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Full Length Research Paper

Pathogens in animal meal and the utilization of salmex in the removal of clostridium perfringen

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The microbiological quality of the ingredients used in feed production is extremely important for animal and public health because food can transmit pathogens. Chemical additives, such as Salmex®, may be used to control these pathogens, since many sporulated microorganisms are resistant to conventional heat treatment. For these reasons, through the conventional bacteriological methods, this study evaluated the presence of pathogens in animal meal and tests the efficiency of additives to combat highly resistant microorganisms. Of the 180 samples of meal analyzed, 71 (39.4%) were positive for the presence of *Clostridium perfringens*, and 41 (22.8%) were positive for the presence of *Salmonella* spp. The additive tested, Salmex®, was effective in elim inating *C. perfringens,* with significantly decreased bacterial counts 24 h after treatment and total absence of *C. perfringens* after five days of treatment in all the samples tested. The presence of the pathogens *Salmonella* spp. and *C. perfringens* in animal meal endanger both the public and animal health. The efficiency of Salmex® in eliminating *C. perfringens* is a major breakthrough for the poultry industry.

Key words: Poultry, microbiology, public health, animal health.

INTRODUCTION

Throughout the processing of broiler chickens in abattoirs, there is a product loss of approximately 35%, generating a significant amount of waste. One way to utilize the waste is through processing, resulting in products that can be used in animal feed (Nunes et al., 2005). Even with technological advances, animal by-products are often subject to bacterial contamination, especially by microorganisms of the genera *Salmonella* and *Clostridium* that negatively affect animal health (Bellaver, 2002).

Salmonella spp. is Gram-negative pathogen and is known to be detrimental to human and animal health because they are frequently isolated as the etiologic agents of food borne illnesses (Siqueira et al., 2003). C*lostridium perfringens* is a Gram positive and produces several types of toxins that can cause many diseases, including avian necrotic enteritis (Schocken-Iturrino and Ishi, 2000; Bignarde et al., 2008).

The control of necrotic enteritis is considered to be one of the greater challenges for the poultry industry. In addition to an effective biosecurity program, the nutrition of animals is essential to successful control of this disease because bird feed is the main source of *C. perfringens* contamination. Feed ingredients have been identified as environmental niches for this microorganism (Dekich, 1998; Keyburn et al., 2006).

With the prohibition of the indiscriminate usage of antibiotics as growth promoters in conjunction with the approval of using additives in animal feed and its raw materials, several agents have been used to control the contamination of animal by-products. Among these, organic acids and formaldehyde are the most commonly used (Ricke et al., 2005).

For *Salmonella* control, there is a wide range of commercially available, scientifically tested products,

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Table 1. Number of animal meal samples positive for the presence of *Clostridium perfringens* and *Salmonella* spp.

Types of meal	*C. perfringens* (%)	*Salmonella* spp. (%)
Meat and bone	22 (36.7)	28(47.7)
Blood and feather	33 (55)	5(8.3)
Viscera	16 (26.6)	8 (13.3)
Total	71 (39.4)	41(22.8)

a: Chi-square *C. perfringens* = 10.3734. DF=2. P=0.00561. b: Chi-square *Salmonella* spp.= 29.6263. DF=2. *P<0.0001*.

including Salmex® (Btech, Brazil), with varying mechanisms of action (Wales, 2010). Salmex® is a formulated product with formaldehyde and propionic acid, presenting broad-spectrum of antimicrobial additive effectively to control the bacterial contamination. Its formula is approved by the FDA (Food and Drug Administration, USA) for use in animal feed and ingredients for all animal species, and presented by the EFSA (European Food Safety Authority) as the best alternative in the control of Salmonella in feed production chain.

However, there have been few reports on the control of *C. perfringens* by products used by animal feed manufacturers. One reason for this may be related to the fact that this microorganism forms spores; as a result, it has high resistance to many conventional chemical and heat treatments (Richardson, 2008).

Based on this information, the current study was designed to examine the presence of *Salmonella* spp. and *C. perfringens* in animal meal and also to test the efficacy of Salmex® on the growth inhibition of *C. perfringens* in animal by-products.

MATERIALS AND METHODS

The analyses were conducted at the Laboratory of Bacteriology, Department of Veterinary Pathology, Faculty of Agriculture and Veterinary Sciences, UNESP, Jaboticabal Campus. The studies were conducted from February to October, 2010. In total, 60 samples of meat and bone meal (MBM), 60 samples of blood meal and feathers (BFM) and 60 samples of viscera meal (VM) were analyzed to detect the presence of *Salmonella* and *C. perfringens*. These samples were collected from several establishments in the state of São Paulo.

Pathogens isolation

Salmonella spp. isolation was performed following the protocol of Apha (2001). After selective enrichment and isolation, characteristic colonies were selected from brilliant green phenol red lactose-sucrose (BPLS) agar, which are colorless on pink, opaque and slightly translucent, whose medium was pink; and from Mac Conkey agar were selected colonies colorless with medium straw color. Then, these colonies were seeded in inclined Triple Sugar Iron (TSI) tubes and incubated at 37°C for 24 h. After thi s period, the cultures were subjected to slide agglutination test with *Salmonella* polyvalent serum, and those confirmed to be positive were sent to the Oswaldo Cruz Foundation for serotyping.

For *C. perfringens* isolation, 25 g of each sample were homogenized in 225 ml of 1% peptone water and subjected to heat shock to eliminate contaminants and spore germination. For the heat shock, the samples were heated in a water bath at 80°C for 10 min and then cooled in ice water. Next, 1 ml of each sample was inoculated in Petri dishes containing Sulfite Polymyxin Sulfadiazine (SPS) agar. The plates were incubated in anaerobic jars using the Gaspak® System (BBL, USA) at 37°C for 72 h. Black colonies were transferred to tubes containing BHI broth and incubated at 37°C for 24 h. Then, the cultures were subjected to biochemical tests: lactose, maltose, sucrose, salicin, indole, nitrate, gelatinase and motility and H_2S (Carter et al., 1995).

Chemical additive test

To this test was used Salmex® (Btech, Brazil), a formulated product with 330 g/kg of formaldehyde and 90 g/kg of propionic acid. To perform the test, an inoculum was prepared. The colonies were subcultured in Brain Heart Infusion (BHI) tubes, and the culture obtained was transferred to BHI broth in an Erlenmayer flask and incubated for 24 h at 37°C. To count the number of b acterial cells in the inoculum, a serial dilution was performed to a level of 10^{-6}. Then, 1 ml of each dilution was inoculated on the plates with SPS agar and incubated in anaerobic jars using the Gaspak® System at 37°C for 24 h. After incubation, the number of colon y forming units per milliliter was counted (CFU / ml).

A total of 24 samples, each with 3 kg, were used to test the efficacy of Salmex®. Of these, 12 samples were treated, including four MBM samples, four BFM samples and four VM samples. Additionally, there were 12 control samples. All samples were autoclaved at 121°C for 15 min to remove any contami nation. After cooling, the samples received an inoculum concentration of 10 ml/500 g, containing 1 x 10^6 CFU/ml of *C. perfringens*, and were incubated at room temperature for 24 h. Next, all the samples, except the control samples, received treatment with Salmex® at a concentration of 6 kg/t. Microbiological counts were determined during two periods, that is, 24 h and five days after Salmex® treatment. These times correspond to the minimum and maximum period of flour storage in factory silos.

A statistical analysis between the independent variables was performed using the Chi-square test, with a significance level of 1% considered to be statistically significant.

RESULTS

Of the 180 meal samples analyzed for the presence of *Salmonella* spp., 41 (22.8%) were positive. In total, 28 of the 60 MBM samples (47.7%), 5 of the 60 BFM samples (8.3%), and 8 of the 60 VM samples (13.3%) were positive for the presence of *Salmonella* spp., as shown in Table 1.

Of the 180 meal samples analyzed, 71 (39.4%) were positive for the presence of *C. perfringens*. Specifically, 22 of the 60 MBM samples (36.7%), 33 of the 60 BFM samples (55%), and 16 of the 60 VM samples (26.6%) were positive for the presence of *C. perfringens* (Table 1).

Serotyping of the *Salmonella* spp. positive samples, which was conducted by the Oswaldo Cruz Foundation, showed that of the 41 strains isolated, 44% were

Table 2. Levels of *Clostridium perfringens* in Salmex® treated samples and control samples 24 h after treatment.

Sample	Control	CFU/ml	Salmex®	CFU/ml
1	MBM	$3.1x10^{5}$	MBM	0
2	MBM	$5.1x10^{5}$	MBM	0
3	MBM	$5.4x10^{5}$	MBM	0
4	MBM	$4.3x10^{5}$	MBM	0
5	BFM	$3.3x10^{5}$	BFM	0
6	BFM	$6.3x10^{5}$	BFM	0
7	BFM	$3.6x10^{5}$	BFM	0
8	BFM	$2.3x10^{5}$	BFM	0
9	VM	$2.3x10^{5}$	VM	0
10	VM	$3.6x10^{5}$	VM	$1.9x10^{4}$
11	VM	$5.3x10^{5}$	VM	$2x10^{4}$
12	VM	$3.7x10^{6}$	VM	$2.6x10^{4}$

a: MBM, Meat and bone meal. b: BFM, blood and feather meal. c: VM, Viscera meal.

Table 3. Levels of *Clostridium perfringens* in Salmex® treated samples and control samples 5 days after treatment.

Sample	Control	CFU/ml	Salmex®	CFU/ml
1	MBM	$2.7x10^{5}$	MBM	0
2	MBM	$2.1x10^{5}$	MBM	0
3	MBM	$2.3x10^{5}$	MBM	0
4	MBM	$5.3x10^{5}$	MBM	0
5	BFM	$2.3x10^{5}$	BFM	0
6	BFM	$5.4x10^{5}$	BFM	0
7	BFM	$4.4x10^{5}$	BFM	0
8	BFM	$7.3x10^{5}$	BFM	0
9	VM	$6.4x10^{5}$	VM	0
10	VM	$4.8x10^{5}$	VM	0
11	VM	$3.7x10^{5}$	VM	0
12	VM	$2.2x10^{6}$	VM	0

a: MBM, Meat and bone meal. b: BFM, blood and feather meal. c: VM, viscera meal.

identified as *S. Montevideo*, 22% were S. Senftenberg, 20% were *S. Infantis*, 12% were *S. Cerro,* and 2% were *Salmonella typhimurium*.

Salmex® efficiently reduced *C. perfringens* contamination. As observed in Table 2, the control samples in all the types of meals analyzed had counts ranging from 2.3×10^{5} to 5.3×10^{6} CFU/ml at the 24 h time point. However, the treated MBM and BFM samples showed no bacterial growth. The treated VM samples showed a growth range from zero to 2.6×10^{4} CFU/ml.

At the five day time point, the control samples in all the types of meals analyzed had counts ranging from 2.1×10^{5} to 2.2×10^{6} UFC/ml. In contrast, all the samples from the three types of meals with treated Salmex® showed a total absence of microbial growth, as illustrated in Table 3.

DISCUSSION

Our results reinforce the need for greater care in the production of ingredients that are incorporated into animal feed. The risk of contamination exists for both the handlers of these products and for consumers, because *Salmonella* contamination in animal feed can be transferred to the human food chain via meat and eggs (Davies et al., 2001; Calixto et al., 2002). As a result, the contamination of animal feed products is a risk to public health.

Santos et al. (2000), who analyzed the contamination of animal meals from Lavras, Minas Gerais, observed that 90% of the samples were contaminated with *Salmonella.* They concluded that the raw materials were the main source of pathogen transportation to the feed. This rate of occurrence, although higher than the levels described in this study, shows that there is considerable contamination of samples beyond the accepted standards (Brasil, 2008).

In a study on 120 samples of animal meal, Calixto et al. (2002) identified 25 samples positive for *Salmonella* spp. The authors emphasized that the use of animal meal as a protein source in poultry feed is viable; however, they noted that the production of these products must be closely monitored.

In a technical statement (Zanotto et al., 2007) analyzing meat and bones and floated particles from waste water (FP), from Embrapa Swine and Poultry of Santa Catarina in Brazil, the presence of *C. perfringens* was detected in all the samples of FP. However, when the FP was incorporated into the MBM and the mixture was thermally processed in a digester, the contamination ceased to exist. Although, the author claims that the drop in incidence is due to the cooking process, this result is different from what was observed in the current study, that all three types of meal analyzed were positive for *C. perfringens*. This discrepancy can be explained in two ways. One hypothesis is that the microorganism may have withstood the heat treatment; in this study, the process of cooking the meal, which according to Garcia et al. (2001) was the most important risk factor in *C. perfringens* food safety. Alternatively, there may have been failures during processing.

In Japan, Shirota et al. (2001) found that 58.5% of the serotypes isolated in commercial eggs were identical to the strains found in rations. These results confirmed that microbiological quality of meal is essential for consumer health, because the meal can transmit the pathogens to ration, eggs and then to the humans.

The presence of *Salmonella* Typhimurium found in this study stands out because this serovar is directly linked to poultry product contamination that leads to human disease, making it a risk to public health (Cortez, 2006).

Of the 180 samples analyzed for the presence of *Salmonella* spp. in this study, 22.8% were expressed at levels higher than accepted based on current legislation, establishing the absence of the organism in 25 g of product (Brasil, 2008). Because chicken can be a reservoir of this microorganism, these results suggest that the processing of animal meal was inadequate and requires more rigorous control.

The legislation that established the limits for counts of *Clostridium perfringens* in animal by-products (Brasil, 2003) was repealed and the current legislation does not mention this microorganism (Brasil, 2008). This might be because it is a sporulated pathogen, which makes it resistant to many treatments and difficult to control. However, because its presence was observed at significant levels and also the contamination is undesirable, studies involving the inhibition of *C. perfringens* have become important and necessary.

The presence of *C. perfringens* 24 h after Salmex® treatment in only one type of analyzed meal (VM) is likely because the efficiency of the chemical control of contaminants varies with the applied dose and the exposure time to the product. In addition, formaldehyde is less likely to be inactivated by organic matter; however, it requires several hours to reach its full effect (Arts et al., 2006).

The reason for only one type of meal not reaching 100% bacterial inhibition may be due to the texture of the samples. Of the three types of meal, only VM had compressed groats, while MBM and BFM were farinaceous. This feature of the VM meal may caused a difficulty in product penetration, suggesting that grain size and animal meal texture directly influence microbiological quality (Bellaver, 2002).

It is important to mention that several research groups have been developing products based on combinations of chemical agents, including Salmex®. The combination of propionic acid and formaldehyde causes a synergistic effect, thus eliminating problems related to palatability, corrosion and volatility (EFSA, 2008; Khan et al., 2003; Longo et al., 2010).

In the literary review, no studies were found on the chemical evaluation of the growth inhibition of *Clostridium perfringens*; thus, was decided to discuss the findings related to the heat treatment analysis.

Mazutti et al. (2010) evaluated two methods of animal meal sterilization, one on a pilot scale and the other on an industrial scale; both with the injection of saturated steam. Regarding the removal of *C. perfringens*, the pilot-scale process was inefficient, with counts of up to 6.0×10^2 CFU/ml. The industrial scale process reduced but did not eliminate the pathogen, with counts of up to 9.8×10^1 CFU/ml. These results illustrated that heat treatment is less effective when compared to the chemical treatments used in this study, in which was observed a total elimination of the pathogen.

It is noteworthy that heat treatments may not be effective for controlling spoilage pathogens (García et al., 2001). Because heat treatment is not 100% effective in eliminating the pathogen, heating can have a stimulating effect on the multiplication of these microorganisms. In addition, overheating negatively influences palatability and affects the availability of essential amino acids in animal meal (Bellaver, 2002).

Overall, the use of chemicals to inhibit the growth of *C. perfringens* should be taken into account because many studies has shown the presence of this microorganism in animal meal but did not suggest solutions to the problem. This study demonstrated that Salmex® inhibits the growth of this undesirable pathogen.

Conclusions

The presence of *Salmonella* spp. and *Clostridium perfringens* in samples showed that the production of animal meal is a sub-sector that needs technological support to reduce bacterial contamination. In addition to causing economic losses to producers, this conta-mination is also a risk to public health. The efficacy of Salmex® shows that it is possible to inhibit the growth of *C. perfringens*, making the findings here a major breakthrough for the poultry industry.

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