STUDY OF EFFICIENCY FOR *LACTOBACILLUS FERMUNTUM* ON *CRYPTOSPORIDIUM PARVUM*

Shaima D. Salman and *Sabaa T. Mohammed*

*Biology Department, College of Science, Al-Mustansyria University, Baghdad, Iraq.

**ABSTRACT**

This study include collected 60 feces sample from calves suffering from severe diarrhea, *Cryptosporidium parvum* oocyst isolation and purification from feces samples, then the oocysts were used to induce experimental infection in the Immuno suppressed mice. The mice divided into two groups: treatment group and preventive group, the treatment groups inoculated with suspension of bacteria *L. fermentum* (1×10^8 cell / 0.1ml), supernatant (0.1ml) and (0.01 ml) of spiramycin drug, the preventive groups had inoculated with suspension of servant bacteria for a period of 10 days before induced infection. The results showed that the preventive groups had a highest efficiency treatment reached (85.91%) and stopped shedding oocysts on the (7th) day after the treatment, while groups treated with suspension of *L. fermentum* had efficiency treatment was (81.44 %) and stopped shedding oocysts of parasite on the (7th) day but in groups treated with supernatant of bacteria had efficiency treatment (76.33%) and stopped oocysts shedding on the 10th day, treatment groups with spiramycin drug stopped shedding of parasite oocysts till 11th day after treatment and recorded less efficiency treatment reaching (65.14%). Histopathological study, showed that small intestine tissue in the preventive group return to normal forms. The treatment groups with bacteria and it supernatants had simple changes included hydropic degeneration and increase in the number of goblet cells when compared to a group spiramycin which suffered from damage in the mucous layer and the shorten in lengths of the villi with necrosis.

**KEYWORD:** *Cryptosporidium parvum, L. fermentum, probiotic.*
1. INTRODUCTION
Cryptosporidiosis is a zoonotic and anthroponotic disease which caused by protozoan parasites genus *Cryptosporidium parvum*, it has a worldwide major pathogens causing diarrheal diseases in children.\(^1\) It has a complex homogenous life cycle, it is able to complete its life cycle in a single host\(^2\), development of parasite occurs within the brush border of the mucoepithelial cells of the small intestine.\(^3\) Infection by oocysts orally through contaminated food or water, the disease can be asymptomatic or cause acute diarrhea or persistent diarrhea that can last for a few weeks, it is usually watery with mucus.\(^4\)

The common therapy consists Nitazoxanide and Spiramycin can help shorten the amount of time oocysts are passed as well as the duration of diarrhea.\(^5\) The term probiotic is currently used to name ingested microorganisms associated with beneficial effects to humans and animals,\(^6\) many studies have provided evidence that probiotics can also effectively modulate the gut immune system in health and disease.\(^7\) *Lactobacillus* friendly bacteria that normally live in our digestive, urinary, and genital systems without causing disease.\(^8\) It used for treating and preventing diarrhea, including rotaviral diarrhea in children and traveler's diarrhea.

*L. fermentum* is a Gram-positive species of bacterium in the genus *Lactobacillus* it has double functional properties: antimicrobial activity against intestinal pathogens and high total antioxidative activity.\(^9\) The aim of this research was to study the effect of bacterial suspension and supernatants from *Lactobacillus fermentum* as a treatment of *C. parvum* infection and using the same bacteria as a prevent from the *C. parvum* infection.

2. MATERIALS AND METHODS
2.1 Feces Samples
Sixty stool samples were collected from calves suffering from severe diarrhea, in Veterinary college of Baghdad university. Stool samples of animal’s rectum were collected directly and saved in tightened plastic cold container till arrived to the lab.

2.2 Microscopic Examination
Glass slides were prepared from each fecal sample by direct smear method, the positive slide fixed by methanole and stained by modified cold Zehil Neilsen stain\(^10\), then examined under oil immersion(1000 X) to detect the presence of oocyst.
2.3 Isolation and purification oocysts
The oocyst were isolated and purified from the positive sample by using saturated salt solution as Ungar et al method 1986.[11] The oocysts were counted and adjusted in (1x10^4 oocyst /0.1 ml) by using hemocytometer.

2.4 Preparation of free bacterial cells and supernatant
*Lactobacillus fermentum* obtained from Dr. Jehan Abed Al-satar assistant prof. in Department of biology, collage of science, Al mustansiriyah University which isolated from women vagina.

Two bacteria *L. fermentum* inoculated in MRS broth and incubated for 18hr at 37c in un aerobic jar, then the culture was centrifuged at (10000 rpm) for 10min. Supernatant was removed and filtered through (0.22-Mm) pore size filters and concentrated[12], while bacterial cells were taken, washed and suspended in to contain (1×10^8 cell / 0.1ml), then stored in refrigerator to use later.[13]

2.5 Experimental study

2.5.1 Animals
Males of albino mice were obtained from pharmacy college – Baghdad university, their ages were between 12 -14 weeks with weights between (16-22 gm). Mice feces were examined before starting experiment to insure the intestinal vacancy of parasitic infections.

2.5.2 Experimental design
A thirty six mice were Immunosuppressed by dexamethazone according to Regh[14], after (5 days), then the mice divided into six groups. (30) mice of them were inoculated orally by stomach tube with (0.1ml) contain (1x 10^4 oocysts) which prepared previously and keeping twelve of them healthy and not infected as negative control group and Preventative group. After 3 days of inoculation, stool of the mice examined separately to prepare direct smear, after confirmation of infection the infected mice which sub divided into three groups (treatment groups) each one contain(6) mice. The mice put in separated cages, then Groups inoculated as following.

* Group (G1)
The mice of this group were given (0.1ml) of *Lactobacilus fermentum* which contain (1x10^8cell/ml/day) for the duration of the experiment days by using stomach tube.[15]
* **Group (G2)**
The mice of this group were given (0.1ml) of supernatants *Lactobacillus fermantium* for the duration of the experiment days.

* **Group (G3)**
Mice in this group were inoculated with (0.1ml) of spiramycin for the duration of the experiment days.

* **Group (G4)**
The mice of this group were given (0.1ml) of *Lactobacillus fermantium* which contain (1x10^6 cell/ml) for (7) days, then inoculated orally with (1x 10^4 oocysts) to infect mice after 3 days return inoculated with (0.1ml) of *Lactobacillus fermantium*.

* **Group (G5)**
The mice of this group were given orally (0.1ml) of normal saline and consider as a positive control to histopathological study.

* **Group (G6)**
The mice were given orally (0.1ml) of normal saline. This group not infected as negative control.

- During period of experiment the following data had been recorded:
  1- recording any clinical obvious sign.
  2- recording perpant period.
  3- feces examination to calculate of oocyst in one gram every day by using equation of Ryan *et.al.*[16]
  4- sufficient treatment for bacterial, supernatant and spiramycin was measured according to Xiao *et.al.*, 1996.[17]

**2.6 Histopathological study**
All mice in six group were sacrificed then small intestine was removed aseptically, fixed in 10% buffered formalin, processed stained with haematoxylin and eosin and were examined under the light microscope.[18]
2.7 Statistical analysis
The Statistical Analysis System- SAS (2012) was used to effect of different factors (group and day) in number of c. parvum oocysts raised x $10^2$. Least significant difference –LSD test was used to significant compare between means in this study.

3. RESULT AND DISCUSSION
In this study no clinical signs were record in infected or treated groups, and preplant period recorded in all infected mice was between (3-4) days for all groups. The results noticed that orally inoculation of L.fermentum for infected mice led to reduced the shedding of parasite in stool of mice since the first day of treatment in each groups were (12.16, 13.00, 9.83 and 12.50 x$10^2$ cell /gm) in group (G1,G2,G4 and G3) respectively, and continue to decrease gradually with days till stopped shedding of parasite and became (zero) in 7th day post inoculation in ( G1 and G4) , G2 in day 10th while G3 in 11th day post inoculation with comparative to G5 which continued oocysts shedding until the end of experiment .

There was high significant difference between control group and treated group(P≤0.05). additionally, there was statistical difference between treated group (G1,G2,G3,and G4) (P≤0.05) .

The results reported in figure (1) showed the sufficient treatment for L.fermentum, and spiramycin, which calculated depended on number of oocysts that shedding during the period of treatment for each group, regardless of the time it takes to cure or numbers of days the mice stopped shedding of oocysts. that the highest percentage of treatment efficiency was for preventative group of L.fermentum (85.91%) followed by L.fermentum cells and (81.44%) while the supernatant groups recorded only(75.28 % ), the lowest treatment efficiency for spiramycin was (65.13%).

In general the activity of probiotics to prevented and treated pathogen represented by covering the surface of epithelial tissue for intestine ,Secretion of active molecules (e.g. bacteriocins, antibiotics, free fatty acids and hydrogen peroxide), Modulation of the intestinal environment and changing pH.\[19\]

oral administration L. fermentum can enhance T cell differentiation and induce ileum cytokine expression and could modulate immune function in piglets.\[20\]
Lactobacillus fermentum releases surface-active components which can inhibit adhesion of uropathogenic bacteria.\cite{21} Pickerd and Tuthill\cite{22} found that daily ingestion of L. reuteri was efficient to prevent C. parvum intestine colonization and tissue lesions in a host with a deficient immune system.

The study done by Mohammed 2013\cite{23} reported that the oral administration of Bifidobacterium 7 days before histolytica inoculation reduced the cyst excretion in mice since first day and became histolytica free by day 8\textsuperscript{th} post inoculation.

Table (1) Number of C. parvum oocysts in L. fermentum, supernatant, Spiramycin and control groups x 10\textsuperscript{2}.

<table>
<thead>
<tr>
<th>Group</th>
<th>G1</th>
<th>G2</th>
<th>G3</th>
<th>G4</th>
<th>G5</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12.16 ± 0.47</td>
<td>13.00 ± 0.57</td>
<td>12.50 ± 0.42</td>
<td>9.83 ± 0.31</td>
<td>12.50 ± 0.56</td>
</tr>
<tr>
<td>2</td>
<td>9.83 ± 0.40</td>
<td>10.66 ± 0.33</td>
<td>11.83 ± 0.40</td>
<td>7.50 ± 0.42</td>
<td>13.16 ± 0.40</td>
</tr>
<tr>
<td>3</td>
<td>7.50 ± 0.22</td>
<td>8.83 ± 0.31</td>
<td>10.33 ± 0.21</td>
<td>5.33 ± 0.49</td>
<td>15.83 ± 0.40</td>
</tr>
<tr>
<td>4</td>
<td>4.83 ± 0.31</td>
<td>6.83 ± 0.31</td>
<td>9.33 ± 0.42</td>
<td>3.83 ± 0.31</td>
<td>17.50 ± 0.34</td>
</tr>
<tr>
<td>5</td>
<td>2.50 ± 0.22</td>
<td>5.50 ± 0.43</td>
<td>8.50 ± 0.22</td>
<td>2.00 ± 0.25</td>
<td>19.83 ± 0.31</td>
</tr>
<tr>
<td>6</td>
<td>1.00 ± 0.25</td>
<td>2.83 ± 0.31</td>
<td>7.17 ± 0.47</td>
<td>0.66 ± 0.21</td>
<td>19.33 ± 0.61</td>
</tr>
<tr>
<td>7</td>
<td>0.00 ± 0.00</td>
<td>1.50 ± 0.22</td>
<td>5.83 ± 0.31</td>
<td>0.00 ± 0.00</td>
<td>21.16 ± 0.65</td>
</tr>
<tr>
<td>8</td>
<td>0.00 ± 0.00</td>
<td>1.33 ± 0.21</td>
<td>3.66 ± 0.49</td>
<td>0.00 ± 0.00</td>
<td>21.83 ± 0.60</td>
</tr>
<tr>
<td>9</td>
<td>0.00 ± 0.00</td>
<td>0.67 ± 0.21</td>
<td>2.16 ± 0.31</td>
<td>0.00 ± 0.00</td>
<td>20.83 ± 0.54</td>
</tr>
<tr>
<td>10</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.83 ± 0.16</td>
<td>0.00 ± 0.00</td>
<td>22.33 ± 0.66</td>
</tr>
<tr>
<td>11</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>22.66 ± 0.80</td>
</tr>
<tr>
<td>Mean</td>
<td>37.82</td>
<td>51.15</td>
<td>72.14</td>
<td>29.15</td>
<td>206.96</td>
</tr>
</tbody>
</table>

* (P≤0.05).

Figure (1): Comparison between different groups in treatment efficiency.
Histopathological study was done to determine the affection of *L. fermentum* on intestinal tissue which occur in mice infected by *C. parvum* compared with affection of spiramycin. The occur of parasite on the brush border of intestine epithelial layer of control positive group, with simple hydropic degeneration were seen in figure (2) in comparison with control negative group see figure (3).

Inoculated mice with *L. fermentum* or its supernatant caused simple changes includes hydropic degeneration and increase the number of goblet cell. See fig.(4), while in preventive groups the tissues return to normal forms see fig.(5) compared with spiramycin group which caused severe damage in mucosal tissue, infiltration in lymphocytes occur and hydropic degeneration see figure (6), also the villi length became shorter with necrosis see figure (7). Peran et al.\[24\] observed that administration of the probiotic *L. fermentum* facilitates the recovery of the inflamed tissue in the rat colitis, an effect associated with increased levels of glutathione and improvement of the production some mediators which involved in the inflammatory response of the intestine, such as TNF alpha. Toumi et al.\[25\] showed that modification of micro flora by the *Lactobacillus* sp. played a beneficial role in maintaining the integrity of the intestinal mucosal barrier and promoted tissue repair. Also Pelicano et al.\[26\] observed that birds fed with *Bacillus subtilis* as probiotic were higher villi in jejunum and ileum and Greater crypt depths comparative with the control group.

![Figure (2): Hydropic degeneration in small intestine of infected mice control positive (H&E; 400X).](image-url)
Figure (3): Section in normal small intestine of mice (contral–ve) (H&E; 100X).

Figure (4): Increase in number of goblet cells after treated with (bacteria or supernatant) (H&E; 400X).
Figure (5): small intestine look normal in preventive group (H&E; 400X).

Figure (6): Infiltration in lymphocyte and necrosis in mice intestine treated with(sprimycin) (H&E; 400X).
Figure (7): damage in mucosa tissue and villi became shorter with hydropic degeneration in mice intestine (H&E; 400X).

REFERENCES


15. Alak,J.,I.B.W.; Wolf,E.G.and Mdurvwa, A. ESupplementation with Lactobacillus reuteri or L. acidophilus reduced intestinal shedding of Cryptosporidium parvum oocysts in immunodeficient C57BL/6 mice. Cellular and Molecular Biology., 1999; 45(6): 855–863,


