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Effect of different levels of tryptophan on productive performance, egg quality, blood biochemistry, and caecal microbiota of hens housed in enriched colony cages under commercial stocking density

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ABSTRACT A study was conducted to determine the tryptophan (**Trp**) requirement of brown hens housed in enriched colony cages. A corn and wheat-based diets with 8 levels of standardized ileal digestible (**SID**) Trp (0.10, 0.13, 0.16, 0.19, 0.22, 0.25, 0.28, and 0.31% of the diet) were manufactured. The diet containing SID Trp 0.10% had no supplemental Trp and was treated as control. A total of 1,344 hens were randomly allocated to 8 treatments, each having 8 replicate cages with 21 hens per cage. Body weight gain (**BWG**), egg production (**EP**), feed conversion ratio (**FCR**), egg quality, blood biochemistry, caecal microbial profile, and concentration of indoles were determined over a period of 16 wk. The EP was linearly improved by supplementing diet with Trp and was highest in 0.25% SID-Trp group compared to control. Trp supplementation improved ($P < 0.05$) FCR, overall BWG, egg shell characteristics compared to control. The microbial shift in the caecum in response to Trp supplementation was significant in response to higher than current recommendations (0.22% of SID Trp) and indicated a microbial shift towards beneficial bacteria. Indole and skatole concentrations were only significantly different ($P < 0.05$) when hens in control group were compared with those containing highest levels of SID-Trp. This study demonstrates that when hens are at its peak production and are reared in enriched colony cages their Trp requirement is higher than current National Research Council (1994) recommendations and 0.22% of the SID-Trp in diet can be considered as an optimal level based on regression analysis.

Key words: tryptophan, enriched cage, microbial profile, egg production, egg quality

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INTRODUCTION

Tryptophan (**Trp**) is an essential amino acid in monogastric animals as it cannot be synthesized by the body. In monogastric animals, Trp has been associated with several physiological roles such as protein synthesis (Ruan et al., 2014); a tool to improve growth performance (Cortamira et al., 1991), feed intake (**FI**) (Corzo et al., 2005), and feed utilization (Wu, 2009); a facilitator to reduce anxiety and stress (Koopmans et al., 2012); an enhancer to promote egg production (**EP**) (Harms and Russel, 2000); and a regulator of bone mass (Ducy and Karsenty, 2010).

Tryptophan is metabolized through 3 diverse pathways (Yao et al., 2011). The first pathway for Trp metabolism is via the synthesis of serotonin in the brain. The second pathway is the deamination and decarboxylation to produce indole acetic acid; however, the third and most dominant pathway for Trp catabolism is the kynurenine pathway (Yao et al., 2011; O'Mahony et al., 2015). The kynurenine is the major pathway in which 95% of dietary Trp is metabolized (Davis and Liu, 2015). Metabolism of Trp through this pathway primarily results in the production of quinolinic acid via the production of 3-hydroxykynurenine which when metabolized further produces either nicotinamide or nicotinic acid (Yao et al., 2011). Nicotinic acid is an essential component of monogastric diets, with the main function being cell respiration; however, it is also utilized in the metabolism of proteins, carbohydrates, and lipids (Andres, 2012).

Tryptophan can be degraded into 2 volatile lipophilic compounds, indole and 3-methylindole (skatole) via a 2-step process. In step 1, Trp is converted to indole-3-acetic acid by intestinal bacteria such as *E. coli* and *Clostridium* and then in step 2, the several bacteria (genera *Clostridium* and a *Lactobacillus* strain) produce skatole by decarboxylating indole-3-acetic acid to skatole (Hengemnehle and Yokoyama, 1990; Jensen et al., 1995). The toxicity of indoles is due to their function as uncouplers of the proton gradient across mitochondrial membranes which inhibits oxidative phosphorylation and influences the Adenosine Tri-Phosphate

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production and consequently has negative effect on performance of the animal (Chimerel et al., 2013).

The existing database regarding the Trp requirement for laying hens is lacking consistency and the level of supplemental Trp varies from 0.16 to 0.23% in diet for laying hens kept in conventional cages (NRC 1994; Coon and Zhang 1999; Harms and Russel, 1999, 2000; Peganova et al., 2003). There is paucity of data on Trp requirement for brown hens housed in enriched colony cages. Therefore, the objective of the current experiment was to establish an optimum level of standardized ileal digestible (SID) Trp for laying hens reared under commercial stocking density in enriched colony cages based on EP, egg quality, growth performance, blood biochemistry, caecal microbial profile, and caecal concentration of indole and skatole in hens over an experimental period of 16 wk.

MATERIALS AND METHODS

All the animal experimental procedures in the current study were carried out under the Animals Scientific Procedures Act (1986) and approved by the ethical review committee of Scotland's Rural College (**SRUC**).

Birds, Feed, and Management

A total of 1,344 Lohmann brown hens, aged 22 wk old, were randomly allocated to 8 treatments. Each of the treatments had 8 replicate cages with 21 hens per replicate cage. The enriched colony cages had a 750 sq. cm floor area and 11.48 cm feed through space per bird as required by European Commission (1999) for the protection of laying hens. Each cage was equipped with a nest, scratch mat, and perches. No additional furniture was provided in the cages. The hens were allocated to cages to ensure that the initial body weight of hens was similar across treatments. The hens were housed in a facility that enabled regulation of temperature and humidity. The temperature and relative humidity was approximately 20°C and 65%, respectively.

A corn and wheat-based diet deficient in supplemental tryptophan (0.00% L-Trp) was manufactured (Table 1) and then the additional 7 treatments were generated by the addition of 0.03, 0.06, 0.09, 0.12, 0.15, 0.18, and 0.21% L-Trp, respectively to provide 0.10, 0.13, 0.16, 0.19, 0.22, 0.25, 0.28, and 0.31% SID Trp of diet (Table 2). Diets were formulated on the basis of SID amino acids. Amino acids were calculated at 105% of AminoHen® (2011) recommendations for a daily FI of 110 g except for Trp which was deficient in the control diet at 0.10 SID Trp (59% of AMINOHen® recommendation; 0.12% total Trp). Feed and water were provided on an *ad libitum* basis for the duration of the experiment. Birds were weighed at the beginning and end of the experiment, whereas FI was recorded fortnightly.

All experimental diets were analyzed for dry matter, nitrogen, ether extract, and Trp. Total nitrogen

Table 1. Ingredient and chemical composition of control (0.10% SID Trp) group.

Ingredients	%
Corn	51.39
Wheat	10.00
Limestone ($CaCO_3$)	9.70
Corn starch	8.18
Corn gluten meal, 60% CP	7.00
Rapeseed meal	5.00
Soybean meal, 48% CP	5.00
Mono calcium phosphate	1.26
L-Lysine HCl	0.51
Premix[1]	0.50
Salt (NaCl)	0.50
DL-Methionine	0.26
L-Arginine	0.23
L-Isoleucine	0.17
L-Threonine	0.16
L-Valine	0.14
Total	100
Calculated composition	
ME[2], kcal/kg	2825
Crude protein	15.22
SID[3] Lysine	0.80
SID Methionine	0.50
SID M+C[4]	0.72
SID Threonine	0.56
SID Tryptophan	0.10
SID Arginine	0.83
SID Isoleucine	0.63
SID Leucine	1.43
SID Valine	0.69
SID Phenylalanine	0.31
SID Histidine	1.11
Calcium	4.00
Available phosphorous	0.38
Analyzed chemical composition	
Dry matter	89.1
Ether Extract	2.2
Crude protein	14.7
Crude Fiber	2.3
Ash	12.2
Total Lysine	0.77
Total Methionine	0.69
Total Arginine	0.79
Total Threonine	0.58
Total M+C	0.69

[1]Premix = Provided per kilogram of diet: Mn, 80 mg; Zn, 60 mg; Fe, 10 mg; Cu, 5 mg; Se, 0.15 mg; I, 1.0 mg; vitamin A, 6000 IU; vitamin D3, 3000 IU; vitamin E, 5 IU; vitamin B12, 25 μg; vitamin K, 1.0 mg; niacin,10 mg; folic acid, 0.30 mg; pantothenic acid, 4 mg.
[2]ME = metabolizable energy.
[3]SID = standardized ileal digestible.
[4]M+C = methionine plus cysteine.

Table 2. Level of Trp in the experimental diets.

	L-Trp[1] (% of the diet)		
Supplemented	Calculated		Determined
	SID[2]	Total	Total
0.00	0.10	0.12	0.13
0.03	0.13	0.15	0.16
0.06	0.16	0.18	0.18
0.09	0.19	0.21	0.19
0.12	0.22	0.24	0.23
0.15	0.25	0.27	0.30
0.18	0.28	0.30	0.32
0.21	0.31	0.33	0.40

[1]Trp = Tryptophan.
[2]SID = standardized ileal digestible.

content of diet was determined by the combustion method (method 968.06; AOAC International, 2006) whereas ether extract was determined in a soxhlet extractor (method 922.06; AOAC International, 2006). The total Trp was analyzed by HPLC with fluorescence detection (extinction 280 nm, emission 356 nm), after alkaline hydrolysis with barium hydroxide octahydrate for 20 h at 110°C (Commission Directive, 2000).

Egg Production

Total eggs laid per cage were collected and weighed daily to determine egg mass and for calculation of hen day production.

Egg Quality Assessment

Egg quality was assessed every 4 wk (at days 28, 56, 84, and 112). Two eggs were randomly selected from the eggs produced per cage and were used for internal egg quality assessment. All the egg quality assessments were done using egg quality equipment TSS QCM+Range (Eggware EW400, Technical Services and Supplies Ltd (TSS), York, England). The egg qualities assessed by the equipment were albumen height, Haugh unit, shell density, shell thickness, shell weight, and yolk color.

Blood Biochemistry

Sequential blood samples were taken from 2 birds per replicate from 4 treatment groups (0.10, 0.16, 0.22, and 0.31% SID Trp) at day 28, 56, 84, and 112 day of the experiment. Blood (2 to 3 mL) samples were taken from the wing and from the same birds (leg bands were used for identification) using 5 mL heparin tubes with a gel-separation. The tubes were kept at room temperature for 30 min to ensure clotting before they were centrifuged at 3,000 rpm for 10 min. The blood cells were separated below the gel and the serum above the gel. The blood tubes were then sent to Synlab.vet GmbH lab in Germany for analysis. The blood biochemistry included albumin, gamma-glutamyl transferase (**GGT**), aspartate amino transferase (**AST**), alkaline phosphatase (**AP**), total protein, glucose, calcium, cholesterol, uric acid, corticosterone analysis.

Total Microbial Community Analysis

At the end of the study, 1 bird per replicate cage was randomly selected from 4 treatment groups (0.10, 0.16, 0.22, and 0.31% SID Trp) and were euthanized using an over dose of sodium pentobarbitone via intraperitoneal injection. Eight caecal samples from each treatment were sent to Alimetrics for total microbial community analysis. A culture-independent DNA-based method was employed to determine percent guanine + cytosine (%G+C) profiling as described by Apajalahti et al., 1998 and 2001.

Caecal Indole and Skatole Analysis

Caecal digesta samples were collected from same birds as used for microbial G+C profiling (8 replicate samples for each of treatment group having 0.10, 0.16, 0.22, and 0.31% SID Trp) and were sent to Alimetrics for analysis of indole and skatole concentrations.

Statistics

The study design was a randomized complete block design with 8 blocks and 8 treatments. Data was subjected to ANOVA using a GenStat 16 statistical software package (IACR, Rothamstead, Hertfordshire, UK). Quadratic regression analyses were used to determine the optimum level of SID Trp for commercially important traits (such as EP and egg shell quality) in laying hens. Cage was the experimental unit. Statistically significant means were separated using orthogonal polynomial contrast. In case of microbial profiling the statistical analysis consisted of two-tailed *t*-tests for 1% G+C increments from 20 to 70% G+C. The tests were performed pairwise for each treatment combination. All statements of significance are based on the probability level of $P \leq 0.05$.

RESULTS

Nutrient composition of basal diet and determined Trp levels of all experimental diets were within the expected range and are presented in Tables 1 and 2, respectively.

Effect of SID Trp on Egg Production

The EP data over the experimental period of 16 wk is presented in Table 3. During period 1 (wk 22–26), egg weight, egg mass, and percent EP started to increase in response to SID Trp supplementation, however, the differences were only significant ($P < 0.05$) in hens fed diets containing 0.25% SID Trp compared to those having 0.10% SID Trp in diet. After hens were fed experimental diets for 28 d a linear increase in egg mass and EP was observed as dietary levels of SID Trp increased from 0.10 to 0.31%. Egg mass and percent EP was consistently lowest ($P < 0.05$) in control group having 0.10% SID Trp compared to all other groups. The overall period (wk 22–38) depicted the same trends and showed a significant increase ($P < 0.05$) in hens fed Trp supplemented diets compared to unsupplemented control group. Despite the fact that the highest EP was achieved in hens fed diets containing 0.25% SID Trp, the regression analysis showed that the optimal

Table 3. Egg production of hens fed diets containing different levels of SID Trp.

Dietary	Period 1 (wk 22–26)			Period 2 (wk 27–30)			Period 3 (wk 31–34)			Period 4 (wk 35–38)			Overall Period (wk 22–38)		
SID Trp (%)	Egg mass (g/hen)	[1]EW (g)	[2]EP (%)	Egg mass (g/hen)	EW (g)	EP (%)	Egg mass (g/hen)	EW (g)	EP (%)	Egg mass (g/hen)	EW (g)	EP (%)	Egg mass (g/hen)	EW (g)	EP (%)
0.10	48.1	52.5	93.2	48.2	55.9	86.5	51.4	57.5	90.2	51.0	59.8	87.1	49.3	56.0	89.3
0.13	48.7	52.2	96.1	53.1	59.0	91.2	56.1	58.9	96.2	56.7	60.2	95.2	53.6	57.4	94.7
0.16	49.4	53.1	95.6	52.3	57.5	91.8	56.0	59.3	95.5	56.5	60.8	94.2	53.5	57.6	94.3
0.19	49.9	52.6	97.4	52.1	57.3	92.3	56.9	59.2	97.4	56.1	60.5	94.5	53.7	57.3	95.4
0.22	49.7	53.1	96.3	52.0	57.5	91.4	54.9	59.3	94.7	54.7	60.9	92.2	52.8	57.6	93.7
0.25	50.5	53.3	97.4	53.5	57.5	94.3	58.5	59.3	99.4	58.4	60.9	97.0	55.2	57.7	97.0
0.28	48.8	53.5	93.3	52.2	57.8	91.0	55.5	59.6	94.6	57.9	63.8	92.5	53.3	58.4	93.1
0.31	49.8	53.3	95.8	52.5	57.9	91.7	56.7	60.0	95.3	57.6	61.8	94.5	53.9	58.0	94.4
[3]SEM	1.012	0.396	1.734	1.256	1.234	1.993	1.490	0.527	2.192	1.907	1.524	2.017	1.158	0.647	1.701
P-value	0.362	0.019	0.116	0.005	0.485	0.035	0.002	0.002	0.012	0.009	0.25	0.001	<0.001	0.035	0.005
Linear	0.108	<0.001	0.589	0.014	0.470	0.026	0.005	<0.001	0.079	0.002	0.023	0.022	0.001	0.002	0.035
Quadratic	0.120	0.653	0.029	0.014	0.630	0.013	0.017	0.163	0.007	0.172	0.843	0.012	0.008	0.225	0.005

[1]EW = egg weight; [2]EP = egg production.
[3]SEM = standard error of the mean.

inclusion level of SID Trp to achieve the highest percentage of EP was 0.22%.

Effect of SID Trp on Growth Performance

The effect of inclusion levels of SID Trp on FI, feed conversion ratio (**FCR**) and body weight gain (**BWG**) of the laying hens during different experimental periods is presented in Table 4. In general there was no significant ($P < 0.05$) effect of Trp supplementation on FI except in hens fed diets containing 0.25% SID Trp where hens tended ($P \leq 0.1$) to have 3.0, 4.1, and 2.7% increase in FI compared to those on 0.10% SID Trp in diet during period 1, 3, and the overall period (1 to 4), respectively. The egg FCR significantly improved ($P < 0.05$) when laying hens were fed diets supplemented with Trp compared to unsupplemented control during period 2, 3, and 4. The data for the overall period showed an overall improvement of 6.6% in the FCR when Trp-supplemented groups were compared with unsupplemented control group. However, no differences ($P > 0.05$) were observed between Trp-supplemented groups. The overall weight gain (**OWG**) data showed similar trend and was only significantly ($P < 0.05$) lower for hens in unsupplemented control group compared to all Trp-supplemented groups. Although OWG was highest in laying hens fed 0.25% SID Trp, however, the differences were not significant ($P > 0.05$) when compared with other groups containing supplemental Trp.

Effect of SID Trp on Egg Quality

The data on quality of eggs from hens receiving diets containing different levels of SID Trp during the 16 wk period is shown in Table 5. No differences ($P > 0.05$) were observed in albumen height, haugh unit, and yolk color of eggs from birds fed different levels of SID Trp. The egg shell quality was affected by Trp supplementation. Egg shell weight, shell density, and shell thickness was significantly ($P < 0.05$) improved by supplementing diet with Trp. Shell weight at day 28, 84, and 112 was low ($P < 0.05$) in hens fed diet containing 0.10% SID Trp compared to those having 0.16, 0.22, 0.25, and 0.28% SID Trp. Shell density and thickness data showed significant differences between treatments at day 84 and 112. At day 84 shell density and thickness was lowest ($P < 0.05$) in hens having 0.19% SID Trp compared to those having 0.13, 0.16, 0.25, 0.28, and 0.31% SID Trp in the diet. Whereas, at day 112 shell density was lowest ($P < 0.05$) in hens having 0.10 and 0.13% SID Trp compared to 0.10% SID Trp in the diet. The regression analysis results revealed that the optimum dosage to improve the quality of egg shell (shell thickness and density) was 0.22% SID Trp.

Table 4. Feed intake, feed conversion ratio, and overall weight gain of hens fed diets containing different levels of SID Trp during 22 to 38 wk of age.

Dietary	Period 1 (wk 22–26)		Period 2 (wk 27–30)		Period 3 (wk 31–34)		Period 4 (wk 35–38)		Overall period (wk 22–38)		
SID Trp (%)	[1]FI (g)	[2]FCR (g/g)	[1]FI (g)	[2]FCR (g/g)	[1]FI (g)	[2]FCR (g/g)	[1]FI (g)	[2]FCR (g/g)	[1]FI (g)	[2]FCR (g/g)	[3]OWG (kg)
0.10	105.3	2.190	113.3	2.353	114.5	2.230	117.7	2.318	112.0	2.271	0.008
0.13	106.0	2.177	112.5	2.129	116.7	2.080	122.3	2.158	114.3	2.133	0.218
0.16	106.3	2.154	112.3	2.149	112.8	2.020	120.2	2.132	112.7	2.110	0.179
0.19	106.2	2.129	113.5	2.181	116.1	2.040	120.2	2.144	113.9	2.122	0.137
0.22	106.9	2.152	115.2	2.218	114.3	2.090	118.0	2.169	113.6	2.152	0.105
0.25	108.5	2.152	115.1	2.152	119.2	2.040	117.8	2.019	115.1	2.087	0.251
0.28	104.3	2.142	114.5	2.199	113.6	2.050	119.6	2.097	112.6	2.114	0.182
0.31	107.5	2.162	115.6	2.206	115.5	2.040	121.7	2.119	114.8	2.131	0.184
[4]SEM	1.530	0.036	2.210	0.061	2.520	0.036	3.981	0.085	1.835	0.038	0.049
P-Value	0.300	0.768	0.703	0.022	0.274	<0.001	0.904	0.084	0.641	<0.001	<0.001
Linear	0.300	0.286	0.082	0.264	0.692	<0.001	0.881	0.014	0.286	0.003	0.006
Quadratic	0.487	0.166	0.867	0.020	0.761	<0.001	0.723	0.103	0.706	0.003	0.072

[1]FI = feed intake (g/bird/day);
[2]FCR = feed conversion ratio (Egg mass per hen (g/g)/feed intake (g/hen/day);
[3]OWG = Overall weight gain (kg) day 0 to 112.
[4]SEM = Standard error of the means.

Effect of SID Trp on Blood Biochemistry, Caecal Microbial Profile and Concentration of Indoles in the Caecum

Among the 8 levels of SID Trp used in the current study, 4 levels were selected (0.01, 0.16, 0.22SID Trp, and 0.31% SID Trp in diet) to investigate the effect of Trp supplementation on blood biochemistry, caecal microbiota composition, and residual concentration of indoles in the caecum.

The blood biochemistry profile of hens at day 28, 56, 84, and 112 is presented in Table 6. Total protein and albumin level in the blood were significantly lower ($P < 0.05$) in 0.10% SID Trp group compared to all other SID Trp groups (0.16, 0.22, and 0.31% SID Trp) throughout the experimental period of 16 wk. Significant differences in blood calcium were also noted during the first 12 wk when the level of calcium was significantly higher ($P < 0.05$) in all SID Trp groups (0.16, 0.22, and 0.31% SID Trp) compared to control group (0.10% SID Trp). The level of corticosterone between treatments was similar ($P > 0.05$) throughout the experimental period except at day 84 where higher levels were observed in 0.10% SID Trp group compared to 0.16% SID Trp. The uric acid was lowest and highest ($P < 0.05$) in 0.10% SID Trp group compared to all other SID Trp groups (0.16, 0.22, and 0.31% SID Trp) at day 28 and 112, respectively.

The effect of SID Trp on the bacterial metabolites of Trp fermentation (indole and skatole) showed that when Trp concentration in the diet was increased, residual indole and skatole responded differently (Table 7). It appeared that indole concentration dropped while skatole concentration increased as a response to elevated dietary SID Trp. However, the differences were only significant ($P < 0.05$) when diets containing 0.10% SID Trp was compared with 0.31% SID Trp.

The microbial shifts in caecum as a response to SID Trp were relatively small (Figure 1–3). A tendency to reduce the abundance of bacteria with G+C 39 ± 2% ($P < 0.1$) was observed when hens fed diets containing 0.10% SID Trp were compared with those having 0.16% SID Trp in diet (Figure 1). Hens fed diets containing 0.10% SID Trp tended to increase the abundance of bacteria with %G+C 62 ± 3% ($P < 0.1$); however, the overall differences were very small and non-significant. Figure 2 illustrated microbial shift ($P < 0.1$) from high %G+C (~61 ± 2%; $P < 0.05$) towards bacteria with %G+C below 50 when hens in 0.10% SID Trp group were compared with those fed 0.22% SID Trp in diet. Whereas, when 0.31% SID Trp group was compared with 0.10% SID Trp group (Figure 3) data showed significant increase in bacteria with % G+C content 38 ± 3% ($P < 0.05$) and a concomitant reducing trend in bacteria with %G+C 52 to 60.

Table 5. Effect of diets containing different level of SID Trp on egg quality.

Period (day)	Item	Dietary SID Trp (%)								[1]SEM	*P*-value	Linear	Quadratic
		0.10	0.13	0.16	0.19	0.22	0.25	0.28	0.31				
28	Albumen Height (mm)	10.2	10.1	10.8	10.3	10.4	10.7	10.4	10.6	0.370	0.633	0.283	0.528
	Haugh Unit	101.1	100.5	103.1	100.9	101.5	103.0	101.0	102.1	1.566	0.746	0.471	0.741
	Shell Density (mg/cm^2)	76.1	79.2	80.1	80.6	80.5	82.8	81.2	80.0	1.887	0.580	0.010	0.020
	Shell Thickness (μ)	341.8	353.7	360.1	357.1	354.6	367.0	358.0	354.6	8.660	0.235	0.110	0.055
	Shell Weight (g)	5.1	5.4	5.6	5.6	5.6	5.7	5.6	5.5	0.154	0.002	<0.001	0.001
	Yolk Color (Roche)	8.9	9.1	9.0	9.3	9.3	9.2	9.1	9.5	0.239	0.306	0.026	0.932
56	Albumen Height (mm)	10.3	10.2	10.2	10.0	10.0	10.2	10.5	9.8	0.366	0.745	0.504	0.929
	Haugh Unit	100.9	100.1	99.9	99.2	98.8	99.6	101.0	97.9	1.611	0.574	0.244	0.771
	Shell Density (mg/cm^2)	78.5	81.5	77.7	81.8	79.4	82.3	81.0	82.1	1.923	0.123	0.060	0.981
	Shell Thickness (μ)	353.2	360.3	347.4	360.9	354.4	356.0	357.9	368.0	8.060	0.343	0.134	0.287
	Shell Weight (g)	5.5	5.8	5.6	5.9	5.7	5.9	5.8	6.0	0.193	0.122	0.009	0.605
	Yolk Color (Roche)	9.3	9.4	9.4	9.3	9.1	9.4	9.4	9.6	0.217	0.663	0.363	0.200
84	Albumen Height (mm)	9.8	9.6	9.5	9.6	9.4	9.5	9.6	9.6	0.384	0.964	0.601	0.306
	Haugh Unit	98.4	96.8	96.2	97.5	95.4	96.8	97.0	97.2	1.838	0.862	0.651	0.252
	Shell Density (mg/cm^2)	79.2	81.7	81.5	74.7	80.2	81.2	81.4	81.3	1.891	0.005	0.304	0.160
	Shell Thickness (μ)	358.0	362.3	362.9	336.1	357.7	363.0	359.0	361.4	9.180	0.076	0.707	0.196
	Shell Weight (g)	5.7	6.0	6.0	5.4	5.9	5.8	5.9	5.9	0.176	0.004	0.484	0.603
	Yolk Color (Roche)	9.2	9.5	9.2	9.3	9.3	9.3	9.4	9.3	0.213	0.880	0.994	0.823
112	Albumen Height (mm)	9.7	9.5	9.4	8.7	9.2	9.3	10.0	9.3	0.451	0.198	0.950	0.084
	Haugh Unit	97.2	96.4	95.4	92.0	94.4	95.3	98.4	95.3	2.150	0.147	0.961	0.056
	Shell Density (mg/cm^2)	77.4	80.6	84.2	82.9	82.3	82.8	81.2	80.3	1.770	0.011	0.264	<0.001
	Shell Thickness (μ)	353.4	350.6	375.1	366.1	369.9	371.0	366.0	357.2	8.420	0.039	0.232	0.004
	Shell Weight (g)	5.6	5.9	6.3	6.1	6.1	6.1	6.0	5.9	0.157	0.008	0.233	<0.001
	Yolk Color (Roche)	9.3	9.0	9.2	9.1	9.4	9.2	8.9	9.1	0.203	0.289	0.283	0.777

[1]SEM = Standard error of means.

Table 6. Effect of different levels of SID Trp on blood biochemistry profile of hens.

Period (day)	Item	Dietary SID Trp (%)				[4]SEM	*P*-Value	Linear	Quadratic
		0.10	0.16	0.22	0.31				
28	Albumin (g/L)	14.4	17.2	16.8	17.1	0.591	<0.001	<0.001	0.002
	GGT[1](IU/L)	22.1	20.4	20.1	18.0	2.6	0.492	0.133	0.980
	AST[2] (IU/L)	193.5	190.4	197.2	192.3	9.87	0.918	0.955	0.826
	AP[3] (IU/L)	641	514	553	506	116	0.642	0.337	0.618
	Total Protein (g/L)	41.8	49.0	46.6	46.5	2.041	0.008	0.100	0.018
	Glucose (mmol/L)	13.9	13.6	14.6	13.0	0.528	0.040	0.201	0.080
	Calcium (mmol/L)	5.3	6.9	6.6	6.3	0.344	<0.001	0.039	<0.001
	Cholesterol (mmol/L)	2.7	3.7	3.1	3.2	0.539	0.407	0.603	0.333
	Uric acid (μmol/L)	246.0	292.1	236.1	223.6	24.30	0.037	0.093	0.210
	Corticosterone (ng/mL)	2.6	2.6	2.8	2.7	0.341	0.924	0.63	0.864
56	Albumin (g/L)	15.0	17.3	17.6	17.7	0.700	<.001	<.001	0.014
	GGT[1](IU/L)	21.3	19.2	17.8	19.6	2.159	0.461	0.425	0.172
	AST[2] (IU/L)	188.9	175.2	190.3	200.2	10.18	0.117	0.108	0.185
	AP[3] (IU/L)	483	527	591	413	146	0.667	0.661	0.278
	Total Protein (g/L)	44.3	51.1	49.2	48.8	2.175	0.019	0.138	0.023
	Glucose (mmol/L)	13.3	13.1	13.8	14.0	0.468	1.670	0.050	0.670
	Calcium (mmol/L)	5.4	6.2	6.4	6.3	0.342	0.022	0.012	0.065
	Cholesterol (mmol/L)	2.8	3.5	3.4	3.1	0.621	0.685	0.871	0.276
	Uric acid (μmol/L)	288.5	273.6	275.5	329.6	29.9	0.220	0.141	0.134
	Corticosterone (ng/mL)	3.8	3.2	2.9	3.5	0.591	0.476	0.613	0.141
84	Albumin (g/L)	14.5	17.1	17.7	17.5	0.620	<.001	<.001	<0.001
	[1]GGT (IU/L)	21.9	20.8	21.3	19.3	2.289	0.699	0.295	0.794
	AST[2] (IU/L)	203.2	189.6	196.9	201	22.04	0.931	0.962	0.606
	AP[3] (IU/L)	420	340	341	452	67.2	0.245	0.520	0.055
	Total Protein (g/L)	45.5	51.6	51.1	49.5	2.469	0.069	0.211	0.029
	Glucose (mmol/L)	11.6	11.0	10.9	12.5	0.643	0.064	0.126	0.027
	Calcium (mmol/L)	5.3	6.5	6.5	6.6	0.369	0.002	0.003	0.016
	Cholesterol (mmol/L)	2.8	3.9	3.3	3.4	0.642	0.409	0.644	0.311
	Uric acid (μmol/L)	246.1	273.3	286.0	274.5	31.8	0.640	0.383	0.341
	Corticosterone (ng/mL)	4.56	2.45	4.7	3.85	0.82	0.034	0.981	0.470
112	Albumin (g/L)	15.7	18.4	17.3	17.1	0.652	0.002	0.174	0.004
	GGT[1](IU/L)	18.8	17.9	19.2	20.6	2.299	0.691	0.320	0.575
	AST[2] (IU/L)	175.5	190.4	178.8	207.1	16.8	0.240	0.102	0.582
	AP[3] (IU/L)	472.0	451.0	331.0	548.0	101.8	0.211	0.587	0.084
	Total Protein (g/L)	50.8	54.4	51.5	48.5	2.448	0.131	0.159	0.106
	Glucose (mmol/L)	13.2	12.4	13.6	13.0	0.608	0.278	0.769	0.874
	Calcium (mmol/L)	6.3	6.9	6.5	6.2	0.318	0.160	0.422	0.067
	Cholesterol (mmol/L)	3.3	3.6	2.8	3.0	0.374	0.173	0.156	0.719
	Uric acid (μmol/L)	312.2	260.8	266.2	214.3	24.91	0.003	<0.001	0.851
	Corticosterone (ng/mL)	3.7	3.1	4.4	4.1	0.766	0.392	0.387	0.950

[1]GGT = Gamma-glutamyl transferase.
[2]AST = Aspartate amino transferase.
[3]AP = Alkaline Phosphatase.
[4]SEM = Standard error of means.

DISCUSSION

The objective of the current study was to assess the response of laying hens to corn and wheat-based diets containing 8-graded levels of SID Trp (0.10, 013, 0.16. 0.19, 0.22, 0.25, 0.28, and 0.31%) on EP, feed utilization, body weight, and egg quality. Out of these 8 levels of SID Trp, 4 levels (0.10, 016, 0.22, and 0.31% SID Trp) representing diets with no supplemental Trp, NRC (1994) current recommendations, higher than current recommendations (based on Helmbrecht and Elwert, 2015), and highest SID Trp level, respectively, were further selected to assess the impact of SID Trp levels on blood biochemistry parameters, caecal microbiota composition and bacterial metabolites of SID Trp fermentation (indole and skatole concentration).

Trp requirements in hens is predominantly targeted on EP and feed efficiency variables, which are more associated with the economics of EP. The current study showed that SID Trp had a significant effect on the EP. Overall EP data (over the period of 16 wk) clearly showed that hens fed diets containing no supplemental Trp (0.10% SID Trp) had lowest ($P < 0.05$) EP compared to all hens having Trp supplementation. The optimum EP was recorded in 0.25% SID Trp group where the overall production was 8.6, 2.4, 2.8, 1.7, 3.5, 4.1, and 2.7% higher when compared with 0.10, 0.13, 0.16, 0.19, 0.22, 0.28, and 0.31% SID Trp, respectively. Supplementation of Trp in laying hen diet has been proven to significantly increase EP, it has been indicated that this is achieved as Trp enhances gonadotrophin release and protein availability (DongYou et al., 2010). Dong and Zou (2017) suggested that the optimum level of

Table 7. Effect of different levels of SID Trp on residual caecal concentration of indole and skatole.

SID Trp (% of the diet)	Indole (mg/kg fresh weight)	Skatole (mg/kg fresh weight)
0.10	0.114	0.049
0.16	0.075	0.230
0.22	0.067	0.031
0.31	0.045	0.316
[1]SEM	0.030	0.107
P-Value	0.180	0.032
Linear	0.038	0.061
Quadratic	0.580	0.428
0.10 vs. 0.16	0.215	0.102
0.10 vs. 0.22	0.137	0.867
0.10 vs. 0.31	0.033	0.019

[1]SEM = Standard error of means.

total dietary Trp ranged from 0.19 to 0.21% for Xinyang green shell hens and that improvement in laying performance and egg quality was due to antioxidant function of Trp. Similar to our results Russell and Harms (1999) observed that only levels of Trp lower than or equal to 0.13% promoted reduction in the EP, egg mass, and body weight produced by hens of 53 to 59 wk of age. However, contrary to our results, previous studies have suggested requirements similar to NRC (1994) recommendations (Jensen et al., 1990; Russell and Harms, 1999). This lower requirement of Trp compared to current study could be due to the reason that hens used in those studies were either housed individually or in pairs. Our study suggests that hens aged 22 to 38 wk old can benefit from higher Trp supplementation when reared in colony cages under commercial stocking density.

There is evidence that serotonin affects active intestinal electrolyte transport by increasing the intestinal plasma membrane permeability to calcium and thus increases the intracellular calcium (Donowitz et al., 1980). Thus, it can be hypothesized that supplementation of Trp in hen diets positively facilitated the calcium absorption/availability and thus improved egg shell quality as evidenced in the current study.

The linear increase in the FCR and OWG in response to different levels of SID Trp in layer diet in the current study could be due to enhanced protein digestibility. It has been reported that there is positive correlation

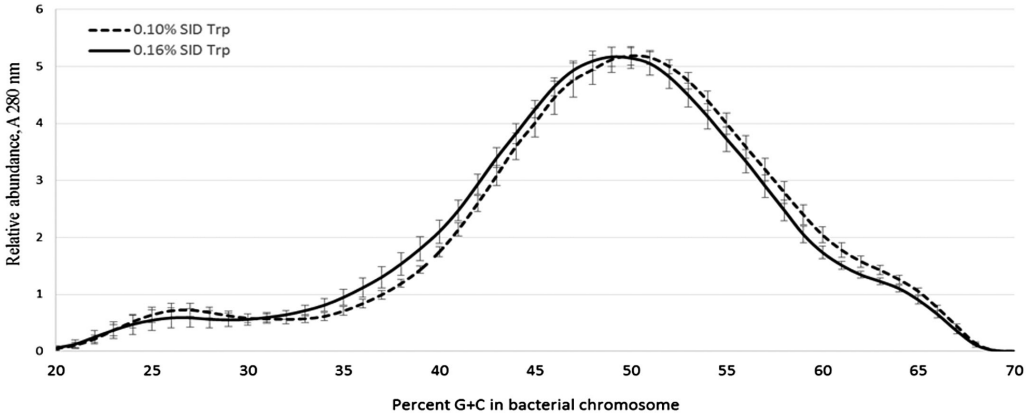
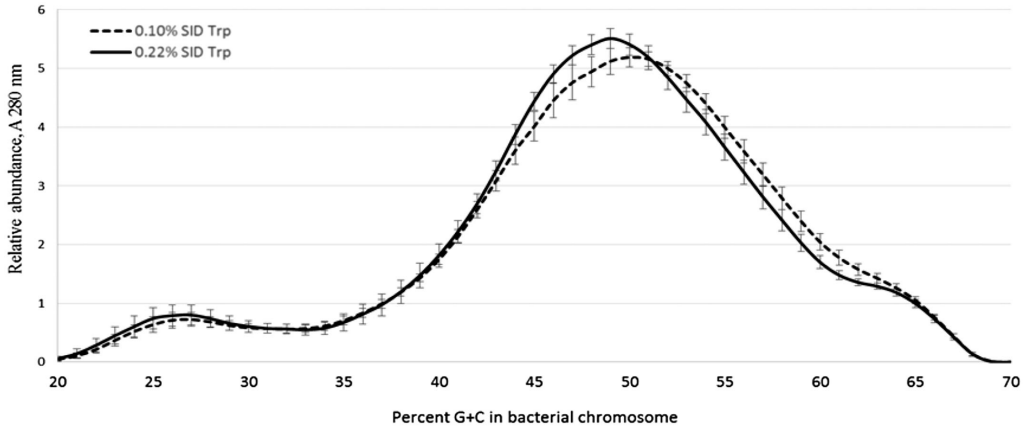
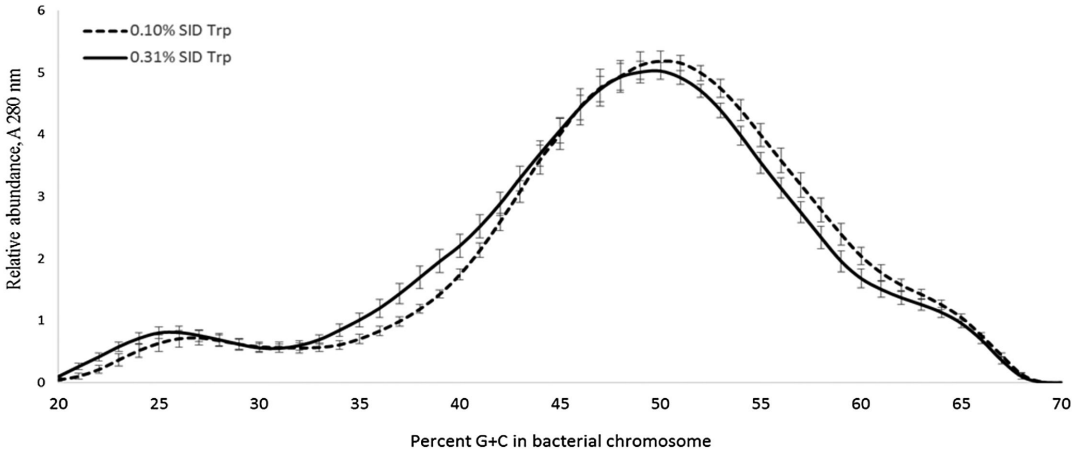


Figure 1. A pairwise comparison showing caecal microbiota composition measured by %G+C profiling of the total chromosomal DNA. The dotted line present caecal microbiota of hens fed diets containing 0.10% standardized ileal digestible Tryptophan whereas, the solid line present 0.16% standardized ileal digestible Tryptophan group. Each line represents the mean of the %G+C profiles of 8 replicate samples and the bars are standard errors of the mean.



Figures 2. A pairwise comparison showing caecal microbiota composition measured by %G+C profiling of the total chromosomal DNA. The dotted line present caecal microbiota of hens fed diets containing 0.10% standardized ileal digestible Tryptophan whereas, the solid line present 0.22% standardized ileal digestible Tryptophan group. Each line represents the mean of the %G+C profiles of 8 replicate samples and the bars are standard errors of the mean.



Figures 3. A pairwise comparison showing caecal microbiota composition measured by %G+C profiling of the total chromosomal DNA. The dotted line present caecal microbiota of hens fed diets containing 0.10% standardized ileal digestible Tryptophan whereas, the solid line present 0.31% standardized ileal digestible Tryptophan group. Each line represents the mean of the %G+C profiles of 8 replicate samples and the bars are standard errors of the mean.

between ileal protein digestibility and bird performance (Cowieson and Bedford, 2009; Cowieson and Roos, 2014). This may suggest that when the diet becomes deficient in any one of the essential amino acids the protein digestibility decreases and as a consequence it is natural for the bird to reduce weight (Apajalahti and Vienola, 2016). This could be the likely reason why in the current study hens fed on 0.10% SID Trp having no supplemental Trp had reduced OWG. Another possible reason for suppressed growth and efficiency could be due to bypass of soluble protein from the ileum to the caecum and thus could have caused a shift in mucin production, epithelial cell sloughing, and consequently increased the concentration of harmful metabolites (Apajalahti and Bedford, 2000).

It has been shown that Trp decreases the aggressive behaviors (Shea et al., 1990) in birds by increasing central levels of the neurotransmitter serotonin (Raleigh et al., 1985). Serotonin is reported to regulate hypothalamic-pituitary-adrenal axis, because activation of the serotonergic system causes increased secretion of adrenocorticotrophic hormone and corticosteroids in mammals (Fuller, 1981). However, in the current study hens fed different supplemental levels of Trp over a 16 wk period did not show any signs of stress in hens as indicated by no differences ($P > 0.05$) in serum corticosterone levels ($P > 0.05$) and plumage and comb pecking feather pecking score data presented elsewhere (Dewart, 2014).

There were significant differences ($P < 0.05$) in total protein, albumin, glucose, calcium, and corticosterone between treatments (0.16, 0.22, 0.21 SID Trp vs. 0.10% SID Trp). However, all differences were within the normal physiological ranges (Hrabcakova et al., 2014). The increase in total protein and albumin in Trp-supplemented hens is reported to be due increase in the serum insulin like growth factor (IGF-1) which either increases the absorption of amino acids (Jacob et al., 1989) or inhibits protein degradation by inhibiting the expression of cathepsin B and 20S protease and plasma cortisol secretion (Simmons et al., 1984).

Percent G+C profiling is a molecular technique where the outcome is not a list of quantified bacterial species but a profile indicating the relative abundance of bacteria with different DNA base composition (percentage of G+C of the total base pairs in the chromosome). This method depicts the entire bacterial community and, hence enables the detection of putative alterations at the community level without any bias common for molecular analysis techniques which are dependent on pre-designed primers or probes. In the current study, hens fed diets containing 0.01% SID Trp tended to reduce lactic acid bacteria such as members of genera *Lactobacillus*, *Streptococcus,* or *Enterococcus* (G+C 39 ± 2%) compared to those in 0.16% SID Trp group showed a slight increase in the abundance of bacteria belonging to genus *Bifidobacterium* (G+C 62 ± 3%). It was noted that hens fed diet containing 0.22% SID Trp showed significant effect ($P < 0.05$) on caecal microbial profiles. The observed shift may indicate reduction in numbers of *Ruminococcaceae* spp. and *Bifidobacterium* spp. and concurrent increase in numbers of *Lachnospiraceae* spp. (Apajalahti et al., 1998; Apajalahti and Vienola, 2016). Many members of the latter are butyrate producers and considered beneficial for intestinal health. However, hens fed diets containing 0.31% SID Trp showed significant increase in lactic acid bacteria (G+C 38 ± 3%) belonging to genera *Lactobacillus*, *Streptococcus,* or *Enterococcus* and concomitant reducing trend in bacteria with %G+C 52 to 60 belonging to family *Ruminococcaceae* (Clostridial cluster IV) as reported by Apajalahti and Vienola, (2016).

Amino acids produce toxic end-products when fermented by putrefactive bacteria. The indole compounds are considered to be uremic toxins among the Trp metabolites (Saito et al., 1980). In this study, it was hypothesized that SID Trp excess would be bypassed to caecum and there it would be converted into potentially

harmful indole and/or skatole. When these fermentation products are produced in high concentrations they may have adverse effects on chicken growth and performance due to their function as uncouplers, which prevent the formation of ATP in oxidative phosphorylation mitochondria (Apajalahti and Vienola, 2016). Some of the skatole shifts were consistent with changes in the low G+C clostridia which contain among other bacteria a skatole producer, *Clostridium scatologenes* and the causative agent of necrotic enteritis, *Clostridium perfringens*. Although both indole and skatole are end products of Trp fermentation, however, they may be produced by different bacteria. Therefore, it is possible that diet composition shifted bacterial activities so that skatole producers were favored at the expense of indole producers. Among the reported skatole producers are *Clostridium scatologenes* and *Clostridium drakei* both of which are obligate anaerobes found in habitats such as intestinal tract (Apajalahti and Vienola, unpublished data).

In conclusion, the data from the current study suggests that the requirement of the SID Trp is higher than the current NRC recommendations if laying hens are housed in enriched colony cages under commercial stocking density. Considering that EP rate is the main criteria for egg producers and that the quality of egg shell is important not only for the egg industry but also for the hatchery and consumer's perspective this study recommends the inclusion level of SID Trp in brown hens aged 22 to 38 wk old to be 0.22%.

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