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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,

embedding, sectioning and staining of sections [26]. Stained sections were viewed in the TEM at high magnification such that the cell walls and any extracellular polysaccharide were clearly visible. Digital photomicrographs of sections at right angles to the cell walls of each culture were recorded.

2.6. Data management and statistical analyses

Statistical analyses were performed using GraphPad Prism 5.0 software. Epidemiological cut-off values were determined by visual inspection of the MIC distributions. The MIC50 is the median MIC. The MIC90 is the value for which 90% of the isolates have MICs below or equal to it and 10% of the isolates have MICs above it. Differences in resistance rates between host species were tested using Fisher's exact test. For significant results, pairwise comparisons of resistance rates between species were calculated using the 2-sided Fisher's exact test. The nonparametric Kruskal–Wallis method was used to compare MIC distributions among the isolates from different host species. Pairwise associations between antimicrobial resistances were determined using Pearson correlation coefficients and the exact Pearson chi-square test for significance; Spearman's rank order correlation was used to measure the strength of association between antimicrobials' MIC values with chi-squared tests of association to determine significance. To compare the resistance level of isolates from different host species the MAR index was calculated as described in previous works [9].

3. Results

3.1. Antimicrobial susceptibility patterns

Thirty *C. perfringens* isolates were tested for susceptibility to antimicrobials commonly used in the local poultry industry as growth promoters. Concentration range, MIC50 and MIC90 of the isolates from poultry and cattle origin are showed in Table 1. The isolates were classified as susceptible or resistant based on MIC distributions. Isolates with a MIC above the MIC50 are considered resistant. Unimodal distributions with low MICs (4–16 mg/L) were observed for enramycin, flavomycin and virginiamycin and all isolates where categorized as susceptible when MICs were below 4, 8 and 4 mg/L, respectively (Fig. 1A). The MICs of avilamycin, bacitracin and lincomycin all showed a bimodal distribution (Fig. 1B) and isolates were categorized as susceptible when MICs were below 8, 125 mg/L and 70.5 mg/L, respectively. Isolates from poultry farms showed higher MICs compared with bovine isolates to avilamycin ($P < 0.01$), bacitracin ($P < 0.05$), josamycin ($P < 0.05$) and lincomycin ($P < 0.05$) (Table 1). MICs distribution of chestnut and quebracho were multimodal and differences in MICs between isolates from different animal species were significant only for chestnut (Fig. 1C). Disc diffusion method was used to determine *C. perfringens* strains susceptibility to therapeutic antimicrobials. Inhibition zones were measured and resistance breakpoints were determined according to BSAC as mentioned before. According to these criteria all isolates were categorized as susceptible. Despite poultry isolates showed a reduced susceptibility to ampicillin and trimetoprim-sulphamethoxazole and bovine isolates were less susceptible to tetracycline.

3.2. Multi-resistance and pairwise associations between AGPs and others antimicrobials

To define and compare multi-resistance of the isolates from poultry or cattle, MAR (multiple antimicrobial resistances) indices were calculated. The MAR index for an isolate is the total number on antibiotics to which the isolate is resistant/total number of antibiotics tested. The average MAR index was 0.397 when all the isolates were considered, when separating the results by animal species, the MAR index was 0.543 for poultry isolates and 0.133 for cattle isolates. Considering all of the tested isolates, 71% and 50% were resistant to 2 and 5 or more AGPs respectively. Considering the results by animal species, 89% and 78% of the poultry isolates were considered resistant to 2 and 5 or more AGPs respectively, while 40% of the bovine isolates were considered resistant to 2 AGPs and none of the bovine isolates were resistant to 3 or more AGPs. Many significant pairwise associations were observed between MICs of different AGPs (Table 2), but their exact relevance is difficult to evaluate. Strong associations (high Spearman correlation coefficient supported by a highly significant *P*-value) between antimicrobial agents were observed, like bacitracin with avilamycin, josamycin with enramycin and lincomycin, avilamycin with josamycin, and lincomycin with josamycin. Other relatively strong associations were seen between chestnut extract and quebracho tannins (Table 2). Correlation coefficients were also determined between AGPs and therapeutic antimicrobials. Strong associations were seen between enrofloxacin with bacitracin and josamycin, and between tetracycline with lincomycin. In this analysis between AGPs and therapeutic antimicrobials, negative associations were only considered because the methods used to test susceptibility were different. No significant correlation was observed between tannins and tested AGPs or therapeutic antimicrobials (Table 2). MAR index was not calculated for therapeutic antimicrobials because all tested isolates were defined as susceptible despite the reduced susceptibility of the poultry strains.

3.3. Selection of AGPs resistant sub-populations

One *C. perfringens* isolated from cattle and one from poultry were used for selection of resistant sub-populations. Both strains

Table 1
Comparison of antimicrobial susceptibility between animal species.

	Poultry				Cattle				(P)
	Range	MIC50	MIC90	% Resistance	Range	MIC50	MIC90	% Resistance	
Avilamycin	4–512	512	512	66.7%	4	4	4	0.0%	0.0037(**)
Bacitracin	4–256	128	256	77.8%	4–16	8	16	0.0%	0.0392(*)
Enramycin	4–8	4	4.8	11.1%	4	4	4	0.0%	0.4561
Flavomycin	4–16	8	16	44.4%	8	8	8	0.0%	0.2367
Josamycin	4–64	32	38.4	11.1%	4–32	4	20.8	0.0%	0.0235(*)
Lincomycin	4–256	256	256	0.0%	4–256	8	179.2	0.0%	0.0234(*)
Virginiamycin	4	4	4	0.0%	4	4	4	0.0%	–
Chestnut	4–128	64	128	33.3%	8–512	256	409.6	60.0%	0.1746
Quebracho	32–512	256	512	22.2%	32–512	256	512	40.0%	0.4893

Antimicrobials concentrations are expressed as mg/L.
*P < 0.05, **P < 0.01.

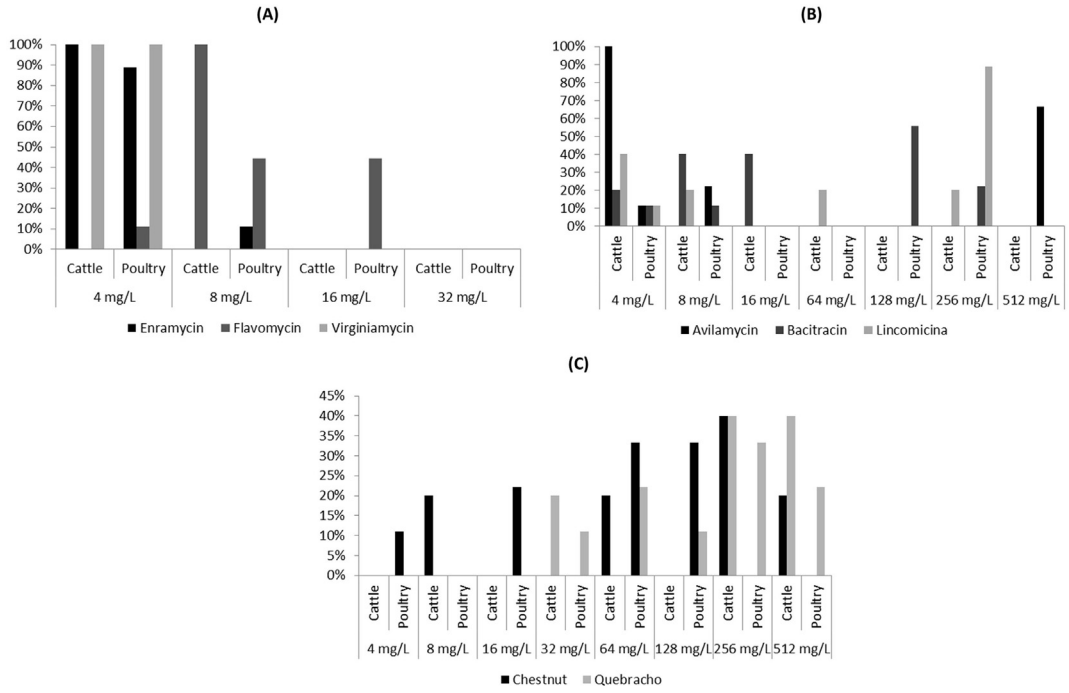

Fig. 1. Distribution of MICs of 2 tannins based extracts and 9 AGPs. (A) Unimodal distributions with low MICs were observed for virginiamycin, flavimycin and enramycin. (B) Bimodal distributions of MICs were observed for avilamycin, lincomycin and bacitracin, poultry isolates shows higher MICs compared with cattle isolates. (C) MICs distribution of chestnut and quebracho were multimodal and no significant difference was seen between isolates from cattle and poultry.

Table 2
Pairwise associations between antimicrobial minimal inhibitory concentrations (MIC).

	Avilamycin	Bacitracin	Josamycin	Lincomycin	Chestnut	Quebracho
Bacitracin	0.631	–	–	–	–	–
	0.015	–	–	–	–	–
Enramycin	–	0.534	–	–	–	–
	–	0.049	–	–	–	–
Josamycin	0.536	0.646	–	0.659	–	–
	0.048	0.012	–	0.0103	–	–
Lincomycin	–	0.552	–	–	–	–
	–	0.040	–	–	–	–
Chestnut	–	–	–	–	–	0.704
	–	–	–	–	–	0.005
Enrofloxacin	–	−0.723	−0.664	–	–	–
	–	0.0179	0.036	–	–	–
Streptomycin	–	–	–	–	–	–
	–	–	–	–	–	–
Chloramphenicol	–	–	–	–	–	–
	–	–	–	–	–	–
Tetracycline	–	–	–	0.608	–	–
	–	–	–	0.027	–	–

(–) no statistically significant association detected (P > 0.05); first number in each cell is the Spearman correlation coefficient and second is the *P* value for each for the respective specific pairwise association.

were chosen because they have the lowest MICs for the tested inhibitory compounds. Following selection for resistance, chosen sub-population of both strains displayed augmented level of resistance to avilamycin (MIC increased 2 times for bovine strain and 8 times for poultry strain after selection) and bacitracin (MIC increased 4 times for bovine and poultry strains after selection). No increased tolerances to tannins were measured in any of the colonies previously exposed to chestnut or quebracho. The results are summarized in Table 3.

3.4. Growth in the presence of tannins

Tolerance of isolates of *C. perfringens* after 20 subculture cycles with or without hydrolyzable and condensed tannins in the culture media was investigated. Since these commercial tannin extracts are not pure, sugars or other constituents may have facilitated bacterial growth. All tested strains were able to improve growth rate during the early exponential phase of growth in the presence of tannins but growth rate diminished significantly in the exponential phase compared to controls.

3.5. TEM

TEM was employed to examine the cell morphology (especially cell wall structure) of *in vitro* tannins-adapted isolates of *C. perfringens*. Bacterial cells growing in media supplemented with

Table 3
MIC of selected *C. perfringens* subpopulations previously exposed to antimicrobials compounds.

Treatment		MIC (mg/L)	
		Cattle strain	Poultry strain
Avilamycin	GT 0	4	4
	GT 400	8	32
Bacitracin	GT 0	4	8
	GT 400	16	32
Quebracho	GT 0	16	0.2
	GT 400	16	0.2
Castaño	GT 0	4	32
	GT 400	4	32

GT: generation time.

0.5× MIC were harvested. The isolates harvested at 0.5× MIC were subjected to chemical fixation prior to TEM examination. For bacteria exposed to both tannins, the TEM photographs analysis showed that there was an alteration in the thickness of the cell wall when the bacteria were exposed to hydrolyzable or condensed tannins, such changes were visible at both ×49,000 and ×9300 magnification. The observed morphology of the cell wall in control bacteria was composed of two compact laminae: a thin and intensely electro dense inner layer that lies near the cytoplasmic membrane and an electron lucen outer layer. In contrast, bacteria grown in the presence of chestnut derived o quebracho derived showed a thick electro dense layer where the above layers are not distinguished. As an example TEM photographs are shown in Fig. 2.

4. Discussion

The available information about the inhibitory activity of tannins against *C. perfringens* and other avian pathogens in addition to the experimental use in animal infection models [21] and the impact on the productive parameters in commercial farms suggest that these compounds can be an interesting alternative to AGPs [29]. However, information about the development of resistance in *C. perfringens* against tannins is not available, as it would be useful to support their use as a better alternative to AGPs. Tannins are widespread throughout the plant kingdom with diverse biological and biochemical functions such as protection against pathogenic attack from bacteria and fungi [24]. Herbivores are dependent on plants for food and have co-evolved mechanisms to obtain this food. For example ruminants that regularly browse condensed tannins contained in plants have developed tolerance to tannins, at least partly, through the presence of tannin-resistant ruminal microorganisms. Some bacteria belonging to the *Streptococcus* genus, isolated from feral goat rumen samples, showed to be resistant to tannic acid at concentrations up to 7% (w/v) and to condensed tannins up to 4% (w/v) [17,18,28]. Later these isolates were identified as *Streptococcus gallolyticus* (*Streptococcus caprinus*). In contrast, growth of the more common *Streptococcus bovis* was inhibited by tannins at concentrations 10-fold lower. Although the isolation of tannins resistant/tolerant bacteria (other than *C. perfringens*) from the rumen of grazing ruminants has been reported previously, tannin susceptibility pattern of *C. perfringens* bovine isolates revealed no statistically significant differences in MICs when compared with strains isolated from poultry. This result suggests that long term exposure of the gastrointestinal microbiota to tannins is not favoring the selection of less susceptible strains of *C. perfringens*.

Moreover, some authors claim that morphology changes could be one of the most probable resistance mechanisms in bacteria. In *S. gallolyticus*, electron microscopy studies show that cells grown with condensed tannins are interconnected by condensed extracellular material which is absent in cells grown without condensed tannins [20]. It has been hypothesized that these systems prevent cytosolic and membrane damage caused by condensed tannin fractions, although the mechanisms of resistance appear to be very complex. In *C. perfringens*, no extracellular material could be observed in electron microscopy analysis, although some morphological changes were observed in the cell surface of bacteria cultured with hydrolyzable or condensed tannins. These changes would not be associated with increased tannins resistance but possible with part of the mechanism of antibacterial activity. Changes in cell wall permeability to cations (lead citrate and uranyl acetate) seem to be a potential explanation for the changes observed in cell wall electrodensity, suggesting that hydrolyzable or condensed tannins can affect membrane synthesis or repair mechanisms [6,22,26].

The experimental selection of resistant sub-populations of *C. perfringens* suggests that development of resistance against tannins is, at least, more difficult than against AGPs like avilamycin and bacitracin in both, avian and ruminant strains. The antimicrobial activity of hydrolyzable and condensed tannin-rich extracts against *C. perfringens* has been shown to be bactericidal and bacteriostatic, respectively, suggesting that antibacterial activity of tannins in *C. perfringens* involves different mechanisms. However, hydrolyzable or condensed tannins were unable to select clones with increased resistance, particularly in cattle isolates which are regularly exposed to both types of tannins. In contrast, the regular use of avilamycin and bacitracin in commercial poultry farms could be a possible explanation for the higher drop in susceptibility against these AGPs observed in avian strains. The mechanism of decreased susceptibility to bacitracin after repeated experimental exposure of *C. perfringens* strains to AGPs could be explained by the

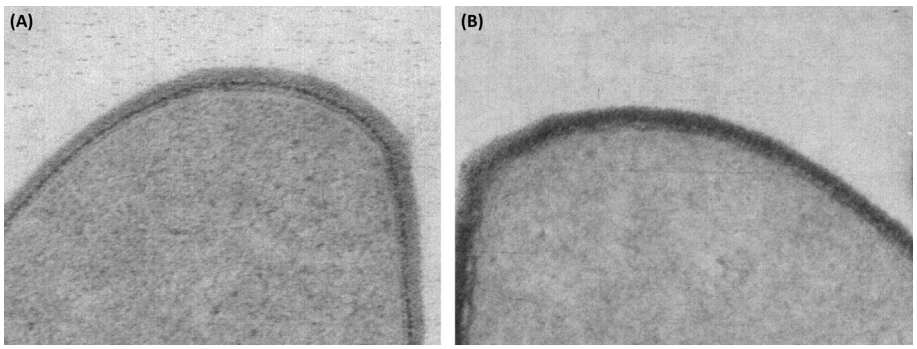

Fig. 2. TEM results at ×49,000 magnification. (A) Unexposed *C. perfringens* strain Cp088. (B) *C. perfringens* strain Cp088 cultured with 0.5× MIC of quebracho.

findings of Charlebois and colleagues [3] which identified and described a bacitracin ABC transporter and showed that the *bcrABDR* gene cluster has an inducible expression when *C. perfringens* cells are subjected to bacitracin stress.

Accordingly with other numerous reports, *C. perfringens* isolates analyzed in the present report showed a decreased susceptibility to several antimicrobial agents of practical relevance for therapy and prevention of human and animal diseases. Also, it was possible to determine that susceptibilities were not distributed evenly across the isolates, suggesting that the specific use of antimicrobials as AGPs in routinely practices decreases the susceptibility of *C. perfringens*. For example, bovine *C. perfringens* isolates showed a unimodal distribution of MIC with low values for bacitracin, whereas bimodal distributions with predominance of high MIC were observed in chicken isolates. These findings are in agreement with previously published reports on bimodal distribution of MIC values for bacitracin in poultry *C. perfringens* isolates [25,27]. However, although clear bimodal distributions of MICs were noted in all of these studies, there were slight differences in actual MIC values depending on the testing method. Decreased susceptibility to bacitracin in poultry isolates is not surprising given the frequent use of bacitracin for prevention of intestinal diseases as *C. perfringens* necrotic enteritis. In agreement with presented results, Silva [25] and Slavic [27] reported 49% and 64% resistant *C. perfringens* strains, while Johansson et al. [11] found a low rate of resistance in isolates of *C. perfringens* in Swedish and Denmark, countries in which bacitracin was no longer in use.

The result of the present report shows that all the isolates tested remain susceptible to therapeutic antimicrobials like ampicillin or enrofloxacin. However, poultry isolates show a decrease in sensitivity to these antimicrobials which was highly correlated with an increased resistance to bacitracin or others AGPs. Traditionally, a specific antimicrobial was selected as growth promoter if it lacks of analogue compounds or if it has low use in human medicine, assuming there is no cross-resistance with other antibiotics [5]. However, recent field investigations have shown that animal husbandry use of antimicrobial increases the probability of domestic animal bacteria to develop resistance or cross-resistance to drugs approved for use in human medicine. Co-existence of various resistance genes in the same plasmid or transposon results in the incidental transfer of the whole group, and may explain why exposure to one antimicrobial agent may co-select bacteria that are resistant to several agents. Several examples are reported, like maintenance of glycopeptide resistance in porcine enterococci by the use of macrolides [14]. Furthermore, the non-use of this antimicrobial does not necessarily result in a decrease in resistance. Bager et al. [1] report that glycopeptide resistance in *Enterococcus* spp. from broilers decreased significantly after the ban of avoparcin, while remained in pig isolates at similar levels due to the co-selection of multiresistance plasmids by the use of tylosin.

5. Conclusions

The results presented here suggest that the antibacterial activity of tannins against *C. perfringens* could be effective during long periods of time because it would be difficult for these anaerobic Gram positive bacteria to develop resistance. Besides the benefits over productive parameters and animal health, tannins added to feed could be a better alternative to antimicrobials growth promoters as the rotation due to increased resistance would not be necessary. Further studies of tannin resistance of *C. perfringens* isolates obtained from farm animals after prolonged use of tannins as feed additives would be crucial to support *our in-vitro* findings, and to reinforce the observed difficulty of *C. perfringens* to develop resistance against tannins.

Competing interests

Some of the authors provide consulting services to companies related with poultry nutrition.

Authors' contributions

LMR, JED and MEFM participated in the design of the study. LMR, JED, BCR and EAR performed the experiments. LMR, JED and MEFM analyzed the data. LMR and MEFM collected *C. perfringens* strains. LMR, JED and MEFM wrote the paper. All authors contributed to the critical revision of the manuscript for important intellectual content and have seen and approved the final draft. All authors read and approved the final manuscript.

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