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Effects of Feeding Different Levels of Low Crude Protein Diets with Different Levels of Amino Acids Supplementation on Layer Hen Performance

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ABSTRACT

The use of synthetic amino acids (methionine, lysine, threonine) makes the formulation of diets with minimum levels of crude protein possible. The objective of this study was to determine the effects of low crude protein diet with amino acids supplementation on the performance, egg production, small intestine villus height and faecal LAB and ENT count in laying hens. A total of 144 16-week old layer hens of Hisex brown were assigned to four dietary treatments: 17.5% CP (control); (ii) 17.5% CP; (iii) 17% CP; and (iv) 16.5% CP supplemented with amino acids. Treatment group supplemented 17% CP was significantly higher ($P<0.05$) in egg production than other groups. Nonetheless, there were no significant differences ($P>0.05$) in egg quality. Results showed significant differences ($P<0.05$) in both villi height and crypt depth in duodenum, jejunum and ileum. The treatment supplemented with 17% CP showed a significant reduction ($P<0.05$) in faecal pH compared to the other groups. There was a significant different ($P<0.05$) in LAB and ENT count and LAB/ENT ratio among the dietary treatments. In conclusion, 17% CP treatment supplemented with amino acids demonstrated the best effects in improving the hens' egg production, small intestine villus height and even promoting beneficial effects of faecal microflora.

Keywords: Methionine, lysine, egg production, egg quality, microflora, morphology

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INTRODUCTION

Inclusion of synthetic amino acid in poultry rations is becoming very common in poultry industry as chickens can only utilise approximately 40% of the dietary protein (NRC, 1994; Saki et al., 2012) and it has been proven that decreasing crude protein level in the diet has many advantages. However, commercial amino acid must be supplemented to meet the requirements of limiting amino acid as the dietary protein is decreased (Mousavi et al., 2013). In corn soybean meal diets for laying hens, methionine, lysine and threonine are generally the first and next limiting amino (Gheisar et al., 2011). Numerous studies on low crude protein diet with amino acid supplementation have been conducted on various classes of poultry essential amino acids such as methionine, lysine, threonine and tryptophan that have become economically available in recent years due to technological advances (Yakout, 2010). Recent reports indicated that promising results in terms of maintaining birds' performance and maximising profitability can be obtained by the use of low protein diets with supplementation of amino acid for laying hens (Zeweil et al., 2011; Kashani et al., 2014).

According to Rao et al. (2013), low protein diet with supplementation of synthetic amino acid is able to minimise nitrogen excretion, production cost, intestinal disorder and amino acid excess. Reducing dietary crude protein increased the efficiency of utilisation of dietary crude protein and improved poultry tolerance to high ambient temperature (Zeweil, 2011). Laudadio et al. (2012a) also stated that the dietary protein level reduction in the broilers' rations under hot environmental conditions could be advantageous compared to the conventional feeding programmes.

Thus, it has become more evident that synthetic amino acid inclusion in diets allows nutritionists to further decrease crude protein while more effectively meeting the birds' amino acid requirements for maintenance and tissue accretion (Gheisar et al., 2011). Several investigators have reported that the requirements for certain essential amino acids for laying hens increase with the increase in dietary level of protein (Adeyemo et al., 2012).

Commercial amino acids must be used in order to meet the requirements of limiting amino acids due to the dilution of amino acids as the dietary protein is reduced. Inadequate knowledge about the essential amino acid requirements of laying hens, essential amino acid content of feed ingredients, digestibility and bioavailability of amino acids in feed ingredients and proper ratio between essential amino acids in low protein diets may have been the reasons for the inferior performance of hens fed a low protein, amino acid-supplemented diet (Mousavi et al., 2013). A few studies have been done with layer hen fed with 17.5% CP as control and 16.5 % CP for lower level in early laying phase. The current trial was carried out to determine how much dietary crude protein could be reduced, while supplementing diets with constant amount of synthetic amino acids such as

DL-methionine, L-lysine and L-threonine on production performance, egg quality, villus height and crypt depth and faecal pH, LAB and ENT count of commercial laying hens during production phase, i.e. from 20 to 32 weeks of age.

MATERIALS AND METHODS

In present experimental study, 16 week-old birds were kept in Ladang 2 Poultry Unit, Universiti Putra Malaysia, Serdang. A total of 144 Hisex Brown birds were randomly divided into four treatment groups with 6 replications of 36 birds per treatment. The feeding period lasted for 17 weeks, starting from the time when the birds were 16 weeks of age and ending when they were 32 weeks of age, with the addition of four weeks for acclimatisation purpose. Four dietary treatments consisted of: (T1) 17.5% CP (without supplementation of lysine and threonine; (T2) 17.5% CP diet; (T3) 17% CP and (T4) 16.5% CP diets supplemented with methionine, lysine and threonine. Diets were formulated to meet or exceed the minimum amino acid standards, as recommended by Hisex Brown Management Guide (2008), and water was provided *ad-libitum*. The compositions of the basal diet are shown in Table 1.

The individual feed intake was recorded weekly, while feed conversion ratio and live body weight gain were calculated at the end of the study. Eggs from individual cages were collected daily and weighed. The hen/day egg production and feed conversion ratio were calculated as the rate of production per hen per day and feed intake/egg mass. Live body weight gain (LBWG) was calculated as the difference between the initial and final body weights. At the end of the experiment, twelve birds of each treatment were randomly selected and slaughtered for digesta and small intestine samples for further analysis.

A total of 30 eggs per treatment were randomly collected and used to determine the egg quality. These measurements involved Haugh unit, eggshell thickness, eggshell weight, yolk percentage and albumen percentage. The Haugh unit was calculated using the egg analyser ORKA EggAnalyzer®. Eggshell thickness was measured in micrometer (μm) using a digimatic micrometre on the large end, equatorial region and small end. Meanwhile, albumen percentage was calculated as albumen weight over egg weight meanwhile yolk percentage was calculated as yolk weight over egg weight.

Different sections of small intestine were obtained for the morphometric analysis, as described by Choe et al. (2012). Segments of about 5cm long were removed from the small intestines (duodenum, jejunum and ileum) at the following locations: (i) from gizzard outlet to the end of pancreatic loop, (ii) segment between pancreatic loop and Meckel's diverticulum, and (iii) segment between Meckel's diverticulum and ileo-caecal junction was removed. The intestinal segments were flushed with 10% neutral buffered formalin solution and then used for the morphometric analysis. Then, the segments were fixed in 10% neutral buffered formalin solution overnight.

Table 1
Ingredients and composition of the experimental diets

Parameter	Dietary Treatment[1]			
	T1	T2	T3	T4
Crude Protein (%)	17.5	17.5	17.0	16.5
Corn	46.30	46.34	47.00	47.24
Crude Palm Oil	3.45	3.45	3.44	3.52
Wheat Pollard	11.80	11.97	12.45	12.77
Soy Bean Meal	22.86	22.67	21.75	21.64
Fish Meal	4.30	4.34	4.00	3.24
Dicalcium Phosphate	0.80	0.79	0.85	0.99
Calcium Carbonate	8.00	8.00	8.00	8.00
Vitamin Premix[2]	0.55	0.55	0.55	0.55
Mineral Premix[3]	0.55	0.55	0.55	0.55
Salt	0.06	0.06	0.06	0.08
Antioxidant	0.55	0.55	0.55	0.55
Toxin Binder	0.55	0.55	0.55	0.55
L-Lysine	0.00	0.01	0.04	0.07
DL-Methionine	0.15	0.16	0.16	0.17
L-Threonine	0.00	0.01	0.03	0.05
Calculated Analysis [4] (%)				
Crude Protein	17.5	17.5	17.0	16.5
Energy kcal/Kg	2811	2811	2811	2811
Fat	5.65	5.67	5.66	5.61
Fiber	3.61	3.68	3.68	3.68
Calcium	3.62	3.63	3.61	3.66
Available Phosphorus	0.43	0.43	0.43	0.43
Arginine	1.10	1.09	1.05	1.02
Lysine	0.97	0.98	0.97	0.98
Methionine + Cysteine	0.73	0.74	0.73	0.73
Methionine	0.46	0.47	0.46	0.47
Threonine	0.64	0.65	0.65	0.65
Tryptophan	0.20	0.20	0.20	0.19

[1] T1: 17.5% CP without essential amino acid supplementation (negative control);
T2: 17.5% CP with amino acid supplementation (positive control);
T3: 17% CP with amino acid supplementation;
T4: 16.5% CP with amino acid supplementation.
[2] Provided per kg diet: Fe 100mg; Mn 110mg; Cu 20mg; Zn 100mg; 12mg; Se 0.2mg; Co 0.6mg.
[3] Provided per kg diet: Vitamin A 6,667 IU; Vitamin D 1,000 IU; Vitamin E 23 IU; Vitamin K3 1.33mg; Cobalamin 0.03mg; Thiamin 0.83mg; Riboflavin 2mg; Folic Acid 0.33mg; Biotin 0.03mg; Pantothenic Acid 3.75mg; Niacin 23.3mg; Pyridoxine 1.33mg.
[4] The diets were formulated using Feedlive International Software (Thailand).

Intestinal samples were then excised, dehydrated in a tissue processing machine (Leica Microsystems K. K., Tokyo, Japan) and embedded in paraffin wax. Sections of 4 mm were cut from each of the sample, fixed on slides, stained with haematoxylin and eosin, mounted and examined under light microscopes. The distance from the tip of the villus to the villus crypt junction represents villus height, while crypt depth was defined as the depth of the invagination between adjacent villi. The villi height and crypt depth were measured using an image analyser.

Fresh faecal droppings were collected aseptically once a week using a sterile plastic bag from every treatment group and stored in the chiller prior to the laboratory analysis. The faecal lactic acid bacteria (LAB) and *Enterobacteriaceae* (ENT) population were determined using the method described by Foo et al. (2003) and Loh et al. (2010). The faecal samples were diluted in sterile peptone water to 10% (w/v) and left at room temperature for an hour prior to tenfold serial dilutions (v/v). Enumerations of LAB were carried out on Lactobacillus agar DE Man, ROGOSA and SHARPE (MRS-agar, Merck, KgaA, Darmstadt, Germany). The plates were incubated anaerobically at 30ºC for 48 hours. ENT were spread plated and enumerated on Eosin-methylene-blue lactose sucrose agar plates (EMB-agar, Merck, KgaA, Darmstadt, Germany) and incubated aerobically for 24 hours at 37ºC. The number of colony forming units (CFU) was expressed as the base 10 logarithm of CFU (log10 CFU) per gram. All the samples were repeated in triplicates and the LAB/ENT ratio was also calculated. One gram of fresh faecal sample was mixed homogeneously with 9ml of deionised distilled water in a sterile tube. The pH value was measured using a Mettler-Toledo pH meter with a glass electrode (Mettler-Toledo, England). The faecal pH values were determined as stated by Choe et al. (2012).

All the collected data were subjected to SAS statistical analysis. The means and their standard errors were computed using One Way Analysis of Variance (ANOVA). Duncan's Multiple Range Test System was used to compare the significant difference between the treatments at $P<0.05$. The results were expressed as the mean ± standard error of mean.

RESULTS

The growth performance for the layer hen fed low crude protein diet supplemented with commercial amino acid is shown in Table 2. In particular, the LBWG and egg weight of hens had no significant differences ($P>0.05$) when fed with different levels of low crude protein diets. Treatment 3 (17% CP) had significantly higher ($P<0.05$) egg production and egg mass than other groups. As for the feed intake, there were significant differences ($P<0.05$) among the treatment groups. T4 had the lowest feed intake and T1 had the highest feed intake. FCR was significantly different ($P<0.05$) among the treatments, with T3 having the lowest FCR at 1.79.

The egg quality of layer hen fed with low crude protein diet supplemented with amino acids is shown in Table 3. There were no significant differences (P>0.05) in Haugh units, yolk and albumen weight percentage, eggshell weight and thickness among the treatment groups.

The intestinal villus height and crypt depth for birds fed with low crude protein diet with amino acid supplementation are presented in Table 4. The results showed significant differences (P<0.05) in both villi height and crypt depth in duodenum, jejunum and ileum. Duodenal, jejunum and ileum villi height in T1 and T3 were significantly higher (P<0.05) than T2 and T4. The duodenal, jejenum and ileum crypt depth showed similar results as the villi

Table 2
Production performance of layer hen at different levels of crude protein diets with amino acid supplementation

Parameter	Dietary Treatment[1]			
	T1	T2	T3	T4
HDEP[2] (%)	82.77 ± 0.04[b]	75.76 ± 0.05[c]	87.79 ± 0.01[a]	82.77 ± 0.04[b]
Egg weight (g)	55.15 ± 0.64	54.99 ± 0.65	55.44 ± 0.59	55.50 ± 0.56
Egg mass (g/hen/day)	45.65 ± 0.02[c]	41.66 ± 0.03[d]	48.68 ± 0.01[a]	45.94 ± 0.02[b]
Feed intake (g/hen/day)	87.67 ± 0.02 [a]	85.55 ± 0.05[c]	87.04 ± 0.04[b]	82.94 ± 0.04[d]
LBWG[3] (kg)	0.33 ± 0.03	0.29 ± 0.02	0.28 ± 0.03	0.28 ± 0.02
FCR[4]	1.92 ± 0.01[b]	2.06 ± 0.01[a]	1.79 ± 0.01[d]	1.81 ± 0.01[c]

[abcd] Means in the same row not sharing a common superscript are significantly different (P<0.05).
[1]T1: 17.5% CP without supplemented essential amino acid (negative control)
T2:17.5% CP with amino acid supplementation (positive control)
T3: 17% CP with amino acid supplementation
T4: 16.5% CP with amino acid supplementation
[2]HDEP: Hen day egg production
[3]LBWG: Live body weight gain
[4]FCR: Feed conversion ratio

Table 3
Egg quality of layer hen at different levels of crude protein diets with amino acid supplementation

Parameter	Dietary Treatment[1]			
	T1	T2	T3	T4
Haugh units	50.31 ± 0.86	50.66 ± 0.78	50.60 ± 0.73	50.47 ± 0.92
Yolk weight (%)	25.53 ± 0.55	25.60 ± 0.09	26.68 ± 0.59	27.30 ± 0.92
Albumen weight (%)	65.43 ± 0.92	65.76 ± 0.28	64.01 ± 0.93	63.86 ± 0.68
Eggshell weight (g)	5.26 ± 0.03	5.10 ± 0.01	5.28 ± 0.15	4.87 ± 0.09
Eggshell thickness (mm)	0.59 ± 0.01	0.63 ± 0.01	0.59 ± 0.03	0.58 ± 0.02

[1]T1: 17.5% CP without supplemented essential amino acid (negative control)
T2:17.5% CP with amino acid supplementation (positive control)
T3: 17% CP with amino acid supplementation
T4: 16.5% CP with amino acid supplementation

height, whereby the values of T1 and T3 were significantly higher (P<0.05) than T2 and T4. For VH/CD ratio, however, there were no significant differences (P>0.05) in duodenum, jejunum and ileum between all the groups.

The faecal pH, LAB and ENT counts are presented in Table 5. The lowest (P<0.05) faecal pH was observed in T3 and T4 compared to the rest of the treatment groups. However, the treatment supplemented with 17% CP showed the lowest reduction (P<0.05) in faecal pH compared to the other groups. There was a significant difference (P<0.05) in the LAB and ENT count and LAB/ENT ratio between the dietary treatments. T3 showed significantly higher (P<0.05) LAB counts compared with other treatment groups. T3 also had the lowest (P<0.05) ENT counts. As for the LAB/ENT ratio, T3 had the highest ratio among all the treatment groups.

DISCUSSION

The egg weight and live body weight gain (LBWG) were not significantly different among the treatment groups. This finding suggests that low protein is well fortified with essential amino acids and has adequate level of total nitrogen that can maintain egg weight. Zou and Wu (2005) reported that the no response of egg weight to protein might be due to over consumption of feed of hens fed with low protein diet. In addition, a study conducted by Khajali et al. (2007) revealed that egg weight was not significantly

Table 4
Villus height and crypt depth of layer hen at different levels of crude protein diets with amino acid supplementation

Parameter	Dietary Treatment[1]			
	T1	T2	T3	T4
Villi height, µm				
Duodenum	668.59 ± 44.15[a]	149.99 ± 7.81[b]	726.29 ± 91.59[a]	182.55 ± 16.25[b]
Jejunum	496.17 ± 31.80 [a]	107.24 ± 7.92 [b]	433.71 ± 52.87 [a]	127.86 ± 14.19 [b]
Ileum	361.00 ± 21.88[a]	81.01 ± 3.00[b]	317.73 ± 36.44[a]	111.78 ± 14.60[b]
Crypt depth, µm				
Duodenum	123.24 ± 11.17[a]	23.86 ± 2.31[b]	138.07 ± 15.27[a]	31.61 ± 4.30[b]
Jejunum	105.32 ± 5.91[a]	29.93 ± 1.37[b]	110.69 ± 11.03[a]	29.70 ± 6.22[b]
Ileum	90.51 ± 6.23[a]	21.70 ± 1.64[b]	74.18 ± 6.70[a]	24.12 ±3.59[b]
Villi height to crypt depth ratio				
Duodenum	2.87 ± 0.74	2.98 ± 0.64	3.75 ±1.31	3.38 ± 1.04
Jejunum	2.43 ± 0.57	2.31 ± 0.47	2.22 ± 0.70	2.70 ± 0.83
Ileum	2.20 ± 0.53	2.47 ± 0.52	2.27 ± 0.71	2.99 ± 1.05

[ab] Means in the same row not sharing a common superscript are significantly different (P<0.05).
[1]T1: 17.5% CP without supplemented essential amino acid (negative control)
T2:17.5% CP with amino acid supplementation (positive control)
T3: 17% CP with amino acid supplementation
T4: 16.5% CP with amino acid supplementation

Table 5
Faecal pH and faecal LAB and ENT count of layer hen at different levels of crude protein diets with amino acid supplementation

Parameter	Dietary Treatment[1]			
	T1	T2	T3	T4
Faecal pH	6.83 ± 0.09[a]	6.68 ± 0.05[a]	6.37 ± 0.06[b]	6.46 ± 0.04[b]
LAB counts (log CFU/g)	5.71 ± 0.09[c]	5.91 ± 0.07[b]	6.31 ± 0.04[a]	6.03 ± 0.01[b]
ENT counts (log CFU/g)	5.82 ± 0.02[a]	5.75 ± 0.01[b]	5.61 ± 0.02[c]	5.47 ± 0.03[d]
LAB/ENT ratio	0.98 ± 0.01[c]	1.03 ± 0.01[b]	1.12 ± 0.01[a]	1.10 ± 0.01[a]

[abcd] Means in the same row not sharing a common superscript are significantly different (P<0.05).
[1]T1: 17.5% CP without supplemented essential amino acid (negative control)
T2:17.5% CP with amino acid supplementation (positive control)
T3: 17% CP with amino acid supplementation
T4: 16.5% CP with amino acid supplementation

affected by feeding reduced crude protein diet. This result is in agreement with a study conducted by Yakout (2010), which observed no difference in egg weight and body weight gain when birds were fed with low crude protein diets supplemented with amino acid. Meanwhile, no significant changes were observed in the initial, final and change in body weight of hens at 21 to 33 weeks of age fed different dietary treatments, indicating that different dietary treatments with higher levels of protein and methionine are not sufficient to make alteration in the body weights (Kumar et al., 2012). In the current study, higher hen day egg production was observed in the T3 (17% CP) treatment group compared to other dietary groups. This could be due to the basic amino acid and dietary protein fulfilment in the dietary treatment. This was supported by a previous research that indicated better performance was observed in animals fed with the low dietary crude protein supplemented with amino acid (Banarjee et al., 2013). Results of this experiment are also in disagreement with those reported by Liu et al. (2004) and Wu et al. (2005), who claimed that reducing dietary protein would reduce egg production.

In both these diets supplemented with amino acids in this study, there were increases in the feed intake when birds were fed with 17% CP compared with those fed with 17.5% CP diet supplemented with amino acid. Our results are in agreement with Kidd et al. (2001), who reported that 20% CP diet with amino acids supplementation fed to broilers had significant increase in feed intake than those fed a diet with 23% CP. Feed intake was increased when reduced protein diets were fed to chickens. This happened as the result of an amino acid "appetite" that occurred when feeding amino acids marginal diets. In terms of FCR, 17% CP had significantly lower value compared to hens fed with higher level of dietary protein treatments. According to Novak et al. (2006), feed efficiency of

birds improved as dietary protein decreased. Variation in FCR could also be attributed to the differences in egg mass as the resultant effects of feed intake (Adeyemo et al., 2012). In a study conducted by Kumar et al. (2012), FCR was similar in hens fed medium (16%) or high (18%) protein diets during 20 to 43 weeks of age.

In our study, egg qualities are not affected by reducing dietary crude protein. This was supported by a previous study (Adeyemo et al., 2012), which revealed egg qualities were not affected by reducing protein level in poultry ration. There were no differences observed in Haugh units in the early phase (20-43 weeks of age) of the production cycle when feeding low protein diets (Novak et al., 2006). There was no effect of lysine on the variables Haugh unit, yolk and albumen percentage, as reported by Torki et al. (2014), Figueiredo et al. (2012) and Jardim et al. (2008, 2010). Eggshell percentage and thickness were not influenced by the dietary treatments, which could be explained by the fact that eggshell synthesis requires only a small amount of protein (Praes et al., 2014).

The dietary nutrient utilisation efficiency is affected by gastrointestinal tract development and can be assessed through measurements of villi height and crypt depth (Swatson et al., 2002). Meanwhile, Tufarelli et al. (2010) found that the changes in gut morphology influenced the nutrient utilisation and performance of rabbits. The intestinal villi and cells in chickens are affected by dietary components (Incharoen & Yamauchi, 2009; Incharoen et al., 2009). Small intestine is the predominant site for protein digestion. Dietary protein not only plays a major role in poultry growth and reproductive performance, but also on the gastrointestinal tract features (Laudadio et al., 2012). Maximum digestion and absorption are believed to occur as the villus height to crypt depth ratio increases in weaned pigs. The increased villus height to crypt depth ratio produced an intestinal structure more oriented to absorption (Xu et al., 2012). Villi are important structures in the small intestine that involves mainly in nutrient absorption. Therefore, an increased villus height would increase the surface area for nutrient absorption (Choe et al., 2012). Lengthening of villi may increase total luminal villus absorptive area and subsequently result in satisfactory digestive enzyme action and higher transport of nutrients at the villus surface. However, deeper crypt indicates faster tissue turnover and high demand for the renewal of the villus, which suggests that the host's intestinal response mechanism is trying to compensate for normal sloughing or atrophy of villi due to inflammation from pathogens and their toxins (Incharoen et al., 2010). The villus height to crypt depth ratio is a very useful measure to estimate the absorption capacity of the small intestine. Higher villus height to crypt depth ratio is a positive aspect as it results in a decreased turnover of the intestinal mucosa and leads to lower maintenance requirement, which ultimately leads to a higher animal growth rate (Tufarelli et al., 2010; Laudadio et al., 2012).

The faecal pH, LAB count, ENT count and LAB to ENT ratios were significantly different among the treatment groups. The decrease in the faecal pH with low CP regimens provides a more favourable pH environment for digestive activity, allowing for greater digestion and absorption of nutrients, particularly in the small intestine. The acidic environment favours growth of LAB. Results for the ENT count showed a clear alteration of faecal microflora in low level of CP. It has been proven that increased LAB count could decrease ENT count (Loh et al., 2007; Choe et al., 2012). According to Loh et al. (2007), LAB has antagonistic effect on harmful bacteria either *in vitro* or *in vivo* studies. These beneficial bacteria have been shown to be able to reduce the faecal ENT count. Intestinal LAB is able to produce antimicrobial compounds such as bacteriocin and organic acids, which are bacteriostatic against pathogenic bacteria. The undissociated organic acid decreases the pH when it passes through. The LAB competes against enteropathogens for nutrient, binding and receptor sites. They have strong inhibitory effects in preventing the adherence, establishment and replication of pathogenic bacteria. The LAB is important component for a balanced microflora in the gastrointestinal tract. In this study, 17% CP has beneficial effects on laying hens, and this is most likely due to the optimal intestinal environment as confirmed by the faecal pH (Laudadio et al., 2012).

CONCLUSION

The findings of this study indicate that reducing dietary protein level supplemented with essential amino acids in layer ration could be advantageous compared to conventional feeding programmes. Furthermore, the diet containing 17% CP with addition of synthetic amino acids offers an advantage on improving the hen/day egg production and shows positive effects such as increased small intestine villus height and promoted beneficial effects of faecal microflora. These findings indicate that the response of layers to lowered levels of dietary crude protein can be used in feeding layer hens.

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