Effect of *Lactobacillus paracasei* (CNCM1-1572) Against *Escherichia coli* O157:H7 Isolated from Sheep

Ali Jamal Turkey Al-Saadi*, and Sahar Mahdi Hayyawi Al-Rubay
Department of Microbiology, College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq.

*Correspondence: mal452@gmail.com; Tel.: +964 780 023 2313

Abstract
This study was based on the importance of the effect of *L. paracasei* against *E. coli* O157:H7 that was isolated from sheep suffering from diarrhea in many areas of Baghdad (Abu-Ghraib, AL-Mahmoudia and AL-Yosifiya). All samples were cultivated on MacConkey agar, Eosine Methylene Blue and Sorbitol MacConkey agar for *E. coli* isolation and then identified by biochemical tests. Out of 101 diarrhea samples, 100 isolates gave positive *E. coli* results. The isolates of *L. paracasei* were taken and cultured on conditions at 37°C for 24 hours in Man Rogosa Sharpe broth and incubated under CO₂ (5-10%) for 24 hours, then recultured on MRS agar, examined by gram stain and then confirmed diagnosis by Vitek2. *Lactobacillus paracasei* was examined against *E. coli* O157:H7 by well diffusion method and measured the diameters of the inhibition zone around colonies. Mice (white Balb) were used as laboratory animal models to investigate the effect and efficacy of *L. paracasei* in treating diarrhea caused by *E. coli* O157; 50 mice were divided into five groups. The histopathological examination of the intestine noticed changes during infection with *E.coli* O157:H7 treated with probiotics.

Keywords: Vitek2; Laboratory technique; MacConkey agar; Histopathology; Iraq.

Introduction
*Escherichia coli* is a normal flora in most animals' and humans' intestines. *Escherichia coli* O157: H7 toxin-producing bacteria Shiga has become a significant food and waterborne pathogen that causes diarrhea, hemorrhagic colitis and hemolytic uremic syndrome (HUS) in humans. These pathogens bind themselves by surface-associated structure to the mucosal surface and then cause further damage through enterotoxins. Principal pathogenic factors of EHEC are intimate for adherence of bacteria and Shiga toxin-like bacteria (Stx). Probiotics are currently understood to be living microbes that, when given in adequate amounts, impart a benefit for the host's health. It consists of non-spore-forming, Gram-positive, microaerophilic, or facultatively anaerobic rods. Not only are lactobacilli widely employed in food fermentation processes, but they are also used to prevent and treat various intestinal diseases brought on by harmful bacteria. The immunomodulatory effects of probiotic strains limos lactobacillus have also reduced allergy, and necrotizing enterocolitis is two diseases where the inflammatory response occurs.

Materials and Methods
Samples collection
101 samples were collected from sheep infected with diarrhea of both sexes, located in three areas (Abu-Ghraib, Al-Mahmoudia and AL-Yosifiya) from October to December (2021).

Cultural characteristic
The naked eye examined the growing colonies on Blood agar, MacConkey agar, and Sorbitol MacConkey agar. They included the color, shape and size.

Preparation of L. paracasei isolates
It was prepared as 20 mg of L. paracasei powder (commercial) inoculated in the MRS broth by adding Tween 80 after 48 hr taken by a sterile loop from broth, cultured on the MRS agar to identify and conformation of the bacterium.

Susceptibility test of L. paracasei against E. coli
The experiment aimed to assess E. coli growth inhibition by lactic acid bacteria (LAB). This modified method has been obtained as described previously 10.
1. *Escherichia coli* were cultured in nutrient broth.
2. Each *Lactobacillus* strain (CNCM I-1572) was grown in MRS stock by adding Tween 80. CFS’s probiotic lactobacilli inhibitory properties have been studied through a well-diffusion method 11.
3. Transfer 0.1ml of the nutrient broth containing *E. coli* after incubation for 24 hr to the Muller Hinton medium, and using a cotton swab (sterile swab) spread the bacteria completely over the medium. After that, the dishes were removed from the incubator and pierced with a sterile cork borer into several holes (6mm), then drilled pieces were removed.
4. The holes agar was filled with 100 μl a quantity of *L. paracasei* extract, and then the dishes were transferred to the refrigerator, left for two hours, and incubated at 37ºC for 24 hours. After that, the diameter of the inhibition area was measured.

Vitek 2 compact
The VITEK2 system was utilized by the guidelines provided by the manufacturer, Bimerieux, and following Fritsche et al. 12 in the diagnosis of isolates. A total of 1 ml of the isolates was put into sterile test pieces with three milliliters of ordinary saline solution. The turbidity meter (DensiChek TM) included with the VITEK 2 was used to measure the turbidity of the bacterial suspension in each tube to ensure that it was equivalent to McFarland tube number 0.5, which equals 1. 5×10^8 CFU/ml.

Bacterial count
One selected isolate from *E.coli* O157:H7 was diagnosed by molecular detection of PCR, was cultured in EMB agar, then incubated at 37ºC for 24 hr. after the cell was harvested by phosphate buffer saline and washing three times with PBS and centrifuged at 1250 rpm for 10 minutes. The sediment was cultured in EMB agar at 37ºC for 24 hr. The bacterial number was 1x10^7 CFU/ml at dose 0.25 ml 13. Fifty mice were divided into five groups: the first group (ten mice) fed orally with PBS solution, the second group positive infected mice orally with *E. coli* (1x10^7 CFU/ml) for 4 days, the group infected mice orally with *E.coli* for 4 days then treated orally with *L. paracasei* 2x10^8 CFU/ml at dose 0.25 ml 14. The fourth group enhanced the protection of the mice by giving *L. paracasei* orally for 8 days, after that, infected orally with *E.coli* for 4 days, then treated *L. paracasei* orally for 8 days, and the fifth group conferred health benefits to mice by giving orally, only *L. paracasei* for 8 days.
**Histological examination**

Samples measuring 1 x 1 x 1 cm were taken from the gut, and tissues were immediately preserved in a solution containing 10% formaldehyde. The specimens were washed in tap water after the fixation had been done for 72 hours, and processing was then carried out as per standard procedure using a set of increasing alcoholic concentrations from 70% to absolute 100% for 2 hours in each concentration to remove water from the tissues. Then, clearance was carried out using xylol, and the specimens were then infiltrated with semi-liquid paraffin wax at 58° in two stages. Finally, blocks of specimens were made with paraffin wax, and sections of Hematoxylin and Eosin (H & E) stain were applied to all tissues, and under a light microscope, the histopathological changes were examined.

**Statistical analysis**

This study's data results were analyzed using Graph Pad Prism 8 software and Microsoft Excel 2013 for each biological replicate. The probability level at P values below ≤ 0.05 was used to identify a significant difference.

**Results**

Isolation of E.coli on MacConkey and EMB agars

All isolates of *E.coli* gave a pink color on MacConkey agar due to the fermentation of lactose, which will produce acid by products that lower the pH, transforming the neutral red indicator into the rose. The Second stage was done for *E.coli* isolates on EMB agar, which was used to distinguish *E.coli* from other Gram-negative bacteria. It was used to identify *E. coli*, which produced metallic green colonies on the media, as shown in Figure 1a. The results agree with Hanoun and Al-Samraee.

The positive isolates on EMB agar were selected and cultivated on SMAC agar and appeared as colorless colonies, which indicates that *E.coli* O157:H7 did not ferment sorbitol by Enterohemorrhagic *E.coli* (EHEC), Figure 1b.

![Figure 1](image_url)  
Figures 1. (a) Isolates of *E.coli* on EMB agar colonies appeared as green metallic sheen; (b) Isolation of *E.coli* on sorbitol MacConkey (SMAC) agar.

**Biochemical testing**

The biochemical identification of the IMViC test gave positive results for the indole methyl red, while it gave a negative result for the voges procedure and Simmon citrate, as shown in Figure 2.
Identification of *L. paracasei*

The colonies of *L. paracasei* appeared as white or milky, circular shapes with smooth entire edges and shiny, smooth surfaces Figure 3 a. The result of the microscopic examination of *L. paracasei* appeared as Gram-positive, bacilli, long or short rod shape, chain, arranged in single or pairs and non-spore-forming Figure 3b.

![Figures 2 and 3](image)

**Figure 2.** Biochemical test (IMViC) of *E. coli*.

**Figure 3.** (a) Colonies of *L. paracasei* on MRS agar; (b) Gram positive bacilli- shaped *L. paracasei* under light microscope (100X).

Confirmation of *L. paracasei* by VITEK 2

This test used a VITEK2 compact system that used up a Gram-positive (GP ID) card (Biomerieux-France). This system technology has improved the field of bacterial examination by providing more reliable technology, high speed, and high sensitivity for bacterial identification, with as high as 99% accuracy results. The result was a 99% probability of *L. paracasei*, as listed in Figure 4.
Figure 4. Result of VITEK 2 compact system for L. paracasei.

Inhibitory effects of E. coli against L. paracasei growth in vitro

The mean zone diameter of inhibition depended on the concentration of Lactobacillus paracasei (1.5×10^8 CFU/ml), Figure 5. The result showed that E.coli bacteria did not grow around the wells containing Lactobacillus paracaseiand this indicates the growth-inhibitory effect of L. paracasei.

Figure 5. Inhibitory effect of L. paracasei against E. coli in Muller Hinton agar.

Histological examination

The current histological results among mice groups were illustrated according to each group with organ. For group 1, the standard histological structure of the small intestine, including mucosa with villi and Lamina proper, submucosa and muscularis, Figure 6a. The results demonstrated that the gut in the control group was normal throughout, free of any histological abnormalities as reported recently 30. In group 2, moderate necrotic findings were recognized in intestinal villi with surface sloughing of epithelial lining accompanied by mild leukocytic infiltration and few apoptotic enterocytes. Other villi appeared with surface structure and moderate villus atrophy, as shown in Figure 6b) After swallowing E. coli O157: H7, the Shiga toxin is released when the bacteria attach to the intestinal mucosa. The toxin, in turn, prevents the epithelial cells lining the intestinal mucosa from making protein, which results in cell death.; mucous collapse, and possibly bloody diarrhea.
Figure 6. (a) Histopathological tiny intestinal segment from the negative control group appears within structural limit villi and Lamina propria, submucosa and muscularis (H and E stain 10X); (b) Histopathological section of small intestine of control positive infected group E.coli O157-H7 at 4 days post infection shows moderate villus atrophy with surface structureless (H & E stain X10); (c) Histopathological section of small intestine of (G3) four days after infected E. coli and treated with L. paracasei shows moderate mucosal sloughing with villus stunting (H & E stain 10X); (d) Histopathological section of small intestine of (G4) enhanced protection the mice by giving L. paracasei orally for 8 days , thereafter infected orally with E.coli for 4 days, then treated orally L. paracasei for 8 days shows prominence of MALT with moderate elongation intestinal villi (H & E stain 10X); (e) Histopathological section of small intestine (G5) confer health benefits of mice by giving orally, only L. paracasei for 8 days shows no clear pathological with mild submucosal cellular infiltration (H & E stain 10X).

Concerning group 3, there was moderate mucosal sloughing with evidence of villus stunting. Also, the examined intestinal tissue showed mild submucosal mononuclear cell (MNC) infiltration with capillary dilation. In addition, moderate degeneration findings may be recorded in the submucosal gland (Figure 6c). In this
study, it appeared when probiotics were given. It will lead to the secretion of IgAs against Shiga toxins, decreasing the concentration of Shiga toxins in the intestine. This will reduce the damage that occurs in the intestine. In group 4, the main intestinal finding characterized by moderate enlargement of MALT as well as moderate distention of Lamia proper with mononuclear cells (MNCs) may detect in some villi also moderate elongation of intestinal villi with the prominence of MALT, Figure 6d. Regarding group 5, no apparent pathological alterations were recognized in either small intestine or colonic tissue except mild infiltration of MNCs in submucosal tissue, as shown in Figure 6e. As well as a maximum increase in body weight was demonstrated in the group receiving probiotics plus a standard dose compared to the control group (group 1) and challenge groups with or without probiotic supplementation.

Discussion
The results of the isolation of E. coli on MacConkey and EMB agars agreed with the findings of other studies 19,20,21. The results of the biochemical identification of the IMViC test were identical to those of other studies 22,23,24,25. The colonies of L. paracasei appeared as white or milky, circular shapes with smooth entire edges and shiny, smooth surfaces (Figure 3a), which agreed with other studies 26,27. The colonies of L. paracasei appeared as white or milky, circular shapes with smooth entire edges and shiny, smooth surfaces were similar to those observed by Lengkey et al. 28. The results of the VITEK2 compact system that used up a Gram-positive (GP ID) card were agreed with manufacturer's standers and with other researchers 29,30, regarding the inhibitory effects of E. coli against L. paracasei growth in vitro, pathogenic E. coli bacteria isolated from diarrhea-infected sheep agreed with other studies 31,32.

For histology, the result of the intestinal section of group 6 (A) also agreed with other authors 33,34,35,36. In this group, studies showed that when probiotics are given before infection, they enhance the immune response to Shiga toxin, so when probiotics are given after infection, it will lead to an increase in the secretion of IgAs, and this will prevent the secretion of Shiga toxin, so we will notice the intestines gradually returns to the normal state, and this agreed with others 37. The results of group 6e studies have shown how probiotics can help avoid health issues, particularly digestive issues like diarrhea brought on by infections 38. Also, when probiotic was administered only, no apparent pathological alteration was recognized in the small intestine and colonic tissue except mild infiltration of MNCs in submucosal tissue as observed by other researchers 39.

Conclusions
This is the first study in Iraq that demonstrated the effectiveness of L. paracasei as a probiotic bacteria in the treatment of diarrhea in sheep as an alternative to antibiotic treatment. Escherichia coli O157:H7 was the most common cause of diarrhea in sheep. The general appearance of the 5 groups of mice was the best in health, activity, and weight assessment. The administration of L. paracasei proved to give high protection against E. coli O157:H7 infection.

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