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Study on effect of antibiotic, probiotic and/or organic acids on experimental infection with *Salmonella entertidis* field isolate in broiler chicken

Kh.M.Elbayoumi, Hoda M. Mekky, Eman R. Hassan, M.A.Bosila and Zeinab M.S. Amin Girh

Department of Poultry Diseases, Veterinary Research Division, National Research Centre, P.O. Code 12311 Dokki, Giza, Egypt

Abstract: *Salmonella enteritidis* (*S.enteritidis*) was isolated from field infected clinically diseased broiler chicken and identified by biochemical tests, serotyping and PCR. An experimental study were fulfilled in order to compare current efficiency of antibiotic colistinesulphate, *B.subtilis* and acidifiers mixtures on control of field isolate *S. Enteritidis* in broiler chickens. A total number of 290 Cobb broiler chicks were used, 1- day old, ten examined bacteriologically to prove their freedom from *S. Enteritidis*, 280 chicks were divided into 7 equal groups, 40 chicks in each. Groups 1, 2 and 3 received organic acids start from five days of age till two weeks post challenge, groups 1 and 4 treated with colistinesulphate start from 2nd day post challenge for 5 successive days while groups 2 and 5 treated with *B.subtilis* from 5 days of age till two weeks post challenge while group 6 and 7 kept as control negative non treated group and positive challenged group respectively. All birds groups except control negative group 6 were challenged orally by 0.5 ml containing 10^9 CFU/ml *S. Enteritidis* at 21 days of age. Clinical diagnosis, performances together with histopathological examination of liver and spleen were studied. Results of mortality rate revealed that the highest mortality rate was group 7 (control positive group) which was 35%, followed by group 3 (organic acid group) which was 17.5%, then group 5 *(B.subtilis)* which was 15%, followed by group 2 (Bacilussubtilis – organic acid groups) which was 12.5%, followed by group 4 (colistine) which was 10%, then group 1 (colistine – organic acid) which was 5%, and finally group 6 (control negative) showing no mortalities. Samples from dead birds including liver, heart, spleen and cecum were aseptically collected from each group post challenge for *S. Enteritidis*r e-isolation. Which indicate positive for *S.entertidis* microorganism which considered suspected cause for mortalities. Concerning ABW, it was found that the lowest is group 7 (infected control positive) which was 1831gm by the end of the experiment when compared with control negative group 6 which was 2050 gm. on the other hand the highest ABW by the end of the experiment was group 2 (received organic acids and Bacillus subtilis which was 2160 gm followed by group 1 (colistinesulphate and organic acids) which was 2130 gm , then followed by group 4 (colistine only) which was 2110 , followed by group 5 (*B. subtilis*) which was 2100 gm, then followed by group 3 (organic acids) which was 2095 gm. FCR group 7 (infected non treated) was the highest (bad FCR) which was 1.75 , followed by group 4 (colistinesulphate) which was 1.62, then followed by control negative group 6 (1.61), followed by group 3 (organic acid) and 5 (*B. subtilis*) which both have the same results which was 1.60 , followed by group 1 (colistine and organic acid) which was 1.59 and finally the best feed conversion rate was group 2 (organic acids and *B. subtilis*) which was 1.58. Histopathological changes it was found that liver and spleen of control negative group 6 show normal histology while group 7 (infected) liver showed

congestion of the sinusoids, area of coagulative necrosis infiltrated with lymphocytes and the hepatocytes suffering from hydropic degeneration in the cytoplasm, other histopathological sections showed showing congestion of the sinusoids, area of coagulative necrosis infiltrated with lymphocytes with fatty changes. Spleen of group 7, there was severe congestion of the red bulb and area of hemorrhages, later severe depletion of the lymphoid follicle takes place, on the other hand groups 1 (colistine + organic acid), 2 (organic acid + *B.subtilis*) and group 4 (colistine) show mild pathological changes in liver. groups 3 (organic acid) and 5 (*B.subtilis*) showing moderate changes in liver.
It could be concluded that *S enteritidis*has great economic importance in poultry industry and could be controlled with new antibiotic alternative such as organic acids and prebiotics preventing hazard and improve poultry performance.
Key words: broiler performance, prebiotic, *Salmonella enteritidis*, colistine, Bacillus subtilis , organic acid, PCR.

Introduction

Bacterial poultry disease cause severe economic losses in poultry industry, one of these important diseases is *S. enteritidis* infection Which is the causative agent for Salmonellosis in poultry and food poisoning in human[1], the disease transmitted by rodents, wild birds, insects and reptiles without showing any clinical disease[2]. *S. enteritidis*in poultry causes increase mortality rate and decrease in egg productionworldwide[3]and in Egypt[4]. In broiler chickens *S. enteritidis* causes variable mortality 20-96% [5]especially in vertical transmitted chicks, characteristic pericarditis with necrotic foci and petechial hemorrhage on liver [6].

Many risk factors can settle *S. enteritidis* infection and colonization such as poor biosecurity, sub-minimal treatment protocols, poor management practices, poor chick quality[7]. Strict hygienic measures for rodent, wild birds and insect together with treatment of infectious strain help in control the disease. Colistinesulphate is one of used antibiotics in order to control *S. enteritidis* infection efficiently in broiler as it was found that it decrease the rate of infection of flocks and contamination of carcasses together with improving live weight gain increases by 14% and the feed conversion rate by 8% [8]. Unfortunately this antibiotic showed multi-drug resistance nowadays together with antimicrobial genetic elements that can be exchanged between intestinal bacteria[9,10] this give rise for use of new products safe and efficient such as Probiotics and prebiotics for prophylaxis and control of *S. enteritidis*[11,12] as it could control *S. enteritidis* safe and efficiently. Probiotics such as *Bacillus* spp. and *B.subtilis* spores as it was found that it significantly reduced the average Salmonella load of cecum samples of the chickens, by 3 log units this help not only prevent infection by decrease dose of infectious agent but also aid on the processing side by decreasing the amount of Salmonella entering the facility and improving food safety [13]. Other researchers noticed that *Bacillus* spp. and *B. subtilis* spores may be successful competitive exclusion agents, the organism modulates the intestinal microbiota and favors the growth of lactic acid bacteria with putative health-conferring properties[14]**,** recently it was found that broiler fed dietssupplemented with *B. subtilis* had 4.4% greater body weight gain than those fed non- probiotic diets and thus improve the growth performance of broiler chickens [15]moreover [16]noticed that *B. subtilis* containing diet leads to increased efficiency of intestinal digestion in the host animal. Not only *B.subtilis* hasbeneficial role as a preventive measure for Salmonella spp. Infection but also organic acid has a great role in decrease population of pathogenic microorganisms as it showing a significant reduction in total number of Salmonella spp. positive cecal tonsils, and reducing the number of Salmonella microorganism in the crop when compared with control negative infected broiler chickens [17].

Also it was found that organic acids improves poultry performance including average live weight, average daily gain, average daily feed consumption and mortality rate compared with the control group[18,19]. From the above mentioned data our trail was designated in order to compare effect of antibiotics alternatives probiotic and prebiotic against well known used antibiotic colistinesulphate in control of recent field isolate *S.entertidis* in broiler chickens.

Material and methods

1. Experimental Chickens

A total 290, day-old Cobb broiler chicks of mixed sex were used. The chicks were taken from a breeder flock free from Salmonellosis. The birds were kept under complete observation for whole experimental (32 days) in separate thoroughly cleaned and disinfected houses and provided with feed and water adlibitum. All the birds were vaccinated against Newcastle disease (ND) using live Hitchner B1 and La Sota vaccine strains at 5 and 16 days of age, respectively, against infectious bronchitis (IB) disease using live H 120 strain at 1 day old by coarse spray and also against avian influenza (AI) disease using inactivated H5N1 reasortant virus vaccine strain a 7 days old. Vaccination against infectious bursal disease (IBD) was applied using live intermediate strain (Bursine plus) at 14 days. of age. All the vaccines were given via eye drop instillation except (AI) vaccine which given through subcutaneous route at the back of the neck.

2. Ration:

Commercial starter and grower broiler chicken ration were given till 21 and 32 days of age, respectively. The used commercial balanced ration based on yellow corn or soybean that met the National Research Council (NRC) (1984) broiler chicken requirements. The starter ration contained crude protein-not less than 21%, crude fat-not less than 2.94%, crude fibers-not less than 2.35%, metabolizing energy-not less than 3054 Kcal/kg ration and used for the first 3 weeks of age. The grower ration contained crude protein-not less than 17.15%, crude fat-not less than 2.5%, metabolizing energy-not less than 3020 Kcal/kg ration and used for the remaining of the experimental period. The ration contained coccidiostate (Semiduramicin) while no antibiotics were added to it.

3. Used Probiotic:

FIVE-MEN SONG Companywater soluble powder
Composition: each 100 g contain *Bacilus subtilis* (min) 8.4×10^6 CFU Sorbitol sodium (min) 400gm.Vitamin B1 (min) 200gm, Glucose up to 100gm.
Administration and dosage
0.5-1g per 1L of drinking water or 300g/ 1 ton of feed
Lot number : 20150306
Exp date 20/3/2017

4. Used prebiotic (organic acids) :

- " Acidofort" –Batch No. : 0141207.
- component citric acid, lactic acid, phosphoric acid and sodium citrate

5. Antibiotic colistinesulphate used:

Colistinsulphate 6 MIU: each gm contains 6000.000 IU colistinesulphate
Green Vet company- Batch No. 2986.

6. Isolation and purification of *Salmonella spp. :*

Twenty gm of poultry droppings was pre-enriched in 180 ml of selective enrichment media (Selenite Faeces (SF) broth), incubated at 37°C for 24 hours, then subcultured on Salmonella – Shigella agar (SSA) media and incubated at 37°C for 24-48 hours suspected colonies were identified morphologically (transparent colonies with black centre on SSA) and biochemically sugar fermentation test and motility test as described by [20,21].

7. Molecular identification: this carried out according to [22]:

7.1. **DNA extraction.** DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56^{O}C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged

following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

7.2. Oligonucleotide Primer. Primers used were supplied from **Metabion (Germany)** are listed in table (1).

7.3. PCR amplification. Primers were utilized in a 25- µl reaction containing 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl of DNA template. The reactions were performed in an Applied biosystem 2720 thermal cycler.

7.4. Analysis of the PCR Products.

The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH). Electrophoresis was done in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the PCR products were loaded in each gel slot. A Gelpilot 100 bp DNA Ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Table (1): Primers sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondar denaturation	Annealing	Extension		
sefA	GCAGCGGTTACTATTGCAGC	310	94°C 5 min.	94°C 30 sec.	52°C 30 sec.	72°C 40 sec.	72°C 7 min.	Akbarmehr *et al*., 2010
	TGTGACAGGGACATTTAGCG							

8. preparation of challenge inoculums:

The Challenge Inoculum Broth culture of *S. Enteritidis* field strain was centrifuged at 3000 r.p.m for 10 min. Sediment was diluted with sterile buffer saline and adjusted using MacFerland matching tube to contain 10^9 CFU/ml. The challenge inoculum was prepared according to the method of [23]. At 21 days of age, each bird in the experimentally infected groups was inoculated orally with 0.5 ml/ containing 10^9 CFU/ml *S. Enteritidis*[24].

9. Re-isolation of S. Enteritidis from dead challenged birds:

Dead birds from each group post challenge were collected and the liver, heart, spleen and caecum were used for *S. Enteritidis* re-isolation. Samples were inoculated into tetrathionate broth, incubated at 37°C for 24 hr, streaked onto S.S agar and incubated at 37°C for 24 hr. Suspected colonies were identified morphologically and biochemically

10. Experimental Design.

After isolation, biochemical characterization and moleculer identification of *S. enteritidis* field isolate strain from clinical field case, A total number of 290 one day old Cobb broiler chicks were used in protection study, at day old, ten chicks were sacrificed and examined bacteriologically to prove their freedom from *S. Enteritidis* infection then 270 chicks were divided into 7 equal groups, 40 chicks in each as shown in table (2). Groups 1, 2 and 3 received organic acids start from five days of age till two weeks post challenge, groups 1 and 4 treated with colistinesulphate start from 2nd day post challenge for 5 successive days while groups 2 and 5 treated with *B.subtilis* start from 5 days of age till two weeks post challenge days post challenge while group 6 and 7 kept as control negative non treated group and group positive challenged group respectively. All birds groups except control negative group 6 were challenged orally by 0.5 ml containing 10^9 CFU/ml *S. enteritidis* at 21 days of age. Clinical Signs, mortalities and gross lesions in the challenged groups were observed daily for two weeks post challenge, poultry performance including FCR, together with samples for histopathological examination were studied.

Table (2): Treatment of chicken groups infected with *S.entertidis*

Group	Infection with *S.entertidis*	Type of treatment
1	+ve	Colistine + organic acids
2	+ve	*B.subtlis* + organic acids
3	+ve	Organic acids
4	+ve	Colistine
5	-ve	*B. subtlis*
6	-ve	No treatment
7	+ve	No treatment

10.Histopathological Studies:

Tissue specimens from liver and intestine of experimental birds of each group chicks were fixed in 10% neutral formalin solution and the specimens were routinely processed in paraffin embedding method, sectioned and stained with Haematoxylin and Eosin (H&E) for light microscopic examination according to [25]

11- Feed conversion rate (FCR):

It was calculated by total weight/g of food consumption / birds of specific group during a given period over total weight gain /g of the same group birds during a given period [including weight gain of birds which died during the given period] according to [26].

Results and Discussion

Isolation of *S. entertidis* from samples collected from clinical field case were fulfilled on specific media together with assist our results by biochemical reaction .further identification was conducted using polymerase chain reaction PCR that ensure our isolate is *S.entertidis* strain as shown in photo (1)

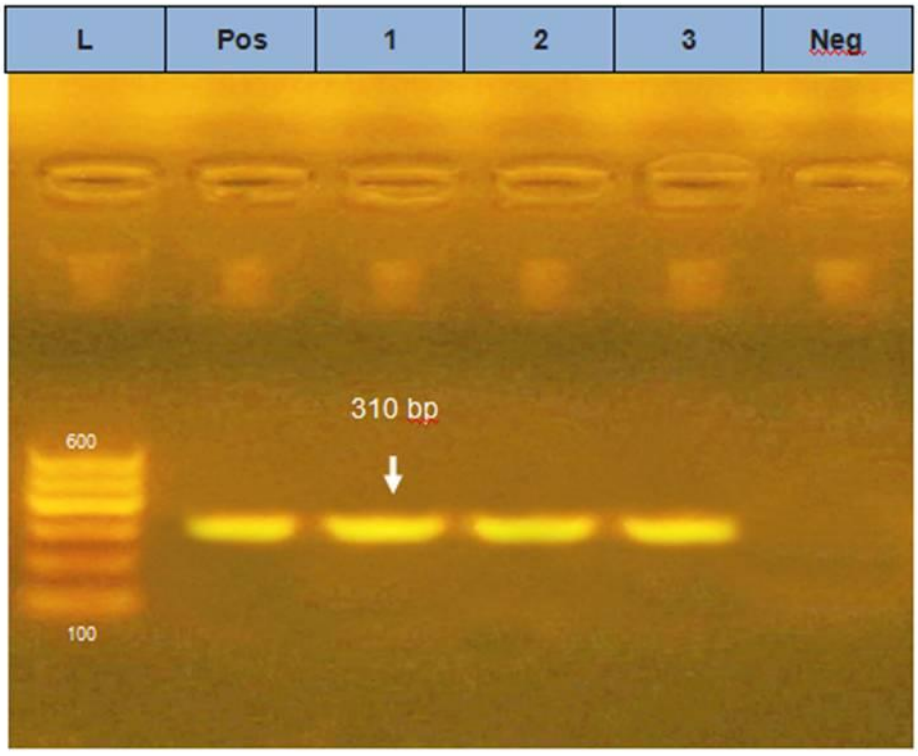

Photo (1): positive *S. entertidis* band At 310bp

Positive 1,2and 3 tested samples at 310bp when compared with positive control

As it was found that a 310 bp fragment within the sefA gene is specific for Salmonella Enteritidis[27] which is supporting our results, similar results was found also by [28]

Results of mortality rate are shown in table (3) , the highest mortality rate was group 7 (control positive group) which was 35%, followed by group 3 (organic acid group) which was 17.5% , then group 5 (*Bacillus subtilis* group) which was 15%, followed by group 2 (*Bacilus subtilis* – organic acid groups) which was 12.5%, followed by group 4 (colistine group) which was 10%, then group 1 (colistine – organic acid group) which was 5%, and finally group 6 (control negative group)showing no mortalities. Samples from dead birds including liver, heart, spleen and cecum were aseptically collected from each group post challenge for *S. Enteritidis* re-isolation. Which indicate positive for *S.entertidis* microorganism which considered suspected cause for mortalities. highest mortality rate was in group 7 which indicate that *S.entertidis* cause high mortality experimentally in broiler chicks, this results was matched with result found by [29,30]who reported that *S entertidis*one of the a causative bacteriological agent for high mortalities in broiler chickens. the lowest mortality rate was group 1 (received colistinesulphate and organic acids) this maybe the synergisticantibacterial effect induced by both colistinesulphate and organic oil, as it was found that addition of colistinesulphate in broiler diet decrease the rate of infection of flocks and contamination of broiler carcasses with *S enteritidis*[8], recently [31]noticed that *S. enteric spp.* that isolated from imparted duckling showing 100% sensitivity to colistinesulphate. on the other hand it was found that most of the tested essential oils and compounds exhibited good antibacterial and antifungal effect[32], recently it was found that organic acid showing a significant reduction in total number of Salmonella spp. positive cecal tonsils, together with reducing the number of Salmonella organism in the crop when compared with controls[17].Mortality rate was also improved in groups 2 (organic acids and *B. subtilis*), 5 *(B.subtilis)*, and 3 (received organic acid alone) which was 12.5%, 15% and 17.5% respectively, this improvement against control positive group maybe due to the single and dual synergistic effect of both organic acids and probiotics. As organic acids many researcher found that probiotic *B.subtilis* probiotic has antibacterial effect as it was found that feeding *B. subtilis* significantly reduced the average Salmonella load of cecum samples of the chickens, by 3 log units that decrease bacterial load and preventing disease together with decreasing the amount of Salmonella entering the facility and improving food safety in processing side[13]**,** this may be due to this probiotics reduce colonization of opportunistic microorganisms in the gastrointestinal tract by competitive exclusion phenomena [33,34,35].Yet, understanding of how probiotics mediate these health benefits, specifically reduction of Salmonella infection, is very limited and need further investigations.

Table (3): Mortality rate in *S.enteritidis* infected treated and control chicken group.

Group	Total number of birds/each group	Number of dead birdspost challenge	Mortality rate
1	40	2	5%
2	40	5	12.5%
3	40	7	17.5%
4	40	4	10%
5	40	6	15%
6	40	0	0
7	40	14	35%

Table (4): Performance of treated and control *S.enteritidis* infected broiler chicken groups

Group Number	Age/ week	Treatment	Infection	ABW	AFI	FCR
1	1	Colistinesulphate + Organic acids	+	165.50	180	0.70
	2			435	470	1.08
	3			855	1125	1.31
	4			1415	2110	1.49
	5			2130	3390	1.59
2	1	*B,subtilis* + Organic acids	+	160	140	0.87
	2			445	475	1.06
	3			875	1130	1.29
	4			1460	2150	1.47
	5			2160	3420	1.58
3	1	Organic acids	+	161	195	0.76
	2			430	460	1.06
	3			845	1110	1.31
	4			1380	2080	1.50
	5			2095	3370	1.60
4	1	Colistine only	+	164	198	0.81
	2			422	450	1.06
	3			815	1095	1.34
	4			1385	2110	1.52
	5			2110	3420	1.62
5	1	*B. subtilis*	+	163	188	0.73
	2			425	455	1.070
	3			840	1095	1.31
	4			1375	2080	1.51
	5			2100	3375	1.60
6	1	Non treated	- ve	161	140	0.86
	2			420.5	451	1.072
	3			810	1090	1.34
	4			1340	2030	1.51
	5			2050	3320	1.61
7	1	Non treated	+	162	142	0.87
	2			419	450	1.07
	3			815	1095	1.34
	4			1274	2030	1.59
	5			1831	3220	1.75

Concerning ABW, it was found that the lowest is group 7 (infected control positive group) which was 1831gm by the end of the experiment when compared with control negative group 6 which was 2050 gm, this result was matched with [36]who noticed that chickens infected at day one and those infected at 3 weeks of age with *S.enteritidis* organism had lower body weight compared to the controls. on the other hand the highest ABW by the end of the experiment was group 2 (received organic acids and *B.subtilis* which was 2160 gm followed by group 1 (received colistinesulphate and organic acids) which was 2130 gm , then followed by group 4 (received colistine only) which was 2110 , followed by group 5 *(B.subtilis)* which was 2100 gm, then followed by group 3 (received organic acids) which was 2095 gm, the highest ABW in group received both *B. subtilis* and organic acids this may be due to dual synergistic effect of probiotic *B.subtilis* and organic acids, *B.subtilis* significantly reduces Salmonella in broiler chickens [13]which considered good alternatives to antibiotics in promoting growth resulting from a beneficial modulation of the intestinal micro flora, which leads to increased efficiency of intestinal digestion [16]while organic acids lead to significantly higher ($p<0.05$) in average live weight and poultry performance [18]and could replace antibiotic as growth promoter safely[37], moreover organic acids showing a significant reduction in total number of Salmonella spp. positive cecal

tonsils, and reducing the number of Salmonella microorganism in the crop when compared with control negative infected broiler chickens, resulting in decrease amount of infectious dose and improve performance. FCR group 7 (positive infected non treated) was the highest (bad FCR) which was 1.75 , followed by group 4 (received colistinesulphate) which was 1.62, then followed by group 6 (control negative) which was 1.61, followed by group 3 (received organic acid) and 5 (received *Bacillus subtilis*) which both have the same results which was 1.60 , followed by group 1 (received colistine and organic acid) which was 1.59 and finally the best feed conversion rate was group 2 (received organic acids and *Bacillus subtilis*) which was 1.58. the latest (group 2) was the best this maybe due to positive synergistic beneficial effect by *B.subtilis* and organic acids, as *B.subtilis* colonize intestine and by competitive exclusion prevent colonization of pathogenic microorganisms[33]thus prevent infection, also organic acids alter media in intestine which become unfavorable for pathogenic bacteria and improve digestion [38]therefore organic acids and *B.subtilis* together or when used alone has positive effect on FCR.

Histopathological changes it was found that liver and spleen of control negative group 6 show normal histology while group 7 (control positive infected) liver showed congestion of the sinusoids, area of coagulative necrosis infiltrated with lymphocytes and the hepatocytes suffering from hydropic degeneration in the cytoplasm (fig 1) , other histopathological sections showed showing congestion of the sinusoids, area of coagulative necrosis infiltrated with lymphocytes with fatty changes (fig 2). concerning spleen of group 7, there was severe congestion of the red bulb and area of hemorrhages (fig 3), later severe depletion of the lymphoid follicle takes place (fig 4). This pathological finding was parallel with results found by [39,40]who report similar pathological changes in liver and spleen due to Salmonella infection on the other hand groups 1 (colistine + organic acid), 2 (organic acid + *B.subtilis*) and group 4 (colistine) show mild pathological changes in liver in the form of light congestion of the central vein (fig 5) together with light depletion of lymphoid follicles (fig 6). Groups 3 (received organic acid) and 5 *(B.subtilis)* showing moderate histopathological changes in liver in the form of congestion of the central and portal vein the hepatocytes suffering from vacuolar degeneration in the cytoplasm (fig 7). Light or moderate pathological changes maybe resulted from the used antibiotic colistinesulphate, probiotic *B.subtilis* and/or organic acids used as it was found that *S.enteritidis* sensitive to many antibiotics specially colistinesulphate[31]resulting in control infection and prevent colonization in intestine , organic acids found to have antibactertial and antifungal activities resulting in decease infection bolus [32] resulting in decrease total Salmonella bacterial count[17], moreover organic acids produce unfavorable media resulting in decrease colonization in small intestine and improve performance[41]. Probiotic Bacillus subtilis also competing pathogenic Salmonella spp. on active site in small intestine and produce unfavorable metabolitis for this pathogenic microorganisms resulting in decrease population and preventing infection which help in better intestinal health [13,14]unfortunately mode of action of both *B.subtilis* and organic acid need further investigations.

It could be concluded that *S. enteritidis* has great economic importance in poultry industry and could be controlled with new antibiotic alternative such as organic acids and prebiotics preventing hazard and improve poultry performance.

Histopathological stained liver and spleen sections of Chicken (H&E X 200) showing:

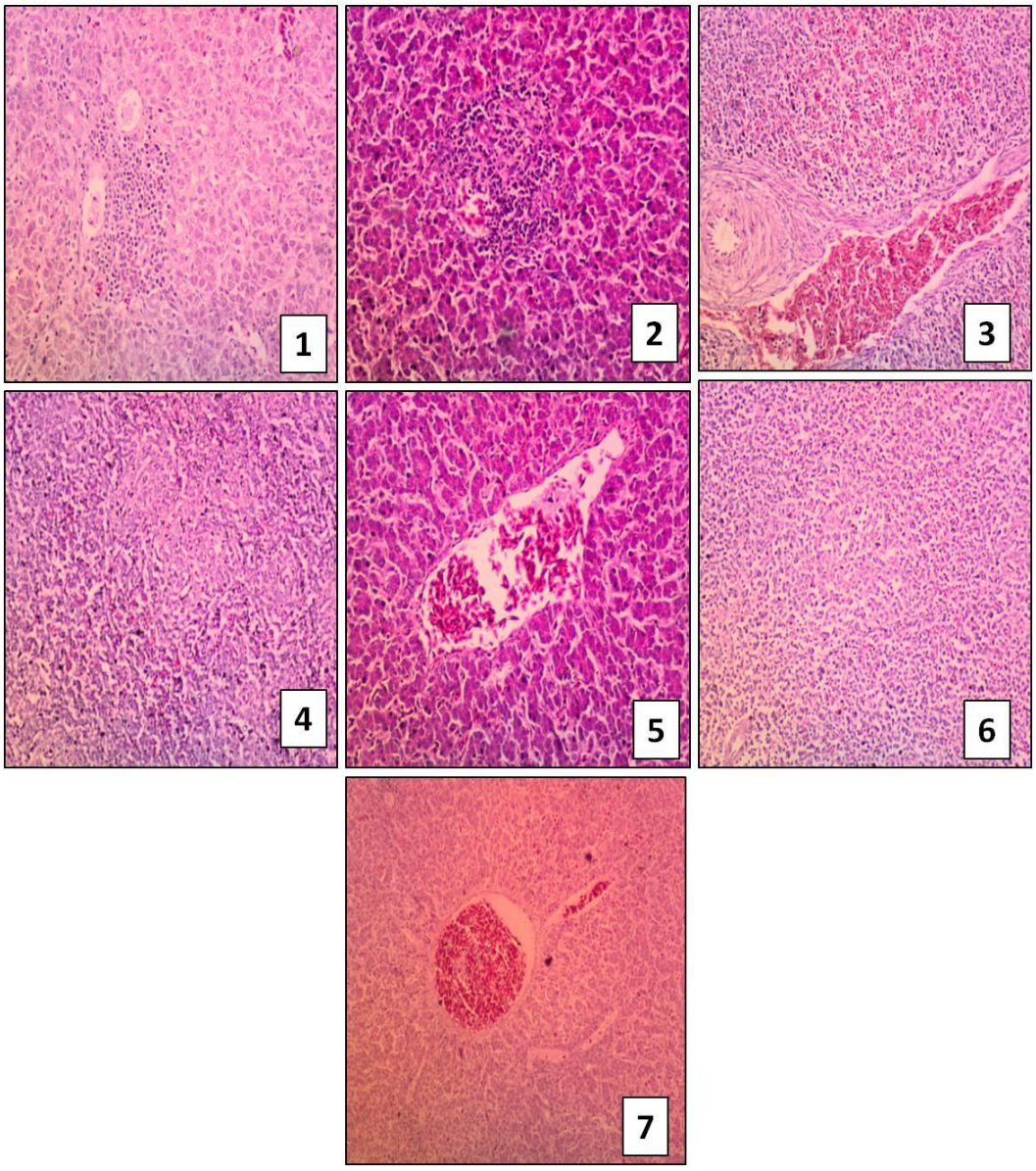

Fig (1): liver showing congestion of the sinusoids, area of coagulative necrosis infiltrated with lymphocytes and the hepatocytes suffering from hydropic degeneration in the cytoplasm.
Fig (2): liver showing congestion of the sinusoids, area of coagulative necrosis infiltrated with lymphocytes and some fatty changes.
Fig (3): spleen showing severe congestion of the red bulb and area of hemorrhages.
Fig (4): spleen showing severe depletion of the lymphoid follicle.
Fig (5): liver showing light congestion of the central vein.
Fig (6): spleen showing light depletion of the lymphoid follicle.
Fig (7): liver showing congestion of the central and portal vein the hepatocytes suffering from vacuolar degeneration in the cytoplasm.

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