

**THE EFFECIENCY OF ENTERIC *LACTOBACILLUS* IN PREVENTING
HEMORRHAGIC COLITIS AND BLOCKING SHIGA TOXINS
PRODUCTIONS IN RATS MODELS INFECTED WITH
ENTEROHEMORRHAGIC *ESCHERICHIA COLI* (EHEC)**

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ABSTRACT

The objective of this study was to investigate the prophylactic roles of human enteric derived *Lactobacillus plantarum* L1 (L1) and *Lactobacillus paracasei* L2 (L2), on EHEC O157:H7 infection in rodent models (*In vivo*). The *Lactobacillus* suspensions (L1 and L2) were individually and orally administered to experimental rats at a daily two consecutives of 100 µl (10⁸ CFU/ ml/rat) for up to two weeks. Thereafter, on the 8th day of experiment rats were orally challenged with one dose infection of EHEC (10⁵ CFU/ml/rat). Animals mortality and illness symptoms have been monitored. There was no fatal EHEC infection in rats that had been pre-colonized with the *Lactobacillus* strains, while most of EHEC infected rats were died (90%). The Stx1 and Stx2 levels were significantly lower (14 and 12 folds) in the L1 and L2 pre-inoculated rates respectively, compared with those in the EHEC colonized group. Histological sections were proven the prophylactic roles of L1 and L2, whereas, no effective histological upsets were detected in *Lactobacillus* + EHEC- colonized rats. The cytopathic symptoms were predominant in kidney and intestinal sections of EHEC infected rats. The kidney sections cytopathy manifested to lining membrane ulceration, infiltration of mononuclear cells and glomerular and tubular epithelium necrosis. The striking attaching and effacing (A/E) lesions were prominent in intestinal sections of EHEC infected animal models.

Key words: Diarrhegenic *E. coli*, hemolytic uremic syndrome (HUS), intestinal microbiome, probiotic *Lactobacillus*.

الوندائي والركابي

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التناول المسبق لعصيات الملبنة المعوية تقي من الإصابة بالتهاب القولون النزفي وتفيد انتاج سموم الشيجا في جردان
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المستخلص

الهدف من الدراسة الحالية هو التحري عن الدور الوقائي لكل من الملبنة المعوية (*Lactobacillus plantarum* L1 (L1) و *Lactobacillus paracasei* L2 (L2) ضد الاصابة ببكتريا القولون النزفي في الجردان (داخل الجسم الحي). تم تطعيم جردان التجارب فمويًا بعالقي كل من بكتريا L1 و L2 بعدد خلايا 10⁸ خلية / مل / جرد و بشكل منفرد ولمرتين يوميًا ولفترة أسبوعين و بالتزامن و ابتداء من اليوم الثامن للتجربة تم تطعيم الجردان بجرعة فموية واحدة من عالقي بكتريا EHEC و بعدد خلايا 10⁵ / مل/ جرد و تم مراقبة الاعراض المرضية الظاهرة خلال فترة التجربة مع تثبيت نسب نفوق الحيوانات. لم تظهر إصابات مرضية شديدة في الحيوانات المطعمة مسبقًا ببكتريا *Lactobacillus* بينما نفقت معظم الحيوانات (بنسبة 90%) الملقحة ببكتريا EHEC و انخفضت مستويات سموم الشيجا Stx1 و Stx2 بشكل ملحوظ و بمعدل 12 و 14 مرة في خروج الحيوانات المعاملة مسبقًا ببكتريا L1 و L2 على التوالي عند المقارنة مع الحيوانات الملقحة ببكتريا EHEC و بينت فحوصات المقاطع النسيجية للكلى والأمعاء التأثيرات الوقائية لتطعيم الجردان مسبقًا ببكتريا *Lactobacillus* حيث لم يلاحظ وجود اعتلالات مرضية نسيجية واضحة في حين كانت التغييرات النسيجية واضحة في المقاطع النسيجية للحيوانات الملقحة ببكتريا EHEC و قد تجلى الاعتلال الكلوي في تفرح النسيج المبطن للكلى وترشح خلايا الدم الوحيدة النواة مع تخر البطانة الطلائية للكبيبات و النبيبات الكلوية و السمة المرضية المميزة لالتهاب القولون النزفي و المتمثلة بطمس و اندماج الزغابات المعوية (A/E) لوحظت في المقاطع النسيجية لأمعاء الحيوانات المعاملة ببكتريا EHEC.

الكلمات المفتاحية: بكتريا القولون المسببة للإسهال، متلازمة انحلال الدم اليوريمي، الفلورا المعوية الطبيعية، المعززات الحيوية لعصيات الملبنة،

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INTRODUCTION

The enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 or shiga toxin producing *E. coli* (STEC) is food-borne and zoonotic pathogen, belonging to diarrheagenic *E. coli*. Ruminants, particularly cattle, are considered the primary reservoir of EHEC and the main source of human infection (19). EHEC is one of the most world's common intestinal pathogen, and its illnesses are typically severe infections, manifestation ranging from diarrhea and hemorrhagic colitis (HC) to the possibly fatal hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) (20). Shiga-like toxins (Stxs) are the key virulent pathogenic factors in EHEC infections, and closely related to the pathogenicity of charring strains. Once EHEC enter host body start Stxs production, through type III secretion system (28). The genes encoding Stxs are located within temperate lysogenic phages integrated to the host bacterial genome (16). Stxs are subdivided into two major classes Stx1 and Stx2, each one contains several subclasses. EHEC strains may secrete one or more Stxs subclasses, strains of serotype O157:H7 the most common etiological agent of HUS, often producing both of Stx1 and Stx2. Stx2 has been found to be associated more frequently with severe illness (17). Stxs is an AB₅ toxin family that selectively binds through its B subunit to the globotriaosylceramide (Gb3), which is a Stxs receptor expressed in the enterocyte's epithelium and on the surface of endothelial cells (12). Stxs binding mediates the distraction of electrolytes channels in the intestinal epithelial membrane, resulting massive loss of ions and water, thus inhibiting protein biosynthesis and inducing cell apoptosis, leading to organ damage (22,31). In addition to presentation of Stxs, the fully virulent phenotypic strains of EHEC include intimin as an additional virulence factors that involved for attaching and effacing (A/E) lesion on host intestinal microvilli (15). Antimicrobial agents are not recommended for treatment of EHEC infections as they are known to stimulate bacterial cell lysis and the release of lysogenic toxins, and thus raising the risk of HUS (10). The management of an EHEC infection depends mainly on the

supportive care and hydration. Various therapeutic alternative approaches are available, one of novel and promising treatment practices is uses of probiotics for management of EHEC infections (9). Probiotics are live beneficial microorganisms that provide the host with a health benefit when ingested in adequate amounts (8). Many probiotic strains are members of the intestinal microbiota, some of which have been increasingly incorporated into foods and supplements to preserve gut balance and strength the functions of gastrointestinal tract (5). The association between specific intestinal microbiota, and EHEC infection has been explored in several *in vitro* and *in vivo*. Cascade of pathways were attributed to the probiotic's protective strategies and their interactions to EHEC. Probiotics probably are likely to be able to reorganize host-infectious agent/toxin interactions, making these interactions unfavorable (27). Probiotic bind and occupy receptors expressed by enterocytes, and create decoy receptors that have trapped toxins, or are capable of modifying the intestinal microenvironment, make such interactions unfavorable (13). *Lactobacillus* members represents the predominant Firmicutes groups of gastrointestinal microbiotas, strains belonging *Lactobacilli* have established safety record and have been given generally recognized as safe (GRAS) status by the United States Food and Drug Administration (14). Strains of enteric *Lactobacillus* are highly relevant for the protection of tissue invasion by enteropathogenesis, by utilizing different defensive mechanisms of host colonization by pathogens, and modulating host immune response (11). The extent of lactobacilli protective mechanisms is strain-dependent. For instance, the production of organic acids by the *L. delbrueckii*UFV-H2b20 has been shown to be an important antagonistic factor, and the production of short chain fatty acids, like butyrate may be significant as well (16). An *in vitro* research showed that co-cultivation of EHEC with assorted probiotics culture include, *Bifidobacterium*, *Pediococcus*, and *Lactobacillus* strains results in reduction of Stx2 production. This behavior attributed to pH dropping due to the organic acids released

by these agents (7). The objectives of the present study were, the *in vivo* assessment the prophylactic mechanisms of two potentially probiotic strains of enteric Lactobacilli, *L. plantarum* and *L. paracasei* isolated from infants' feces against Enterohemorrhagic *E. coli* (EHEC) in rodent model.

MATERIALS AND METHODS

Microorganism and growth conditions

Two potentially probiotic isolates of enteric *Lactobacillus* were used in the study, *L. plantarum* L1 (L1) and *L. paracasei* L2 (L2), both isolates were previously isolated from human infants' feces, and suggested to be promising probiotic isolates with regards bactericidal activity. *Lactobacillus* strains were recovered from glycerol stock cultures (30%) at -80°C on de-Man, Rogosa and Sharpe (MRS) agar medium (Himedia, India), under anaerobic conditions (anaerobic jar supplied with Gas Pak, BioMerieux, France) cultures were incubated at 37°C for 48h. Using of MRS agar, a few subculture steps were carried out to obtain pure cultures. The diagnosis of both L1 and L2 was confirmed by DNA sequencing of the 16S rRNA, GenBank BLAST analysis confirmed the identification of both strains with accession No. of HM101329.1 and MF423812.1 respectively (2). *E. coli* O157:H7 (EHEC) was refreshed from glycerol stock culture (20%) at brain heart infusion agar and diagnosis was

confirmed by culturing on sorbitol MacConkey agar (SMAC).

Animals: Six week- old male Albino rats with an average weight of 112.48±0.02 g was used in the trails, they were purchased from National Control Center for Drugs and Since Researches (NCCDR) / Iraq. Rats were kept in plastic cages (2 animals/cage), and maintained on a 12:12 light: dark cycle at regulated temperature 25 ± 2°C, the animals were given free access to food and water during the entire experiment duration.

Experimental design

The experimental animals, rats (n= 40) were assigned randomly to four different experimental groups (A, B, C, D), 10 rats/group (Table. 1). The group A animals considered as negative control group, were left uninoculated, and left on normal rodent chew diet for two weeks. Stable colonization of gastrointestinal tract (GIT) of group B animals was achieved by two consecutives oral doses of 100 µl (10⁸ CFU/ ml/ rat) of L1 suspension daily for two weeks. Same inoculation protocol was followed for colonization the GIT of group C animals with L2 suspension. A week later of *Lactobacillus* colonization (on the 8th day), animals in groups B, C, D were orally challenged with one dose infection of EHEC (10⁵ CFU/ ml/ rat). Throughout the study, the illness symptoms, survival rate (number of alive/total number of rats), and animal's weight was reported daily.

Table 1. Experimental animals groups design

Experimental groups	Group descriptive
Group A	Control group (not inoculated)
Group B	Orally L1 pre-inoculated + EHEC infected group
Group C	Orally L2 pre-inoculated + EHEC infected group
Group D	Orally EHEC challenged group

Detection of Stxs in rats' fecal samples

The Stxs titers were qualified using a sandwich enzyme-linked immunosorbent assay (ELISA Kit/ antibodies, R-Biopharm AG, Germany). In brief, after EHEC challenge the freshly collected samples of feces were suspended in PBS (1:1) at time intervals of 1, 3, 5 and 7 days, fecal suspensions were centrifuged (900 x g, 10 min, at 25°C), and the supernatant was used for Stxs levels detection.

Gastrointestinal tract colonization

Lactobacillus colonization of rats GIT, was tracked by viable cell count (CFU) in freshly collected fecal samples, at time intervals of 2, 4, 6, 8, 10, 12 and 14 days of the experiment, while, EHEC rat's GIT colonization was tracked at time intervals of 1, 3, 5 and 7 days following the pathogen challenge. Fecal samples were serially diluted (10-fold) and 100 µl aliquot inoculated on MRS agar for *Lactobacillus* counts and SMAC agar for EHEC counts, plates incubated for 48 h at

37°C (MRS plates were incubated anaerobically and SMAC plates under aerobic conditions). The identification of feces recovered *Lactobacillus* were confirmed using API 50 (BioMerieux, France), and CFU was expressed as (\log_{10} CFU/ gm feces). All the counting was done triplicates.

Histopathological analysis

Selected survival animals from each experimental group were killed and dissected, thereafter kidneys and intestines were removed, the obtained organs kept in 10% formaldehyde at room temperature (~25°C) overnight. Tissues were stained with hematoxylin and eosin, and sections examined under compound microscopes.

Ethics approval and agree to participate

The experimental protocol was carried out in compliance with Dept. Biology / College of science / Baghdad University, research ethics committee (approval No. BEC/0620/0028).

Statistical analysis

The Statistical Analysis System- SAS, program was used to detect the effect of difference factors in study parameters. Least significant difference –LSD test was used to significant compare between means in this study.

RESULTS AND DISCUSSION

In vivo prophylactic strategies of *Lactobacillus* pre-feeding against EHEC infection, was analyzed in rodent model. Animals weight were tracked throughout the study as general wellbeing index. Non-statistically significant difference ($P>0.05$) was observed between the weight gain of animals in B and C groups, in contrast, the rat's weight in group D decreased significantly ($P<0.05$) compared to other experimental groups, rats infected with EHEC lost >15% of their initial weight by the day 8 of infection (Table .2). The survival rate (number of alive/ total number of rats) was reported daily, no deaths occurred in groups A animals, one mortality was recorded during inoculation sessions in group B and two in group C animals. The survival rate was drastically declined to 10 % in group D, nine of animals in the group died at the end of trail. The group D animals (EHEC challenged group) were associated with the main identified symptoms of illness. It was limited to the severe diarrhea, lethargy, hair loss, and

movement anxiety disorder. In group B and C animals, the aforementioned clinical symptoms were almost invisible, except the lethargic and inactivity of few animals. It was clear the pre- inoculation of *Lactobacillus* L1 and L2 were able to confer defense against infection with EPEC, as demonstrated by the absence of visible disease symptoms and high survival rate, in groups B and C animals relative to animals of group D, and may indicated that the involvement of potentially probiotic *Lactobacillus* could colonize the rats digestive tract with good intestinal lining furnishing and consequently started to exert antagonistic behavior, that reduced the incidence of EHEC infection, the mortality rate and improved the general health scores (11). Antimicrobial activity against various pathogens is an essential attribute of probiotic members of *Lactobacillus* for preserving a healthy microbial balance in the GIT. The protective mechanisms of *Lactobacillus* are strain specific and considered to be multifactorial mechanisms assign to the production of different metabolites such as organic acids, hydrogen peroxide, ethanol, acetaldehyde, acetoin, carbon dioxide, and bacteriocins. In addition, the competitive exclusion, immune modulation, stimulation of host defenses and the development of signaling molecules that may trigger changes in expression of genes (16,18).

Table 2. Experimental rats body weight (g)

groups	Initial wt.(g)	Final wt.(g)
A	112.5	292.0
B	111.19	287.31
C	111.68	284.1
D	112.04	93.86
LSD	24.094	41.772

Detection of Stxs in rats fecal samples

In the *Lactobacillus* pre-colonized rats' groups, the levels of Stx1 and Stx2 were significantly suppressed compared with those in the EHEC-challenged group (group D), furthermore, no major differences were observed in Stx1 and Stx2 levels between the L1 and L2 pre-colonized groups. By contrast, the Stx1 and Stx2 levels have been noticeably rose from third day and up in the feces of group D animals. The Stx2 exhibited >14-fold higher levels at day 7 compared with rates colonized by L1, and >12-fold higher

compared with rats colonized by L2. While, the level of Stx1 increased at lower rate, on day 7 it exhibited >9-fold higher compared

with rats colonized by L1 and >7-fold higher compared with rats colonized with L2 (Table 3).

Table 3. Detection the levels of Stxs (measured by ELISA) in the fecal samples of experimental rat's challenge with EHEC

Experimental groups	Stxs level (ng. ml ⁻¹) in rats' fecal samples							
	Stx1				Stx2			
	day1	day3	day5	day7	day1	day3	day5	day7
Control group (group A)	nd	nd	nd	nd	nd	nd	nd	nd
L1+EHEC colonized group (group B)	nd	12	10.6	11	nd	18.8	nd	21
L2+EHEC colonized group (group C)	nd	nd	8.4	10.2	nd	13	37	25
EHEC-colonized group (group D)	nd	86	122	153	nd	165	210	300
LSD	nd	5.85	11.64	16.52	nd	19.74	18.55	26.31

(n = 8-10 per group). Data are presented as mean ±SD. (P<0.05). (nd, not detected)

The high productivity of Stx2 may it reflects the pathogenicity of used EHEC strains and as it is also approved by high rates of mortality (90%) in group D animals. There is growing evidence in impact of Stx2-producing EHEC strains in the pathogenesis of HC and HUS (17). The obtained results from previous experiment in this study demonstrated that the co-inoculation of L1 and L2 with EHEC effectively decreased the lethality of EHEC infection. The mechanism by which the probiotics protected host animals from EHEC infection were not fully elucidated, may this be attributed even partially to the suppression of Stxs production. Shiga toxins are the main virulence factor of EHEC strains (16). Stxs produced by EHEC promotes apoptosis of intestinal epithelial lining (31). Inhibition of Stxs output and circulation in the body is the key role in prevention of lethal EHEC infection (23). Commensal bacteria may influence the concentration of Stxs in the gut by inhibiting the growth of EHEC. The researches have been shown that the pure cultures of assorted probiotic strains inhibit *Stx* transcription in laboratory media (21). In the streptomycin-treated mouse model of EHEC infection, human derived intestinal *Bifidobacterium* inhibited the production of mitomycin C-induce Stxs, without interfering with EHEC growth (3). The pH reduction due to acetate production was proposed to mediate the restrictive impact of *Lactobacillus* strains on Stxs biosynthesis (7). The molecular cross talk via or independently of the quorum sensing that controls the expression of Stxs

may be the underlining the molecular mechanism (29).

Gastrointestinal tract colonization

Two consecutive daily doses of 10⁸/CFU viable L1 and L2 were administrated to experimental animals in the groups B and C for weeks, in individual bases. The colonization of rats GIT was monitored by re-isolation of viable lactobacilli from animals' fecal samples intervals. The review of obtained data showed that the involved inoculation protocol was sufficient to establish proper GIT furnishing. The L1 and L2 was sufficiently achieved GIT colonization and maintained high population level, the account of L1 and L2 were almost doubled, increasing by almost two log cycles. With no substantial difference (P>0.05) in the number of viable cells between the two experimental groups B and C throughout the feces sampling sessions. Whereas, L1 and L2 were re-isolated from rats' feces at average levels 2.24-5.29 and 1.88-4.92 log₁₀ CFU/gm feces respectively (fig.1). The antibacterial effectiveness of *Lactobacillus* is highly dependent on its survival, persistence and proliferation in GIT. The attachment and adhesion capacity can be considered as a standard biomarker for the selection of potential probiotic strains, as proliferation will not advance without this requirement being met. The exclusion and displacement of pathogens, as well as the competition profile, depends on adhesion and proliferation (4). The probiotic *Lactobacillus* members inhibition of pathogen adhesion showed variability and indicated that it is simply a strain dependent

property. Studies have documented that probiotics compete with pathogens for the adhesion sites, since both probiotics and pathogens have common kind of adhesins on their surfaces (25). From the analysis of L1 and L2 feces shedding numbers we can assure that these isolates have met the condition of adhesion and good proliferation in rats GIT. Otherwise, the pathogen EHEC recovery from the feces of rats was substantially and gradually reduced in *Lactobacillus* + EHEC inoculated rats. Complete clearance level was not performed. The L2 pre-fed (group C) showed a significant decline in the viability of EHEC at the last experimental day (1.89 log₁₀ CFU/gm of faces) compared to viability recorded of group D animals. group B animals (L1 pre-fed) also had shed a lower count of EHEC (2.01 log₁₀ CFU/ gm of faces) in the fecal samples. In contrast the pathogen proliferated aggressively in rats infected with EHEC alone (group D), as the shedding number of EHEC in the feces samples was

increased significantly ($P < 0.05$), with a viable count rising by approximately three log cycle and reaching 6.12 log₁₀ CFU/gm feces at day 14 of experiment (fig.2). Studies suggested that in the early stage of EHEC infection the pathogen count is crucial factor influencing the severity of infection and enables adhesion to the epithelial cells that leading to establishment of disease (32). Several studies have reported that enteric *Lactobacillus* strains exclude pathogenic *E. coli* from GIT adhesion sites in different cell lines and animal models. The probiotic *Lactobacillus paracasei* was reported to inhibit the adhesion of enterotoxigenic *E. coli* K99 to the intestinal mucosa in gnotobiotic lambs (29). Oral pre-feeding of live *Lactobacillus. Delbrueckii* UFV-H2b20 has been shown to significantly protects against infection of *E. coli* O157:H7 in mice (19) Similarly, *Lactobacillus plantarum* promoted cell adhesion reduction of *E. coli* O157:H7 in murine model(4).

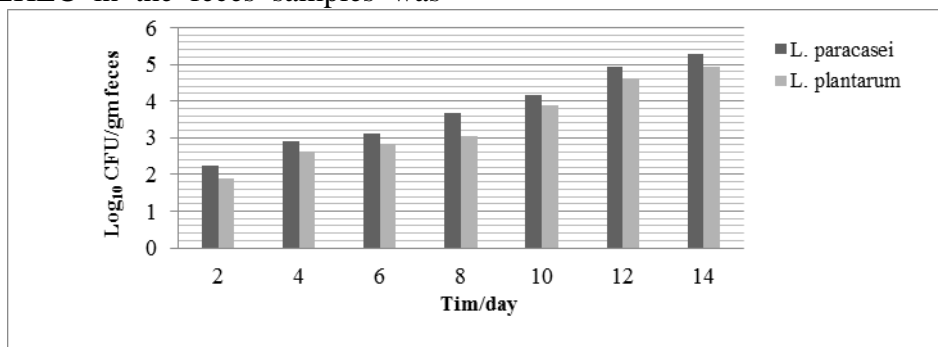


Figure 1. Viability (CFU/gm feces) of *L. plantarum* (L1) and *L. paracasei* (L2) in faces samples of rats in two evaluated groups B and C. (n = 8 -10 / group). Data are presented as mean \pm SD. ($P < 0.05$).

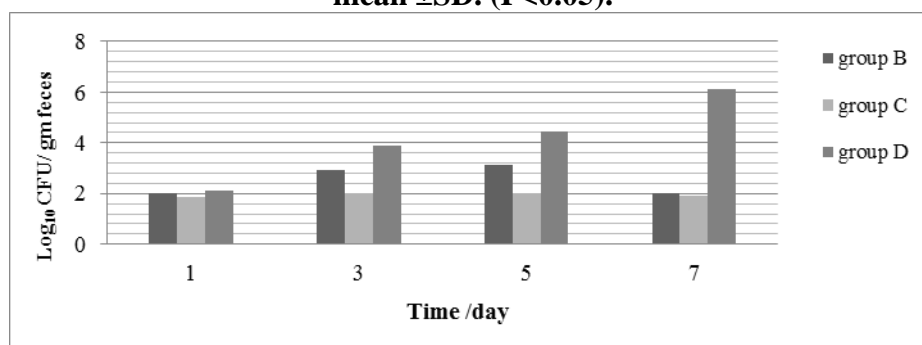


Figure 2. viability (CFU/gm) of EHEC (*E. coli* O157:H7) in fecal samples of experimental rats in three evaluated groups B, C, and D. (n = 1 – 10 / group). Data are presented as mean \pm SD, ($P < 0.05$).

Histopathological study

The protective effects of rat's pre-colonization by *Lactobacillus* isolates was declared in histological analysis of intestine and kidney sections. L1 and L2 Pre-feeding protected the

host animals from the development of pathogenic lesions and almost completely repressed the inflammatory phenomena caused by EHEC inoculation. The cytopathic signs have clearly been detected in the kidneys and

intestine of EPEC-colonized group (group D). No cytopathic lesions were detected in kidney section of group B animals (fig. 3 A), while, there was some neutrophils infiltration in kidney histological sections of group C (fig.3 B). The cytopathic signs were obviously observed in the kidneys of EPEC-colonized group (group D), histopathological lesions were indicated with endothelial cell swelling and hyperplasia, infiltration of mononuclear cells, necrosis of glomerular and renal tubules, and ulceration of lining membrane, with no evidence of glomerular thrombi (fig.3, C and D). The acute tubular necrosis without tubular thrombi was also observed in streptomycin-treated mice model involved for demonstration of EHEC pathogenesis (25). Not noticing of glomerular thrombi in the examined section indicated that the study used strain of EHEC did not mediated HUS (30), furthermore, the glomerular damage emphasized the cytopathic effects of Stx2 on capillary endothelial cells (23). The effacement of microvilli, depletion of gobletted cell and neutrophils infiltration, were the main cytopathic lesions that observed in the intestinal sections of group D animals. The characteristic attaching and effacing (A/

E) lesions also was clear in intestinal epithelia. All the mentioned cytopathic lesions were not detected in intestinal sections of group B, except some ulceration was detected in group C intestinal sections, this may be due to technical mistakes in preparation procedure and staining process (fig.4, A, B and C). The observation of A/E of intestinal microvilli phenomenon may confirm the adherence of EHEC to the enterocytes was mediated by intimin the outer-membrane adhesin proteins, that play a crucial role in EHEC infection initiation (1, 24).

Conclusion

The current study demonstrated that orally administration of human derived enteric *Lactobacillus* prevented EHEC infection in rats' model. It suggested the protective strategies may be associated with shiga toxin production suppression. Regarding to *L. plantarum* and *L. paracasei*, GIT colonization, the competitive exclusion also plays a role in protective mechanisms. Further studies are needed to explain the mechanism that underlies the protective function of *L. plantarum* and *L. paracasei* against EHEC infection.

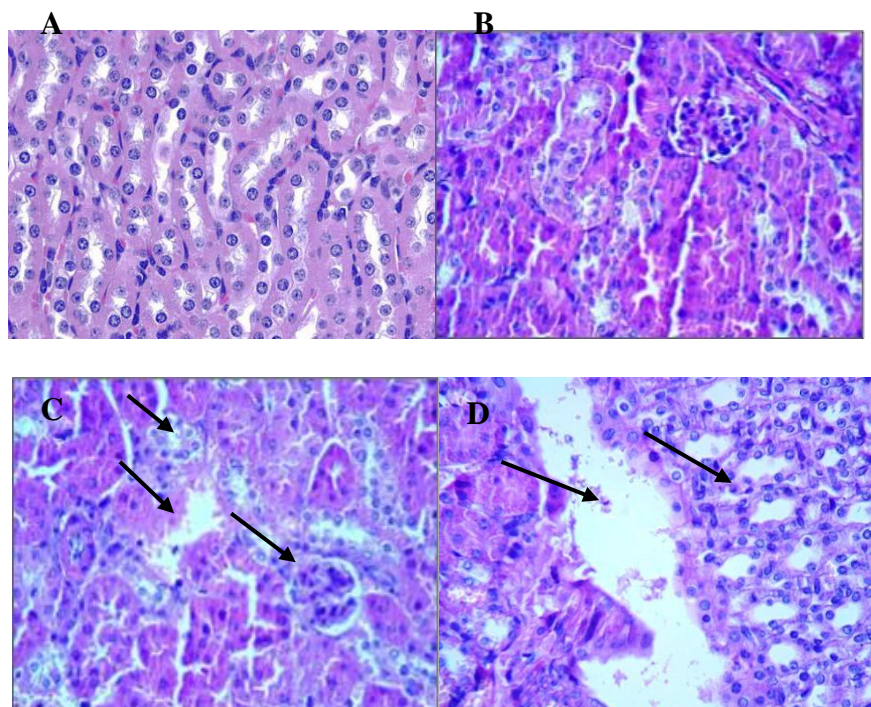


Figure 3. Histopathological analysis of rat's kidney sections, hematoxylin and eosin staining (40X). A, represents *L. plantarum* + EHEC colonized rat kidney. B, represents *L. paracasei* +EHEC colonized rat kidney. C and D, represents EHEC- mono-colonized rat kidney, showed clear cytopathic lesions pronounced by necrosis of glomerular and renal tubules, ulceration of lining membrane and infiltration of mononuclear cells

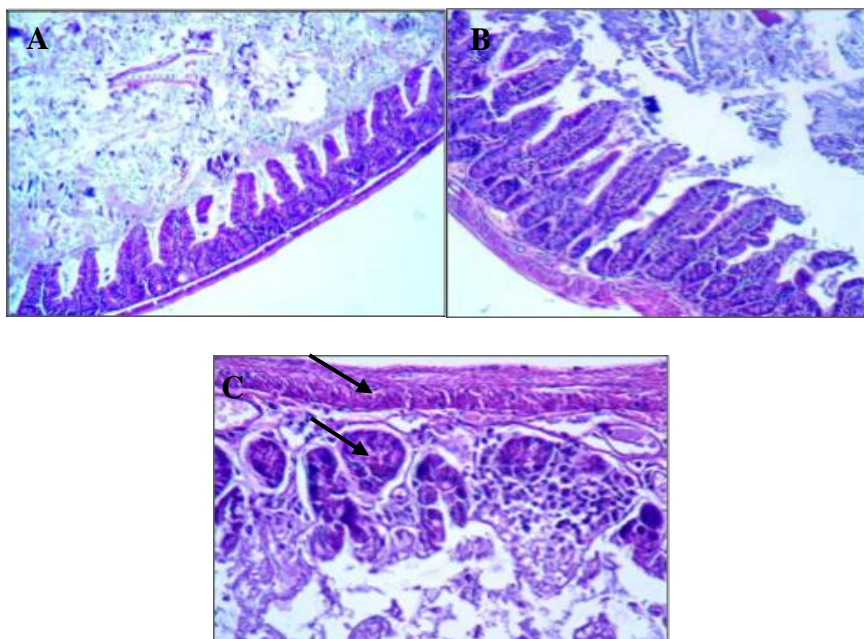


Figure 4. Histopathological analysis of rat's intestinal sections, hematoxylin and eosin staining (40X). A, represents *L. plantarum* + EHEC colonized rat intestine. B, represents *L. paracasei* + EHEC colonized rat intestine. C, represents EHEC- mono-colonized rat intestine, showed clear cytopathic lesions pronounced by effacement of microvilli, depletion of goblet cell and neutrophils infiltration

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