An Electron Microscopic Study of the Effect of (*Saccharomyces cerevisiae*) on the ability of *E. coli* 0157:H7 to attach and efface healthy young broilers

Abstract

The present study was carried out to determine the influence of dietary probiotic source feed (fungal-yeast; *Saccharomyces cerevisiae*) on the ability of *E. coli* 0157:H7 to attach and efface healthy young broilers at different feeding periods (7, 10, 14, 21, and 28 days of age). There were reductions in bacterial attachment of broilers fed various yeast levels. Twenty one days post-challenging about 87% of the (G1); positive control, chicks fed control diet containing 0.00% baker yeast and challenged with *E. coli* 0157:H7, showed both cecal attachment and effacement. On the other hand 37% of the (G2); chicks fed control diet containing 0.75% baker yeast and challenged with *E. coli* 0157:H7, showed cecal attachment. And only 16% of the (G3); chicks fed control diet containing 1.00% baker yeast and challenged with *E. col*i 0157:H7 showed cecal attachment. The results of this study suggest adding yeast at 1.0% into broilers diets causing a significant (P<0.005) decrease in bacterial attachment and may enhance the productive performance and nutrients utilization via the inhibitory effect of yeast against pathogenic bacteria *E. coli* 0157:H7.

Keywords: *Escherichia coli*, Probiotic, Broiler, fungal-yeast; *Saccharomyces cerevisiae*, AEEC

Introduction

Escherichia is a genus of Gram negative facultative anaerobic, rod-shaped bacteria of the tribe Escherichieae, family Enterobacteriaceae, found in the large intestine of warm-blooded animals and are members of the "coliform" group of bacteria (Perna et al., 2001). Attaching and effacing *Escherichia coli* (AEEC) is Entero-haemorrhagic *E. coli* (EHEC) belongs to a family of pathogenic bacteria that attach closely to host enterocytes and efface the microvilli of the cells to colonize host intestinal mucosa (Agin et al., 2005). Attachment of EHEC to the apical epithelial surface leads to recruitment of cytoskeletal proteins and

injection of effector molecules directly into the host cell (Gao et al., 2009). *E. coli* strain O157: H7 has emerged in the past decade as an important food-borne pathogen with haemolytic uremic syndrome, neurologic symptoms and haemolytic colitis outbreaks (Krystle, 2010). It was first isolated from a patient in1975; (Nataro and Kaper, 1998). This EHEC serovar of *E. coli* produces Shiga-like cytotoxins (SLT I or SLT I1 or both) that cause host cell death by inhibiting protein synthesis (Leroy et al., 1994). *E. coli* 0157:H7 also release verotoxins in the intestine, translocated across the gut epithelium into the circulation, and transported to microvascular endothelial cells and can cause microvascular endothelial injury. (Schüller, 2011). *E. coli* 0157:H7 is regarded as the third most frequently isolated pathogen from stools, after Campylobacter and Salmonella (Harris et al., 2003).

Chickens are more susceptible to colonization by pathogens (Callaway, 2009). Antibiotics used in sub-therapeutic level as growth promoter in poultry diets are no longer desirable because of concerns about their bacterial resistance and residual problems in tissues and eggs of birds (Wallace, 2004). Therefore, the development of new antimicrobial compounds and growth promoter have been carried out, such as dietary use of probiotics (Van Immerseel et al., 2009). Probiotic supplements may have a potential effect on modulation of intestinal microflora and pathogen inhibition in hosts (Mountzouris et al., 2010). Many studies support that introduction of probiotics to the gastrointestinal tract (GI) in poultry can maintain normal intestinal microflora by competitive exclusion and antagonism alter the enteric microflora in hosts, which in turn has a broad mechanistic effects on intestinal defense mechanisms, including; (i) inhibiting microbial pathogenic growth by altering metabolism , increasing digestive enzyme activity and decreasing bacterial enzyme activity and ammonia production, (ii) improving feed intake and digestion, (iii) increasing epithelial cell tight junctions and permeability, (iv) stimulating the immune response of the intestinal mucosa, (v) increasing the secretion of antimicrobial products, (vi) eliminating pathogenic antigens, (vii) does not causes microbial mutation, (viii) have no residual effect and (ix) have no withdrawal time (Kabir, 2009; Kizerwetter-Swida, and Binek, 2009; Mountzouris, et al., 2010).

Probiotics often contain some nonpathogenic yeast (Foligné, et al., 2010). The yeast *Saccharomyces cerevisiae* (Baker's yeast*)* has been known as a probiotic in feed animals (Brown, 2011). *S. cerevisiae* is considered as probiotic that, when administered through the digestive tract, have a positive impact on the hosts health through its direct nutritional effect.

Moreover, *S. cerevisiae* could act as bioregulalor of the intestinal micro flora and reinforcing the host natural defenses, through the sanitary effect by increasing the colonization resistance and stimulation of the immune response (Hassanein and Soliman, 2010). The beneficial effect of *S.cerevisiae* is attributed to the fact that it is a naturally rich source of proteins, minerals and B complex vitamins (Shareef, and AL- Dabbagh, 2009). It was reported that yeast, and its cell wall extract containing 1,3-1,6 D-glucan and Mannan oligosaccharide are the important natural growth promoters for modern livestock and poultry production (Hassanein and Soliman, 2010). The yeast also has been shown to survive gastric acid in the stomachs of mammals suggesting that it might survive passage through the low pH environment of the proventriculus and gizzard of chickens to reach the intestines and ceca (Zhang et al., 2005).

The objective of this study was to study the effects of *S. cerevisiae* as a dietary probiotic source feed on the ability of *E. coli* 0157:H7 attaching and effacing healthy young broilers at different feeding periods (10, 14, 21, and 28 days of age); demonstrated by Transmission Electron Microscopic study and bacterial enumeration.

Materials and Methods:

i. **Bacterial strain and growth conditions.** *E. coli* serotype 0157:H7 (ATCC 43889), obtained from the American Type Culture Collection (Rockville, Md.), used to challenge chicks, was grown on Trypticase soy agar (TSA; Sigma, USA) at 37°C for 24 h and then in 50 ml of Trypticase soy broth (Sigma, USA) and incubated in a rotary shaker (100 rpm) at 37°C for 6 h. in 250-ml Erlenmeyer flasks. Bacteria were washed three times and resuspended in 0.01 M phosphate-buffered saline (PBS) (pH 7.5). Bacteria suspensions in Trypticase soy broth adjusted to concentrations of approximately 10^9 cells per ml were used as an inoculum.

ii. **Chickens and Bacterial challenge.** 1- Day-old male Ross-308 chicks (n = 180), weighing approximately 40 g, were obtained from (Agricultural Research Station, King Faisal University, Al-hassa, Saudi Arabia). Chickens were distributed randomly among 3 groups 60/treat with three replicates of 20 chicks per replicate**.** Chicks were assigned to one of the following treatments; group1 (G1); positive control, chicks fed control diet containing 0.00% baker yeast and challenged with *E. coli* 0157:H7, group2 (G2); chicks fed control diet containing 0.75% baker yeast and challenged with *E. coli* 0157:H7, and group3 (G3); chicks fed control diet containing 1.00% baker yeast and challenged with *E. col*i 0157:H7. Five chicks in each replicate /group served as controls and received per-orally 1.5 ml of

Trypticase soy broth into the crop via a 20-gauge cannula, the remaining chicks received 1.5 ml of 1.9 x 10^9 *E. coli* 0157:H7 inoculum by a 20-gauge cannula. All chicks were held individually in cages that did not allow feeding on excrement. Feed and water provided *ad libitum*. Weekly body weight, body gain, feed intake, feed conversion ratio and mortality rate recorded from 0-28 d of age. Chicks challenged at 7 d of age with *E. coli* (1.50 mL of broth containing 1.9 x 10^9 colony forming units (CFU) for each chick). Chicks from each group (Fifteen *E. coli* 0157:H7-challenged and five control chicks at each sampling time/replicate) were killed by bleeding after anesthetization with chloroform at 7, 10, 14, 21, and 28 days post-treatment. Birds were housed 15 per cage in plastic cages measuring 00 inches (length) by 00 inches (width) by 00 inches (height) (ca. 177.5 by 177.5 by 40.6 cm). Housing temperature was maintained at 35^0C for the first 4 to 5 days and then kept at 25^0C for the remaining growth period.

Examination of chicks and tissues. From inoculation until sacrifice, the chicks were monitored for abnormal behavioral signs, diarrhea, and weight changes. Immediately after sacrifice the internal organs of G 1, G 2, and G 3 chicks were surgically exposed and examined for gross pathological abnormalities of the heart, liver, gallbladder, spleen, kidneys, and gastrointestinal tract.

Bacterial adhesion assay. The method was adapted from (Brinn and Pickett, 1979) assay to confirm that the attached bacteria were *E. coli* 0157:H7. Specimens taken from these organs were fixed in 10% phosphate-buffered formalin containing 0.5% acetyl-tri-methyl-ammonium bromide for 48 h at room temperature. Specimens were embedded in plastic by (Cold Glycol Methacrylate (GMA) (JB-4; Polysciences, Warrington Pa.). The JB-4 plastic tissue sections were stained with hematoxylin and eosin and azure A (Lillie, R. D., and H. M. Fulmer. 1976; Woodruff, J. M., and S. A. Greenfield. 1979), then were microscopically examined. Sections that showed bacterial attachment were treated by 0.1% trypsin (1:250; Sigma, USA) in 0.05 M Tris hydrochloride (pH 7.4) for 30 min at 37°C.. Sections were washed in PBS, and the trypsin activity was suppressed by exposure to IgG-free 5% fetal calf serum (GIBCO) for 15 min at 4°C. The sections were washed twice in PBS, and endogenous peroxidase activity was stopped with 0.3% H202 for 1 h. After wash with PBS, the sections were overlaid with *E. coli* 0157- specific rabbit antiserum (1:25 dilution; *E. coli* Reference; Department of Microbiology and Parasitology, College of Veterinary Medicine, King Faisal University, Al-Ahsa, Saudi Arabia) for 2 h in a high-humidity chamber. After washed twice

in PBS, the sections were covered with goat anti-rabbit IgG -conjugated with horseradish peroxidase (1:100 dilution; Sigma Chemical Co., St. Louis, Mo.) for 45 min, washed twice in PBS, immersed for 15 min in Karnovsky mixture (Graham, Jr., and Karnovsky, 1966) containing 0.01 M imidazole (Sigma, USA), and washed twice in 0.05 M Tris hydrochloride (pH 7.6). The sections were processed through different concentrations of ethanol to xylene, mounted in resin (Sigma, USA), and examined microscopically.

Bacterial Enumeration. Immediately after sacrifice, the internal organs of G1, G2, and G3 chicks were surgically exposed, and the heart, liver, kidneys, spleen, gizzard, small intestine (cut into three equal segments), colon, and cecae were aseptically removed and individually placed in preweighed conical plastic sterile tubes (50 ml; Becton Dickinson Labware, Oxnard, Calif.). Each tissue was weighed, diluted (1:10) in cold PBS, and aseptically homogenized for 1 min at 4°C with a Polytron tissue homogenizer (Brinkmann Instruments, Inc., N.Y.). Samples were serially diluted 10-fold in PBS, and each dilution was spread on duplicate in MacConkey agar (Difco Laboratories, Detroit, Mich.). The plates were incubated at 37°C for 24 h, and lactose-positive colonies, identified as *E. coli* by standard biochemical tests were counted. Ten lactose-positive colonies, identified as *E. coli* strain were selected at random from each culture at the highest dilution of tissue and were serologically confirmed as *E. coli* 0157:H7 by slide agglutination with 0157:H7 -specific antiserum produced by immunizing rabbits (*E. coli* Reference; Department of Microbiology and Parasitology, College of Veterinary Medicine, King Faisal University, Al-Ahsa, Saudi Arabia). The number of organisms was counted.

Scanning electron microscopy (SEM). The samples obtained from the cecum were used for scanning electron microscopy. The replicate tissue samples were fixed in 3.0% glutaraldehyde-phosphate buffer (pH 7.4) at 4°C for 3 h and washed three times for 15 min each in 0.1 M phosphate buffer (pH 7.4). Sections were postfixed in 1% osmium tetroxide-phosphate buffer at 4°C for 1 h. After dehydration through a graded series of ethyl alcohol-water mixtures, infiltrated with isopentyl acetate, and dried in a critical-point drying; carbon dioxide with a Polaron E-3000 critical point dryer (Polaron Equipment Pty. Ltd., Watford, England), they were fixed to the brass stub and coated with a thin layer of gold about 27.0 nm of gold in a (Dynavac SC150 sputter coating unit) (Dynavac High Vacuum Ltd., Victoria, Australia). Samples were examined with a scanning electron microscope (JEOL JSM-5510LV) operated with an accelerating voltage of 15 kV.

Transmission electron microscopy (TEM). The samples obtained from the cecum were used for transmission electron microscopy. Replicate Sections were fixed in 3.0% glutaraldehyde, postfixed in 1% osmium tetroxide at 4^0C in sodium phosphate buffer (0.1 M, pH 7.4)., and embedded in epoxy resin (Ted Pella, Inc., Redding, CA, USA). Thin sections stained with toluidine blue were examined to select areas for electron microscopy. Ultrathin sections were cut, stained with uranium acetate and lead citrate, and examined with a transmission electron microscope (JEOL JEM-1010) operated at 100 kV.

Statistical Analysis

One-way ANOVA used to determine the effect of different doses of yeast dietary feed additives on bacterial attachment. Threshold for significance was $P \leq 0.05$.

RESULTS:

The physical response of chickens challenged orally with *E. coli* 0157:H7 was not different from that of control animals in that there was no apparent loss of appetite, neither blood nor mucus was observed in the feces, no reduction in weight gain, no alteration in locomotion, and no diarrhea or respiratory distress. All animals, both inoculated and control, appeared healthy.

Gross pathology. The cecae of inoculated chickens were the only segment of the gastrointestinal tract that showed observable lesions. Gross pathological examination of vital organs showed changes beginning 14 to 21 days in the cecae of all *E. coli* 0157:H7-challenged chickens. The Cecae were swelled due to the presence of gas and edema of the mucous membrane, and the cecal tonsils of the challenged chickens were swelled and remained swelled at 28 days post-challenging. Cloacal bursae of inoculated and control chickens appeared normal. Splenic enlargement was observed in challenged chickens 28 days post-challenging.

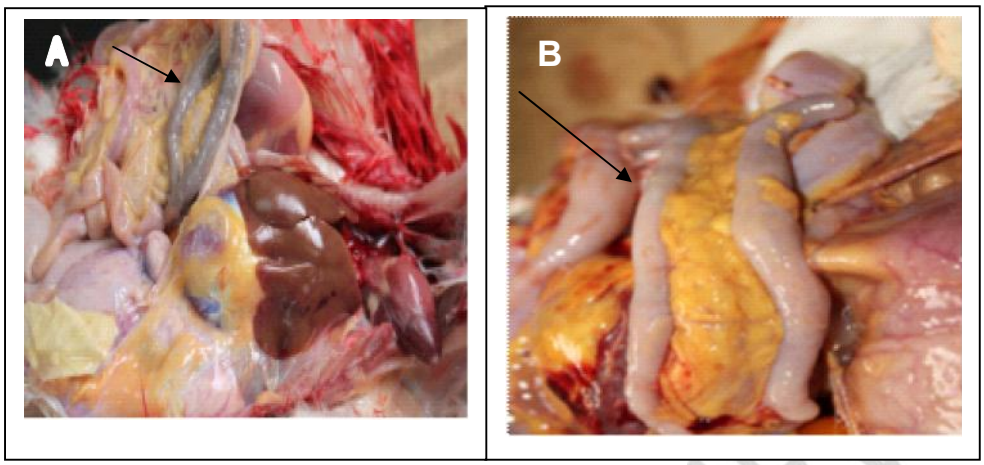

Figure 1: Photo-- micrograph of chicken cecal surface epithelium 21 days old. (A) Normal control chick's cecum (arrow). (B) swelled chick's cecum (arrow) post inoculation with *E. coli* 0157:H7.

Adherence characteristics of AEEC *E. coli* 0157:H7. To confirm the attachment and effacement of *E. coli* 0157:H7, cecal tissue sections were treated with an immunoperoxidase stain with *E. coli* 0157-specific antiserum. Results showed that 21 days post-challenging about 87% of the G1 inoculated chicks showed both cecal attachment and effacement. On the other hand 37% of the G2 inoculated animals showed cecal attachment. And only 16% of the G3 inoculated animals showed cecal attachment. Maximal effacement involving >80% of the cecal surface epithelium. of the G1 inoculated chicks.

Electron microscopy of the intestinal epithelia. By SEM and TEM, The cecae of inoculated chickens were the only portion of the gastrointestinal tract that had observable lesions. Major differences were noted between chickens inoculated with the *E. coli* 0157:H7 and those inoculated with *E. coli* 0157:H7 in conjunction with the yeast. The epithelial lesions were more severe in chickens studied 21 to 28 days post-challenging compared with those examined after 10 days. The largest numbers of organisms were present in the *E. coli* 0157:H7 challenged chicken ceacum. Lower numbers of organisms were seen in the chicken cecum, inoculated with *E. coli* 0157:H7 in conjunction with the yeast. Thin sections from the chickens indicated that bacteria were observed almost exclusively in cecal tissue. **SEM** was used to visualize the *E. coli* O157:H7 bacteria inhabiting the epithelial surfaces of the cecum of chicks. By 21 days post-challenging, SEM showed the bacteria adhering in clusters throughout the ceacum epithelium, and irregular surface lesions on the mucosal surface (Fig. 2B). Mucus was visible throughout the surface epithelium and was closely associated with

aggregates of bacteria. Some bacteria were beginning to burrow through the epithelium. Some intact microvilli were still present on the cell surface with no attached bacteria at some portions of them (Fig. 2C). By 28 days post-challenging, mucus-bacterial aggregates covered a large portion of the villous surface, and there was damage to the epithelium in foci in which bacteria were adherent (Fig. 2D). Many microvilli were lost from the surface of enterocytes, to which *E. coli* O157:H7were attached. The cecal epithelium of the chickens in the control group appeared healthy with intact cells and microvilli and showed no observable lesions (Fig. 2A). Group2, and group3 on the other hand; showed lesser of bacterial attachment in the cecae 7, 10, 14, and occasional observable bacterial attachment at 21, and 28 days of age post-challenging with elongated microvilli, at the site where bacteria were attached (Fig. 2E) A mucosal surface with no associated bacteria is intact (Fig. 2F). In addition, a greater amount of mucus covering the cecum of yeast-fed birds could be observed. Several goblet cells not associated with any bacteria colonies.

Transmission electron microscopy micrographs of chicken cecal surface epithelium are showed in Figures 3, 4, 5, 6 and 7. (Fig. 3A); shows 28 days old control chickens striated border apical (luminal) surface and the enterocytes were closely packed showing the striated microvillus brush border and tight junction with no bacteria attached. The luminal border was regular and smooth. (Fig. 3B;) shows Cecum of *E. coli* 0157:H7 -inoculated chickens14 days and continuing in an advanced state to 28 days post-challenging, viewed with the TEM showed that randomly arranged bacteria were intimately associated with apical plasma membranes of crypt and striated border of the surface epithelial cells within the lumen and microvilli are effaced and fused within the lumen. No bacteria were seen free in the lumen. The luminal border of the epithelium appears irregular when compared with that from control. (Fig. 3C); shows 28 days old control chickens cecal villi with the columnar epithelium consisting of enterocytes with striated border. The epithelium presents a clear striated border on the apical (luminal) surface and the enterocytes are closely packed. The luminal border is regular and smooth. There were no bacteria attached. The glycocalyx was clearly observed covering the microvilli cells without signs of atrophia were seen and there were no visible epithelial protrusions. (Fig. 3D); shows cells that was identified as lymphocytes observed in the small cecal cylinder lumen after 14 days of infection. (Fig. 3E); shows 21 days old control chickens cecum columnar epithelium striated brush border. (Fig. 3F); shows cross section of the bacteria intimately associated with apical plasma membranes

of the surface epithelial cell 21 days post-challenging. (Fig. 4A); shows infected chick cecum (14-day-old) with some of the bacteria entrapped in the mucin layers along the epithelial border, and others were intimately adherent to luminal apical plasma membrane of crypt. The microvilli were effaced and fused. Tight junctions were thickened and distorted. (Figure 4B); shows the bacterial cell wall in direct contact with the irregular shaped superficial epithelial cell 28 days post-challenging. (Figure 4C) shows cross section *of E. coli* 0157:H7 with a typical intimate-adherence pattern with irregularity of two of the associated epithelial cells. (Figure 4D); shows Electron-dense damaged cells devoid of microvilli in areas where the bacteria were present in direct contact with the cell membrane 28 days post challenging. (Figure 4E); shows that the bacteria were frequently associated with projections (A pedestal-like protrusion), and (Figure 4F); shows invaginations of plasma membrane at the bacterial attachment sites 28 days post challenging. (Figure 5A) shows irregular shape and arrangement of superficial mucosal epithelial cells with microvillar marked distortion effacement adjacent to the lesion with multi intimately attached *E. coli* 0157:H7 at attachement sites 28 days post challenging. (Figure 5B); shows macrophage cell (MQ) (a type of white blood cell) engulfing the bacteria in a process that is called phagocytosis, 28 days post challenging, The luminal superficial epithelial cells were irregular in shape, with typical intimate-adherence pattern of transverse section of *E. coli* 0157:H7 bacteria. (Figure 5C); shows Section of the *E. coli* 0157:H7 infected cecum 28 days post challenging showing; Enterocyte without microvilli, irreversible nuclear injury of enterocyte with attached E. *coli* 0157:H7 of the necrotic cells. (Figure 6A); shows dense surface epithelial cells of the cecum of the infected chicken 28 days post challenging. There was loss of normal microvillus pattern with an almost spherical nucleus and irregularly shaped mitochondria with the *E coli* 0157:H7 bacteria adherent, forming attaching-effacing lesions with accumulation of actin at the site of attachment at the intercellular junction. Macrophage was also identified. A concentration of electron-dense material was seen beneath some adherent organisms. (Figure 6B) shows irregular shaped Epithelial cell with multi vacuoles infiltrated with multi *E. coli* 0157:H7 bacteria 21 days post-challenging. (Figure 6C); shows irregular shaped Luminal superficial epithelial cells, with loss of microvilli, frequently showing erosion 28 days post challenging with typical intimate-adherence pattern of transverse section of *E. coli* 0157:H7 bacteria. Also seen bacteria penetrated the cells. Normal tight junctions were demarcated in the control micrographs, while electron dense aggregations were observed in the infected ones. (Figure 6D); shows a transverse section of *E. coli* 0157:H7 bacteria 28 days post

challenging infiltrating the epithelial cells with damaged regions having large cytoplasmic vesicles and vacuoles. There was a dense coat of irregular appendages that extend between the bacteria**.** (Figure 6 E); Shows many bacteria intimately attached to irregularly in shape luminal superficial epithelial cells with multi protrusion detachments in more damaged regions with large cytoplasmic vesicles and vacuoles loaded with cellular debris and occasional bacteria at the upper edge of the enterocyte beneath the attached bacteria 28 days post challenging. (Figure 6F); shows lost Microvilli from the surface of enterocytes with progressive atrophy and the associated cytoskeletons were disrupted., degenerated, and necrotic, frequently showing erosion .Transverse section of *E. coli* 0157:H7 bacteria intimately attached to the cells 28 days post challenging, with probably *an E. coli* 0157:H7 vesicle are also shown. (Figure 7A); shows Group2 treated chickens ceum lumen enterocytes were mostly seen with typical ultrastructural characteristics, however, they become taller in size with some minor alterations of disoriented or elongated microvilli in compare to control one with 21 days post- challenging. (Figure 7B); shows; Group2 treated chickens cecum with the length of villi in the treated birds seemed slightly longer and bulged at some parts than ones in the yeast-free fed birds. (Figure 7C); shows Group3 treated chickens cecum showing a typical intimate-adherence pattern with the apparent lengthening of microvilli by day 21 of challenging. (Figure 7D); shows higher magnification of Group3 treated chickens cecum .showing bulged villi. There were no indication of epithelial effacement or bacterial penetration in group2 and 3 infected chickens. In addition, a greater amount of mucus covering the intestines of yeast-fed birds could be observed.

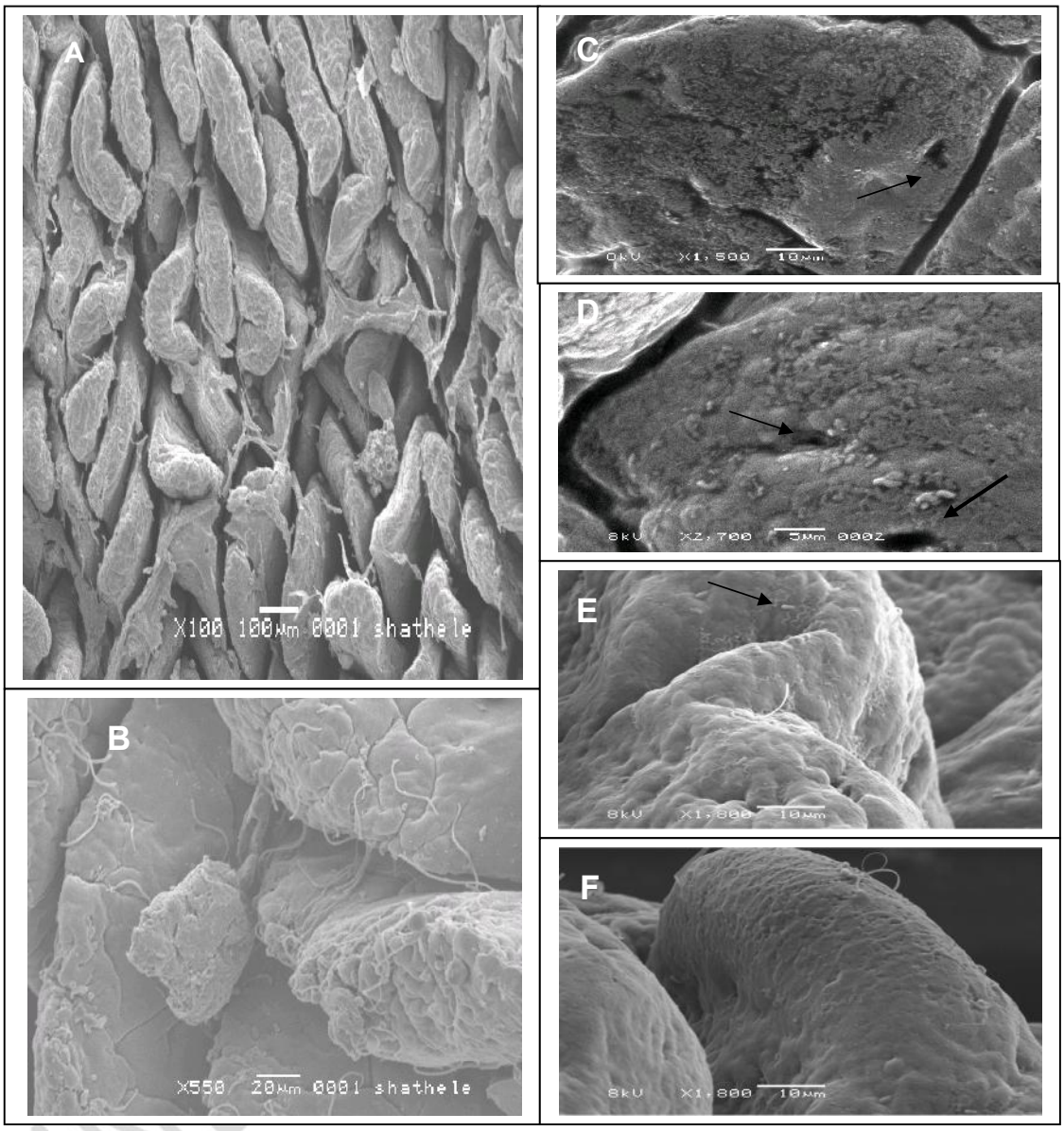

Figure 2. Scanning electron micrographs of cecal villi in broiler chickens at 21 d of age. (A) cecal mucosa of control chicken with no lesions observed, Columnar epithelial cells are regular in arrangement, and aligned microvilli are evenly present on the surface of epithelial cell membranes, Bar, 100 µm. (B) Cecal mucosa of control chicken, , with no lesions observed, identified are the cilia, at 28 d of age, Bar, 20 µm. (C) Cecal mucosa from chicken infected with *E. coli* O157:H7, showing many bacteria attached to rounded enterocytes. Also visible are pore like openings within the epithelium in areas of colonization which is irregularly shaped (arrows). Some portions of some intact microvilli were still present on the cell surface with no attached bacteria21 days post- challenging, Bar, 10µm. (D) Higher magnification of *E. coli* O157:H7-infected cecum 28 days post-challenging with bacteria masking rounded cecal enterocytes. Microvilli are disoriented, elongated, or reduced in number. Also visible are pore like openings within the epithelium in areas of colonization which is irregularly shaped (arrows). Bar, 5 µm. (E) Group2, and group3 showed occasional observable bacterial attachment at 21, and 28 days of age post-challenging (arrow) with elongated microvilli, at

the site where bacteria were attached Bar, 10 µm. (F) A mucosal surface with no associated bacteria is intact in G3 treated chicken 28 days post- challenging, Bar, 10 µm.

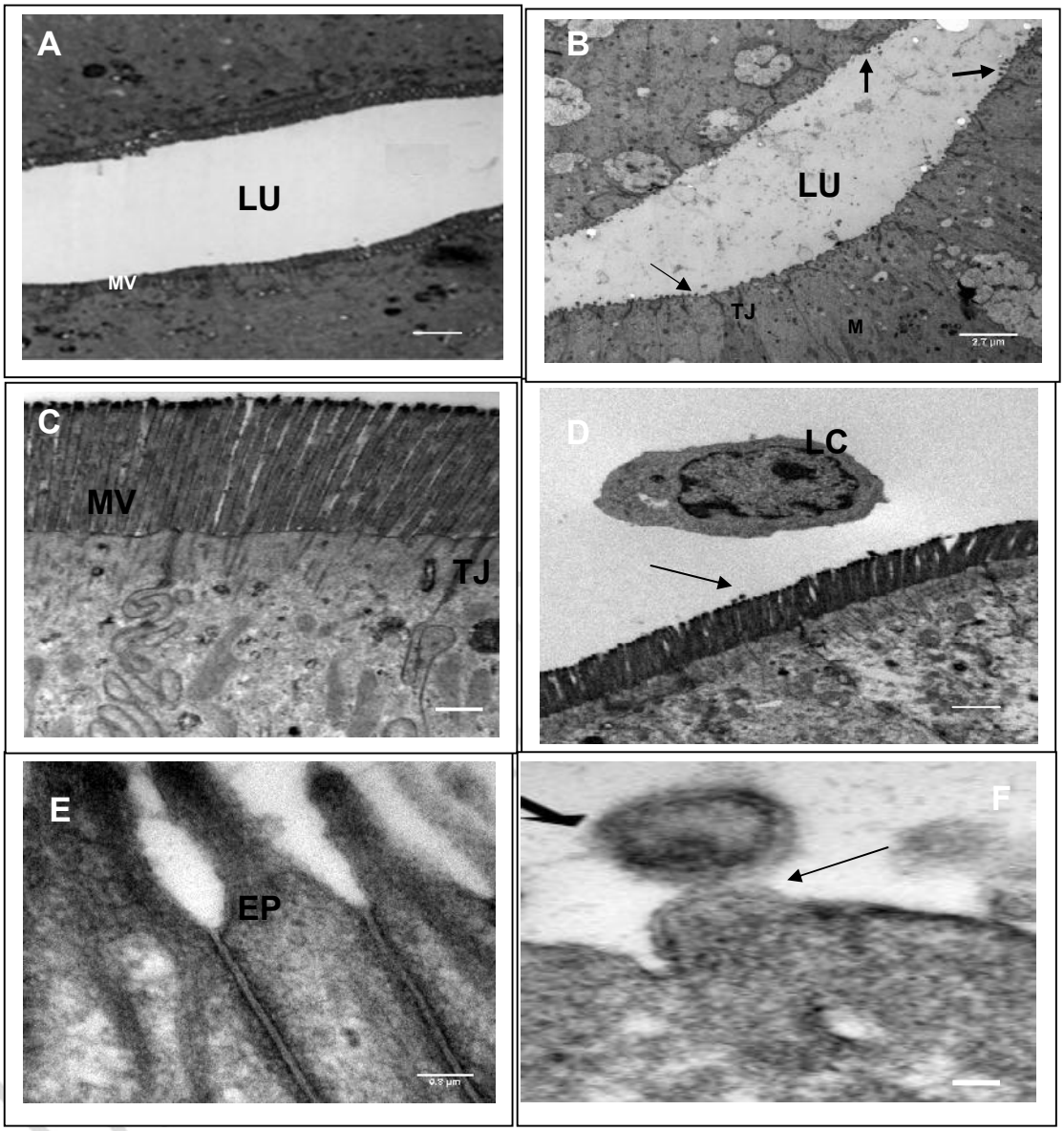

Figure 3: Transmission electron microscopy micrograph of chicken cecal surface epithelium; (A) A 28 Day's old control chickens apical (luminal) surface (LU), identified are: the enterocytes which are closely packed (showing the striated microvillus brush border (MV) and tight junction (arrow). There are no bacteria attached. The luminal border is regular and smooth. Bar, 2.7 µm, (B) A 28 days old infected chickens apical (luminal) surface (LU), identified are; *E. coli* 0157:H7 bacteria (arrows) intimately associated with apical plasma membranes of crypt, the microvilli are effaced and fused with loss of striated microvillus brush border within the lumen, mitochondria (M), and thickened tight junction(TJ), Bar, 2.7 µm. (C) Higher magnification of 28 days old control chickens cecum, identified are; the striated microvillus brush border (MV) and tight junction (TJ) with no bacteria attached. The glycocalyx was clearly observed covering the microvilli. Bar, 1.1 µm. (D) Cells identified as lymphocytes (LC) in the cecal lumen 14 days post infection. Cells without signs of atrophia were seen

and there were no visible epithelial protrusions. *E. coli* 0157:H7 bacteria (arrow) intimately associated with apical plasma membranes, Bar, 1.6 µm, (E) Higher magnification of 21 days old control chickens cecal columnar epithelium (EP) striated brush border. Bar, 0.8 µm. (F) transverse section of *E. coli* 0157:H7 (short arrow) with fimbria (arrow) intimately associated with apical plasma membranes of the surface epithelial cell 21 days post-challenging, Bar, 1.6 µm.

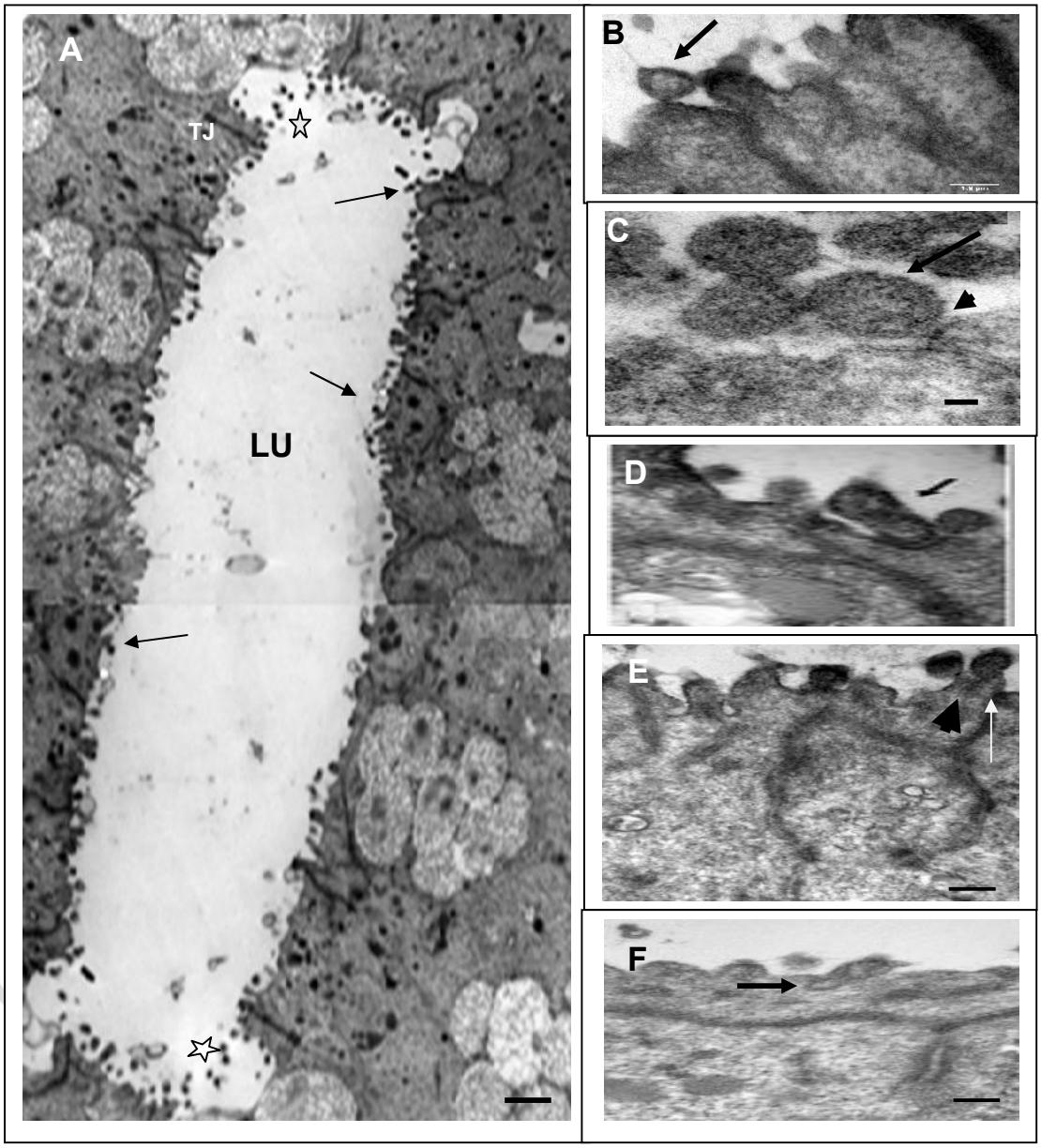

Figure 4: Transmission electron microscopy micrograph of an infected chick cecum. (A) Apical (luminal) surface (LU) 21 days post-challenging, identified are: some of the bacteria entrapped in the mucin layers along the epithelial border(stars), and others (arrows) are intimately adherent to luminal apical plasma membrane of crypt, loss and displacement of the epithelial brush border, Necrotized surface epithelial cells, are also present (microvilli are effaced and fused) and tight junctions (TJ) are thickened and distorted, Bar, 2.7 µm. (B) Arrow indicate the bacterial cell wall in direct contact with two of the irregular shaped superficial epithelial cell 28 days post-challenging, Bar, 1.6 µm. (C) *E.*

coli 0157:H7 transverse section with a typical intimate-adherence pattern (arrow) to two of the associated irregular shaped epithelial cells28 days post-challenging, (arrowhead) indicated bacterial fimbria, Bar, 1.6 µm. (D) Electron-dense damaged cells devoid of microvilli in areas where the bacteria (arrow) present in direct contact with cell membrane 28 days post challenging. Bar, 1.6 µm. (E) A pedestal- like protrusion, (arrowhead) at the bacterial (arrow) attachment sites 28 days post challenging. Bar, 0.3 µm. (F). Cell membranes with invagination, at the bacterial attachment site (arrow) 28 days post challenging. Bar, 0.3 µm.

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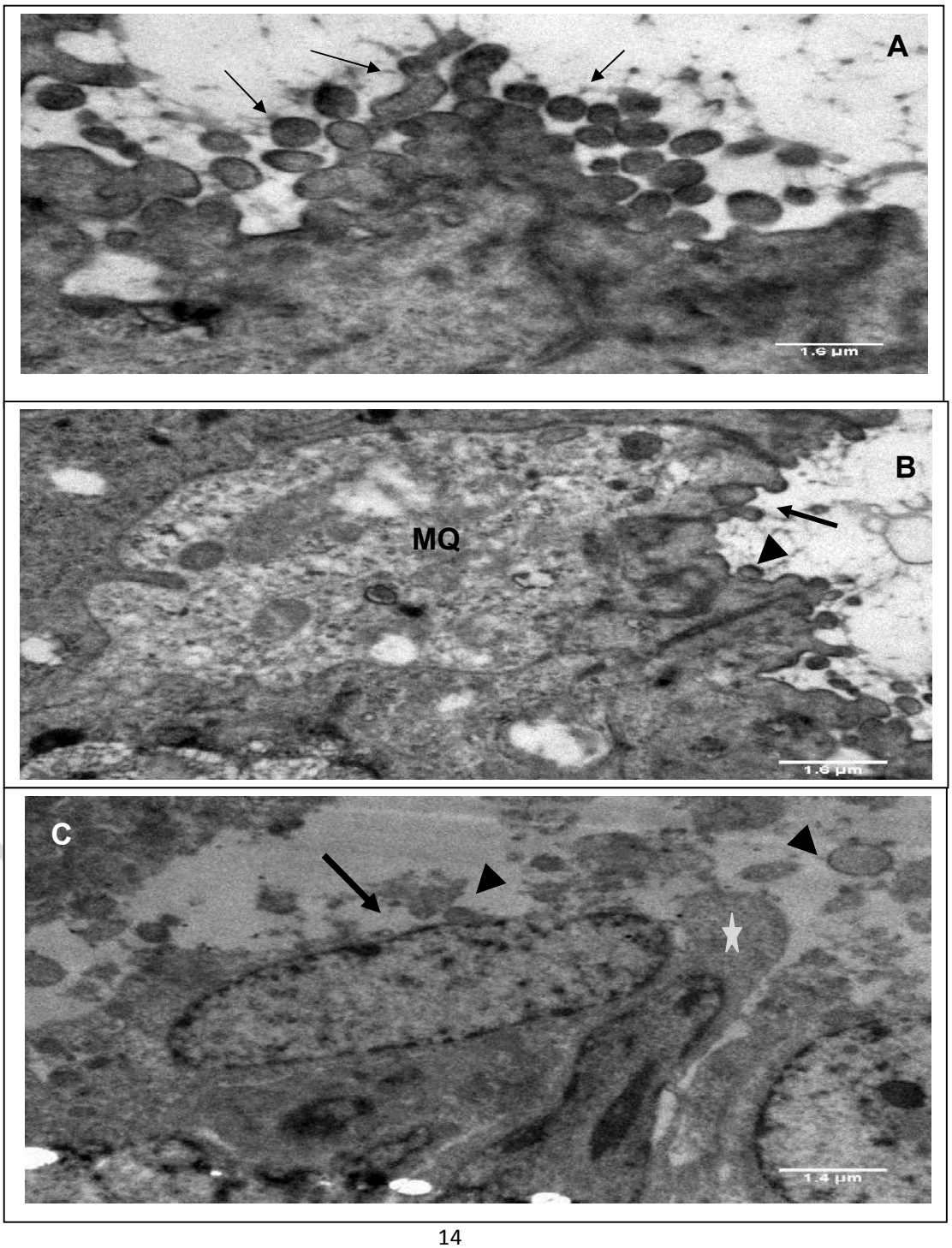

Figure 5: Transmission electron microscopy micrograph of an infected chick cecum. (A) Epithelial cell death and sloughing, with multi *E. coli* 0157:H7 intimately attached (arrows), 28 days post challenging, Bar, 1.6 µm. (B) Macrophage cell (MQ) engulfing the bacteria (arrow), 28 days post challenging, The luminal superficial epithelial cells were irregular in shape, with typical intimate-adherence pattern of transverse section of *E. coli* 0157:H7 bacteria (arrowhead). Bar, 1.6 µm. (C). *E. coli* 0157:H7 infected cecum 28 days post challenging showing; Enterocyte without microvilli (star), irreversible nuclear injury of enterocyte (arrow) and transverse section in *E. coli* 0157:H7 (arrow heads), Bar, 1.4 µm.

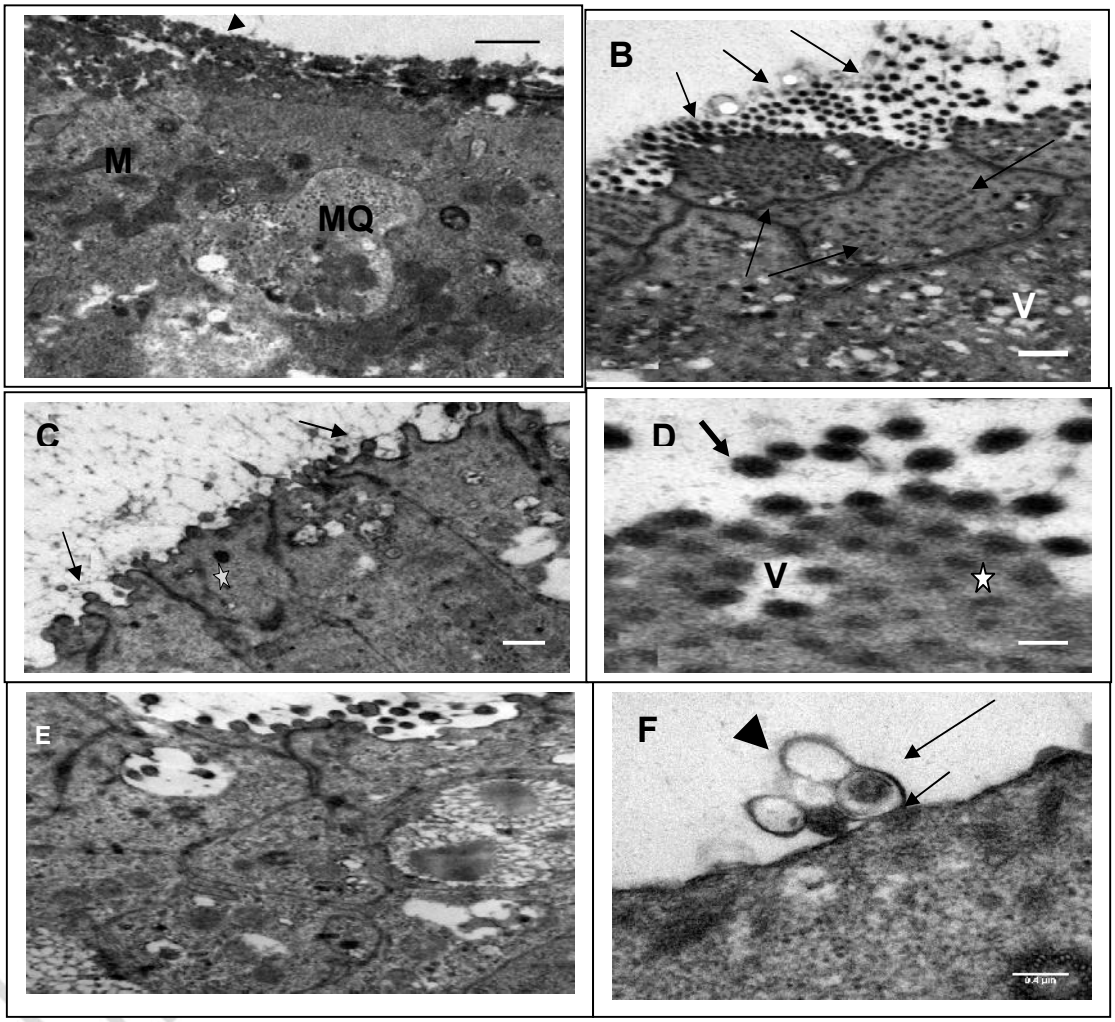

Figure 6: Transmission electron microscopy micrograph of an infected chick cecum. (A) Dense surface epithelial cells of the cecum of the infected chicken 28 days post challenging showing loss of normal microvillus pattern and adherent bacteria (arrow head) forming attaching-effacing lesions with accumulation of actin at the site of attachment at the intercellular junction and irregularly shaped mitochondria (M). Macrophage (MQ) is also identified. A concentration of electron-dense material was seen beneath some adherent organisms. Bar, 1.6 µm. (B) Irregular shaped epithelial cell with multi vacuoles (V) infiltrated with multi *E. coli* 0157:H7 bacteria (arrows) 21 days post-challenging, Bar, 0.7 µm. (C) Luminal superficial epithelial cells irregular in shape, with loss of microvilli, frequently showing erosion 28 days post challenging with typical intimate-adherence pattern of transverse section of *E. coli* 0157:H7 bacteria (arrow).Also seen bacteria penetrated the cells (star). Normal tight junctions are demarcated in the control micrographs, while electron dense aggregations (TJ) observed in the infected ones. Bar, 1.6 µm (D) A damaged region of epithelial cells 28 days post

challenging; (short arrow) indicate a transverse section of *E. coli* 0157:H7 bacteria infiltrating the cell. There is a dense coat of irregular appendages that extend between the infiltrating bacteria **(**star**).** Large cytoplasmic vesicles and vacuoles is observed (V). Bar, 0.7 µm. (E) Many bacteria(arrow) intimately attached to irregular in shape luminal superficial epithelial cells with multi protrusion detachments in more damaged regions with large cytoplasmic vesicles and vacuoles (V) loaded with cellular debris and occasional bacteria within cytoplasmic vacuoles at the upper edge of the enterocyte beneath the attached bacteria 28 days post challenging. Bar, 0.7 µm. (F). Transverse section of *E. coli* 0157:H7 bacteria (arrow) intimately attached to epithelial cells (short arrow) with progressive atrophy and the associated cytoskeletons were disrupted., degenerated, and necrotic, frequently showing erosion 28 days post challenging, (arrowhead) probably indicate *E. coli* 0157:H7 vesicle, Bar, 0.4 µm.

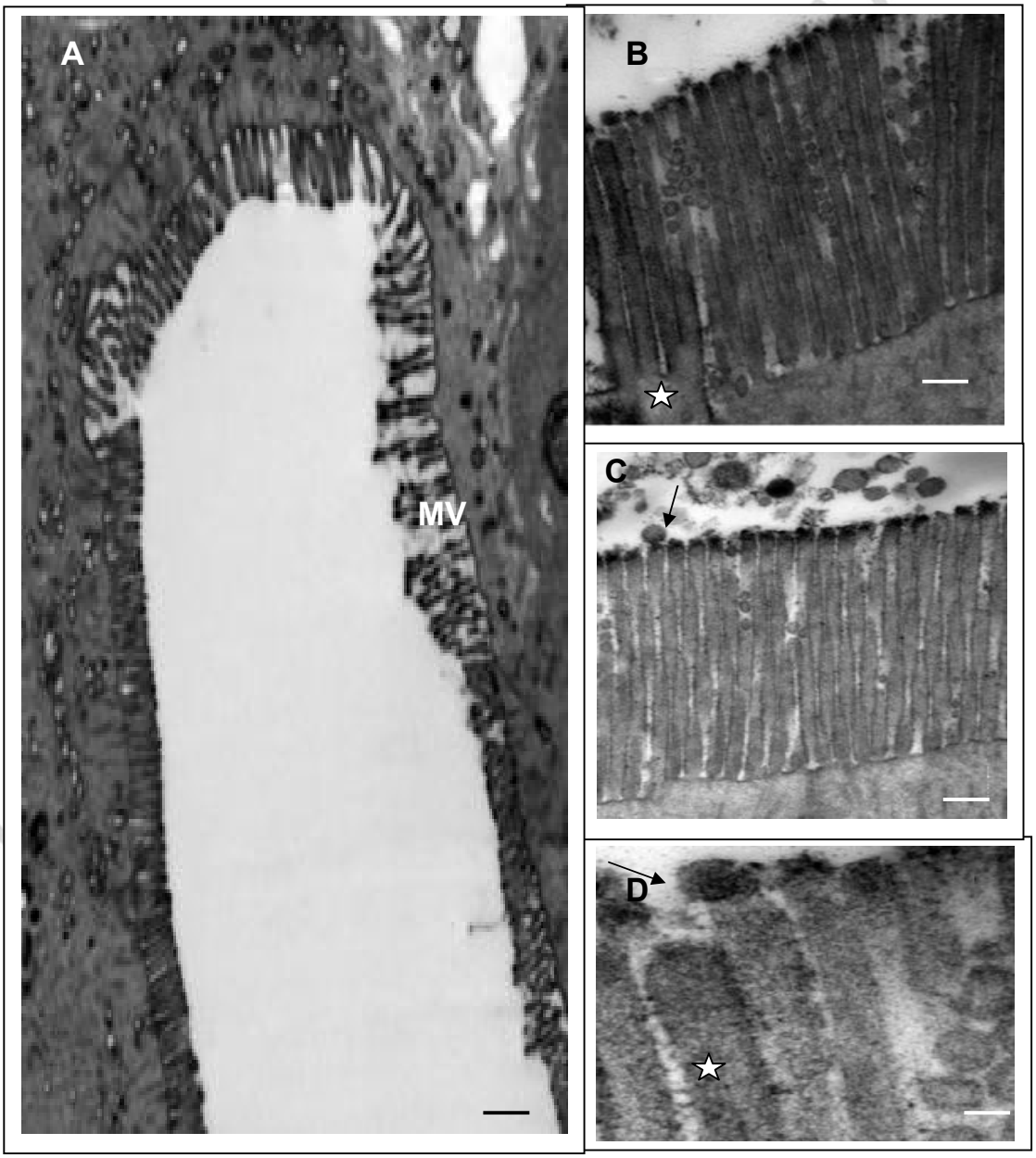

Figure 7. Transmission electron microscopy micrograph of an infected (Group2, and Group3) chick cecum (A) shows Group2 treated chickens cecum lumen enterocytes mostly seen with typical ultrastructural characteristics, however, they become taller in size with some minor alterations of disoriented or elongated microvilli (MV) in compare to control one with 21 days post-challenging. Bar, 1.4 µm. (Figure 7B) shows; Group2 treated chickens cecum with the length of villi in the treated birds seemed slightly longer and bulged (star) at some parts than ones in the yeast-free fed birds 21 days post-challenging. Bar, 0.3 µm. (Figure 7C) shows; Group3 treated chickens cecum with a typical intimate-adherence pattern of *E. coli* 0157:H7 bacteria (arrow) with the apparent lengthening of microvilli by day 21 of challenging Bar, 0.3 µm. (Figure 7D) shows higher magnification of Group3 treated chickens cecum with bulged villi (star), and intimately adherent *E. coli* 0157:H7 bacteria (arrow) 21 days post-challenging. Bar, 1.6 µm. There were no indication of epithelial effacement or bacterial penetration in group2 and 3 infected chickens.

***E. coli* 0157:H7 distribution in the intestine**. G1 CFU Count of the post-challenged chickens with *E. coli* 0157:H7 was the highest in the cecae, where $>10^7$ CFU / g were present (Table 1). (Table 1 and Fig. 8) Show that the number of CFU ranged from 10^5 to 10^7 per g. The mean ± standard deviation of the $\log_{10}$ CFU ranged from5.45 *to* 7.93 for the cecae in group1 treatment. *E. coli* 0157:H7 was present in smaller numbers (<1.0 to >3 $\log_{10}$/g than in cecae), in the colon. Fewer *E. coli* 0157:H7 bacteria were present in the cecae of chickens 21 days post-challenging than in chickens assayed 10,14 and at 28 days post-challenging; whereas, less bacteria were present in colon 28 days post-challenging than at 10 to 21 days post-challenging (TABLE 1). *E. coli* 0157:H7 was not detected in the heart, liver, kidneys, spleen, or gizzard of chickens 10 to 28 days post-challenging, nor in any organs of the control animals. (Table 2) show that around (>500 CFU/g) *E. coli* 0157:H7 were present in different parts of the small intestine. G2 CFU Count of the post-challenged chickens with *E. coli* 0157:H7 was the highest in the cecae (Table 2 and Fig. 8). The mean ± standard deviation of the $\log_{10}$ CFU ranged from 1.36 to 1.98 for the cecae in group2 treatment. However, the mean ± standard deviation of the $\log_{10}$ CFU ranged from 1.05 to 1.49 for the colon in group2 treatment. G3 on the other hand showed drastic diminishing in the mean ± standard deviation of the $\log_{10}$ CFU count and there was effect yeast on bacterial total count which was sharply reduced when supplemented yeast level increased with the most reduction was recorded for the birds fed 1.0% yeast (Table 3 and Fig. 8). Figure 9 shows the dietary yeast effect on distribution of *E. coli* 0157:H7 in broilers ceacae. All data points are average count ± standard deviation SD values from 3 groups 60/treat with three replicates of 20 chicks per replicate. ($P \leq 0.05$). G1= 0.00% baker yeast and peroral challenged with *E. coli* 0157:H7. G2= 0.75% baker yeast and peroral challenged with *E. coli* 0157:H7. G3= 1.00% baker yeast and peroral challenged with *E. coli* 0157:H7. There was a dramatic decrease effect of dietary

yeast intake on the attachment of the *E. coli* 0157:H7. The clear effect was at 1.0% baker yeast intake.

The ANOVA test clearly showed a significant effect of baker's yeast on *E. coli* O157:H7 attachment.. Overall, it was found that there existed a clear negative relationship between baker's yeast and *E. coli* O157:H7 attachment. A least square linear regression analysis was run utilizing data from Tables 2 and 3 which showed a significant negative relationship between the baker's yeast on *E. coli* O157:H7 attachment ($R^2 = 0.16$). Although the relationship was very poor, yet the data showed that the baker's yeast affects negatively the *E. coli* O157:H7 attachment.

TABLE 1. Distribution of E. coli 0157:H7 in chicken organs after peroral inoculation in G1 positive control; chicks fed control diet containing 0.00% baker yeast and challenged with *E. coli* 0157:H7.

Organ	Log_{10} *E. coli* 0157:H7/g of contents at post-challenging times[1]			
	10 days	14 days	21 days	28 days
Cecum	7.93 ± 0. 20	7.26 ± 1.65	5.45 ± 1.94	7.39 ± 0.42
Colon	5.60 ± 1.62	5.97 ± 2.33	4.59 ± 1.43	4.20 ± 2.58
Small intestine; Proximal segment	3.66 ± 0.70	2.56 ± 1.63	2.4 ± 0.23	2.40 ± 1.7
Small intestine; intermediate segment	4.99 ± 0.77	1.8 ± 1.53	2.0 ± 1.36	2.20 ± 1.6
Small intestine; Distal segment	4.14 ± 0.44	2.76 ± 1.86	1.9 ± 1.40	3.8 ± 1.75

[1] Each value represents the Mean CFU count ± standard deviation.

TABLE 2. Distribution of E. coli 0157:H7 in chicken organs in G2 chicks fed control diet containing 0.75% baker yeast and peroral challenged with *E. coli* 0157:H7.

Organ	Log_{10} *E. coli* 0157:H7/g of contents at post-challenging times[1]			
	10 days	14 days	21 days	28 days
Cecum	1.98± 1.50	1.81± 0.08	1.36± 0.05	1.85± 0.07

Colon	1.4± 0.05	1.49± 0.04	1.15± 0.04	1.05± 0.06
Small intestine; Proximal segment	<1.0	<1.0	<1.0	<1.0
Small intestine; intermediate segment	1.25± 0.03	<1.0	<1.0	<1.0
Small intestine; Distal segment	1.03± 0.05	<1.0	<1.0	<1.0

[1] Each value represents the Mean CFU count ± standard deviation

TABLE 3. Distribution of E. coli 0157:H7 in chicken organs in G3 chicks fed control diet containing 1.00% baker yeast and peroral challenged with *E. coli* 0157:H7.

Organ	Log_{10} *E. coli* 0157:H7/g of contents at post-challenging times[α]			
	10 days	14 days	21 days	28 days
Cecum	<1.0	<1.0	<1.0	<1.0
Colon	<1.0	<1.0	<1.0	<1.0
Small intestine; Proximal segment	<1.0	<1.0	<1.0	<1.0
Small intestine; intermediate segment	<1.0	<1.0	<1.0	<1.0
Small intestine; Distal segment	<1.0	<1.0	<1.0	<1.0

[1] Each value represents the Mean CFU count ± standard deviation

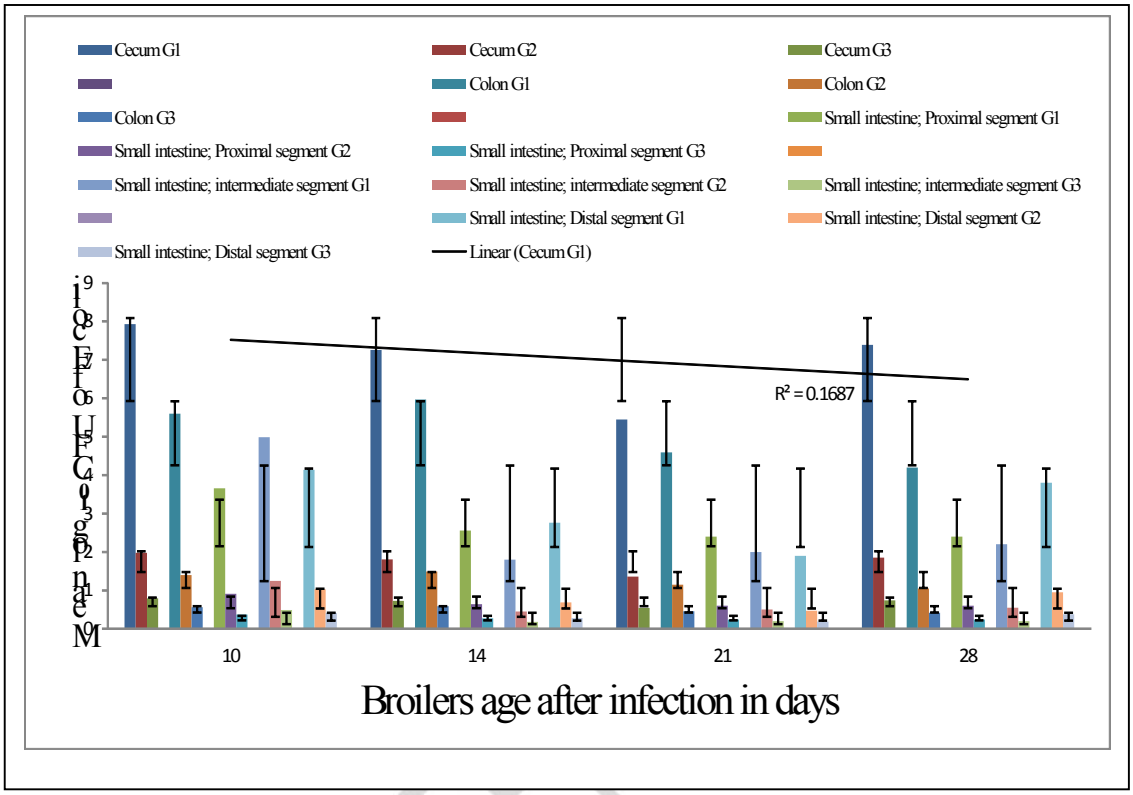

Figure 8. Dietary yeast effect on distribution of *E. coli* 0157:H7 in broilers intestine organs. All data points are average count ± standard deviation SD values from 3 groups 60/treat with three replicates of 20 chicks per replicate. ($P \leq 0.05$). G1= 0.00% baker yeast and peroral challenged with *E. coli* 0157:H7. G2= 0.75% baker yeast and peroral challenged with *E. coli* 0157:H7. G3= 1.00% baker yeast and peroral challenged with *E. coli* 0157:H7.

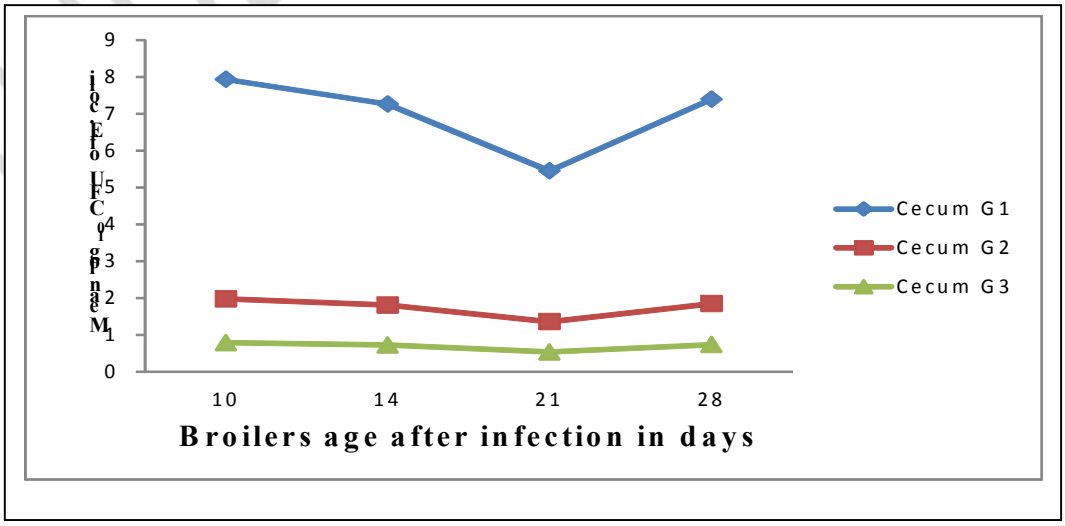

Figure 9. Dietary yeast effect on distribution of *E. coli* 0157:H7 in broilers ceacae. All data points are average count ± standard deviation SD values from 3 groups 60/treat with three replicates of 20 chicks per replicate. ($P \leq 0.05$). G1= 0.00% baker yeast and peroral challenged with *E. coli* 0157:H7. G2= 0.75% baker yeast and peroral challenged with *E. coli* 0157:H7. G3= 1.00% baker yeast and peroral challenged with *E. coli* 0157:H7.

DISCUSSION:

Bacterial enumeration, and the ultrastructure study, showed the ability of *Escherichia coli* ATCC 43889 (0157:H7) to specifically colonize chicken cecae for up to 28 days post-challenging after a peroral challenge at 7 days of age. With the exception of cecal damage and colonization, no other organ systems or portions of the gastrointestinal tract were affected by the bacteria. The aggregative adherence of bacteria to the cecal apical membrane, associated multifocal attaching and effacing lesions with a loss of microvilli and the rounding of the apical membrane suggests that this tissue may have specific receptor sites that allow attachment to take place. However; our study showed that A/E lesions and penetration of the cecal surface epithelium appeared at 21 days post-challenging. In earlier studies, adherent bacteria have been reported from 18 h post- infection in a calf model (Dean-Nystrom EA, et al., 1997) and up to 90 day post infection in a chick model (Schroeder, et al., 2004). In the present study, the intimate attaching and effacing (A/E) lesions found in the cecum of infected chicks is in agreement with (Nagano et al., 2003). Effacement was characterized by the thinning and ultimate disappearance of the epithelial cell striated border and the appearance of epithelial cell cytoplasmic vacuoles. Although *E. coli* 0157:H7 has not been isolated from chickens, this organism is able to colonize well in chickens, caeca being the primary site of colonization , this is in agreement with (Best, et al., 2005). Our study showed that some cell membranes developed an invagination, a pedestal- like protrusion at the AEEC attachment sites along the apical epithelial cell membranes (Fig.4 E). This is in agreement with (Tzipori et al 1989). The present study showed severe surface epithelium cell damages, Vacuolation of the cecum, disruption of cell membrane, degenerative swelling of endoplasmic reticulum and mitochondria, and irreversible nuclear injury (Fig.5 C). Widened intracellular spaces between surface epithelial cells may indicate active fluid secretion by these cells and the undergoing necrosis and vacillations probably due, at least in part, to the action of Shiga-like toxin produced by the bacteria (Xin-He et al., 1991). Our findings of severe epithelial damage are in agreement with light microscopic evidence of surface epithelial necrosis described in earlier animal models (Kelly et al., 1990). We showed presence of bacterial fimbriae (Fig.3 F). This in agreement with (Taylor et al., 1989) who

showed that EHEC strains possess a 60-MDa plasmid which encodes fimbriae that mediate bacterial attachment to cultured INT407 cells and may be involved in virulence. However; (Salanitro et al., 1978) reported no filaments connecting bacteria to the epithelium or microbial penetration of the mucosa were seen in SEM or TEM of tissue sections from the duodenum, ileum, or cecum. Ultrastructure features of the A/E lesion have been described before (Dean-Nystrom et al., 1997) and could account for the loss of normal absorptive function. We showed that some parts of the ceacum in chicks were seem to be sparsely inhabited with bacteria. A possible explanation is that the bacteria were associated with mucin layers which were easily removed from the epithelial surface when tissue sections are processed for electron microscopy. The probiotic treatment implemented in this project did have a significant inhibitory effect on the investigated *E. coli* bacteria in the chicken's intestine. These findings are in agreement with those of (Wakwak et al., 2003; Kabir et al.,2005; Nava et al., 2005; Li et al., 2008), they stated that the counts of *E.coli* bacteria were lower due to adding baker's yeast into broiler chicks' diets. And *S. cervisiae* supplementation of broilers to the level of 1, 1.5 and 2%, were significantly beneficial (Shareef and AL-Dabbagh, 2009). The morphology examination showed that probiotics had beneficial effect on cecal morphology causing an increase in villus height and villus surface area compared to control and group1 treatment. This is in agreement with Santin et al., 2001; Laagered and Bauer 2004. It has been reported that several harmful pathogenic bacteria exhibit a binding specific for the sugar mannose of the yeast's cell wall that thought to block the attachment of pathogenic bacteria to the animal's intestine and colonization and that the adherence of *bacteria* to enterocytes of the small intestine of chicks, in vitro, was inhibited in the presence of yeast's mannose (Laegreid and Bauer, 2004). Later, they found that inclusion of mannose in the drinking water of chicks reduced bacterial colonization of the cecum and that because yeast has been demonstrated not to permanently colonize animals, the yeast and any yeast-bound pathogens pass out in the bird excretion and bacterial colonization is diminished. On the other hand there are research studies reporting the lack of effect of probiotics on broiler performance (Maiolino et al., 1992). The chicks used in this study appeared to be very susceptible to infection after inoculation with *E. coli* O157:H7; Chicks receiving only one oral dose of these strains became infected and the cecum enterocytes respond to AEEC, by allowing intimate attachment and forming pedestal-like protrusions. This suggests that; chickens may serve as hosts and possibly as reservoirs for *E. coli* 0157:H7. The failure of this strain to cause diarrhea may have been related to the extent of bacterial inoculations; More

than one oral inoculations or suitable conditions of experimental infection may be necessary for these AEEC organisms to induce diarrhea in a young chick. Obtained results confirmed the fact reported by many researchers that the gastrointestinal tract can adapt and react morphologically to external factors related to dietary changes, i.e. addition of probiotics (Van der Klis and Van der Voorst, 1993). These changes were represented by elongated villi and a higher villi/crypt ratio, which indicate a lower rate of enterocyte-cell migration from the crypt to the villus. It was reported that the intestine can change its surface by growing to length, and/or by increasing or decreasing the height of its villi (Uščebrka et al., 2005; Žikiæ et al., 2008). It can be concluded from the present study that adding lower level of probiotic yeast supplementation did show a tendency to inhibit pathogenic Enterohemorrhagic *E. coli* O157:H7- binding and internalization of adherent bacterial effacement when compared to the un-supplemented control chickens. This is in agreement with (Kabir et al., 2004) who reported that probiotics, once established in the gut, may produce substances with bactericidal or bacteriostatic properties (bacteriocins) such as lactoferrin, lysozyme, hydrogen peroxide as well as several organic acids. Studies are in progress to determine probiotic yeast effect on layers and to determine the most effective source of probiotic and its dose.

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