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Different dosages of SALMEX® to control *Clostridium perfringens* in poultry feed ingredients

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Ingredients of animal origin are important for the animal feed industry because they contain significant amounts of nutrients, minerals, and vitamins. However, the use of these raw materials is a problem due to pathogenic bacterial contamination, especially *Clostridium perfringens* and *Salmonella* spp. One way to control contamination is the addition of chemical products during ingredient production. Thus, the objective of this study was to evaluate a formaldehyde and organic acid-based product (SALMEX®) for two periods of action after experimental challenge with *C. perfringens* in two poultry feed ingredients. Microbiological analyses to enumerate the pathogen were conducted using colony-forming units per mL (CFU/mL) after incubation on SPS agar at 37°C for 48 h in anaerobic jars using the GasPak® system. The results show that there were significant differences among the dosage treatments and ingredients. With respect to the action time of the product, there were no significant differences observed between 24 h and 5 days, but there was a reduction in bacterial count with doses above 3 kg/t. This reduction was greater in the five-day SALMEX® treatment when compared to the 24-hour period. Thus, we can conclude that a higher product dose and a longer incubation time leads to more efficient product action.

Key words: Animal health, microbiology, nutrition, poultry industry, pathogen.

INTRODUCTION

Since the 1950s, the Brazilian poultry industry has undergone modernization, especially in the areas of genetics, animal management, nutrition, equipment and animal health, and has become highly productive (Tinôco, 2001). This sector is an important chicken meat exporter (Tavares and Ribeiro, 2007); according to ABEF 2014, Brazil is the world's third largest producer, behind only the USA and China, with 12.31 million tons produced, and the top exporter, with 3.918 million tons exported.

Ingredients originating from animals are made from meat byproducts that are not fit for human consumption, such as bone, feathers and blood. Because these byproducts are rich in nutrients, minerals and vitamins, they are important for the production of animal feed (Costa et al., 2008). However, these ingredients are also ideal environments for the proliferation of microorganisms, especially pathogens (Mazutti et al., 2008).

The main microorganisms present in such ingredients

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are *Salmonella* spp. and *Clostridium perfringens*, which are a problem because they can cause diseases in both animals and humans (Santos et al., 2008). Along with *Salmonella* spp. and *E. coli,* researchers have found *C. perfringens* in many types of feed ingredients, including meat meal, fish meal, corn, barley, wheat, and sunflowers (Prió et al., 2006).

C. perfringens is a Gram-positive, anaerobic bacterium that is capable of producing endospores (Schocken-Iturrino et al., 2009). It can be found throughout the environment and is frequently found in the intestines of domestic animals. It is responsible for various diseases, such as food poisoning and gas gangrene in humans and necrotic enteritis in poultry, which are caused by the toxins that this bacterium produces. Thus, adequate microbiological control in both the raw material used for feed and the final feedstuff product is important (Longo et al., 2010).

The primary method used to reduce animal feed contamination is to monitor and control bacterial contamination of ingredients and equipment used in the manufacturing and processing of the raw material (Wales et al., 2010). However, microbiological control can also be achieved with the addition of chemical products to the feed (Dibner and Buttin, 2002). Some chemicals that can control bacterial proliferation are organic acids (acetic acid, propionic acid, and salts of citric and formic acid), ethanol, formaldehyde, alcohol, zinc propionate, and zinc acetate, but the efficiency of these compounds can vary (Wales et al., 2010).

This study investigated the effect of different dosages (1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 kg/t) of SALMEX®, a mixture of formaldehyde and organic acids, on the control of *C. perfringens* in two poultry feed ingredients (meat and bone meal; vegetable mix) for two periods of time (24 h and 5 days) after experimental challenge.

MATERIALS AND METHODS

Experimental tests with different dosages of chemical additive

This experiment used sixteen (16) samples of meat and bone meal and 16 samples of vegetable mix (40% soybean meal and 60% corn) at two different time-periods (24 h and five days), for a total of 64 samples. Four samples of each meal were used as positive controls for both 24 h and 5 days; these samples did not receive a bacteria inoculum, the chemical, nor the sterilization treatment. The remaining samples were placed in bags, sterilized at 121°C for 15 min in an autoclave and cooled by manual agitation to avoid compaction. After this process, the meal was inoculated with a bacterium and treated with chemical product, as detailed below.

The inoculum was obtained from a standard culture of *C. perfringens,* ATCC 13124. These cells were grown in Brain Heart Infusion (BHI) broth, incubated at 37°C for 48 h in anaerobic jars using the GasPak® System (BBL, USA), and centrifuged at 2,991 x *g* for 5 min to increase the cells concentration to 10^6 CFU/mL. The sediment was collected and the cells counted by serial dilution (to 10^{-6}) on Sulfite Polymyxin Sulfadiazine (SPS) agar and incubated under the same conditions as the BHI broth. The inoculum was maintained at 4ºC until the time of challenge (APHA, 2001).

For the SALMEX® treatments, each type of meal was divided into four units weighing 3 kg per unit for each dosage level. The SALMEX® (Btech, Brazil) contained 9% propionic acid, 33% formaldehyde and terpenes such as dispersants and surfactants. Next, 60 mL of *C. perfringens* inoculum was mixed with each meal portion to yield a final concentration of 10^4 CFU/mL.

The feed ingredients and SALMEX® were mixed in an experimental mixer that sprays the products while the ingredients are under rotation. This experimental machine was provided by the SALMEX® manufacturer and was specifically made for dosing and mixing fluids. Before each ingredient was added to the mixer, the equipment was cleaned and disinfected with 70% alcohol.

The dosages of SALMEX® were 1.0, 2.0 and 3.0 kg/t for the vegetable mix and 4.0, 5.0 and 6.0 kg/t for the animal meal. All samples were stored in the laboratory at room temperature for either 24 h or 5 days.

At the end of each period, 25 g of each sample was transferred to 225 mL of 1% peptone water, and serial dilutions were made to 10^{-6} for CFU enumeration. Each diluted sample was heat-shocked at 80°C for 10 min to allow the spores to germinate and to remove contaminants and then cooled in ice water. An aliquot of 1 mL of each dilution was transferred to a Petri dish, and SPS agar was added by the pour plate method. The plates were then incubated in anaerobic jars using the GasPak® System at 37°C for 48 h (APHA, 2001). Colonies suggestive of *C. perfringens* were transferred into test tubes containing BHI and subjected to the following biochemical tests: lactose, maltose and sucrose fermentation, salicin, indole, nitrate, gelatinase, motility and H_2S production (Carter et al., 1995).

Statistical analysis

The data from the count of colony forming units (CFU/mL) were statistically analyzed using an analysis of variance and means with a comparison by 8x2 factorial trial. The *F*-test was also performed, and the significance levels at 5% were determined. The statistical analysis was performed using AgroEstat, Version 1.0 (Barbosa and Maldonado Jr, 2010).

RESULTS AND DISCUSSION

Microorganisms, such as *Salmonella* spp. and *C. perfringens*, proliferate in ingredients of both animal and vegetable origin, and this contamination happens mostly in raw material (Cardozo et al., 2012; Casagrande et al., 2013). In the positive controls, those without chemical treatment *C. perfringens* growth were found. The average *C. perfringens* population for the vegetable mix was 4.28 log CFU/mL at 24 h and 4.64 log CFU/mL after 5 days. For the meat and bone meal control, the average was 4.46 and 4.45 log CFU/mL at 24 h and 5 days, respectively.

The three SALMEX® dosages for the vegetable mix were 1.0, 2.0 and 3.0 kg/t. After 24 h of chemical action, the population means were 3.27, 3.59, and 4.04 log CFU/mL, respectively. After five days of the product's action, the average population was 4.11 log CFU/mL for the dose of 1.0 kg/t, 3.43 log CFU/mL for 2.0 kg/t, and 2.57 log CFU/mL for 3.0 kg/t, as shown in Table 1.

For the meat and bone meal, the mean bacterial counts 24 h after the application of the chemical were 2.10 log CFU/mL for the dose of 4.0 kg/t, 3.48 log CFU/mL for 5.0 kg/t, and 4.14 log CFU/mL for 6.0 kg/t. On the fifth day of

Table 1. The levels of *Clostridium perfringens* in experimentally inoculated vegetable meal, and meat and bone meal, for periods of 24 hours and 5 days after the action of the SALMEX® product at predetermined dosages, presented in log CFU/mL.

Type of meal	Dosage of SALMEX® (Kg/t)	24 h	5 days	Type of meal	Dosage of SALMEX® (Kg/t)	24 h	5 days
VM[1]	0.0	4.18	4.51	MBM[2]	0.0	4.65	4.08
VM	0.0	4.08	4.32	MBM	0.0	4.51	4.60
VM	0.0	4.48	5.70	MBM	0.0	3.95	4.41
VM	0.0	4.30	6.08	MBM	0.0	4.48	4.54
Mean	-	4.28	4.64	Mean	-	4.46	4.45
SEM	-	0.07	0.38	SEM	-	0.13	0.10
VM	1.0	3.00	0.00	MBM	4.0	0.00	0.00
VM	1.0	3.64	0.00	MBM	4.0	0.00	0.00
VM	1.0	2.48	4.51	MBM	4.0	0.00	0.00
VM	1.0	3.23	4.32	MBM	4.0	2.70	0.00
Mean	-	3.27	4.11	Mean	-	2.10	0.00
SEM	-	0.21	1.10	SEM	-	0.58	0.00
VM	2.0	3.43	2.60	MBM	5.0	0.00	0.00
VM	2.0	0.00	3.88	MBM	5.0	0.00	0.00
VM	2.0	4.08	0.00	MBM	5.0	3.60	3.00
VM	2.0	2.88	3.48	MBM	5.0	3.90	3.30
Mean	-	3.59	3.43	Mean	-	3.48	2.88
SEM	-	0.78	0.76	SEM	-	0.94	0.79
VM	3.0	0.00	3.18	MBM	6.0	3.93	3.40
VM	3.0	4.49	0.00	MBM	6.0	4.34	0.00
VM	3.0	4.04	0.00	MBM	6.0	4.36	0.00
VM	3.0	3.54	0.00	MBM	6.0	3.34	0.00
Mean	-	4.04	2.57	Mean	-	4.15	2.80
SEM	-	0.89	0.69	SEM	-	0.21	0.74

[1]VM = vegetable mix; [2]MBM = meat and bone meal.

SALMEX® action, the dosage of 4.0 kg/t inhibited the growth of *C. perfringens*, while the doses of 5.0 and 6.0 kg/t only resulted in lower growth, with respective values of 2.88 log and 2.80 log CFU/mL (Table 1). This unexpected result may have occurred because of ingredient compaction and poor homogenization in the manual application of the *C. perfringens* culture used for challenge. Some compaction of the ingredients may have occurred due to the heat and humidity produced during the autoclave sterilization. This could explain the agent's survival ability, as the chemical product cannot penetrate this compaction.

C. perfringens has the capacity to form spores (Schocken-Iturrino et al., 2009) which supports its resistance to chemicals. Therefore, a longer chemical exposure is required for the product to act on the bacterial cell. This can explain the fact that the average bacterial counts increased for the 24-h period. In addition, Ricke (2003) found that the type of microorganism, the change in superficial tension, and spore formation, all determine bacterial sensitivity to organic acid antimicrobial agents.

Animal meals provide a better environment for pathogenic microorganism development and present a higher risk of contamination (Longo et al., 2010; Mazutti et al., 2008) when compared to vegetable meals. Therefore, the samples of animal origin were treated with higher dosages of SALMEX® (4.0, 5.0 and 6.0 kg/t) than those of vegetable origin.

When used for the control of *Salmonella* spp., the SALMEX® product was effective and prevented the growth of this microorganism at dosages ranging from 1.0 to 6.0 l/ton (Albuquerque et al., 1998). In another study on Enterobacteriaceae control in swine feed, treatments using a mixture of propionic acid and formaldehyde were performed. That study analyzed three different concentrations (0.0, 1.0, 2.0, and 3.0 g/kg) and two periods (24 h and 14 days), and enterobacteria reduction was observed in the concentration of 3 g/kg at 14 days (Sbardella et al., 2014). However, in this experiment, we only found lower population counts of *C. perfringens* with SALMEX® dosages over 3.0 kg/t. Furthermore, complete control was observed only with the SALMEX® dose of 4.0 kg/t and then, only in the bone and meat meal, not the vegetable meal.

Table 2. The comparison between the statistical means of the positive control and the products receiving different dosages, in log CFU/mL.

Treatment[(1)]	Mean
1	2.824[abc]
2	2.722[bc]
3	2.259[c]
4	0.949[c]
5	2.201[c]
6	2.688[bc]
7	4.705[a]
8	4.404[ab]
F-test	7.91 (p<0.0001)
MSD [(2)] (5%)	1.931
Period	Mean
24 h	3.144[a]
5 days	2.544[a]
F-test	3.88 (p=0.0548)
MSD (5%)	0.613
Value of the *F*-test for the interaction	
Treatment *vs* Period	1.71 (p=0.1285)

[a, b, c] Means within a column with unlike superscripts differ significantly ($P < 0.05$). [(1)]1= Vegetable Mix with 1.0 Kg/t SALMEX®; 2= Vegetable Mix with 2.0Kg/t SALMEX®; 3= Vegetable Mix with 3.0 Kg/t SALMEX®; 4= Meat and Bone Meal with 4.0 Kg/t SALMEX®; 5= Meat and Bone Meal with 5.0 Kg/t SALMEX®; 6= Meat and Bone Meal with 6.0 Kg/t SALMEX®; 7= Positive Control Vegetable Mix; 8= Positive Control Meat and Bone Meal. [(2)]MSD= Minimum Significant Difference for means comparison.

Our *C. perfringens* colony counts in the SALMEX® treated samples demonstrated that the product was most effective at higher doses and with longer periods of action. This is similar to two studies found in literature. The first one by Cardozo et al. (2012) determined that SALMEX® at a dosage of 6 kg/t was effective in inhibiting *C. perfringens* in animal meal. The second one by Casagrande et al. (2013) evaluated the efficiency of different products containing formaldehyde and organic acids in the elimination of the same pathogen at concentrations of 3.0 and 6.0 kg/t in animal and vegetable ingredients of poultry feed.

Bacteria inhibition by organic acids occurs through the inside of cells and the dissociation of cations and anions. Cations are responsible for reducing the bacteria's internal pH, consuming vital energy, and causing the death of these cells. The anionic form diffuses freely through the cell wall, and becomes toxic in this dissociated form (Lambert and Stratford, 1999). Finally, the antimicrobial action of organic acids is specifically related to the acid concentration, the pH of the environment and the type of microorganism (Wales et al., 2010; Dibner and Buttin, 2002).

Formaldehyde is a potent chemical product because of the way it operates in the cells. According to Tortora et al. (2005), formaldehyde has the ability to inactivate cellular constituents such as protein and nucleic acid. Thus, this product results in the death of the cell and is more effective in eliminating bacteria.

Statistical analysis of this experiment showed that, among the evaluated variation factors, the only significant difference was found between the treatments with p<0.0001. The product action period did not further affect the final result (p=0.0548). The interaction between treatment and time was not significant (p=0.1285), demonstrating that these factors are independent of each other. The statistical ANOVA showed a mean of 2.84 log CFU/mL, an SD of 1.22 and a CV of 42.865.

The statistical average for the different time-period treatments (24 h and 5 days) demonstrated that treatment of the vegetable mix with the dosage of 2.0 kg/t and the meat and bone meal with the dosage of 6.0 kg/t were statistically equivalent. The vegetable mix (3.0 kg/t) and the meat and bone meal (4.0 and 5.0 kg/t) treatments all obtained satisfactory and similar results (Table 2).

The positive controls for both the vegetable mix and the animal meal had higher average scores than the other treatments and were statistically equal. Interestingly, the treatment of the vegetable mix with the dosage of 1.0 kg/t showed results similar to all others. For the period of product action there were no significant differences observed between the 24 h and 5-day periods. In a study by Carrique-Mas et al. (2007), in which the periods of product action were 24 and 72 h, the effect of time also interfered with the effectiveness of treatments.

A study by Albuquerque et al. (1998), comparing different commercial organic acid compounds at different doses during experimental inoculation of *Salmonella* spp. in animal feed, concluded that organic acids show different bactericidal behaviors because their effectiveness depends on the product and the concentration used.

Conclusion

SALMEX® reduced *C. perfringens* populations in samples that received doses above 3.0 kg/t. After 24 hours of product action, there were no counts in 29% of the samples, and after a period of five days, this percentage increased to 63%. This demonstrates that time is an important factor for SALMEX® action.

Conflict of Interests

The authors did not declare any conflict of interests.

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