Control of Cryptosporidiosis by Probiotic Bacteria

Magda M. Sanad, Jamila S. Al-Malki, and Areej G. Al-Ghabban

Abstract—Cryptosporidiosis, an opportunistic parasitic disease, has become a topic of great interest being life-threatening among immunocompromised patients worldwide. To date, there is no totally effective therapy other than a healthy intact immune system. Probiotics, widely reported to stimulate both innate and acquired immunity at mucosal and systemic levels, were investigated in the present study for the therapeutic effects of daily administration of a mixture of L. acidophilus, L. helveticus and Bifidobacterium bifidum against Cryptosporidium parvum (genotype 2) infection in an immunosuppressed mouse model. Although complete eradication of the parasite was not achieved, but the used probiotics mixture induced significant reduction in parasite burden, ultrastructural changes in relation to parasite attachment, and internalization to epithelial cells, partial reparation of the parasite-induced intestinal mucosal damage and increase in the serum level of IFN-γ. These results reveal beneficial effects of probiotics upon cryptosporidiosis and indicate that they could help in reducing the risks of severe disease in immunosuppressed patients. Hopping for a more beneficial role against cryptosporidiosis, further studies are recommended to exploit the role of specific strains in therapeutic intervention, and possibility of their combination with chemotherapeutic agents.

Keywords—Cryptosporidium parvum (genotype 2), immunosuppressed mouse model, probiotic bacteria, parasite burden, intestinal mucosa, electron microscopy, IFN-γ.

I. INTRODUCTION

C. parvum has gained recognition as a prominent enteropathogen of humans and animals. It is an obligate intracellular protozoan parasite of worldwide distribution in the environment, and proven to have potential to cause watery diarrhea among children, immunocompromised adults and newly born animals particularly calves. The severity of clinical signs depends on the immune status of the patient. In immunocompetent individuals, the infection is self-limiting and patients can clear parasites spontaneously within a few weeks (1). In immunocompromised and immunosuppressed hosts such as HIV patients, the infection is more severe; leading to prolonged and persistent diarrhea with extensive alterations of the intestinal epithelium and can be life threatening (2). Protection against this parasite has been largely associated with cell-mediated immune response and production of interferon gamma (IFN-γ) (3-4). Epithelial cells of the intestinal mucosa surface has been shown to play an important role in initiation of the mucosal immune response (5,6). Epithelial cells, once infected, have increased expression of inflammatory chemokines and cytokines and demonstrate antimicrobial killing mechanisms, including production of NO and antimicrobial peptides (7). Besides the definite role of immunity, there is a clear evidence that resistance to C. parvum infection can be mediated by non-specific mechanisms associated with the presence of intestinal flora. In other words, colonization of the intestine by Cryptosporidium depends on the intestinal microflora because newborn or adult germ-free mice are far more susceptible to this parasite than flora-bearing adult mice (8).

To date, there is no totally effective and approved therapy for cryptosporidiosis and a healthy intact immune system seems to be the only solution. Probiotics, defined as live microorganisms that when administered in adequate amount confer a health benefit on the host (9), have been shown to stimulate and regulate natural and acquired immune responses and to enhance the mucosal barrier (10-13). The use of probiotics in the treatment of specific diseases has evolved into an extremely valuable option yet to be optimally used in clinical medicine. Although modulation of Cryptosporidium parvum infection by various probiotic bacteria species had been controversially reported in immunodeficient (14) and immunocompetent (15) animal models and in cell cultures (16), but probiotics had been successfully used for treatment of a girl with cryptosporidiosis (17). Promising health benefits and efficacy of probiotics for preventing and treating a variety of diseases attract increasing attention to use probiotics and probiotic-derived factors for novel therapies. So, the aim of the present work is to study the therapeutic efficacy of consumption of a mixture of L. acidophilus, L. helveticus and Bifidobacterium bifidum on experimental cryptosporidiosis using immunosuppressed mouse model.

II. MATERIALS AND METHOD

A. The parasite

C. parvum (genotype 2) oocysts (originally isolated from fecal samples of infected calves) were maintained in suckling Swiss albino mice at Zoology Department, Faculty of Science, Al Taif University. Oocysts from feces of infected mice were collected and concentrated by the sugar centrifugal floatation method after being stored in 2.5% potassium dichromate solution at 4°C to be used within one month.

B. Experimental animals

A total of 60 Laboratory-bred Swiss albino mice, 1-2

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months old and free from intestinal parasitic infections were immunosuppressed (I.S.) by hydrocortisone acetate (25 mg/ml.) in a daily intramuscular dose of 0.1 ml/mouse for 5 weeks.

C. Experimental infection

Oocysts were washed with phosphate buffered saline (pH 7.4) by centrifugation at 670 g for 7 minutes just before inoculation. Infection was done on the 14th day of immunosuppressive therapy by oral inoculation of $10^5$ oocysts/mouse in 0.1 ml PBS.

D. Administration of the probiotics

A commercial mega Acidophilus, obtained from GNC Inc., Saudi Arabia, was provided as capsules each containing 1.5 x $10^9$ CFU of a mixture of three probiotic bacteria species: Lactobacillus acidophilus; Lactobacillus helveticus and Bifidobacterium bifidum. The orally inoculated daily dose / mouse was freshly prepared and adjusted to a concentration of 20 x$10^5$ CFU in 0.1 ml PBS.

E. Animal groups

A total of 60 immunosuppressed mice were maintained in the laboratory as 4 groups, 15 mice each: Gr. I (infection control): only infected, Gr. II: (treatment control): only treated, once daily for 3 weeks from the 14th day of immunosuppression therapy until sacrifice, Gr.III (early treatment): infected and treated once daily from the 1st to the 21st post-infection (P.I), Gr. IV (late treatment): infected and treated once daily from the 7th to the 21st post-infection (P.I) day. All procedures including euthanasia procedure were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, Institute for Laboratory Animal Research (NIH Publications No. 80-23; 1996) and the Ethical Guidelines of the Experimental Animal Care Center (Al Taif University, Saudi Arabia)

III. ASSESSMENT OF THERAPEUTIC EFFICACY OF THE PROBIOTICS

1. Determination of intensity of infection by:

   a. Determination of Cryptosporidium oocysts count:

      Three mice from each of the infected groups were subjected to collection of fresh fecal samples of each individual mouse on days 6, 11, 16, 21 P.I. Counting of shed oocysts in 10 microscopic fields (x400) of a modified Zeil Neelsen stained smear, calculation of the mean oocyst count and the percent reduction in each group was determined.

   b. Determination of parasite count in stained ileal sections:

      Three mice from each of the infected groups were dissected on days 6, 11, 16, 21 P.I. and 1-cm fragments from the distal ileum were collected, fixed in 10% buffered formalin, processed to paraffin sections and hematoxylin and eosin staining. Parasite stages were counted in 10 microscopic fields (x 400).

2. Quantification of mouse IFN-γ in the serum:

   At the end of the probiotics treatment period, exsanguinations of mice from all groups (3/group) were subjected for separation of sera. Concentrations of IFN-γ were determined by commercially available ELISA Kit (Genzyme, Thermo Fisher Scientific, USA). Detection limit = 100 pg/ml. IFN-γ assay in duplicate.

3. Histopathological study:

   At the end of the probiotics treatment period, hematoxylin and eosin stained ileum sections from mice of all groups (3/group) were examined microscopically for histopathological changes.

4. Transmission electron microscopy (TEM) study:

   At the end of the probiotics treatment period, small tissue samples from the distal ileum of sacrificed mice (3/group) were collected and immediately fixed in cold buffered glutaraldehyde (2.5 %) in 0.1 M phosphate buffer (pH 7.4) for 1-4 days. Post-fixation was done in 1% O$_3$O$_4$ in the same buffer at 4°C for 2-3 hours, transferred to 1% uranyl acetate and dehydrated in ascending grades of ethyl alcohol to be embedded in Epon resin. Ultra-thin sections were stained with uranyl acetate and lead citrate and examined for morphological changes in invasive parasitic stages using a Zeiss EM 906 transmission electron microscope.

IV. STATISTICAL ANALYSIS

Data were computerized and statistically analyzed using the arithmetic mean and standard deviation, Chi square test and one way ANOVA.

V. RESULTS

Oocysts count (Tables 1 & 2):

In the present study, the infecting dose of $10^5$ oocysts/mouse achieved 100% take up of infection. The early treated mice (Gr.III) showed significant (P<0.05) decrease in the number of oocysts shed on 6, 11, 16 and 21 P.I. days in comparison with the non-treated (Gr. I) and the late-treated (Gr.IV) groups. In addition, the early treated group showed significantly (P<0.05) percent reduction in oocysts shedding in comparison to the late treated one.

<table>
<thead>
<tr>
<th>TABLE I</th>
<th>CRYPTOSPORIDIUM OOCYSTS COUNT AMONG IMMUNOSUPPRESSED (I. S.) MICE TREATED WITH PROBIOTICS VERSUS UNTREATED CONTROL AT DIFFERENT POST-INFECTION DAYS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Groups</td>
<td>I.S., untreated (Gr. I)</td>
</tr>
<tr>
<td>P.I day</td>
<td>oocysts count 10 HFP (mean = SD)</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------</td>
</tr>
<tr>
<td>6</td>
<td>a 110.33 ± 16.81</td>
</tr>
<tr>
<td>11</td>
<td>a 121.66 ± 33.82</td>
</tr>
<tr>
<td>16</td>
<td>a 173.72 ± 22.10</td>
</tr>
<tr>
<td>21</td>
<td>a 98.56 ± 14.65</td>
</tr>
</tbody>
</table>

No of examined mice at each time point / group = 3 (a, b, c): means bearing different letter within the same day are significantly different
Table II

Percent reduction in Cryptosporidium oocyst shedding among I.S. treated groups at different post-infection days

<table>
<thead>
<tr>
<th>P.I. days</th>
<th>I.S. treated early (Gr.III)</th>
<th>I.S. treated late (Gr.IV)</th>
<th>Statistical analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>b 57.2 ± 0.3</td>
<td>a 79.2 ± 0.6</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>11</td>
<td>b 46.3 ± 1.7</td>
<td>a 79.2 ± 0.6</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>16</td>
<td>b 46.4 ± 1.5</td>
<td>a 79.2 ± 0.6</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>21</td>
<td>b 70.0 ± 0.9</td>
<td>a 26.4 ± 0.5</td>
<td>P &lt; 0.05</td>
</tr>
</tbody>
</table>

(a, b): means bearing different letter within the same day are significantly different

Table III

Number of parasitic stages in ileum sections of immunosuppressed (I.S.) groups treated with probiotics versus untreated control at different post-infection days

<table>
<thead>
<tr>
<th>P.I. days</th>
<th>No. of parasitic stages in ileum sections (mean ± SD)</th>
<th>Statistical analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>1.9 ± 0.3</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>11</td>
<td>1.9 ± 0.2</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>16</td>
<td>1.9 ± 0.1</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>21</td>
<td>1.9 ± 0.0</td>
<td>P &lt; 0.05</td>
</tr>
</tbody>
</table>

No of examined mice at each time point / group = 3 (a, b, c): means bearing different letter within the same day are significantly different

Histopathological changes (Table IV and Figs 1-3):

The non-treated infected control (Gr. I) showed severe mucosal and submucosal inflammatory changes represented by shortening, broadening and fusion of villi; hydropic degeneration, edematous and exudative inflammation, desquamation and sloughing of intestinal epithelial cells and evident vacuolated epithelial cells due to complete cell necrosis. In both early and late treated groups, partial regression of edematous and necrotic changes in intestinal epithelial cells and improvement in villous architecture were noticed at the end of the probiotics administration period. In the non-infected treated control (Gr.II), no pathological changes were noticed.

Table IV

Number of parasitic stages in ileum sections of immunosuppressed (I.S.) groups treated with probiotics versus untreated control at different post-infection days

<table>
<thead>
<tr>
<th>Animal group</th>
<th>Lesion</th>
<th>Decrease villus height</th>
<th>Increase crypt depth</th>
<th>Epithelial cell sloughing</th>
<th>Villous fusion</th>
<th>Cellular infiltration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infection control (Gr.I)</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Treatment control (Gr.II)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Infected &amp; treated early (Gr.III)</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Infected &amp; treated late (Gr.IV)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

-absent; + mild to absent; + mild; ++ moderate; +++ severe

Serum level of mouse IFN-γ (Table V):

Sera of treated mice (Gr.II, Gr.III, Gr.IV) showed insignificant (P > 0.05) increase in the level of IFN-γ in comparison to the recorded level in sera of the infected non-treated control (Gr. I).

Table V

IFN-γ level in sera of different I.S. studied groups

<table>
<thead>
<tr>
<th>Mouse groups</th>
<th>IFN-γ level (pg/ml) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infection control (Gr.I)</td>
<td>a 234 ± 6.4</td>
</tr>
<tr>
<td>Treatment control (Gr.II)</td>
<td>a 437 ± 15.1</td>
</tr>
<tr>
<td>Infected &amp; treated early (Gr.III)</td>
<td>a 416± 18.2</td>
</tr>
<tr>
<td>Infected &amp; treated late (Gr.IV)</td>
<td>a 388 ± 35</td>
</tr>
</tbody>
</table>

Statistical analysis P > 0.05

(a): means bearing the same letter within the same column are insignificantly different
Electron microscopy results (Figs 4-5):

In the non-treated control group zoites were mostly located among the microvilli, attached to the enterocytes surface and surrounded by a four-membrane complex (MC), the most inner one appeared more electron-dense at its apical end. In attached forms, the parasite pellicle was mostly separated from the host cell membrane at the base of the parasite, membrane fusion was seen at the parasite-cell contact (with some serrations) and the pellicle and host cell membrane became unclear. The margin of attachment area showed high electron density (DB). A vacuole was detected in the cytoplasm at the base of the zoite "representing feeder organelle" (FO) attached to the host cell membrane. Each parasite was existed in a parasitophorous vacuole (PV) and completely covered with a double-layered membrane derived from numerous microvilli (MV) that elongated besides and around the parasite.

In treated groups a lot of extracellular stages were noticed and evident ultrastructural changes were seen in the invasive non-attached zoites. These were represented as lacking normal integrity with degeneration of their contents, a fuzzy uneven outer surface with more electron dense serrations in some zones, ill-defined nucleus, ill-defined parasite membrane, less electron dense area surrounding the cytoplasm and incomplete covering by markedly redundant microvillous membranes.

Fusion of parasite and host-cell membranes was seen at the parasite-cell contact forming a dense band (DB). An eccentric rounded to ovoid nucleus, nucleolus and rough endoplasmic reticulum were seen. Bar = 2μm

Fig 4-d Transmission electron micrograph showing section of C. parvum zoite from the non-treated I. S. group (Gr.I), attached to the enterocytes membrane. A vacuole was detected in the cytoplasm at the base of the zoite representing feeder organelle (FO) attached to the host cell membrane. Bar = 1μm

VI. DISCUSSION

To date, the infection of cryptosporidiosis in the immunocompromised patients is largely out of treatment. Immunocompetent individuals can get rid of the infection because parasite eradication relies on innate and acquired immunity. So, in the preset study, mouse model was maintained immunosuppressed throughout the experiment and probiotic treatment was used to promote human health due to...
their effects on luminal microbial ecology and immune modulation, particularly through balance control of pro-inflammatory and anti-inflammatory cytokines (18). Also, probiotics are important to stimulate the proliferation of mucosal epithelial cells which are considered as the first line of defense against intestinal pathogens (19). In the present study, a mixture of Lactobacillus acidophilus; Lactobacillus helveticus and Bifidobacterium bifidum was assessed because these species are most widely used to prevent and treat intestinal disorders (20, 21). The significant reduction in the number of oocysts and in percent reduction in oocysts shedding detected in fecal samples collected from the early treated group on days 6- 21 P. I. in comparison to the late treated mice, together with the significant decrease in the number of endogenous parasite stages in ileum sections along the intestinal mucosal surface of the early treated mice in comparison with the non-treated, all these results confirm the effective therapeutic role of probiotic bacteria used in the present study against Cryptosporidium and are in agreement with Mc Donald,et al (7) and Alak et al (14). Histologically, morphological and cellular alteration of microvillus membrane integrity revealed that probiotic administration ameliorated the mucosal damage in both early and late probiotic- treated I S mice, compared with the severe microvillus damage, edematous and vacuolated epithelial cells in non-treated mice. These results clearly show the anticryptosporidial effect of the probiotics in vivo by modulating the gut cells to inhibit the colonization and multiplication of Cryptosporidium, thus reducing the severity of cryptosporidiosis.

Gupta and Grag (22) mentioned that probiotics induce modulation of the intestinal tract by having the capacity to control the proliferation of surrounding microorganisms. Wollowski et al (23) and Madsen (24) reported that probiotic bacteria influence the mechanisms of innate immunity and the activation of B cells for mucosal immunity important for protection against pathogens, prevention of the penetration by foreign antigens, and maintenance of mucosal homeostasis. Close results were reported by Alak et al (25) since daily ingestion of L. reuteri was efficient to prevent C. parvum intestine colonization and tissue lesions in immunodeficient mice. Waters et al. (26) suggested that protection was due to secretion of as yet unidentified antimicrobial products. Arvola et al (27) added that probiotics have been shown to modulate release of cytokines (TNF-α IFN-γ, IL-10, IL-12) which play a central role in maintaining the delicate balance between necessary and excessive defense mechanisms. Gill (28) reported that the underlying mechanisms are however not clear, involving stimulation of different subsets of immune system cells to produce cytokines, which in turn play a role in the induction and regulation of the immune response, and to enhance intestinal IgA immune responses and increase intestinal mucin production. Similarly, Borchers et al. (29) found that IL-10 and secretory IgA response, important actors in an efficient anti-Cryptosporidium immune response, have been shown to be induced by some probiotic strains. In the present study, the increased serum level of IFN-γ inflected treated groups is in agreement with Mead and You (30) and Riggs (31) who found that protection against this parasite has been largely associated with production of IFN-γ; a major player not only in cell-mediated immunity, but in early innate immune responses as well. In vitro studies have demonstrated that IFN-γ directly prevents the parasite from invading host cells [32]. Additionally, a case of persistent cryptosporidiosis has been described in a child with primary IFN-γ deficiency [33], suggesting that this cytokine is also important in human infections. The important protective roles for IFN-γ may also include apoptosis induction in infected intestinal epithelial cells, and modulation of mucosa epithelial integrity [6].

In the present work, the ultrastructural changes seen in the invasive non-attached zoites may suggest interference with gliding motility of the sporozoites and affection of the membrane-associated proteins located in apical organelles of C. parvum sporozoites. Chen et al (34) and Sibley (35) provided evidence that blockage of apical discharge by the inhibition of parasite actin and tubulin polymerization and the chelation of intracellular calcium in C. parvum sporozoites significantly decreased the gliding motility of the parasite.

In conclusion, this study provides evidence that a mixture of the probiotics; L. acidophilus, L. helveticus and Bifidobacterium bifidum; is a good choice to improve the mucosal immune system and may have implication in the therapy of cryptosporidiosis during immunosuppressive states. However, further investigations are needed to move forward in this direction, probiotics have to be precisely characterized at a strain level and mechanisms through which they work have to be elucidated.

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