

PROBIOTIC ANTAGONISTIC EFFECTS OF *Lactobacillus collinoides* FROM COW MILK ON COW-DIARRHEA-RECOVERED ENTEROTOXIGENIC *E. coli*: AN *IN VIVO* STUDY

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ABSTRACT

Probiotics have been recorded to introduce vital changes in the functions of various body organs of humans or animals. The current *in vivo* study was launched to unveil the antagonistic effects of *Lactobacillus collinoides*, lactic acid bacteria, isolated from cow milk on the induced infection by enterotoxigenic *Escherichia coli* (ETEC) in mice. Twenty-four albino mice (Balb/C) were divided randomly into six groups (four animals/group) designated as a negative control (NC) group which received nothing, an ETEC-infected (EE) group at 0.1ml of 10⁵cfu/ml/orally for two days (positive control), a *L. collinoides* exposed (LC) group at 0.1ml of 10¹⁰cfu/ml/orally for two days, and an ETEC-exposed, at 0.1ml of 10⁵cfu/ml, pre-treated with *L. collinoides* at 0.1ml of 10¹⁰cfu/ml/orally for two days (EE+LC) group. After the experiment period was done, blood samples were collected from all animals for understanding liver functions via glutamic oxaloacetic transaminase (GOT) and glutamate-pyruvate transaminase (GPT) tests. Then, the mice were sacrificed (10 days after the last dose received by the animals) for doing histopathological examination that targeted the liver, intestine, and stomach. When compared with the NC group, significant ($p < 0.05$) increases in the serum levels of GOT and GPT were seen in the EE group induced after the ETEC infection. For the LC group and when compared to the NC results of GOT and GPT, significant ($p < 0.05$) decreases in the serum levels of these enzymes were noticed in the LC group after the insertion of *L. collinoides*. However, dosing animals of the EE+LC group with *L. collinoides* then inducing infection with ETEC revealed significant ($p < 0.05$) decreases in the serum GOT and GPT levels in comparison to those in EE group with no significant ($p > 0.05$) differences in those levels from the EE+LC group when compared to those in the NC group. The histopathological findings demonstrated that ETEC caused necrosis, degeneration, and infiltration of inflammatory cells in the tested organ tissues; however, dosing EE+LC animals with *L. collinoides* protected those organ tissues from the cytotoxic effects of the ETEC. This study indicates powerful effects of the *Lactobacillus collinoides* against the induced infection by enterotoxigenic *E. coli*.

Keywords: Enterotoxigenic *E. coli*, ETEC, *Lactobacillus collinoides*, probiotics.

How to cite this article: Mhysn AS, Atiyah WR, Abd Sharad A (2019): Probiotic antagonistic effects of *Lactobacillus collinoides* from cow milk on cow-diarrhea-recovered Enterotoxigenic *E. coli*: An in vivo study, *Ann Trop Med & Public Health*; 22(IV): S357. DOI: <http://doi.org/10.36295/ASRO.2019.22124>

INTRODUCTION

Probiotics have a very long history of connection with health. It's been more than a hundred years since Tissier noted that intestinal microbial members of breast-fed healthy children were prevailed by bifidobacteria that were missing from diarrheic-non-breast-fed babies. A number of research have since endorsed this partnership, but they were poorly-constructed and monitored and encountered practical difficulties such as specificity of the strains used, and slow probiotic development in media other than human milk. With more recent developments, they have sometimes successfully accumulated more significant proof that bacteria from probiotics can participate in an individual health. This information corresponded with a growing understanding among customers on the connection between diet and health to create a helping atmosphere for developing the active nutrition idea that describes food or its ingredients with positive impacts beyond their nutritional importance on the wellness of customers ^(1,2). Live microorganisms; probiotics, can bring wellness advantages to a host when consumed in adequate levels. This concept of probiotics was agreed in 2001, later became the reference word for scientific research and regulations, by the World Health Organization (WHO) and the United Nations Food and Agriculture Organization (FAO). As a result of continuously generating study proof showing probiotic prospective safety advantages to customers, the needs for food comprising probiotics grows worldwide ^(3,4). As defensive cultivations, Lactic acid bacteria (LAB), are popular probiotic microorganisms which owing to particular features are well known for their safety. *Enterococcus*, *Leuconostoc*, *Lactobacillus*, *Bifidobacterium*, *Lactococcus*, *Streptococcus*, and *Pediococcus* are the major genera of LAB. These organisms reduce gastrointestinal illnesses through increased development of beneficial microorganisms and decreasing the demographic processes of pathogens ^(5,6). The most prevalent source of child diarrhea is enterotoxigenic *Escherichia coli* (ETEC). The main ETEC applicant vaccines are the LT enterotoxin and colonization factors (CFs). ETEC must adhere to the small intestine epithelial cells by using CFs in order to induce a disease. Because of impacts of enterotoxins, watery diarrhea is generated. The current *in vivo* study was launched to unveil the antagonistic effects of *Lactobacillus collinoides*, lactic acid bacteria, isolated from cow milk on the induced infection by ETEC in mice.

MATERIALS AND METHODS

Bacterial culture (isolation and identification)

Twenty samples of local dairy products including cow and buffalo milk were collected from different areas of Al-Diwaniyah City, south of Iraq. Two grams of each sample was transferred to a flask containing MRS broth as enrichment media in which 100ml of distilled water (DW) was added to it and incubated at 37°C for 24hrs. After incubation was done, 100µl of enriched samples was spread out on a MRS agar followed by anaerobic incubation at 37°C for 48hrs. Bacterial purification was performed by subsequent sub-cultivation. Gram-staining and biochemical tests such as catalase, oxidase, indole, at 15°C based growth, fermentation of carbohydrates (fructose, arabinose, galactose, mannitol, lactose, salicin, trehalose, and sucrose), motility were performed to identify the bacterium ⁽⁷⁾. The ETEC was isolated from cows that had severe diarrhea using MacConkey agar and eosin methylene blue (EMB) agar.

Laboratory Animals:

Twenty-four healthy female mice (strain BALB/c) were obtained from the Laboratory Animal's House in Biotechnology Research Centre/AL-Nahrain University. The age of animals was ranged between 6 to 8 weeks, and their weight was about 25-30g. The mice were placed as four mice in each plastic cage, measured at (29×12.5×11.5cm). Floors of the cages were covered with the soft crushed wood shaving. Hygiene standards were followed throughout the study period using (24-26°C) of room temperature and 14:11hr of light: dark ratio/day. The animals were fed standard pellets. The food and water were supplied ad libitum.

Experimental Design:

Twenty-four albino mice (Balb/C) were divided randomly into six groups (four animals/group) designated as a negative control (NC) group which received nothing, an ETEC-infected (EE) group at 0.1ml of 10^5 cfu/ml/orally for two days (positive control), a *L. collinoides* exposed (LC) group at 0.1ml of 10^{10} cfu/ml/orally for two days, and an ETEC-exposed, at 0.1ml of 10^5 cfu/ml, pre-treated with *L. collinoides* at 0.1ml of 10^{10} cfu/ml/orally for two days (EE+LC) group.

Sampling

Collection of blood samples

The mice were anesthetized by chloroform for collecting blood samples using cardiac puncture and insulin needle and syringes coated with heparin from inside to prevent clotting. After blood was collected from the mice, separation of the serum was performed using 10min centrifugation at 5000rpm. The serum samples were stored at 5°C until use (8). Glutamic oxaloacetic transaminase (GOT) and glutamate-pyruvate transaminase (GPT) tests were performed on the blood samples.

Collection of organ tissues

Then, the mice were sacrificed (10 days after the last dose received by the animals) for doing histopathological examination that targeted the liver, intestine, and stomach. Pieces of those organs were collected placed in petri dishes that contained salty solution used for removing of unnecessary tissues such as fat. Later, Bouin's solution was used to fix the samples in suitable cans, and the process of tissue slide preparation was followed according to⁽⁸⁾. Tissue slides were examined in the Department of Pathology, College of Veterinary Medicine, University of Al-Qadisiyah, Iraq.

RESULTS

Bacterial identification

The bacterial features resulted for the isolated *L. collinoides* and ETEC are shown in table (1) and (2), respectively.

Table 1: *L. collinoides* resulted growth and biochemical features

| Biochemical test | Results |
|-----------------------------|---------|
| Growth in Litmus milk media | + |
| Gelatinase Test | + |
| Oxidase Test | - |
| Catalase Test | - |
| Indol | - |
| Motility | - |
| Growth at 15 C | + |
| Growth at 45 C | - |
| Acid production from | |
| Arabinose | + |
| Galactose | + |
| Mannitol | - |
| Maltose | + |

Table 2: ETEC resulted growth and biochemical features

| Biochemical Test | Result | |
|------------------------------|------------------|---------------|
| Indole Test | + | |
| Simmon´s Citrate Test | - | |
| Urease Test | - | |
| Triple Sugar Iron (TSI) Test | Slant/Butt | Color |
| | Acid/Acid | Yellow/Yellow |
| | H ₂ S | - |

Liver function

When compared with the NC group, significant ($p < 0.05$) increases in the serum levels of GOT and GPT were seen in the EE group induced after the ETEC infection. For the LC group and when compared to the NC results of GOT and GPT, significant ($p < 0.05$) decreases in the serum levels of these enzymes were noticed in the LC group after the insertion of *L. collinoides*. However, dosing animals of the EE+LC group with *L. collinoides* then inducing infection with ETEC revealed significant ($p < 0.05$) decreases in the serum GOT and GPT levels in comparison to those in EE group with no significant ($p > 0.05$) differences in those levels from the EE+LC group when compared to those in the NC group. These data are demonstrated in table (3).

Table 3: Levels of GOT and GPT enzymes in the study groups

| Group | GPT (Mean±SEM U/L) | GOT (Mean±SEM U/L) |
|-------|--------------------|----------------------------|
| NC | 61.25 ± 1.88 | 106.00 ± 3.02 |
| EE | 99.50 ± 4.29* | 270.00 ± 6.94 [□] |
| LC | 36.25 ± 2.68* | 90.50 ± 2.21 * |
| EE+LC | 63.00 ± 0.57 ** | 114.00± 1.32 ^{□□} |

*: $p < 0.05$ as compared with NC group.

** : $p < 0.05$ as compared with EE group.

□: $p < 0.001$ as compared with NC group.

□□: $p < 0.001$ as compared with EE group.

Histopathological findings

The histopathological findings demonstrated that ETEC caused necrosis, degeneration, and infiltration of inflammatory cells in the tested organ tissues; however, dosing EE+LC animals with *L. collinoides* protected those organ tissues from the cytotoxic effects of the ETEC.

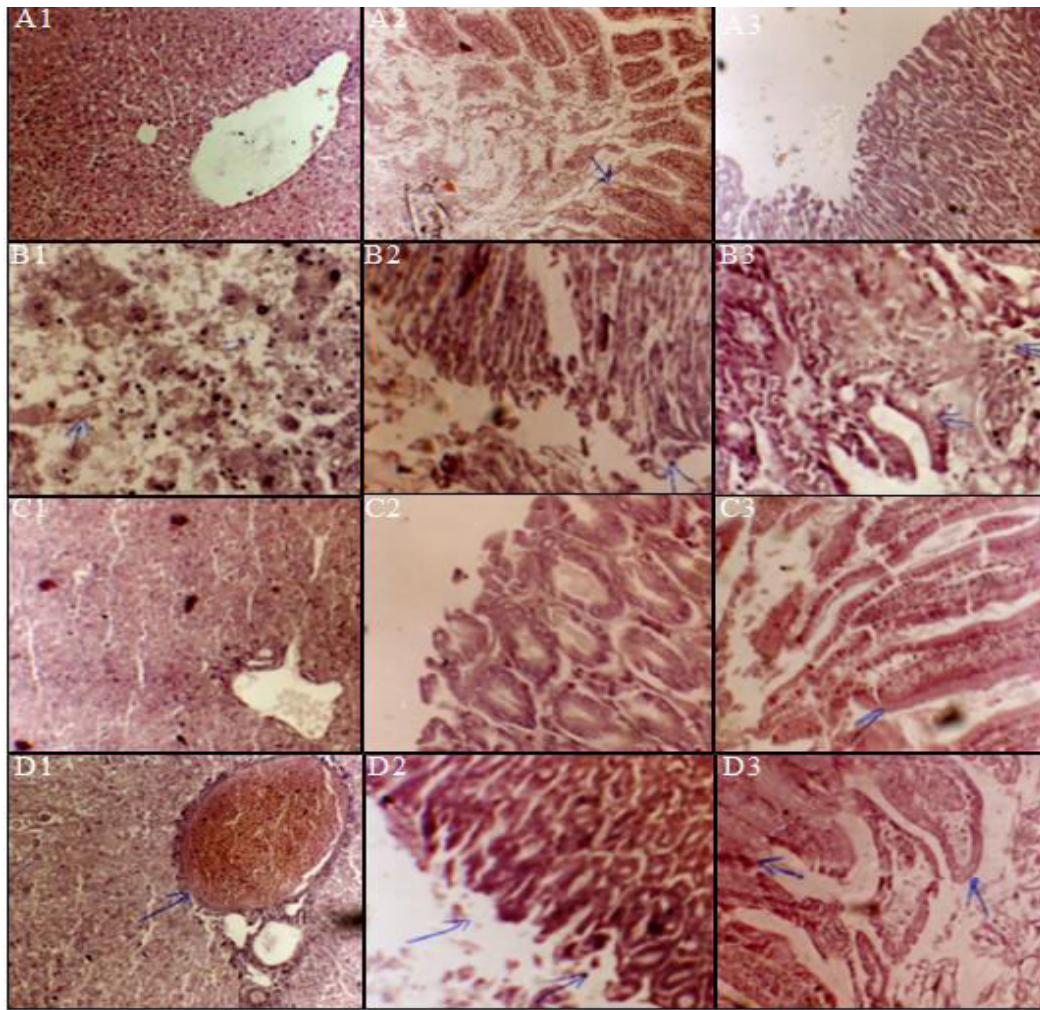


Figure 1: Histological findings in the study mice; each row (1: liver, 2: Stomach, and 3: Intestine mucosa). A. NC group: shows normal tissues, H&E, 100x, 200x, and 100x, respectively. B. EE group: reveals necrosis, degeneration, and infiltration of inflammatory cells in the tested organ tissues, H&E, 200x, 100x, and 200x, respectively. C. LC group: demonstrates normal tissue appearance, H&E, 100x, 200x, and 100x, respectively. D. EE+LC group: unveils normal looking appearance of liver tissue with remaining portal blood vascular congestion (arrows) and intestinal villus regeneration (arrows), H&E, 200x, 100x, and 200x, respectively.

DISCUSSION

The concept of the safety and advantageous use of probiotics is not new. In latest years, however, confidence in the use of probiotics has been considerably improved. Statista.com states that the US revenues of probiotics are more than \$1,1 billion in 2014; probiotics revenues around the world are \$25 trillion. The majority of customers believe that probiotic functions and can be used for various wellness circumstances. The market shelves around the world are packed with products of probiotics. Since not all probiotic products are similarly efficient, it is increasingly difficult to choose a specific brand for a specific disease ⁽⁹⁾. The results demonstrated that dosing animals with *L. collinoides* then inducing infection with ETEC revealed significant decreases in the serum GOT and GPT levels in comparison to those in EE group with no

significant ($p>0.05$) differences in those levels from the EE+LC group when compared to those in the NC group. These data indicate the importance of *L. collinoides* in fighting the pathological changes initiated by the ETEC displayed via levels of the liver function enzymes. This agrees with the fact that *L. collinoides* is one of the LAB bacteria that have been identified to improve the health status of humans and animals. It has been detected that *L. collinoides* strains were capable of generating 4-ethylcatechol and 4-ethylphenol which are volatile phenols⁽¹⁰⁾. It is well established that phenolic compounds play important roles in defeating various health conditions via their antioxidant activities⁽¹¹⁾. The structural and functional damages caused to major biomolecules such as nuclear acids, proteins, and lipids are based upon oxidative stress. In reality, many illnesses, such as bacterial infections^(12,13), and metabolic and cardiovascular disorders are triggered by these injuries. The imbalance generated between free radical manufacturing and the protection of antioxidants may not only happen under illness circumstances but also under normal physiological functions such as intense physical activity. The oxidant effects can be counteracted by the exogenous use of dietary antioxidants^(14,15). According to those available vital data, *L. collinoides* can protect body tissues against the pathological changes induced by the ETEC, and this was also confirmed in the current study results of the histopathological examination that revealed significant protecting effects of *L. collinoides* against the pathogenic influence of the ETEC. It has also been revealed that *L. collinoides* can secrete exopolysaccharides⁽¹⁶⁾ which have increasingly been used in medical industries such as in wound and surgical dressing⁽¹⁷⁾.

CONCLUSION

This study indicates powerful effects of the *L. collinoides* against the induced infection by enterotoxigenic *E. coli*. This conclusion is based on the restored liver function demonstrated via the levels of the liver enzymes. Restoring the histological features of the ETEC-affected tissues after the treatment with *L. collinoides* also comes to support this vital conclusion.

ETHICAL CLEARANCE

The Research Ethical Committee at scientific research by ethical approval of both environmental and health and higher education and scientific research ministries in Iraq

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

FUNDING: Self-funding

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