Influence of Diets Low in Protein or Lysine on the Intestinal Flora of Chicks with Reference to Cecal Contents

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Summary To determine the effect of a certain diet on the intestinal flora of chicks, the cecal flora of chicks fed on a low protein or low lysine diet was examined. The cecal flora of chicks fed on the low protein diet was similar to that of chicks fed on a normal protein diet, but the total count of bacteria, Eubacterium and Enterobacteriaceae in the cecal content of chicks fed on the low lysine diet containing a formulated amino acid mixture minus lysine was significantly lower than that of chicks fed on the control diet.

The total count of Lactobacillus in the cecum was remarkably reduced by feeding the amino acid diet, especially the low lysine diet. Levels of most free amino acids in the cecal contents of the low protein group were significantly lower than those of the control. Lysine, leucine, phenylalanine, methionine, histidine, glycine and tyrosine of the cecal contents in the low lysine group were significantly lower than those of the control group.

Key Words cecal flora, low protein diet, low lysine diet, cecal contents, free amino acids

It is well known that intestinal microflora of chicks has a beneficial effect on the host of sparing nitrogen, under the condition of a nitrogen-free diet (1, 2) or a low lysine diet (3). On the other hand, there are some competitive interactions between the host and its intestinal microflora under the condition of an adequate diet (4). However, little is known about the intestinal flora of chicks given a low protein diet or a low lysine diet. In this work, intestinal flora and nitrogen compounds of chicks given a low protein diet and also a low lysine diet were examined, in order to determine the relationship between the intestinal flora and nitrogen-sparing action.
Animals and diets. Twenty-six female and male broiler chicks of White Rock strain were used in all experiments. One-day-old chicks supplied by a commercial broiler facility were fed on a commercial diet (CLEA JAPAN, Inc.) without chick antibiotics for 2 weeks, and then fed on test diets ad libitum for 2 weeks under the conditions of constant temperature (24 ± 1°C) and relative humidity (55 ± 5%). The compositions of four test diets, i.e. a low protein diet and its control diet, and a low lysine diet and its control diet are given in Tables 1 and 2.

Sampling procedure. After feeding the test diets, each chick was anesthetized with ethyl ether between 11:00 and 12:00 a.m.

Table 1. Composition of low protein diet.

| Ingredient            | Low protein (%) | Normal protein (%) |
|-----------------------|-----------------|--------------------|
| Corn starch           | 76              | 59                 |
| Whole egg protein     | 3               | 20                 |
| Lard                  | 5               | 5                  |
| Soybean oil           | 5               | 5                  |
| Mineral mixture       | 5               | 5                  |
| Cellulose powder      | 5               | 5                  |
| Vitamin mixture       | 1               | 1                  |

a Purified egg protein made by Taiyo Foods Co., Ltd. (Yokkaichi, Japan) was used.
b,c Composition is the same as that in diets used by Ishibashi (9).

Table 2. Composition of low lysine diet.

| Ingredient                | Low lysine (%) | Normal lysine (%) |
|---------------------------|----------------|-------------------|
| Corn starch               | 59             | 59                |
| Amino acid mixture        | 19             | 19                |
| Lard and soybean oil      | 10             | 10                |
| Mineral mixture           | 5              | 5                 |
| Cellulose powder          | 5              | 5                 |
| Vitamin mixture           | 1              | 1                 |
| Lysine                    | 0.2            | 1                 |
| Glutamic acid             | 0.8            |                   |

a One kilogram of the diet contained the following amino acids (g): L-Arg-HCl, 12.7; L-His-HCl, 4.3; L-Trp, 2.1; L-Thr, 6.4; L-Val, 11.0; L-Ile, 11.4; L-Leu, 14.3; Gly, 14.5; L-Glu, 83.3; L-Phe, 8.8; L-Tyr, 6.8; L-Met, 5.8; L-Cys, 3.7; L-Pro, 5.0. b,c Composition is the same as that in diets used by Ishibashi (9).
Crop and cecal contents were weighed and aliquots thereof were transferred into anaerobic transport media \((5)\) and cultured promptly. The remaining aliquots were used for the analysis of nitrogen compounds.

**Bacterial procedure.** Bacterial techniques used were essentially the same as those of Mitsuoka et al. \((4)\) with some modification. Media and cultivation are summarized in Table 3. The compositions of the media have been described in previous papers \((4-7)\). Five different non-selective media and 10 different selective media were used for the isolation of bacteria. All media except modified Medium 10 and \((\text{NH}_4)_2\text{SO}_4\) medium were poured into ordinary Petri dishes. Modified Medium 10 and \((\text{NH}_4)_2\text{SO}_4\) medium were prepared by the “plate-in-bottle” method \((4)\). Samples were weighed and homogenized with 10 or 100 volumes of sterile anaerobic diluent in a homogenizer for about 30 sec in a \(\text{CO}_2\) atmosphere. From appropriate dilutions, 0.05 ml of sample was spread with a sterile glass rod over a half to a quarter of the whole surface of each agar medium, and incubated under the conditions given in Table 3.

After incubation, the number of colonies of each recognizable type was
counted for all plates. A colony of each type was selected and identified. Organisms were subjected to Gram staining and were classified into broad groups on the basis of morphological and biochemical characteristics. In a suitable dilution for each culture plate, the number of colonies of the same bacterial group was counted and expressed as counts per g of wet material. When the count on the non-selective media was higher than that on the selective media, the former was regarded as the accurate viable count of the corresponding bacterial group. A direct microscopical count was also measured using the procedure for direct milk count.

Chemical analysis. Total nitrogen and 5% trichloroacetic acid (TCA)-soluble nitrogen (non-protein nitrogen, NPN) of cecal contents were determined by the micro-Kjeldahl procedure. Ammonia nitrogen was determined by Conway’s method (8). Free amino acids were determined using an automatic amino acid analyzer (JEOL, JLC-6AH).

RESULTS

Body weight gain and cecal contents

Results for body weight gain and cecal contents are shown in Tables 4 and 5. Final body weights and body weight gains in the low protein and low lysine groups were significantly lower than those in the respective control group, but cecal contents of the deficient groups were not significantly lower.

Table 4. Body weight and cecal contents of chicks fed on a low protein diet and a normal protein diet.

|                | Low protein   | Normal protein |
|----------------|---------------|----------------|
| Body weight    |               |                |
| Initial (g)    | 330 ± 5a      | 310 ± 12       |
| Final (g)      | 276 ± 36**b   | 553 ± 7        |
| Body weight gain (g) | −54 ± 25***  | 243 ± 3        |
| Cecal content (mg/head) | 515 ± 147  | 803 ± 406      |

* Means ± SD for 6 chicks. ** t-test analysis, *** p < 0.001.

Table 5. Body weight and cecal contents of chicks fed on a low lysine diet and a normal lysine diet.

|                | Low lysine   | Normal lysine |
|----------------|--------------|---------------|
| Body weight    |               |               |
| Initial (g)    | 300 ± 15a    | 276 ± 22      |
| Final (g)      | 311 ± 10**b  | 448 ± 58      |
| Body weight gain (g) | 11 ± 15***  | 172 ± 46      |
| Cecal content (mg/head) | 525 ± 124  | 750 ± 323     |

* Means ± SD for 6 chicks. ** t-test analysis, *** p < 0.01, **** p < 0.001.
Table 6. Crop flora of chicks fed on a low protein diet and a normal protein diet.

| Bacterial group      | Low protein          | Normal protein        |
|----------------------|----------------------|-----------------------|
| Total bacteria       | 8.59 ± 0.46a         | 8.61 ± 0.74           |
| Enterobacteriaceae   | 7.94 ± 0.93          | 7.64 ± 0.11           |
| Streptococcus        | 6.57 ± 0.52          | 6.97 ± 1.37           |
| Staphylococcus       | 3.83 ± 0.58 (3)b     | 4.73 ± 1.04 (5)       |
| Lactobacillus        | 8.30 ± 0.49          | 8.16 ± 1.20           |
| Bifidobacterium      | 7.18 ± 1.67 (3)      | 7.67 ± 0.06 (2)       |
| Eubacterium          | 6.66 ± 0.98 (4)      | 5.71 ± 3.65 (2)       |
| Bacteroidaceae       | 5.31 ± 2.05 (4)      | 7.45 ± 0.44 (4)       |
| Peptococaceae        | 6.19 ± 0.04 (3)      | 7.80 ± 0.42 (3)       |
| Clostridium          | 5.32 ± 2.28 (4)      | 7.25 ± 0.60 (2)       |

*a Mean ± SD of log bacterial counts/g (when present). b Figures in parentheses refer to the numbers of chicks harboring the organism.

Table 7. Cecal flora of chicks fed on a low protein diet and a normal protein diet.

| Bacterial group      | Low protein          | Normal protein        |
|----------------------|----------------------|-----------------------|
| Total bacteria       | 11.28 ± 0.20a        | 11.31 ± 0.33          |
| Enterobacteriaceae   | 10.08 ± 0.65         | 9.66 ± 0.82           |
| Streptococcus        | 8.61 ± 0.56 (5)b     | 8.89 ± 0.90           |
| Staphylococcus       | 3.69 ± 0.36 (5)      | 3.73 ± 0.57 (3)       |
| Lactobacillus        | 8.41 ± 1.33          | 7.88 ± 1.59           |
| Bifidobacterium      | 10.44 ± 0.24 (5)     | 10.29 ± 0.83 (4)      |
| Eubacterium          | 10.40 ± 0.52         | 10.39 ± 0.93          |
| Bacteroidaceae       | 10.84 ± 0.33         | 10.90 ± 0.40          |
| Peptococaceae        | 10.18 ± 0.63         | 10.00 ± 1.25          |
| Clostridium          | 8.92 ± 1.20 (5)      | 9.03 ± 0.71 (5)       |
| Gemmiger             | 9.77 ± 0.27 (4)      | 9.96 ± 0.21 (3)       |
| Curved rod           | 9.52 ± 0.27 (4)      | 9.54 ± 0.53 (5)       |

*a,b See Table 6.

Effect of the low protein diet on microbial flora of chicks

Microbial floras of crops and ceca of chicks fed on the low protein diet are shown in Tables 6 and 7 respectively. No significant difference was found between the flora of the low protein group and that of the controls. Figure 1 shows the counts for growth on (NH₄)₂SO₄ medium, expressed as percentages of total bacterial counts in the cecal contents of chicks. The counts obtained from the low protein group were greater than those of the control.

Effect of the low lysine diet on microbial flora of chicks

The cecal flora of chicks fed on low lysine diet is shown in Table 8. Total counts
of bacteria, *Enterobacteriaceae*, and *Eubacterium* in cecal contents were significantly lower than those of chicks fed on the control diet. The counts for the other bacterial groups under the condition of feeding the low lysine diet seemed to be lower than those on feeding the control diet.

The number of lactobacilli in the cecal contents of chicks receiving the protein diets was about $10^8$ (Table 7), and the number of lactobacilli decreased remarkably on feeding the amino acid diets, especially the low lysine diet (Table 8).

**Effect of the low protein and the low lysine diets on nitrogenous compounds of cecal contents**

Results for nitrogen compounds in cecal contents are summarized in Tables 9–12. Concentrations of total nitrogen and ammonia nitrogen in the cecal contents

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**Table 8. Cecal flora of chicks fed on a low lysine diet and a normal lysine diet.**

| Bacterial group       | Low lysine   | Normal lysine |
|-----------------------|--------------|---------------|
| Total bacteria        | 10.56 ± 0.34*** | 11.42 ± 0.22 |
| *Enterobacteriaceae*  | 8.34 ± 0.21**  | 10.42 ± 0.84 |
| *Streptococcus*       | 8.16 ± 1.15   | 9.28 ± 1.69   |
| Yeast                 | 6.45 ± 3.75 (2) | 5.95 ± 2.16 (4) |
| *Lactobacillus*       | 3.5 (2)b | 5.95 ± 2.16 (4) |
| *Bifidobacterium*     | 9.20 ± 0.20 (2) | 10.2 ± 0.15 (2) |
| *Eubacterium*         | 9.62 ± 0.61* | 10.6 ± 0.42 |
| *Bacteroidaceae*      | 10.1 ± 0.27 | 11.0 ± 0.35 |
| *Peptococaceae*       | 9.72 ± 0.37 | 9.88 ± 0.40 |
| *Clostridium*         | 4.70 ± 2.29 | 6.10 ± 2.42 (4) |
| Gemmiger              | 9.55 ± 0.15 (2) | 10.7 ± 0.72 (3) |
| Curved rod            | 9.05 ± 0.25 (2) | 10.1 ± 0.15 (2) |

*a,b* See Table 6. Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. 
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Table 9. Nitrogen compounds in the cecal contents of chicks fed on a low protein diet and normal protein diet (mg N/g).

|                      | Low protein     | Normal protein |
|----------------------|-----------------|----------------|
| Total nitrogen       | 10.9 ± 1.5a     | 22.0 ± 8.0     |
| Non-protein nitrogen | 2.25 ± 1.15*    | 3.98 ± 0.21    |
| Ammonia nitrogen     | 0.53 ± 0.15     | 0.73 ± 0.16    |

*a Values are means ± SD for 6 chicks. *p < 0.05.

Table 10. Nitrogen compounds in the cecal contents of chicks fed on a low lysine diet and a normal lysine diet (mg N/g).

|                      | Low lysine      | Normal lysine  |
|----------------------|-----------------|----------------|
| Total nitrogen       | 18.0 ± 4.5a     | 27.0 ± 6.1     |
| Non-protein nitrogen | 3.70 ± 0.70     | 4.20 ± 0.80    |
| Ammonia nitrogen     | 0.46 ± 0.17*    | 0.83 ± 0.27    |

*a Values are means ± SD for 6 chicks. *p < 0.05.

Table 11. Free amino acid pattern of cecal contents of chicks fed on a low protein diet and a normal protein (μmol/g).

| Amino acid | Low protein | Normal protein |
|------------|-------------|----------------|
| Val        | 1.50 ± 0.89*** | 4.60 ± 0.28    |
| Lys        | 0.85 ± 0.62*   | 2.15 ± 0.45    |
| Leu        | 0.56 ± 0.39*** | 2.00 ± 0.17    |
| Thr        | 0.75 ± 0.45**  | 2.18 ± 0.48    |
| Ile        | 0.26 ± 0.18*** | 1.05 ± 0.10    |
| Phe        | 0.33 ± 0.18    | 0.32 ± 0.01    |
| Met        | 0.85 ± 0.56**  | 1.70 ± 0.11    |
| His        | 0.14 ± 0.05*** | 0.39 ± 0.03    |
| Arg        | 0.09 (1)       | 0.27 ± 0.17    |
| Ala        | 3.27 ± 2.02*   | 5.80 ± 0.36    |
| Glu        | 2.21 ± 1.45**  | 12.3 ± 5.12    |
| Gly        | 0.89 ± 0.44**  | 1.77 ± 0.24    |
| Asp        | 0.75 ± 0.49**  | 2.00 ± 0.71    |
| Pro        | 1.30 (1)       | 4.54 (1)       |
| Ser        | 0.76 ± 0.49**  | 1.78 ± 0.46    |
| Tyr        | 0.30 ± 0.21    | 0.23 ± 0.09    |
| Cys        | —              | —              |
| Orn        | 0.22 ± 0.13**  | 0.48 ± 0.02    |
| GlcN       | 0.52 ± 0.24*   | 1.66 ± 0.02    |
| GalN       | 6.21 ± 3.53    | 2.57 ± 0.63    |

*p < 0.05, **p < 0.01, ***p < 0.001 (n = 6).

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of the low protein group were not significantly lower than those of the control group. The non-protein nitrogen concentration of the low protein group was significantly lower, and most of the free amino acids except phenylalanine, arginine, proline, and tyrosine, were significantly lower than those of the control (Table 11).

Total nitrogen and non-protein nitrogen in the cecal contents of chicks fed on the low lysine diet were not significantly lower than those of the control. Lysine, leucine, phenylalanine, methionine, histidine, glycine and tyrosine of cecal contents of chicks fed on the low lysine diet were significantly lower than those of the control (Table 12). Ammonia nitrogen concentration in the cecal contents of the low-lysine group was significantly lower than that of the control.

**DISCUSSION**

Intestinal flora and fecal flora of chicks have already been examined by Ochi et al. (12) and Kimura et al. (13), and it is well known that intestinal flora become established 2–4 weeks after feeding (12). In our present data, the counts of total bacteria and Enterobacteriaceae were higher and those of lactobacilli were lower, than those of the earlier data (12). The counts for the other bacterial groups in our

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present experiment were of the same level as that of the earlier data (12). These differences were thought to derive from our use of purified diets.

The body weight gain and the concentrations of non-protein nitrogen and of most amino acids were significantly reduced by the intake of the low protein diet, but the cecal flora was not significantly changed. Most of the predominant bacteria (80%) isolated from the cecal contents of chicks given the low protein diet were grown in the (NH_4)_2SO_4 medium. In contrast, only a few bacteria (8%) isolated from the cecal contents of chicks given the normal protein diet were grown in the same medium (Fig. 1). These results suggest that the predominant bacteria of the cecal contents of chicks given the low protein diet were adapted to the low nitrogen environment in the cecum, and acquired an ability to grow on the medium which contains ammonia and small amounts of amino acids and proteins.

At a low level of lysine, total bacteria, Enterobacteriaceae and Eubacterium counts were reduced. It is uncertain whether the decrease in these bacterial counts is a cause or a result of the beneficial effect of nitrogen reutilization of the host fed on the low lysine diet (3). A decrease in bacterial counts in the cecal contents of chicks was observed only at a low level of lysine in this experiment, but the concentrations of nitrogenous compounds in the low lysine group were not the lowest among the four diet groups (normal protein diet, low protein diet, normal lysine diet and low lysine diet). Therefore, the decrease in the bacterial counts could not be explained by the decrease of the nitrogenous compounds. Lactobacilli, which were reported to exist in counts of 10^9 per g wet weight of the cecal content of chicks (12), were reduced or not detected in the cecal contents of the amino acid diet groups, the low lysine group in particular (10^3.5).

Barnes (14) suggested that the cecum of chicks is a site for reabsorption of water and non-protein nitrogen, and Salter and Fulford (15) concluded that the intestinal flora has little influence on the digestion of dietary proteins but may play an important role in the degradation of endogenous proteins. Proteins in the cecum of chicks given amino acid diet were endogenous, because the test diets did not contain proteins. Proteins in the cecum of chicks given the protein diets were also thought to be endogenous because proteins in the cecal contents of the chick given the protein diets were as few as those of the lysine diets (Tables 9 and 10). These results support the suggestion of Salter and Fulford (15). Protein and amino acids in the test diets given to chicks might be digested and absorbed up to the time of the ingesta reaching the cecum, only endogenous proteins existing in the cecum.

Remarkable differences of cecal content weights existed between the low and normal protein diets and between the low and normal lysine diets but were not significant, because individual variation was great.

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