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Studies on Factors to Increase Mold Inhibitor Effectiveness in Livestock Rations[1)]

I. Effects of Particle Size and the Levels of Protein in the Ration on the Usage of a Mold Inhibitor

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배합사료에 대한 항곰팡이제의 효과적인 처리 방법과 사료내 영양소 보전 방법

I. 사료의 입자 크기와 사료내 단백질 수준이 항곰팡이제의 작용에 미치는 영향

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적　요

단백질 수준이 각각 18%(초생추 사료)와 12% (비육우 사료)인 사판 사료를 사료 입자 크기로 나누어서 1.19㎜이하가 전사료에 대한양의 80%와 40%인 것으로 만든 다음 각각 다른 단백질 수준과 입자 크기가 배합 사료내에 함유된 항곰팡이제(0.1% W/W)의 효능에 어떻게 영향을 미치는지에 관하여 연구 하였다.

시험 사료내에 함유된 수분의 함량은 12.6% (초생추 사료)와 12.7%(비육우 사료)였다. 시험 사료를 각 처리 별로 1㎏씩 나누어 프라스틱통에 넣은후 뚜껑을 약간 열어둔 상태로 상대습도가 85% 저장 온도를 29±1℃ 되게 유지 시킨채 5일에서 40일 동안 보전한후 곰팡이수, 생성된 탄산 가스양 그리고 생성된 아플라 톡신의 양을 측정 하였다.

형성된 곰팡이 수($P<0.05$)와 탄산 가스 발생양($P<0.01$)은 항곰팡이제가 가해졌을때 사료의 입자 크기가 1.19㎜이하인 것이 전사료의 40%인 구에서 현저히 높았으나 단백질 수준의 차이는 항곰팡이제의 작용에 영향을 미치지 못했다.

그러나 아플라 톡신의 생성은 사료중의 단백질 수준이 높을 수록 많았고($P<0.05$) 또 입자가 큰것이 많은 사료 일수록 현저히 높았다($P<0.05$).

I. INTRODUCTION

Feeds stored under the Korean climatic conditions, especially during the rainy seasons of summer, often become heated and spoil as the result of fungal activity. Since most of the feed grains are imported into Korea, preservation of

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feeds against fungal spoilage is of great importance. Inhibition of fungal growth can be accomplished by regulating moisture, temperature, and the atmosphere of stored grains and grain products. The method of inhibition most appropriate to the animal and feed industries as they are structured today is the use of chemical preservatives. The inhibitors available for practical use are mainly organic acids such as propionic, acetic, benzoic, sorbic, and formic acid (Goering and Gordon, 1974 ; Britt et al., 1975).

It has appeared to be a not uncommon complaint that mold inhibitors do not work consistently and uniformly (Hamilton, 1984). There would be several plausible explanations, but one of the more attractive hypothesis resulted from a survey in which the pH of poultry feed was found to vary by at least 1.5 units (Tabib et. al. 1981). It is characteristic of inhibitory organic acids that the protonated form is more active then the ionized, salt form (Bell, 1958 ; Goering and Gordon, 1973 ; Sauer and Burroughs, 1974). Because the degree of ionization of the carboxyl group is dependent on the pH of the menstrum which contains it, the mold inhibitory properties of organic acids would be dependent on factors which influenced their ionization.

Another attractive hypothesis is factors relating to the logical and practical necessity for an inhibitor to come into direct contact with the fungus before inhibition can ensue. It seems logical that particle size of the substrate and the inhibitor can be added to list of factors influencing the activity of mold inhibitors in solid systems such as grain and feed.

Dixon and Hamilton(1981[a]) studied the effeet of single protein source (15% moisture content) in the corn meal on the activity of mold inhibitors. They conducted research on exprimental mixtures of ingredients that were made by adding to corn meal the amount of a single ingredient (soybean meal or fish meal of poultry by-product or corn gluten meal or fat or limestone) required to give the highest concentration needed and then diluting portions of this with additional corn meal to provide the other desired concentration.

They reported that antagonism was observed with soybean meal, fish meal, poultry by-product meal, and limestone. Dixon and Hamilton (1981 [b]) also reported the effect of particle sizes of corn meal and a mold inhibitor on mold inhibition.

Corn meal which they used in their research contained 20, 25, and 35% H_2O on a wet wight basis. They found that the smaller the corn meal particles, the better the inhibition observed.

The present study was undertaken to document the influence of particle size of substrate and the different levels of protein in the diets on the effectiveness of mold inhibitors, which are typically a commercial complex mixture of varing composition, under the conditions similar to the Korean rainy season.

II. MATERIALS AND METHODS

1. Diet and experimental procedure

The experiment for mold count, CO_2 production, and mycotoxin production in the stored feed was conducted with a commercial chick starter and beef ration containing 18% protein and 12% protein each (Table 1).

Table 1. Composition of two diets

Item	Composition(%)	
	Chick starter	Beef ration
Corn, yellow	36.0	35.0
Sorghum	9.0	
Wheat	16.8	27.0
Soybean meal, 44%	16.8	
Rapeseed meal	2.5	0.63
Wheat bran	6.37	19.0
Defatted rice bran	4.0	10.0
Fish meal, 62%	4.0	
Urea		0.42
Molasses		3.5
Limestone	1.2	4.0
Bone meal	2.0	
Salt	0.2	
Premix	1.13	0.4
Calculated analysis : crude protein	18.0	12.0

The experiments were on the effect of particle size of two different levels of protein (18% for chick starter and 12% for beef ration) on the inhibitor and the interactions between particle size and protein levels on the inhibitor. The part of experimental diets were passed through a 1㎜ mesh and seperated into two particle size ranges : (1) 80% of particles in the experimental ration less than 1.19㎜, (2) 40% of particle size in the experimantal ration less than 1.19㎜.

Moisture content of the feed was determined gravimetrically after heating 10± 0.01g in an oven at 100± 2℃ for 18 to 20 hrs.

A commercial fungistat was mixed in the treated diets at the level of 0.1% (W/W). The powder fungistat was obtained from the mold inhibitor company directly. The experimantal diets treated and untreated with mold inhibitors were divided into plastic cans of 1kg each and the cans were closed (not hermitically) and placed in the incubator which was converted from egg incubator with on evaporate, forced air humidifier at 85% and a controlled-temperature of 29± 1℃. Relative humidity was measured with a wet-bulb thermometer. Rations were incubated for 5, 10, 20 or 40 days. After incubation for 5, 10, 20 or 40 days, 250g of sample was taken from each plastic can and transported to the laboratory in plastic bags and frozen at −25℃ until analyzed.

A 2×2 factorial experimental design was composed of 4 treatments and 4 replicates per treatment. The means were calculated by analysis of variance in which an F-ratio was calculated. If significant($P<0.05$), the least significant difference among means was calculated(Barr et al., 1976).

2. Mold Counts

The total mold count of each sample was made by the method of Stewart et al., (1977) modified by Zhanet et al.(1981). Samples were suspended in 100mℓ of 0.01% Triton X−100 (Fisher Scientific CO., Fair Lawn, NJ) in 0.85% saline and shook for 20 min. on a wrist-action shaker. Further dilutions were made with 0.05% Trition X-100 in 0.85% slaine after permitting the larger particles to settle for 1 min. The high concentration of the nonionic detergent (Triton X-100) in the initial suspension was required to obtain maximal counts. The dilution series utilized a lower concentration for the same reason. Molds were counted in pour plates of Sabouraud's Dextrose Agar (Difco) containing chloramphenicol (40ug/mℓ) to inhibit bacteria and rose bengal (50ug/mℓ) to inhibit overgrowth of spreading mold colonies. The plates were incubated 48hr at 25℃ before counting on a Quebec colony counter (New Brunswick Scientific Co., New Brouswick, Nj). It was recognized that a mold count does not distingish spores, sporangia, and hyphal fragments ; thus, a mold count is a count of propagules or colony forming units that may have very poor correlation with total fungal biomass.

3. Determination of carbon dioxide

Samples of 50g of the diets were taken from the centers of each plasticc can and placed in 100mℓ erlenmeyer flasks which were then hermitically sealed using rubber septa. The flasks were stored at 26+1 C ; after 24h an air sample was removed through each septum with a gas-tight syringe and analysed by gas chromatography (Navarro and Donahye, 1972).

4. Mycotoxin determination

The assay for aflatoxin B_1, B_2, G_1 and G_2 was carried out using the method of AOAC 26.031 (Horowitz, 1980) and the assay for zearalenone was done with the modifications of Eppley (1968) and Howell and Taylor (1981), as follows : A grinded sample of 50g was weighed out and extracted with 150mℓ of methanol-water(90 : 60) for 5 min. Filtration was carried out using an Whatman #4 filter paper. The filtrate was added to 100mℓ of hexane two times. The mixtre of the filtrated and hexane was swirled and the hexane layer was discarded. Iron hydroxide gel was added to the hexane layer for further clean up and it was allowed to stand for about 5-10 min. It was filtrated again through Whatman #4 filter paper. 1.5mℓ of 3M HCI was poured into the filtrated in a 250mℓ seperatory funnel and

the seperatory funnel was rotated for 2 min. The mixture was extracted with 100mℓ beacker and evaporated to near dryness. The wall of the beaker was rinsed with the few drops of dichloromethane-methanol (3 : 1) and transferred to a test tube. The residue in the test tube was evaporated using an N-evaporator to less than 0.5mℓ. The sample in the test tube was loaded by using the micro-syringe on the precoated silica gel 60 thin layer chromatography (TLC) plates. Plates were developed in chloroform : acetone (90 : 10, V : V) for aflatoxin (Anonymous. 1980), and in bezene : methanol (97 : 3, V : V) for zearalenone (Eppley, 1968). The plates were viewed, after drying, under ultraviolet light for fluorescent spots and Rf typical to each mycotoxins.

III. RESULTS AND DISCUSSION

Without a mold inhibitor the experimental diets showed an increase in the number of visible colonies after 5 days storage, and after an additional 5 and 15 days, a further increase was observed(Table 2).

Mold inhibitor prevented mold proliferation in each experimental diet, and the mold count in these feeds did not markedly increase even after 20 days. The number of colonies in the feed treated with mold inhibitor after 20 days of storage was almost identical to that of fresh feed. The effectiveness of mold inhibitor, however, was less in each treated diet which contained the higher amount of large particles during all storage durations ($P<0.05$, Table 2).

Schwarger and Adams (1983) reported that severe overgrowth in the ration occurred after 4 days and worsened by the 12th day without mold inhibitor present. But mold inhibitor at the rate of 0.1% controlled the production of mold for the first two weeks. After two weeks mold inhibitor continued to control the production of toxin even though some mold growth occurred (Nelson, 1983 : paster at al., 1985). Table 2 also showed that the different levels of protein in the ration did not show any difference in the number of visible colonies in both the treated and untreated diets with mold inhibitors. 16.8% soybean meal in the rations as a main protein source in this research might not be enough to show the buffering capacity against mold inhibitor. The results of Table 2 show that the amount of mold inhibitor added to the mixed ration should be different depending on the particle size of the feed. When the particles in the ration were smaller the inhibition observed was better (Dixon and Hamilton, 1981[b]).

Accumulation of CO_2 in the plastic cans filled with untreated feed and in plastic cans contai-

Table 2. Mold count in the experimental diets at 5, 10 and 20 days storage

Protein (%)	1.19mm particle size(%)	Moisture content (%)	Mold Count(colonies/g feed)						
			fresh feed	5 days		10 days		20 days	
				UT	T	UT	T	UT	T
18	80	12.6	110	350	130^{A}	8,000	130^{A}	14,000	140^{A}
	40	12.6	117	330	166^{B}	7,000	155^{B}	14,000	180^{B}
12	80	12.7	82	350	128^{A}	7,000	140^{AB}	15,000	140^{A}
	40	12.7	85	350	160^{B}	7,000	150^{A}	13,000	160^{AB}
Mean protein (%)	18			340	148	7,500	143	14,000	160
	12			350	144	7,000	145	14,000	150
1.19mm particle size(%)	80			350	129^{C}	7,500	135^{C}	14,500	140^{C}
	40			340	163^{D}	7,000	153^{D}	13,500	170^{D}

UT : Untreated diet. T : Treated diet.

^{A,B}Values with different superscripts within the 4 treatments are significantly different($P<0.05$).

^{C,D}Values with different superscripts within the different particle sizes are significantly different($P<0.05$).

ning feed treated with mold inhibitor after 10, 20 and 40 days of storage is shown in Table 3.

Content of CO_2 in feed treated with mold inhibitor was uniformly low (below 1%) up to

Table 3. Carbon dioxide concentrations at 10, 20 and 40 days storage

Protein (%)	1.19㎜ particle size(%)	Moisture content (%)	CO_2 cocentration(%)					
			10 days		20 days		40 days	
			UT	T	UT	T	UT	T*
18	80	12.6	5.50	0.29^A	6.10	0.27^A	10.40	0.29^A
	40	12.6	5.26	0.39^B	6.00	0.42^B	10.50	0.41^B
12	80	12.7	5.80	0.25^A	6.50	0.25^A	10.90	0.27^A
	40	12.7	5.30	0.41^B	6.00	0.41^B	10.50	0.47^B
Mean protein (%)	18		5.38	0.34	6.05	0.35	10.45	0.35
	12		5.55	0.33	6.25	0.33	10.70	0.37
1.19㎜ prrticle size(%)	80		5.65	0.27^C	6.30	0.26^C	10.65	0.28^C
	40		5.28	0.40^D	6.00	0.42^D	10.65	0.44^D

UT : Untreated diet. T : Treated diet.

^{A,B}Values with different superscripts within the 4 treatments are significantly different($P<0.05$).

^{C,D}Values with different superscripts within the different particle sizes are significantly different($P<0.01$).

*Protein X particle size interaction was significant ($P<0.05$).

40 days of storage, whereas in the untreated feed CO_2 concentration reached a peak of 10.9% after 40 days. Production of CO_2 has been used to evaluate mold inhibitors in silage (Daniel et al., 1970), in storage tanks of poultry feed (Paster, 1979), and in the evaluation of some organic acids as mold inhibitors (Dixon and Hamilton. 1981[c]).

Paster et al.(1987) studied the antifungal activity of calcium propionate (0.3%), Agrosil (0.2%), and liquid or powdered Adofeed(0.2%) in poultry feed. They found that carbon dioxide started to accumulate in control bins after 15 days of storage and in bins that received calcium propionate or powdered Adofeed it started to rise gradually after 40 days of storage. There were no differences ($P>0.05$) on the production of CO_2 concentrations between 18% and 12% protein levels in the feeds treated with mold inhibitor.

Dixon and Hamilton (1981[b]) reported that the mold inhibitory properties were depressed when soybean meal was added to corn meal.

They found that CO_2 production was inhibited 81% without soybean meal while in the presence of 50% soybean meal CO_2 production was inhibited only 32%. Their research suggests that the CO_2 production would be decreased in the proportion to the content of soybean meal. 16.8% soybean meal in the starter diet of the current research might not be a large enough amount to show the different concentration of CO_2 production. High protein content in the rations have good buffering capacity (Lehninger, 1975). Protein-containing substances would be substances that were neutral or alkaline in reaction (Table et al. 1981).

Table 3 indicated that the particle sizes of the substrate for fungal activity had an influence on the inhibitory properties of the mold inhibitor ($P<0.01$). The effect of particle size was such that the smaller the particle size of the ration the greater the activity displayed by the mold inhibitor. On a practical basis these findings suggest that mold inhibitors will be more efficacious if the milling process produces a finely ground mill. Dixon and Hamilton(1981[b]) conducted research about the effect of particle size of corn meal on the inhibition by propionic acid, which was determined by measuring CO_2 production. They showed that the inhibition was size dependent at the three H_2O levels treated

(20, 25 and 35%) and at the higher concentrations of propionic acid (1.0 and 2.0mg/g of meal). Their findings and the current research results may be explained by the fact that dispersion of inhibitor in the substrate and the ability of an inhibitor to penetrate the substrate are important attributes of mold inhibitors. In the present study protein levels × particle size interaction in the feed treated with mold inhibitor was significant ($P<0.05$) in the feed stored for 40 days (Table 3).

The mold ration in the present study did not contain detectable amounts of aflatoxin B_2, G_1, G_2 and zearalenone (Table 4).

Under the conditions of 29+1℃ and 85% relative humidity a trace amount of ration aflatoxin B_1 was formed after 10 days of storage in the untreated ration. After 40 days of storage the concentration of aflatoxin was sharply increased.

Table 4. Mycotoxin (aflatoxin B_1) production at 10, 20 and 40 days storage

Protein (%)	1.19mm particle size(%)	Moisture content (%)	CO_2 cocentration(%) fresh Feed	10 days UT	10 days T	20 days UT	20 days T	40 days UT	40 days T
18	80	12.6	0.0	2.2	0.0	2.7	1.3	6.8	4.5^{A}
	40	12.6	0.0	2.0	0.0	3.0	1.1	6.8	4.9^{A}
12	80	12.7	0.0	2.1	0.0	2.8	1.1	6.4	3.6^{B}
	40	12.7	0.0	2.4	0.0	2.9	1.3	6.5	4.5^{A}
Mean protein (%)	18		0.0	2.1	0.0	2.8	1.2	6.8	4.7^{C}
	12		0.0	2.3	0.0	2.9	1.2	6.5	4.1^{D}
1.19mm	80		0.0	2.2	0.0	2.8	1.2	6.6	4.0^{F}
prrticle size(%)	40		0.0	2.2	0.0	3.0	1.2	6.4	4.7^{E}

UT : Untreated diet. T : Treated diet.

^{A,B}Values with different superscripts within the 4 treatments are significantly different($P<0.05$).

^{C,D}Values with different superscripts within the different particle sizes are significantly different($P<0.05$).

^{E,F}Values with different superscripts within the different particle sizes are significantly different($P<0.05$).

*Protein X particle size interaction was significant ($P<0.05$).

Schroeder (1969) stated that the three most important factors influencing aflatoxin formation in stored field crops were moisture, relative humidity, and temperature. It is generally accepted that feed stored below 14% will not permit the growth of A. flavus and A. parasiticus. But the present study showed that this assumption is clearly untrue. The production of aflatoxin in the complete rations of the current research appeared under 12.7% moistuer content and 85% relative humidity which agrees with the reports of Jones et al., (1982), and under the temperature of 29+1℃ which was higher than those reported for stored grain (Schroeder and Hein, 1967 ; Jones et al., 1982).

In samples treated with mold inhibitor, aflatoxin production started from 20 days of storage. After 40 days storage the concentration of aflatoxin found in the treated feed was oven four times that found in the feed stored for 20 days.

The concentration of aflatoxin in the treated feed which contain 12% protein and 80% of the particles below 1.19mm was the lowest level, 3.6 ppb, among treatments ($P<0.05$). The different particle size and protein levels in feed affected ($P<0.05$) the formation of aflatoxin after 40 days of storage (Table 4). In the current research aflatoxin production was affected ($P<0.05$) by the protein levels in rations while the fungal counts and CO_2 production were not affected by the protein levels in ration. It is known that the A. flavus-paraciticus group in the Fungi Imperfecti produce aflatoxin (Landecker, 1972).

In is also known that toxin is produced be-

cause the mycotoxin is metabolized, competing fungi use essential nutrients, the environment does not permit toxin formation, and nontoxic strains predominate over toxic strains (Tuite, 1979). We emphasize that the usage level of mold inhibitor in the storage of the commercial ration for preventing the formation of mycotoxin should be modified depending on the conditions, such as particle size or protein level of the feed itself.

Ⅳ. SUMMARY

The effect of two particle size ranges (80% of the particles in the ration less than 1.19㎜ ; and 40% of the particles in the ration less than 1.19㎜) and two different levels of protein (18% and 12%) on the activity of mold inhibitors in commercial ration was determined by measuring mold count, CO_2 production and aflatoxin concentration. A commercial fungistat was mixed in the treated diets at the level of 0.1% (W/W). Two types of experimental diets (18% & 12% protein) which contained 12.6 and 12.7% moisture content each were stored under the forced air humidifier at 85% humidity and a controlled-temperature of 29+1℃ for 5 to 40 days.

Mold count and CO_2 levels in the feed treated with mold inhibitor were significantly higher ($P<0.05$ and $P<0.01$ respectively) when 40% of the ration's particle size was<1.19㎜. The different protein levels in the ration treated with mold inhibitor did not have a significant effect ($P>0.05$) on the mold count and CO_2 production.

Protein level × particle size range interaction on the CO_2 production was significant ($P<0.05$).

Aflatoxin production in the experimental diet with mold inhibitor was significantly ($P<0.05$) affected by the levels of protein and the different particle size ranges. The interaction of protein levels and particle size ranges on the aflatoxin production was significant ($P<0.05$) at 40 days of storage.

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