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Hydrolyzable and condensed tannins resistance in *Clostridium perfringens*

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ABSTRACT

Tannins added in the diet are being used to improve nutrition and health in farm animals as an alternative to antibiotic growth promoters and to control enteric clostridial diseases. However, the capacity of *Clostridium perfringens* to develop resistance under the selective pressure of tannins is unknown. The purpose of this study was to determine if *C. perfringens* possess the ability to develop resistance against tannins in comparison with antimicrobial agents. Susceptibility for 7 AGPs (antimicrobial growth promoters), 9 therapeutic antimicrobials and 2 tannin based extracts was determined for 30 *C. perfringens* strains isolated from poultry and cattle. Two susceptible strains were selected and cultured in presence of sub-inhibitory concentrations of tannins and AGPs for resistant sub-populations selection. Tannin resistance of *C. perfringens* isolates from both animal species revealed no statistically significant differences in MICs (minimum inhibitory concentration). Poultry isolates showed higher MICs to several AGPs compared with cattle isolates. All isolates were susceptible to the therapeutic antimicrobials tested, but avian isolates showed a significantly lower susceptibility to these antimicrobials which was highly correlated with an increased resistance to bacitracin and others AGPs. *In-vitro* selection of resistant clones suggests that *C. perfringens* was unable to develop resistance against tannins at least compared to AGPs like bacitracin and avilamycin. Avian origin strains, which were previously exposed to antibiotics showed higher resistance, compared to cattle origin strains. These results suggest that the evolution of resistance against tannins in *C. perfringens* would be more difficult and slower than to the determined AGPs.

1. Introduction

Antimicrobial growth promoters (AGPs) in feed has been widely used since the 1950's to improve feed efficiency and animal growth through the modulation of the gut microbiota and the host's immune response [19] as well as to reduce morbidity and mortality due to clinical and/or subclinical diseases [8]. In the last years, the potential risk of generation and transmission of resistance led to the banning of the use of antibiotics as growth promoters in determined countries, although AGPs are still widely used in many others [23]. The reduction in the use of these AGPs was almost immediately followed by health problems in broiler flocks, causing high impact on *Clostridium perfringens* infections epidemiology [30].

C. perfringens is a Gram-positive, rod-shaped, anaerobic, spore-forming bacterium that is commonly found in soil, sewage and in the gastro-intestinal tract of animals and humans as a member of the normal gut microbiota. According to the current classification, *C. perfringens* isolates are divided into five types (A–E) on the basis of the production of four major toxins (alpha, beta, epsilon and iota). *C. perfringens* can cause gas gangrene and food poisoning in humans; necrotic enteritis in poultry; enterotoxemia, hemorrhagic enteritis and sudden death in cattle. Avian necrotic enteritis (NE) is

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among the most important diseases in the poultry industry [30]. Resistance of animal isolates of *C. perfringens* to several antibiotics including bacitracin, tetracycline, clindamycin, lincomycin, and erythromycin has been reported in numerous countries [11,13]. Therefore, the existing challenge is to implement new alternatives to AGPs without affecting the production performances of livestock and also preventing the increase of antimicrobial resistance.

Plant tissues are rich in a wide variety of secondary metabolites such as tannins, terpenoids, alkaloids and flavonoids, which have been found to have *in-vitro* antimicrobial properties. Some of these plant derived compounds seem to be promising candidates to replace AGPs [4,10]. Tannins are polyphenolic compounds produced by plants, ranging in concentrations from <2% to more than 20% of dry weight and may protect plants from herbivore, increase resistance against pathogens, or protect tissues such as wood against decay [24]. Tannins can be separated into two groups; hydrolyzable and condensed tannins [16] depending on their chemical structure. Previous studies have verified the antimicrobial activity of several tannins against different poultry pathogens [2,12]. *In-vitro* [7] and *in-vivo* [29] results suggest that two of the most abundant and common sources of tannins, chestnut (*Castanea sativa*; hydrolyzable tannins) and quebracho (*Schinopsis lorentzii*, condensed tannins) extracts, are effective to reduce and control infection, particularly in poultry. An additional benefit of the use of tannins as alternative to AGPs, is the hypothetical difficulty of bacteria to develop resistance against the diverse range of molecules that contain these plant compounds.

Therefore, the objective of this study was to determine if continuous exposure of *C. perfringens* to plant extracts can diminish the antimicrobial effect of tannins. In this aim, we comparatively evaluated the susceptibility of both poultry and cattle *C. perfringens* isolates to tannins and antimicrobial agents used in therapy, prophylaxis, and/or growth promotion, and challenged the concept that development of tannins resistance is difficult.

2. Materials and methods

2.1. Bacterial isolates and growth conditions

A total of 30 *C. perfringens* isolates of bovine (n = 15) and chicken (n = 15) origin were tested. The isolates were collected from fecal samples recovered from dairy and poultry farms with apparently healthy animals over the period 2012/2013. This study did not involve endangered or protected species. All samples used in this study were collected from the cages or pens which did not involve handling of animals. Birds or bovines were not sacrificed for this study. Due to above reasons, animal ethics approval was not required. Farms were randomly selected based on the willingness of producers to participate in the study and specific permission was not required for sample collection. The isolates were identified as *C. perfringens* type A by standard biochemical tests and multiplex PCR [15]. Strains were stored at −80 °C in 50% glycerol: 50% brain heart infusion. From the freezer stock, all isolates of *C. perfringens* were plated in blood agar plates and incubated overnight at 37 °C under anaerobic conditions.

2.2. Determination of minimal inhibitory concentration (MICs)

MICs were determined for avilamycin, bacitracin, virginiamycin, flavomycin, lincomycin, josamycin and enramycin. Selected antimicrobial are commonly used in poultry commercial farms as growth promoters. Also, two different commercially available tannin-based supplements were used: i) chestnut (*C. sativa*) derived tannins (80% hydrolyzable tannins) and ii) quebracho (*S. lorentzii*) derived tannins (75% condensed tannins) they are both presented as powders to be mixed with fed by producers and were supplied by Silvateam & Cecil S.A. (Argentina). MICs were estimated by broth microdilution assays as previously described [27]. Briefly, sterile 96-well microplates U-bottom with well capacities of 300 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. Two hundred microliters of the tannin and AGPs stock solution were added to each well of the first column using a multichannel 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. Overnight cultures of bacteria grown in BHI were diluted to achieve a 0.5 McFarland turbidity and inoculated in each well of the plate. The microplate was incubated in an anaerobic jar at 37 °C overnight. Bacterial growth was determined by the change in absorbance after reading the microplates at 600 nm (OD600) in a spectrophotometer reader and compared with visual observation. *C. perfringens* ATCC 13124 was included as a control with every batch tested. MICs were defined as the lowest concentration that inhibits visible growth after overnight incubation. The determinations were repeated 3 times and results were expressed as mean values.

2.3. Antimicrobial susceptibility testing by disc diffusion method

Susceptibility of selected *C. perfringens* strains for antimicrobials commonly used in veterinary and human medicine as therapeutic were determined by disc diffusion method according to the recommendations of the British Society for Antimicrobial Chemotherapy (BSAC). The antimicrobial agents tested included: ampicillin (10 μg), cephalothin (30 μg), cefuroxime (30 μg), trimetropim-sulfamethoxazole (1.25 + 23.75 μg), enrofloxacin (5 μg), tetracycline (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg) and streptomycin (10 μg) (Neo-sensitabs, Rosco Diagnóstica A/S, Denmark). *C. perfringens* ATCC 13124 was used for quality control purposes. Antibiotic resistance was determined after dilution an overnight culture of each strain in fresh BHI to achieve a 0.5 McFarland turbidity, streaked on Mueller-Hinton agar plates to which antibiotic discs were applied at previously mentioned doses. Plates were incubated at 37 °C for 24 h, under anaerobic conditions. The inhibition zone was measured for each antibiotic and resistance breakpoints were determined according to BSAC methods for antimicrobial susceptibility testing (version 10.2, May 2011).

2.4. Selection of resistant sub-populations

Susceptible strains with the lowest MICs were chosen to test if sub-inhibitory concentrations of AGPs (avilamycin and bacitracin) or tannins (chestnut and quebracho) could induce the development of resistance. Resistant clones were selected by successive subculturing (10^6 cells) in BHI broth supplemented with 0.5× MIC of each compound under anaerobic conditions at 37 °C until growth was observed. Non-supplemented BHI broth was used as control. After 20 subculture cycles, MICs were defined for each selected clone and compared with the original wild type.

2.5. Transmission electron microscopy (TEM)

TEM was employed to examine cell morphology (especially cell wall structure) of *C. perfringens* strains before and after exposure to tannins at concentrations above the previously determined MIC. For each strain, *in vitro* tannin-exposed isolates that had stable tannin MICs were used in the TEM study. TEM was carried out using standard methodology that included fixation, dehydration,