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Probiotics as a Promising Treatment of Experimental Cryptosporidiosis in an Immuno suppressed Mouse Model

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A B S T R A C T

Cryptosporidium parvum causes disease both in immune competent and immune compromised people. Probiotics are viable nonpathogenic micro organism that have beneficial effects in the prevention and treatment of pathological condition. The concept of using probiotics therapy has opened up new angles on the role of gut microflora in disease prevention. *Lactobacillus casei* are commonly found in fermented dairy products such as yogurt which exhibit probiotic properties. The aim of this research was to gain insight into the potential immune modulating effects of *lactobacillus casei* and yogurt on *C. parvum* infection in immuno suppressed mice. In this study, seventy mice were used; Sixty mice were immuno suppressed and infected with *Cryptosporidium* oocysts and ten mice were left immuno competent and not infected. Then, mice were divided into three groups; group (1) was infected and treated with *Lactobacillus casei*, group (2) was infected and treated with yogurt and group (3) infected and not treated (control). The progress of cryptosporidiosis was assessed by counting oocyst in the stools of mice and developmental stages in histopathological sections of ilea. The results showed that daily administration of yogurt and *Lactobacillus casei* was able to decrease the parasitic burden in mice as compared with non-treated group. It was observed that the yogurt was more efficient than *L. casei* as group (2) stopped oocyst shedding earlier than *L. casei* and also oocyst counts were lower along the duration of experiment as compared with group (1) treated with *L. casei*. The concept that probiotics could control the development of parasites is promising and hopeful.

Introduction

Cryptosporidiosis is classified by the Centers for Disease Control and Prevention as an emerging infectious pathogen (Guerrant, 1997). Initially, *Cryptosporidium parvum* was believed to be only an occupational hazard of people exposed to infected animals, but it is now known to cause illness or disease both in immune competent and immune compromised people. Cryptosporidiosisis one of the most common and certainly the most devastating gastrointestinal infection in people with AIDS (Mac Kenzie *et al.*, 1994).

Most human infections are thought to be caused by *C. parvum* or *C. hominis* although other *Cryptosporidium* species are reported to cause human infection, particularly inimmuno compromised individuals (Caccio, 2002). The propensity of the parasite to survive and be transmitted through water sources makes this important public health threat (Flynn, 2003).

In patients with AIDS, cryptosporidiosis is self-limited in individuals with CD4 cells count higher than 180 cells per cu.mm, chronic in patients with CD4 cell depletion to less than100 cells per cu.mm, and fulminant in some of those with count below 50 cells per cu.mm. (Colford *et al.,* 1996).

AS the numbers of immune suppressed patients from transplantation or chemotherapy increasing, cryptosporidiosis become an increasingly common problem and on a worldwide scale in malnourished infants and children. Management of cryptosporidiosis has been hampered by the look of effective drugs. Paromomycin and Nitazoxanide which can be proposed for the treatment of cryptosporidiosis, have only limited efficacy and cannot eradicate the parasite completely especially in immune suppressed patients (Smith *et al.,* 1998).

Besides the determinant role of immunity, there is clear evidence that colonization of the intestines by *Cryptosporidium* depends on the intestinal microflora as newborn mice or adult mice raised in germ free conditions are far more susceptible to this parasite than conventional adult mice (Foster *et al.,* 2003). These arguments altogether support the hypothesis that cryptosporidiosis can be controlled by administration of probiotics which seem able to modulate both innate and acquired immunity at the mucosal and systemic levels (Laura *et al.,* 2009).

Probiotics are viable nonpathogenic microorganism that, when ingested have beneficial effects in the prevention and treatment of pathological condition (Duggan *et al.,* 2002). The concept of using probiotics therapy has opened up new angles on the role of gut microflora in disease prevention. *Lactobacillus casei* are lactic acid bacteria (LBA) commonly found in fermented dairy products such as yogurt which exhibit probiotic properties (Canganella *et al.,* 1998).

It was documented that *Lactobacillus casei*, when given orally to mice, increases the immune response against pathogens such as *Salmonella* and *E. coli.* Also, *Bifidobacteria* have also been found to aid in anti-tumor activity in the host by stimulating the hosts immune response, lowering cholesterol level, synthesize vitamins, thiamin as well as folic acid (Seidavi *et al.,* 2008).

A good probiotic strain should confer a beneficial property (immune stimulation, protection against pathogens, metabolism, etc), be nonpathogenic, resistant to low pH and acids, thereby persisting in the intestine, and able to adhere to the gut epithelium. Most probiotic organisms are gram positive bacteria, isolated from the human gut microflora or various dairy products such as curd, lassi, and kulfi. However, probiotic beneficial effects have been more often demonstrated in model animals than by direct clinical evidences and depend largely on the dose ingested (Gupta and Garg, 2009).

The aim of this research was to gain insight into the potential immune modulating effects of *lactobacillus casei*and yogurt on *C. parvum* infection in immune suppressed mice.

Materials and Methods

Preparation of the *Cryptosporidium* inocula

Cryptosporidium oocysts were obtained from the faces of diarrheic calves. Stool specimens were examined for *Cryptosporidium* oocysts using safranin-methylene blue technique (Baxby *et al.*, 1984). According to Reese *et al.* (1982), freshly collected positive samples were suspended in saline, then concentrated by Sheather's sucrose floatation method and washed three times in phosphate-buffered saline (PBS) by centrifugation. Purified oocysts were kept at 4ºC until use. The number of oocysts in the suspension was adjusted to approximately 10^6oocysts/ml (Pavlasek, 1982).

Preparation of *Lactobacillus casei*

Lactobacillus casei was isolated from probiotic yogurt available in local markets according to Wood and Holzaptel(1995) method. *L. casei* were obtained by culturing on selective de Man-Rogosa-Shape (MRS) media (Oxoid) and identification was performed by colony morphology, Gram staining and API 50CHL kit with AB plus 4.0 software (BioMerieux, France) (Alak *et. al.*, 1999). Then ($1x10^8$) cell/ml of *Lactobacillus casei* suspension and its supernatants were prepared according to Contrerus *et al.* (1997).

Animals

In this study, laboratory inbred male albino Swiss mice were obtained, their ages were between 6–8 weeks with weights between 20–25gm. Mice feces were examined before starting experiment to insure the intestinal vacancy of parasitic infections.

Experimental Design

Seventy mice were used in this research. Sixty mice were immune suppressed and infected with *Cryptosporidium*oocysts and ten mice were left immune competent and not infected (acts as immune competent non-infected control group). Mice were immune suppressed by injection with (0.1)ml of dexamethasone/mice/day according to Regh (1996). Then, mice were divided into three groups each containing 20 mice:

Group (1): The mice of the first group were given 0.1ml of suspension of *Lactobacillus casei* which contain ($1x10^8$) cell/ml (for 10 days) before infection by using stomach tube. The dose is determined according to Alak *et al.* (1997). Then, mice were inoculated with ($1x10^4$) *Cryptosporidium* oocyst. Inoculation of *Lactobacillus casei* continued till the end of experimental period.

Group (2): The mice of the second group were inoculated with o.1ml of probiotic yogurt for (10 days) before infection. Then each mouse was inoculated with ($1x10^4$) oocyst, their feeding with yogurt continued till the end of experimental period.

Group (3): Mice in this group were inoculated with 0.1 ml of PBS for (10) days, then inoculated with suspension *Cryptosporidium* oocyst which contain ($1x10^4$) oocyst/ml inoculation the PBS continued till the end of experimental period. This group served as infected immune suppressed non-treated control group.

The effect of probiotics was assessed by counting *Cryptosporidium* oocysts shedded in the stools of mice. Counting started one day after infection and repeated every three days until stoppage of oocyst shedding.

Faces of each mouse were collected every three days, suspended in 10% formalin and homogenized. Smears were prepared and stained with safranin-methylene blue stain. The Smears were examined by light microscope and the numbers of oocysts per high power field (H.P.F.) was recorded. For each animal, ten microscopic fields were examined and the arithmetic mean of oocysts/H.P.F. was calculated and for each group of animals, results were given as the arithmetic mean of oocysts/H.P.F.

Also, one mouse of each group was sacrificed with every stool examination. Histopathological examination of the terminal ileum was done with counting of the developmental stages in the enterocytes of the intestinal villi. Ten microscopic fields were examined (Lemeteil *et al.,* 1992). The arithmetic mean of developmental stages /H.P.F. was calculated. Histopathological examination continued until no developmental stages were detected in ileum.

Statistical comparisons between groups were done. The data was coded and data entry was done using SPSS version 22. Frequencies, percentages, means and standard deviation were calculated. Inferential statistics such as t- test were used to compare the effect of treatment used between different groups.

Results and Discussion

In this study, we investigated the therapeutic effect of probiotic bacteria on the development and progression of experimental *Cryptosporidium* infection in immune suppressed mice. The efficacy of treatment was assessed by counting oocysts shedded in the stool of mice and developmental stages in the ilea of mice till the end of experiment.

The results showed that daily administration of yogurt and *Lactobacillus casei* was able to decrease the parasitic burden in mice as compared with non-treated group (Fig. 1).In group (1), treated with *L. casei*, the mean numbers of oocysts in stool decreased gradually until shedding completely stopped 15 days after infection.

Also, the mean numbers of the developmental stages in the ilea decreased with the same pattern as oocysts (Fig. 3) until could not be detected at the same period (15days post-infection) as compared with the control group (Fig. 2). In group (2), treated with yogurt, the mean numbers of oocysts in stool decreased gradually until shedding completely stopped 12 days post-infection. Also, the mean numbers of the developmental stages in the ilea decreased (Fig. 4) with the same pattern as oocysts until could not be detected at the same period (12 days post).

It was observed that oocyst shedding and developmental stages were lower in group (2) treated with yogurt than group (1) treated with *L. casei* (but with no significant differences between gr. 1 & gr. 2 regarding the mean numbers of oocyst counts and developmental stages at different days of investigation, $P > 0.05$). On the other hand, there were significant differences between group (1) and group (3) regarding the mean numbers of oocyst counts and developmental stages at different days of investigation (except the first day and the same between groups (2) & (3) but it was more significant than gr. (1) & (3) ($P < 0.0001$).

Table (1) & (2) showed the mean numbers of oocysts/H.P.F. in the stools of all groups and the mean numbers of developmental stages/H.P.F. in ilea of all groups along the duration of experiments with P values.

In this study, the effect of probiotics on the development and progression of cryptosporidiosis was investigated in immune suppressed mice that fed daily with *lactobacillus casei* bacteria and yogurt starting (10 days) before the infection and continued until clearance of the parasite.

It was observed that yogurt stopped oocyst shedding in stool earlier (12^{th} day) than *L. casei* (at 15^{th} day) and also oocyst counts were lower with yogurt. This may be attributed to the presence of more than one type of probiotic bacteria in yogurt like *Bifidium* and lactic acid bacteria (LAB)which act simultaneously giving more effect (Mohammed *et al.,* 2011).

In other studies, it was shown that, *Lactobacillus acidophilus* and *L. veuteri* have reduced the duration and number of *C. Parvum* oocyst shed in feces of experimentally infected mice [Alak *et al*., (1999), Heyman, (2000) and Foster *et al*., (2003)]. They suggested that certain LAB may possess potential therapeutic properties against *C. parvum*. Foster *et al.,* (2003) found that *Lactobacilli* and *Bifidobacterium* & their supernatant have a reduction effect on the counts and viability of *C. parvum* oocysts.

Researches on immune deficient mice have also suggested that treatment with probiotics can reduce the parasite burden in the intestinal epithelium during cryptosporidiosis (Waters *et al.,* 1999). Beneficial effects of probiotics upon cryptosporidiosis have been demonstrated in other studies. Waters *et al*. (1999) suggested that protection was due to secretion of unidentified antimicrobial products. In a study of Alak *et al*. (1999), adult mice fed daily with *Lactobacillus reuteri* strains 4000 and 4020 or *L. acidophilus* NCFM presented reduced oocyst shedding. They mentioned that this partial protection was not associated with an immune restoration (cytokines production). Daily ingestion of *L. reuteri* was also efficient to prevent *C. parvum* intestinal colonization.

In human, a single case of resolution of prolonged cryptosporidiosis by a probiotic treatment was documented. In view of the benefits of probiotics in infectious diarrhea, Pickerd and Tuthill (2014) recorded a case report of a 12-years old girl that suffered from cryptosporidiosis with severe diarrhea. She was started on a four week course of *Lactobacillus* GG 109 units/day (Culturelle, CAG Functional Foods, Omaha, USA) and *Lactobacillus casei Shiro*ta 6.56109 units/day (Yakult UK Ltd, London, UK) treatment. A repeat stool sample four weeks after starting treatment with probiotics was clear of *cryptosporidium* oocysts. They suggested that this treatment will be a promising therapy.

Also, a beneficial effect of administration of the probiotic yeast *Saccharomyces boulardii* in association with antibiotics was reported in acute amoebiasis due to *Entamoeba histolytica*, with significant decrease of the duration of symptoms (diarrhea, fever, abdominal pain) and presence of cysts in stools (Mansour-Ghanaei *et al*., 2003).

Moreover, the first proposal of the use of probiotics to control infections by *Giardia* came from the discovery that isogenic mice presented a variable susceptibility to *Giardia* infection depending on their intestinal flora. Interestingly, resistance to *Giardia* infection could be transmitted from mouse to mouse by common housing and was abrogated by using antibiotics, such as neomycin, active against the resident anaerobic flora (Singer & Nash, 2000).

Table.1 Mean Numbers of *Cryptosporidium oocyst*/H.P.F ± Standard Deviation in the Stools of all Groups at the Durations of Examination

Groups	**1st day P.I.**	**3rd day P.I.**	**6th day P.I.**	**9th day P.I.**	**12th day P.I.**	**15th day P.I.**
Group (1): treated with L. casei	133±55.32	98±44.25	77±23	44±21.45	18±11.23	0
Group (2): treated with yogurt	120±32.21	87±23.16	65±33.55	31±54.20	15±10.51	0
Gr. (1) & (2) P value	0.3695*	0.3309*	0.1979*	0.3249*	0.3885*	
Group (3): infected & non-treated (control)	178±68.15	213±89.14	268±122.24	301±176.13	475±188.46	622±221.24
Gr.(1)&(3) P value	0.0275**	0.0001**	0.0001**	0.0001**	0.0001**	0.0001**
Gr. (2&3) P value	0.0001**	0.0001**	0.0001**	0.0001**	0.0001**	0.0001**

No significance difference (P> 0.05)
**** :Significance difference (P<0.0001)**

Table.2 Mean Numbers of *Cryptosporidium* Developmental Stages/H.P.F. ± Standard Deviation in the Ilea of all Groups at the Durations of Examination

	1st day P.I.	**3rd day P.I.**	**6th day P.I.**	**9th day P.I.**	**12th day P.I.**	**15th day P.I.**
Group (1): treated with L. casei	65±20.11	46±58,39	24±58.39	16±35.13	9±23.55	0
Group (2): treated with yogurt	559±33.12	33±56.33	18±35.25	8±18.32	0	0
Gr. (1) & (2) P value	0.4928*	0.4780 *	0.6962 *	0.3743 *	0.0956 *	
Group (3): infected &nontreated	96±88,36	109±89.30	177±122.26	204±145.36	266±231.28	309±277.19
Gr.(1)&(3) P value	0.134*	0.0119**	0.0001**	0.0001**	0.0001**	0.0001**
Gr. (2&3) P value	0.0536*	0.0026**	0.0001**	0.0001**	0.0001**	0.0001**

No significance difference (P> 0.05)
**** :Significance difference (P<0.0001)**

Fig.1 *Cryptosporidium oocysts* in the Stools of an Immuno suppressed and Infected Non-Treated Mouse (Group 3, Control)Safranin-Methylene Blue Stain, X 1000

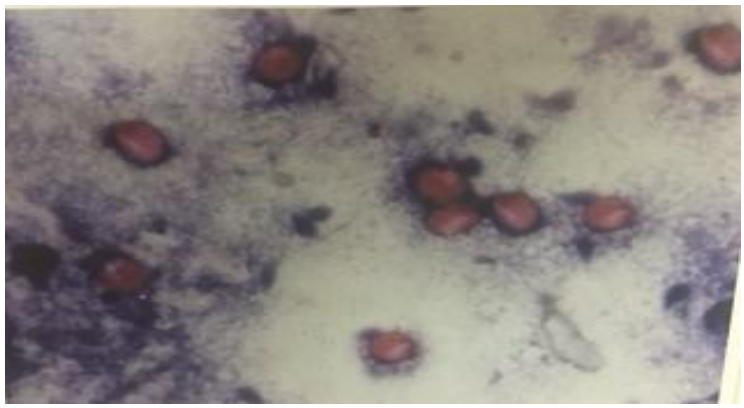


Fig.2 Histopathological Section of Ileum of Immuno suppressed Infected Non Treated Mouse (Group 3) Showing Severe Colonization of *Cryptosporidium* Developmental Stages in the Brush Borders of Villi (Hx& E, 400)

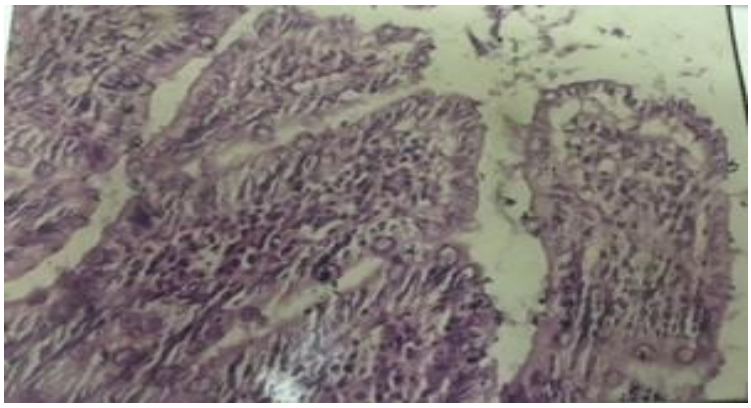


Fig.3 Histopathological Section of the Ileum of Immuno suppressed Infected Treated Mouse with Lactobacillus Casei(Group 1) Showing Little Colonization of *Cryptosporidium* (Hx& E, 400)

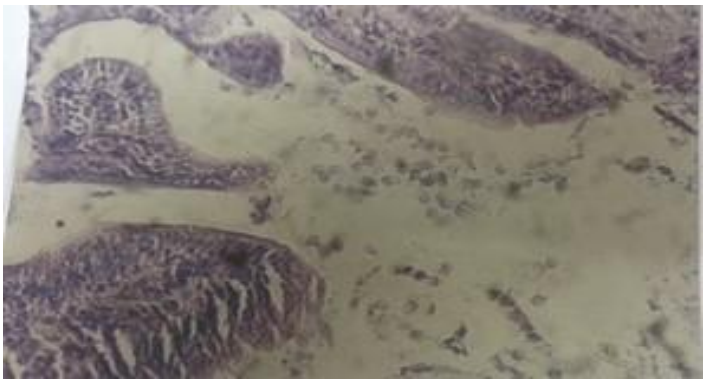
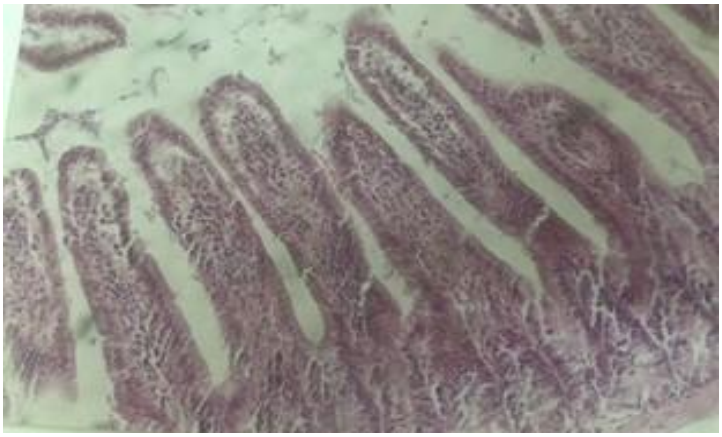


Fig.4 Histopathological Section of Ileum of Immuno suppressed Infected Mouse Treated with Yogurt (Group 2) Showing No Parasites in the Brush Borders but with Still Lengthening of The Villi With Inflammatory Cells (Hx& E, 400)



Proposing probiotics as alternatives to classical treatments, such as drugs or vaccines, against parasites appears unreasonable; a complementary therapeutic approach to reduce risks of infestation or to sustain classical treatments seems more realistic. For the moment, studies of probiotic effects on parasites are still in their infancy, and further investigations are needed to move forward in this direction. Another factor of variability that has to be taken into account is the gut microflora of the experimental animal models. While it is obvious that the animal genetic background is important, the environmental factors, such as hygiene conditions, feed quality, and stress, can also affect the established microflora, influencing the results of the studies (Oelschlaeger, 2010).

In depth understanding of the molecular mechanisms sustaining probiotic action is required to properly design future probiotic treatments. As probiotics can kill pathogens through secretion, inhibit their adhesion or invasion, inactivate toxins, or compete for nutriments, most studies focused on intestinal pathogens with the hypothesis of a local probiotic efficiency. But in vivo studies on non-gut pathogens (*Plasmodium*, *Trypanosoma*, *Babesia*, etc.) support a remote effect provided by probiotics probably through a nonspecific immune stimulation. In all cases, a lot of effort needs to go in the elucidation of the mode of action of the promising organisms. A better understanding of molecular mechanisms underlying the beneficial effects of probiotic on the parasite infection is essential to validate the approach. Further deeper investigations are thus needed using more defined protocols ((Marie-Agnès *et al.*, 2011).

The concept that probiotics could control the development of parasites is promising and hopeful. Therapeutic approaches with probiotics have a definite role in reduction of infestation by specific parasites or their combination to the anti-parasite treatments. The beneficial effects of probiotic on the parasite infection can be understood through molecular studies. Further deeper investigations are thus needed using more defined protocols (specific probiotics and experimental models), as well as extended clinical investigations. We cannot ignore the dramatic effects of probiotics on parasitic infestation.

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