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Microbiological analyses of poultry litter used for ruminant feeding

(Análises microbiológicas da cama de frango utilizada na alimentação de ruminantes)

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ABSTRACT

The aims of this study were to estimate the changes in total bacterial counts (TBC) in poultry litter samples, consisting of rice hulls, after storage, and to identify pathogenic bacteria. For the countings Plate Count agar (Difco) was used. Enrichment and seletive media such as blood agar, MacConkey, Baird Parker, brain and heart agar, and egg yolk solid media, and cooked meat and brain and heart infusion, incubated under aerobic or anaerobic conditions were used to isolate *Staphylococcus aureus, Salmonella sp, Clostridium perfringens, C. botulinum, C. chauvoei, Camylobacter sp, Escherichia coli,* and *Corynebacterium sp.* Litter samples were collected from the houses of the Veterinary School experimental aviary. A fully randomized experimental design was used with four treatments and four replications, for a total of 16 samples. A decrease in TBC was detected when treatment T1 (zero days of storage) was compared with treatments T2 (14 days of storage). On the other hand the treatments T3 (28 days of storage) and T4 (42 days of storage) presented significantly superior counting in relation to treatment T1. Some pathogenic bacteria of *Enterobacteriaceae* such as *Escherichia coli, Proteus, Arizona, Providencia, Edwardsiella,* as well as *Staphylococcus aureus, S. epidermidis,* different species of genus *Clostridium* as *C. perfringens, C. sordelli, C. chauvoei, C. tetani* and *C. novyi* as well as some strains of *Corynebacterium pyogenes* were isolated.

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The enteric group was found to show the highest sensitivity to changes in enviromental conditions, as a function of the different periods of storage.

Key words: Microbiological analyses, poultry litter, ruminant feeding

RESUMO

O presente trabalho teve por objetivos avaliar a variação da contagem total de bactérias em amostras de "cama" de frangos compostas por casca de arroz, após o armazenamento, assim como identificar aquelas bactérias potencialmente patogênicas presentes. Para as contagens foi utilizado o ágar para contagem padrão (Difco), os meios utilizados na identificação foram ágar sangue, MacConkey, Baird-Parker, e para as anaeróbicas tubos com infusão de cérebro e coração, meio de carne cozida (Difco) e placas de ágar Clostrisel, BHI ágar e ágar gema de ovo, incubados em anerobiose com o sistema Gas-Pak (BBL). A metodologia para a identificação bacteriana foi dirigida para a procura de *Staphylococcus aureus, Salmonella sp, Clostridium perfringens, C. botulinum, C. chauvoei, Campylobacter sp, Escherichia coli,* e *Corynebacterium sp.* Foram colhidas amostras da "cama" provenientes de galpões de frangos de corte. O delineamento experimental utilizado foi inteiramente casualizado com quatro tratamentos e quatro repetições, totalizando dezesseis amostras. Os resultados da avaliação demonstraram haver decréscimo no número total de bactérias (NTB) quando se compararam os tratamentos T1 (zero dias de estocagem) e T2 (14 dias de estocagem). Em contraposição os tratamentos T3 (28 dias de estocagem) e T4 (42 dias de estocagem) apresentaram contagens de NTB significativamente superiores em relação ao tratamento T1. Constatou-se a presença de bactérias patogênicas primárias ou secundárias da família das enterobactérias (*Escherichia coli, Proteus, Arizona, Providencia, Edwardsiella),* assim como *Staphylococcus aureus, S. epidermidis,* além de diversas espécies de *Clostridium* como *C. perfringens, C. sordelli, C. chauvoei, C. tetani* e *C. novyi,* e algumas cepas de *Corynebacterium pyogenes.* Entre toda a flora bacteriana determinada, o grupo das enterobactérias, demonstrou maior sensibilidade às modificações do meio, em função dos diversos tempos de estocagem testados.

Palavras-chave: Análise microbiológica, cama de frango, alimentação, ruminantes

INTRODUCTION

The importance of poultry litter in animal feeding has been considered over the last few years because of its nutritional value and availability in different regions of Brazil. However, the studies carried out thus far on the pathogenic flora and bacterial toxins of poultry litter are insufficient to guarantee its safe use in animal

feeds, since the literature mainly contains reports about the saprophytic flora (Halbrook et al., 1951; Schefferle, 1965, 1966).

Recent studies have shown the risk of adding poultry litter contaminated with botulin toxin to cattle feed (Schocken-Iturrino & Avila, 1991), with animal intoxication and death occurring after ingestion. Poultry litter without previous heat treatment is also considered to be a possible vehicle of *Salmonella* dissemination (Fanelli et al., 1970).

The objectives of the present study were to determine the variation in bacterial counts in poultry litter submitted to four different periods of storage, and to isolate and determine the variation in the population of some pathogenic microorganisms. The total number of bacteria present in poultry litter was determined and verified whether natural fermentation of the litter would have an effect in the number of bacteria. It was also determined whether litter contained potentially pathogenic bacteria such as *Staphylococcus aureus, Streptococcus sp, Clostridium sp, Salmonella sp and Corynebacterium sp.*

MATERIAL AND METHODS

For the study of the bacterial flora present in poultry litter utilized as an ingredient of cattle feed, the material was submitted to four different periods of storage. The experiment was developed at the experimental aviary of Faculdade de Ciências Agrárias e Veterinárias de Jaboticabal - UNESP, SP, Brazil.

An experimental pile containing feces from three poultry-raising units (approximately 10 ton) was prepared and subdivided into 4 smaller piles, stored at a site protected from the weather and submitted to periods of storage of zero, 14, 28 and 42 days. The material used to line the floor of the poultry-raising units was rice hulls submitted to no previous desinfection, corresponding to 0.5kg material per fowl, with a mean thickness of 8cm. The analysis was carried out on 16 samples of 5kg, each representing one of the four periods of storage. The samples were collected at 2-week intervals, in which the total bacterial counts were performed, including yeast, and the presence of pathogenic bacteria was determined. A fully randomized design with four treatments and four replications was used in the analysis of microorganism counts. For analysis of variance, the bacterial counts were divided by 1000 and later transformed into logarithm $Log_{10}(x)$.

Aliquots (100g) of each sample were diluted out in ten-fold series with sterile saline up to 10^{-6} and then immediately plated in duplicate onto agar for standard counts (Difco), and incubated at 37°C for 24 to 48 hours. The plates containing 30 to 300 colonies were submitted to bacterial counts with a digital counter (Phoenix) by the method of Speck (1976).

For the isolation of pathogenic bacteria, 1ml of the first dilution was plated onto sheep blood agar, MacConkey agar and Baird-Parker agar and incubated at 37°C for 24 to 48 hours. After the growth of typical colonies, smears were obtained and stained by the Gram method and submitted to screening for classification in terms of shape (cocci, rods) and Gram positivity or negativity. The material was then inoculated into nutrient agar tubes and stored for biochemical identification. Gram-positive cocci were submitted to the coagulase test in tubes containing citrated rabbit plasma, to the DNAse test, and to the thermonuclease test (Lachica et al., 1971). The other microorganisms were submitted to the following tests: indole, motility, H_2S, TSI, oxidase, Simmons citrate, methyl red, Voges-Proskauer, urea, gelatinase, nitrate reduction, and carbohydrate fermentation.

For the isolation of sporulated anaerobic bacteria the material in the tubes was submitted to a thermal sochk at 7°C for 10 minutes and then rapidly cooled to 35°C in ice water. After this shock, the material was inoculated into tubes containing cooked meat medium and brain-heart infusion (BHI, Difco) and incubated in anaerobic jars using the Gas-Pak system (BBL) at 37°C for 24 to 48 hours. After incubation, the tubes with growth were plated onto Clostrisel agar, BHI agar and egg yolk agar and incubated anaerobically under the conditions mentioned above.

Typical colonies were submitted to Gram staining and reinoculated into tubes containing cooked meat medium and BHI for the biochemical tests and for the toxigenicity tests. Sporulated Gram-positive rods were submitted to the catalase test for separation between the genus *Bacillus* and the genus *Clostridium*. After this separation, pure *Clostridium* cultures were submitted to the following biochemical series of tests: gelatinase, motility and H_2S production, nitrate reduction, carbohydrate fermentation, indole, lecithinase, urease, and hemolysis. Clarified extracts of pure cultures were inoculated into mice for the detection of toxin by the method of Sterne & Hobbs (1978).

RESULTS AND DISCUSSION

The bacterial counts and the mean values for the poultry litter samples are presented in Tab. 1 as number of microorganisms per gram of material analyzed.

Tab. 1 shows that at time zero (at the beginning of the poultry litter storage), the mean absolute number of bacteria was higher compared to the period of 14 days, probably due to the natural flora present in the gastrointestinal tract of fowl. By the beginning of the storage period, we believe that, due to changes in pH brought about by the release of excessive ammonia, by the fermentation of the piled material and by the natural dehydration occurring with time, there was a reduction in absolute bacterial numbers at the 14-day time point.

Table 1 Mean total counts of number of bacteria per gram in samples of poultry litter as a function of time of time of storage and replications

Treat/Repl.	R1	R2	R3	R4	Mean
T1 (zero)	54638	56905	78320	70484	64884a*
T2 (14 d)	38918	31781	27726	17918	29086b
T3 (28 d)	41744	56630	70031	61092	57374a
T4 (42 d)	65820	77609	79655	85468	77138a

* Means followed by different letters on the column, differed significantly (P<0.01).

According to Junior & Baliana (1982), through the microbial flora natural selection process, the decreased moisture as well as the elevation of temperature in the pile are the major reasons for the fall in the number of bacteria present in the material. In another study, Schocken-Iturrino et al. (1992) also detected a decreased in number of bacteria after 14 days of storage in feces of laying hens.

On the other hand, a significant increase in total number of bacteria was detected after 28 and 42 days of storage compared to 14 days. These results are in agreement with those obtained by Freitas (1989) who observed a decrease followed by an increase in microbial counts in feces of laying hens as a function of time of storage.

The results on the identification of bacteria and yeasts present in poultry litter after the various periods of storage are presented in Tab. 2, 3 and 4.

As can be seen from the results, these studies permit the isolation of some potential pathogens such as members of *Enterobacteriaceae, (Escherichia coli, Proteus, Arizona, Providencia, Edwardsiella),* as well as *Staphylococcus aureus* and *S. epidermidis*, different species of *Clostridium* that may be pathogenic to animals such as *C. perfringens, C. sordelli, C. chauvoei, C. tetani,* and *C. novyi.* Some strains of *Corynebacterium pyogenes* were also isolated. These data were similar to those observed by Alexander et al. (1968) who reported that some bacteria can survive in poultry litter for significant periods of time as a result of the elimination of competing agents and of material decomposition. This may partially explain the results obtained in the present study in terms of the changes in number (Tab. 1) and species (Tab. 2, 3 and 4) of the bacteria detected after different period of storage. Tab. 2 shows that most of Gram-positive aerobic bacteria continued to the present over the various periods of storage. However, Tab. 3 shows changes in the species of enterobacteria isolated over the different periods of storage, except for *Escherichia coli.*

Table 2 Gram-positive aerobic bacteria and yeasts isolated from poultry litter samples as a function of time of storage and replications

Treat/Repl.	R1	R2	R3	R4
T1 (zero)	*Bacilococcus* *Bacillus sp* *S. epidermidis* *Corynebacterium* *Lactobacillus*	*S. epidermidis* *S. aureus*	*Bacillus sp* *S. aureus* *Sarcina* *S. epidermidis*	Yeasts *S. epidermidis* *Bacillus sp*
T2 (14 d)	*Bacillus sp* Yeasts *S. epidermidis* *Corynebacterium pyogenes*	*Micrococcus sp* *S. epidermidis* *Lactobacillus sp*	Yeast *Micrococcus sp* *S. aureus*	*Bacillus sp* *D. epidermidis* Yeasts
T3 (28 d)	*Micrococcus sp* *S. epidermidis* *S. aureus*	*Micrococcus sp* *Bacillus sp*	*Bacillus sp* *Micrococcus sp* *S. epidermidis* Yeast	Yeast *Micrococcus sp* *S. aureus* *Bacillus* *Lactobacillus*
T4 (42 d)	Yeast *S. epidermidis* *Lactobacillus*	*Bacillus sp* *Micrococcus sp* *S. epidermidis* *S. aureus* Yeast	*Micrococcus sp* *S. epidermidis* *Bacillus sp* *Lactobacillus*	Yeast *Micrococcus sp* *S. epidermidis* *S. aureus*

Table 3 Enterobacteriaceae present in poultry litter samples as a function of time of storage and replications

Treat/Repl.	R1	R2	R3	R4
T1 (zero)	*Providencia* *E. coli* *Arizona* *Citrobacter* *Edwardsiella*	*Providencia* *E. coli* *Arizona* *Citrobacter* *Edwardsiella* *Proteus*	*Providencia* *E. coli*	*Providencia* *E. coli* *Enterobacter*
T2 (14 d)	*Providencia* *E. coli* *Proteus*	*Providencia* *E. coli* *Enterobacter*	*Providencia* *E. coli* *Enterobacter* *Proteus*	*Providencia* *E. coli* *Arizona* *Citrobacter*
T3 (28 d)	*Providencia* *E. coli* *Proteus*	*Providencia* *E. coli* *Proteus*	*Providencia* *E. coli* *Proteus*	*Providencia* *E. coli*
T4 (42 d)	*E. coli* *Proteus*	*Providencia*	*Providencia* *E. coli* *Proteus*	*E. coli*

Table 4 Clostridia isolated from poultry litter samples as a function of time of storage and replications

Treat/Repl.	R1	R2	R3	R4
T1 (zero)	*Clostridium sp* *C. perfringens* *C. sordelli* *C. sporogenes*	*Clostridium sp* *C. perfringens* *C. septicum* *C. chauvoei*	*Clostridium sp* *C. chauvoei*	*Clostridium sp* *C. perfringens* *C. sporogenes*
T2 (14 d)	*Clostridium sp* *C. novyi* *C. tetani*	*C. chauvoei* *C. sporogenes* *Clostridium sp*	*C. septicum* *Clostridium sp* *C. haemolyticum* *C. chauvoei*	*C. sordelli* *C. sporogenes* *C. septicum*
T3 (28 d)	*C. perfringens* *C. chauvoei* *C. novyi* *C. sporogenes*	*C. sporogenes* *C. septicum* *Clostridium sp*	*C. chauvoei* *C. sporogenes* *C. septicum*	*C. tetani* *C. sporogenes* *Clostridium sp*
T4 (42 d)	*Clostridium sp* *C. septicum* *C. sporogenes*	*Clostridium sp* *C. novyi* *C. chauvoei* *C. sporogenes*	*Clostridium sp* *C. sporogenes* *C. novyi*	*C. septicum* *C. perfringens* *Clostridium sp*

According to Tab. 4, most of the *Clostridium* species isolated, by being sporulated, continued to be present over the periods of storage tested. Only *Clostridium botulinum* was not isolated from the samples analyzed; if this microorganism had been present, it might have produced toxin and would have caused botulism, as demonstrated by Schocken-Iturrino & Avila (1991) in a study on poultry litter added to the feed of confined cattle.

CONCLUSIONS

On the basis of the present results, it is possible to conclude that the total bacterial and yeast colony forming microorganism counts in poultry litter varied as a function of the different periods of storage due to the physicochemical changes in the material, demonstrating a possible selective process of this microbial flora. Differential changes occurred in some species of microorganisms isolated according to time of storage, with enterobacteria proving to be more sensitive to environmental changes. *Clostridium botulinum* was not isolated from the poultry litter samples analyzed.

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