www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2021; 10(7): 1280-1284

www.thepharmajournal.com
Received: 18-04-2021
Accepted: 29-05-2021

**Harsha M**
P.V. Narsimha Rao Telangana Veterinary University, Hyderabad, Telangana, India

**Swathi B**
P.V. Narsimha Rao Telangana Veterinary University, Hyderabad, Telangana, India

**Radhakrishna Pulikanti**
P.V. Narsimha Rao Telangana Veterinary University, Hyderabad, Telangana, India

**Rajanna Neeradi**
P.V. Narsimha Rao Telangana Veterinary University, Hyderabad, Telangana, India

**Kannaki TR**
ICAR- Directorate of Poultry Research, Hyderabad, Telangana, India

**Venkata Rama Rao S**
ICAR- Directorate of Poultry Research, Hyderabad, Telangana, India

**Priyanka E**
ICAR- Directorate of Poultry Research, Hyderabad, Telangana, India

**Corresponding Author:**
**Harsha M**
P.V. Narsimha Rao Telangana Veterinary University, Hyderabad, Telangana, India

# Effect of dietary antioxidant and aloe vera leaf powder supplementation on gut health and immuno-competence of Vanaraja birds under heat stress

**Harsha M, Swathi B, Radhakrishna Pulikanti, Rajanna Neeradi, Kannaki TR, Venkata Rama Rao S and Priyanka E**

**Abstract**
A total of 180 Vanaraja birds aged 8 weeks were divided into six groups *viz.*, three control groups fed on basal feed (BF), *Oxycure* supplemented basal feed (OX) and *Aloe vera* supplemented basal feed (AV) and their corresponding treatment groups BFHS, OXHS and AVHS. Treatment birds were subjected to heat-stress (38°C and 40% Relative Humidity (RH); Temperature-Humidity Index, THI=86), for four hours daily for a period of four weeks in an environment-controlled chamber. Control birds were maintained at ambient conditions throughout the trial period (mean THI=78.29). The magnitude of humoral immunity decreased significantly ($P \leq 0.01$) after four weeks of heat-stress and dietary supplementation of *Oxycure* had minimal impact. The cell mediated immunity also decreased significantly ($P \leq 0.05$) on exposure to heat-stress and dietary supplementation of either *Oxycure* or *Aloe vera* failed to improve it. Both duodenal and ileal mean villi heights were significantly ($P \leq 0.01$) greater in control birds fed on *Aloe vera* supplemented feed. Supplementation of feed with either *Aloe vera* or *Oxycure* yielded relatively higher duodenal and ileal villi heights and villi height to crypt depth ratios in heat-stressed subjects. Furthermore, heat-stress has significantly increased the number of harmful (*E. coli* and *Salmonella*) and decreased the number of probiotic (*Lactobacillus*) bacteria in the intestines. Dietary *Aloe vera* supplementation has also positively influenced the gut-microbiota in heat-stressed birds.

**Keywords:** Heat-stress, Vanaraja, temperature-humidity index (THI), gut health, immunity, *Aloe vera*, *Oxycure*

## Introduction

Increased demand for poultry products has led to the application of various breeding strategies to develop high yielding poultry varieties. These modern poultry genotypes are fast growing with higher metabolic activity and sensitivity to temperature changes (Settar *et al*., 1999; Deeb and Cahaner, 2002) [26, 6]. As a result, and with climate change becoming a reality, heat stress has emerged as a major concern in poultry industry (IPCC, 2007) [11].

Although accurate data on economic losses incurred by Indian poultry industry due to heat-stress are not available, studies suggest that these losses are alarming and require immediate and comprehensive approach to deal with. Therefore, it is imperative to begin focusing on climate resilient agriculture systems to deal with heat-stress and climate change to ensure fair productivity even under environmental adversities.

Heat-stress in birds leads to altered behavioural, physiological and immunological responses which generally result in decreased productivity and immune-competence (Lara and Rostagno, 2013; Shakeri *et al.*, 2018 and Wang *et al.* 2018) [13, 27, 30]. Higher environmental temperatures alter the activity of the neuro-endocrine system of poultry, resulting in the activation of Hypothalamic-Pituitary-Adrenal (HPA) axis, with elevated plasma corticosterone concentrations (Garriga *et al.*, 2006; Star *et al.*, 2008; Quinteiro Filho *et al.*, 2010 and Quinteiro Filho *et al.*, 2012) [9, 29, 20, 21].

Modulation of immune response by central nervous system (CNS) is mediated by a complex network between the nervous, endocrine and immune systems. Lymphocytes, monocytes or macrophages, and granulocytes exhibit receptors for many neuro-endocrine products of the HPA and SAM (Sympathetic-Adrenal-Medullary) axes, such as cortisol and catecholamines (released during stress), which can affect cellular trafficking, proliferation, cytokine secretion, antibody production and cytolytic activity (Downing and Miyan, 2000; Padgett and Glaser, 2003; Butts *et al.*, 2008 and Marketon and Glaser, 2008) [8, 18, 5, 15].

However, major immuno-suppressive effects of heat stress include reduced weights of lymphoid organs such as spleen, thymus and bursa (Quinteiro-Filho *et al.*, 2010 and Ghazi *et al.* 2012) [20, 10] and low levels of circulating antibodies (Bartlett and Smith, 2003) [3].

Vanaraja is a multi-colored, dual-purpose, backyard chicken variety developed to suit the free-range farming conditions of rural India. Males of Vanaraja grow up to 2 kg by 12 weeks, while the female chickens produce about 110 eggs up to 72 weeks under free range conditions (Zuyie *et al.*, 2009) [3]. These birds can perform well on diets poor in Metabolizable Energy (ME) and protein, unlike the commercial varieties, provided they are given balanced feed during the initial 6 weeks of age (Rama Rao *et al*., 2005) [23].

Although Vanaraja has displayed a promising performance in the field till date, there always is a need to re-evaluate the performance of these birds under adverse conditions owing to the dynamic nature of climate change. Thus, the present study was taken up to assess the impact of heat-stress exclusively on gut health and immuno-competence of Vanaraja birds along with subsidiary evaluation of heat-stress ameliorative properties of *Oxycure* (a vitamin and mineral supplement) and *Aloe vera* leaf powder (0.5%).

## Materials and Methods

### Experimental Birds

Vanaraja birds were used as experimental subjects in this study. Vanaraja is a dual-purpose chicken variety suitable for backyard rearing, developed by ICAR-DPR, Hyderabad.

### Feeding of Birds

All the birds were given standard Maize-Soya based basal diet supplemented with *Oxycure* (a mineral and vitamin supplement) and *Aloe vera* powder (0.5%) in certain treatments (Tables 1a and 1b).

### Temperature-Humidity Index (THI)

The ambient temperature and relative humidity (RH) inside the shed were recorded daily using a Traceable® Thermometer/Clock/Humidity Meter (*Cat. No. 4040*). The ambient temperature and RH readings were used to calculate the THI as per Moraes *et al*. (2008) [16].

A total of 180 birds aged 8 weeks were divided into six groups (n=30; 6 replicates × 5 birds) namely BF (fed on basal feed alone), BFHS (fed on basal feed and exposed to heat-stress), OX (fed on *Oxycure* supplemented basal feed), OXHS (fed on *Oxycure* supplemented basal feed and exposed to heat-stress), AV (fed on *Aloe vera* supplemented basal feed) and AVHS (fed on *Aloe vera* supplemented basal feed and exposed to heat-stress). Birds were subjected to heat-stress by exposing them to 38°C temperature and 40% RH (THI= 86) in an environment-controlled 'walk-in' humidity chamber (Newtronic®) daily for 4 hours from 9 to 12 weeks of age. Feed and water were provided *ad-libitum*.

Birds were vaccinated against Newcastle Disease Virus on day 5 (LaSota), day 28 (LaSota) and at the end of 9$^{th}$ week ($R_2B$). Serum and whole blood were collected for haemagglutination inhibition assay and lymphocyte transformation test, respectively. Antibody titre in each of the serum samples was recorded and expressed as the $\log_2$ value. These data were subjected to one-way ANOVA to test the effect of heat stress period on the humoral immune response of Vanaraja birds against Newcastle Disease Virus. Further, the significant differences were compared using Duncan's Multiple Range Test (DMRT) as modified by Kramer (1957) [12]. Lymphocyte transformation test was carried out as per Mosmann, 1983 [17].

### Histo-Morphometry of Duodenum and Ileum

At the end of the trial, 6 birds from each group were randomly selected and humanely slaughtered. Segments from the duodenum (midpoint), and ileum (≈10 cm proximal to the ileo-caecal junction) were taken and fixed in 10% formaldehyde solution and later embedded in paraffin wax. Histological studies were performed on 5 µm sections (4 cross-sections for each sample), stained by haematoxylin and eosin, and examined by Olympus® AX70 microscope fitted with an Olympus® DP27 digital camera. Tissue processing and staining were carried out as per the protocol laid by Luna, 1968. The villus height was measured from the villus-crypt junction to the villus tip, while crypt depth was defined as the depth of the invagination between 2 villi *i.e.*, downwards from villus-crypt junction to the end of mucosal layer (Awad *et al*., 2008), as shown in the fig. 1 and 2. The measurements were made using Olympus cellSens™ microscope imaging software.

### Intestinal Bacterial Count

At the end of the trial, a total of 36 birds (n=6) were randomly selected and humanely slaughtered. Intestinal contents from ileum and caecum were separately collected from each bird. The collected intestinal contents were processed and cultured under specific growth conditions in order to estimate the number of colony forming units (cfu) of *Escherichia coli* using EMB Agar- M317 (HiMedia®), *Lactobacillus species* using Lactobacillus MRS Agar- M641 (HiMedia®) and *Salmonella species* using Xylose-Lysine Deoxycholate (XLD) Agar- M031 (HiMedia®) in a known quantity of sample as per Wise (2006) [31] and Sanders (2012) [25], as shown in fig. 3, 4 and 5. The number of cfu is proportional to the number of viable bacterial cells present.

Data were subjected to one-way ANOVA to test whether these parameters differed significantly between the groups. Further, significant differences were compared using Duncan's Multiple Range Test (DMRT), as modified by Kramer (1957) [12].

## Results

### Haemagglutination Inhibition (HI)

No significant differences in the mean HI dilutions (Table 2) were observed among the groups even after three successive weeks of heat-stress exposure. However, at the end of fourth week of heat-stress exposure, significant ($P \leq 0.01$) differences between the groups were observed where all non-heat-stressed groups displayed better humoral immune response than that of the heat-stressed groups, *i.e.*, significantly ($P \leq 0.01$) greater mean HI dilution values in groups AV, BF and OX than in their corresponding heat stressed groups AVHS, BFHS and OXHS. Also, no significant differences were observed between the mean HI dilution values of all groups of heat-stressed birds (AVHS, BFHS and OXHS). Unlike, AV and BF groups, whose mean HI dilution values are significantly ($P \leq 0.01$) higher than that of their heat-stressed counterparts, the mean HI dilution value of OX group did not differ significantly with its heat-stressed counterpart at the end of 4$^{th}$ week of heat-stress exposure.

**Lymphocyte Transformation Test**

The results as depicted by the stimulation index values (Table 2) showed significantly ($P \leq 0.05$) higher values in birds subjected to heat-stress. However, the indices were similar among BF and BFHS. Higher index values indicate reduced cell mediated immune response and vice-versa.

**Histo-Morphometry of Duodenum and Ileum**

The results of the histo-morphometric study are presented in the table 3. In duodenum, significantly ($P \leq 0.01$) lower mean villi heights were observed in heat-stressed birds than in non-heat-stressed birds. Least mean villi heights (1597.58 ± 5.95 µm) were observed in BFHS, followed by BF and OXHS (1682.08 ± 6.31 and 1703.51 ± 3.17 µm, respectively), while the crypt depth was significantly ($P \leq 0.01$) higher in heat-stressed groups except in AVHS, whose values were comparable with all control groups.

In ileum, the mean villi height was significantly low in heat-stressed birds compared to control. It was least in BFHS (1161.93 ± 24.67 µm) and improved upon supplementation either with *Aloe vera* (1242.46 ± 4.40 µm) or with *Oxycure* (1232.74 ± 7.95 µm). Crypt depth differed significantly ($P \leq 0.01$) between AVHS and BFHS groups and between their corresponding control groups. However, in heat-stressed and control birds supplemented with *Oxycure*, mean crypt depth did not differ significantly.

Villi height-crypt depth ratios of both ileum and duodenum were significantly ($P \leq 0.01$) lower in heat-stressed birds than in control birds.

**Intestinal Bacterial Count**

The results pertaining to bacterial count are presented in table 4. Significant influence of treatment was observed on the counts of *E. coli* and *Salmonella* in caecum and counts of *Lactobacilli* in ileum. The number of *E. coli* was significantly higher in the caecum of heat stressed birds fed basal feed and *Oxycure* supplemented feed. The counts were intermediate in birds fed *Aloe vera* supplemented feed. While, significantly lesser number of *E. coli* were observed in the caeca of control birds fed basal feed and basal feed supplemented with *Oxycure*. Although statistically not significant, ileal *Salmonella* count was numerically higher in heat-stressed birds with and without supplementation, while caecal *Salmonella* count was highest in *Aloe vera* supplemented groups with 0.42 ± 0.12 million cfu/m coL (in AV) and 0.38 ± 0.10 million cfu/mL (in AVHS). Caecal count was significantly ($P \leq 0.05$) lower in control birds fed on basal feed and *Oxycure* supplemented basal feed. The ileal *Lactobacilli* counts significantly ($P < 0.05$) differed between the groups, with highest count recorded in AV (1.41 ± 0.28 million cfu/mL), followed by OXHS (1.10 ± 0.15 million cfu/mL).

**Discussion**

No significant influence of heat stress was observed on the humoral immune response even after three successive weeks of heat stress exposure. However, there was significant decrease in humoral immune response at the end of fourth week of heat stress in all the experimental groups, when compared to their respective controls. However, the decrease was non-significant among the two *Oxycure* supplemented groups indicating heat ameliorative influence of *Oxycure*. Similar beneficial effect of *Oxycure* was reported in the Annual Report of ICAR-DPR (2017-18).

The results of Lymphocyte Transformation Test as depicted by the calculated stimulation index values revealed that birds subjected to heat stress showed significantly poor cell mediated immunity when compared to their respective controls. Higher index value indicates reduced CMI response and *vice versa*. The results also suggest non-significant impact of supplementation of either *Oxycure* or *Aloe vera* on CMI response of heat stressed birds. The present observations are in agreement with the previous findings. Regnier and Kelley (1981) [24] reported suppressed CMI response in chicken exposed to either heat or cold stress. On the contrary, Puthpongsiriporn *et al.* (2001) [19] reported that CMI response was greater in hens fed with vitamin E and C.

The mean villi heights in duodenum and ileum were significantly lower in all the groups subjected to heat stress when compared to controls. Similarly, villi height-crypt depth ratios in duodenum and ileum decreased significantly in heat stressed birds compared to their respective controls. The present findings on alteration of histomorphometry are in agreement with the previous reports. For instance, Burkholder *et al.* (2008) [4] reported a significant decrease in crypt depth in heat stressed broilers compared with control group. While Deng *et al.* (2012) [7] observed reduced villi heights and villi height to crypt depth ratios in ileum and caecum of heat stressed birds. Further, Sohail *et al.* (2012) [28] reported significantly lowered villi heights and crypt depths in heat stressed birds. The present study showed a beneficial effect of *Aloe vera* supplementation to basal feed with respect to the morphometry of duodenum and ileum. Similarly, there is a marginal improvement in morphometry with *Oxycure* supplementation both in control and heat stressed groups. This is evident from among the heat stressed birds, those which were fed on *Aloe vera* and *Oxycure* had significantly ($P < 0.01$) greater duodenal and ileal villi heights than those fed on basal feed alone. Also, the supplemented groups showed significantly reduced intestinal epithelial cell turnover than those fed on basal feed alone. Several earlier studies reported beneficial effects of probiotic supplementation on the tropic effect of intestinal micro architecture (Wong *et al.*, 2006 and Rahimi *et al.*, 2009) [32, 22]. The feed supplements used in the present study may have acted as a prebiotic that supported the growth of probiotic microflora eventually showing improvement of micro architecture.

In the present study, ileal and caecal *E. coli, Salmonella* and *Lactobacilli* were counted to understand the impact of heat stress on gut microflora. Maximum number of *E. coli* in caecum was recorded in heat stressed birds fed on basal feed (0.65 ± 0.18 million cfu/mL) and *Oxycure* supplemented basal feed (0.67 ± 0.13 million cfu/mL) indicating no effect of supplementation of *Oxycure* on caecal *E. coli* count in heat stressed birds. Similarly, the caecal *E. coli* population in both the *Aloe vera* supplemented groups showed no difference indicating its positive influence on gut health. Though there was no significant difference in ileal *Salmonella* count among all the groups, the counts were numerically high in heat stressed birds. Ileal *lactobacilli* counts were highest in non-heat stressed birds supplemented with *Aloe vera*. The quality of microflora in terms of ileal *Lactobacilli* did not deteriorate in birds subjected to heat stress upon supplementation with *Oxycure* indicating the beneficial effect of *Oxycure* in heat stress conditions.

In general, evidence has been generated in this study to affirmatively conclude that gut health and immunocompetence of Vanaraja birds are affected under heat-stress and dietary supplementation of *Oxycure* and *Aloe* *vera* (0.5%) has marginally improved their performance. Supplementation of feed with either *Oxycure* or *Aloe vera* has had no impact on the magnitude of immune response under heat-stress. Conversely, both *Oxycure* and *Aloe vera* had beneficial effect on the gut health of heat-stressed birds as evidenced by the increased villi height-crypt depth ratios (both in duodenum and ileum). Heat-stress, in this study has significantly altered the quality of microflora and dietary supplementation of *Aloe vera*, but not *Oxycure*, was found to be beneficial in improving the quality of microflora.

**Acknowledgements**
The authors gratefully acknowledge the support of ICAR-DPR in extending all the facilities for the study.

**Statement of Animal Rights**
All activities involving birds including their slaughter were performed in compliance with the ethical standards with prior approval of Institutional Animal Ethics Committee, ICAR-Directorate of Poultry Research.

**Funding Sources**
This research received grants from National Innovations in Climate Resilient Agriculture (NICRA), a network project of the Indian Council of Agricultural Research (ICAR).

**Conflict of Interest Statement**
The authors declare that they have no conflict of interest.

**References**
1. Annual Report, 2017-18.. ICAR-Directorate of Poultry Research Rajendranagar, Hyderabad-500 030, Telangana, India 2018.
2. Awad WA, Ghareeb K, Bohm J. Intestinal structure and function of broiler chickens on diets supplemented with a symbiotic containing Enterococcus faecium and Oligosaccharides, International Journal of Molecular Sciences 2008;9:2205-16.
3. Bartlett JR, Smith MO. Effects of different levels of zinc on the performance and immunocompetence of broilers under heat stress. Poultry Science 2003;82:1580-88.
4. Burkholder K, Thompson K, Einstein M, Applegate T, Patterson J. Influence of stressors on normal intestinal microbiota, intestinal morphology, and susceptibility to Salmonella enteritidis colonization in broilers, Poultry Science 2008;87:1734-41.
5. Butts CL, Sternberg EM. Neuroendocrine factors alter host defense by modulating immune function, Cellular Immunology 2008;252:7-15.
6. Deeb N, Cahaner A. Genotype by environment interaction with broiler genotypes differing in growth rate 3. Growth rate and water consumption of broiler progeny from weight selected versus non selected parents under normal and high ambient temperatures, Poultry Science 2002;81:293-301.
7. Deng W, Dong XF, Tong JM, Zhang Q. The probiotic Bacillus licheniformis ameliorates heat stress-induced impairment of egg production, gut morphology, and intestinal mucosal immunity in laying hens, Poultry Science 2012;91:575-82.
8. Downing JEG, Miyan JA. Neural immunoregulation: Emerging roles for nerves in immune homeostasis and disease, Immunology Today 2000;21:281-89.
9. Garriga C, Hunter RR, Amat C, Planas JM, Mitchell MA, Moreto M. Heat stress increases apical glucose transport in the chicken jejunum, American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 2006;290:195-201.
10. Ghazi SH, Habibian M, Moeini MM, Abdolmohammadi AR. Effects of different levels of organic and inorganic chromium on growth performance and immunocompetence of broilers under heat stress, Biological Trace Element Research 2012;146:309-17.
11. IPCC (Intergovernmental Panel on Climate Change), Climate Change: Impacts, Adaptation and Vulnerability, Summary for Policy Makers 2007, www.ipcc.cg/SPM13apr07.pdf
12. Kramer CY. Extension of multiple range tests to group correlated adjusted means, Biometrics 1957;13:13-18.
13. Lara LJ, Rostagno MH. Impact of Heat Stress on Poultry Production, Animals 2013;3:356-69.
14. Luna LG. Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology, 3rdEdn. McGraw-Hill, New York 1968, 4-8.
15. Marketon JIW, Glaser R. Stress hormones and immune function, Cellular immunology 2008;252:16-26.
16. Moraes SRP de, Yanagi Junior T, Oliveira ALR de, Yanagi S de NM, Café MB. Classification of the temperature and humidity index (THI), aptitude of the region, and conditions of comfort for broilers and layer hens in Brazil. Proc. of International Livestock Environment Symposium - ILES VIII, Iguassu Falls City, Brazil, August 31 to September 2008;4:2008.
17. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, Journal of Immunological Methods 1983;65:55-63.
18. Padgett DA, Glaser R. How stress influences the immune response, Trends in immunology 2003;24:444-48.
19. Puthpongsiriporn U, Scheideler SE, Sell JL, Beck MM. Effects of vitamin E and C supplementation on performance, *in vitro* lymphocyte proliferation, and antioxidant status of laying hens during heat stress, Poultry Science 2001;80:1190-200.
20. Quinteiro, Filho WM, Ribeiro A, Ferraz de Paula V, Pinheiro ML, Sakai M, Sa LR, *et al*. Heat stress impairs performance parameters, induces intestinal injury, and decreases macrophage activity in broiler chickens, Poultry Science 2010;89:1905-14.
21. Quinteiro Filho WM, Rodrigues MV, Ribeiro A, Ferraz de Paula V, Pinheiro ML, Sá LR, Ferreira AJ. Acute heat stress impairs performance parameters and induces mild intestinal enteritis in broiler chickens: role of acute hypothalamic-pituitary-adrenal axis activation, Journal of Animal Science 2012;90:1986-94.
22. Rahimi S, Grimes JL, Fletcher O, Oviedo E, Sheldon BW. Effect of a direct-fed microbial (Primalac) on structure and ultrastructure of small intestine in turkey poults, Poultry Science 2009;88:491-503.
23. Rama Rao SV, Panda AK, Raju MVLN, Shyam Sunder G, Bhanja SK, Sharma RP. Performance of Vanaraja chicken on diets containing different concentration of metabolizable energy at constant ratio with other essential nutrients during juvenile phase, Indian Journal of Poultry Science 2005;40:245-48.
24. Regnier JA, Kelley KW. Heat- and cold-stress suppresses *in vivo* and *in vitro* cellular immune responses of chickens, American Journal of Veterinary Research

1981;42:294-99.
25. Sanders ER. Aseptic Laboratory Techniques: Plating Methods, Journal of Visualized Experiments 2012;63:e3064.
26. Settar P, Yalcin S, Turkmut L, Ozkan S, Cahanar A. Season by genotype interaction related to broiler growth rate and heat tolerance, Poultry Science 1999;78:1353-58.
27. Shakeri M, Cottrell JJ, Wilkinson S, Ringuet M, Furness J, Band Dunshea FR. Betaine and Antioxidants Improve Growth Performance, Breast Muscle Development and Ameliorate Thermoregulatory Responses to Cyclic Heat Exposure in Broiler Chickens, Animals (Basel) 2018, 8. Doi: 10.3390/ani8100162.
28. Sohail MU, Hume ME, Byrd JA, Nisbet DJ, Ijaz A, Sohail A, *et al*. Effect of supplementation of prebiotic mannan-oligosaccharides and probiotic mixture on growth performance of broilers subjected to chronic heat stress, Poultry Science 2012;91:2235-40.
29. Star L, Decuypere E, Parmentier HK, Kemp B. Effect of single or combined climatic and hygienic stress in four layer lines: 2. Endocrine and oxidative stress responses, Poultry science 2008;87:1031-38.
30. Wang Y, Saelao P, Chanthavixay K, Gallardo R, Bunn D, Lamont SJ, *et al*. Physiological responses to heat stress in two genetically distinct chicken inbred lines, Poultry Science 2018;97:770-780.
31. Wise K. Preparing Spread Plates Protocols 2006. www.microbelibrary.org/index.php /component/resource/laboratory-test/3085-preparing-spread-plates-protocols.
32. Wong JMW, de Souza R, Kendall CWC, Emam A, Jenkins DJA. Colonic health: Fermentation and short chain fatty acids, Journal of Clinical Gastroenterology 2006;40:235-43.
33. Zuyie R, Sharma VB, Bujarbaruah KM, Vidyarthi VK. Performance of Vanaraja birds under extensive system of rearing at different altitudes in Nagaland, Indian Journal of Poultry Science 2009;44:411-413.