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## Research Article

# Immune-Modulating Effects of Aviboost® Nucleotides on The Intestinal Epithelium of Broiler Chickens

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**ABSTRACT**

This study investigated the effect of dietary supplementation of nucleotides on growth performance, intestinal histomorphology and immunity response in broiler chickens. Four hundred one day old Cobb broiler chickens were randomly assigned to two separated groups, nucleotides treated group (NTG) and control group (CG). In NTG, Aviboost Poultry Tonic (with nucleotides, 5000 mg/liter) was given with the recommended dose for 16 hours daily in the first 3 days and continuously in drinking water at days 4 and 5 of age and at days 18, 19 and 20 of age, and repeated at day 25 till 30 days of age. Feed intake and mean body weight were calculated weekly till 5$^{th}$ week of age (35 days). Serum and intestinal samples were collected at 22 and 31 days for cytokine and intestinal histomorpholgical analysis, respectively. The mean body weight gain of the CG was 1.950 kg per bird compared with 2.112 kg per bird in NTG at 35 days of age. Feed conversion ratio (FCR) was improved in NTG (FCR = 1.67) compared to CG (FCR = 1.8). In NTG, Interleukin-6 and Interferon-γ mean levels were higher than the CG at both 22 and 31 days. In NTG, the absorptive cells of the villus demonstrated changes compared to CG in form of hyperplasia of the cells and increased the average height of the microvilli from 1.4 micron in CG to 1.82 and 2.4 micron at 22 and 31 day old group, respectively in NTG. The lamina propria of the villus of NTG depicted numerous lymphoid and plasma cells in 22 day old group and the population of the plasma cells increased in 31 day old group in number and activation. In conclusion, supplementation of diet with nucleotides stimulated the immunity and increased villi height and body weight gain of broilers.

**Key words:** Broilers-Immune-modulatory- Histomorphology and Innate immunity

## INTRODUCTION

The world population is expected to reach more than 9 billion by 2050, imposing food security challenges particularly for developing countries. The livestock vector is one of the fastest growing agricultural sectors contributing about 40 percent of the global value of agricultural production (Bruinsma, 2003). Despite the benefits to many of increased livestock production, this has created two major public health issues. First, sub-therapeutic use of antibiotics as growth promoters in animal feed has evoked widespread concern, with their use banned in many countries, due to the potential to develop antibiotic resistance in microbial populations associated with human and animal diseases. Second, some of the foods borne zoonotic diseases are serious public health concerns around the world. Probiotics are becoming increasingly popular as one of the alternatives to Antibiotic Growth Promoters (AGP). The most important objectives for using probiotics in animal feed are to maintain and improve the performance of animal and prevent and control enteric pathogens. Increasing numbers of probiotic products are being developed and used in animal nutrition. Fuller, 1989; defined probiotics as "live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance." The joint Food and Agriculture Organization (FAO) and World Health Organization (WHO) Working group defined probiotics as live micro-organisms which when administered in adequate amount confer a health benefit on the host (FAO/ WHO, 2001) which is the most widely accepted definition (Hill *et al*., 2014).

Nucleotides are low molecular weight intracellular compounds that participate in numerous biochemical processes. They consist of a nitrogenous base (pyrimidine or purine) linked to a pentose (ribose or deoxyribose) sugar to which one, two or three phosphate groups are attached. Nucleotides are the constitutive units of DNA

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and RNA nucleic acids frequently used to improve the disease resistance, to decrease the mortality and to promote the growth rate. Nucleotides participate to different important physiological processes in the body. As carriers, they allow the transport of ATP the main energetic molecule for the living cells. Moreover, they play a vital role in several enzymatic reactions affecting proteins, fats and carbohydrates metabolism. Some cells at high turn-over rate, such as gut and immune system ones (lymphocyte and bone marrow cells) are not able to produce enough nucleotides to cover their need. Furthermore, nucleotides promote the proliferation of bone marrow cells, stimulate the production and secretion of interleukins and interferon gamma and increase the cytotoxicity of the natural killer cells (Yu *et al*., 2002 and Sauer *et al.*, 2011). Dietary nucleotides act as a growth factors on the gut cells, promoting their differentiation and maturation, a higher thickness of intestinal mucosa and of villi and an increased activity of the digestive enzymes are subsequent to the benefit on the intestinal cells. Besides; higher villi surface and crypts depth increase upon inclusion of nucleotides in the diet (Gutierrez *et al.*, 2007, Domeneghini *et al*., 2006). This work was planned to investigate the effect of orally administered nucleotides on the intestinal health, size and cell population of villi as well as Interleukin-6 (IL-6) and Interferon-gamma (INF-γ) serum concentration in broiler chickens.

## MATERIALS AND METHODS

**Chickens:** Four hundred one day old Cobb broiler chickens were reared on letter in 2 separated groups. Balanced rations (with anticoccidial feed additive) and clean drinking water were given continuously, also, the following vaccination program was applied: Hitcher B1 at 6 days of age, IBD (Intermediate plus strain) at 13 days of age and lasota strain at 18 days of age.

**Nucleotides:** Aviboost Poultry Tonic, animal health, Bayer, Reg.No.23083 (Act 36/1947) with nucleotides "5000 mg/liter (w/v)". The product also contains essential fatty acids (500 mg/l) and zinc (3000 mg/l) in a water soluble non sugar base.

**Experimental design**

**First group (G1):** comprising 200 chicks, anti-mycoplasma drug (tilmycosin phosphate,275 mg) was given in the first 3 days for 8 hours daily. On the other hand, Aviboost Poultry Tonic was given with the recommended dose for 16 hours daily. At days 4 and 5 of age, Aviboost was given for 2 successive days in drinking water continuously. At days 18, 19 and 20 of age, Aviboost was given continuously and repeated at day 25 till 30 days of age (for 6 days continuously). Feed intake and mean body weight were calculated weekly till 5$^{th}$ week of age (35 days).

**Second group (G2, control group):** comprising 200 chicks and given anti-mycoplasma in the first 3 days for 8 hours daily. Feed intake and mean body weight were calculated weekly till 5$^{th}$ week of age (35 days).

**Serum Samples:** were collected at 22 and 31 days of age from the two groups for measurement of Interleukin-6 (IL-6) and Interferon-gamma (INF-γ) concentrations.

**Intestinal samples:** were collected at 22 and 31 days of age from the two groups for Electron Microscope.

**Interferon-gamma (INF-γ) assay** (Karakolev *et al.*, 2015). INF-γ concentrations were determined by immunoenzymatic assay. Chicken INF-γ ELISA kits (Novatein Bio, Massachusetts, USA) were used. In the wells of the ELISA plate, 7 standards were added with concentrations of 0, 6.25, 12.5, 25, 50, 100 and 200pg/ ml. Absorptions were measured at wavelength of 450nm. Interferon concentrations were calculated from the standard curve by means of a software product.

**Interleukin-6 (IL-6) assay:** It was determined using Sandwich-ELISA where the Micro-ELISA plate provided has pre-coated with antibody specific to chicken IL-6. The optical density (OD) was measured spectrophotometrically at a wavelength of 450nm. The OD value is proportional to the concentration of chicken IL-6. The concentration of IL-6 in the sample was calculated by comparing the OD of the sample to the standard curve according to Helle *et al.* (1991).

**Feed intake and feed efficiency:** Feed intake and feed efficiency were calculated during the period of experiment (till 5 week of age) according to Afsharmanesh and sadaghi, 2014; Landy, N. & Kavyani, A. 2013. The outcome may be increase feed intake without significant improvement in feed conversion ratio (FCR) or increase feed intake along with significant improvement in FCR.

**Body weight gain:** (Abdel-Rahman *et al*, 2013) Body weight gains were calculated during the period of experiment (till 5$^{th}$ week of age).

**Histomorphology:** (Bozzola and Russell, 1991) 5 – 10 small pieces 1 X 1 mm in size ware taken from each specimens and fixed in 5% cold glutaraldehyed immedialy after diseecting the animal for 24 – 48 h. The specimens ware then washed in cacodylate buffer ( PH 7.2 ) 3 – 4 times for 20 minutes every time and post fixed in 1% O4 s4 for 2 howrs, after that washed in the same buffer four times. Dehydration by ascending grades of alcohol (30 – 50 – 70 – 90 and 100% 2 hours) of each were done and were embedded in epon – araldite mixture according to the protocol of E.M. unit, Assiut University (Bozzol and Russell, 1991). From the embedded blocks semithin sections by L K B ultramicrotom in thickness of 0.5 – 1 micron were prepared for orientation of the tissue and photographed by sc30 Olympus camera and then ultrathin section in thickness of 500 – 700 A were made using leica AG ultramicrtome and contrasted in uranyl acetate and lead citrate, as usual examined by JEM 100 CXII electron microscope at 80 KV and photographed by CCD digital camera Model XR- 41.

**Statistical analysis:** The results were presented as means ± SD. All given parameters were compared between the control group and the vaccinated groups using the one way ANOVA with fixed effects of the factors using statistica 6.0 (Start Soft INC.). Differences were considered significant at $P \leq 0.05$.

## RESULTS

### Results of Interferon-gamma (INF-γ) and Interleukin-6 (IL-6) assay

Table 1 presented the average INF-γ concentrations in control and nucleotide treated group. In nucleotide treated group INF-γ mean levels in the blood were 170±10 and 420±50 pg/ml versus 125±0.56 and 290±20 in controls at 22 and 31 days, respectively., while the average IL-6 concentrations in control and nucleotide treated group. In nucleotide treated group IL-6 mean levels in the blood were 25±3 and 32±2 pg/ml 16±0.76 and 21±3in controls at 21 and 31 days, respectively.

### Feed intake and feed efficiency

The feed intake of the two groups was 7080 kg per group at 35 days of age (5$^{th}$ week).

### Body weight gain

The mean body weight gain of the control group was 1.950 kg per bird compared with 2.112 kg per bird in nucleotides treated groups at 35 days of age.

### Results of histomorpholgical changes of the duodenal mucosa in control and nucleotides treated group

### Control group

The control groups depicted the normal morphological structure of the duodenal mucosa (Fig. 1 and Fig. 4). The surface epithelium of the villas formed by absorptive cells of high columnar cells having microvilli in the luminal surface of average length 1.42 micron (Fig. 2) and did not changed in 22 and 31 day old (Fig. 5). In between the absorptive cells numerous Goblet cells were present (Fig. 5). In 31 day old group, slight focal hyperplasia of the epithelial cells were evident. Few small endocrine cells having small amount of electron dens granules were situated mostly on the basement membrane. The lamina propria of the villi of normal morphological appearance which formed by small vasculature, few connective tissue cells and fibers without any pathological reaction in 22 day old group (Fig. 3) and few small plasma cells were evident in the 31 day old group (Fig. 6).

**Table 1:** INF-γ and IL-6 concentrations in the control and nucleotides treated group

| Parameter | INF-γ conc. (pg/ml) | | IL-6 conc. (pg/ml) | |
|---|---|---|---|---|
| Groups | Control | Nucleotides treated | Control | Nucleotides treated |
| 22 day | 125±0.56 | 170±10 | 16±0.76 | 25±3 |
| 31 days | 290±20 | 420±50 | 21±3 | 32±2 |

**Table 2:** The mean body weight gain of the control and nucleotides treated group

| Mean body weight /week | Control group | Nucleotides treated group |
|---|---|---|
| 1 | 111.37 g | 131.3 g |
| 2 | 343.43 g | 348.5 g |
| 3 | 871 g | 900.66 g |
| 4 | 1470 g | 1550 g |
| 5 | 1995 g | 2112 g |
| Feed conversion rate | 1.8 | 1.67 |

### Nucleotide treated group

The absorptive cells of the villus depicted changes compared to control groups in form of hyperplasia of the cells (Fig. 7 and Fig. 10). Moreover, the average height of the microvilli increased to 1.82 micron in 22 day old group (Fig. 8) and 2.4 micron in 31 day old group (Fig. 11). The surface epithelium of the villus demonstrated light electron dens cells containing numerous membrane bounded fat globules (absorbed fat) in their cytoplasm (Fig. 11). The lamina propria of the villus depicted numerous lymphoid and plasma cells in 22 day old group (Fig. 9) and the population of the plasma cells increased in 31 day old group in number and activation (Fig. 12).

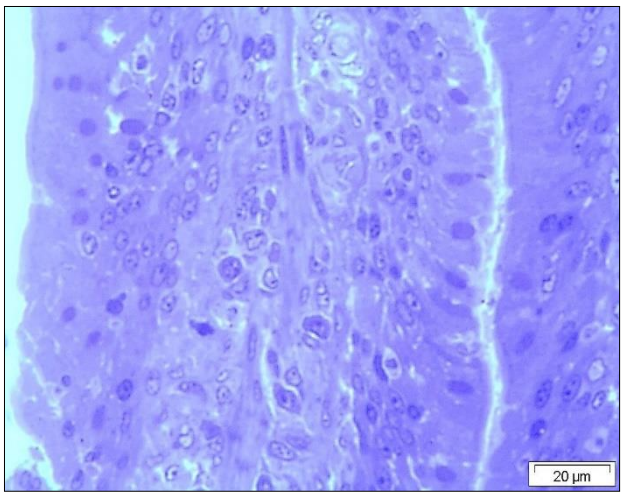


**Fig. 1:** Semithin section of duodenal villi formed by thin connective tissue core covered by absorptive epithelial cells of high columnar type with oval vesicular nucleus situated mostly at the base .Also contain numerous goblet cells found in between the absorptive cells but mostly in the upper portion.

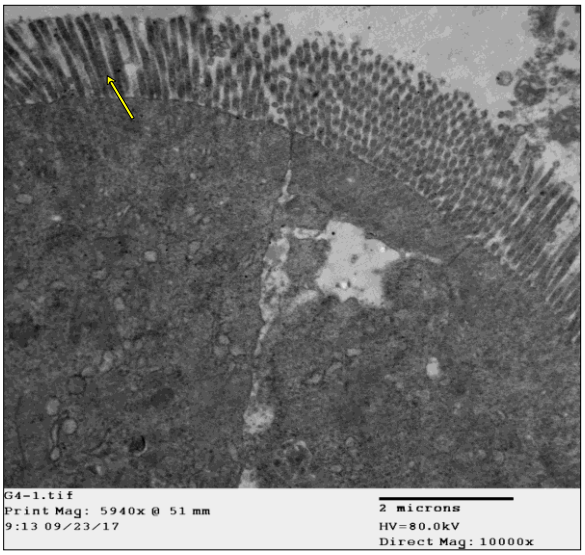


**Fig. 2:** T.E .microscopy of the absorptive cells (A) having microvilli (arrow) in average 1.42 micron in length. The cytoplasm contain mitochondria, free ribosomes, absorptive vacuole (v) and few electron dens granules.

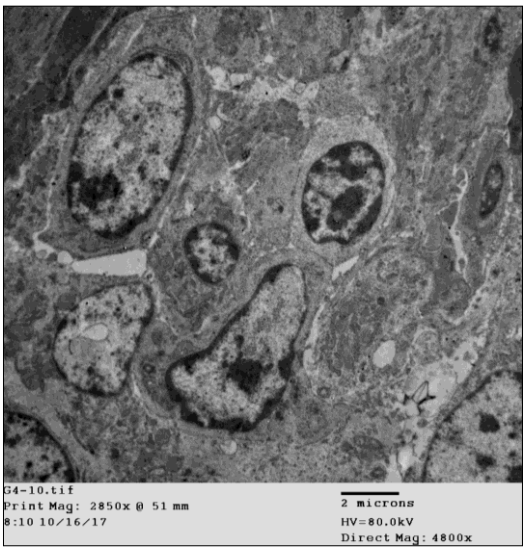


**Fig. 3:** T.E .microscopy of the villus core showing presence of macrophages, lymphoid cells (L), slight edema and few collagen fiber (Co).

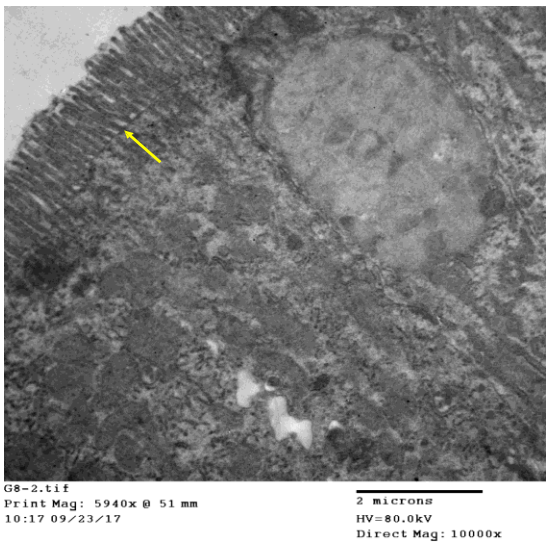


**Fig. 5:** T.E .microscopy showing the absorptive cells (A) having microvilli (arrow) about 1.42 micron length. The cytoplasm contain mitochondria, free ribosomes, RER, absorptive vacuoles (v) and electron dens granules (g). The goblet cell (G) destined with mucus globule and found embedded in between the absorptive cells.

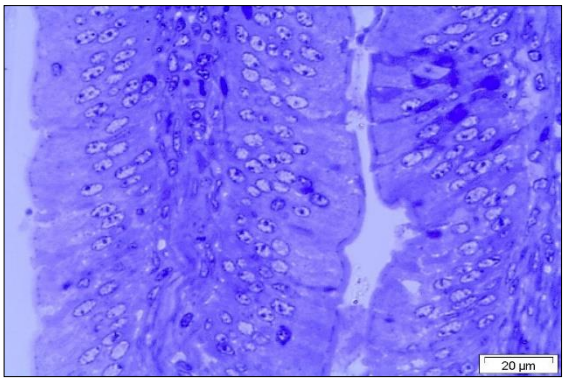


**Fig. 7:** Semithin section of duodenum showing thickening of the connective tissue core of the villi due to increases of the cell infiltration mostly of lymphoid type. The absorptive epithelial cells of the villi showing slight to moderate hyperplasia.

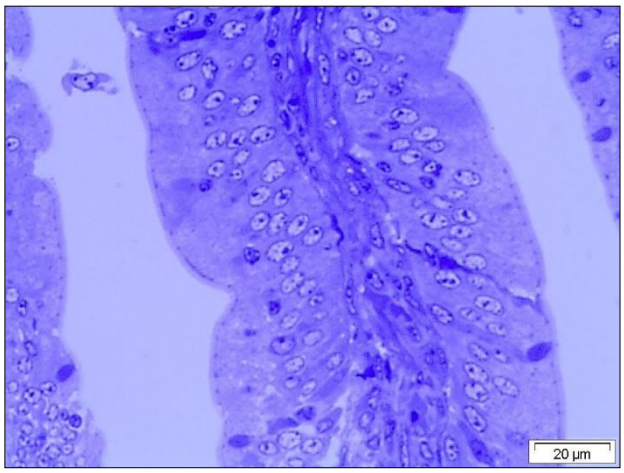


**Fig. 4:** Semithin section of duodenum villus more or less similar to the **fig. 1** by light microscopy with mild and focal hyperplasia of the absorptive cells.

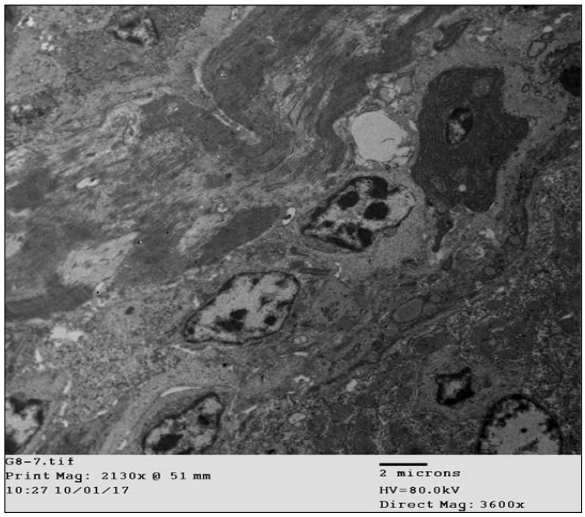


**Fig. 6:** T.E .microscopy of the connective tissue core of the villus contain collagen fiber (Co), few lymphocyte (L) macrophage (M) and plasma cell (P).

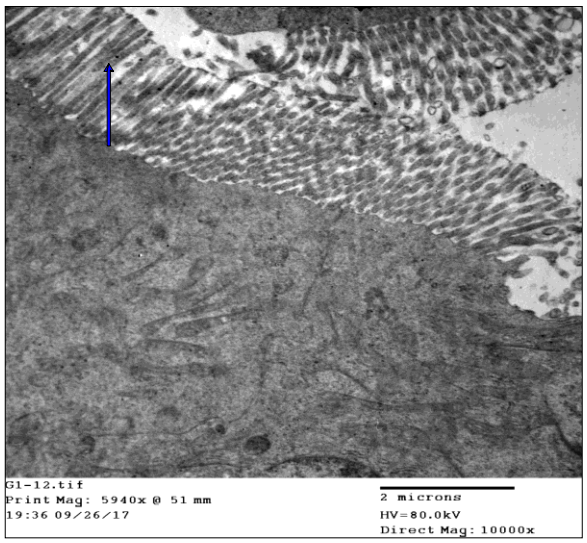


**Fig. 8:** T.E .microscopy of the absorptive epithelium (A) showing microvilli (arrow) of 1.82 micron length and their cytoplasm contain mitochondria, RER, free ribosoms and few small electron dens granules (g).

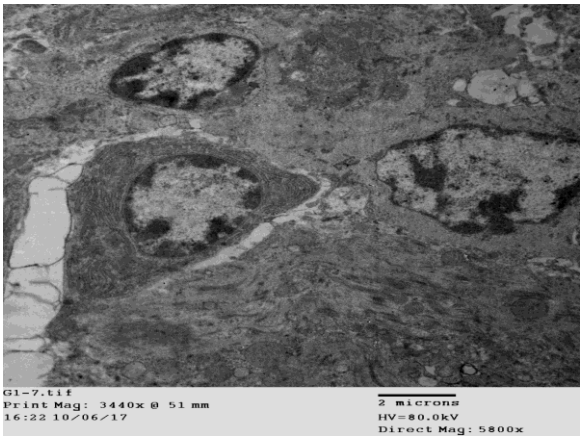


**Fig. 9:** T.E .microscopy of the connective tissue core of the villus of the duodenum showing edema with presences of few plasma cells (P), macrophages (M) and lymphoid cells (L).

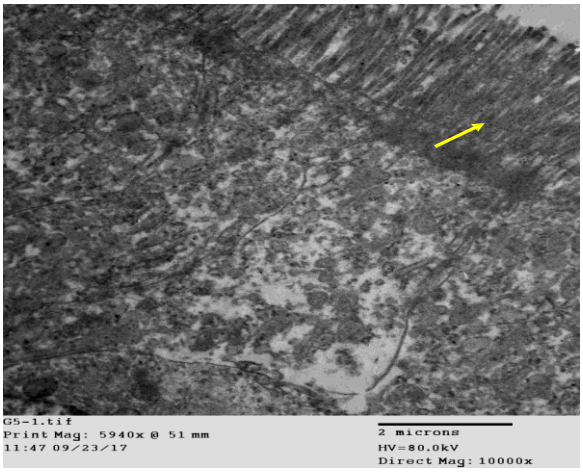


**Fig. 11:** T.E .microscopy of the absorptive cell (A) of the villi showing vacuolation (v) of the cytoplasm with presence of small fat vacuoles (f), mitochondria, RER and free ribosomes in their cytoplasm, The cell surface have erected microvilli (arrow) about 2,4 micron in length.

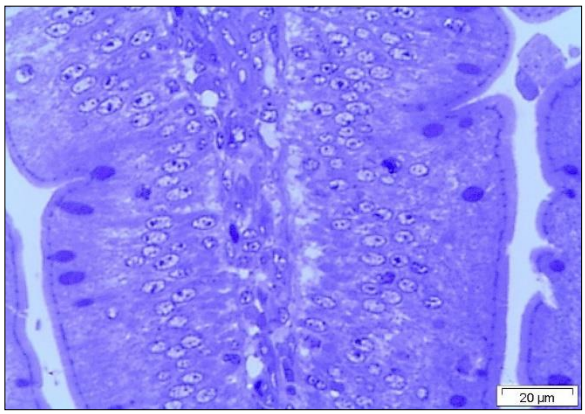


**Fig. 10:** Semithin section of duodenum showing the absorptive cells moderately hyperplasic with increases of their highs. The goblet cells become numerous. The connective tissue core of the villi showing slight edema with increases of the cellularity.

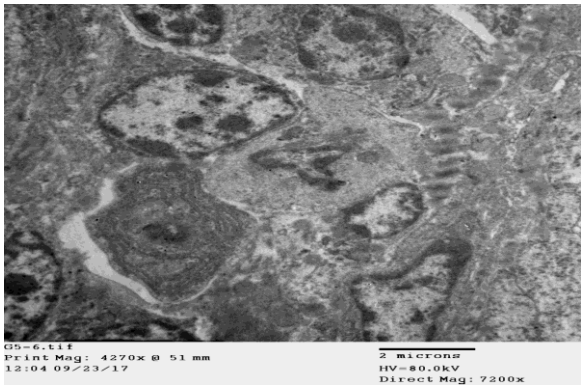


**Fig. 12:** T.E. microscopy of the villus core of the duodenum showing presence of numerous macrophages (M), lymphoid cells (L) and plasma cells (P).

## DISCUSSION

Nucleotides are well known for modulation of the immune system and improvement of gut health (Holen *et al*., 2004; Hess *et al*., 2012). In the current study, the effects of nucleotides supplementation on growth performance and modulation of the immune system and improvement of gut health of broiler chickens was investigated. In nucleotide treated group, Interleukin-6 and Interferon-γ mean levels were higher than the control group at both 22 and 31 days. The average mean levels of INF-γ mean levels in the blood were 170±10 and 420±50 pg/ml in nucleotide treated group versus 125±0.56 and 290±20 in controls at 22 and31 days, respectively.

Macrophages respond to a range of different cell products during the innate and acquired immune responses. Of these, INF-γ (originally called Macrophage- activating factor) is among the most important (Schroder *et al.*, 2004). INF-γ is the sole type II INF. It is structurally unrelated to type I INFs, binds to a different receptor, and is encoded by a separate chromosomal locus. Initially, it was believed that CD4+ T helper type I (Th1) lymphocytes, CD8+cytotoxic lymphocytes and Natural killer cells (NK) excessively produced INF-γ (Fischer *et al.*, 2015 and Sachdeva *et al.*, 2015). On the other hand, IL-6 mean levels in the blood were 25±3 and 32±2 pg/ml in nucleotide treated group versus16±0.76 and 21±3in controls at 21 and 31 days, respectively. Cytokines are secreted proteins involved with cell recruitment and regulation of both innate and adaptive responses. They are essential for an effective host immune response to pathogens. Chicken interleukin-6 (IL-6) has been confirmed to have a role in proinflammatory response (Kaiser *et al.*, 2000). An early stage of inflammation involves secretion of chemokines CXCLi2 (interleukin-8) as a chemotaxin for chicken heterophils which are comparable to mammalian neutrophils (Kogut, 2002 and Kaiser *et al.*, 2006).

The broilers supplemented with nucleotides demonstrated higher immune responses than control broilers. Although the exact molecular mechanisms by which nucleotides modulate the immune system are not completely understood, upregulation of toll-like receptors

by nucleotide containing diet might be related to the role of these products in the development and proliferation of tissues with rapid cell turnover such as enterocytes, and lymphocytes (Carver, 1999). In broiler chickens, toll-like receptor TLR21 was shown to act as a functional homologue to mammalian TLR9 and is involved in recognition of unmethylated CpG oligonucleotides (Keestra *et al*., 2010).

Dietary nucleotides might influence protein synthesis by modulation of the intercellular nucleotide pool (Bueno *et al*., 1994; Uauy *et al*., 1994; Holen and Jonsson, 2004). It has been reported that dietary nucleotides are capable of increasing cell-mediated immunity and improving host resistance to bacterial infections (Maldonado *et al*., 2001; Frankic *et al*., 2006; Hess *et al*., 2012). Therefore, inclusion of nucleotides in the diet of broiler chickens might be beneficial for activation of the local innate immunity of broilers under microbial challenge. For example, Thanissery *et al*. (2010) demonstrated that the diet containing nucleotides reduced *C. perfringens* level compared with the control diet on d 1 and 7 post-challenge.

In the present study, the mean body weight gain of broilers fed with nucleotides was higher than control broilers. Furthermore, feed conversion ratio (FCR) was improved in nucleotides treated group (FCR = 1.67) compared to control group (FCR = 1.8). These results in agreement with Bruno (2009) who evaluated the effect of different nucleotides levels (0 to 0.05%) on broiler performance and observed a linear improvement during the period of 1 to 21 days of age; however, the same benefit of nucleotides was not observed during the subsequent periods. Rutz *et al*. (2006) also verified broiler performance improvement when broilers were fed yeast extracts and attributed the better performance to the beneficial effect of the nucleotides present in the yeast extract.

The structure of the intestinal mucosa is an important determinant of intestinal function (digestive and absorptive) affecting growth performance of poultry. Generally, increase in villus height and villus height: crypt ratio increases the absorption of nutrients due to a larger surface area (Afsharmanesh and Sadaghi, 2014). Dietary nucleotides also have an essential role in the development and proliferation of tissues and cells with a rapid cell turnover such as the intestine and lymphocytes where *de novo* synthesis of nucleotides cannot meet their demand in such rapidly proliferating tissues. Therefore, adding nucleotides to diets may spare the energetic cost of *de novo* synthesis (Bueno *et al*., 1994; Uauy *et al*., 1994). Dietary nucleotides in poultry diets can affect the histology of the intestinal mucosa. In the present study, the absorptive cells of the villus of nucleotides treated broilers demonstrated changes comparing with the control in form of hyperplasia of the cells. Moreover, the average height of the microvilli increased from 1.4 micron in control group to 1.82 and 2.4 micron at 22 and 31 day old group, respectively in nucleotides treated group. The lamina propria of the villus of nucleotides treated group depicted numerous lymphoid and plasma cells in 22 day old group and the population of the plasma cells increased in 31 day old group in number and activation.Our results were in agreement with previous studies. For example, Tsujinaka *et al*., 1993 and Dell'Orto *et al*., 2002 demonstrated the beneficial effect of nucleotides on intestinal cell integrity, development, and turnover, with significant proliferation of crypt cell. Yu *et al*., 2002 observed that nucleotides also increase intestinal villi length, and improve the immune response of broilers, promoting nutrient absorption and increased weight gain. McCauley *et al*., 1998 found that nucleotides also have significant effects on enterocytes during intestinal development, maturation, and repair after damage caused by stress or pathogens. The villus height and the villus:crypt ratio in the intestinal mucosa were increased by dietary nucleotides and acceleration of intestinal epithelium repair after lesions caused by pathogens (Bueno *et al*., 1994).

**Conclusions**

Supplementation of diet with nucleotides stimulated the immune responses and improved body weight gain of broilers due to increase of the height of intestinal villi.

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