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Physical and Immunological Effects of *Lactobacillus acidophilus* La-5 against *Escherichia coli* O157:H7

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Abstract: Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 causes diarrhea in calves, and shed in their feces. It is a zoonotic food-borne pathogen reaches human food chain resulting in life threatening hemolytic uremic syndrome. So we studied the prevalence of *E. coli* O157:H7 in diarrheic calves, investigated their ability to produce Shiga toxins, reported their antimicrobial resistance profile and evaluated the physical and immunological effects of *Lactobacillus acidophilus* La-5 against it. *E. coli* O157:H7 prevalence among 100 diarrheic calves in El-Fayoum Governorate was 5%. All the isolated strains were Shiga toxigenic on *Vero* cell line and multidrug resistant. *L. acidophilus* La-5 was active against *E. coli* O157:H7 physically and immunologically. Physically, *L. acidophilus* La-5 cells and culture free spent medium co-aggregated and agglutinated *E. coli* O157:H7 independently. Immunologically in experimental murine model, *L. acidophilus* La-5 significantly increased the phagocytic activity, phagocytic index and humoral immune response (immunoglobulin G) in the lactobacillus treated group compared with infected group. In addition, *L. acidophilus* La-5 prevented the attaching and effacing lesions in ceca and reduced the damage in renal tissues. Moreover, *L. acidophilus* La-5 significantly reduced the load and time of *E. coli* O157:H7 shedding in Lactobacillus treated group in comparison with infected group. Finally, *L. acidophilus* La-5 overwhelmed *E. coli* O157:H7 pathogenicity against intestinal and kidney tissues in murine model via know-hows physical and immune modulating effects.

Key words: *Lactobacillus acidophilus* La-5 · EHEC O157 · Shiga Toxin · Phagocytic Index · ELISA · Electron Microscopy

INTRODUCTION

Probiotics are live microorganisms that when administered in adequate amounts confer health benefits on the host [1]. Probiotics act physically through microbe-microbe interactions; microbial barrier formation and microbe-host interactions via strengthen the intestinal barrier function. In addition they modulate the innate and adaptive immune systems [2].

Lactobacilli exhibit antagonistic activity against both Gram positive and Gram negative bacteria through antimicrobial compounds they produce [3]; definitely *Lactobacillus acidophilus* La-5 exerts antimicrobial activities against food-borne pathogens [4].

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is a zoonotic food-borne pathogen [5] of worldwide significance; elicits possibly life-threatening hemorrhagic colitis, hemolytic uremic syndrome and/or bloody diarrhea [6].

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Cattle are considered the major EHEC asymptomatic reservoir [7]**.** Nevertheless, EHEC O157:H7 intimidates calves' health; it induces cytoskeletal rearrangement in infected enterocytes, results in attaching and effacing lesions and subsequently diarrhea that may be bloody [8-10].

Treatment of infected calves with antibiotic emerges resistant difficultly treated strains. Resistant strains could transfer their resistant genes to the other ecosystem microorganisms that threats animals' and humans' health [11, 12]. Moreover, *E. coli* isolates from calves showed a statistically higher prevalence of virulence genes compared with isolates from older animals [13]. To prevent *E. coli* O157:H7 infection in humans, its load in animals must be reduced with special reference to diarrheic calves.

So this study aimed to probe *E. coli* O157:H7 burden in diarrheic calves; and emphasis the extent to which *L. acidophilus* La-5 could obligate multidrug resistant *E. coli* O157:H7 not to harm the experimentally infected BALB/c mice and investigate *L. acidophilus* La-5 immune modulating effects.

MATERIALS AND METHODS

Fecal Samples: One hundred fecal swabs were collected from neonatal diarrheic calves aged up to one month from El-Fayoum Governorate in the period from January till May 2013. Fecal swabs were submitted to the laboratory in ice chest and processed on reception.

Isolation and Biochemical Identification of *E. coli* O157:H7: It was carried out as outlined by Collee *et al*. [14].

Serological Identification of *E. coli* O157:H7: It was carried out as delineated by Ewing [15].

Antimicrobial Sensitivity Profile: *E. coli* O157:H7 isolates were tested according to Bauer-Kirby [16] technique against common antibiotics used for diseases treatment in livestock and humans. Antimicrobial disks were amikacin (30 µg), amoxicillin+clavulanic acid (20+10 µg), ampicillin (10 µg), ampicillin + sulbactam (10+ 10 µg), cefepime (30 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), gentamicin (10 µg), imipenem (10 µg), tetracycline (30 µg), trimethoprim+sulfamethoxazole (1.25+ 23.75 µg) from Oxoid and judged as outlined by Clinical Laboratory Standard Institute (CLSI) [17] guidelines.

Cytotoxic Activity of *E. coli* O157:H7 on *Vero* Cells: We tested *E. coli* O157:H7 isolates for Shiga toxin production by *Vero*-cytotoxicity assay as previously detailed by Pai *et al*. [18].

Probiotic Strain: *L. acidophilus* La-5 was obtained from the culture collection of the Canadian Research Institute for Food Safety, Canada.

In vitro Effects of *L. acidophilus* La-5 on *E. coli* O157:H7

Co-aggregation Assay of *L. acidophilus* La-5 to *E. coli* O157: This assay was carried out as designed earlier by Reid *et al*. [19] and read as (1 to 4) degree of aggregation in ascending manner.

Co-aggregation Inhibition Assay of *L. acidophilus* La-5 to *E. coli* O157:H7 by Mannose Sugar: The assay is identical as described previously by Reid *et al*. [19] with amendment that *L. acidophilus* La-5 was suspended in phosphate buffer saline (PBS) pH 7.2 containing 2% mannose.

Plate Agglutination of *L. acidophilus* La-5 Culture Free Spent Medium (CFSM) to *E. coli* O157:H7: Dense suspension of *E. coli* O157:H7 was prepared by centrifugation of 24 h culture in tryptone soy broth and washed twice in PBS pH 7.2. Then equal volumes of *E. coli* O157:H7 and CFSM were mixed.

Plate Agglutination Inhibition Assay of *L. acidophilus* La-5 CFSM to *E. coli* O157:H7: As previously mentioned except, *E. coli* O157:H7 was washed twice in PBS pH 7.2 containing 2% mannose sugar. Then equal volumes of *E. coli* O157:H7 and CFSM were mixed.

***In vivo* Effects of *L. acidophilus* La-5 on *E. coli* O157:H7 in Murine Model**

Experimental Design: Forty five BALB/c mice (20-25 g, each) were purchased from Research Institute of Ophthalmology Financial Management (Giza, Egypt) to be used in this study. Mice were divided randomly into three groups, 15 each and kept seven days without any treatment for acclimatization. Group C (control group); it was kept as control negative without any treatment; group I (infected group); each mouse of the group was orally inoculated with a single dose of 150 µL containing 10^{10} CFU of *E. coli* O157:H7 as elected by Nagano *et al*. [20]. In addition, mice were allowed to suckle by pipetting

to emulate the natural exposure route and preceded by 16 h starvation, as prolonged dietary restriction promotes *E. coli* O157:H7 colonization [21]; and group L (*L. acidophilus* treated group); *L. acidophilus* La-5 was given in 10^9 CFU/mL of drinking water containing milk base diet as recommended by Madureira *et al*. [22] for consecutive 15 days before infection as previously described in infected group and then continued on drinking water containing *L. acidophilus* La-5 the remained period of the experiment to confirm a continuous exogenous probiotic effect [23].

Mortality Follow Up: Number of dead mice was reported daily till the end of the experiment at the 21st day post infection.

Fecal Bacterial Count: Enumeration of *E. coli* O157:H7 was carried out according to Roxas *et al*. [24].

Assessment of Peritoneal Macrophage Function: Phagocytic activity and phagocytic index were determined as outlined by Zhang *et al*. [25].

Assessment of Humoral Immune Response by ELISA: Determination of immunoglobulin G (IgG) titer in serum samples was based on ELISA described by De Herdt *et al*. [26] and Shu *et al*. [27].

Transmission Electron Microscopy: Ceca were retrieved from C, I and L groups and examined by transmission electron microscopy in EM unit Faculty of Science, Ein Shams University in a blinded fashion.

Histopathological Examination: Kidneys were retrieved from C, I and L groups and examined histopathologically according to Banchroft *et al*. [28].

Statistical Analysis: Data were expressed as the mean ± standard deviation of the mean. Data comparisons were made with Student's *t* test. Differences were considered significant when the *P* value was ≤ 0.05.

RESULTS

Isolation, Biochemical and Serological Identification of *E. coli* O157:H7: Five isolates belonged to *E. coli* O157:H7 (5%) were recovered from 100 diarrheic calves.

Antimicrobial Sensitivity Profile: The examined *E. coli* O157:H7 showed multidrug resistance behavior; they showed resistance against tetracycline (30 µg), ampicillin (10 µg), trimethoperim +sulphamethoxazole (1.25+ 23.75 µg), chloramphehicol (30 µg), gentamicin (10 µg), ampicillin + sulbactam (10+ 10 µg), and cefepime (30 µg) with (100, 100, 80, 60, 40, 40, and 20% respectively) and sensitive to amikacin (30 µg), amoxicillin+clavulanic acid, ciprofloxacin (5 µg) and imipenem (10 µg).

The strain used in experimental infection was resistant to tetracycline (30 µg), ampicillin (10 µg), ampicillin + sulbactam (10+10 µg), and cefepime (30 µg) and sensitive to amikacin (30 µg), imipenem (10 µg), trimethoprim + sulfalmethoxazole (1.25+23.75 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg) and amoxicillin + clavulanic acid (20+10 µg).

Cytotoxic Activity on *Vero* Cells: *Vero* cells exhibited pathological changes (rounded, wrinkled, detached and floated in the medium) 48 h post inoculation with supernatant of 24 h cultured *E. coli* O157:H7 isolates.

Co-Aggregation and Co-Aggregation Inhibition Assays: We observed moderate co-aggregation occurred between *L. acidophilus* La-5 and the examined five isolates of *E. coli* O157:H7 ranging from 1- 2 degree and partially inhibited by inclusion of mannose sugar.

Plate Agglutination and Plate Agglutination Inhibition of CFSM to *E. coli* O157:H7: We demonstrated rapid flocculation of the bacterial homogenous suspension by their mixing with CFSM. Mannose 2% partially reduced this flocculation when incorporated with the bacterial cell suspension.

Mortality Follow Up: Five mice (33.3%) out of the 15 mice in the infected group died till the 6th day post *E. coli* O157:H7 oral inoculation and the reminder continued alive till the end of the experiment 21st day post infection. No mortality was recorded in control or in lactobacillus treated groups till the end of the experiment.

Fecal Bacterial Count: Table (1), shows significant reduction in *E. coli* O157:H7 shedding post infection in *L. acidophilus* La-5 treated group when compared with infected group at 2nd and 7th days post infection. *E. coli* O157:H7 shedding reported with infected group till the end of experiment while *L. acidophilus* La-5 treated group ceased shedding at 14th day post infection.

Table 1: *E. coli* O157:H7 shedding in infected and *L. acidophilus* La-5 treated groups at 2nd, 7th, 14th and 21st days post infection

	I				L			
Group								
Days post infection	2nd	7th	14th	21st	2nd	7th	14th	21st
Shedding in $\log_{10}$	7.1±0.1	6.6±0.2	6.6±0.1	5.4±0.2	6.6±0.2*	4.2±0.1*	0*	0*

I: *E. coli* O157:H7 infected group.

L: Lactobacillus treated group.

* Means the difference is statistically significant regarding infected group and lactobacillus treated group at the same time post infection $p \leq 0.05$.

Table 2: Phagocytic activities and Phagocytic indexes of peritoneal phagocytes in control, infected and lactobacillus treated groups at 0 day; and 3th and 7th days post infection

	C	I		L		
Group						
Days post infection	0	3rd	7th	0	3rd	7th
Phagocytic activity	40.0±4.1	54.6±3.6	61.0±6.0	64.0±5.2*	86.3±1.46*	81.7±4.7*
P.I	0.7±0.3	1.2±0.2	1.5±0.2	1.8±0.3*	2.5±0.3*	2.2±0.3*

C: Control group.

I: *E. coli* O157:H7 infected group.

L: Lactobacillus treated group.

P.I: Phagocytic index

* Means the result is significantly different comparing infected group and lactobacillus treated group at the same time post infection $P \leq 0.05$.

Table 3: ELISA immunoglobulin IgG titers in control, infected and lactobacillus treated groups at 7th, 14th and 21st days post infection

	I			L		
Group						
Day post infection	7th	14th	21st	7th	14th	21th
ELISA titers in $\log_{10}$	6.3±0.2	6.7±0.2	6.9±0.3	7.1±0.4*	6.5±0.4	5.2±0.2*

I: *E. coli* O157:H7 infected group.

L: Lactobacillus treated group.

* Means the result is significantly different by comparing infected group and lactobacillus treated group at the same time post infection $P \leq 0.05$.

Assessment of Peritoneal Macrophage Function: Phagocytic activity and index were significantly higher in lactobacillus treated group before infection at zero day than those of control group without any treatment. Post infection, lactobacillus treated group showed higher phagocytic activities and indices at the 3rd and 7 th days when compared with infected group at the same time as Table (2) shows.

Assessment of Humoral Immune Response by ELISA: IgG was significantly higher in *L. acidophilus* La-5 treated group than infected group at the 7th and 14th days post infection and it decreased significantly at the 21st day post infection as Table (3) illustrates.

Transmission Electron Microscopy: Electron micrograph images of *E. coli* infected group show detached villi, hyper stimulated goblet cells and abnormal enterocytes structure at 44 h post infection (Fig. 1-A). At the 7th day post infection pathological lesions became intensive and continued to hurt goblet cells as Figure (1-B) reveals. On the other hand, lactobacillus treated group show normal villi with normal enterocytes structure at 44 h and at the 7th day post infection (Fig. 1C and D). Figure 1-E shows normal villi and enterocytes of the control mice cecal tissues.

Histopathological Examination: Examination of the *E. coli* infected group revealed focal inflammatory cells aggregation and focal hemorrhages at the degenerated and necrotic tubules in mouse kidney died 4th day post infection (Fig. 2-A) while Figure (2-B) shows congested blood vessels and congested tufts of the glomeruli at the 7th day post infection.

Kidney lesions appeared as mild swelling and vacuolization in the lining endothelium of the glomerular tufts in *L. acidophilus* La-5 treated mouse 44 h post infection (Fig. 2-C), whereas at the 7th day post infection (Fig. 2-D) *L. acidophilus* La-5 kept healthy kidney structures when matched with those of control mouse kidney (Fig. 2-E).

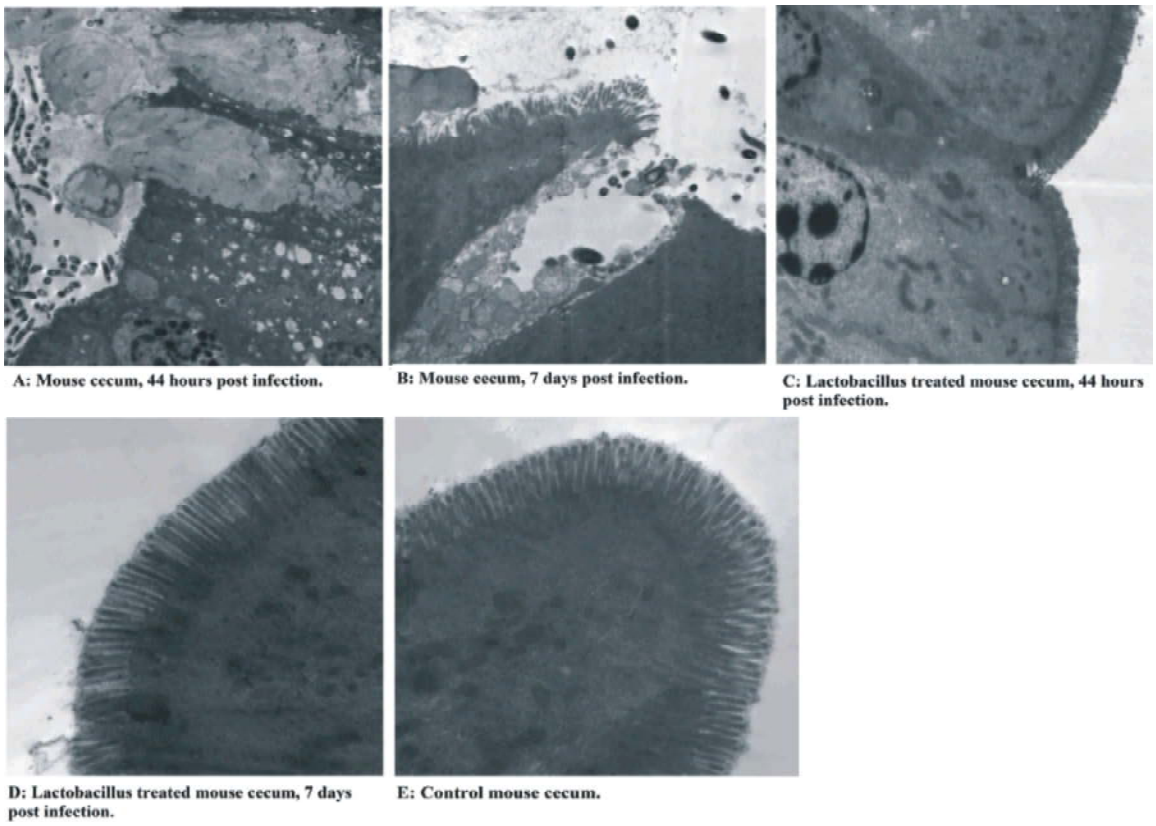

Fig. 1: Cecal electron micrograph images of *E. coli* O157:H7 infected mice, *L. acidophilus* La-5 treated mice and control mice. At 44h post infection with *E. coli* O157:H7, there is absence of glycoclayx, enterocytic vacuolization, swollen mitochonderia, and hyper stimulated goblet cells with profuse secretion (A) and 7 days post infection (B); cecum of *L. acidophilus* La-5 treated mice (C & D) 44 h and 7 days post infection and control mouse cecum (E)

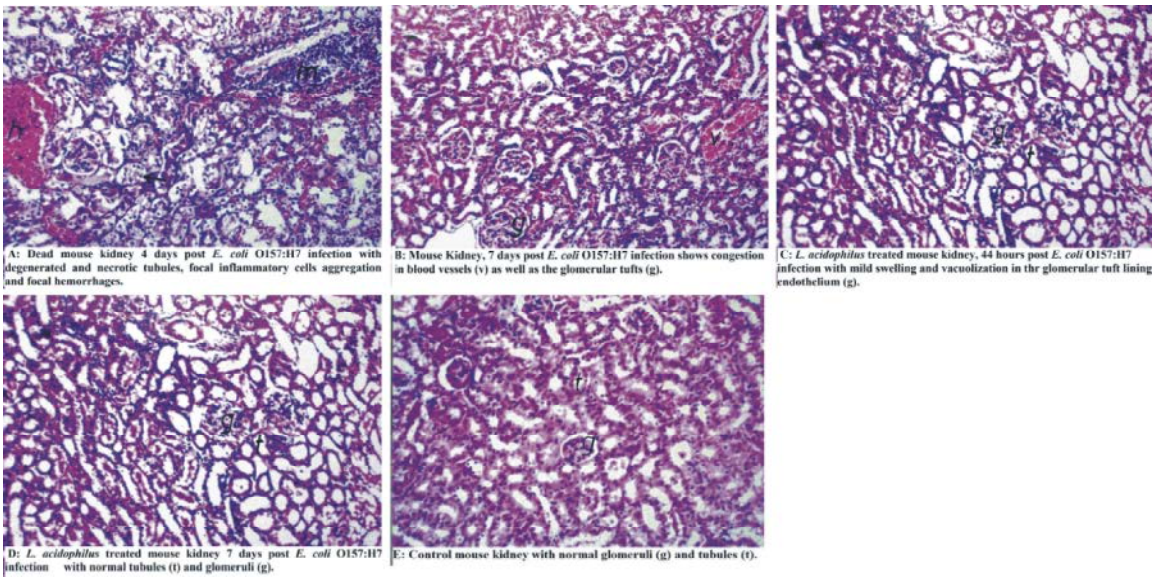

Fig. 2: Histopathological images of *E. coli* O157:H7 infected mice kidney at 4th and 7th days post infection (A & B); kidney of *L. acidophilus* La-5 treated mice (C & D) 44 h and 7th days post infection and control mouse kidney (E)

DISCUSSION

In this study we aimed to probe *E. coli* O157:H7 burden in diarrheic calves; and to emphasis the extent to which *L. acidophilus* La-5 could obligate multidrug resistant *E. coli* O157:H7 not to harm experimentally infected BALB/c mice and investigate *L. acidophilus* La-5 immune modulating effects. Our results showed *E. coli* O157:H7 to be one of the causative agents of calves' diarrhea in El-Fayoum Governorate. *L. acidophilus* La-5 possessed the upper hand over *E. coli* O157:H7 in murine model.

Our *E. coli* O157:H7 isolation rate (5%) is lower than that reported by Badawy *et al*. [29] who isolated *E. coli* O157:H7 from 17.3% of diarrheic calves. While it is higher than the isolation rate 3.57% reported by Abd Al-Azeem *et al*. [30] and this may be due to incorporation of calves older than those of our study. *E. coli* O157:H7 was isolated in higher rates from calves less than 2-weeks old in Kang *et al*. [9] study.

All the isolated *E. coli* O157:H7 injured *Vero* cell line that agrees with Irshad *et al*. [31], who isolated 10 *E. coli* O157 and all of them were verocytotoxigenic. *Vero* cells became rounded, wrinkled, detached and floated in the medium [18]. This declares the high public health significance associated with *E. coli* O157:H7 shedding in diarrheic calves.

The examined five *E. coli* O157:H7 isolates showed multidrug resistance behavior against tetracycline, ampicillin, trimethoperim + sulphamethoxazole, chloramphehicol, gentamicin, ampicillin + sulbactam, and cefepime with (100, 100, 80, 60, 40, 40, and 20% respectively). Multidrug resistance behavior could be due to the empirical use of these antibiotics in livestock [32].

L. acidophilus La-5 could be an alternative competitor to antibiotics for *E. coli* O157 infection treatment. Physically, *L. acidophilus* La-5 and its (CFSM) co-aggregated and agglutinated *E. coli* O157:H7 that could protect enterocytes by masking or blocking the surface of the pathogenic cell and reduce the toxins activity [33]. Likewise, co-aggregated and agglutinated *E. coli* O157:H7 reserved loose in the intestinal lumen allowing intestinal peristalsis to sweep them outside the body.

Mannose sugar partially reduced the co-aggregation and agglutination of *E. coli* O157:H7 by *L. acidophilus* La-5 cells and its CFSM. These findings support the hypothesis stated by Servin and Coconnier [34] that probiotics could compete with pathogens for the same enterocyte carbohydrate receptors. In addition we could expect that *L. acidophilus* La-5 extracellular products act in synergistic action with their cells.

In our experiment, *E. coli* O157:H7 oral inoculation in mice destroyed intestinal villi, hyper stimulated goblet cells and degenerated enterocytic components (mitochondria and nuclei) as Figures 1-A and 1-B illustrate. Lu and Walker [35] previously explained these lesions as *E. coli* secrete a receptor (type III secretion) into the microvillus surface of enterocytes that disrupts the microvillus and alters its actin structure to form a dome-like anchoring site. Moreover, Kaper *et al*. [36] enlightened the ability of *E. coli* O157:H7 to induce cytoskeletal rearrangements in the infected epithelial cells resulting in attaching and effacing lesions.

E. coli O157:H7 produces Shiga toxins in situ that cross the intestinal barrier to blood stream and attack the vascular epithelium possessing globotriaosyl ceramide (Gb3) receptors. Shiga toxins induce epithelial cell death, resulting in focal hemorrhages at the degenerated and necrotic renal tubules [21]; Figures 2-A and 2-B show Shiga toxins pathogenicity in infected mice kidneys.

Immunologically, *L. acidophilus* La-5 orchestrated innate and adaptive immunity to eliminate *E. coli* O157:H7 from mice after oral inoculation with minimal suffers. *L. acidophilus* La-5 administration 15 days pre-infection prepared phagocytes well [37] for the battle against *E. coli* O157:H7. Further to infection phagocytes sustained with high phagocytic activity and phagocytic index after infection when compared with mice infected group without *L. acidophilus* La-5 treatment at 0, 3rd and 7th day post infection (Table 2).

Then adaptive immunity pursued phagocytic activation, spread its militaries and significant humoral immune response (IgG) that eliminated the infection entirely by the 14th day post infection (Table 3) that is in concordance with Shu and Gill [38].

We found that phagocytic activity, phagocytic index and humoral immune response inversely correlated with bacterial shedding (Table 1). *L. acidophilus* La-5 significantly reduced *E. coli* O157:H7 shedding in *L. acidophilus* treated group than those in infected group at 2nd and 7th days post infection and then totally eliminated the infection at the 14th day. Finally *L. acidophilus* La-5 protected mice life in the lactobacillus treated group against 33.3% mortality in the infected group.

We could conclude that multi-resistant *E. coli* O157:H7 threats calves' health. *L. acidophilus* La-5 overwhelmed *E. coli* O157:H7 pathogenicity against intestinal and kidney tissues in murine model via know-hows physical and immune modulating effects. Subsequently food inclusion of *L. acidophilus* La-5 could improve calves' health and reduce *E. coli* O157:H7 shedding; potential foodborne pathogen that could jeopardize human life.

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