PAPER

Efficacy test of a hydrolysable tannin extract against necrotic enteritis in challenged broiler chickens

Giovanni Tosi,[1] Paola Massi,[1] Mauro Antongiovanni,[2] Arianna Buccioni,[2] Sara Minieri,[2] Luigi Marenchino,[3] Marcello Mele[4]

[1]Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna, Forlì (FC), Italy
[2]Dipartimento di Scienze delle Produzioni Agroalimentari e dell'Ambiente, Università di Firenze, Italy
[3]Gruppo Mauro Saviola Srl, Viadana (MN), Italy
[4]Dipartimento di Scienze Agrarie, Alimentari e Agro-Ambientali, Università di Pisa, Italy

Abstract

A hydrolysable tannin extracted from chestnut (SaviotaN®) was tested for efficacy in controlling the proliferation of *Clostridium perfringens* in the gut of broiler chickens challenged via oral gavage first with coccidia (*Eimeria tenella, Eimeria acervulina, Eimeria maxima)* at the age of 10 days, and then with *Clostridium perfringens* at the age of 15 days. We randomly allocated 150 broiler chickens within 5 poultry isolators (30 birds each). Dietary treatments consisted of a basal diet (C) composed of corn [575 g/kg on dry matter (DM)] and soybean meal (100 g/kg DM), barley bran (220 g/kg DM), corn gluten feed (30 g/kg DM), soybean oil (25 g/kg DM), vitamin mineral premix (49.5 g/kg DM), and four other diets obtained by adding chestnut tannin extract (1.5, 3, 5, and 12 g/kg during week 1, 10.0 g/kg during week 2, and 8.0 g/kg during the last two weeks, respectively) to C. At the age of 20 days, 15 birds/group were euthanised and individually examined for the level of gut infection by counting *Clostridium perfringens* and macroscopic gut lesions. Results demonstrated that chestnut tannin gave significant results even at low concentration levels in the feed (1.5 to 3.0 g/kg), but was actually efficient in controlling necrotic enteritis at levels ≥5.0 g/kg. The treatment (12.0 g/kg during the first week and 8.0 g/kg during the last two weeks of age) resulted very efficient in controlling the proliferation of *Clostridium perfringens* and in reducing the severity of gut damage compared to the untreated infected group.

Introduction

Necrotic enteritis is one of the world's most prominent and severe diseases in chickens and, in particular, in broiler chickens (McDevitt *et al.*, 2006). The disease is responsible for high mortality rates and for a significant depression of performance parameters. The disease usually occurs in broiler chickens of 2 to 6 weeks of age and is caused by the overgrowth of *Clostridium perfringens* (type A and, to a lesser extent, type C) in the small intestine and by the production of extracellular toxins damaging the intestine. Although *Clostridium perfringens* is recognised as the etiologic agent of necrotic enteritis (Elwinger *et al*., 1992), other co-factors are usually required to precipitate an outbreak, including environment, climate, management of hygiene and diet. In commercial production, coccidiosis is another important predisposing factor for triggering outbreaks of necrotic enteritis. Experimental induction of intestinal damage to cause necrotic enteritis in broilers has been successfully accomplished by co-infection with *Eimeria* spp. (Persia *et al*., 2006).

In many countries, necrotic enteritis is controlled by the use of antibiotics in feed or drinking water. The European Union has enforced a ban on the use of in-feed antibiotics and consumer pressure may force similar restrictions on antibiotic use. Therefore, alternative strategies for the control of necrotic enteritis are needed to limit the economic impact of the disease. Possible alternatives include probiotics, prebiotics, organic acids and products extracted form plants. In human medicine, plants are known to be a source of bioactive compounds useful as alternative to drugs, but the applications in veterinary medicine are very limited. Tannins are a complex mixture of polyphenolic compounds characterised by a high variability in molecular structure. In particular, hydrolysable tannins (HT) are characterised by the presence of a core of glucose esterified with gallic and hexahydroxydiphenic acids. Tannins extracted from chestnut wood (*Castanea sativa Miller)*, commonly found in the central Mediterranean area, are an example of HT. In literature, a considerable number of publications demonstrated the antinutritional effects of tannins in poultry feeding, inducing a worsening of productive performances as a consequence of a decrease in organic matter digestibility, especially for the protein component (Chang and Fuller, 1964; Ahmed *et al.*, 1991; Garcia *et al.*, 2004; Barroga *et al.*, 1985; Longstaff and McNab, 1991a, 1991b). In contrast, it is evident from several *in vitro* studies that the microorganisms in the gastro-intestinal tract are strongly sensible to the presence of HT and that tannins from chestnut wood are efficient against coccidiosis and necrotic enteritis in poultry (Elizondo *et al.*, 2010). Hence, it seems very interesting to test *in vivo* the efficacy of this kind of compounds as protective agents to control the intestinal diseases produced by the most common bacteria and parasites of chickens.

The aim of the present trial was the evaluation of the efficacy of an industrial HT, extracted from chestnut wood (commercial name SaviotaN®; Gruppo Mauro Saviola s.r.l., Viadana, Italy), in controlling necrotic enteritis and multiplication of *Clostridium perfringens* in the gut of broiler chickens. The study was designed using *Eimeria* spp. infection as experimental model to produce clinical infection of *Clostridium perfringens* in experimentally challenged broiler chickens.

Corresponding author: Prof. Marcello Mele, Dipartimento di Scienze Agrarie, Alimentari e Agro-Ambientali, Università di Pisa, , via del Borghetto 80 56124, Pisa, Italy.
Tel. +39.050.2218938 - Fax: +39.050.2218970.
E-mail: marcello.mele@unipi.it

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Materials and methods

Animals

The broiler chickens used in this study were female Ross 308 chicks, purchased from a local hatchery. The chicks were vaccinated against Marek disease in the hatchery, but not against coccidiosis. One hundred and fifty birds at one day of age were allocated randomly within

poultry isolators (Allentown®; Allentown, NJ, USA) equipped with air filtration systems both inward and outward. The poultry isolators were 5, with 30 birds each.

Diets

Feed and drinking water were administered *ad libitum* throughout the whole trial by means of hopper feeders and bell-shaped drinkers.

Dietary treatments, formulated according to animal requirement (National Research Council, 1994), consisted of a basal diet [control (C)] composed by corn meal [575 g/kg on dry matter (DM)], soybean meal (100 g/kg DM), barley bran (220 g/kg DM), corn gluten feed (30 g/kg DM), soybean oil (25 g/kg DM) vitamin mineral premix (49.5 g/kg DM with 10 g/kg of Lysine and 3 g/kg of Methionine), and of four other diets obtained by adding to C the chestnut tannin extract (CE) SaviotaN® (equivalent to 570 g of gallic acid/kg DM) in different doses:

CE1.5: with 1.5 g/kg SaviotaN®, throughout the whole trial;

CE3: with 3.0 g/kg SaviotaN®, throughout the whole trial;

CE5: with 5.0 g/kg SaviotaN®, throughout the whole trial;

CE12: with 12.0 g/kg SaviotaN® during the first week, 10.0 g/kg during the second week, and 8.0 g/kg further on for the last two weeks.

The chemical characterisation of CE (SaviotaN®) is reported by Romani *et al.* (2012).

Proximate analysis of feed samples

Samples of C were oven dried at 60°C for 24 h. The dry samples were analysed for crude protein (CP), ash, ether extract (EE) and crude fibre (CF), according to the 954.01, 954.05, 920.39 and 962.09 procedures of AOAC (1990), respectively. The chemical composition of the control diet was: DM, 895; CP, 205; EE, 55; CF, 45; and ash, 75 g/kg. All chemical fractions are expressed on DM basis. The value of gallic acid equivalents in the extract powder was determined according to Romani *et al.* (2012).

Experimental challenge

At the age of 10 days all the animals were challenged via oral gavage with low dosages of coccidia (3000 oocysts/bird of a mixture of *Eimeria tenella, Eimeria acervulina* and *Eimeria maxima* in 1 mL of phosphate-buffered saline). The three *Eimeria* spp. had been isolated from clinical cases of coccidiosis in unvaccinated broiler flocks. Oocysts were recovered from the intestinal contents of affected birds using saturated NaCl solution and centrifugation at 2000 rpm for 10 min. Collected oocysts were washed with distilled water, centrifugated and sporulated at 28-30°C for 48-72 h with forced aeration using a pump. Finally, the sporulated oocysts were resuspended in 2% (w/v) potassium dichromate solution and stored at 4°C. Before inoculation, the potassium dichromate solution was removed by washing and repeated centrifugations in water. The number of oocysts in the suspension was calculated using a McMaster's chamber and the final concentration of oocysts was obtained adjusting the volume of the suspension by addition of water.

At the age of 15 days all the birds were challenged via oral gavage with 10^7 cfu/bird of a toxigenic strain of *Clostridium perfringens* type A (ATCC® 13124; LGC Standards-ATCC, Teddington, UK). All birds were examined twice daily and dead chickens were immediately collected for *post-mortem* analysis.

At the age of 20 days, 15 birds per group were euthanised and individually examined for the level of gut infection by means of the count of *Clostridium perfringens* (expressed as cfu/g of gut content and converted to $\log_{10}$ to be processed) and for macroscopic gut lesions. A section of the small intestine about 10 cm in length, cranial to Meckel's diverticulum, was removed. Fresh digesta samples (1 g) were transferred into sterile plastic bags containing 10 mL of buffered peptone water. The suspension was homogenised for 2 min using a stomacher laboratory blender and serially diluted in 10-fold increments in buffered peptone water; 0.1 mL of each dilution were plated on blood agar base containing 5% sheep blood and 100 mg/L neomycin for the enumeration of microbial populations. All plates were incubated anaerobically at 37°C for 48 h. Alfa and beta-hemolytic colonies, microscopically confirmed as gram-negative rods, were counted as *Clostridium perfringens*. The count of *Clostridium perfringens* was expressed as cfu/g of gut content and converted to $\log_{10}$ to be statistically processed. Macroscopic gut lesions were evaluated according to Keyburn *et al.* (2006): the intestine was opened at the infection site (duodenum and jejunum) and the degree of damage to the epithelium was determined according to the following scheme: score 0=no gross lesions; score 1 = thin or friable walls; score 2= focal necrosis (1 to 5 foci); score 3=focal necrosis (5 to 15 foci); score 4=focal necrosis (16 or more foci); score 5=patches of necrosis 2 to 3 cm long; score 6=diffuse necrosis.

The same measurements were performed on the other 15 birds euthanised at 25 days.

Statistical analysis

All experimental data have been processed for statistical significance of the effect of SaviotaN® in controlling the proliferation of *Clostridium perfringens* in the gut of the birds. Data of colony forming units (cfu) were transformed into logarithms and analysed by a two way ANalysis Of Variance (ANOVA) model including tannin supplementation (5 levels: 0, 1.5, 3.0, 5.0, and 12.0 g/kg) and days of treatments (2 levels: 20 and 25 days), with interaction, as main fixed factors. Data of lesion score were compared by the χ^2 test according to McCann *et al.* (2006) using SAS software (1999).

Results and discussion

The results of this trial suggested that chestnut tannins used as feed additives could be useful in controlling clostridial infection in broiler chickens.

In fact, the count of *Clostridium perfringens* (Table 1) decreased significantly as the level of tannin was increased in the feed, both at 20 and 25 days. Furthermore, the level of *Clostridium perfringens* increased dramatically in the last 5 days of the trial in C, C1.5 and C3.0 groups. Starting from C5.0, this trend was inverted, becoming significant with the last

Table 1. Enumeration of *Clostridium perfringens* in gut content, expressed as $\log_{10}$ of the cfu/g of intestinal content.

	Diets					
	C	CE1.5	CE3	CE5	CE12	SEM
20 days	5.73^{A*}	5.46^{AB*}	5.33^{B*}	4.41^{C}	3.75^{D*}	0.09
25 days	7.66^{A*}	6.31^{B*}	5.72^{C*}	4.15^{D}	3.24^{E*}	0.15

C, control; CE1.5, basal diet with 1.5 g/kg SaviotaN®; CE3, basal diet with 3.0 g/kg SaviotaN®; CE5, basal diet with 5.0 g/kg SaviotaN®; CE12, basal diet with 12.0 g/kg SaviotaN® during the first week, 10.0 g/kg during the second week, and 8.0 g/kg for the last two weeks. $^{A-E}$Means with different letters within the same row are statistically different ($P<0.01$); means with asterisks within the same column are statistically different ($P<0.01$).

Table 2. Lesion score response to SaviotaN® treatment (number of birds).

	Negative (score 0-1)	Positive (score 2-3)	Positive (score 4-6)
C, 20 days	0	9	6
C, 25 days	0	8	7
CE1.5, 20 days	0	13	2
CE1.5, 25 days	0	6	9
CE3, 20 days	0	13	2
CE3, 25 days	0	10	5
CE5, 20 days	4	11	0
CE5, 25 days	2	13	0
CE12, 20 days	8	7	0
CE12, 25 days	10	5	0

C, control; CE1.5, basal diet with 1.5 g/kg SaviotaN®; CE3, basal diet with 3.0 g/kg SaviotaN®; CE5, basal diet with 5.0 g/kg SaviotaN®; CE12, basal diet with 12.0 g/kg SaviotaN® during the first week, 10.0 g/kg during the second week, and 8.0 g/kg for the last two weeks.

Table 3. Mean values of lesion scores treated with Student's *t*-test.

	Diets					
	C	CE1.5	CE3	CE5	CE12	SEM
20 days	3.13^{A*}	2.87^{A**}	2.73^{Aa}	2.00^{Bb}	1.40^{Bc}	0.30
25 days	3.93^{Aa*}	3.53^{A**}	3.13^{Ab}	2.13^{B}	1.33^{C}	0.36

C, control; CE1.5, basal diet with 1.5 g/kg SaviotaN®; CE3, basal diet with 3.0 g/kg SaviotaN®; CE5, basal diet with 5.0 g/kg SaviotaN®; CE12, basal diet with 12.0 g/kg SaviotaN® during the first week, 10.0 g/kg during the second week, and 8.0 g/kg for the last two weeks. ^{a,b}Means with different letters within the same row are statistically different at P<0.05. ^{A,B}Means with different letters within the same row are statistically different at P<0.01. Means with asterisks within the same column are statistically different (*P<0.05; **P<0.01).

group. Our data agree with those of Elizondo *et al.* (2010), who in an *in vitro* trial, studied the effectiveness of CE on the growth of *Clostridium perfringens*. In fact, in our trial the growth of *Clostridium perfringens* in the gut of challenged broiler chickens was reduced in a dose-dependent manner in presence of this kind of CE. Elizondo *et al.* (2010) found that chestnut tannins are characterised by antimicrobial activity higher than that of quebracho; these authors showed, moreover, that quebracho tannins are able to induce a decrease in the antimicrobial capacity of chestnut tannin if mixed with CE. These properties are linked to the ability of tannins to inhibit alpha and epsilon toxins produced by bacteria (Elizondo *et al.*, 2010; Buzzini *et al.*, 2008). In contrast, the mechanism of action against coccidia is not well known. Mansoori and Modirsanei (2012) supposed that tannic acid is able to inhibit several enzymes responsible for life cycle of parasites and that tannins may inhibit the assumption of nutrients, essential for the growth of parasites (Min and Hart, 2003). Unlike what found for *Clostridium* spp., the ability of HT to penetrate the cell membrane of *Eimeria* spp. at different stages of development causing the loss of intracellular components accompanied to a damage of cytoplasm was not demonstrated (Mc Sweeney *et al.*, 2001).

Lupini *et al.* (2009) demonstrated antiviral activities of CE against avian reovirus and avian metapneumovirus replication because in samples containing CE a reduction of the viral cytopathic effect (CPE) was induced about 80%. The evaluation of lesion score, found in this trial, put in evidence that birds classified with score 0 and 1 were absent in the groups treated with less than 5 g/kg of tannin, but were 10 out of 15 in the last group at 25 days (Table 2). The lightly affected ones, scored 2 and 3, were greatly represented in the medium tannin groups, while the highly affected chickens, scored 4 to 6, were absent in the treated groups 5 g/kg and upward. Thus, the concentration of 5 g/kg appears the border level to be adopted to protect broiler chickens from necrotic enteritis.

Lesion scores figures were treated with the Student's *t*-test as well (Table 3). Even though this is not completely correct, it may be useful to confirm that the response of the chickens to tannin is level-dependent and indicates that it protects the animals in the last 5 days, from 0.5% upward, unlike the lower levels, as highlighted by the count of *Clostridium perfringens* cfu/g (Table 1).

The health situation of the birds at day 25 resulted worse than that recorded 5 days earlier, with the exception of the two CE5 and, better, CE12, with highly significant differences. When increasing the concentration from 3 up to 12 g/kg and comparing 20 to 25 days, an inversion of trend can be observed: the health conditions of the birds worsened severely and significantly in the first two groups, but were decidedly better in CE3 upward. In any case, the beneficial effect of tannin started to be significantly detectable even at the level of 3 g/kg.

In literature, controversial observations are reported about the adverse effects of tannins in chicken welfare, but information prevalently regards condensed tannins (CTs). In fact, several authors reported that CT in chickens are able to damage the intestinal mucosa binding themselves to intestinal lumen and causing shortening, distortion and atrophy of villi with a proliferation of mucous secreting goblet (Waghorn, 1996; Ortiz *et al.*, 1994). Other authors, in contrast, demonstrated that CT from mimosa, quebracho or grape seed are characterised by an interesting efficacy as antimicrobial agents on gut microflora, thus to be used as potential alternative to antibiotics (Shanmugavelu *et al.*, 2006; Harborne, 2001; Wink, 2004). Nevertheless, few data are reported about HT effect on poultry growth performance (Min and Hart, 2003).

Recently, Schiavone *et al.* (2008) showed that the use of CE in poultry feeding does not influence feed digestibility, carcass quality and nitrogen (N) balance; in fact, it has a positive influence on growth performance if included in the diet up to 2 g/kg (on DM).

Conclusions

During the last decades, public awareness of environmental pollution associated with the use of antibiotics has been publicised with the aim to find alternatives to drugs in animal husbandry, increasing the use of bioactive plant extracts to prevent several animal diseases not only in organic but also in conventional livestock. The results of the present trial are to be considered very interesting from the point of view of prevention and, possibly, therapy of necrotic enteritis in broilers in replacement for the chemicals recently banned in Europe as preventing factor. Chestnut tannin extract may help to control pathogen colonisation of the chicken gut without the development of bacteria resistance that commonly occurs when synthetic antimicrobial growth promoters are used. Due to the complexity of poultry digestive tract, further investigations are required to discover the mode of action against bacterial proliferation.

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