

Comparative Efficacy of Metronidazole in Combination ColistinSulphate and Probiotics against *Clostridium Perfringens* and Their Effects on Histopathological Features of Visceral Organs in Broiler

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Abstract

Gastro enteric diseases are an important threat to the poultry industry due to reduced productivity, increased death rate, reduced welfare of birds and the associated contamination of poultry products for human consumption. Necrotic enteritis is a widespread and economically disturbing bacterial disease in modern broiler flocks. The primary etiologic agent of necrotic enteritis is *Clostridium perfringens* type A. The current study was designed to determine the histopathological changes in broilers challenged by *Clostridium perfringens* and determine the comparative efficacy of colistinSulphate, metronidazole and probiotics on histopathological changes. A total of 100 birds were divided into 5 groups (A, B, C, D and E) each having 20 birds. Each group was treated according to its trial. Birds in all groups (except group E) were inoculated orally with *Clostridium Perfringens* 1 ml (3x10¹⁰ CFU/ml/bird) on day 9th, 10th, 11th, 12th, 13th of chick's age twice a day. Chicks in group A were administered probiotics 1ml/L in drinking water for the first seven days and 2ml/L for rest of the 35 days. Chicks in Group B & C were administered ColistinSulphate 2 g/lit drinking water and Metronidazole Syrup 4 ml/1 lit drinking water after two days of inoculation for five continuous days. Group D and E represented positive and negative control groups respectively. Birds were observed twice a day for clinical signs and mortality. Weight of birds was measured at 20th, 25th, 30th and 35th day. Most sensitive antibiotic was found metronidazole as it gave 95 % efficacy on day 5th of the treatment while colistinSulphate gave 70 % recovery on the same day and probiotic gave only 50 % recovery. Four (n=4) birds from each group were slaughtered at 20th, 25th, 30th and 35th day for necropsy to determine score of gross pathological lesions and sample collection (Liver, Kidney and Intestine) for histopathological studies. The results had significant difference among different groups on different days. Findings of the study revealed that metronidazole effectively abolish the adverse effects of *Clostridium perfringens*. Use of Antibiotics especially metronidazole along with probiotics can be useful in the treatment and control of *Clostridium perfringens* infections in poultry birds. Moreover, metronidazole is better choice for the treatment and control of clostridial infections in poultry birds as compared to ColistinSulphate.

Key words: Histopathological, *Clostridium perfringens*, Efficacy, Metronidazole, Clostridial.

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Introduction

Necrotic enteritis is widespread bacterial ailment that international poultry segment bears major production losses [16]. Disease occurs due to increased amount of harmful *Clostridium perfringens* microbes and assembly of its large amount of detrimental toxins in the chick's GIT tract [17]. In present broiler flocks necrotic enteritis has an important place as widespread and economically destructive bacterial disease [17]. The cause of necrotic enteritis is *Clostridium perfringens* type A [8]. *C. perfringens* type A is the normal inhabitant of intestinal microflora in the intestine that includes a hundred of bacterial species [7]. Depending on production of four main toxins (as alpha, beta, epsilon and iota toxins) *Clostridium perfringens* strains categorized into five types (A, B, C, D and E). The principle toxin of *Clostridium perfringens* is type A. *C. perfringens* type A strains produce alpha toxin, type B strains produce alpha, beta and iota toxin, the type C strains

produces alpha and beta toxin, type D strains produce alpha and iota toxin and type E strains produce alpha and iota toxin [6]. An alpha toxin of *C. perfringens* is the etiological agent of necrotic enteritis in broilers and a number of severe lesions that is similarly compared with wild-type strain [15].

Materials and methods

One day old broiler chicks were purchased from commercial hatchery (Islamabad hatchery). The birds in all groups were housed in the same management and environmental conditions at experimental sheds of Microbiology UVAS, Lahore. Chick starter crumbs as basal diet and clean water was offered ad libitum (Table 3.1-3.2). By using live vaccine at the age of 5 and 23 days Newcastle disease (ND) Vaccine (VRI, Lahore) was used in chicks and at the age of 8 and 21 infectious bursal disease (IBD) (Merial) was used.

Preparation of inoculum

A freeze dried *Clostridium perfringens* type D ampule were procured from VRI Lahore and reconstituted in cooked meat medium and incubated overnight at 37 C under anaerobic conditions. The cultured tubes were checked for any contamination. The contaminated tubes were discarded and tubes having pure culture of *Clostridium perfringens* were taken and kept for experimental infection. Mice inoculation test was also be performed for the confirmation of this culture [12].

Experimental design

A total of 100 birds were divided into 5 groups (A, B, C, D and E) each having 20 birds. Each group was treated according to its trial as below (Table 3.3). Birds in all groups (except group E) were inoculated orally with *Clostridium Perfringens* @ 1 ml (3x10¹⁰ CFU/ml/bird) [11] on day 9th, 10th, 11th, 12th, 13th of chicks age twice a day. Birds were kept off feed before the inoculation of infection.

Chemotherapeutic trial

To check the comparative efficacy of probiotics, colistinesulphate and metronidazole the following procedure was adopted. From day first of experiment chicks in group A were administered probiotics (Green Pakistan Nature Farming (Pvt.) Ltd) 1ml/L in drinking water for the first seven days and 2ml/L in drinking water for rest of the days of experiment. Probiotic contains *Lactobacillus plantarum*; *Lactobacillus casei*, *Lactobacillus acidophilus*; *Lactobacillus fermentum*; *Lactobacillus delbrueckii*; *Bacillus subtilis*; *Saccharomyces cerevisiae*; *Actinomycetes*; *Fungi ray*. Chicks in Group B and C were administered Colistin Sulphate (Prix pharmaceutical, Pakistan) 2 g/1lit drinking water and Metronidazole Syrup (Sanofii company) 4 ml/1 lit drinking water after 2 days of inoculation for five continuous days. Group D and E represented positive and negative control groups respectively. Birds were observed twice a day for clinical signs and mortality. Weight of birds was measured at 20th, 25th, 30th and 35th day of experiment. .

Four (n=4) birds from each group were slaughtered at 20th, 25th, 30th and 35th day of experiment for necropsy to determine score of gross pathological lesions and sample collection (Liver, Kidney and Intestine) for histo-pathological studies.

Efficacy of drugs

The efficacy of the drugs in group A, B and C was observed at 16th, 17th, 18th, 19th and 20th days of

experiment post treatment on the basis of clinical signs.

Histopathological study

Samples of liver, kidney and intestine (2 cm in size) were preserved in 10 % buffered formalin. The histopathological examination was carried out on all samples at Histopathology Laboratory of University of Veterinary and Animal Sciences, Lahore according to standard procedure described by Bancroft and Gamble [2].

Microscopic study

Slides were observed under 4 X of microscope (Olympus DP20) attached with camera and operated by software. Scale was calibrated using Neubauerhemocytometer. Observing 1 chamber of Neubauerhemocytometer under 4 X gave a magnification factor of 400, ie. Measured size of villi, crypt were divided by 400 to get actual size of villus. The heights of intestinal villus were expressed as micrometers (µm).

Villus size (µm)= $\frac{\text{Measurement of Villi (mm)}}{1000}$ x

400

Results

Clinical findings

The broiler chicks in groups A, B, C, D were challenged with *Clostridium perfringens* 1 ml (3x10¹⁰ CFU/ml/bird) infection (twice a day) for five days at day 9th, 10th, 11th, 12th, 13th. After the inoculation of infection birds of all these groups were closely observed for any clinical sign of disease twice a day. Birds in group E remained normal after infection. They were active and all birds were taking their feed properly. After 48 hrs of infection birds in group A, B, C and D were dull and depressed. Feed intake was reduced and four birds were found dead after 48 hrs of inoculums of group B, 2 were dead in group C and 4 were dead in group D. The birds were showing vent pasting, diarrhea, dullness, depression, anorexia and pyrexia. These changes were more pronounced in group D as compared to other groups. Similarly, two birds were found dead at 16 day of age in group A. Diarrhea and vent pasting was more pronounced in birds of group B, C and D. After the administration of antibiotics in groups B & C, clinical signs dramatically become less severe. Our study showed that metronidazole treated birds (Group C) showed mild clinical signs as compared to colistin sulphate treated birds (Group B). This shows that metronidazole has more efficacy against *Clostridium*

perfringens as compared to Colistine sulphate. On the other hand, the birds of group D showed clinical signs more severe as compared to group A birds. These finding showed that probiotics has some inhibitory effects for the growth of *Clostridium perfringens* in the gut mainly due to competitive exclusion effects for receptor bindings.

After treatment it has been observed that after administration of antibiotics at 20th day the lesions were more severe in A, B, C and D group so post mortem was performed at 20th day and sample of liver, kidney and intestine were collected in 10 % buffered formalin. At day 25th, 30th, 35th clinical signs, histopathological lesions and gross lesions were less severe as compared to start of infection and 20th day of experiment. It showed that antibiotics have good effect and have inhibitory effect on the growth of bacteria. After administration of antibiotics in group B & C lesions were less severe and clinical signs were fade up. In group A mild clinical signs and lesions were observed as compared to group B & C. In group D after 20th day more pronounced clinical signs were observed and lesions were more severe. In group E all the birds remained normal and healthy.

Table 1 Scores of clinical signs of broiler chicks at 20th day of age.

Clinical signs and behavior	Score range	Groups			
		A	B	C	D
Alertness normal-depressed	0-3	2	3	3	3
Attraction to feed normal to less interest	0-3	3	2	2	3
Feces consistency normal formed watery	0-3	2	2	1	3
Feather normal shiny –ruffled broken	0-3	2	1	1	3
Cumulative score	-	9	8	7	12

0=Healthy, 1= Mild signs, 2= Moderate, 3=Severe

Chemotherapeutic trial

To check the comparative efficacy of probiotics, colistinesulphate and metronidazole, the mentioned drugs were administered orally for 5 consecutive days. Metronidazole gave good results in treatment trials as it gave 95 % of the recovery on the day 5th of the trial while Cholistine was on 2nd, it gave 70 % recovery on the day 5th and probiotic gave 50 % recovery on the day 5th. Day-wise recovery of the birds after giving chemotherapy is explained in the table 4.2. This was not significant statistically.

Body weight

Data of weekly Body weight gain (BWG) of treatments are presented in Table 4.3. It can be seen from the results that maximum decrease in weight was in chicks of group D followed by chicks of group A, B, C and E. Chicks in group C showed relatively more weight gain as compared to group B which shows that metronidazole is more effective against

Clostridium perfringens as compared to Colistinsulphate. In the same way, probiotic treated chicks (group A) also showed more weight gain as compared to Groups D chicks which indicate beneficial effects of probiotics on gut health for digestion & absorption.

Gross pathology

Scores of gross lesions of different organs can be seen in table 4.4 that chicks of group D showed most severe gross lesions in the form of hemorrhages, edema, congestion and necrosis followed by chicks of group A, B, and C while chicks in group E did not showed any gross lesion. Chicks in group C showed relatively mild gross lesion as compared to group B which shows that metronidazole is more effective against *Clostridium perfringens* as compared to Colistinsulphate. In the same way, probiotic treated chicks (group A) also showed mild gross pathological lesions as compared to Groups D chicks which indicate inhibitory effects of probiotics for the growth of *Clostridium perfringens* on gut mucosa.

Histopathological lesions

At the end of 35 days, all birds of each group were slaughtered for the conduction of postmortem and histopathological studies. Histopathological lesions were observed in liver, intestine and kidney. Histopathological picture showed mild to moderate vaculation and degeneration of cytoplasm of hepatocytes. Mild congestion was observed in liver. In intestine atrophy and necrosis of intestinal villi along with infiltration of mononuclear inflammatory cells. There was mild degeneration and sloughing of epithelial villi. Blood spots, hemorrhages, degeneration of tubules and congestion was observed in kidney. Birds of group E showed normal morphological pattern of histological structure of liver. A normal lobular structure of liver with hepatocytes having centrally placed neuclei and normal cytoplasm was observed. In the same way, histological picture of Intestine showed normal villi, normal crypts, sub mucosa, tunica muscularis and serosa.

It can be seen from the results that chicks of group D showed most severe histopathological lesions in the form of hemorrhages, edema, congestion, degeneration of tubules and necrosis followed by chicks of group A, B, and C while chicks in group E did not showed any histopathological lesion. Chicks in group C showed relatively less severe histopathological lesion as compared to group B

Table 2 Comparative efficacy of chemotherapeutic trials in poultry birds.

Chemotherapeutical trials	Groups	No. of birds	Day 1	Day 2	Day 3	Day 4	Day 5	p-value
Probiotics	A	20	0(0 %)	4 (20 %)	7(35 %)	8(40 %)	10(50 %)	0.08
ColistineSulphate	B	20	1(5 %)	6 (30 %)	9(45 %)	13(65%)	14(70 %)	
Metronidazole	C	20	2(10%)	7 (35 %)	13(65%)	18(90%)	19(95 %)	

Table 3 Average weight on weekly basis of broiler chicks at different days of experiment (gm).

Days	A	B	C	D	E	p-value
20	299	466	533	259	569	0.99
25	740	760	790	730	800	
30	1056	1112	1139	1036	1167	
35	1407	1422	1436	1385	1452	

which shows that metronidazole is more effective against *Clostridium perfringens* as compared to Colistinsulphate. In the same way, probiotic treated chicks (group A) also showed less histopathological lesions as compared to Groups D chicks which indicate inhibitory effects of probiotics for the growth of *Clostridium perfringens* on gut mucosa.

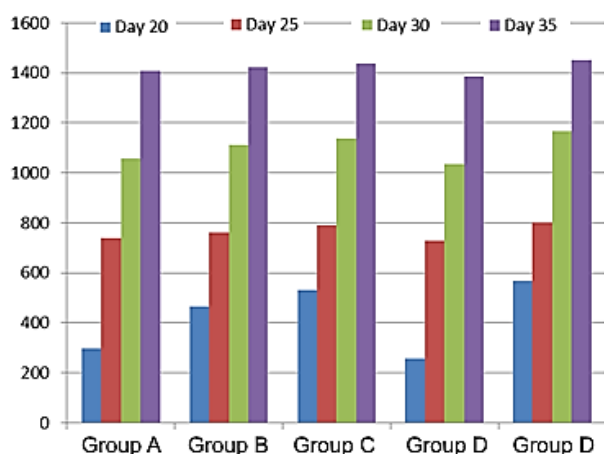


Fig. 1: Graphical presentation of avg weight on weekly basis of broiler chicks at different days of experiment (gm).

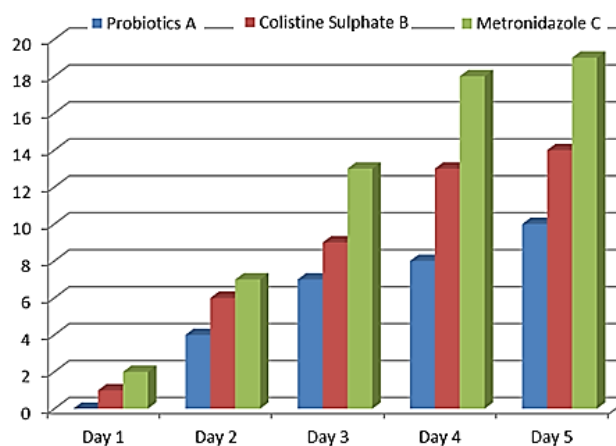


Fig. 2: Graphical presentation with respect to treatments.

The villus architecture showed that if villi height and width was more or increase then surface area of intestine increases and more absorption of nutrients along with more digestion take place. As showed in table group E have more height and width as compared to group A, B, C and D. Group D (infected group) have less height and width as compared to treated groups. This table showed that metronidazole treated group (A) has more villi height and width as compared to colistinesulphate (group B) and probiotic (group A) treated group.

Discussion

Songer [14] reported that *Clostridium perfringens* is the causes of cost-effective important diseases to poultry industry. Van Immerseel et al. [17] and Williams [19] also reported that the major emerging disease in poultry sector is necrotic enteritis in Pakistan like other developing countries of World. Knarreborg et al. [10] reported that in European countries growth promoting antibiotics for broiler feed were prohibited and in broiler feed exclusion of these growth promoting antibiotics is considered a main reason which alters the micro floral environment of the gut of broiler chicks and it has become clear with the help of many studies that extraction of these antibiotic growth promoters become the cause of retarded growth and increased outbreak cases of necrotic enteritis as studied by Williams [19].

The present study was conducted on *C. perfringens* in broilers to check the comparative efficacy of probiotics and antibiotics against clostridialinfection. The experimental birds were divided into five different group. After 48 hours of *C. perfringens* infection, the birds in group A, B, C and D were dull and depressed. While birds in the group E remained normal after *C. perfringens* infection. They were active and taking their feed properly. These findings are in line with study of Riddell et al. [13].

After 48 hours, the feed intake was reduced and four birds were found dead after 48 hrs of inoculums of group B and C. The birds were showing vent pasting,

Table 4: Villus architecture on 20th day of age in broilers.

Villus architecture Day on day 20					
Parameters	Group A	Group B	Group C	Group D	Group E
Villi height (µm)	668.89	725.44	985.4	648.34	1002.2
Width (µm)	112.20	127.7	143.2	106.3	152.15

Table 5: Scores of gross lesions of different organs of broiler chicks at 20th day of age

Organ	Lesion	Max. possible score	Groups				
			A	B	C	D	E
Liver	Enlargement	18	13	14	7	15	0
	Friable	18	12	10	6	9	0
	Hemorrhages	18	14	11	9	17	0
Total score liver		54	39	35	22	41	0
Intestine	Enlargement	18	12	7	6	13	0
	Hemorrhages	18	14	12	7	16	0
Total score intestine		36	26	19	13	29	0
Kidney	Hemorrhages	18	10	14	7	14	0
	Congestion	18	14	12	9	16	0
Total Score kidney		36	24	26	16	30	0
Cumulative score (liver + intestine + kidney)		126	89	80	51	100	0

diarrhea, dullness, depression, anorexia and pyrexia. These changes were more pronounced in group D as compare to other groups. Similarly, two birds were found dead at 16 day of age in group A. Diarrhea and vent pasting was more pronounced in birds of three groups B, C and D. The most farmers' complaints were the quick wetting of litters. Craven [4] study shows that the diarrhea has been identified as a common clinical signs relating to *C. perfringens* infection among the poultry professionals.

After the administration of different antibiotics in groups B and C, clinical signs dramatically become less severe. Present study showed that metronidazole treated birds (Group C) showed mild clinical signs as compared to colistin sulphate treated birds (Group B). This shows that metronidazole has more efficacy against *Clostridium perfringens* as compared to colistin sulphate. Metronidazole significantly reduces the lesions caused by necrotic enteritis lesion scores along with mortality as compared to control groups. These findings are in line with Vissienon et al. [18]; and Brennan et al. [3]. On the other hand, Group A birds showed clinical signs of relatively less severity as compared to Group D birds. This finding reveals that probiotics has some inhibitory effects on the growth of *Clostridium perfringens* in the gut mainly due to competitive exclusion effects for receptor bindings.

In the present study, the results shows that the maximum decrease in weight was in chicks of group D followed by chicks of group A, B, C and E. Chicks in group C showed relatively more weight gain as compared to group B which also shows that metronidazole is more effective against *Clostridium perfringens* as compared to colistin sulphate. In the same way, probiotic treated chicks (group A) also showed more weight gain as compared to group D chicks which indicate beneficial effects of probiotics on gut health for digestion & absorption.

Elwinger et al. [5] and Kaldhusdal et al. [8] studied that the damaging nature of toxins produced by *Clostridium perfringens* may be the cause of necrotic spots on intestinal mucosa. *C. perfringens* causes chronic damage to the intestinal mucosa, which leads to impairment of digestion and nutrients and absorption, reduced weight gain and increased feed conversion ratio. The bridging of technology transfer from labs to field application requires lots of efforts, especially in case of most innovative approaches in today science [19-21]

In the present study, our results shows that the chicks of group D showed more severe gross lesions in the form of hemorrhages, edema, congestion and necrosis followed by chicks of group A, B, and C while chicks in group E did not showed any gross lesion. These findings are in line with Williams [22]. Chicks in group C showed relatively less gross lesion as compared to group B which shows that metronidazole is more effective against *Clostridium perfringens* as compared to colistin sulphate. In the same way, probiotic treated chicks (group A) also showed less gross pathological lesions as compared to groups D chicks which indicate inhibitory effects of probiotics for the growth of *Clostridium perfringens* on gut mucosa as reported by Keyburn et al. [9].

At the 35 days, all birds of each group were slaughtered for the conduction of postmortem and histopathological studies. Histopathological lesions were observed in liver and intestine. Histopathological picture showed mild to moderate vacuolation and degeneration of cytoplasm of hepatocytes. Mild congestion was observed in liver. In intestine atrophy and necrosis of intestinal villi along with infiltration of mononuclear inflammatory cells. Epithelial cell proliferations were reduced and there was mild degeneration and sloughing of epithelial villi. Our findings of intestinal cell degradation are in line with previous studies of Ahmed et al. [1]. This degradation of cells and changes in the shape of cells is a result of harmful toxins produced by *Clostridium perfringens*. Liver of

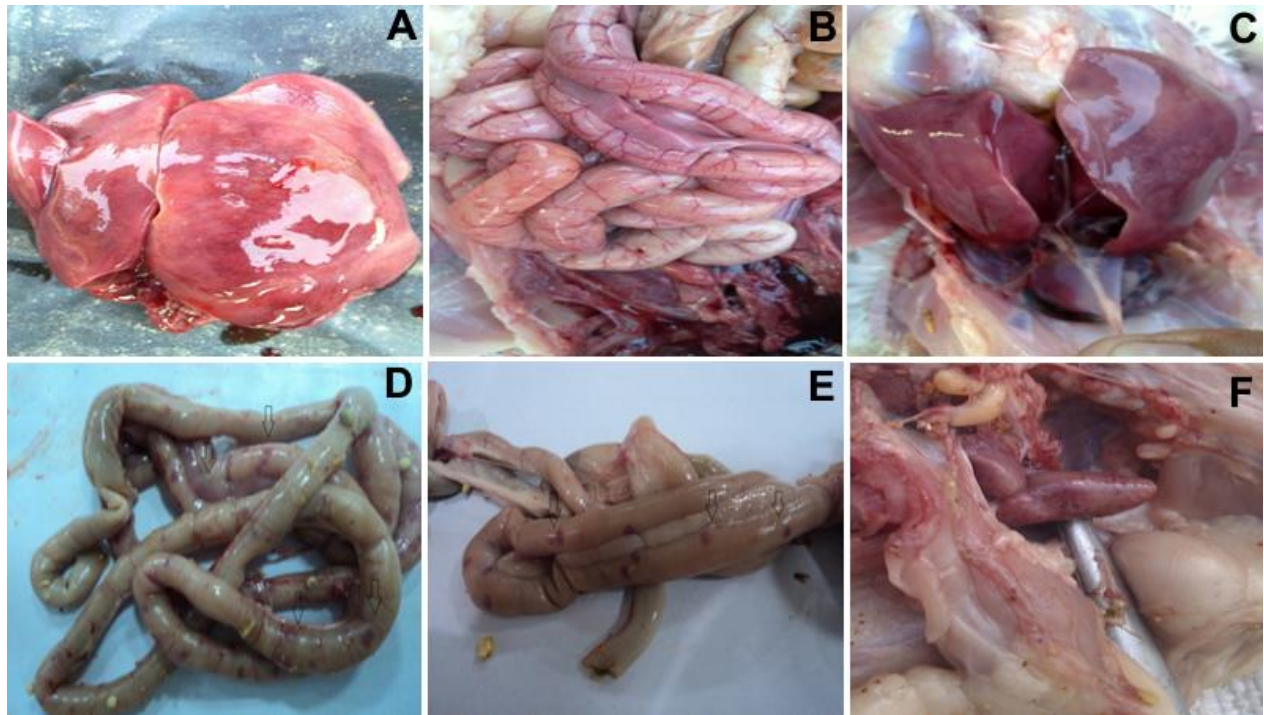


Fig. 3: Liver showing hemorrhages (A), congestion Intestine showing normal appearance (B) birds at day 20 of age in group E and enlargement in group A at day 20.
Liver showing hemorrhages (C) and small arrows show the gross necrotic enteritis necrotic spots in group B at day 20 of age lesions in intestine (D) of broiler at day 20 of age (group D).
Arrows show the gross necrotic enteritis Hemorrhagic kidney (E) of broiler bird and lesions in intestine (F) of broiler bird at day 20th of age (group D) at day 20 of age (group C)

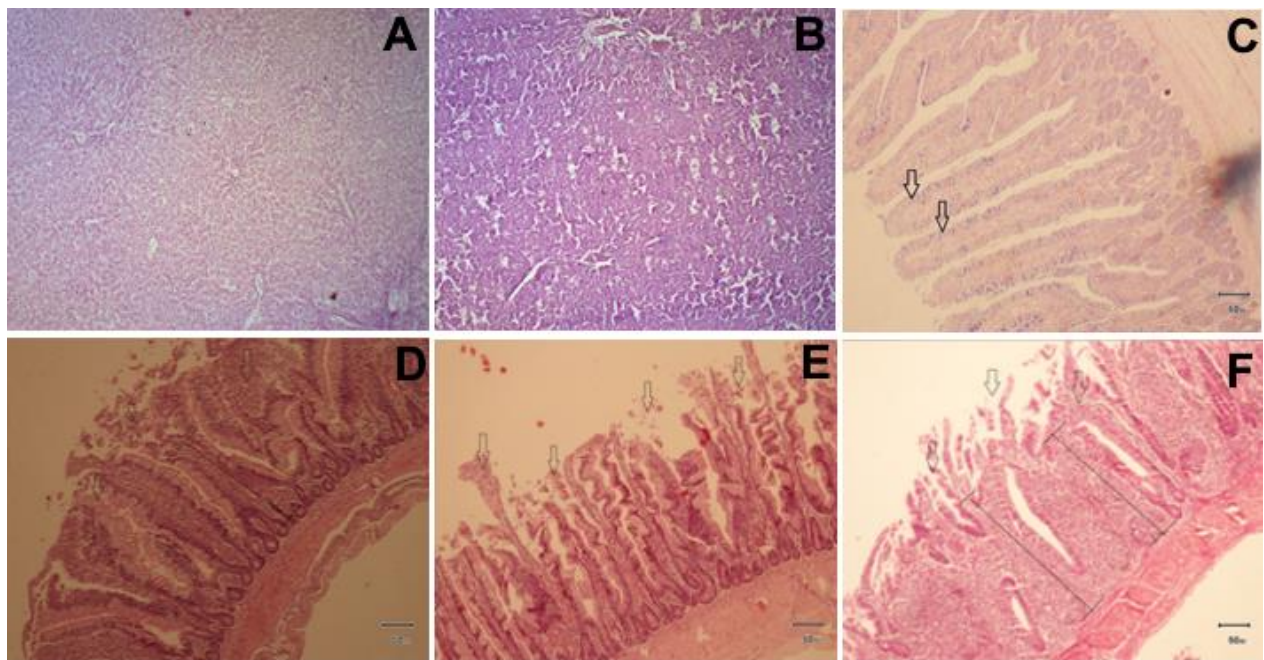


Fig. 4: Liver at day 20 of age (group E) (A), Congested structure of broiler liver with damaged hepatocytes at day 20 of age (group D) (B).
Normal villi length and width in intestine of broiler birds at day 20 of age (group E) (C), Degenerated & bursted villi in intestine of broiler birds at age (group C) (D).
Arrows showing degenerated & bursted Pronounced degenerated & bursted in intestine of broiler birds at day 20 (E) villi in intestine of broiler birds at of age (group B) 20 day of age (group D) (F).

birds of group E showed normal morphological pattern of histological structure of liver. A normal lobular structure of liver with hepatocytes having centrally placed nuclei and normal cytoplasm was observed. In the same way, histological picture of intestine showed normal villi, normal crypts, sub mucosa, tunica muscularis and serosa. It can be seen from the results that chicks of group D showed most severe histopathological lesions in the form of hemorrhages, edema, congestion and necrosis followed by chicks of group A, B, and C while chicks in group E did not showed any histopathological lesion. Chicks in group C showed relatively mild histopathological lesion as compared to group B which shows that metronidazole is more effective against *Clostridium perfringens* as compared to colistin sulphate.

In the same way, probiotic treated chicks (group A) also showed less histopathological lesions as compared to group D chicks which indicate inhibitory effect of probiotics for the growth of *Clostridium perfringens* on gut mucosa. *Clostridium perfringens* type D is an emerging pathogen for poultry birds in Pakistan. It put adverse effects on the health of intestinal mucosa leading to mal-digestion and mal-absorption of nutrients. Metronidazole effectively abolishes the adverse effects of *Clostridium perfringens*. Use of antibiotics especially metronidazole along with probiotics can be useful in the treatment and control of *Clostridium perfringens* infections in poultry birds. Moreover, metronidazole is better choice for the treatment and control of clostridial infections in poultry birds as compared to colistin sulphate. There is need for further studies on other animals and birds species to find out the pathogenesis of *Clostridium perfringens*. There is also need to study the effective control of necrotic enteritis and treatment of necrotic enteritis in birds.

Colistine sulphate is not absorbed from the intestine thus having good results in the treatment of gastrointestinal infection with Gram-negative bacteria without producing residues in the various tissues of the target animals. Probiotic have no withdrawal period or any residual effect. Metronidazole at this dose rate also did not have any residual effect and dose rate is significant for the use of broilers.

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