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Effect of Supplementation of Fermented Yeast Culture During Summer on Plasma Leptin and Ghrelin and Expression of their Receptors in Different Tissues and on Production Performance During-Post Summer Period in PD 3 Chicken Line

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ABSTRACT

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The present experiment was conducted to observe the effects of supplementation of fermented yeast (*Saccharomyces cerevisiae*) culture (FYC, a commercial product at 1.5×10^7 cfu/g) on physiological blood parameters, expression of genes for hormone receptors and on production performance of layers during summer season. The control group was devoid of supplementation of FYC. A total of 150 layers (PD 3 chicken line; 17 weeks age) were divided into three equal groups each comprising of 10 replicates of 5 birds. The dietary treatments included supplementation of FYC to the basal diet at 0 (C; control), 0.5 (T1) and 1.25 (T2) g/kg. The results indicated that the concentration of the hormones leptin and ghrelin was significantly ($P<0.01$) greater for the control group. Further, the plasma concentration of malondialdehyde (MDA) ($P<0.01$) and total cholesterol ($P<0.05$) were significantly greater at 49d of the experiment in the control group when compared with the respective parameters in the group of birds supplemented with the higher dose (1.25 g/kg) of yeast culture (T2). The expression of leptin- and ghrelin-receptors was down-regulated in the treatment groups significantly ($P<0.05$) in brain, liver (T2) and magnum (portion of reproductive tract) tissues (T1). The histopathological evaluation of the intestinal tissues indicated that the severity of the necrosis of the villi of the jejunum was mild for the treatment groups when compared with that of the control (C) group. Hence, it can be concluded that supplementation of yeast culture @ 1.25 g/kg was beneficial in lowering stress markers like cholesterol, MDA and further reducing the severity of necrosis resulting in increased egg production, fertility and hatchability.

Keywords: Egg production, Fermented yeast culture, Hormone, PD3 chicken line, Summer.

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INTRODUCTION

Heat stress remains a perpetual challenge for the poultry enterprises in tropical climate of India. During growth phase, chickens have higher metabolic activity (Freitas *et al.,* 2014), and the stress experienced during summer will be more. Heat stress interferes with the bird's comfort and suppresses productive efficiency. There is increased excretion of electrolytes and catabolism of amino acids, which contribute to increments in body's heat. The stress experienced during growth phase, may have an impact on egg production and feed efficiency in the later part of the season coinciding with the laying period. These are the two important parameters which govern the economics of the layer production. Heat stress also leads to cellular oxidative stress, which causes damage to cell membranes. As a consequence of exposure to higher ambient temperature during summer, birds reduce their feed intake (Geraert *et al.,* 1996; Garriga *et al*., 2006) in order to diminish the metabolic heat for maintaining their body temperature.

Leptin, probably because of its sensitivity to heat stress, has been suggested to play a key role in regulation of energy metabolism under heat stress conditions (Morera *et al*., 2012). Leptin exerts its effect by binding to a receptor which belongs to the cytokine receptor super-family (Newman and Gonzalez-Perez, 2013). Its expression at the level of the ovary (Ohkhubo *et al.,* 2000) suggests that leptin might act directly on the ovary to regulate chicken reproductive function. Exogenous leptin treatment modulates physiological process including feed intake, immune response and ovarian functions in birds (Ohkubo and Adachi, 2008, Paczoska-Eliasiewicz *et al*., 2003). A direct effect of ghrelin on basic ovarian functions including apoptosis, proliferation, steroid and peptide hormone secretion has been reported (Sirotkin and Grossman, 2008). Plasma ghrelin comes essentially from the stomach. Significant levels of ghrelin are also expressed in other tissues including placenta, testis, kidney, pituitary, small intestine, pancreas, lymphocytes, brain, lung, and ovary (Gualillo *et al*., 2003). Likewise, adenosine monophosphate-activated protein kinase (AMPK) is an important enzyme that, when activated, is effective at cranking up the metabolism and burning fat (Walter *et al.,* 2010). However, no information is available on the above-mentioned parameters in case of chicken.

Supplementation of dried yeast containing *Saccharomyces cerevisiae* as a pure culture to high fiber diets containing palm kernel meal significantly ($P < 0.05$) improved BW gain and feed efficiency of broiler chicks (Onifade and Babatunde, 1996). Miles and Bootawala (1991) and Liu *et al.* (2000) reported improvement in feed efficiency and egg components quality on supplementation of yeast culture.

It was hypothesized that, supplementation of fermented yeast culture may improve production performance, through modulation of physiological parameters during pre-laying period. The present study was taken up to study the effect of summer season on hormones and their receptors. Fermented yeast culture used in the present study, is a commercial product composed of yeast and the media on which

it is grown, and is a source of vitamins, and amino acids, etc. (Yalcin *et al.,* 2008). Fermented yeast culture is a source of amino acids and minerals, which may partially compensate for the loss of these elements during stress and improve production performance. Further, since leptin and ghrelin modulate feed intake, and summer season is known to affect feed intake, it was of interest to study the effect of these hormones on different parameters during summer period.

There are no reports available in the chicken, especially layers, on the effect of heat stress on leptin, ghrelin and their receptors. Further, reports are also not available with respect to the effect of supplementation of FYC in alleviation of heat stress, on metabolites, hormones and their receptors. Supplementation of yeast culture has beneficial effects on production and health (Gao *et al.*, 2008; Song *et al.*, 2014), and thus it was hypothesized that supplementation of FYC to PD3 chickens during pre-laying and laying period coinciding, respectively with summer and post-summer may prove beneficial by increasing the egg production.

MATERIALS AND METHODS

The experiment was conducted at poultry farm of the Directorate of Poultry Research. The summer period considered was from mid-April to mid-June. The duration of the summer period was for eight weeks (with the age of the birds from 17 to 24 weeks). The study during post-summer period was extended from 25 to 32 weeks of the age of the birds. During summer the temperature varied between 39-29 °C, and relative humidity varied between 40-60%.

Management and feeding of birds

A total of 150 birds of PD 3 chicken line were divided equally into three groups. Each group consisted of 50 numbers of birds, there were ten replicates and each replicate consisted of five birds. The birds were housed in individual cages (30.5×46×58 cm) and maintained on a 16L: 8D photo-period and standard conditions of temperature and ventilation as per the

Table 1. Ingredient composition (%) of the diets† for the experimental layers

Ingredients	Diet	
	Layer grower	Layer breeder
Maize	56.05	61.39
Soyabean meal	24.09	24.74
De-oiled rice bran	15.3	0.49
Stone grit	1.86	10.9
Di-calcium phosphate	1.66	1.5
Salt	0.35	0.35
Sodium bicarbonate	0.1	0.1
DL-methionine	0.11	0.1
L-Lysine	0	0
Trace minerals	0.1	0.1
Vitamin premix	0.015	0.1
Vitamin B-complex	0.015	0.1
Antibiotic	0.05	0.05
Choline chloride	0.1	0.1
Toxin binder	0.1	0
Tylosin	0.05	0
Coccidiostat	0.05	0

†Detailed composition are as per Panda *et al.* (2012)

farm's standard operating procedures. Feed intake and BW of the birds were recorded at 15d intervals between 17-32 weeks of age of the birds. Water was supplied *ad libitum*. Fermented yeast culture (FYC; Diamond V XP, USA; www.diamondv.com) was supplemented at three different concentrations to the basal diet namely, at 0 (C; control), 0.50 (T1) and 1.25 (T2) g/kg, respectively, to the three groups of birds. Feed was provided *ad libitum* to all the groups. The composition of the basal diet is given in Table 1. During grower's phase (16-20 weeks of age) the CP of the diet was 18% and ME 2800 kcal/kg. During layer's phase (21-32 weeks) CP was 15% and ME was 2500 kcal/kg. Basal diet was offered to hens as per the composition followed by Panda *et al*. (2012). Supplementation of FYC was initiated at the beginning of 17-week age of the birds and continued till they attained 32 weeks of age. Blood samples were collected during summer period only for seven weeks. In the post-summer period blood samples were not collected, because it was intended to observe the effect of physiological parameters observed during summer season on egg production.

Estimation of hormones

Leptin and ghrelin hormones were estimated by competitive enzyme immunoassay method, using commercial EIA kits (BlueGene Biotech, Shanghai, China), in the blood samples collected at 2-weeks intervals (starting from d7) from six randomly selected birds from each group. The blood samples were collected from the brachial vein of the chicken. After collection of blood samples in to heparinised tubes, samples were centrifuged at $3000 \times g$ for 15 min, plasma was separated and stored at -20°C for the assay of hormones and metabolites. For the assay of the hormones, HRP enzyme was used in the enzyme immunoassay method. At the end of the assay the absorbance of the coloured product formed was measured at 450nm. The intra- and inter-assay coefficient of variation was $<8\%$.

Gene expression studies

For quantification of hormone receptors, five birds from each group were selected at random, and were sacrificed on 35d (21 weeks of age) of the summer period. Liver, brain and magnum tissues were excised out. After collection of tissues, approximately 100g of the tissues were taken, cleaned and rinsed in saline and stored at -40°C for gene expression studies. For the brain tissue, samples were pooled together. Total RNA was extracted from liver, brain and magnum tissues by using TRIZOL (In Cell Technologies, Hyderabad, India) and further converted to cDNA using First strand cDNA synthesis kit (Thermo Fisher Scientific, India). It was amplified with respective primers (Table 2).

Table 2. Primers used for gene expression study

Name of the gene	Sequence of the primers
Leptin Receptor	5' – GTGGCTGAAGACTGTGATTGGTGTA – 3'-FP
	5' – TACGGCATCGGTACAGGCTCAGA – 3'-RP
Ghrelin Receptor	5' – CTGCAAGCTCTTCCAGTTCATCAGC – 3'-FP
	5' – CCAGAGGATGAGGATGACCAGCTTG–3'-RP

The primers were got synthesized at Chromous Co. Pvt. Ltd., Bangalore. Actin was taken as house-keeping gene. Once the PCR protocol with different primers was standardized, the cDNA was subjected to qPCR for quantification of leptin- and ghrelin-receptors.

Assay of plasma metabolites and enzyme

Estimation of plasma total proteins, total cholesterol, AMP kinase, malondialdehyde (MDA) (BlueGene Biotech Co., Shanghai, China) and protein carbonyl (Cayman Co. USA) was done using commercially available kits. The MDA was assayed based on the reaction of MDA with a chromogenic agent thiobarbituric acid (TBA) at high temperature under acidic conditions to form MDA-TBA adduct. The complex absorbance was measured at 540 nm. Concentration of plasma AMP kinase was estimated by competitive enzyme immunoassay method. Horse radish peroxidase enzyme was used and in the final step, the absorbance of the coloured complex formed was measured at 450 nm. The estimation of plasma total cholesterol was based on the methods of Zlatkis *et al*. (1953).

Processing of tissues for histopathology

For histopathological studies on jejunum, five birds from each group were randomly selected and sacrificed, and jejunum portion of the digestive tract was excised out and cleaned. The contents of the jejunum were gently removed. The tissue was rinsed in saline and fixed in 10% formaldehyde. Intestinal segments were kept in Bouin's solution and dehydrated in a graded series of alcohols. Finally, each specimen was embedded in paraffin wax. Sections were fixed on glass slides and stained with haematoxylin-eosin. Further the slides were observed under light microscope for the observation of the morphology and necrosis of the villus. The severity of the necrosis was graded as severe (+++), medium (++), mild (+) and normal (±).

Recording of egg parameters

Laying rate and egg weight were recorded daily for all the groups from 25-32 weeks of age during post-summer period. The fertile eggs were detected by the procedure of candling on the 18th day of incubation, and percentage of fertility was calculated. The percentage of hatchability was calculated as the percentage of eggs which actually hatched out as live young.

Statistical analysis

The data were analyzed using two way-ANOVA, with treatment and period as main factors using general linear model procedure (GLM) of SPSS 10 for Windows. The effects of dietary treatment on plasma hormones, metabolites and receptor expression were analysed using one-way ANOVA, for observing the significant effect of treatments on parameters for a particular time period. LSD was utilized for pair-wise comparison of the least square means $P < 0.05$ (SPSS user guide).

Table 3. Effect of supplementation of fermented yeast culture on levels of plasma leptin and ghrelin in PD 3 chicken line during summer season

Days	Dietary treatments[†]			Significance[‡]
	C	T1	T2	
Leptin (ng/ml)				
7	$1.657^{a}\pm0.09$	$1.608^{a}\pm0.15$	$1.252^{b}\pm0.05$	T, P, T×P
21	$1.824^{a}\pm0.06$	$1.788^{c}\pm0.08$	$1.345^{b}\pm0.15$	
35	$1.683^{a}\pm0.08$	$1.665^{a}\pm0.06$	$1.319^{b}\pm0.06$	
49	$1.524^{a}\pm0.10$	$1.536^{a}\pm0.12$	$1.397^{b}\pm0.10$	
Ghrelin (pg/ml)				
7	62.01 ± 3.20	60.23 ± 1.25	54.78 ± 2.11	T, P, NS
21	$77.57^{a}\pm2.10$	$74.23^{a}\pm2.19$	$67.56^{b}\pm2.01$	
35	$68.70^{a}\pm2.20$	$67.43^{a}\pm2.53$	$54.62^{b}\pm2.38$	
49	$58.29^{a}\pm2.39$	$59.45^{a}\pm2.34$	$49.75^{b}\pm2.93$	

[†]Basal diet alone (C; control) or supplemented with fermented yeast culture at 0.50 (T1) and 1.25 (T2) g/kg.

[‡]Significant ($P<0.05$) effects of the dietary treatment (T), period (P) and their interaction (T×P); NS, non-significant ($P>0.05$)

[abc]Values with different superscripts in a row are significantly different ($P<0.01$). NS-not significant.

RESULTS AND DISCUSSION

Observations of the birds during the summer period revealed that the hens panted, and spread the wings away from the body, which, according to Furlan and Macari (2002), is behaviour of birds suffering from heat stress. Such behaviour was observed, but could not be differentiated between the groups. Birds are homoeothermic and are able to maintain body temperature, but when the ambient temperature is high the dissipation of heat is less (Yahav, 2009). Some supplement can be given along with the feed, which may modulate factors associated with heat stress and alleviate it.

The data on plasma levels of hormones are presented in Table 3. The plasma leptin concentration differed significantly ($P<0.01$) between the control and T2 group at 7d and 21d of the experiment (Table 3). Similar results were observed with respect to the concentration of plasma ghrelin which decreased from 21d to 49d in all the groups (Control, T1 and T2), the difference in the concentration of ghrelin at any time from 21-49d was significant ($P<0.01$), when compared between the groups, it being higher in the control and T1 group vs. T2 group. The supplementation of lower dose of FYC could not significantly reduce the concentration of ghrelin. A significant ($P<0.05$) effect of the period and the treatment was observed on the level of both hormones leptin and ghrelin, whereas for leptin, a significant ($P<0.05$) interaction between the two main factors was also apparent indicating that the treatment effect was not uniform over the time.

The expression fold of mRNA of the leptin and ghrelin receptors in liver, brain and magnum portion of the reproductive tract in both the treatment groups (Fig 1A, 1B) was significantly ($P<0.05$) lower when compared with their control counterparts. In the T2 group, the expression of the receptors were more down-regulated when compared with the expression of the receptors in the T1 group, except for the

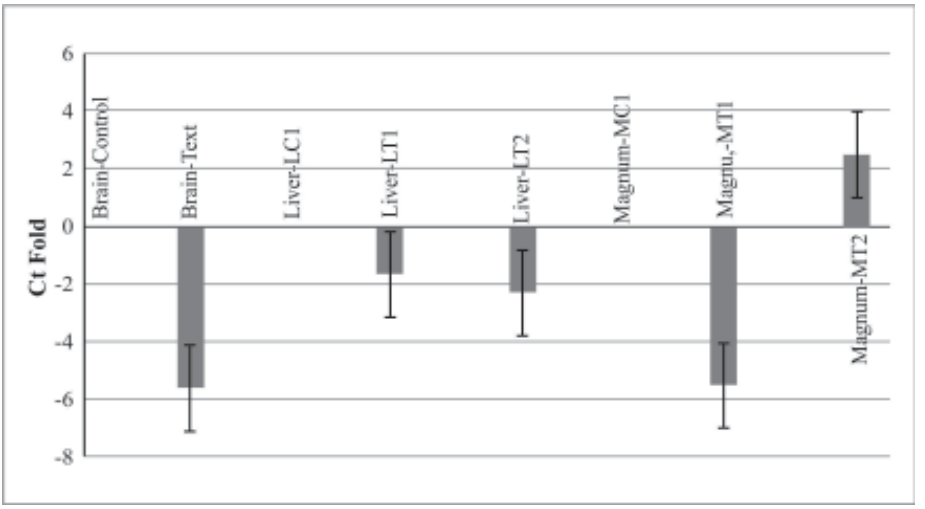

Fig. 1A. Relative down regulated gene expression of Leptin receptor in treatment groups T1 (0.5g/kg) and T2 (1.25g/Kg) with respect to control (C1) and normalized with respect to actin gene. The test values are significantly different ($P<0.05$) from control. C1 Control, T1 and T2 Treatment. N=5

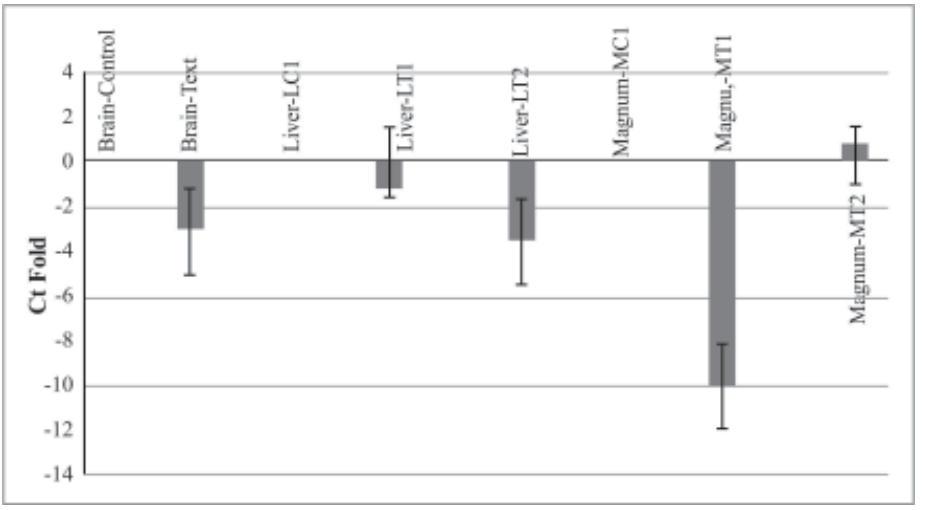

Fig. 1B. Relative down regulated gene expression of Ghrelin receptor in treatment groups T1 (0.5g/kg) and T2 (1.2 5g/Kg) with respect to control (C1) and normalized with respect to actin gene. The test values are significantly different from control ($P<0.05$). C1-Control, T1 and T2 Treatment. N=5.

Table 4. Effect of supplementation of fermented yeast culture on levels of different plasma parameters in PD 3 chicken line during summer season.

Parameters	Dietary treatments†			Significance‡
	C	T1	T2	
Total cholesterol (mg/dl)				
d7	$211.9^{a}\pm7.18$	$197.32^{b}\pm2.13$	$193.8^{b}\pm5.20$	T×P
d49	$234^{a}\pm9.23$	$222^{b}\pm7.28$	$163^{c}\pm5.36$	
MDA (μMol/ml)				
d7	$140.09^{a}\pm10.0$	$135.21^{a}\pm2.32$	$80.54^{b}\pm2.69$	T, P, T×P
d49	$100.2^{a}\pm5.2$	$108.00^{a}\pm4.5$	$58.00^{b}\pm1.58$	
Total protein (mg/ml)				
d7	3.60 ± 0.05	3.59 ± 0.05	3.54 ± 0.04	T, T×P
d49	$3.19^{a}\pm0.04$	$3.32^{a}\pm0.04$	$3.96^{b}\pm0.04$	
Protein carbonyl (nmol/mg)				
d7	321.19 ± 10.05	315.65 ± 4.32	320.62 ± 9.45	NS
d49	328.60 ± 2.3	320.10 ± 2.10	315.20 ± 3.10	
AMP kinase (ng/ml)				
d7	$0.99^{a}\pm0.03$	$0.85^{b}\pm0.02$	$0.88^{b}\pm0.02$	T, P, T×P
d49	$1.58^{a}\pm0.03$	$1.38^{b}\pm0.05$	$1.22^{b}\pm0.03$	

†Basal diet alone (C; control) or supplemented with fermented yeast culture at 0.50 (T1) and 1.25 (T2) g/kg.

‡Significant ($P<0.05$) effects of the dietary treatment (T), period (P) and their interaction (T×P); NS, non-significant ($P>0.05$).

[abc]Values with different superscripts in a row are significantly different ($P<0.01$).

magnum tissue, where it was up-regulated, when compared with the control or T1 group. The reason is not known. In the control group, the higher concentration of plasma leptin may be due to higher temperature present during the summer period, similar reports are available in mice (Morera *et al.,* 2012). It is known that soluble receptors are present in plasma for leptin (Martin *et al.,* 2008) and may be higher in the control group, which might have led to non-availability of peripheral leptin to the organs like hypothalamus and liver. Less amount of leptin and ghrelin in the tissues might have led to significantly higher expression ($P<0.05$) of hormone receptors in the control group when compared with the T2 group.

The data on plasma levels of metabolites and antioxidant indices are given in Table 4. The concentration of plasma total cholesterol was significantly greater ($P<0.05$) in the control group at 7d of the experiment when compared with the concentration of either treatment groups. However, at 49d the decrease in the level

of cholesterol of the treatment groups was dose dependant. The decrease in the T2 was greater ($P<0.05$) when compared with the levels of T1 group. Higher plasma cholesterol levels in broilers under chronic heat stress have been reported (Xie *et al.*, 2015). Supplementation of either dose of FYC could decrease the level of cholesterol significantly. It has been reported that yeast has substances which inhibit synthesis or lower cholesterol level in broilers (Shareef and Al-Dabbagh, 2009). It has been reported that β-glucan extracted from yeast cell wall has cholesterol lowering effect in mice (Kusumaiti and Dhewantara, 2016). It is reported that, higher ambient temperature increases the concentration of plasma cholesterol (Ismail *et al.,* 2013). In the present study also similar results were obtained, concentration being higher in the control group when compared with the treatment group. The treatment and period did not have any effects on cholesterol level, but there was a significant ($P<0.05$) interaction evident between the two factors.

Table 5. Effect of supplementation of fermented yeast culture on egg parameters in PD 3 chicken line during post summer season

Parameters / Dietary treatments†	C	T1	T2
Egg production, %	60	62	64
Fertility, %	86	89	91
Hatchability (FES), %	90	92	97

†Basal diet alone (C; control) or supplemented with fermented yeast culture at 0.50 (T1) and 1.25

Similarly, the concentration of MDA was significantly greater ($P<0.01$) in the control and T1 group at both 7d and 49d when compared with the T2 group (Table 4). The lower dose of FYC was not effective in causing a significant decrease the concentration of plasma MDA. Both the treatment and period had significant ($P<0.01$) effect on plasma MDA levels. Further, there was a significant ($P<0.01$) interaction between the period and treatment on plasma MDA level with an uniform decrease in the concentration of MDA over time. Cyclic chronic heat exposure during summer period affected the metabolic heat stress parameters positively and supplementation of FYC (1.25 g/kg) could reduce this effect. Higher plasma leptin and MDA levels have been reported under the effect of heat stress (Al-Azraqi, 2008; Ismail *et al.*, 2013). When there is lipid peroxidation, plasma MDA levels tend to be high. The difference in the concentration of plasma protein carbonyls was not significantly different at 7d or 49d when compared between control and with either of the treatment groups or between the treatment groups. It indicates that protein oxidation did not result in significant difference of the concentration of protein carbonyls between the groups.

The concentration of plasma total protein was similar among the groups at 7d. However, higher dose of FYC supplementation increased the level of plasma total protein significantly in the T2 group at 49d (Table 4). It is known that under chronic heat stress, the catabolism as well synthesis of proteins is reduced (Ma *et al.,* 2015). Hence, it might not have caused significant difference in the concentration of total plasma proteins between the groups at an earlier stage. However higher dose of FYC

caused a significant increase in the level of protein in the T2 group when compared with the other two groups. Treatment had a significant ($P<0.05$) effect on increasing the concentration of plasma total protein accompanying a significant ($P<0.05$) interaction between the treatment and period indicating that the treatment effect was not uniform i.e., the higher dose (1.25 g/kg) could only increase the concentration of plasma total protein. The concentration of plasma AMP kinase was significantly greater ($P<0.01$) in the control group when compared with the T1 and T2 groups at 7d as well as on 49d (Table 4). The difference in the concentration of plasma AMP kinase between T1 and T2 groups was not significant. It indicates that both doses of FYC were equally effective in reducing concentration of plasma AMP kinase. Cellular AMP kinase enzyme is known as energy sensor of the cell. Role of plasma AMP kinase is not well known. Higher concentration of plasma AMP kinase at higher ambient temperatures has been reported in dairy cows (Min *et al.,* 2015). It was suggested that it may be due to disturbances in liver metabolism. In the present study also, the concentration of plasma AMP kinase was higher at 49d in the control when compared with the T2 group. The effect of period and treatment were significant ($P<0.05$) for plasma AMP kinase level. There was a significant ($P<0.05$) interaction between the two factors as well. An increasing trend was observed in all the groups over a period of time but treatment could decrease the level of plasma AMP kinase at 49d of the experiment. During post-summer period, it was observed that supplementation of FYC (T2), had positive effect in increasing egg production performance, fertility and hatchability parameters of birds during 26-32 weeks of age (Table 5). It is well known that, heat stress decreases egg production (Al-Saffar and Rose, 2002; Saint-Pierre *et al.,* 2003). The egg weights of different groups did not differ significantly from each other. There is no report on effect of heat stress during pre-laying period on egg production during post summer period. Increase in egg production performance has been reported by Yalcin *et al.* (2008) on using the same supplement used in the present study, but the dose offered was higher. The difference in BW between the groups during or post-summer period was not significant (data not given)

Studies on histopathology of the jejunum revealed that upon supplementation of FYC the severity of enteritic necrosis was mild (70%) for the treatment (T2) group during post-summer period (Fig 2A). In the control and T1 groups the severity observed was at medium level with none exhibiting normal condition of the villi (Fig 2B, 2C). It was reported by Lambert *et al.* (2002) that higher ambient temperature causes increase in intestinal permeability and damage to the membranes due to oxidative stress. It was reported by Song *et al.* (2014) that feeding probiotic mixtures have beneficial effect on the integrity and morphology of intestine in broilers. Yeast cell wall improves ileal villus development (Zhang *et al.*, 2005; Shareef and Al-Dabbagh, 2009). Shareef and Al-Dabbagh (2009) have also reported that there is decrease in skin and meat TBARS concentration in broilers on supplementation of YC.

Fig. 2 (A, B & C). Mild (+) (A: T2) and medium necrosis (++) (B: Con; C: T1) of villi in the jejunum as observed under light microscope at 400x magnification.
C-control, T1-0.5g/kg, T2-1.25g/kg. Img 3—— Mild (+) (2A, for T2 group)
Img 4 and Untitled.300dpi in folder represent medium necrosis (++) (2B,2C; for C and T1)

CONCLUSIONS

Hence according to the hypothesis, supplementation of FYC (1.25 g/kg) to PD3 chickens during pre-laying and laying period coinciding, respectively, with summer and post-summer periods, proved beneficial effect by altering the level of hormones, metabolites, expression of receptors and increased the production performance. It is concluded that supplementation of FYC @ 1.25 g/kg had a positive effect on production performance and possibly helped the hens acclimatised to higher temperatures at an earlier time through its effect on plasma hormones, their receptors and metabolites.

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