

Effect of Diet on Adhesion and Invasion of Microflora in the Intestinal Mucosa of Chicks^{1, 2} (40331)

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Published results based on experiments with young chicks (1, 2) do not show conclusive evidence about active microbial participation in modifying the nutritional response of young chicks to a given diet. Our earlier work done in this laboratory (unpublished) with different cereal grains did, however, suggest that counts of microbes in the lumen of the gut differ with age and diet and, depending upon the diet, could be involved in the response of chicks to antibiotic-supplemented diets.

Adhesion of microflora to the intestinal wall in young pigs (3), man (4), and chicks (5) has been observed. The *Lactobacilli* are known to adhere to the epithelium of the crop and bursa in chicks soon after hatching, but no penetration into deeper tissues has been observed (6). Implantation of *Lactobacilli* through the oral route suppressed *Enterococci* in the small intestine and ceca and promoted growth in young chicks (7).

The present investigation was designed to characterize the nature and distribution of intestinal microbes which might adhere to the intestinal epithelium, penetrate the mucosa or become translocated to other organs. The growth response of young chicks on different diets, with and without supplemental penicillin, was also determined.

Materials and methods. Three replicate groups of 10 (five each of male and female) 1-day-old broiler chicks were randomly assigned to each of the eight different diets (Table I) under study. The chicks were

housed in electrically heated battery brooders with wire floors and free access to feed and water. Birds were reared up to 2 weeks of age, and effects of different diets on weekly body weights, feed consumption, and mortality were recorded and analyzed (8).

Microbiological examinations of intestine and tissues. At 10 days of age, three chicks from each treatment (one chick selected randomly from each of the three replicates) were fasted for 16 hr, sacrificed by electrocution, and immersed in a disinfectant (1% septsol) to minimize contamination of internal organs. The livers were then aseptically exposed, the surface of the right lobe was cauterized by a hot metal spatula, and samples were taken from the site (1.25 cm below the surface) with an inoculating loop for subsequent culturing on blood agar plates and incubation at 37° under aerobic and anaerobic conditions. Bacterial cultures were made similarly from the left kidneys. Bacterial isolates from livers and kidneys were identified by morphological characteristics and biochemical tests (9).

A section of small intestine (2.5 cm long) immediately below the yolk-stalk was removed without contaminating the exterior and transferred to preweighed sterile bottles containing 50 ml of phosphate-buffered saline, pH 7.1 (PBS). Each gut sample was opened with sterile scissors, washed with four changes of PBS (50 ml for the first washing, 10 ml for the subsequent washings), weighed, and then ground using a Thomas glass tissue grinder to make a 5% homogenate in sterile reinforced clostridial medium (RCM, BBL 11565) without agar. Serial dilutions of the fourth washing and the intestinal homogenates were made in liquid RCM, and each of at least five serial dilutions was plated in triplicate for bacterial counts. Pour plates of standard method agar (BBL 11638) containing 0.1% starch, 0.5% dextrose, and 5% horse blood and brilliant green bile agar (BBL

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11078) were used for enumeration of aerobes and coliforms, respectively. RCM agar with 5% (v/v) horse blood (with second layer without blood) was used for culturing anaerobes in anaerobic jars with a Gaspak and catalyst system. Microbial adhesion to the intestinal lining was considered to have occurred if the number of colony-forming units (CFU) recovered from the homogenized intestine sig-

nificantly ($P < 0.05$) exceeded that of the fourth washing (12).

Results. Effects of diets on growth, feed, efficiency, and mortality. The chicks fed a diet containing corn gained significantly higher body weights ($P < 0.05$) than chicks fed diets containing either rye or beans (raw or cooked) (Table II). The chicks fed a diet containing rye grew significantly better ($P <$

TABLE I. COMPOSITION OF DIETS AND OUTLINE OF THE EXPERIMENT.

Ingredients (%)	Treatment No.							
	1	2	3	4	5	6	7	8
Premix								
Soybean meal	22.09	22.09	22.09	22.09	3.7	3.7	3.7	3.7
Fish meal	5.00	5.00	5.00	5.00	5.3	5.3	5.3	5.3
Meat and bone meal	2.50	2.50	2.50	2.50	—	—	—	—
Dried whey product	2.00	2.00	2.00	2.00	—	—	—	—
Dehydrated alfalfa	2.50	2.50	2.50	2.50	—	—	—	—
Iodized salt	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Limestone	0.73	0.73	0.73	0.73	1.00	1.00	1.00	1.00
Dicalcium phosphate	1.00	1.00	1.00	1.00	2.00	2.00	2.00	2.00
Vitamin premix ^a	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Mineral premix ^b	0.05	0.05	0.05	0.05	0.10	0.10	0.10	0.10
DL-Methionine	0.04	0.04	0.04	0.04	0.35	0.35	0.35	0.35
Animal fat	—	—	—	—	7.00	7.00	7.00	7.00
Experimental diets								
Premix	36.46	36.46	36.46	36.46	20.00	20.00	20.00	20.00
Yellow corn	63.54	63.54	—	—	—	—	—	—
Rye	—	—	63.54	63.54	—	—	—	—
Raw Red Mexican beans	—	—	—	—	80.00	80.00	—	—
Autoclaved Red Mexican beans ^{c, d}	—	—	—	—	—	—	80.00	80.00
Procaine penicillin (ppm)	—	0.50	—	0.50	—	0.50	—	0.50

^a Vitamin premix at 0.25% of the diet supplies the following per kilogram of the diet: vitamin A, 5500 I.U.; vitamin D₃, 1650 I.C.U.; vitamin E, 4.4 I.U.; riboflavin, 3.3 mg; calcium pantothenate, 4.4 mg (or pantothenic acid, 4.05 mg); niacin, 22 mg; choline chloride, 577 mg; vitamin B₁₂, 0.011 mg; and ethoxyquin, 62.2 mg.

^b Mineral premix at 0.05% of the diet supplies the following per kilogram of the diet: Mn, 50 mg; Fe, 50 mg; Cu, 5 mg; Zn, 50 mg; I, 1.5 mg; Ca, 60 mg; and Co, 0.5 mg.

^c Red Mexican beans were autoclaved at 1.06 kg/cm² of pressure for 30 min and oven-dried at 70°F.

^d Red Mexican beans (*Phaseolus vulgaris* Linneaus var. Othello) were used during these studies.

TABLE II. BODY WEIGHTS, FEED EFFICIENCY, AND MORTALITY OF CHICKS FED DIFFERENT DIETS.^a

Diets	Average body weights (g)		Average feed efficiency (g)	Mortality (%)		
	Age			1 week	2 weeks	Total
	1 week	2 weeks	0-1 week			
Corn	106 ^a	224 ^{a, b}	1.52 ^d	0	0	0
Corn + procaine penicillin	108 ^a	232 ^a	1.47 ^d	0	0	0
Rye	82 ^{c, d}	168 ^c	1.85 ^{c, d}	0	0	0
Rye + procaine penicillin	91 ^b	199 ^b	1.49 ^d	0	0	0
Raw beans	52 ^e	63 ^e	6.25 ^a	11	72	83
Raw beans + procaine penicillin	60 ^d	75 ^e	3.13 ^b	14	52	66
Autoclaved beans	78 ^d	131 ^d	2.30 ^c	2	0	2
Autoclaved beans + procaine penicillin	87 ^{b, c}	162 ^c	1.84 ^{c, d}	0	0	0

^a Means within each column followed by superscripts having common letters are not significantly different ($P = 0.05$) when calculated with Duncan's multiple range test.

0.05) than the chicks fed a diet with raw beans. When autoclaved, the bean diet supported chick growth that was better ($P > 0.05$) than that obtained with the diet containing rye, though it was still significantly lower ($P < 0.05$) than that with the diet containing corn. Penicillin added at a 50 ppm level gave significantly higher body weights ($P < 0.05$) over controls with diets containing rye or beans, but not in chicks fed a diet containing corn.

Birds on diets containing rye or autoclaved beans (Table II) were less efficient than those fed a corn diet. The efficiency of feed conversion was poorest in the chicks fed the diet containing raw beans during the same period. Replacing raw beans with autoclaved beans resulted in improved feed efficiency of chicks. Addition of penicillin to all of these diets markedly improved the feed efficiency.

During the 2-week period, 83% mortality was observed in chicks fed the diet containing raw beans (Table II). When procaine penicillin (50 ppm) was supplemented to this diet, mortality was reduced to 66%. In chicks fed the diet containing autoclaved beans, the mortality was only 2%, and supplement of penicillin to this diet prevented mortality completely. No mortality resulted in chicks fed diets containing corn or rye.

Effects of diets on intestinal microbes. In

chicks fed diets containing corn 10^4 aerobic organisms/g of wet sample from the lumen of the ileum were enumerated (Table III). Replacing rye with corn in the chick diet resulted in a significant decrease ($P < 0.05$) in the viable counts of aerobes in lumen material of the gut and a significant increase ($P < 0.05$) in the viable counts of aerobes adhered to the epithelial wall of the intestine. Feeding of diets containing raw beans to chicks significantly increased ($P < 0.05$) the luminal and epithelial counts of viable coliforms, total aerobes, and total anaerobes. Compared to raw beans feeding of autoclaved beans resulted in a significant decrease ($P < 0.05$) in the viable counts of coliforms and total anaerobes in lumen and of those aerobes adhered to the epithelial wall. Supplementing the diets containing either corn or rye with penicillin (50 ppm) resulted in an insignificant ($P > 0.05$) decrease in viable counts of total anaerobes (excluding coliforms) and total anaerobes. A significant decrease ($P < 0.05$) in viable counts of coliforms and anaerobes was observed in chicks fed diets containing raw or autoclaved beans.

Adhesion of aerobes to the epithelial wall was not affected by feeding diets containing corn, while chicks fed diets containing rye or raw beans showed a significant increase ($P < 0.05$) in adhesion of aerobes to the gut wall.

TABLE III. INFLUENCE OF DIFFERENT DIETS ON THE NUMBERS OF INTESTINAL BACTERIA FREE IN THE LUMEN AND ADHERED TO GUT WALL (ONE INCH BELOW YOLK-STALK) OF CHICKS AT TEN DAYS OF AGE.

Diets	No. of viable organisms/g of wet tissue (log)					
	Coliforms		Total aerobes		Total anaerobes	
	In lumen ^a	Adhered ^b	In lumen	Adhered	In lumen	Adhered
Corn	2.1 ^c	2.4	4.7	4.3	4.9	5.2
Corn + procaine penicillin	2.1	2.2	4.2	3.8	4.7	4.8
Rye	2.0	2.3	3.8	5.1	4.7	5.0
Rye + procaine penicillin	2.0	2.5	3.8	4.7	4.6	4.6
Raw beans	2.9	3.9	5.4	6.4	6.1	6.8
Raw beans + procaine penicillin	2.2	3.4	5.2	5.8	5.6	6.2
Autoclaved beans	2.1	2.9	5.1	5.1	5.4	6.0
Autoclaved beans + procaine penicillin	2.5	3.7	4.9	4.5	5.5	5.7

^a In the fourth washing solution of the sampled intestine.

^b In the 5% homogenates of the sampled intestine that had been washed four times.

^c Least significant difference ($P = 0.05$) for:

	Coliforms	Total aerobes	Total anaerobes
Means within each diet	0.5	0.62	0.44
Means within "in lumen" and "adhered" organisms in each diet	0.31	0.53	0.25

Means within each diet that differ by more than the stated value are significantly different ($P = 0.05$ or less).

Autoclaving of beans prior to feeding reduced the adhesion of aerobes. No significant ($P < 0.05$) adhesion of coliforms to the gut wall was evident in chicks fed diets containing either corn or rye; however, feeding diets containing beans (raw or autoclaved) resulted in a significant increase ($P < 0.05$) in adhesion of coliforms and total anaerobes. Supplementation of these diets with penicillin significantly ($P < 0.05$) reduced the adhesion of aerobes (in chicks fed diets containing corn, rye, or autoclaved beans) and significantly ($P < 0.05$) increased the adhesion of coliforms in chicks fed the diet containing rye.

Colonies isolated from the liver and kidney of the chicks fed diets containing raw beans were identified as *Escherichia coli* and *Streptococcus fecalis*. No bacteria were isolated from livers and kidneys of chicks fed diets containing either corn or rye. No bacteria were observed in the liver and kidney of chicks fed the autoclaved beans.

Discussion. The results of the present study support earlier observations (1, 2) which showed that the organisms adhering to or inside the gut wall might be significantly related to growth and survival. In the present studies, the aerobes either adhered to or penetrated the ileal wall in chicks fed diets containing rye or raw beans, and a major group among these adhering organisms was coliforms. Attachment of *E. coli* to the intestinal wall has been reported earlier (10-12). In chicks fed a diet containing raw beans, an adhesion of anaerobes to the gut wall was evident.

Adhesion of aerobes (excluding coliforms) to the gut wall in the chicks fed the diet containing rye suggested that these microbes play a role in modifying the response of chicks to dietary antibiotics. MacAuliffe and McGinnis (13) obtained a much greater growth improvement with antibiotic supplement to a diet containing rye than to a similar diet containing corn.

The present results show that beans, when included in the raw form in a chick diet, cause poor growth that is ameliorated by autoclaving the beans. These observations extend and support earlier findings (14-17). Feeding autoclaved beans reduced adhesion of aerobes to the gut wall. The isolation of *E.*

coli and *S. fecalis* from the livers and kidneys of chicks fed diets containing raw beans is highly suggestive of microbial involvement in the heavy mortality observed in chicks on these diets and confirms our recent observation (17) that these organisms cause mucosal tissue damage, penetrate the epithelium and cause septicemia, organ invasion, and death. Feeding a diet containing autoclaved beans caused adhesion of coliforms to the intestinal wall, but no organisms were isolated from livers and kidneys. This indicates that the factors in raw beans which permit microbial penetration of the gut wall are heat-labile. Earlier Jayne-Williams and Hewitt (18) implicated strains of *E. coli* being responsible for the lethal effects of raw beans. Furthermore, they postulated that hemagglutinins (or possibly other heat-labile toxic factors) may interfere with normal body defense mechanisms, thereby allowing the normal intestinal bacteria to pass through lumen to other body tissues. The findings of the present study support the above hypothesis (19, 20). The increase in the microbial adhesion to the intestines of chicks fed diets containing either rye or raw beans could be the result of lectin-mediated attachment of bacterial cells to the intestinal wall since lectins are known to combine with bacteria as well as intestinal mucosal cells. Our observations confirming it to be so will be reported in a separate publication.

A significant reduction in mortality of chicks (from 83% on diets containing raw beans to 2% on diets containing autoclaved beans) observed in this study is similar to that reported for Japanese quail (18). Penicillin added to the diet containing raw beans did not prevent mortality in chicks completely.

In our earlier work (unpublished) on the influence of dietary levels of raw beans on growth of chicks, a 46% dietary level resulted in 7% mortality versus 83% mortality in chicks fed at a level of 80% raw beans.

From the above observations and related earlier work in this laboratory (17), it is postulated that feeding diets containing raw beans causes the normal intestinal microflora to colonize on the intestinal wall in young chicks, and its magnitude is proportional to the level of raw beans in the diet. The more extensive colonization or damage to the in-

testinal mucosa due to the components of raw beans enables the microorganisms to become more invasive, as evidenced by the presence of aerobic organisms in the livers and kidneys. Further work on pathological examination of liver and kidney of chicks fed diets containing raw beans is in progress.

Summary. Compared to chicks fed a diet containing corn, those fed a diet containing rye showed significantly lower growth that was ameliorated by antibiotic supplement to the diet. Adhesion of aerobes (excluding coliforms) and anaerobes to the intestinal wall was indicated in the chicks fed the diet containing rye which was reduced by penicillin supplementation. There was no mortality in chicks fed diets containing corn or rye, whereas the poorest growth and a very high mortality resulted in chicks fed diets containing raw beans. Such adverse effects were alleviated by dietary antibiotic supplement. High numbers of aerobes, mainly coliforms, were found adhered to the mucosal wall of the chicks fed diets containing raw beans, and *E. coli* and *S. fecalis* organisms were isolated from their kidneys. Autoclaving the beans greatly improved growth, reduced mortality, and caused no adhesion of intestinal aerobes to the mucosal wall. A penicillin supplement to the diet resulted in further improvement of growth and reduction of mortality.

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