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Effect of Different Levels of Citrus Waste (*Kinnow sp.*) on Duodenal Morphology of Broiler Birds Without and With Cocktail of Enzymes

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ABSTRACT

The study was carried out to determine the effect of different levels of citrus waste (*Kinnow sp.*) as replacement of whole ration on intestinal morphology in broilers. The experiment consisted of 256 meat type birds. The day old birds were distributed into 8 groups and each group was replicated four times. Eight dietary treatments included Control (T_1), positive control (T_5) and Citrus waste was supplemented at 2.5, 5.0 and 7.5 percent level in diet T_2 to diet T_4 and citrus waste with cocktail of enzymes was added from diet T_6 to diet T_8 during starter (0-14 days), grower (15-21 days) and finisher (22-42 days of age) phases. The birds of equal body weight were slaughtered on 42nd day. After thorough examination sections of duodenum were collected and processed. Images were taken to measure villi height and crypt depth. Study showed that the inclusion of citrus waste up to 5% level improved ($P<0.05$) duodenal morphology with higher villi length and crypt depth. More number of polymorphonuclear cells was observed at places in mucosa suggesting better immunomodulatory effect due to inclusion of citrus waste in the diet. However, supplementation of enzyme (100 g/q) didn't showed any significant ($P<0.05$) difference between the groups. It can be concluded that supplementation of citrus waste decreased the villi height and crypt depth at higher citrus waste inclusion level.

Keywords: Citrus waste, *kinnow*, duodenal morphology, villi height, crypt depth

Poultry industry requires 10.9 million MT of broiler feed to satisfy the nutritional requirement of increasing broiler production which is becoming a tough task due to decrease in the availability of conventional feed resources. In poultry industry, feed alone cost approximately 60 - 70% of the total cost of production (Thirumalaisamy *et al*., 2016). Increases in the prices of feed ingredients have direct impact on the broiler industry both in terms of economics and demand. In order to meet the increased demand for poultry feed, search for novel feed resources, particularly those not competing with human food, is the key for sustainable development of the poultry industry. The food processing sector generate approximately 1.81 million tonnes of fruit and vegetable wastes in India which are either composted or dumped in landfills or rivers, causing environmental hazards (Wadhwa and Bakshi, 2013). One of the major contributions of financial health of food industry is the efficient utilization of waste produced during different stages of processing. These wastes left after processing are rich in essential nutrients that have the potency to be supplemented in animal diets as by-products. Citrus waste of the citrus processing industry is available in huge quantities in India which includes 60−65% peel, 30−35% internal tissues and up to 10% seeds which constitutes 50% of processed citrus (Crawshaw, 2004). The term citrus covers oranges, sweet lemon/lime, lemon, kinnow, grapefruit, tangerine, etc. The major by-products of processed citrus are dried pulp, molasses, washed pulp solids and essential oils. Dried citrus pulp contains almost 5–10% CP, 6.2% EE, 10–40% soluble fiber (pectins), 54% water-soluble sugars, 1–2% calcium and 0.1% phosphorus (Wadhwa and Bakshi, 2013). Citrus waste is abundantly available in India and has no interference with human requirements. This can be used as a good alternate feed ingredient for broilers. Use of citrus waste will not only help in preventing environmental pollution but will be a cost effective alternative feed ingredient for economical broiler production.

MATERIALS AND METHODS

Three hundred day old broilers chicks (IBL-80) were procured. Each bird was weighed on arrival and randomly assigned to eight different dietary treatments using completely randomized design. The feeding was done in three phases i.e. starter (0-14 days), grower (15-21 days) and finisher (22-42 days) phase. At the end of experiment i.e. on 42nd days, one bird /replicate making four birds per treatments with equal sex ratio and identical body weight were sacrificed. Dressing was done and after thorough gross examination, small representative pieces (approximately 0.5 cm length) of duodenum was collected from each sacrificed bird and fixed in 10% neutral buffered formalin. After proper fixation for 3-4 days, tissues were cut into thinner sections (1-2 mm thick). The tissues were washed in running water for 7- 8 h, dehydrated in ascending grades of ethyl alcohol, cleared in benzene and embedded in paraffin wax (Luna, 1968). The paraffin blocks prepared were cut at 4-5 µ thickness sections with a hand operated microtome. The paraffin embedded sections were then passed through sequential steps of deparaffinisation in xylene, rehydration through descending grades of ethyl alcohol to running water. Sections were stained by routine haematoxylin and eosin stain. Through microphotography, images were taken by Nikon 80i microscope with attached camera and photography unit. The histometrical measurements of duodenum viz. Villi height and Crypt depth was measured using the Image J software.

The collected data were analyzed using software package for social sciences (SPSS Version 24.0) at 95% significant level using Duncan's level of significance values.

RESULTS AND DISCUSSION

Effect of citrus

Longest (P<0.05) average villi height (1884.93µm) and average crypt depth (1596.57µm) was found in the group fed with 5% citrus waste irrespective of level of supplementation of enzyme (Table 1). However, results obtained were comparable with control group. Shortest (P<0.05) average villi height (1039.69µm) and average crypt depth (771.86 µm) was observed for 7.5% citrus fed group. Non-significant (P<0.05) difference was found for villi height to crypt depth ratio (V/C) between non-supplemented citrus waste group and the groups fed with either 2.5 or 5.0% citrus waste. Poorest V/C ratio was observed at highest citrus waste fed group.

Effect of enzymes

Higher average villi length (1735.07 µm) and higher average crypt depth (1449.53 µm) was observed for groups fed diet supplemented without enzyme (Table 1). Addition of enzyme could not support the improvement in duodenal morphology. No specific reasons could be ascertained.

Effect of Citrus waste × Enzyme

The average length of villi and average depth of crypt were higher (P<0.05) and showed better morphology in T_7 group supplemented with diet containing 5% citrus waste with enzyme but was non-significant with control group where no citrus waste was supplemented (Table 1).

Table 1: Effect of feeding different levels of citrus waste on the duodenal morphology of meat type bird

Treatment	Citrus (%)	Enzyme (G/Q)	Villus height (µm)	Crypt depth (µm)	Villus height: Crypt depth ratio
Effect of Citrus waste					
—	0	—	1765.13^{ab}	1439.07^{a}	1.25^{b}
—	2.5	—	1635.11^{b}	1459.29^{a}	1.13^{b}
—	5.0	—	1884.93^{a}	1596.57^{a}	1.19^{b}
—	7.5	—	1039.69^{c}	771.86^{b}	1.43^{a}
—	Pooled SE		40.891	32.847	0.044
Effect of Enzymes					
—	—	0	1735.07^{a}	1449.53^{a}	1.21^{a}
—	—	100	1427.36^{b}	1183.86^{b}	1.28^{a}
	Pooled SE		28.915	23.226	0.031
Effect of Citrus waste × Enzymes					
T_1	0	0	1858.87^{a}	1647.49^{ab}	1.13^{cd}
T_2	2.5	0	1800.69^{ab}	1552.14^{bc}	1.18b^{cd}
T_3	5.0	0	1797.79^{ab}	1445.58^{cd}	1.24^{bcd}
T_4	7.5	0	1482.92^{c}	1152.93^{e}	1.30^{bc}
T_5	0	100	1671.38^{b}	1230.65^{e}	1.36^{b}
T_6	2.5	100	1469.53^{c}	1366.44^{d}	1.08^{d}
T_7	5.0	100	1972.08^{a}	1747.56^{a}	1.13^{cd}
T_8	7.5	100	596.4587^{c}	390.7837^{e}	1.55^{a}
	Pooled SE		57.829	46.453	0.062

a, b, = Means bearing different superscripts in a column differ significantly (P≤0.05)

Group 1: Control birds

Mucosa of duodenum of control birds was thrown in finger like slender, unbranched longitudinal villi (Fig. 1A). The villi were broader near the base and conical at the apex. The lamina Propria was consisted of loose

connective tissue. The muscularis mucosa was marked by longitudinally arranged smooth muscle fibers towards the side of the submucosa and vertically arranged smooth muscle fiber strands on the side of the lamina Propria. The villi of the duodenum were lined by simple columnar epithelium. The columnar cells had elongated nuclei (Fig. 1B). The epithelium comprised of multiple cell types i.e. the chief cells, goblet cells and the enterochromaffin cells. Few polymorphonuclear cells were also observed at places in mucosa. Similar observations were recorded by Nasrin *et al*. (2012) in broilers and Rana *et al.* (2016) in Uttara fowl. The average length of villi (1858.87 µm) and average depth of crypt (1647.49 µm) (Table 1) shows normal morphology of duodenum (Fig. 1A).

Group 2: Treatment birds (supplemented feed containing 2.5% citrus waste)

Lining mucosa and epithelium were similar to the control groups. The average height of the villi (1800.69 µm) and depth of the crypt (1552.14 µm) (Table 1) showed non-significant difference from control group (Fig. 2A). The number of polymorphonuclear cells was visibly similar in number as compared to the control groups (Fig. 2B).

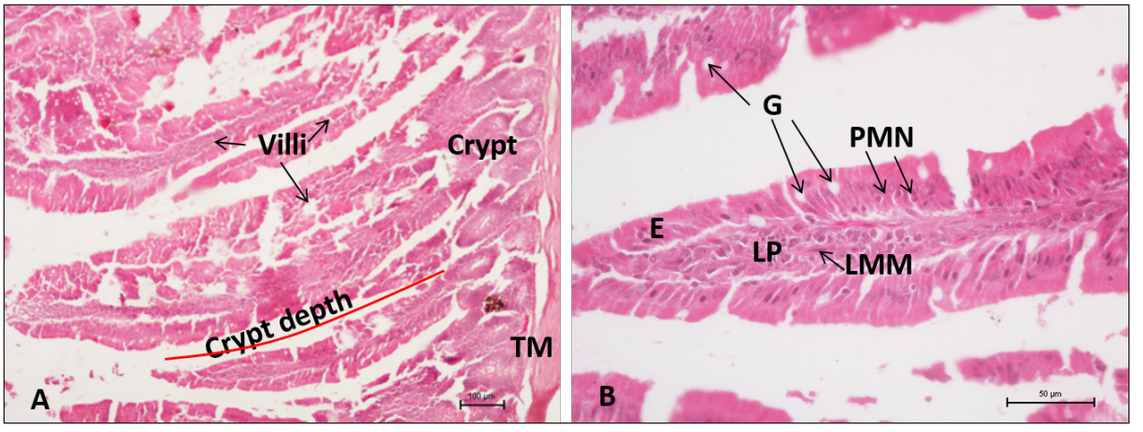

Fig. 1: Paraffin section of duodenum of control birds stained with haematoxylin and eosin showing, **(A)** duodenal villi, crypt, crypt depth, tunica muscularis (TM), Bar length 100µm; **(B)** lining epithelium (E), lamina Propria (LP), Lamina muscularis mucosa (LMM), Goblet cells (G) and Polymorpho nuclear cells (PMN), Bar length 50 µm

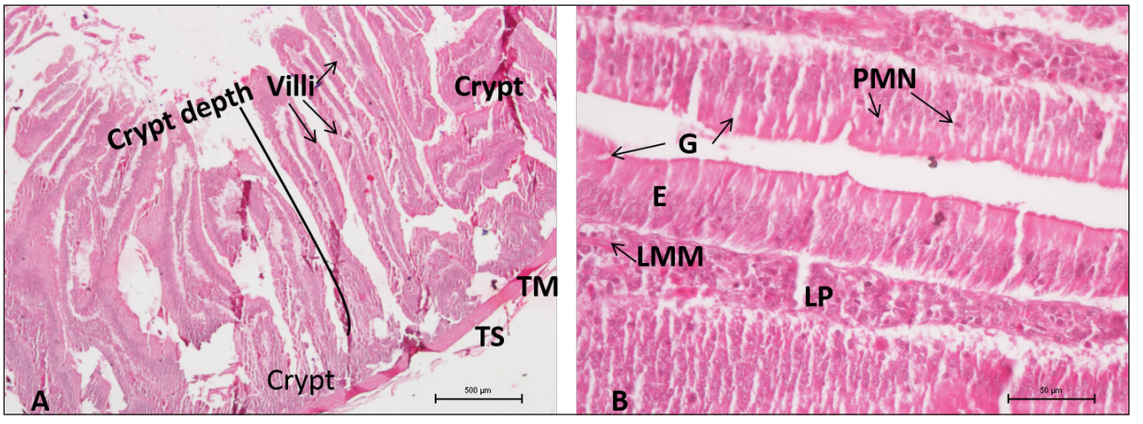

Fig. 2: Paraffin section of duodenum of treatment birds supplemented feed containing 2.5% citrus waste stained with haematoxylin and eosin showing, **(A)** duodenal villi, crypt, crypt depth, tunica muscularis (TM), Tunica Serosa (TS), Bar length 100 µm; **(B)** lining epithelium (E), lamina Propria (LP), Lamina muscularis mucosa (LMM), Goblet cells (G) and Polymorpho nuclear cells (PMN) **(B)**, Bar length 50 µm

Group 3: Treatment birds (supplemented feed containing 5% citrus waste)

Lining mucosa and epithelium were similar to the control groups. The villi were thrown into the mucosa showed finger like projection with more conical apex, thick body and base (Fig. 3A) as compared to control group with no significant difference in the villi length (1797.79µm) but differs significantly ($P<0.05$) with relation to crypt depth (1445.58 µm) (Table 1). The goblet cells were fewer in the epithelial layer. The number of polymorphonuclear cells in mucosa (Fig. 3B) showed higher number than that of control and T_2 suggesting effect of citrus waste in the gut mucosa. It showed that increasing level of citrus waste in the diet results in increased the number of polymorphonuclear cells. Higher number of polymorphonuclear cells in mucosa might be suggestive of some immunomodulative effects of citrus waste in the gut mucosa.

Group 4: Treatment birds (supplemented feed containing 7.5% citrus waste)

Lining mucosa and epithelium were different than the control groups (Fig. 4A). Histomorphometry suggested shorter villi length and decreased crypt depth (Table 1) as compared to other groups fed with citrus waste without enzymes. The villi were thrown into the mucosa showed thin projection with blunt apex and slender body as compared to control group. There was difference in the villi length and crypt depth from the control significantly

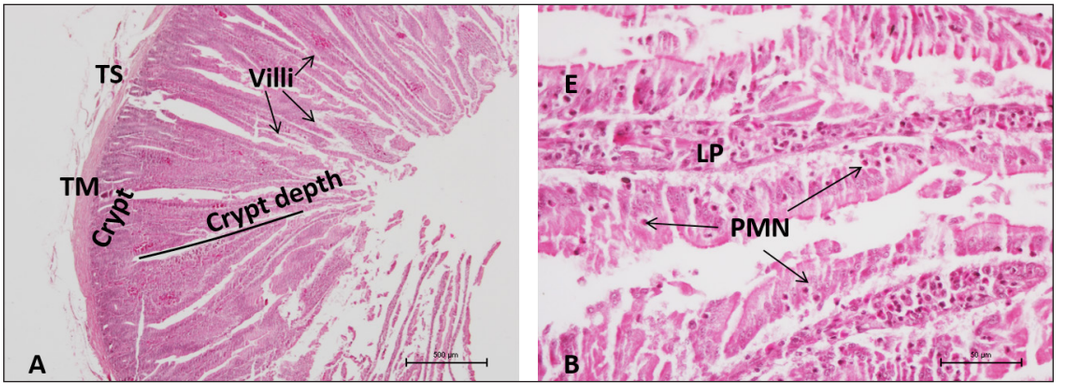

Fig. 3: Paraffin section of duodenum of treatment birds supplemented feed containing 5% citrus waste stained with haematoxylin and eosin showing, **(A)** duodenal villi, crypt, crypt depth, tunica muscularis (TM), Tunica Serosa (TS), Bar length 100µm; **(B)** lining epithelium (E), lamina Propria (LP) and Polymorpho nuclear cells (PMN), Bar length 50 µm

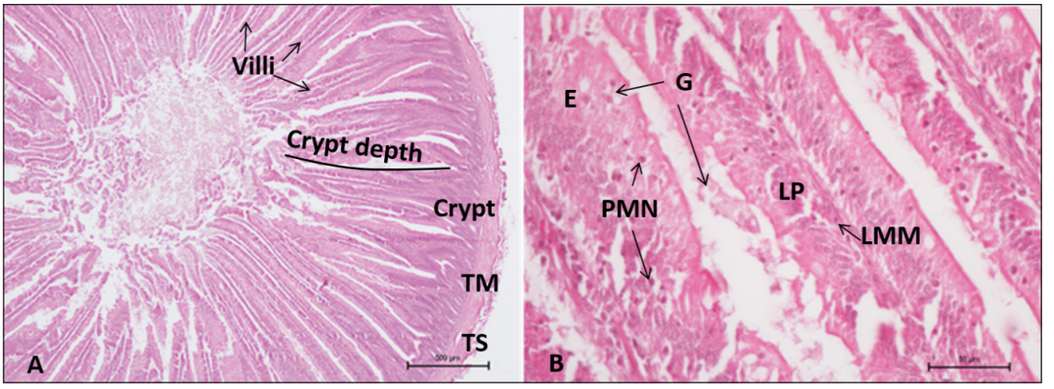

Fig. 4: Paraffin section of duodenum of treatment birds supplemented feed containing 7.5% citrus waste stained with haematoxylin and eosin showing, (A) duodenal villi, crypt, crypt depth, tunica muscularis (TM), Tunica Serosa (TS), Bar length 100µm; (B) lining epithelium (E), lamina Propria (LP), Lamina muscularis mucosa (LMM), Goblet cells (G) and Polymorphonuclear cells (PMN) (4.B), Bar length 50 µm

(P<0.05). The number of polymorphonuclear cells in mucosa (Fig. 4B) shows higher number than that of control and other treatment groups. It showed that increasing level of citrus waste in the diet resulted in increase the number of polymorphonuclear cells.

Higher length of villus might be associated with increased the surface area for absorption of nutrients (Xu *et al.,* 2003). Deeper crypt indicate fast tissue turnover to permit renewal of the villus as needed in response to normal sloughing or due to inflammation from pathogens or their toxins (Yason *et al*., 1987). Heavier chickens were generally associated with longer villi, greater villus width, higher villus surface area as compared to lighter one (Incharoen *et al.,* 2010).

Group 5: Treatment birds (supplemented with enzymes)

Mucosa of duodenum of fowl of positive control fowls was thrown in finger like slender, unbranched longitudinal villi (Fig. 5A). The villi were shorter when compared to control group significantly (P<0.05). The morphology of lamina Propria, muscularis mucosa is having same longitudinally arranged smooth muscle fibers towards the side of the submucosa and vertically arranged smooth muscle fibre strands on the side of the lamina Propria like that of control group. The villi of the duodenum were lined by simple columnar epithelium. The columnar cells had elongated nuclei (Fig. 5B). The goblets cells appeared numerous as compared to the control groups. But the amount of polymorphonuclear cell in mucosa was higher in number when compared with control group. The shorter villi and crypt depth was correlated with the poor growth parameters recorded in the experiment but the exact reason for the same could not be ascertained.

Group 6: Treatment birds (supplemented with 2.5% citrus waste with enzymes)

The height of villi and crypt depth measured was found to be giving similar results with that of T_4 where 7.5% citrus waste without enzyme was supplemented in broiler diet and significantly (P<0.05) different result from T_2 where same level of citrus waste supplementation has been given showing lower crypt depth and villus height (Table 1). The histomorphological features were comparable to the control group of animals (Fig. 6A). The amount of polymorphonuclear cells became lesser in number (Fig. 6B).

Group 7: Treatment birds (supplemented with 5% citrus waste with enzymes)

Mucosa of duodenum of birds of this group was thrown in finger like slender, unbranched longitudinal villi (Fig. 7A). The villi were broader near the base and conical at the apex. The lamina Propria was consisted of loose connective tissue. The muscularis mucosa was marked by longitudinally arranged smooth muscle fibers towards

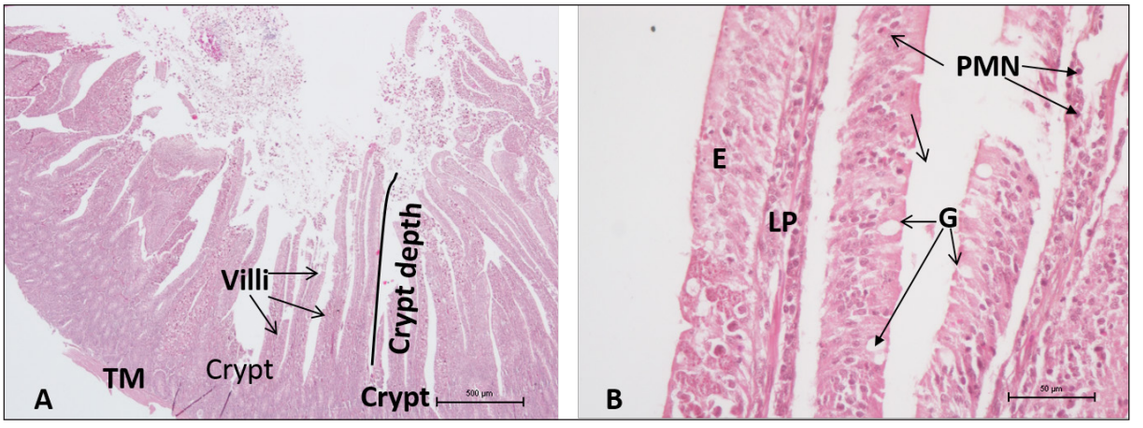

Fig. 5: Paraffin section of duodenum of positive control birds supplemented with enzymes stained with haematoxylin and eosin showing, (A) duodenal villi, crypt, crypt depth, tunica muscularis (TM), Bar length 100µm; (B) lining epithelium (E), lamina Propria (LP), Goblet cells (G) and Polymorpho nuclear cells (PMN), Bar length 50 µm

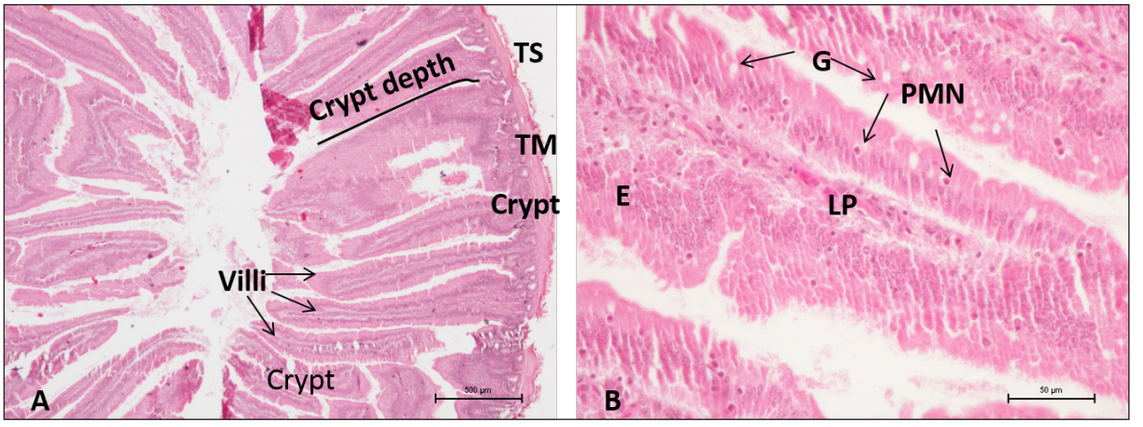

Fig. 6: Paraffin section of duodenum of treatment birds fed feed supplemented with 2.5% citrus waste with enzymes stained with haematoxylin and eosin showing, **(A)** duodenal villi, crypt, crypt depth, tunica muscularis (TM), Tunica Serosa (TS), Bar length 100µm; **(B)** lining epithelium (E), lamina Propria (LP), Goblet cells (G) and Polymorpho nuclear cells (PMN), Bar length 50 µm (B)

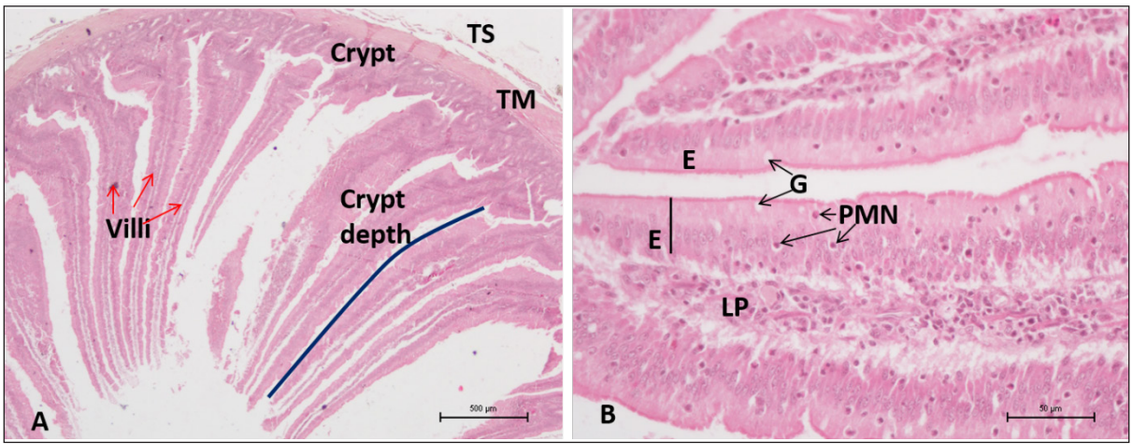

Fig. 7: Paraffin section of duodenum of treatment birds supplemented feed containing 5% citrus waste with enzymes stained with haematoxylin and eosin showing, (A) duodenal villi, crypt, crypt depth, tunica muscularis (TM), Tunica Serosa (TS), Bar length 100µm; (B) lining epithelium (E), lamina Propria (LP), Goblet cells (G) and Polymorpho nuclear cells (PMN), Bar length 50 µm

the side of the submucosa and vertically arranged smooth muscle fibre strands on the side of the lamina Propria. The villi of the duodenum were lined by simple columnar epithelium. The columnar cells had elongated nuclei (Fig. 7B). The epithelium comprised of multiple cell types i.e. the chief cells, goblet cells and the enterochromaffin cells. More number of polymorphonuclear cells were also observed at places in mucosa suggesting better immunomodulatory effect due to inclusion of citrus waste in the diet. The average length of villi (1972.08 µm) and average depth of crypt (1747.56 µm) (Table 1) showed better morphology of duodenum when compared to other treatment groups significantly ($P<0.05$) which was showing better result when comparable with control group (Fig. 7A). This was suggesting higher and greater rate of absorption in the bird which was reflected in their blood parameters showing lower blood cholesterol and triglyceride levels with higher anti-oxidant level in the body.

Group 8: Treatment birds (supplemented feed containing 7.5% citrus waste)

Section of duodenum clearly shows smallest villi with

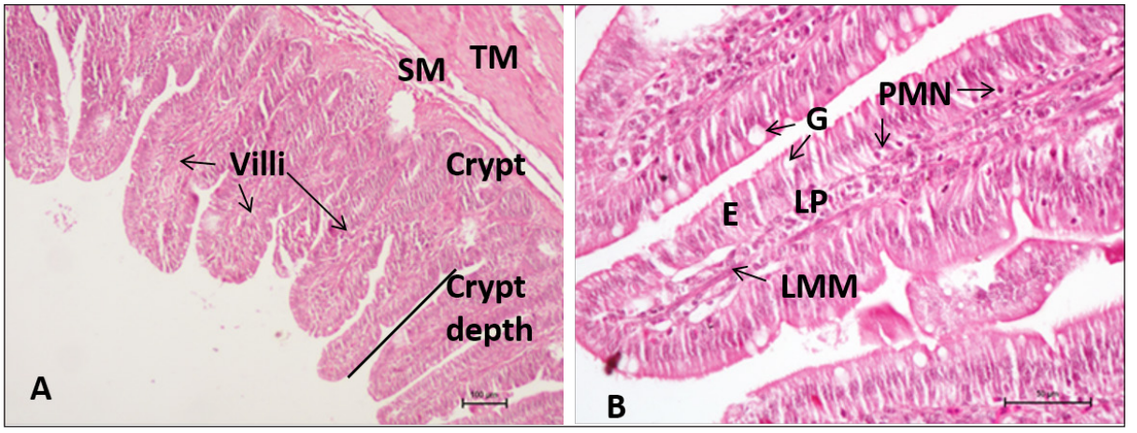

Fig. 8: Paraffin section of duodenum of treatment birds supplemented feed containing 7.5% citrus waste with enzymes stained with haematoxylin and eosin showing (A) duodenal villi, crypt, crypt depth, tunica muscularis (TM), Submucosa (SM), Bar length 100µm; (B) lining epithelium (E), lamina Propria (LP), Lamina muscularis mucosa (LMM), Goblet cells (G) and Polymorpho nuclear cells (PMN), Bar length 50 µm

thick body and base with blunt apex (Fig. 8A). The average villus height (596.46 µm) and crypt depth (390.78 µm) (Table 1) was found to be lowest among all the groups significantly (P<0.05). The number of polymorphonuclear cells was found to be less in number when compared to other group (Fig. 8B). The duodenal morphology might be correlated with the growth performance of the birds. Poor growth performance was seen in this group given with higher level of citrus waste along with enzyme which could be interpreted from the duodenal morphology due to very less absorption surface for nutrient as compared to other treatment. No specific reason could be ascertained for the growth depression.

CONCLUSION

Higher length of villus was associated with increase in the surface area for absorption of nutrients. Deeper crypt indicate fast tissue turnover to permit renewal of the villus as needed in response to normal sloughing or due to inflammation from pathogens or their toxins. The present study was in agreement with Akbarian *et al*. (2013) where proximal intestinal morphology was modified with inclusion of orange peel extract, but without having beneficial effect on growth performance of broilers. Present study was in correlation with Basir and Toghyani (2017) who reported significant increase in the ratio of villus height and crypt depth in jejunum which may be due to the presence of tannins in the dried lemon pulp (P<0.05).

The average length of villi and average depth of crypt were higher and showed better morphology of duodenum in group supplemented with 5% citrus waste when compared to other treatment groups significantly (P<0.05).

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