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BRIEF COMMUNICATION

PROTECTIVE EFFECT OF THE PROBIOTIC *Saccharomyces boulardii* IN *Toxocara canis* INFECTION IS NOT DUE TO DIRECT ACTION ON THE LARVAE

Luciana Farias da Costa de AVILA(1), Paula de Lima TELMO(2), Lourdes Helena Rodrigues MARTINS(3), Thaís Aimeé GLAESER(3), Fabricio Rochedo CONCEIÇÃO(2), Fábio Pereira Leivas LEITE(1) & Carlos James SCAINI(4)

SUMMARY

In a previous study our group found that the probiotic *Saccharomyces boulardii* was capable of reducing the intensity of infection in mice with toxocariasis. In order to assess whether the mechanism involved would be a direct action of the probiotic on *Toxocara canis* larvae, this study was designed. Both probiotics were singly cultivated in plates containing RPMI 1640 medium and *T. canis* larvae. *S. boulardii* and *B. cereus* var. toyoi cultures presented 97.6% and 95.7% of larvae with positive motility, respectively, and absence of color by the dye trypan blue, not representing significant difference to the control group ($p > 0.05$). We conclude that none of the probiotics showed *in vitro* effects on *T. canis* larvae and that the interaction with the intestinal mucosa is necessary for the development of the protective effect of *S. boulardii*.

KEYWORDS: Toxocariasis; Probiotics; Larvicidal action.

Visceral toxocariasis is a chronic parasitic disease of worldwide distribution[12] currently regarded as a public health problem[17]. This parasitosis, commonly caused by the nematode *Toxocara canis,* is characterized by the migration and presence of the parasite larvae in humans. These parasites develop in the intestines of dogs, definitive hosts, that contaminate the soil with the parasite eggs. Humans and other paratenic hosts, such as mice, rats, chickens, lambs, and pigs, among others, acquire toxocariasis by ingesting *T. canis* eggs containing the infective-stage larvae[9]. The larvae hatch in the small intestine, where they perforate the wall and are disseminated by the systemic circulation[14].

The various clinical forms of human toxocariasis complicate treatment, making necessary the search for new alternatives of control and treatment. Although several studies have shown the benefit of the use of probiotics for the prevention and treatment of diseases, few have been conducted to evaluate the preventive effect on protozoosis[10,13,15] and helminthiasis[1,2,3]. The *Saccharomyces boulardii,* yeast characterized as anti-inflammatory and immunostimulant in the intestinal mucosa[3], is also related to the reduction of parasite burden in mice infected with *T. canis*[1], although the mechanism of action is unknown. Besides this fungus, the *Bacillus cereus* var. toyoi is a probiotic related to the stimulation of the immune response and promotes animal weight gain[7,8,11]. There is still a gap in the understanding of the mechanism of action of these and other probiotics. In the present study, we evaluated whether there is any larvicidal effect of the probiotics *S. boulardii* and *B. cereus* var. toyoi on *T. canis* larvae *in vitro*.

T. canis eggs were collected directly from the uterine tubes of adult gravid female parasites obtained after the treatment of puppies with pyrantel pamoate (15 mg/kg). Afterwards, the eggs were incubated in 2% formalin solution at 28 °C, with humidity above 90% and oxygenation for 30 days. Then, the extraction of larvae was performed according to DE SAVIGNY (1975). This study was approved by the Ethics Committee on Animal Experimentation of the Universidade Federal de Pelotas.

The *S. boulardii* yeast was cultivated in the YPD (Yeast Peptone Dextrose) medium, which was incubated in an orbital agitator (150 rpm) at 37 °C for 48 h. *B. cereus* var. toyoi was cultivated in BHI (Brain Heart Infusion) medium and incubated at the same conditions for 24 h. The cultures were centrifuged at 4000 × g for 10 min at 4 °C, and the pellets were washed with phosphate buffered saline (PBS) and quantified by determining colony forming units (CFU).

A culture in RPMI 1640 with L-glutamine was performed, supplemented with penicillin, streptomycin and fungizone (Invitrogen®), of 100 larvae/hole in microculture plates (96 holes - TPP®). *S. boulardii* or *B. cereus* var. toyoi probiotics were then added in triplicate, at

(1) Post-Graduate Program in Parasitology, Universidade Federal de Pelotas (UFPEL); Campus Universitário, Caixa Postal 354, 96010-900 Pelotas, RS, Brazil. Tel.: 55.53.3275-7350, Fax: 55.53.3275-7350.
(2) Center of Technology Development - Biotechnology (UFPEL).
(3) Parasitology Laboratory, Universidade Federal do Rio Grande (FURG).
(4) Post-Graduate Program in Health Sciences (FURG).
Correspondence to: Luciana Farias da Costa de Avila. E-mail: lucostaavila@hotmail.com

concentrations of 1.10^4, 1.10^5, 1.10^6 and 1.10^7 CFU/hole. The RPMI 1640 has been used and works with these probiotics[6,19] and this medium with antibiotics was checked and did not inhibit the probiotics in the concentrations used. Two controls were used, one with live larvae and another with dead larvae, both in triplicate, in the RPMI 1640 culture. In all wells the dye trypan blue was added as a marker of cell viability. Since dead larvae have no motility and present blue staining, the control of dead larvae was used as a standard to assist in the identification of larvae in this condition after *in vitro* test. The material was incubated for 48 hours at 37 °C with 5-10% CO_2. Afterwards, the motility of the larvae was evaluated in a light microscope, considering alive those that presented positive motility and absence of trypan blue staining. The experiment was done twice, with similar results. Data was subjected to an analysis of variance, and the means were compared with Tukey's test, with a significance level of 95%.

The number of recovered live larvae in treatments with *S. boulardii* or *B. cereus* var. toyoi, at all tested concentrations, did not present significant difference for the control of live larvae. An average viability of 97.6% and 95.7% ($p > 0.05$) was observed for the treatment with *S. boulardii* and *B. cereus* var. toyoi, respectively (Table 1). In the control of live larvae an average viability of 96.5% was observed, with triplicate values of 95.9%, 96.7% and 97.0%. In the control of dead larvae, all of them were unviable and stained with trypan blue. The incubation time of 48 h was used because in just two days of infection it was already possible to observe a reduction in the number of *T. canis* larvae in mice treated with *S. boulardii*[1].

Table 1

Average percentage (%) of the larvae that presented positive motility and absence of trypan blue staining at the different concentrations of probiotic. There was no significant difference between the groups ($p > 0.05$)

Group	10^4	10^5	10^6	10^7	Overall percentage
S. boulardii	97.4	97.1	98.9	97.0	97.6
B. cereus var toyoi	96.8	96.1	94.9	95.1	95.7

Despite the absence of *in vitro* larvicidal effect of these two probiotics on *T. canis* observed in this study, a recent work conducted by our team showed a 36.7% reduction in larval burden of mice infected with *T. canis* that received *S. boulardii.* Moreover, this probiotic reduced by 41.9% the average number of larvae recovered from the liver[1], demonstrating that such a probiotic promoted an important reduction in the intensity of the infection by *T. canis* larvae. Regarding the probiotic *B. cereus* var. toyoi, there are no reports of study in experimental models infected with *T. canis*, or other nematode, to do a comparison.

Interesting results were obtained with the probiotic *Lactobacillus casei* inoculated intraperitoneally in NIH mice once a week during six weeks. The authors report that this probiotic induces a total protection against *Trichinella spiralis* infection at low doses[16]. Similar results were obtained in BALB/c mice with inhibited *T. spiralis* infections and invasion of the intestinal mucosa[18]. Regarding toxocariasis, CHIODO *et al.* (2010) showed that the CECT7121 probiotic strain of *Enterococcus faecalis* presented direct action on *T. canis* once it caused the death of *in vitro* larvae after 48-hour incubation. According to this author, the inhibitory activity obtained from the *in vivo* experiments was lower than *in vitro*, indicating a loss of the inhibitory effect of *E. faecalis* in the intestinal lumen; precisely the opposite of what we observed with *S. boulardii,* which increases its effect on the intestinal mucosa.

From these results we can conclude that the contact of the *S. boulardii* yeast with the intestinal mucosa of the host is necessary for the development of their action on *T. canis* larvae and the mechanism of action is not a direct effect of the probiotics on the parasite. All these results suggest that the larvicidal effect and the mechanism of action developed on *T. canis* larvae varies according to the probiotic used. The use of probiotics for the control of parasitic diseases has shown promising results and further studies should be conducted in order to analyze the effect of other probiotics on *T. canis* larvae and other nematodes.

RESUMO

Efeito protetor do probiótico *Saccharomyces boulardii* na infecção por *Toxocara canis* não se deve à ação direta sobre as larvas

Em estudo prévio nosso grupo verificou que o probiótico *Saccharomyces boulardii* foi capaz de reduzir a intensidade de infecção em camundongos com toxocaríase. Este estudo foi elaborado com o objetivo de avaliar se o mecanismo envolvido seria uma ação direta do probiótico sobre as larvas de *Toxocara canis.* Ambos probióticos foram cultivados, separadamente, em placas com meio RPMI1640 e larvas de *T. canis*. As culturas com *S. boulardii* e *B. cereus* var. toyoi apresentaram, respectivamente, 97,6% e 95,7% das larvas com motilidade positiva e ausência de coloração pelo azul de tripan, não representando diferença significativa do controle ($p > 0,05$). Concluímos que nenhum dos probióticos apresentou efeito *in vitro* sobre as larvas de *T. canis* e que a interação com a mucosa intestinal é necessária para o desenvolvimento do efeito protetor de *S. boulardii*.

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