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Effect of tannins on the *in vitro* growth of *Clostridium perfringens*

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ABSTRACT

Vegetable tannins are water-soluble polyphenolic compounds of varying molecular weights that occur abundantly in nature. The diet of many free-ranging wild animals contains significant amounts of tannins. Also, commercial tannins are used in animal industry as food additives to improve animal performance. In order to further determine the capacity of tannins to inhibit the development of intestinal diseases produced by *Clostridium pefringens*, we evaluated here the effect of tannins from quebracho, chestnut or combinations of both on *C. perfringens* and their toxins. The *C. perfringens* (types A, B, C, D and E) growth obtained from the intestine of healthy and diseased animals was reduced in a dose-dependent manner in the presence of quebracho tannins, chestnut tannins, combinations of both or a commercial formula based in these tannins. Although the minimal inhibitory concentration of both tannins varied between isolates, no statistically significant differences were observed between isolates from healthy or sick animals. Comparative analysis showed that the concentrations of quebracho tannin inhibiting the growth of *C. perfringens* were higher than chestnut tannin. In fact, antibacterial effect of quebracho tannin was increased up to 20 times with the addition of 25% of chestnut tannin and 85 times with 75% of chestnut tannin. Antibacterial activity of the commercial product was up to ~50 times higher than quebracho tannin alone. Quebracho tannin showed partial bactericidal activity, whereas chestnut tannin activity was stronger. Both tannins were able to reduce the alpha toxin lecithinase activity and epsilon toxin cytotoxicity in MDCK cells. These results suggest that tannin-supplemented diet could be useful to prevent some clostridial diseases.

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1. Introduction

Vegetable tannins are water-soluble polyphenolic compounds of varying molecular weights abundantly found in nature and have the ability to precipitate proteins (Spencer et al., 1988). Tannins can be classified into condensed and hydrolysable (Haslam, 1996, Scalbert, 1991) and differ in their nutritional significance and toxic effects (Reed, 1995). Condensed tannins are not readily degraded in the gut, while hydrolysable tannins undergo microbial and acid hydrolysis with the release of simpler phenolics (Schneider and Blaut, 2000).

Current scientific evidence suggests that there is significant potential in the use of tannins to enhance animal health in general and that of ruminants such as cattle, deer and sheep in particular. Low to moderate tannin concentrations may improve the digestive utilization on feeding, mainly due to a reduction in protein degradation in the rumen and a subsequent increase in amino acid flow to the small intestine (Hagerman et al., 1992; Tabacco et al., 2006). These effects on nutrition are reflected in a better animal performance.

Chestnut (*Castanea sativa*; hydrolysable tannins) and quebracho (*Schinopsis lorentzii*, condensed tannins) are some of the most common tannins available commercially.

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Due to their beneficial action upon animal performance, these tannins are used as commercial feed additives in ruminants. In order to further determine the capacity of tannins added to ruminant feeding to inhibit the development of intestinal diseases produced by *Clostridium pefringens*, we evaluated the effect of tannins from quebracho, chestnut and combinations of both on the *in vitro* growth of different strains of *C. perfringens* and the activity of their toxins.

2. Materials and methods

2.1. Vegetal extracts

Commercially available chestnut tannins (80% hydrolysable tannins), quebracho tannins (75% condensed tannins) and a commercial feed additive based on these tannins (ByPro®) were tested (supplied by Silvateam & Cecil S.A., Argentina).

2.2. Bacterial cultures

C. perfringens strains of the toxinotypes A, B, C, D and E were randomly selected from a collection of isolates obtained from the faeces of healthy animals and the intestine of diseased animals. One type A strain was selected from an animal with gas gangrene. Stock cultures of *C. perfringens* isolates were prepared in cooked meat medium (CMM) (Difco Laboratories, Detroit, MI) and were stored at −20 °C. Prior to their use in experiments, bacteria were initially cultured overnight at 37 °C in CMM. To ensure culture purity, samples of overnight CMM cultures were streaked on Blood agar base (Oxoid Ltd., Basingstoke, UK) with 5% sheep blood (BAP), which was incubated overnight at 37 °C under anaerobic conditions. One colony of each strain was grown overnight at 37 °C under anaerobic conditions in Brain and Hearth Infusion (BHI) broth (Oxoid Ltd., Basingstoke, UK) and then diluted 1:10 mixed (1 ml) with fresh pre-reduced BHI (10 ml per strain) before their use in the experiments.

2.3. Determination of minimal inhibitory concentration

Minimum inhibitory concentrations (MICs) were determined using a micro-broth dilution assay. Sterile 96-well microplates U-bottom with well capacities of 500 μl were used (Cell Star, Greiner Bio-one, Germany) and 100 μl of fresh pre-reduced BHI broth was added to each well of the plate except for the first column. Tannins were solubilized in BHI and filtered through 0.22 μm filters (Millipore, Bedford, MA). Samples were diluted to twice the desired initial test concentration with BHI. All wells, except the first, were filled with BHI (100 μl). Two hundred microliters of the tannin stock solution were added to each well of the first column using a multi-channel pipettor (Eppendorf AG, Germany). Then 100 μl of the stock solution were removed from the first column and mixed five times with the broth in the corresponding wells of the next column. Subsequently, this doubling dilution was performed in rows across the plate except the last column that was kept for use as control. The dilution procedure resulted in a gradient of tannin concentration from 0 to 10 mg/ml across the plate. Overnight cultures of bacteria grown in BHI were inoculated in each well of the plate (10 μl per well). The microtitre plate was incubated in an atmosphere of 80% N_2 10% H_2 10% CO_2 in an anaerobic jar (Oxoid Ltd., Basingstoke, UK) at 37 °C overnight.

Bacterial growth was determined by the change in absorbance after reading the microplates at 600 nm (OD_{600}) in a spectrophotometer reader (Labsystems, Helsinki) and compared with visual observation. MICs were defined as the lowest tannin concentration that prevented growth in the triplicate wells. The determinations were repeated 3 times and results were expressed as average values.

2.4. Bacterial growth in the presence of tannins

The effect of tannins on the bacterial growth was determined by adding different tannin concentrations to *in vitro* cultures of *C. perfringens*. Overnight-grown *C. perfringens* isolates were inoculated (0.2 ml) into tubes containing 10 ml of BHI broth supplemented with tannins. For comparative analysis of quebracho and chestnut tannins, *C. perfringens* type A (isolated from a healthy calf) or D (isolated from an ovine with enterotoxaemia) were cultured with 0.03, 0.12, 0.5, 2, and 8 mg/ml of tannins. Tubes were cultured anaerobically under N_2–H_2–CO_2 gas at 37 °C and growth was determined via measurement of optical density (OD_{600}) at 120 min intervals in a Cecil CE2021 model spectrophotometer. Uninoculated tubes, receiving equivalent concentrations of tannins, served as blanks for the subtraction of background turbidity caused by tannin–protein interactions. Specific growth rate was calculated as the maximum specific growth rate achieved by each culture, while OD_{max} was the maximum OD achieved by each culture (2 replicates per treatment). The specific growth rate was calculated from consecutive OD_{600} measurements ($\mu = \Delta \ln OD_{600}/\Delta t$, where t is time).

2.5. In vitro bactericidal analyses

A single isolated colony from a BAP streaked with *C. perfringens* isolates A or D was inoculated into 10 ml of BHI broth, which was then incubated overnight at 37 °C. A 0.1-ml aliquot of each overnight culture was then inoculated into 10 ml of BHI and cultured at 37 °C until stationary phase (8 h). At this moment, 2 ml of filtered phosphate buffered saline (PBS; pH 7.2, 0.01 M) with or without 20 or 10 mg of quebracho or chestnut tannins were added to 2 ml of the cultures. Isolates were incubated in this medium up to 18 h under anaerobiosis. Samples taken from cultures at different time points were 10-fold serially diluted in sterile PBS. Dilutions were plated (0.1 ml) on BAP and incubated overnight at 37 °C. Colonies that grew on agar plates after 18 h incubation were directly counted. The percentage of bactericidal effect was calculated from control to 10 or 5 mg of tannins per ml at 18 h.

2.6. Antitoxic activity

Alpha-toxin-mediated lecithinase activity was assayed on the egg yolk salt agar (EYSA) as described by Rigby

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