorus	1.01	0.79	0.59
L-Arginine	0.41	-	-
L-lysine	0.35	0.164	0.210
DL-methionine	0.35	0.236	0.219
Salt	0.18	0.221	0.224
L-threonine	0.12	0.095	0.055
UNE TM premix[1]	0.11	0.11	0.11
UNE Vit premix[2]	0.085	0.085	0.085
Na bicarb	0.078	0.02	0.015
Choline Cl 70%	0.029	0.018	0.017
Quantum Blue 5G	0.01	0.01	0.01
Xylanase powder	0.005	0.005	0.005
Nutrient analysis			
MEn, kcal/kg	3,013	3,100	3,200
Crude protein, %	23	21.5	19.5
Calcium, %	0.96	0.87	0.78
Available phosphorus, %	0.45	0.43	0.39
Sodium, %	0.16	0.16	0.16
Lysine, %	1.28	1.10	1.01
Methionine, %	0.64	0.52	0.48
Methionine+ Cysteine, %	0.93	0.81	0.76

[1]Trace mineral concentrate supplied per kilogram of diet: Cu (sulphate), 16 mg; Fe (sulphate), 40 mg; I (iodide), 1.25 mg; Se (selenate), 0.3 mg; Mn (sulphate and oxide), 120 mg; Zn (sulphate and oxide), 100 mg; cereal-based carrier, 128 mg; mineral oil, 3.75 mg.

[2]Vitamin concentrate supplied per kilogram of diet: retinol, 12,000 IU; cholecalciferol, 5,000 IU; tocopherol acetate, 75 mg, menadione, 3 mg; thiamine, 3 mg; riboflavin, 8 mg; niacin, 55 mg; pantothenate, 13 mg; pyridoxine, 5 mg; folate, 2 mg; cyanocobalamin, 16 mg; biotin, 200 mg; cereal-based carrier, 149 mg; mineral oil, 2.5 mg.

analyzed nutrients contents are presented in Table 2. Birds and feed were weighed on arrival and on days 10, 24, and 35, and mortality incidence was recorded daily. Body weight gain (**WG**), feed intake (**FI**), and mortality-adjusted feed conversion ratio (**FCR**) were calculated for all phases. The lighting, relative humidity, and temperature followed Ross-308 strain guidelines (Aviagen, 2018). All birds had ad libitum access to feed and water throughout the study.

Necrotic Enteritis Challenge

On d 9, challenged groups were inoculated with 1 dose (1 mL/bird) of a field strain of *Eimeria* strains (Eimeria Pty Ltd, Ringwood, Vic, Australia). Each dose of inoculum consisted of 5,000 sporulated oocysts each of *E. maxima* and *E. acervulin*a and 2,500 sporulated oocysts of *E. brunetti* in 1 mL of 1% (w/v) sterile phosphate-buffered saline (**PBS**). To be able to confirm that all birds have been under the same stress of handling and gavage, the nonchallenged groups were inoculated with sterile PBS as a control. Primary poultry isolates of *C. perfringens* (strain EHE-NE18) containing the toxin NetB (Keyburn et al., 2008) were obtained from CSIRO Livestock Industries, Geelong, Australia. The challenge inocula were freshly prepared by growing the bacterial strain separately in 100 mL of sterile thioglycolate (USP alternative, Oxoid, Australia) with added starch (10 g/L) and pancreatic digest of casein (5 g/L); this was incubated overnight at 39°C. On d 14 and 15, birds in the challenged groups were gavaged with approximately 10^8 CFU/mL of *C. perfringens*, whereas the nonchallenged birds were inoculated with sterile thioglycolate broth.

Administration of Bromelain

The proprietary bromelain-based formulation (in powder form), ANR-pf, was sourced from Anatara Lifesciences. This specific bromelain solution was prepared and supplemented to birds according to the instructions of the manufacturer. The powder product (ANR-pf) was mixed with water to prepare the solution. The additive was administered orally via 2 routes, gavage and adding into drinking water as described in Table 1. The amount of product administered to the birds was based on their average weight on the administration day. In gavaged groups, birds were gavaged twice on d 8 and d 13, with either a low dose (0.4 mL/kg per body weight) or a high dose (0.8 mL/kg per body weight). In the group supplemented via drinking water, the volume of average daily water consumption per bird was determined prior to diluting the product in the water on both administration days (d 8 and d 13) to ensure that the birds in all pens received the same dose of medication via drinking water in 24 h. After drinking the supplemented water, the birds were supplied with nonmedicated drinking water. Birds in the nonchallenged groups and challenged birds in the in-water supplemented group, were all gavaged with 1 mL of PBS.

Data and Sample Collection

On d 10, 24, and 35, all birds and leftover feed were weighed and mortality was recorded daily. The average weight gain, feed intake and FCR were calculated based on the collection dates. On d 16, 2 birds per pen from all groups were randomly selected, weighed, and euthanized by cervical dislocation to perform post-mortem analysis and intestinal lesion scoring.

Lesion Score

Two sampled birds were dissected and the intestinal tract (duodenum, jejunum, and ileum) was separated. All sections were based on a previously reported lesion scoring system that ranges from 0 to 6 (Keyburn et al., 2006).

Fecal Eimeria Oocyst Shedding

On d 14 and 19 fresh fecal contents in each pen were collected in a 15 mL container and stored at 4°C for oocyst counts to be conducted. Briefly, the fecal sample in each container was completely mixed with a clean spatula to make sure the sample was homogenized.

10 mg of fecal sample was taken to mix with 900 μL saturated salt solution. Samples were vortexed to mix and left for 1 h in the fridge to allow oocysts to float and sample debris to settle. 600 μL saturated salt solution was added to the Whitelock chamber and 1500 μL of the sample was pipetted from top of the sample and added to the Whitelock chamber. Oocyst were then counted under a microscope using a 10 × lens.

Statistical Analysis

Statistical analyses were performed using Minitab (version 17.0, 2017 Minitab Inc., State College, PA). Performance data and transformed-oocyst count data (Log_{10} transformation) were statistically analyzed by one-way analysis of variance (**ANOVA**), followed by Fisher's Least Significance Difference (**LSD**) test to determine the differences between treatment means where significance was observed. The intestinal lesion scores and mortality data were analyzed by the nonparametric Kruskal −Wallis test, as the data was not normally distributed. Pen served as the experimental unit and there were six replicate pens per treatment. Significant differences between mean values were declared at $P < 0.05$.

RESULTS

Broiler Performance

During the growing phase (d 11−24), birds in both nonchallenged groups showed a significantly higher WG and FCR compared to all challenged birds ($P < 0.001$, Table 3). In addition, challenged birds in the group receiving the treatment in drinking water (CC + 0.8 D) had a significantly higher WG compared to all other challenged birds during d 11 to 24 ($P < 0.001$). Also, in this period, birds receiving the treatment with drinking water (CC + 0.8 D) and birds gavaged with the higher dose of treatment (CC + 0.8 G) had a significantly lower FCR compared to the birds gavaged with the lower dose (CC + 0.4 G) of ANR-pf ($P < 0.001$). Furthermore, the overall performance of birds showed higher WG in both nonchallenged groups compared with all challenged birds ($P < 0.001$), apart from challenged birds receiving ANR-pf in drinking water. In the 0 to 35 d period, FCR of birds in the nonchallenged group was significantly lower than the challenged birds ($P < 0.01$), despite the fact that FCR of challenged birds receiving ANR-pf in drinking water (CC + 0.8 D) was not statistically different from that of the nonchallenged birds receiving 0.8 mL/kg of ANR-pf (NC + 0.8 G). In addition, challenged birds that received the low dose (CC + 0.4 G) of ANR-pf had a significantly higher FCR compared to the challenged birds receiving ANR-pf in drinking water (CC + 0.8 D) in this period ($P = 0.001$).

Table 4. Effect of ANR-pf on lesion score of broilers subjected to subclinical necrotic enteritis challenge on d 16.

	Lesion score		
Treatment	Duodenum	Jejunum	Ileum
NC	0	0[b]	0[b]
NC + 0.8 G	0	0[b]	0[b]
CC	0.083	0.250[a]	0.250[a]
CC + 0.4 G	0.083	0.250[a]	0.167[ab]
CC + 0.8 G	0	0.250[a]	0[b]
CC + 0.8 D	0	0[b]	0[b]
SEM	0.048	0.079	0.063
P-values	0.533	0.026	0.022

[a,b]means in a column not sharing a common letter are significantly different ($P < 0.05$).

Lesion Score

Intestinal lesions show a significantly lower score in the jejunum of challenged birds receiving the medication via water (CC + 0.8 D) compared to all other challenged birds ($P < 0.05$, Table 4). Additionally, the ileum of challenged birds in the nonadditive group (**CC**) showed higher lesions compared to birds that received the low dose (CC + 0.4 G) of ANR-pf and the in-water receiving groups (CC + 0.8 D) ($P < 0.05$).

Oocyst Shedding

Table 5 shows the mean oocyst shedding per pen on d 14 and 19 of the experiment. Both nonchallenged groups did not excrete oocysts with confirmed zero counts. On d 14, challenged groups did not show any difference in the number of the oocyst count ($P > 0.05$). However, on d 19, birds receiving ANR-pf in drinking water (CC + 0.8 D) showed a significantly lower shedding of oocysts compared to the challenged birds that did not receive any supplement (CC) ($P < 0.001$).

Table 3. Effects of different doses and administration methods on performance of broiler chicken under subclinical NE challenge.

	0−10		11−24		25−35		0−35	
Treatment	WG	FCR	WG	FCR	WG	FCR	WG	FCR
NC	264	1.064	933[a]	1.447[c]	1201	1.553	2398[a]	1.510[d]
NC + 0.8 G	262	1.052	944[a]	1.445[c]	1179	1.582	2385[a]	1.527[cd]
CC	258	1.048	747[c]	1.621[ab]	1150	1.582	2155[c]	1.583[ab]
CC + 0.4 G	262	1.045	768[c]	1.580[b]	1179	1.614	2198[bc]	1.581[ab]
CC + 0.8 G	261	1.043	764[c]	1.687[a]	1168	1.566	2202[bc]	1.599[a]
CC + 0.8 W	254	1.043	824[b]	1.556[b]	1214	1.546	2291[ab]	1.551[bc]
SEM	4.60	0.013	17.2	0.023	22.2	0.017	37.6	0.014
P-values	0.675	0.848	<0.001	<0.001	0.398	0.077	<0.001	0.001

[a,b,c,d]Means in a column not sharing a common letter are significantly different ($P < 0.05$).

Table 5. Effect of ANR-pf on faecal oocyst counts in broilers subjected to subclinical necrotic enteritis challenge from d 0 to 35.

	Oocyst shedding counts Log10/g	
Treatment	D 14	D 19
NC	0[b]	0[d]
NC + 0.8 G	0[b]	0[d]
CC	5.04[a]	4.35[a]
CC + 0.4 G	4.99[a]	4.22[ab]
CC + 0.8 G	5.07[a]	4.12[bc]
CC + 0.8 D	5.05[a]	3.94[c]
SEM	0.033	0.070
P-values	<0.001	<0.001

[a,b,c,d]means in a column not sharing a common letter are significantly different ($P < 0.05$).

DISCUSSION

In the present study, 2 different methods (gavage and in-water application) were used for applying bromelain extract product, ANR-pf, to chickens under a subclinical NE challenge. The proprietary supplement used in this experiment (ANR-pf) is a product containing 670 mg/g of bromelain, a complex mixture of thiol proteases and nonprotease, predominantly sourced from pineapple and that is a water-soluble polymer that can be chemically changed via free carboxyl groups (Chakraborty et al., 2021).

A successful subclinical NE infection was introduced in the challenged groups, as typical signs such as mild lesions and impaired FCR and BW were observed in the challenged birds (Jayaraman et al., 2013). In the current study, birds were administered with ANR-pf (d 8 and d 13), either by gavage or in drinking water. Results showed that the overall weight gain (d 0−35) in challenged birds receiving the additive in drinking water was not significantly different from the nonchallenged birds. Those challenged chickens receiving ANR-pf via drinking water showed performance improvements, negative lesion scores and lower faecal oocyst count at d 19. In contrast, the bromelain gavaged birds (either nonchallenged birds or NE challenged) did not show any positive effects of this additive when compared to the drinking water group. The variation in these responses might reveal possible differences in the activity of the additive via the delivery routes. To the best of our knowledge, there has been no available information reporting the effect of the delivery route of bromelain on farm animals. Therefore, underlying reasons of the effectiveness with such a delivery route may require more attention.

It has been shown that significant levels of components of orally ingested bromelain have been found to be absorbed into the bloodstream, thus enhancing the proteolytic and fibrinolytic blood activity for hours (Chakraborty et al., 2021). Many studies have demonstrated that pineapple derived extract has a wide range of antibacterial, antiprotozoal/anticoccidial, and anthelmintic properties (Stepek et al., 2006; Ali et al., 2015; Daiba et al., 2023). A human study showed that the estimated half-life of the proteolytic activity in plasma and of intestinally absorbed bromelain after oral administration was 6 to 9 h and 6.07 h, respectively (Castell et al., 1997). In weaned piglets challenged with $K88^+$ enterotoxigenic *Escherichia coli* (**ETEC**), a common pathogen causing diarrhea in young piglets, continuous oral daily administration (10 d) of bromelain, either 12.5 mg or 125 mg, reduced intestinal $K88^+$ *Escherichia coli* (ETEC) numbers. Furthermore, these authors reported an increase in WG of piglets receiving the additive; however, the positive effects on inhibiting $K88^+$ ETEC receptor activity temporarily lasted for 30 h after treatment (Chandler and Mynott, 1998), consistent with the duration of regeneration of new enterocytes (Chandler and Mynott, 1998). Dosković et al. (2013) recommended that the supplemented enzyme activity in poultry feed must be sufficiently high enough to compensate for the relatively short transit time of digesta through the intestine. This could be the reason of the lack of response in bromelain-gavaged birds, where the gavaging of this product a day prior to the challenge did not give the product sufficient time to be in contact with the gut to exhibit its positive effects. In contrast with our findings, Hale (2004) suggested that administration of bromelain as a concentrated bolus once or several times daily would be expected to result in higher total proteolytic activity, particularly within the lumen of the gastrointestinal tract, compared with timed-release dosing that generated lower peak concentrations. However, the present results showed administration at 0.8 mL/kg via drinking water over a 24-hour period could exert the positive effects of this additive on NE-infected birds, rather than delivering the product via gavage. Previously, following oral administration of bromelain in pigs, the proteolytic activity of this additive has been shown to modify the intestinal receptor attachment sites in the small intestine and demonstrated to prevent attachment of *E. coli* (Mynott et al., 1996). Hence, the consistent positive impacts of the additive on broilers when delivered via drinking water for an extended time, together with the lack of effects in gavaged birds on performance, intestinal lesion score, and oocyst counts suggests the importance of the chosen administration routes and the timing of delivery of the additive for efficacy is significant and needs to be further examined.

It is well-known that intestinal pathogenic infections, such as *Eimeria* spp. or *Clostridium perfringens* can adversely affect intestinal nutrient digestion and absorption in chickens. This is related to the damage caused to the intestinal morphology and integrity, decreased activity of important mucosal enzymes, and adversely disrupted microbiome (Peek et al., 2009; Whelan et al., 2019). The improvement in WG of challenged birds that received the additive via drinking water implies that bromelain application was successful in assisting birds under the intestinal disorders induced by NE challenge when consumed within 24 h.

Bromelain has previously shown high anticoccidial activity against *Eimeria* spp. and the sporulation both *in vitro* and *in vivo* by degrading the coccidial shell wall, softening and destructing the central cytoplasmic (Juasook et al., 2017; Daiba et al., 2022; Daiba et al., 2023).

As mentioned previously, bromelain is a crude aqueous extract rich in cysteine proteases, which can partially degrade the mucus layer, thus impairing the adhesion of pathogens to the mucus (Peek et al., 2009). Therefore, this could partially contribute to the lower oocyst counts observed in bromelain-treated birds on d 19. *Eimeria* infections are known to be a predisposing factor for intestinal disease, as these produce physical changes to the GIT and also cause disruption of gut microbial communities (Stanley et al., 2014; Wu et al., 2014). The results show that the ANR-pf reduced the oocyst counts on d 19 indicating exerted positive effects of ANR-pf administrated by drinking water on d 13. Similarly, Juasook et al. (2017) investigated the effect of pineapple peel extract on 21-day-old broilers infected with 2×10^4 *E. tenella* sporulated oocysts and found a significantly lower number of oocysts in birds treated with the additive. In partial agreement with the current results, Peek et al. (2009) indicated that dietary supplementation with a protease reduced negative impacts of a coccidiosis infection (*E. acervulina, E. maxima,* and *E. tenella*) on body weight gain in broilers but had no effects on lesions and oocyst shedding. The lower jejunal lesion scores in the birds administrated ANR-pf is another indicator of the improvement of gut health. The effectiveness of bromelain on relieving the severity of intestinal lesions in NE-infected birds could be partly related to its antimicrobial properties. Studies have shown bromelain can possess anti-adhesive properties, that can inhibit microbial pathogens such as *B. cereus, S. aureus and E. coli,* from adhering to the glycoprotein receptors in the intestinal tract (Mynott et al., 1996; Praveen et al., 2014; Loon et al., 2018; Van Doan et al., 2021). Broiler chickens also showed lower *E.coli* and increased *Lactobacillus* numbers when supplemented with different bromelain levels from 0.05% to 0.2% (Akit et. al., 2019). In the lactating sows and weanling piglets, the inclusion of bromelain in the diets also reduced faecal *E. coli* population (Begum et al., 2015; Hossain et al., 2015).

CONCLUSIONS

The current study demonstrated a positive effect of the supplementation of this specific bromelain-containing product ANR-pf in the drinking water of broilers, including alleviating the adverse impacts of subclinical NE infection in the birds. Despite the past studies on the effect of bromelain in animals, the understanding of the effectiveness of this additive in poultry is still limited, especially with intestinal disorder conditions, such as NE infection. Therefore, the impact and efficacy of bromelain is worthy of further investigation in relation to nutrient digestibility, favorable microbiome homeostasis and intestinal morphology changes, as well as beneficial immune responses. Further research is warranted to determine the administration routes and ideal doses of this product to achieve optimal outcomes on the performance and intestinal health of broiler chickens.

ACKNOWLEDGMENTS

The study was funded by Anatara Lifesciences Ltd company, QLD, Australia. The authors thank Ms Petrina Young for providing *Eimeria* spp. oocysts and Prof. Robert Moore for providing *C. perfringens* EHE-18.

DISCLOSURES

The authors declared that there are no conflicts of interest.

REFERENCES

Akit, H., N. Zainudin, N. Wahid, S. Zakaria, H. Foo, and T. Loh. 2019. Dietary bromelain progresses nutrient digestibility, digesta viscosity and intestinal villus height as well as reduces intestinal *E. coli* population of broiler chickens. Malaysian Soc. Anim. Pro. 22:1–16.

Ali, A. A., M. A. Milala, and I. A. Gulani. 2015. Antimicrobial effects of crude bromelain extracted from pineapple fruit (Ananas comosus (Linn.) Merr.). Adv. Biochem. 3:1–4.

Arefin, P., S. Habib, A. Arefin, and S. Arefin. 2020. A review of clinical uses of Bromelain and concerned purification methods to obtain its pharmacological effects efficiently. Int. J. Pharm. Res. 12:469–478.

Aviagen. 2018. Ross broiler management handbook. Aviagen Group. Accessed May 2023. https://aviagen.com/assets/Tech_Center/Ross_Broiler/Ross-BroilerHandbook2018-EN.pdf

Begum, M., H. Li, M. Hossain, and I. Kim. 2015. Dietary bromelain-C. 3.4. 22.32 supplementation improves performance and gut health in sows and piglets. Livest. Sci. 180:177–182.

Castell, J. V., G. Friedrich, C. S. Kuhnand, and G. E. Poppe. 1997. Intestinal absorption of undegraded proteins in men: presence of bromelain in plasma after oral intake. Am. J. Physiol. 273:139–146.

Chakraborty, A. J., S. Mitra, T. E. Tallei, A. M. Tareq, F. Nainu, D. Cicia, K. Dhama, T. B. Emran, J. Simal-Gandara, and R. Capasso. 2021. Bromelain a potential bioactive compound: a comprehensive overview from a pharmacological perspective. Life 11:317.

Chandler, D., and T. Mynott. 1998. Bromelain protects piglets from diarrhoea caused by oral challenge with K88 positive enterotoxigenic Escherichia coli. Gut 43:196–202.

Chobotova, K., A. B. Vernallis, and F. A. A. Majid. 2010. Bromelain's activity and potential as an anti-cancer agent: current evidence and perspectives. Cancer letters 290:148–156.

Daiba, A. R., J. M. Kagira, M. Ngotho, J. Kimotho, and N. Maina. 2022. In vitro anticoccidial activity of nanoencapsulated bromelain against Eimeria spp. oocysts isolated from goats in Kenya. Vet World 15:397.

Daiba, A. R., N. Maina, K. J. Maina, K. James, A. I. Youssouf, and M. Naomi. 2023. Assessment of anticoccidial efficacy of chitosan nanoencapsulated bromelain against coccidia in naturally infected goats in Kenya. African J. Biotechnol. 22:19–25.

Dosković, V., S. Bogosavljević-Bosković, Z. Pavlovski, B. Milošević, Z. Škrbić, S. Rakonjac S, et al. 2013. Enzymes in broiler diets with special reference to protease. World's Poult. Sci. J. 69:343–360.

Feijoo-Siota, L., and T. G. Villa. 2011. Native and biotechnologically engineered plant proteases with industrial applications. Food Bioproc. Tech. 4:1066–1088.

Freitas, D., S. Vieira, C. Angel, A. Favero, and A. Maiorka. 2011. Performance and nutrient utilization of broilers fed diets supplemented with a novel mono-component protease. J. App. Poult. Res. 20:322–334.

Hale, L. P. 2004. Proteolytic activity and immunogenicity of oral bromelain within the gastrointestinal tract of mice. Int. Immunopharmacol. 4:255–264.

Hale, L. P., P. K. Greer, C. T. Trinh, and C. L. James. 2005. Proteinase activity and stability of natural bromelain preparations. Int. Immunopharmacol. 5:783–793.

Hossain, M., S. Lee, and I. Kim. 2015. Effects of bromelain supplementation on growth performance, nutrient digestibility, blood profiles, faecal microbial shedding, faecal score and faecal noxious gas emission in weanling pigs. Vet. Med. (Praha) 60:544–552.

Hu, W., A.-M. Wang, S.-Y. Wu, B. Zhang, S. Liu, Y.-B. Gou, and J.-M. Wang. 2011. Debriding effect of bromelain on firearm wounds in pigs. J. Trauma Acute Care Surg. 71:966–972.

Immerseel, F. V., J. D. Buck, F. Pasmans, G. Huyghebaert, F. Haesebrouck, and R. Ducatelle. 2004. *Clostridium perfringens* in poultry: an emerging threat for animal and public health. Avian Pathol 33:537–549.

Jayaraman, S., G. Thangavel, H. Kurian, R. Mani, R. Mukkalil, and H. Chirakkal. 2013. Bacillus subtilis PB6 improves intestinal health of broiler chickens challenged with *Clostridium perfringens*-induced necrotic enteritis. Poult. Sci. 92:370–374.

Juasook, A., W. Aengwanich, T. Chalalai, N. Watwiengkam, T. Asawapattanakul, and W. Promsud. 2017. Changes in sporulation, packed cell volume, malondialdehyde level, faecal oocyst count and histopathology of *Eimeria tenella*-infected broilers treated with pineapple (Ananas comosus) crude extracts. Int. J. Poult. Sci. 16:189–195.

Keyburn, A. L., J. D. Boyce, P. Vaz, T. L. Bannam, M. E. Ford, D. Parker, A. Di Rubbo, R. I. Rood, and R. J. Moore. 2008. NetB, a new toxin that is associated with avian necrotic enteritis caused by *Clostridium perfringens*. PLoS Pathog 4:1–11.

Keyburn, A. L., S. A. Sheedy, M. E. Ford, M. M. Williamson, M. M. Awad, J. I. Rood, and R. J. Moore. 2006. Alpha-toxin of *Clostridium perfringens* is not an essential virulence factor in necrotic enteritis in chickens. Infect Immun 74:6496–6500.

Lin, Y., and O. A. Olukosi. 2021. Exogenous enzymes influenced eimeria-induced changes in caecal fermentation profile and gene expression of nutrient transporters in broiler chickens. Animals 11:2698.

Loon, Y. K., M. H. Satari, and W. Dewi. 2018. Antibacterial effect of pineapple (Ananas comosus) extract towards *Staphylococcus aureus*. Padjadjaran J. Dentist 30:1–6.

Mazorra-Manzano, M., J. Ramírez-Suarez, and R. Yada. 2018. Plant proteases for bioactive peptides release: a review. Crit. Rev. Food Sci. Nutr. 58:2147–2163.

Mynott, T., R. Luke, and D. Chandler. 1996. Oral administration of protease inhibits enterotoxigenic Escherichia coli receptor activity in piglet small intestine. Gut 38:28–32.

Nguyen, D. H., S. I. Lee, J. Y. Cheong, and I. H. Kim. 2018. Influence of low-protein diets and protease and bromelain supplementation on growth performance, nutrient digestibility, blood urine nitrogen, creatinine, and faecal noxious gas in growing−finishing pigs. Canadian J. Anim. Sci. 98:488–497.

NHMRC. 2013. Australian code for the care and use of animals for scientific purposes. Natl. Health Med. Res.. . 8th rev. ed. Council Press, Canberra.

Pavan, R., S. Jain, and K. Shraddha. 2012. Properties and therapeutic application of bromelain: a review. Biotechnol. Res. Int. 2012:976203.

Peek, H., J. Van der Klis, B. Vermeulen, and W. Landman. 2009. Dietary protease can alleviate negative effects of a coccidiosis infection on production performance in broiler chickens. Anim. Feed Sci. Technol. 150:151–159.

Praveen, N., A. Rajesh, M. Madan, V. R. Chaurasia, N. V. Hiremath, and A. M. Sharma. 2014. In vitro evaluation of antibacterial efficacy of pineapple extract (bromelain) on periodontal pathogens. J. Int. Oral Health 6:96–98.

Qaisrani, S., M. Van Krimpen, R. Kwakkel, M. Verstegen, and W. Hendriks. 2015. Dietary factors affecting hindgut protein fermentation in broilers: a review. World's Poult. Sci. J. 71:139–160.

Sahbaz, A., O. Aynioglu, H. Isik, U. Ozmen, O. Cengil, B. D. Gun, and K. Gungorduk. 2015. Bromelain: a natural proteolytic for intra-abdominal adhesion prevention. Int. J. Surg. 14:7–11.

Shojadoost, B., A. R. Vince, and J. F. Prescott. 2012. The successful experimental induction of necrotic enteritis in chickens by *Clostridium perfringens*: a critical review. Vet. Res. 43:1–12.

Stanley, D., S.-B. Wu, N. Rodgers, R. A. Swick, and R. J. Moore. 2014. Differential responses of cecal microbiota to fishmeal, *Eimeria* and *Clostridium perfringens* in a necrotic enteritis challenge model in chickens. PLoS One 9:e104739.

Stepek, G., A. Lowe, D. J. Buttle, I. Duce, and J. M. Behnke. 2006. In vitro and in vivo anthelmintic efficacy of plant cysteine proteinases against the rodent gastrointestinal nematode, *Trichuris muris*. Parasitology 132:681–689.

Van Doan, H., S. H. Hoseinifar, R. Harikrishnan, T. Khamlor, M. Punyatong, W. Tapingkae, et al. 2021. Impacts of pineapple peel powder on growth performance, innate immunity, disease resistance, and relative immune gene expression of Nile tilapia, *Oreochromis niloticus*. Fish Shellfish Immunol. 114:311–319.

Whelan, R. A., K. Doranalli, T. Rinttilä, K. Vienola, G. Jurgens, and J. Apajalahti. 2019. The impact of Bacillus subtilis DSM 32315 on the pathology, performance, and intestinal microbiome of broiler chickens in a necrotic enteritis challenge. Poult. Sci. 98:3450–3463.

Wilkie, D. C., A. G. Van Kessel, L. J. White, B. Laarveld, and M. D. Drew. 2005. Dietary amino acids affect intestinal *Clostridium perfringens* populations in broiler chickens. Canadian J. Anim. Sci. 85:185–193.

Windisch, W., K. Schedle, C. Plitzner, and A. Kroismayr. 2008. Use of phytogenic products as feed additives for swine and poultry. J. Anim. Sci. 86:E140–E148.

Wise, M. G., and G. R. Siragusa. 2007. Quantitative analysis of the intestinal bacterial community in one to three week old commercially reared broiler chickens fed conventional or antibiotic free vegetable based diets. J. Appl. Microbiol. 102:1138–1149.

Wiszniewski, G., S. Jarmołowicz, M. S. Hassaan, E. Y. Mohammady, M. R. Soaudy, J. Łuczyńska, E. Tońska, E. Terech-Majewska, T. Ostaszewska, and M. Kamaszewski. 2019. The use of bromelain as a feed additive in fish diets: growth performance, intestinal morphology, digestive enzyme and immune response of juvenile Sterlet (*Acipenser ruthenus*). Aquacult. Nutr. 25:1289–1299.

Wu, S.-B., D. Stanley, N. Rodgers, R. A. Swick, and R. J. Moore. 2014. Two necrotic enteritis predisposing factors, dietary fishmeal and *Eimeria* infection, induce large changes in the caecal microbiota of broiler chickens. Vet. Microbiol. 169:188–197.

Yenice, G., H. İskender, E. Dokumacioglu, O. Kaynar, A. Kaya, A. Hayirli, and G. Sezmis. 2019. Dietary bromelain supplementation for improving laying performance, egg quality and antioxidant status. Eur. Poult. Sci. 83:1–16.

Yin, D., X. Yin, X. Wang, Z. Lei, M. Wang, Y. Guo, S. E. Aggrey, W. Nie, and J. Yuan. 2018. Supplementation of amylase combined with glucoamylase or protease changes intestinal microbiota diversity and benefits for broilers fed a diet of newly harvested corn. J. Anim. Sci. Biotechnol. 9:1–13.